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Identification of the *znuA*-Encoded Periplasmic Zinc Transport Protein of *Haemophilus ducreyi*

DAVID A. LEWIS,[1,2] JULIA KLESNEY-TAIT,[1] SHERYL R. LUMBLEY,[1] CHRISTINE K. WARD,[1] JO L. LATIMER,[1] CATHERINE A. ISON,[3] AND ERIC J. HANSEN[1]*

Department of Microbiology, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9048,[1] and Department of Genitourinary Medicine and Communicable Disease[2] and Department of Microbiology,[3] Imperial College School of Medicine, St. Mary's Campus, Norfolk Place, London W2 1PG, United Kingdom

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The *znuA* gene of *Haemophilus ducreyi* encodes a 32-kDa (mature) protein that has homology to both the ZnuA protein of *Escherichia coli* and the Pzp1 protein of *H. influenzae*; both of these latter proteins are members of a growing family of prokaryotic zinc transporters. Inactivation of the *H. ducreyi* 35000 *znuA* gene by insertional mutagenesis resulted in a mutant that grew more slowly than the wild-type parent strain in vitro unless $ZnCl_2$ was provided at a final concentration of 100 μM. Other cations tested did not restore growth of this *H. ducreyi* mutant to wild-type levels. The *H. ducreyi* ZnuA protein was localized to the periplasm, where it is believed to function as the binding component of a zinc transport system. Complementation of the *znuA* mutation with the wild-type *H. ducreyi znuA* gene provided in *trans* restored the ability of this *H. ducreyi* mutant to grow normally in the absence of exogenously added $ZnCl_2$. The wild-type *H. ducreyi znuA* gene was also able to complement a *H. influenzae pzp1* mutation. The *H. ducreyi znuA* isogenic mutant exhibited significantly decreased virulence ($P = 0.0001$) when tested in the temperature-dependent rabbit model for experimental chancroid. This decreased virulence was not observed when the *znuA* mutant was complemented with the wild-type *H. ducreyi znuA* gene provided in *trans*.

Chancroid is one of the most prevalent sexually transmitted diseases and a major cause of morbidity in the resource-poor countries of Asia, Africa, and Latin America (53). This disease remains relatively uncommon in the United States and Western Europe (58). Prospective longitudinal and cross-sectional case control studies in Africa have provided substantial evidence that genital ulcer disease, either as a clinical syndrome or as an etiological diagnosis, is a significant risk factor for the heterosexual spread of the human immunodeficiency virus type 1 (HIV-1) even when sexual behavior is controlled for in the statistical analyses (11, 24, 49). In view of the epidemiological synergy that exists between chancroid and HIV-1 infection (63), there has been renewed research effort to elucidate the pathogenic mechanisms and virulence factors of *Haemophilus ducreyi*.

Several potential *H. ducreyi* virulence factors have been reported in the literature, and these include lipo-oligosaccharide (6, 13, 25, 59), a novel pilus (10), a soluble cytolethal distending toxin (16, 17), a hemoglobin-binding outer membrane protein (20, 57), a hemolysin capable of cytotoxicity involving direct contact between the bacterium and the eukaryotic cell (2, 45), and a copper-zinc superoxide dismutase which protects *H. ducreyi* from exogenous superoxide (55). In addition, gene products which may be involved in regulating the expression of *H. ducreyi* virulence factors have been described (14, 46). In contrast, much less is known about *H. ducreyi* gene products involved in the acquisition of essential nutrients and trace elements from the host environment.

Zinc is essential for all organisms due to its various physiological roles, which include maintaining both the structural stability and catalytic activity of several proteins (60), as well as stabilizing motifs in some transcriptional regulatory proteins (15). Zinc, at high concentration, is inherently toxic through inhibition of the aerobic respiratory chain (8, 34), and bacterial cells must regulate their zinc content over a small concentration range. Three factors have made study of zinc transport in bacteria difficult, including the extremely low bacterial requirement for zinc, the nonspecific binding of zinc to bacterial surfaces, and the rapid exchange of zinc between intracellular and extracellular compartments. An export system for cations, including zinc, was recently identified in *Escherichia coli* (7, 51), and a putative zinc uptake system (*adcABC*) was found to be essential for genetic competence in *Streptococcus pneumoniae* (18). The Pzp1 protein of *H. influenzae* was the first specific bacterial zinc transporter to be identified at the biochemical level (39). More recently, Patzer and Hantke (48) described the *znuABC* high-affinity zinc uptake system in *E. coli*.

