

Characterization of the CpxRA Regulon in *Haemophilus ducreyi*^{∇†}

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The *Haemophilus ducreyi* 35000HP genome encodes a homolog of the CpxRA two-component cell envelope stress response system originally characterized in *Escherichia coli*. CpxR, the cytoplasmic response regulator, was shown previously to be involved in repression of the expression of the *lspB-lspA2* operon (M. Labandeira-Rey, J. R. Mock, and E. J. Hansen, *Infect. Immun.* 77:3402–3411, 2009). In the present study, the *H. ducreyi* CpxR and CpxA proteins were shown to closely resemble those of other well-studied bacterial species. A *cpxA* deletion mutant and a CpxR-overexpressing strain were used to explore the extent of the CpxRA regulon. DNA microarray and real-time reverse transcriptase (RT) PCR analyses indicated several potential regulatory targets for the *H. ducreyi* CpxRA two-component regulatory system. Electrophoretic mobility shift assays (EMSAs) were used to prove that *H. ducreyi* CpxR interacted with the promoter regions of genes encoding both known and putative virulence factors of *H. ducreyi*, including the *lspB-lspA2* operon, the *flp* operon, and *dsrA*. Interestingly, the use of EMSAs also indicated that *H. ducreyi* CpxR did not bind to the promoter regions of several genes predicted to encode factors involved in the cell envelope stress response. Taken together, these data suggest that the CpxRA system in *H. ducreyi*, in contrast to that in *E. coli*, may be involved primarily in controlling expression of genes not involved in the cell envelope stress response.

Haemophilus ducreyi is the etiologic agent of chancroid, a sexually transmitted genital ulcer disease (54, 59). In some resource-poor developing countries of Asia, Africa, and Latin America, chancroid is a very common sexually transmitted infection (reviewed in reference 54). In the United States, chancroid is very rarely seen but outbreaks in urban areas have been documented (23, 40) and this disease may be underreported (52). The public health consequences of chancroid are not insignificant, because the presence of genital ulcers caused by *H. ducreyi* increases the likelihood of heterosexual transmission of HIV-1 infection (20, 51).

Despite sustained efforts to study the pathogen, chancroid remains one of the least understood infectious diseases (reviewed in references 7, 30, 36, and 54). How the bacterium causes tissue necrosis and retards lesion healing (54) has not been elucidated, and experimental investigation of its virulence mechanisms *in vitro* has been complicated by the fastidious nature of *H. ducreyi*. Nonetheless, over the past 15 years, a large number of putative virulence factors of this organism have been identified, including numerous proteins and the lipooligosaccharide (LOS). However, subsequent testing in the human challenge model for experimental chancroid (for a review, see reference 31) revealed that only a few of these gene products are truly essential for full virulence of *H. ducreyi*. Mutations in *pal* (21), *hgbA* (1), *dsrA* (8), the *flp* gene cluster (55), *ncaA* (22), and *lspA1* and *lspA2* (29) attenuated the virulence of *H. ducreyi* in human volunteers, whereas mutations in four other genes caused partial attenuation (4, 6, 28, 34).

There is even less information available about the regulatory

mechanisms that control expression of virulence-related genes in *H. ducreyi*. Although *H. ducreyi* is an obligate human pathogen, this biological fact does not prevent it from having to regulate gene expression. The earliest documented example of gene regulation in *H. ducreyi* involved a heme acquisition system (18), and in a later study, mutant analysis indicated that inactivation of the *lspA1* gene would cause increased expression of the LspA2 protein (61). The use of DNA microarray analysis and real-time reverse transcriptase (RT) PCR showed that several hundred *H. ducreyi* genes are differentially regulated in the presence of fetal calf serum (FCS), and subsequent experiments revealed that the *H. ducreyi* CpxR response regulator is involved in negatively controlling expression of the *lspB-lspA2* operon (35).

Two-component signal transduction systems (TCSs) typically comprise a membrane-bound sensor (histidine protein) kinase and a cytoplasmic response regulator (for reviews, see references 38, 65, and 69). Upon sensing the appropriate signal, the sensor kinase autophosphorylates a specific histidine residue. The phosphoryl group is then transferred to a specific aspartate residue in the response regulator, which thereupon becomes a transcriptional activator or repressor. TCSs have been shown to control numerous different genes involved in virulence expression, nutrient uptake, motility, and the response of the bacterial cell to stress (49). In *Escherichia coli*, there are three TCS-based cell envelope stress response systems, including the regulons of BaeSR, regulator of capsule synthesis (RCS), and CpxRA (for a review, see reference 38). Among these TCSs, CpxRA has been the most studied to date (47).

There are now several examples of TCSs that function in other pathogens that naturally infect only humans, including *Bordetella pertussis* (53), *Neisseria gonorrhoeae* (42), and *N. meningitidis* (27). The demonstration of a functional CpxR ortholog in *H. ducreyi* (35), together with the presence of a closely linked open reading frame (ORF) encoding a predicted

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
<i>H. ducreyi</i> strains		
35000HP	Human-passaged <i>H. ducreyi</i> strain	57
35000HP Δ <i>cpxA</i>	35000HP Δ <i>cpxA</i> ::Cm ^r	This study
35000HP Δ <i>cpxR</i>	35000HP Δ <i>cpxR</i> ::Cm ^r	35
<i>E. coli</i> strains		
DH5 α	F ⁻ ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>phoA supE44</i> λ ⁻ <i>thi-1 gyrA96 relA1</i>	Invitrogen
TOP10	F ⁻ <i>mcrA</i> Δ (<i>mir-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74 recA1 araD139Δ(<i>ara-leu</i>)7697 <i>galU galK rpsL endA1 nupG</i></i>	Invitrogen
HB101	F ⁻ <i>thi-1 hsdS20</i> (r _B ⁻ m _B ⁻) <i>supE44 recA13 ara-14 leuB6 proA2 lacY1 galK2 rpsL20</i> (Str ^r) <i>xyl-5 mtl-1</i>	Promega
Plasmids		
pCR2.1	<i>E. coli</i> cloning vector; Amp ^r	Invitrogen
pML117	pCR2.1 containing a 35000HP <i>cpxA</i> gene with a 1.15-kb deletion	This study
pML118C	pML117 with a <i>cat</i> cartridge in the deletion site in <i>cpxA</i>	This study
pLS88	<i>H. ducreyi</i> shuttle vector; Km ^r	66
pML141	pLS88 containing the wild-type 35000HP <i>cpxA</i> gene	This study
pML153	pL141 with the H253Q substitution in CpxA	This study
pML125	pLS88 containing the wild-type 35000HP <i>cpxR</i> gene	35
pML154	pML125 with the D52A substitution in CpxR	This study

CpxA ortholog, led us to construct additional *H. ducreyi* mutants and recombinant *H. ducreyi* strains to determine the extent of the CpxRA regulon in *H. ducreyi*. Our results indicate that, although the *H. ducreyi* CpxR and CpxA proteins are highly similar to the two *E. coli* proteins with the same designations, their functions may have evolved to control a set of genes in *H. ducreyi* that are not involved in the cell envelope stress response. Some of these CpxRA-regulated genes encode proven virulence factors of this pathogen.

MATERIALS AND METHODS

Bacteria and culture conditions. Strains used in this study are listed in Table 1. *H. ducreyi* strains were grown on chocolate agar (CA) plates that were incubated at 33°C in a humidified atmosphere containing 95% air and 5% CO₂. For broth culture, *H. ducreyi* strains were grown in a Columbia broth-based medium (62) containing 2.5% (vol/vol) heat-inactivated fetal calf serum (CB) at 33°C in a gyratory water bath at 100 rpm. Antibiotic selection used with *H. ducreyi* strains involved chloramphenicol (1 μ g/ml) and/or kanamycin (30 μ g/ml). *E. coli* strains including DH5 α , TOP10, and HB101 were used for general cloning procedures and were grown in Luria-Bertani medium supplemented with the appropriate antibiotic (ampicillin, 100 μ g/ml; kanamycin, 50 μ g/ml; or chloramphenicol, 30 μ g/ml). *E. coli* HB101 was transformed with recombinant plasmids derived from pLS88 (66), and the plasmids were then isolated from this strain and introduced by electroporation into *H. ducreyi* for complementation studies.

Development of a polyclonal CpxA antiserum. The peptide DGEGVPESEY KQIFRPFYRVGEAR, corresponding to amino acids 395 to 418 of the *H. ducreyi* 35000HP CpxA protein, was synthesized by the Protein Technology Center at the University of Texas Southwestern Medical Center. This peptide was covalently coupled to maleimide-activated keyhole limpet hemocyanin (Thermo Scientific, Rockford, IL). Immunizations were carried out by Rockland Immunochemicals (Boyertown, PA). SulfoLink coupling resin (Pierce/Thermo Scientific) was used to immobilize the CpxA peptide in order to affinity purify CpxA-specific antibodies from the antiserum. The resulting purified polyclonal CpxA antibody was used at a concentration of 70 ng/ml as a primary antibody in Western blot analysis.

SDS-PAGE and Western blot analysis. Proteins present in whole-cell lysates (derived from 5 \times 10⁷ to 1 \times 10⁸ CFU) of the appropriate *H. ducreyi* strains were resolved by SDS-PAGE in 4 to 20% (wt/vol) polyacrylamide separating gels and electroblotted onto polyvinylidene difluoride (PVDF) membranes as described previously (41). Membranes were then incubated in StartingBlock (phosphate-buffered saline [PBS]) blocking buffer (Thermo Scientific, Rockford, IL) containing 5% (vol/vol) normal goat serum for 1 h at room temperature or overnight

at 4°C, after which they were incubated in primary antibody for 3 to 4 h at room temperature or overnight at 4°C. Membranes were then incubated for 1 h at room temperature in a 1:20,000 dilution of either goat anti-mouse IgG-horse-radish peroxidase (IgG-HRP) or goat anti-rabbit IgG-HRP (Bio-Rad, Hercules, CA). The LspA1-specific monoclonal antibody (MAb) 40A4 (62), the LspA2-specific MAb 1H9 (62), the PAL-specific MAb 3B9 (56), LspB-reactive polyclonal mouse serum (63), DsrA-reactive polyclonal mouse serum (19), Flp1-reactive polyclonal rabbit serum (41), and CpxR-reactive polyclonal rabbit serum (35) have been described previously. Western blots were developed using the Western Lightning Plus chemiluminescence reagent (New England Nuclear, Boston, MA).

Construction and complementation of an *H. ducreyi* *cpxA* mutant. An ~1.2-kb fragment corresponding to the 5' upstream region of the *H. ducreyi* 35000HP *cpxA* ORF was PCR amplified from chromosomal DNA with *Ex Taq* DNA polymerase (Takara Bio Inc., Shiga, Japan) by using primers P1 (35) and P183 (Table 2). A second 1.2-kb fragment corresponding to the 3' downstream region of the *H. ducreyi* *cpxA* ORF was PCR amplified with primers P184 (Table 2) and P2 (35). Primers P183 and P184 carried 24-nucleotide (nt) complementary sequences (Table 2), and a SmaI site (underlined) was included in primer P183. The two PCR fragments were gel purified, and equal amounts were used as the templates in overlapping extension PCR (25) with primers P1 and P2. The resultant 2.4-kb PCR product had a 1,149-bp deletion within the *cpxA* ORF, with a SmaI site in the center. This fragment was cloned into pCR2.1 (Invitrogen) to obtain pML117. A *cat* cartridge from pSL1 (37), modified to contain the native *cat* promoter (35), was digested with SmaI and ligated to SmaI-digested pML117 to obtain pML118C. Primers P1 and P2 were used to amplify an ~3.2-kb fragment from pML118C containing the *cpxA* deletion construct described above. The amplicon was DpnI digested to cut any residual plasmid DNA and then subjected to gel purification. Different amounts of the purified PCR fragment (5 to 100 μ g) were used for electroporation of *H. ducreyi* 35000HP as described previously (35). PCR followed by nucleotide sequence analysis was used to confirm that the desired deletion was in frame in the resultant chloramphenicol-resistant *H. ducreyi* 35000HP Δ *cpxA* mutant. Complementation of the *H. ducreyi* 35000HP Δ *cpxA* mutant was performed as follows: a 100-ng quantity of pML141 (described below) was used for electroporation of 35000HP Δ *cpxA* to obtain the kanamycin- and chloramphenicol-resistant strain 35000HP Δ *cpxA*(pML141).

Recombinant plasmids and site-directed mutagenesis. Plasmid pML125, carrying the wild-type *cpxR* gene from *H. ducreyi* 35000HP, has been described previously (35). To construct a plasmid containing the *cpxA* gene, the wild-type *cpxA* gene from *H. ducreyi* 35000HP together with 268 nt upstream of the translational initiation codon was PCR amplified from chromosomal DNA by using primers P299 and P298 (Table 2). The 1,677-bp amplicon was digested with SacII and ligated to SacII-digested pLS88 (16) to obtain pML141.

Based on sequence homology to well-characterized CpxR and CpxA proteins

TABLE 2. Oligonucleotides used in this study

Primer name	Sequence (5'-3') ^a	Primer name	Sequence (5'-3') ^a
Primers for construction of mutants		Primers for EMSAs	
P183.....	ACCACCACTTTGAGGGTCACGTG CCCCGGGAATAGCTAACATA ATAGCAAATGT	HD0765R.....	CCGGAATATCAACCCATTTC
P184.....	GCACGTGACCGTCAAAGTGG TGGT	HD0266F.....	GGTACCATCGTTTCAATTCTG
P298.....	ACGCCCGCGGAAGCTTAATGT ATTAAATTAT	HD0266R.....	TAAGGCACGTCCAGTACCAC
P299.....	ACGCCCGCGGAGATGTTAATG CATAACCCTG	HD0189F.....	TTGCACACGCTACACGTA
P464.....	CGCGCGGTTGAGCTCCCTA TCAT	HD0189R.....	TAGCTTGAGCAGCTTGCA
P465.....	CATTAACAGGAGCTCGTTCC TGCC	HD0815F.....	CGGAACAAGTTACCAAGCAA
Primers for real-time RT-PCR		HD0815R.....	TTCACCACCAGATGCAATTT
HD0281F.....	TCGTTGAGGCTGCTTGTAGT	HD0812F.....	CGAAATTGTTGGGTTTGATG
HD0281R.....	AAAGGTGGAACATCTCGTCC	HD0812R.....	CTTGCTTCATTCGTGCAATC
HD1629F.....	CAAGCAGGATCGGTATGTT	HD1076F.....	AGCGTTAGTGCCTGGTATG
HD1629R.....	TAACGCCATAGCCATCAGAG	HD1076R.....	TGCAAACCCCTAGCTTTGTG
HD1160F.....	TAGCCACGTGTTAGGCATTC	HD1186F.....	GGCGAACAAGCTATTTGTGA
HD1160R.....	CTTTACGATTACGGGTTGCA	HD1186R.....	TTGTTCCCTGCCTGCAATC
HD0472F.....	ATGCGCGTAGGCAACATA	HD1456F.....	GACACGTTCCGGGTGATATTG
HD0472R.....	TGAGCCACTTCTTATTGTGGA	HD1456R.....	GACGAGCAGCATCATCAGTT
HD0312F.....	CCTTTGGTGCTGCAGTTAGA	HD0436F.....	CCACTTTAATTGGCGGAGAT
HD0312R.....	TCGTATTGTTGTGGTGCAATG	HD0436R.....	TCCTATGGTGCCAGAAACAA
HD0564F.....	GGATTTACTCGGCGAAAGAG	HD1468F.....	TGGATCTTACCTGATTCCA
HD0564R.....	CCATACCACGGACAATTCA	HD1468R.....	CCTCTGCCGTCATTATCAAA
HD0850F.....	ATTGACGTGGCATAACCAGAA	Primers for EMSAs	
HD0850R.....	CTGCTGTGCTGTTCTCAAA	HD1433P(F).....	TTAAGCATTTTTATTTACTTT
HD0405F.....	AAAGATCCGCTACTGCCATT	HD1433P(R).....	GCAAGCAAACGTAGTGAGCCA
HD0405R.....	TGTGCGCTACAATTTGTCA	HD1312P(F).....	AAACCGATCTGGTCTAACCGA
HD0903F.....	TGCAGTAGGTATCCGCATTG	HD1312P(R).....	AAATATAAAAATAAATTTATGTT
HD0903R.....	GCACGATTGAAATCACCAAC	HD0281P(F).....	AAATACTCTAATCAAGTTGACGT
HD0998F.....	TAGCCGCTTCTTTGGGTAAT	HD0281P(R).....	GCTGATATACTTATTGTTCCC
HD0998R.....	TGAGTGAAGCCCGTAATCTG	HD0046P(F).....	TTTTATTTTGCAAATTATAC
HD0349F.....	GTTGTACGGCGTGTATGGAG	HD0046P(R).....	TTCCATTTAATTTTCTCTA
HD0349R.....	CACCTTGTGTTGCGGTATGT	HD0769P(F).....	TGAATTGGAGTGGACCAGGAC
HD1326F.....	CGGGTTAATGGGTTTATTGG	HD0769P(R).....	ATAGTAGAACAAGCTAATCCC
HD1326R.....	CCCAAATGGAGCATATCAGA	HD0260P(F).....	ATGGTGAAAAAATCCCGCTT
HD2013F.....	AGGTTTCAGCGGGTAAATC	HD0260P(R).....	ACACTTAAACCTAAGACTAAT
HD2013R.....	ATATCTTTGCCTTGCGCTTT	HD0638P(F).....	TACATCTGATTGATATT
HD0765F.....	GCTAATTTGCCACACGCTTA	HD0638P(R).....	TTCATTAGTGTTCTTAA
		HD1788P(F).....	TTCATTAGCCCGTAATACGAT
		HD1788P(R).....	ATGTTTTAAATAAACTCATTAA
		HD2025P(F).....	ATTAATAAACAATAGTACTTA
		HD2025P(R).....	GTTTGCATATTGCTTTCCGGCA
		HD1737P(F).....	CAGGGTTGATAATACATCAAT
		HD1737P(R).....	ACTAACGCAAAAAATAAC TTTA
		HD0649P(F).....	ATTCAGTATGCCTAATCCTAT
		HD0649P(R).....	CTTTTTCAGTATGTTTCATAAT

^a Bold text indicates complementary sequences for use in overlapping extension PCR. Underlining indicates restriction sites as described in Materials and Methods.

from enteric bacteria, site-directed mutagenesis with the QuikChange kit (Stratagene) was used to make the following changes in the putative residues involved in phosphotransfer: D52A in CpxR and H253Q in CpxA. For PCR amplification, 50 ng of template pML125 or pML141 DNA was used. The resultant plasmids carrying the mutated *cpxR* (i.e., pML154) or *cpxA* (i.e., pML153) gene were verified by nucleotide sequencing. Plasmids carrying the desired mutations were used for electroporation of 35000HPΔ*cpxR* or 35000HPΔ*cpxA* as described previously (35).

Autoagglutination assay. The abilities of *H. ducreyi* strains to autoagglutinate were evaluated as described previously (61) with some modifications. Briefly, bacterial cells grown on CA plates were used to inoculate 10 ml of CB, and the cells were grown overnight (~16 h). These cells were diluted in fresh Columbia broth to an optical density at 600 nm (OD₆₀₀) of ~0.7 in Klett colorimeter tubes, which were then allowed to remain static at room temperature. The absorbance of the suspensions was measured every 30 min for 3 h.

RNA purification and DNA microarray analysis. The RiboPure bacterial kit (Ambion, Austin, TX) was used to isolate total RNA from broth-grown cultures after 8 h of growth as described previously (35). After RNA isolation, the purity of samples was determined by PCR using oligonucleotide primers specific for *gyrB* (35) to detect possible DNA contamination. Quality assessment was per-

formed using the Agilent 2100 bioanalyzer (Agilent Technologies), which reported RNA integrity numbers (RINs) between 7.8 and 10 for all the samples used. The *H. ducreyi* custom-spotted DNA microarrays used in this study have been described previously (35). For each experiment, 5- to 20-μg quantities of total RNA extracted from cells grown to mid-exponential phase were used for first-strand cDNA synthesis as described previously (35). To avoid gene-specific dye bias, each sample was subjected to reverse labeling (i.e., a dye swap). Differential expression was defined as a minimum of a 2-fold change in expression in the *H. ducreyi* 35000HP Δ*cpxA* mutant relative to that in the wild-type *H. ducreyi* 35000HP or in *H. ducreyi* 35000HP Δ*cpxR*(pML125) relative to that in *H. ducreyi* 35000HP Δ*cpxR*(pLS88). The data were further analyzed so as to include only expression profiles that had a *P* value of ≤0.05 after a one-sample *t* test analysis.

Real-time RT-PCR. Oligonucleotide primers used to amplify ORFs HD2025, HD1643, HD1312, HD0045, HD0046, HD1433, HD0769, and HD0902 were described previously (35); all others are listed in Table 2. Eighteen genes from each of the DNA microarray experiments were randomly selected for further confirmation of their relative transcription levels by two-step real-time RT-PCR. The reverse transcriptase reaction was performed as described previously (35). Assays were performed on three independent biological replicates, using

HD1643 (*gyrB*) to normalize the amount of cDNA per sample. The fold change for each gene was calculated using the $2^{-\Delta\Delta CT}$ method.

Electrophoretic mobility shift assay (EMSA). The ability of purified recombinant CpxR to bind *H. ducreyi* promoters was examined by using the digoxigenin (DIG) gel shift kit from Roche as described previously (35), with the *lspA1* and *gyrB* promoter regions being used as negative controls and the *lspB* promoter region being used as a positive control in each experiment. DNA promoter regions containing the putative consensus CpxR binding sequence in the center of the fragments (~200 bp) were generated by PCR (relevant primer sequences are shown in Table 2).

Microarray data accession numbers. The data from the DNA microarray experiments were deposited in the NCBI Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>) under accession numbers GSE21788 and GSE21789.

RESULTS

Probable structural features of *H. ducreyi* CpxA and CpxR proteins. A simple BLAST search (2) using the product of *H. ducreyi* gene HD1470 returns other CpxA proteins, with the closest homolog being CpxA of *Actinobacillus pleuropneumoniae* (78% identity; E value, 0.0). Structural motifs present in *H. ducreyi* CpxA mark it as the sensor/histidine kinase component of a TCS. No crystal structure for a CpxA protein is known, but a combination of secondary-structure prediction and comparison to known histidine kinase (HK) structures indicates that this CpxA has a short, cytoplasmic part (amino acids [aa] 1 to 17), followed by a transmembrane helix (aa 18 to 38). Residues 39 to 168 comprise a periplasmic domain with mixed secondary structures and probably serve as the sensor (the ligand is unknown). Residues 169 to 188 make up the final transmembrane helix. The cytoplasmic C-terminal domain of this CpxA can be characterized by its similarities to known nuclear magnetic resonance (NMR) and crystal structures. As examples, sequence alignments that take known structures of other proteins and the secondary-structure predictions (44) for *H. ducreyi* CpxA into account indicate that the C terminus of this protein is likely to have a structure similar to those of sensor proteins. In *H. ducreyi* CpxA, aa 189 to 242 likely comprise a so-called HAMP domain (3), which bridges the second transmembrane helix of the protein to the dimerization domain (aa 243 to 303). This conclusion is based on its alignment to the HAMP domain of Af1503 from *Archaeoglobus fulgidus*, the NMR structure of which is known (26). The alignment demonstrates 25% identity and strong conservation of secondary structure between the two proteins (data not shown). H253 is the first residue in the H box (aa 253 to 301) and is the essential residue that serves as an intermediate phosphoreceptor once the protein becomes activated by a stimulus. Residues 304 to 464 comprise the C-terminal ATP binding domain. To examine the likely structure of this domain of CpxA, it is most instructive to compare it to the C-terminal domain of HK853 from *Thermotoga maritima*, the crystal structure of which was recently determined (39). A sequence alignment demonstrates that the two domains have approximately 23% identity and that the secondary structures of the two proteins are highly correlated (data not shown). Given the significant correspondence between the two proteins, it is likely that they have similar three-dimensional structures.