In the present study, an *H. ducreyi* gene (*znuA*) encoding a zinc transport protein was identified and shown to complement a mutation in the equivalent *H. influenzae pzp1* gene. Inactivation of the *H. ducreyi* 35000 *znuA* gene resulted in a mutant exhibiting decreased in vitro growth rates in broth compared to the wild-type parent strain. This growth defect was overcome by the addition of 100 μM $ZnCl_2$. In addition, the isogenic *znuA* mutant exhibited significantly reduced virulence in the temperature-dependent rabbit model for experimental chancroid.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *H. ducreyi* strains were routinely cultivated on chocolate agar (CA) plates containing GC agar base (36 g/liter) (Difco, Detroit, Mich.), bovine hemoglobin (20 g/liter) (Sigma, St. Louis, Mo.), 1% (vol/vol) IsoVitalex (BBL Microbiological Systems, Cockeysville, Md.) supplemented with 100 μM $ZnCl_2$ (Zn). GC-heme agar (56) supplemented with kana-

* Corresponding author. Mailing address: Department of Microbiology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-9048. Phone: (214) 648-5974. Fax: (214) 648-5905. E-mail: hansen01@utsw.swmed.edu.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
E. coli		
DH5α	Host strain for cloning experiments	54
HB101	Host strain essential for propagating plasmids carrying mutated *H. ducreyi* DNA inserts used in electroporation	6, 54
XL1-Blue	Host strain used for screening of the ZAP Express bacteriophage library	Stratagene
XLOLR	Host strain used for excision of recombinant pBK-CMV plasmids from the ZAP Express bacteriophage	Stratagene
H. influenzae		
NTHI 6564	Wild-type parent strain	39
NTHI 6564 *pzp1* mutant	*pzp1* mutant of NTHI 6564	39
NTHI 6564.*pzp1*(pCW170)	NTHI 6564 *pzp1* mutant containing the vector pCW170	This study
NTHI 6564.*pzp1*(pDL6-2)	NTHI 6564 *pzp1* mutant containing pDL6-2 with the *H. ducreyi znuA* gene	This study
H. ducreyi		
35000	Wild-type strain isolated in Winnipeg, Manitoba, Canada	29
35000.901	Isogenic mutant with a *kan2* cartridge inserted into the *Msc*I site in the *znuA* gene	This study
35000.901(pCW170)	35000.901 containing the vector pCW170	This study
35000.901(pDL6-2)	35000.901 containing pDL6-2 with the *H. ducreyi znuA* gene	This study
RO18	Wild-type strain from Kenya	3
Cha-1	Wild-type strain from Dallas, Tex.	31
A77	Type strain from Institut Pasteur	44
1151	Wild-type strain from the Gambia	57
1153	Wild-type strain from the Gambia	Allan Ronald
511	Wild-type strain from Thailand	Allan Ronald
512	Wild-type strain from Thailand	57
Hd12	Wild-type strain from Korea	43
1352	Wild-type strain from Kenya	57
78226	Wild-type strain from Winnipeg, Manitoba, Canada	3
6V	Wild-type strain from Atlanta, Ga.	3
BG411	Wild-type strain from Kenya	3
O41	Wild-type strain from Sweden	57
1145	Wild-type strain from Amsterdam	57
Plasmids		
pUC18	Cloning vector, Amp^r	54
pUC18K2	pUC18 with the *kan2* cartridge containing the promoterless *aphA-3* gene	James Kaper
pBK-CMV	Phagemid excised from the ZAP Express cloning vector	Stratagene
pJKT913	pBK-CMV with a 3.7-kb *H. ducreyi* DNA insert containing the *znuA* gene	This study
pBluescript II KS(−)	Cloning vector, Amp	Stratagene
pJKT933	pBluescript II with a 3.7-kb *Hin*dIII-*Sac*I fragment from pJKT913 containing the *znuA* gene	This study
pDL5-3	pJKT933 with a *kan2* cartridge inserted into the *Msc*I site within the *H. ducreyi znuA* ORF	This study
pLS88	Cloning vector capable of replication in *H. ducreyi*; Kan^r Sm^r Sul^r	19
pCW170	pLS88 with a *cat* cartridge inserted into the *Eco*RI site of this vector	This study
pDL6-2	pCW170 with a 402 nt *Pvu*I-*Xho*I fragment removed from the *kan* gene and a 1.8-kb PCR-derived, *Xho*I-*Pvu*I DNA fragment with the *H. ducreyi znuA* gene inserted in its place	This study

mycin (30 μg/ml) was used to select *H. ducreyi* transformants after electroporation. Nontypeable *H. influenzae* (NTHI) strains were grown on CA-Zn plates or on BHIs plates (brain heart infusion [BHI; 37 g/liter] [Difco], Bacto Agar [15 g/liter] [Difco], 5% [vol/vol] Levinthal's base [1]) with or without 100 μM $ZnCl_2$ (Zn). *E. coli* strains were grown on Luria-Bertani medium (LB) (54). For antimicrobial supplementation, kanamycin (Sigma) was used at 30 μg/ml, and chloramphenicol (Sigma) was used at 2 μg/ml (for *Haemophilus* species) or 30 μg/ml (for *E. coli*).