The HD1469 gene encodes a 240-aa protein with homology to CpxR proteins which are the response regulators in TCSs. The protein with the highest level of homology (90% identity;

E value, 4×10^{-113}) is CpxR of *A. pleuropneumoniae*. Comparing *H. ducreyi* CpxR to other response regulators indicates that this CpxR has two domains: an N-terminal receiver domain (aa 1 to 116) and a C-terminal effector domain (aa 141 to 240). Between these two domains, there is a connector region (aa 117 to 140) that is predicted to have no regular secondary structure. In the receiver domain, the residue (D52) necessary for phosphoryl transfer from CpxA is conserved. An analysis of the primary sequence of and the secondary-structure prediction for *H. ducreyi* CpxR (data not shown) aligned to the sequence of the receiver domain of the structurally characterized YycF from *Bacillus subtilis* (Protein Data Bank [PDB] accession number 2ZWM) indicates that this domain is 50% identical to that of *H. ducreyi* CpxR, suggesting that the structures of the two proteins are likely to be very similar. The C-terminal domain of *H. ducreyi* CpxR very likely binds to DNA, given its sequence homology to other effector domains that have this function. By using a structure-assisted sequence alignment (44), the sequence of the *E. coli* PhoB effector domain was compared to that of *H. ducreyi* CpxR (revealing 33% identity). Nearly every amino acid residue in *E. coli* PhoB that makes sequence-independent contacts with DNA is identical to the corresponding residue in *H. ducreyi* CpxR, with a few exceptions which suggest that *H. ducreyi* CpxR does not bind to the same DNA sequences as PhoB (data not shown). The high level of sequence similarity between these two effector domains implies that they share a common fold.

Characterization of an *H. ducreyi* *cpxA* deletion mutant. Deletion of *cpxA* from *H. ducreyi* 35000HP (see Materials and Methods) (Fig. 1A) did not result in a significant growth defect, although the apparent density of the mutant culture was slightly lower than that of the wild-type parent strain culture after 8 h (Fig. 1B). This difference may have been the result of increased autoagglutination exhibited by the *cpxA* mutant (Fig. 1C and D). In addition, the overall total protein profile of this *cpxA* mutant was significantly different from that of the wild-type parent strain (Fig. 1E). Mass spectrometric analysis indicated that the ~40-kDa protein that was much more highly expressed by the *cpxA* mutant was OmpP2B (46). This same type of analysis revealed that the ~27-kDa protein that was present in the wild-type parent strain and apparently missing in the *cpxA* mutant was DsrA (19), a proven virulence factor of this pathogen (8).

Deletion of *cpxA* affects expression of *H. ducreyi* gene products involved in virulence. In a previous study (35), CpxR, the response regulator in the CpxRA two-component regulatory system, was shown to be a repressor of the *H. ducreyi* *lspB-lspA2* operon and to have no apparent direct effect on the expression of *lspA1* (Fig. 2A, lane 2, and B). Many histidine kinases are known to have phosphatase activity toward their cognate response regulators. A deletion of the sensor kinase should therefore result in the inability of the response regulator to be dephosphorylated, assuming that the regulator could be phosphorylated by other small phosphodonors such as acetyl phosphate (32, 67, 68). Using both real-time RT-PCR and Western blot analyses, we showed that deletion of the *cpxA* ORF resulted in the reduction of expression of both *lspB-lspA2* transcripts (Fig. 2D) and the respective encoded proteins (Fig. 2C, lane 2). These results would be consistent with the presence of high levels of phosphorylated CpxR in the *cpxA* mutant, especially because the apparent level of CpxR

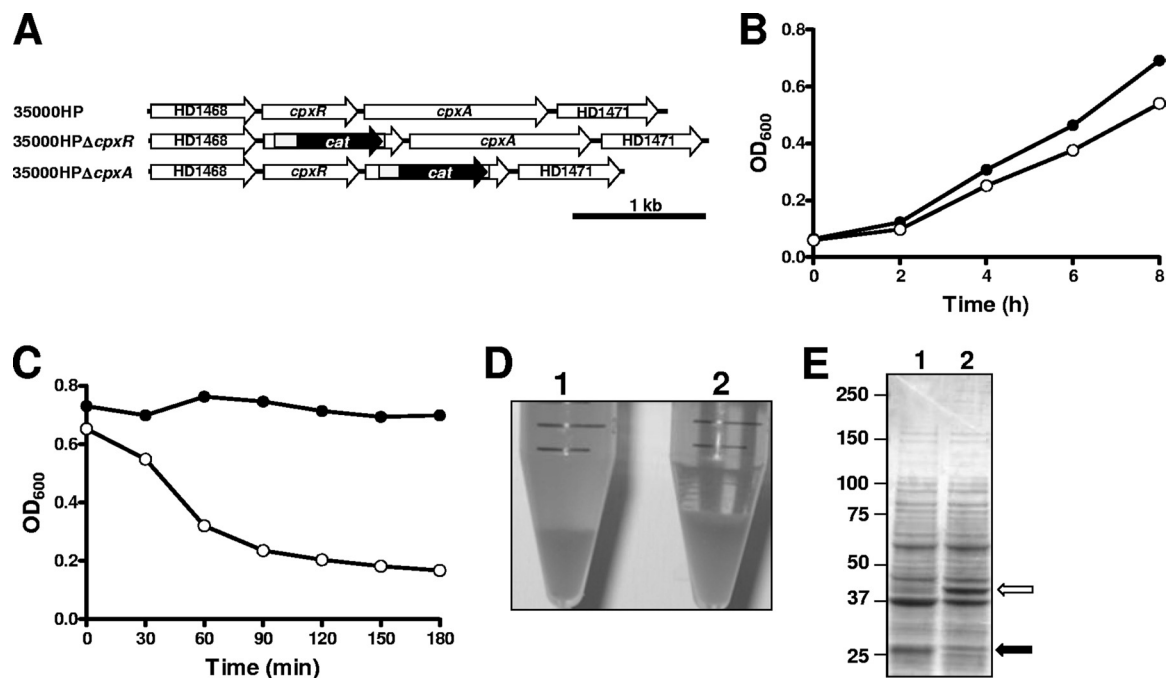


FIG. 1. Characterization of the *H. ducreyi* *cpxA* deletion mutant. (A) Schematic of the *cpxRA* locus in the wild-type strain 35000HP, the 35000HPΔ*cpxR* deletion mutant (35), and the 35000HPΔ*cpxA* deletion mutant. (B) Growth of the wild-type parent strain 35000HP (closed circles) and the *cpxA* deletion mutant (open circles) in broth. Results from a representative experiment are shown. (C) Autoagglutination rates for the wild-type parent strain 35000HP (closed circles) and the *cpxA* deletion mutant (open circles). (D) Photograph of suspensions of the wild-type parent strain 35000HP (1) and the *cpxA* deletion mutant (2) after 3 h (at the end of the autoagglutination assay for which results are presented in panel C). (E) Total cell protein profiles for the wild-type parent strain 35000HP (lane 1) and the *cpxA* deletion mutant (lane 2) as determined by resolving proteins by SDS-PAGE and staining with Coomassie blue. The white arrow indicates the OmpP2B protein, which is overexpressed in the mutant, and the black arrow indicates the DsrA protein, which is present in the wild-type strain and missing in the mutant.

protein in this *cpxA* mutant (Fig. 2C, lane 2) was equivalent to that in the wild-type parent strain (Fig. 2C, lane 1). Based on these results, it is our working assumption that the CpxR protein of this *H. ducreyi* *cpxA* deletion mutant is constitutively phosphorylated and it will be referred to as such for the remainder of this study.

Expression of the *flp1* and *dsrA* genes (Fig. 2D) and their respective encoded protein products (Fig. 2C, lane 2) was also reduced in this *cpxA* mutant. The absence of CpxA also resulted in a reduction in the levels of LspA1 protein (Fig. 2C, lane 2), but the *lspA1* transcript levels remained essentially unchanged (Fig. 2D). This result is consistent with the hypothesis that the effect of the CpxRA regulatory system on LspA1 expression is not direct (35). Provision of a wild-type *H. ducreyi* *cpxA* gene in *trans* (Fig. 2C, lane 3) lifted the repression of expression of the LspB, LspA2, Flp1, and DsrA proteins (Fig. 2C, lane 3), even though expression of CpxA in this recombinant strain (Fig. 2C, lane 3) was substantially lower than that in the wild-type parent strain (Fig. 2C, lane 1). This complemented *cpxA* mutant also exhibited a wild-type autoagglutination phenotype (data not shown).

Effects of site-directed mutations in *cpxR* and *cpxA*. As discussed previously, H253 in CpxA and D52 in CpxR are likely to be the conserved sites for phosphotransfer between this sensor kinase and its response regulator. A D52A substitution in CpxR (encoded by pML154) (Fig. 2A, lane 5) did not result in an increase in expression of LspB, LspA2, Flp1, or DsrA relative to that in the recombinant strain containing the wild-type

cpxR gene in *trans* (Fig. 2A, lane 3), raising the possibility that the overexpression of the mutated CpxR protein observed in this recombinant strain (Fig. 2A, lane 5) might overcome the need for phosphorylation of CpxR (12). Surprisingly, the expression of LspA1 was restored to near wild-type levels (Fig. 2A, lane 5) by overexpression of this mutated CpxR protein. This observation, together with the above data indicating a possible posttranscriptional/translational effect of CpxR on expression of LspA1, suggests that a factor(s) downstream of CpxR requires the phosphorylated state of CpxR for activity.

An H253Q substitution in CpxA (encoded by pML153), which should abolish its kinase activity but maintain its ability to dephosphorylate CpxR (50), did not relieve the repression of expression of LspB, LspA2, Flp1, and DsrA (Fig. 2C, lane 5). It must be noted that the expression of the mutant (H253Q) CpxA protein in the complemented *cpxA* mutant (Fig. 2C, lane 5) was much lower than that of wild-type CpxA in the wild-type strain (Fig. 2C, lane 1) or in the complemented *cpxA* mutant carrying the wild-type *cpxA* gene in *trans* (Fig. 2C, lane 3). These observed very low levels of CpxA expression in these two recombinant strains could result in relatively high levels of CpxR phosphorylation, which would explain why LspB, LspA2, Flp1, and DsrA are still repressed in both of these strains (Fig. 2C, lanes 3 and 5). The very low level of expression of wild-type CpxA and CpxA(H253Q) in these two complemented strains may be related to potential toxicity issues involved in attempted overexpression of an integral membrane protein.

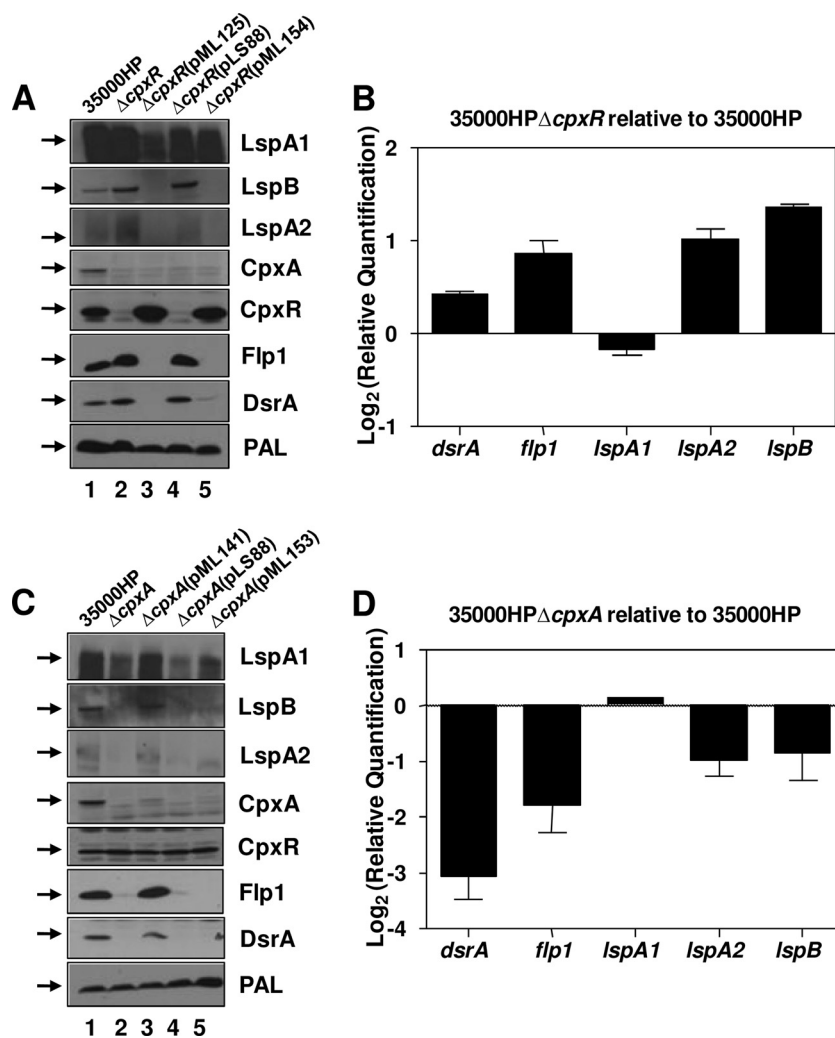


FIG. 2. Deletion of *cpxA* or *cpxR* results in dysregulation of expression of several *H. ducreyi* gene products involved in virulence. *H. ducreyi* strains grown for 8 h in CB were used for preparation of whole-cell lysates or for RNA extraction. (A) Western blot analysis of whole-cell lysates of 35000HP (lane 1), 35000HPΔ*cpxR* (lane 2), 35000HPΔ*cpxR*(pML125) (lane 3), 35000HPΔ*cpxR*(pLS88) (lane 4), and 35000HPΔ*cpxR*(pML154) (lane 5); primary antibodies are indicated to the right of each panel. The PAL monoclonal antibody 3B9 (56) was used as a loading control for both panels A and C. It should be noted here that both LspA1 and LspA2 typically appear diffuse or exhibit a multiple banding pattern in Western blot analysis (35, 61). Black arrows on the left in panels A and C indicate the position of the relevant antigen. (B) Real-time RT-PCR showing transcript levels for *dsrA*, *flp1*, *lspA1*, *lspA2*, and *lspB* in 35000HPΔ*cpxR* relative to 35000HP. (C) Western blot analysis of 35000HP (lane 1), 35000HPΔ*cpxA* (lane 2), 35000HPΔ*cpxA*(pML141) (lane 3), 35000HPΔ*cpxA*(pLS88) (lane 4), and 35000HPΔ*cpxA*(pML153) (lane 5); primary antibodies are indicated to the right of each panel. (D) Real-time RT-PCR showing transcript levels for *dsrA*, *flp1*, *lspA1*, *lspA2*, and *lspB* in 35000HPΔ*cpxA* relative to 35000HP.

The *H. ducreyi* CpxRA regulon. To identify members of the *H. ducreyi* CpxRA regulon, DNA microarray analysis was initially performed with wild-type *H. ducreyi* 35000HP and the isogenic *cpxR* mutant. As observed in similar studies performed with *E. coli* (47), inactivation of the response regulator (i.e., *cpxR*) did not have much effect on overall gene expression (data not shown). With the working assumption that a *cpxA* deletion mutant should have a highly phosphorylated CpxR protein (10, 32, 68), we next compared the global expression profile of the wild-type strain with that of the *cpxA* mutant. Regulated genes were defined as those with a minimum 2-fold transcriptional change ($P < 0.05$). One hundred eighty-one genes were found to be differentially regulated (108 were upregulated and 73 were downregulated) in the *cpxA* mutant

background (see Table S1 in the supplemental material). To validate the DNA microarray results, real-time RT-PCR was performed with a set of selected genes (Fig. 3A) which showed a correlation coefficient R^2 of 0.9648.

We also compared the overall gene expression in 35000HPΔ*cpxR* carrying a wild-type *cpxR* gene in *trans* [35000HPΔ*cpxR*(pML125)] to that in the same mutant carrying the empty plasmid vector [35000HPΔ*cpxR*(pLS88)]. The former strain overexpressed CpxR (Fig. 2A, lane 3), a condition that may overcome the need to have CpxR phosphorylated, as described previously for *E. coli* (12). Under this condition of overexpression of CpxR, a total of 224 genes were differentially expressed, with 90 genes being upregulated and 134 being downregulated (see Table S2 in the supplemental

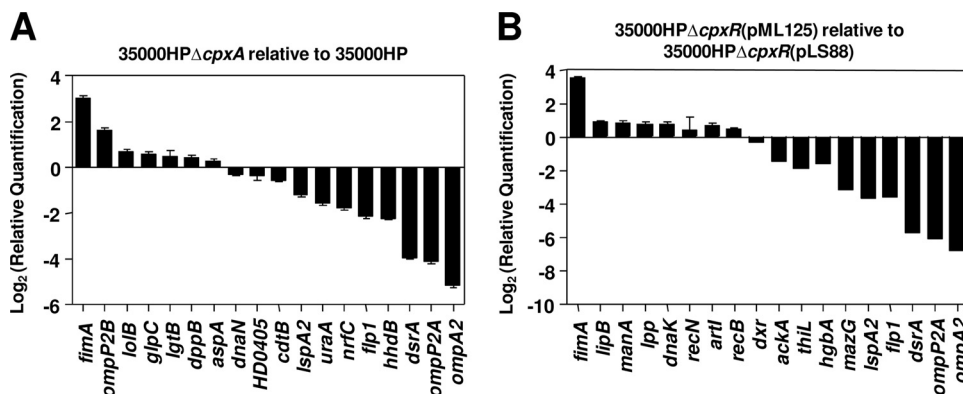


FIG. 3. Real-time RT-PCR analysis of selected gene expression in mutant and recombinant strains of *H. ducreyi*. (A) Genes in 35000HPΔ*cpxA* exhibiting upregulation, downregulation, or no change relative to those in 35000HP in a DNA microarray analysis were analyzed by real-time RT-PCR. (B) Genes in 35000HPΔ*cpxR*(pML125) exhibiting upregulation, downregulation, or no change relative to those in 35000HPΔ*cpxR*(pLS88) in a DNA microarray analysis were analyzed by real-time RT-PCR. These data are the means of results from three independent experiments.

material). A subset of these genes was used to validate the DNA microarray data by using real-time RT-PCR (Fig. 3B), and a correlation coefficient R^2 of 0.9302 was obtained.

A comparison of the two sets of DNA microarray data revealed 116 genes in common (Table 3 lists the 30 most up- and downregulated genes). The most highly upregulated genes in both groups were those of the putative fimbrial operon *fimABCD*, and the most downregulated were *ompA2*, *ompP2A*, members of the *flp* operon, and *dsrA*. The high level of similarity between the expression profile results from these two comparisons [the *cpxA* mutant versus the wild type and 35000HPΔ*cpxR*(pML125) versus 35000HPΔ*cpxR*(pLS88)] supports our hypothesis that CpxR is highly active in both situations, due to constitutive phosphorylation of CpxR in the former case and to overexpression of CpxR in the latter.

In addition to the large number of genes whose expression was affected by both constitutive phosphorylation and overexpression of CpxR (Table 3, columns giving median log₂ ratios of expression levels), there were several genes whose expression was affected only by the constitutive phosphorylation of CpxR in the *cpxA* mutant (Table 4) or only by the overexpression of CpxR (Table 5). The gene whose expression was the most highly upregulated under conditions in which CpxR is constitutively phosphorylated was *ompP2B* (Table 4). It should be noted that protein analysis also showed that the OmpP2B protein was overexpressed by this *H. ducreyi cpxA* deletion mutant (Fig. 1E).

***H. ducreyi* CpxR directly interacts with the promoter regions of genes encoding proven and putative *H. ducreyi* virulence factors.** DNA microarray data only allowed us to infer that these particular genes can be controlled, directly or indirectly, by CpxR. In order to establish a more direct relationship, a subset of genes common to both sets of DNA microarray data described above were selected and their promoter regions were analyzed for the presence or absence of a putative consensus CpxR binding sequence. Based on studies performed with *E. coli*, this sequence consists of two conserved pentamers (i.e., GTAAA) usually separated by 5 nt (14). A recent study indicated that strong CpxR regulation in *E. coli* can be correlated with the presence of this motif within 100 nt in the 5' direction from the transcriptional start site (47). Figure 4A shows the

putative consensus CpxR binding sequences in the promoter regions of *lspB*, *ompA2*, *ompP2A*, *flp1*, *dsrA*, and *fimA*. Four of these six consensus binding sequences were located within 100 nt of the translational start sites of their respective genes. The promoter region of *fimA* contained three GTAAA or similar sequences, while the other five promoter regions contained only two.

EMSA were performed using purified *H. ducreyi* recombinant CpxR (rCpxR)-His, with the promoter regions of *lspA1* and *gyrB* being used as negative controls and the *lspB* promoter region being used as a positive control (35). The former promoter regions did not detectably bind CpxR in the EMSA, whereas the latter promoter region exhibited a readily apparent shift in the EMSA when CpxR was present (Fig. 4B). The *lspB*, *ompA2*, *ompP2A*, *flp*, and *dsrA* promoter regions all exhibited readily apparent decreases in the rate of migration in the EMSA when CpxR was present (Fig. 4B), indicating that CpxR directly interacts with these promoter regions and that the genes are likely to be part of the CpxRA regulon in *H. ducreyi*. In contrast, numerous attempts to demonstrate binding of CpxR to the promoter region of *fimA* by EMSA were unsuccessful. In view of the latter result, although the expression of the *fimABCD* operon changes dramatically when CpxR is in an active state (Table 3 and Fig. 3), the effect exerted by CpxR on expression of the *fimABCD* operon is apparently not direct.

***H. ducreyi* CpxR interaction with the promoter regions of genes likely to be involved in the cell envelope stress response.** The CpxRA system in enteric organisms, including *E. coli*, has been extensively studied (for a review, see reference 47). Although the extent of the CpxRA regulon in *E. coli* remains to be fully defined (47), there are at least seven genes (*degP*, *dsbA*, *ppiA*, *ppiD*, *psd*, *secA*, and *spy*) that have been shown to be involved in the response to cell envelope stress and that are regulated by the CpxRA system in *E. coli*. The *H. ducreyi* 35000HP genome (GenBank accession no. NC_002940) was investigated to find homologs of the aforementioned enteric genes. HD0260 was found to have 53% identity (BLAST probability score, 6×10^{-127}) to *E. coli degP*, HD0638 had 43% identity (BLAST probability score, 1×10^{-47}) to *E. coli dsbA*, HD1737 had 30% identity (BLAST probability score, 8×10^{-76}) to *E. coli ppiD*, HD0649 had 54% identity (BLAST

TABLE 3. Genes whose expression was most affected by the constitutive phosphorylation and overexpression of CpxR as measured by DNA microarray analysis^a

ORF	Gene	Description of gene product	Value from comparison of 35000HP Δ cpxA to 35000HP		Value from comparison of 35000HP Δ cpxR(pML125) to 35000HP Δ cpxR(pLS88)	
			Median log ₂ ratio of expression levels ^b	SD ^c	Median log ₂ ratio of expression levels ^d	SD ^e
HD0281	<i>fimA</i>	Possible fimbrial major pilin protein	3.47	1.05	3.62	0.41
HD0282	<i>fimB</i>	Possible fimbrial structural subunit	3.01	0.32	3.16	0.41
HD0283	<i>fimC</i>	Probable fimbrial outer membrane usher protein	2.55	0.54	2.80	0.41
HD1109		Putative oxalate/formate antiporter	2.41	0.06	3.90	0.25
HD0284	<i>fimD</i>	Probable periplasmic fimbrial chaperone	2.29	0.08	2.27	0.31
HD1512		Acriflavine resistance protein	2.20	0.14	2.12	0.41
HD1163	<i>ribAB</i>	Riboflavin biosynthesis protein RibA	2.19	0.52	1.56	0.30
HD1165	<i>ribH</i>	6,7-Dimethyl-8-ribityllumazine synthase	1.82	0.14	1.28	0.25
HD1161	<i>ribD</i>	Riboflavin-specific deaminase	1.73	0.20	1.58	0.31
HD1513		Putative RND efflux membrane fusion protein	1.69	0.15	1.44	0.34
HD0313	<i>dppC</i>	Dipeptide transport system permease protein	1.66	0.16	1.20	0.23
HD1084		HesB family protein	1.66	0.12	1.59	0.29
HD1142	<i>sdaA</i>	L-Serine dehydratase	1.65	0.54	1.44	0.28
HD0765	<i>manA</i>	Mannose-6-phosphate isomerase	1.62	0.05	1.39	0.33
HD0812	<i>artI</i>	Arginine ABC transporter; periplasmic binding protein	1.61	0.21	1.60	0.31
HD1511	<i>glmU</i>	Bifunctional GlmU protein	1.50	0.08	0.96	0.23
HD0351	<i>prfC</i>	Peptide chain release factor 3	1.42	0.16	1.65	0.23
HD1007	<i>bioB</i>	Biotin synthetase	1.39	0.12	0.89	0.24
HD0564	<i>aspA</i>	Aspartate ammonia-lyase	1.37	0.05	2.11	0.33
HD2012	<i>lipA</i>	Lipoic acid synthetase	1.36	0.20	1.60	0.38
HD0766	<i>manZ</i>	Mannose-specific phosphotransferase system IID component	1.32	0.08	1.40	0.25
HD1146	<i>glpF</i>	Glycerol uptake facilitator protein	1.30	0.06	1.11	0.33
HD2017		Possible rare lipoprotein A	1.29	0.06	1.72	0.23
HD2016	<i>dacA</i>	Penicillin binding protein 5	1.27	0.08	2.02	0.28
HD0767	<i>manY</i>	Mannose-specific phosphotransferase system IIC component	1.23	0.08	1.41	0.26
HD0404	<i>lpxM</i>	Lipid A acyltransferase	1.20	0.13	1.09	0.22
HD1085	<i>hscB</i>	Chaperone protein HscB	1.17	0.10	0.94	0.24
HD1353	<i>ihfB</i>	Integration host factor, beta subunit	1.14	0.16	0.93	0.21
HD0449	<i>fis</i>	DNA binding protein	1.12	0.16	-1.02	0.19
HD0300	<i>grxA</i>	Glutaredoxin	1.11	0.14	0.79	0.24
HD1156	<i>lspA2</i>	Large supernatant protein 2	-1.19	0.12	-2.95	0.20
HD1304	<i>tadA</i>	Tight adherence protein A	-1.20	0.12	-1.80	0.19
HD1327	<i>hhdA</i>	Hemolysin	-1.25	0.06	-1.20	0.11
HD1480	<i>bioD1</i>	Probable dethiobiotin synthetase	-1.28	0.07	-1.66	0.30
HD1857	<i>sgaH</i>	Probable hexulose-6-phosphate synthase	-1.29	0.05	-1.21	0.21
HD1503	<i>guaB</i>	Inosine-5-monophosphate dehydrogenase	-1.31	0.05	-2.43	0.17
HD1305	<i>flpD</i>	<i>flp</i> operon protein D	-1.39	0.09	-1.88	0.17
HD1291	<i>gapA</i>	Glyceraldehyde 3-phosphate dehydrogenase	-1.41	0.13	-1.10	0.26
HD1986	<i>fumC</i>	Fumarate hydratase class II	-1.46	0.05	-1.68	0.22
HD1290	<i>msrB</i>	Peptide methionine sulfoxide reductase MsrB	-1.50	0.11	-1.57	0.31
HD1389	<i>moaE</i>	Molybdopterin converting factor subunit 2	-1.50	0.56	-2.03	0.15
HD1306	<i>rcpB</i>	Rough colony protein B	-1.53	0.13	-2.16	0.20
HD1280		Possible serine protease homolog	-1.63	0.11	-1.94	0.29
HD1390	<i>moaD</i>	Molybdopterin converting factor subunit 1	-1.66	0.62	-2.41	0.17
HD1307	<i>rcpA</i>	Rough colony protein A	-1.83	0.20	-2.26	0.16
HD0347	<i>nrfB</i>	Nitrate reductase; cytochrome <i>c</i> -type protein	-1.92	0.25	-1.94	0.32
HD1308	<i>flpC</i>	<i>flp</i> operon protein C	-2.06	0.09	-2.37	0.17
HD0344	<i>nrfA</i>	Nitrate reductase; cytochrome <i>c</i> ₅₅₂	-2.12	0.19	-2.11	0.33
HD1309	<i>flpB</i>	<i>flp</i> operon protein B	-2.43	0.45	-2.95	0.22
HD1278		Possible serine protease	-2.51	0.87	-2.92	0.26
HD0896	<i>cysK</i>	Cysteine synthase	-2.56	0.12	-3.92	0.23
HD1312	<i>flp1</i>	<i>flp</i> operon protein Flp1	-2.85	0.94	-2.51	0.28
HD0998	<i>uraA</i>	Uracil permease	-2.92	0.26	-2.27	0.42
HD1310	<i>flp3</i>	<i>flp</i> operon protein Flp3	-3.10	0.66	-3.37	0.39
HD1311	<i>flp2</i>	<i>flp</i> operon protein Flp2	-3.11	0.71	-3.66	0.35
HD1985		Possible DNA transformation protein	-3.16	0.61	-2.31	0.44
HD0233	<i>carB</i>	Carbamoyl-phosphate synthase, large subunit	-3.34	0.21	-2.72	0.50
HD0235	<i>carA</i>	Carbamoyl-phosphate synthase, small subunit	-3.63	0.26	-3.21	0.52
HD0769	<i>dsrA</i>	Serum resistance protein DrsA	-3.68	0.15	-4.38	0.32
HD0046	<i>ompA2</i>	Major outer membrane protein homolog	-4.61	0.10	-5.72	0.21
HD1433	<i>ompP2A</i>	Outer membrane protein P2 homolog	-4.68	0.35	-3.49	0.43

^a The table includes the top 30 most up- or downregulated ORFs and does not include ORFs described as encoding hypothetical or conserved hypothetical proteins.

^b Median log₂ ratio of expression levels from comparisons of 35000HP Δ cpxA to 35000HP in three independent experiments ($P < 0.05$).

^c SD for the 35000HP Δ cpxA data set versus the 35000HP data set.

^d Median log₂ ratio of expression levels from comparisons of 35000HP Δ cpxR(pML125) to 35000HP Δ cpxR(pLS88) in three independent experiments ($P < 0.05$).

^e SD for the 35000HP Δ cpxR(pML125) data set versus the 35000HP Δ cpxR(pLS88) data set.

TABLE 4. Genes whose expression was most affected by constitutively phosphorylated CpxR as measured by DNA microarray analysis^a

ORF	Gene	Description of gene product	Median log ₂ ratio of expression levels ^b	SD
HD1435	<i>ompP2B</i>	Outer membrane protein P2 homolog	2.30	0.15
HD1590	<i>deaD</i>	Cold shock DEAD box protein A	2.13	0.15
HD0386	<i>apbE</i>	Thiamine biosynthesis lipoprotein	1.88	0.05
HD1162	<i>ribE</i>	Riboflavin synthase, alpha chain	1.78	0.13
HD0312	<i>dppB</i>	Dipeptide transport system permease protein	1.74	0.17
HD1629	<i>lolB</i>	Outer membrane lipoprotein LolB	1.67	0.15
HD0792	<i>ccmF</i>	Cytochrome <i>c</i> -type biogenesis protein	1.57	0.17
HD1179	<i>ksgA</i>	Dimethyladenosine transferase	1.56	0.57
HD0634	<i>accB</i>	Biotin carboxyl carrier protein of acetyl coenzyme A (acetyl-CoA) carboxylase	1.48	0.17
HD0635	<i>accC</i>	Biotin carboxylase	1.46	0.11
HD0068	<i>ftpA</i>	Fine tangled pilus major subunit	-1.19	0.08
HD1077	<i>ogt</i>	Methylated-DNA-protein-cysteine S methyltransferase	-1.25	0.07
HD1326	<i>hhdB</i>	Hemolysin activation/secretion protein	-1.37	0.44
HD1525	<i>gam</i>	Putative mu phage host nuclease inhibitor protein	-1.38	0.10
HD0740	<i>hflX</i>	GTP binding protein HflX	-1.46	0.12
HD1391	<i>moaC</i>	Molybdenum cofactor biosynthesis protein C	-1.57	0.55
HD1392	<i>moaA</i>	Molybdenum cofactor biosynthesis protein A	-1.80	0.52
HD0232	<i>arcB1</i>	Ornithine carbamoyltransferase	-3.10	0.13

^a The table does not include ORFs described as encoding hypothetical or conserved hypothetical proteins.

^b Median log₂ ratio of expression levels from comparisons of 35000HPΔ*cpxA* to 35000HP in three independent experiments ($P < 0.05$).

probability score, 5×10^{-93}) to *E. coli psd*, and HD1788 had 67% identity (BLAST probability score, 0.0) to *E. coli secA*. *H. ducreyi* apparently lacks homologs of the *E. coli ppiA* and *spy* genes.

Analysis of the expression profiles of the aforementioned genes (exclusive of *ppiA* and *spy*) in the DNA microarray-based experiments in which CpxR was either overexpressed [i.e., in the comparison of 35000HPΔ*cpxR*(pML125) and 35000HPΔ*cpxR*(pLS88)] or constitutively phosphorylated (i.e., in the comparison of 35000HPΔ*cpxA* and 35000HP) revealed that the expression of these five genes did not change significantly in either of the data sets (data not shown). Subsequent real-time RT-PCR analysis of gene expression under conditions in which

CpxR is constitutively phosphorylated (i.e., in 35000HPΔ*cpxA* versus 35000HP) was used to validate the DNA microarray results. In comparison to the changes in expression of *lspB*, *dsrA*, and *ftp1* in the *cpxA* mutant, the changes in expression of *degP*, *dsbA*, *ppiD*, *psd*, and *secA* were very modest and did not meet the 2-fold minimum difference cutoff (Fig. 4C). Analysis of the promoter regions of *degP* and *ppiD* revealed the presence of poor consensus CpxR binding motifs, because they lack highly conserved residues and the linker regions between the two GTAAA repeats are either too long or too short (data not shown). The promoter regions of *dsbA*, *psd*, and *secA* did not have any recognizable CpxR binding motifs.

The interaction of the promoter regions of *degP*, *dsbA*, *ppiD*,

TABLE 5. Genes whose expression was most affected by overexpression of CpxR as measured by DNA microarray analysis^a

ORF	Gene	Description of gene product	Median log ₂ ratio of expression levels ^b	SD
HD0262	<i>argR</i>	Arginine repressor	2.46	0.32
HD0025	<i>dcuB1</i>	Anaerobic C ₄ -dicarboxylate transporter	2.24	0.30
HD0811	<i>artP</i>	Arginine ABC transporter; ATP binding protein	2.16	0.67
HD1814		Probable transport protein	1.89	0.17
HD0285		Possible minor fimbrial subunit	1.86	0.17
HD0266	<i>lpp</i>	15-kDa outer membrane lipoprotein	1.55	0.24
HD0565	<i>clpB</i>	ATP-dependant Clp protease chain B	1.51	0.18
HD0337	<i>rhuB</i>	Probable pseudouridylylase synthase	1.49	0.22
HD0988	<i>prtC</i>	Oligopeptidase A	1.39	0.19
HD1076	<i>recB</i>	Exodeoxyribonuclease V, beta subunit	1.31	0.17
HD1301	<i>tadD</i>	Tight adherence protein D	-1.75	0.12
HD1300	<i>tadE</i>	Tight adherence protein E	-1.78	0.22
HD1303	<i>tadB</i>	Tight adherence protein B	-1.80	0.21
HD1504	<i>guaA</i>	GMP synthase (glutamine hydrolyzing)	-1.92	0.25
HD0577	<i>selD</i>	Selenide; water dikinase	-1.99	0.16
HD1407	<i>efp</i>	Elongation factor P	-2.03	0.15
HD1150	<i>glpT</i>	Glycerol 3-phosphate transporter	-2.18	0.20
HD1394	<i>torZ</i>	Trimethylamine- <i>N</i> -oxide reductase 2	-2.20	0.16
HD0202	<i>slyD</i>	FK506 binding protein (FKBP)-type peptidyl-prolyl <i>cis-trans</i> isomerase	-2.31	0.14
HD1900	<i>fkpA</i>	FKBP-type peptidyl-prolyl <i>cis-trans</i> isomerase FkpA	-2.61	0.15

^a The table does not include ORFs described as encoding hypothetical or conserved hypothetical proteins.

^b Median log₂ ratio of expression levels from comparisons of 35000HPΔ*cpxR*(pML125) to 35000HPΔ*cpxR*(pLS88) in three independent experiments ($P < 0.05$).

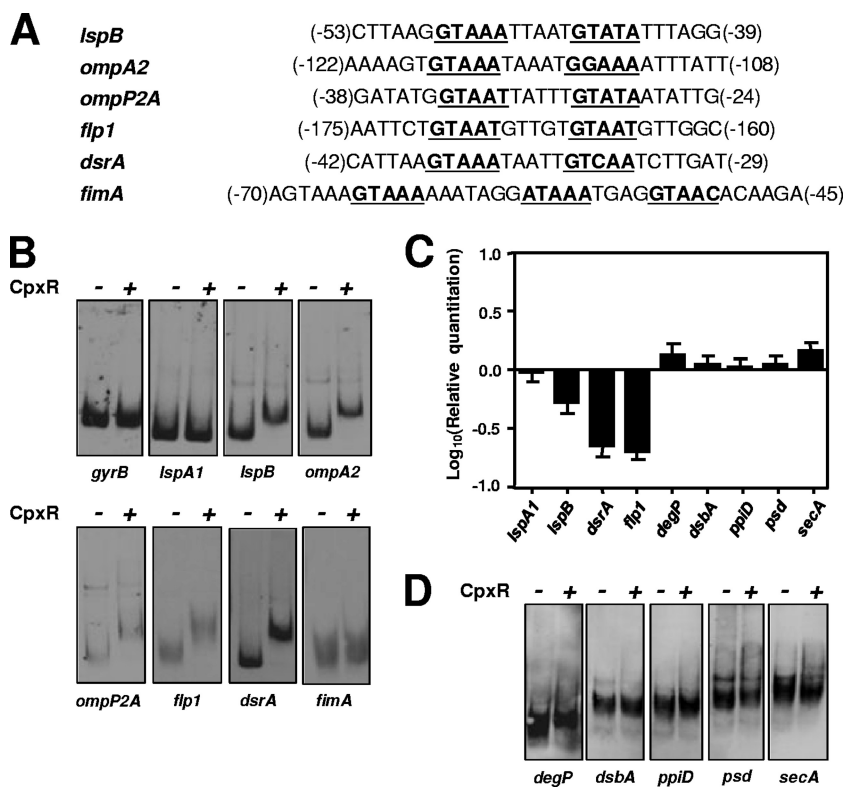


FIG. 4. CpxR interacts with the promoter regions of genes encoding known and putative *H. ducreyi* virulence factors but not with the promoter regions of homologs of *E. coli* cell envelope stress response genes. (A) Nucleotide sequences of the promoter regions for the *H. ducreyi* *lspB*, *ompA2*, *ompP2A*, *flp1*, *dsrA*, and *fimA* genes. Putative consensus CpxR binding sequences are shown in bold and underlined. Numbers in parentheses indicate the position of the first base of the first repeat (left) and the last base of the last repeat (right) relative to the predicted translation initiation codon. (B and D) Results from EMSAs using 50 pmol of purified rCpxR-His together with the DIG-labeled promoter region DNA. Nucleotide sequences of the primers used to PCR amplify the promoter regions are listed in Table 2. (B) Results from EMSAs using the promoter regions of *gyrB*, *lspA1*, *lspB*, *ompA2*, *ompP2A*, *flp1*, *dsrA*, and *fimA*. The *lspB*, *lspA1*, and *gyrB* promoter regions were used as positive (i.e., *lspB*) or negative (i.e., *lspA1* and *gyrB*) controls in every assay. The minus sign indicates that no CpxR protein was present, whereas the plus sign indicates the presence of CpxR protein. (C) Real-time RT-PCR analysis of gene expression from *lspA1*, *lspB*, *dsrA*, *flp1*, *degP*, *dsbA*, *ppiD*, *psd*, and *secA* in *H. ducreyi* Δ *cpxA* relative to wild-type *H. ducreyi*. (D) Results from EMSAs using the promoter regions of the following *H. ducreyi* genes: *degP*, *dsbA*, *ppiD*, *psd*, and *secA*.

psd, and *secA* with *H. ducreyi* rCpxR-His was next tested in the EMSA. Only the *degP* promoter region showed an apparent interaction with the *H. ducreyi* protein, whereas the other four promoter regions did not (Fig. 4D). Repeated attempts to demonstrate an interaction of CpxR with the promoter regions of the rest of these well-known cell envelope stress response genes were unsuccessful. These results suggest either that *H. ducreyi* has a different and presently unidentified set of genes to deal with envelope stress response or that the CpxRA regulon is functionally different from the CpxRA two-component regulatory systems in other bacteria and controls primarily gene products not involved in the cell envelope stress response.

DISCUSSION

Bacteria have multiple different mechanisms for sensing their environment and responding with changes in gene expression. Given the importance of the integrity of the cell envelope to bacterial survival, it is not surprising that five different systems which respond to stresses in the envelope have been identified (for a review, see reference 38). Among these, the CpxRA TCS is perhaps the best studied. At least two

important functions have been ascribed to the Cpx system in enteric bacteria; these include regulating factors that deal with misfolded proteins in the periplasmic space and affecting expression of surface components that mediate attachment to some surfaces. It has also been suggested that the Cpx signaling pathway may play a role in signaling *E. coli* cells present in biofilms to stop making biofilm-related adhesins (17). The signals that activate the Cpx system in *E. coli* are diverse and include alkaline pH, overexpression of certain proteins, interaction with abiotic surfaces, and others (for a review, see reference 38). The Cpx regulon in *E. coli* has been described as involving 34 operons and at least 50 genes, some of which are still uncharacterized (47).

In contrast to the numerous different TCSs present in *E. coli*, the CpxRA system is the only complete TCS in *H. ducreyi* (Robert S. Munson, Jr., personal communication). A previous study showed that growth of *H. ducreyi* in the presence of fetal calf serum results in downregulation of the CpxRA system and that a *cpxR* deletion mutant expresses significantly increased levels of both LspB and LspA2 (35). Additional experiments indicated that a purified recombinant *H. ducreyi* CpxR protein bound to the promoter region of the *lspB-lspA2* operon and

that overexpression of CpxR causes decreased expression of LspB and LspA2 (35). These data indicated that CpxR was likely to be a negative regulator of this small operon. In the present study, we constructed additional *H. ducreyi* mutants and complemented mutant strains and used EMSAs to begin to identify the components of the CpxRA regulon in this sexually transmitted pathogen.

Analysis of the predicted structural features of both the *H. ducreyi* CpxA and CpxR proteins revealed that both of these macromolecules closely resemble their respective homologs in other bacteria. The *H. ducreyi* CpxA and CpxR proteins had 78% and 90% identity to the respective Cpx proteins expressed by *A. pleuropneumoniae*, and the *H. ducreyi* CpxR protein was impressively conserved across different genera. The *H. ducreyi* CpxA protein likely assumes a topology that is frequently observed for the membrane-bound HKs of TCSs (58). There are two membrane-spanning α -helices; a periplasmic domain is located between these helices, and a large cytoplasmic domain dominates the C-terminal portion of the second helix. HKs having this topology are complex, dynamic dimers that likely undergo significant conformational changes during the course of a signaling cycle (11, 26). In other HKs, these changes are induced by the binding of ligands to the periplasmic sensor domain (58). In the case of *H. ducreyi* CpxA, however, the signal is unknown.

The *H. ducreyi* CpxR protein very likely comprises two domains: a receiver domain that accepts a phosphoryl moiety from the activated *H. ducreyi* CpxA and a DNA binding domain whose activity (i.e., relative ability to bind DNA) is modulated by the phosphorylation status of the receiver domain. The *H. ducreyi* CpxR protein appears to be different from other response regulators in that the connector region between the aforementioned two domains is longer than those described previously. The significance of this difference, if any, remains to be determined.

In *E. coli* and other enteric bacteria, the cytoplasmic membrane-bound CpxA histidine kinase is the sensor for the CpxRA TCS, functions as an autokinase, and has both phosphotransferase and phosphatase activities with respect to its cognate response regulator CpxR. In *E. coli*, deletion of *cpxA* removes the CpxA-based phosphatase activity but does not prevent CpxR from being phosphorylated, likely by the small phosphodonor acetyl phosphate (13, 50, 68). In the absence of CpxA phosphatase activity, phosphorylated CpxR should accumulate and exert a maximal effect, in the positive or negative sense, on genes regulated by CpxR (15, 32). We used this as a working assumption when we constructed a *cpxA* deletion mutation in *H. ducreyi* 35000HP. The findings depicted in Fig. 2 and 3 support our working assumption that the *cpxA* mutation in *H. ducreyi* resulted in what appears to be a constitutively active (i.e., phosphorylated) CpxR protein. We reported previously that CpxR acts as a repressor of expression of the *lspB-lspA2* operon in *H. ducreyi* (35). Inactivation of the *cpxA* gene (Fig. 2C, lane 2) resulted in an almost complete elimination of LspB and LspA2 protein expression detectable by Western blot analysis. Real-time RT-PCR analysis (Fig. 2D) confirmed that this inhibitory effect in the *cpxA* mutant was exerted at the level of transcription. It should be noted that CpxR levels in the *cpxA* mutant (Fig. 2C, lane 2) appeared to be equivalent to those in the wild-type strain (Fig. 2C, lane 1),

so the increased repression of *lspB* and *lspA2* is consistent with CpxR's being highly phosphorylated in this mutant. Interestingly, overexpression of CpxR [in the recombinant strain 35000HP Δ *cpxR*(pML125)] (Fig. 2A, lane 3) resulted in a similarly enhanced level of repression of LspB and LspA2 expression. Taken together, these results indicated that the increased CpxR activity in these two strains might be useful in studies using DNA microarray analysis to identify the components of the CpxRA regulon in *H. ducreyi*.

DNA microarray analyses comparing the *cpxA* deletion mutant with the wild-type strain and the recombinant *H. ducreyi* strain overexpressing CpxR [35000HP Δ *cpxR*(pML125)] with a recombinant strain that cannot express CpxR [35000HP Δ *cpxR*(pLS88)] showed that numerous genes were up- or downregulated by both constitutively phosphorylated CpxR and extremely abundant CpxR (Table 3, first and second expression ratio columns, respectively). In addition, there were some genes whose expression was affected under the former condition but not under the latter condition and vice versa. The most upregulated gene when CpxR was constitutively phosphorylated (i.e., in 35000HP Δ *cpxA*) was *ompP2B*. Analysis of whole-cell lysates comparing the wild type to 35000HP Δ *cpxA* (Fig. 1E) also showed the corresponding protein to be highly expressed. The two sets of DNA microarray results are derived from significant alterations of the CpxRA regulatory system and likely include some genes whose expression is not directly affected by CpxR. Inspection of the promoter regions of the 10 most up- or downregulated genes (not including multiple genes apparently linked in an operon) affected by CpxR (Table 3) revealed that only 20% of these genes contained a putative consensus CpxR binding motif (data not shown). This finding makes it likely that the other 80% of these genes are regulated only indirectly by CpxR.

Three interesting findings are contained within these data sets and the accompanying EMSA results. The first is that the putative *fimABCD* operon was highly upregulated when CpxR was either constitutively phosphorylated or very abundant in *H. ducreyi* (Table 3). Whereas the genes encoding the fine tangled pili (9) and other surface proteins of *H. ducreyi* have been described previously, there have not been any previous descriptions of this four-gene cluster. The predicted FimA major structural protein has 30 to 40% identity (at the amino acid sequence level) with fimbrial proteins of other Gram-negative species, including *Providencia rettgeri* (GenBank accession number EF54293.1), *Yersinia pseudotuberculosis* (GenBank accession number ACC90454.1), and *Serratia marcescens* (GenBank accession number AAA26576.1). It must also be noted that the *fimA* promoter region did not exhibit a change in its migration characteristics when incubated with CpxR in the EMSA, a result which indicates that CpxR likely does not directly regulate expression of *fimA*. This finding raises the possibility that CpxR may be part of a regulatory cascade controlling the expression of the *fimABCD* operon or that it may be part of a protein complex needed for binding. Similarly, whether the predicted fimbrial proteins are expressed by *H. ducreyi* either *in vivo* or *in vitro* remains to be determined, and it would be of interest to determine whether inactivation of this operon would reduce the virulence potential of *H. ducreyi*. It should be noted here that phosphorylated CpxR has been shown previously to be involved in the control

of the expression of other surface appendages, including the curli fimbriae (48) and the uropathogenic *E. coli* P pilus (24).

The second result of significance is the observation that CpxR affects the expression of at least three proven virulence factors expressed by *H. ducreyi*. In addition to the LspA2 protein, which can inhibit the phagocytic activity of macrophage-like cell lines *in vitro* (60), the *flp* operon genes and the *dsrA* gene both exhibited significant downregulation of expression when CpxR was either constitutively phosphorylated or very abundant (Table 3; Fig. 2C and D and 3). An intact *flp* gene cluster has been shown to be required for full virulence of *H. ducreyi* in the human challenge model for experimental chancre (55), and inactivation of the *dsrA* gene markedly attenuates the virulence of this pathogen in the same model system (8). In addition to these proven virulence factors, the expression of the genes encoding the presumably surface-exposed outer membrane proteins OmpA2 (33) and OmpP2A (46) was negatively affected by CpxR (Fig. 3), as was expression of the gene encoding the HhdA hemolysin (43). In contrast, the expression of the outer membrane protein OmpP2B (46) was upregulated in the absence of CpxA (Fig. 3A). A detailed analysis of the *ompP2B* promoter region showed the lack of a defined consensus CpxR binding motif, and EMSA studies showed no interaction with purified rCpxR (data not shown). Therefore, it is likely that the effect of CpxR on the regulation of expression of OmpP2B is indirect. The opposing responses of the *ompP2A* and *ompP2B* genes to phosphorylated CpxR (Fig. 3A) is reminiscent of those observed in an *E. coli* *cpxA* mutant in which there was increased transcription of *ompC* and decreased transcription of *ompF* (5).

The third interesting result was the apparent lack of effect of the *H. ducreyi* CpxR protein on expression of *H. ducreyi* homologs of genes that have been established as being regulated by CpxR in *E. coli* (47). The promoter regions of a number of these genes, including *degP*, *dsbA*, *ppiD*, *psd*, and *secA*, were tested for their abilities to bind *H. ducreyi* CpxR in an EMSA. Only *degP* showed apparent binding of CpxR in the EMSA (Fig. 4D). These EMSA-derived data were reinforced by real-time RT-PCR analyses in which these five genes showed relatively little change in expression in a *cpxA* mutant (Fig. 4C). Taken together with the observed effect of CpxR on expression of several *H. ducreyi* virulence factors (discussed above), these data raise the possibility that, in *H. ducreyi*, perhaps another gene product is involved in controlling the cell envelope stress response. However, the apparent absence of other complete TCSs in this pathogen (Robert S. Munson, Jr., personal communication) makes this scenario somewhat unlikely.

The full extent of the CpxRA regulon in *H. ducreyi* remains to be elucidated. However, based on the results presented above, its composition may be quite different from that currently defined in *E. coli*. *In silico* analysis of the *H. ducreyi* genome detected between 100 and 300 loci with homology to the *E. coli* consensus CpxR binding motif, dependent on the stringency of the search parameters (data not shown). How many of these are actually controlled by CpxR is not known, nor is there any information currently available about what environmental signals might activate the CpxA sensor kinase, with the exception that transcription of both *cpxR* and *cpxA* appears to be downregulated in *H. ducreyi* in the presence of fetal calf serum (35). It should also be noted that all of the

experiments in the present study utilized *H. ducreyi* 35000HP, a prototypic class I strain of the pathogen (64). Two clonal *H. ducreyi* populations (i.e., class I and class II) have been described as differing in the expression of certain surface-exposed proteins and lipooligosaccharide (45). Whether gene expression in class II strains of *H. ducreyi* may differ with regard to regulation by CpxR was not investigated in this study and would need to be addressed in the future.

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REFERENCES

- Al-Tawfiq, J. A., K. R. Fortney, B. P. Katz, A. F. Hood, C. Elkins, and S. M. Spinola. 2000. An isogenic hemoglobin receptor-deficient mutant of *Haemophilus ducreyi* is attenuated in the human model of experimental infection. *J. Infect. Dis.* **181**:1049–1054.
- Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**:403–410.
- Aravind, L., and C. P. Ponting. 1999. The cytoplasmic helical linker domain of receptor histidine kinase and methyl-accepting proteins is common to many prokaryotic signalling proteins. *FEMS Microbiol. Lett.* **176**:111–116.
- Banks, K. E., K. R. Fortney, B. Baker, S. D. Billings, B. P. Katz, R. S. J. Munson, and S. M. Spinola. 2008. The enterobacterial common antigen-like gene cluster of *Haemophilus ducreyi* contributes to virulence in humans. *J. Infect. Dis.* **197**:1531–1536.
- Batchelor, E., D. Walthers, L. J. Kenney, and M. Goulian. 2005. The *Escherichia coli* CpxA-CpxR envelope stress response system regulates expression of the porins OmpF and OmpC. *J. Bacteriol.* **187**:5723–5731.
- Bauer, M. E., C. A. Townsend, R. S. Doster, K. R. Fortney, B. W. Zwickl, B. P. Katz, S. M. Spinola, and D. M. Janowicz. 2009. A fibrinogen-binding lipoprotein contributes to the virulence of *Haemophilus ducreyi* in humans. *J. Infect. Dis.* **199**:684–692.
- Bong, C. T., M. E. Bauer, and S. M. Spinola. 2002. *Haemophilus ducreyi*: clinical features, epidemiology, and prospects for disease control. *Microbes Infect.* **4**:1141–1148.
- Bong, C. T., R. E. Throm, K. R. Fortney, B. P. Katz, A. F. Hood, C. Elkins, and S. M. Spinola. 2001. DsrA-deficient mutant of *Haemophilus ducreyi* is impaired in its ability to infect human volunteers. *Infect. Immun.* **69**:1488–1491.
- Brentjens, R. J., M. Ketterer, M. A. Apicella, and S. M. Spinola. 1996. Fine tanded pili expressed by *Haemophilus ducreyi* are a novel class of pili. *J. Bacteriol.* **178**:808–816.
- Carlsson, K. E., J. Liu, P. J. Edqvist, and M. S. Francis. 2007. Influence of the Cpx extracytoplasmic-stress-responsive pathway on *Yersinia* sp.-eukaryotic cell contact. *Infect. Immun.* **75**:4386–4399.
- Casino, P., V. Rubio, and A. Marina. 2009. Structural insight into partner specificity and phosphoryl transfer in two-component signal transduction. *Cell* **139**:325–336.
- Dahl, J. L., B. Y. Wei, and R. J. Kadner. 1997. Protein phosphorylation affects binding of the *Escherichia coli* transcription activator UhpA to the *uhpT* promoter. *J. Biol. Chem.* **272**:1910–1919.
- Danese, P. N., W. B. Snyder, C. L. Cosma, L. J. B. Davis, and T. J. Silhavy. 1995. The Cpx two-component signal transduction pathway of *Escherichia coli* regulates transcription of the gene specifying the stress-inducible periplasmic protease, DegP. *Genes Dev.* **9**:387–398.
- De Wulf, P., A. M. McGuire, X. Liu, and E. C. Lin. 2002. Genome-wide profiling of promoter recognition by the two-component response regulator CpxR-P in *Escherichia coli*. *J. Biol. Chem.* **277**:26652–26661.
- De Wulf, P., and E. C. C. Lin. 2000. Cpx two-component signal transduction in *Escherichia coli*: excessive CpxR-P levels underlie CpxA* phenotypes. *J. Bacteriol.* **182**:1423–1426.
- Dixon, L. G., W. L. Albritton, and P. J. Willson. 1994. An analysis of the complete nucleotide sequence of the *Haemophilus ducreyi* broad-host-range plasmid pLS88. *Plasmid* **32**:228–232.
- Dorel, C., P. Lejeune, and A. Rodrigue. 2006. The Cpx system of *Escherichia coli*, a strategic signaling pathway for confronting adverse conditions and for settling biofilm communities? *Res. Microbiol.* **157**:306–314.
- Elkins, C. 1995. Identification and purification of a conserved heme-regulated hemoglobin-binding outer membrane protein from *Haemophilus ducreyi*. *Infect. Immun.* **63**:1241–1245.
- Elkins, C., K. J. Morrow, Jr., and B. Olsen. 2000. Serum resistance in *Haemophilus ducreyi* requires outer membrane protein DsrA. *Infect. Immun.* **68**:1608–1619.

20. Fleming, D. T., and J. N. Wasserheit. 1999. From epidemiological synergy to public health policy and practice: the contribution of other sexually transmitted diseases to sexual transmission of HIV infection. *Sex. Transm. Infect.* **75**:3–17.
21. Fortney, K. R., R. S. Young, M. E. Bauer, B. P. Katz, A. F. Hood, R. S. Munson, Jr., and S. M. Spinola. 2000. Expression of peptidoglycan-associated lipoprotein is required for virulence in the human model of *Haemophilus ducreyi* infection. *Infect. Immun.* **68**:6441–6448.
22. Fulcher, R. A., L. E. Cole, D. M. Janowicz, K. L. Toffer, K. R. Fortney, B. P. Katz, P. E. Orndorff, S. M. Spinola, and T. H. Kawula. 2006. Expression of *Haemophilus ducreyi* collagen binding outer membrane protein NcaA is required for virulence in swine and human challenge models of chancroid. *Infect. Immun.* **74**:2651–2658.
23. Haydock, A. K., D. H. Martin, S. A. Morse, C. Cammarata, K. J. Mertz, and P. A. Totten. 1999. Molecular characterization of *Haemophilus ducreyi* strains from Jackson, Mississippi and New Orleans, Louisiana. *J. Infect. Dis.* **179**:1423–1432.
24. Hernday, A. D., B. A. Braaten, G. Broitman-Maduro, P. Engelberts, and D. A. Low. 2004. Regulation of the Pap epigenetic switch by CpxAR: Phosphorylated CpxR inhibits transition to the phase ON state by competition with Lrp. *Mol. Cell* **16**:537–547.
25. Horton, R. M., H. D. Hunt, S. N. Ho, J. K. Pullen, and L. R. Pease. 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* **77**:61–68.
26. Hulko, M., F. Berndt, M. Gruber, J. U. Linder, V. Truffault, A. Schultz, J. Martin, J. E. Schultz, A. N. Lupas, and M. Coles. 2006. The HAMP domain structure implies helix rotation in transmembrane signaling. *Cell* **126**:929–940.
27. Jamet, A., C. Rousseau, J. B. Monfort, E. Frapy, X. Nassif, and P. Martin. 2009. A two-component system is required for colonization of host cells by meningococcus. *Microbiology* **155**:2288–2295.
28. Janowicz, D., I. Leduc, K. R. Fortney, B. P. Katz, C. Elkins, and S. M. Spinola. 2006. A DltA mutant of *Haemophilus ducreyi* is partially attenuated in its ability to cause pustules in human volunteers. *Infect. Immun.* **74**:1394–1397.
29. Janowicz, D. M., K. R. Fortney, B. P. Katz, J. L. Latimer, K. Deng, E. J. Hansen, and S. M. Spinola. 2004. Expression of the LspA1 and LspA2 proteins by *Haemophilus ducreyi* is required for virulence in human volunteers. *Infect. Immun.* **72**:4528–4533.
30. Janowicz, D. M., W. Li, and M. E. Bauer. 2010. Host-pathogen interplay of *Haemophilus ducreyi*. *Curr. Opin. Infect. Dis.* **23**:64–69.
31. Janowicz, D. M., S. Ofner, B. P. Katz, and S. M. Spinola. 2009. Experimental infection of human volunteers with *Haemophilus ducreyi*: fifteen years of clinical data and experience. *J. Infect. Dis.* **199**:1671–1679.
32. Klein, A. H., A. Shulla, S. A. Reimann, D. H. Keating, and A. J. Wolfe. 2007. The intracellular concentration of acetyl phosphate in *Escherichia coli* is sufficient for direct phosphorylation of two-component response regulators. *J. Bacteriol.* **189**:5574–5581.
33. Klesney-Tait, J., T. J. Hiltke, I. Maciver, S. M. Spinola, J. D. Radolf, and E. J. Hansen. 1997. The major outer membrane protein of *Haemophilus ducreyi* consists of two OmpA homologs. *J. Bacteriol.* **179**:1764–1773.
34. Labandeira-Rey, M., D. M. Janowicz, R. J. Blick, K. R. Fortney, B. Zwickl, B. P. Katz, S. M. Spinola, and E. J. Hansen. 2009. Inactivation of the *Haemophilus ducreyi luxS* gene affects the virulence of this pathogen in human subjects. *J. Infect. Dis.* **200**:409–416.
35. Labandeira-Rey, M., J. R. Mock, and E. J. Hansen. 2009. Regulation of expression of the *Haemophilus ducreyi* LspB and LspA2 proteins by CpxR. *Infect. Immun.* **77**:3402–3411.
36. Lewis, D. A. 2003. Chancroid: clinical manifestations, diagnosis, and management. *Sex. Transm. Infect.* **79**:68–71.
37. Lukomski, S., R. A. Hull, and S. I. Hull. 1996. Identification of the O antigen polymerase (*rfc*) gene in *Escherichia coli* O4 by insertional mutagenesis using a nonpolar chloramphenicol resistance cassette. *J. Bacteriol.* **178**:240–247.
38. MacRitchie, D. M., D. R. Buelow, N. L. Price, and T. L. Raivio. 2008. Two-component signaling and gram negative envelope stress response systems. *Adv. Exp. Med. Biol.* **631**:80–110.
39. Marina, A., C. D. Waldburger, and W. A. Hendrickson. 2005. Structure of the entire cytoplasmic portion of a sensor histidine-kinase protein. *EMBO J.* **24**:4247–4259.
40. Mertz, K. J., D. L. Trees, W. C. Levine, J. S. Lewis, B. Litchfield, K. S. Pettus, S. A. Morse, M. E. St. Louis, J. B. Weiss, J. Schwelke, J. Dicks, R. Kee, J. Reynolds, D. Hutcheson, D. Green, I. Dyer, G. A. Richwald, J. Novotny, M. Goldberg, J. A. O'Donnell, and R. Knaup for the Genital Ulcer Disease Surveillance Group. 1998. Etiology of genital ulcers and prevalence of human immunodeficiency virus coinfection in 10 US cities. *J. Infect. Dis.* **178**:1795–1798.
41. Nika, J. R., J. L. Latimer, C. K. Ward, R. J. Blick, N. J. Wagner, L. D. Cope, G. G. Mahairas, R. S. Munson, Jr., and E. J. Hansen. 2002. *Haemophilus ducreyi* requires the *flp* gene cluster for microcolony formation in vitro. *Infect. Immun.* **70**:2965–2975.
42. Overton, T. W., R. Whitehead, Y. Li, L. A. Snyder, N. J. Saunders, H. Smith, and J. A. Cole. 2006. Coordinated regulation of the *Neisseria gonorrhoeae*-truncated denitrification pathway by the nitric oxide-sensitive repressor, NsrR, and nitrite-insensitive NarQ-NarP. *J. Biol. Chem.* **281**:33115–33126.
43. Palmer, K. L., and R. S. Munson, Jr. 1995. Cloning and characterization of the genes encoding the haemolysin of *Haemophilus ducreyi*. *Mol. Microbiol.* **18**:821–830.
44. Pei, J., B. H. Kim, and N. V. Grishin. 2008. PROMALS3D: a tool for multiple protein sequence and structure alignments. *Nucleic Acids Res.* **36**:2295–2300.
45. Post, D. M., and B. W. Gibson. 2007. Proposed second class of *Haemophilus ducreyi* strains show altered protein and lipooligosaccharide profiles. *Proteomics* **7**:3131–3142.
46. Prather, D. T., M. Bains, R. E. Hancock, M. J. Filiatrault, and A. A. Campagnari. 2004. Differential expression of porins OmpP2A and OmpP2B of *Haemophilus ducreyi*. *Infect. Immun.* **72**:6271–6278.
47. Price, N. L., and T. L. Raivio. 2009. Characterization of the Cpx regulon in *Escherichia coli* strain MC4100. *J. Bacteriol.* **191**:1798–1815.
48. Prigent-Combaret, C., E. Brombacher, O. Vidal, A. Ambert, P. Lejeune, P. Landini, and C. Dorel. 2001. Complex regulatory network controls initial adhesion and biofilm formation in *Escherichia coli* via regulation of the *csqD* gene. *J. Bacteriol.* **183**:7213–7223.
49. Raivio, T. L. 2005. Envelope stress responses and Gram-negative bacterial pathogenesis. *Mol. Microbiol.* **56**:1119–1128.
50. Raivio, T. L., and T. J. Silhavy. 1997. Transduction of envelope stress in *Escherichia coli* by the Cpx two-component system. *J. Bacteriol.* **179**:7724–7733.
51. Royce, R. A., A. Sena, W. Cates, Jr., and M. S. Cohen. 1997. Sexual transmission of HIV. *N. Engl. J. Med.* **336**:1072–1078.
52. Schulte, J. M., F. A. Martich, and G. P. Schmid. 1992. Chancroid in the United States, 1981–1990: evidence for underreporting of cases. *MMWR Surveill. Summ.* **41**:57–61.
53. Smith, A. M., C. A. Guzman, and M. J. Walker. 2001. The virulence factors of *Bordetella pertussis*: a matter of control. *FEMS Microbiol. Rev.* **25**:309–333.
54. Spinola, S. M., M. E. Bauer, and R. S. Munson, Jr. 2002. Immunopathogenesis of *Haemophilus ducreyi* infection (chancroid). *Infect. Immun.* **70**:1667–1676.
55. Spinola, S. M., K. R. Fortney, B. P. Katz, J. L. Latimer, J. R. Mock, M. Vakevainen, and E. J. Hansen. 2003. *Haemophilus ducreyi* requires an intact *flp* gene cluster for virulence in humans. *Infect. Immun.* **71**:7178–7182.
56. Spinola, S. M., T. J. Hiltke, K. R. Fortney, and K. L. Shanks. 1996. The conserved 18,000-molecular-weight outer membrane protein of *Haemophilus ducreyi* has homology to PAL. *Infect. Immun.* **64**:1950–1955.
57. Spinola, S. M., L. M. Wild, M. A. Apicella, A. A. Gaspari, and A. A. Campagnari. 1994. Experimental human infection with *Haemophilus ducreyi*. *J. Infect. Dis.* **169**:1146–1150.
58. Stock, A. M., V. L. Robinson, and P. N. Goudreau. 2000. Two-component signal transduction. *Annu. Rev. Biochem.* **69**:183–215.
59. Trees, D. L., and S. A. Morse. 1995. Chancroid and *Haemophilus ducreyi*: an update. *Clin. Microbiol. Rev.* **8**:357–375.
60. Vakevainen, M., S. Greenberg, and E. J. Hansen. 2003. Inhibition of phagocytosis by *Haemophilus ducreyi* requires expression of the LspA1 and LspA2 proteins. *Infect. Immun.* **71**:5994–6003.
61. Ward, C. K., J. L. Latimer, J. R. Nika, M. Vakevainen, J. R. Mock, K. Deng, R. J. Blick, and E. H. Hansen. 2003. Mutations in the *lspA1* and *lspA2* genes of *Haemophilus ducreyi* affect the virulence of this pathogen in an animal model system. *Infect. Immun.* **71**:2478–2486.
62. Ward, C. K., S. R. Lumbley, J. L. Latimer, L. D. Cope, and E. J. Hansen. 1998. *Haemophilus ducreyi* secretes a filamentous hemagglutinin-like protein. *J. Bacteriol.* **180**:6013–6022.
63. Ward, C. K., J. R. Mock, and E. J. Hansen. 2004. The LspB Protein Is Involved in the Secretion of the LspA1 and LspA2 Proteins by *Haemophilus ducreyi*. *Infect. Immun.* **72**:1874–1884.
64. White, C. D., I. Leduc, B. Olsen, C. Jeter, C. Harris, and C. Elkins. 2005. *Haemophilus ducreyi* Outer membrane determinants, including DsrA, define two clonal populations. *Infect. Immun.* **73**:2387–2399.
65. Whitworth, D. E., and P. J. Cock. 2009. Evolution of prokaryotic two-component systems: insights from comparative genomics. *Amino Acids* **37**:459–466.
66. Whitton, P. J., W. L. Albritton, L. Slaney, and J. K. Setlow. 1989. Characterization of a multiple antibiotic resistance plasmid from *Haemophilus ducreyi*. *Antimicrob. Agents Chemother.* **33**:1627–1630.
67. Wolfe, A. J. 2010. Physiologically relevant small phosphodonors link metabolism to signal transduction. *Curr. Opin. Microbiol.* **13**:204–209.
68. Wolfe, A. J., N. Parikh, B. P. Lima, and B. Zemitaitis. 2008. Signal integration by the two-component signal transduction response regulator CpxR. *J. Bacteriol.* **190**:2314–2322.
69. Yamada, S., and Y. Shiro. 2008. Structural basis of the signal transduction in the two-component system. *Adv. Exp. Med. Biol.* **631**:22–39.