For measurement of bacterial growth in vitro, *H. ducreyi* strains were inoculated into a filter-sterilized medium containing Columbia broth (35 g/liter) (Difco), Trizma base (1 g/liter) (Sigma), equine hemin (25 μg/ml), 2.5% (vol/vol) heat-inactivated fetal calf serum (HyClone, Logan, Utah), and 1% (vol/vol) IsoVitalex (62). NTHI strains were grown in filter-sterilized BHI broth containing 10% (vol/vol) Levinthal's base. In some experiments, cations including $ZnCl_2$ were added to these broths at various concentrations. Growth was monitored by serial absorbance measurements (λ = 600 nm) by using a Spectronic 20D+ spectrophotometer (Spectronic Instruments, Inc., Rochester, N.Y.). Cultures were incubated at 33°C (*H. ducreyi*) or 37°C (NTHI) at gyrotory speeds of 90 rpm (*H. ducreyi*) or 160 rpm (NTHI). All growth experiments were performed at least twice.

Monoclonal antibody (MAb) production. Concentrated culture supernatant fluid was prepared from cultures of *H. ducreyi* 35000 grown in the presence of human foreskin fibroblasts and collagen in serum-free medium (26, 36a). Splenocytes from mice immunized with this preparation were used in a standard hybridoma fusion protocol (52) to obtain a hybridoma cell line that secreted the *H. ducreyi* ZnuA-directed immunoglobulin G MAb 3F1.

***H. ducreyi* genomic library construction and screening.** An *H. ducreyi* 35000 genomic library (37) constructed in the ZAP Express vector (Stratagene, La Jolla, Calif.) was screened for the presence of recombinant bacteriophages forming MAb 3F1-reactive plaques. The relevant pBK-CMV plasmids containing the *H. ducreyi* DNA inserts were excised by using the manufacturer's protocol. One such plasmid, pJKT913, containing a 3.7-kb *H. ducreyi* DNA insert with the *znuA* gene, was selected for further study. This 3.7-kb *H. ducreyi* insert was removed from pJKT913 by digestion with *Hin*dIII and *Sac*I (which are present in the pBK-CMV multicloning site on either side of the *Bam*HI site); this fragment was ligated into *Hin*dIII- and *Sac*I-digested pBluescript II KS(−) to obtain pJKT933.

Recombinant DNA techniques. Standard techniques, including restriction enzyme digests, ligation, transformation, and plasmid purification, have been described elsewhere (4, 54). A cartridge (*kan2*) containing a promoterless *aphA-3* gene encoding kanamycin resistance was constructed by Karen Jarvis and James B. Kaper, Center for Vaccine Development, University of Maryland, and was utilized for construction of nonpolar mutations as described by Menard et al. (42). This 0.85-kb cartridge was excised from plasmid pUC18K2 by digestion with *Sma*I. The pUCΔECAT-derived chloramphenicol acetyltransferase (*cat*) cartridge used to construct pCW170 was obtained from Bruce A. Green, Wyeth-Lederle Vaccines and Pediatrics, West Henrietta, N.Y. PCR was performed according to manufacturer instructions with either *Taq* DNA polymerase (Promega, Madison, Wis.) or *Pfu* DNA polymerase (Stratagene). Boiled bacterial cell preparations (33) or purified *H. ducreyi* chromosomal DNA (6) were used as templates for PCR.

Nucleotide sequence analysis. Nucleotide sequence analysis was accomplished as described earlier (62). For the purpose of interstrain comparisons, a 1.9-kb PCR product containing the entire *znuA* gene was amplified from the chromosomal DNA of five other *H. ducreyi* strains (RO18, Cha-1, A77, 1151, and 512) with *Pfu* polymerase and the oligonucleotide primers P3 and P4 (see Fig. 2).

N-terminal amino acid sequence analysis. The 32-kDa protein reactive with MAb 3F1 was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene difluoride membrane by the method of Matsudaira (41), and subjected to N-terminal amino acid sequence analysis as described elsewhere (32).

Construction of an isogenic *H. ducreyi znuA* mutant. Plasmid pJKT933 was linearized with *Msc*I, which cut once within the *znuA* gene. The *kan2* cartridge was then blunt-end ligated into pJKT933 to obtain pDL5-3. DNA sequence analysis confirmed the proper construction of the nonpolar mutation in the *znuA* gene. *E. coli* HB101 was transformed with pDL5-3, and plasmid purified from this strain was linearized by digestion with *Pst*I and used to electroporate *H. ducreyi* 35000 (30); transformants were selected on GC-heme agar plates supplemented with kanamycin (30 μg/ml). Transformant colonies were screened by direct PCR-based amplification of chromosomal DNA from single colonies by using oligonucleotide primers P1 (5′-GCGATGTTAGGCGTAACGACAG-3′) and P2 (5′-GCGTGAAAAAATCGTCAGTTCG-3′) and by loss of reactivity with MAb 3F1 in a colony blot radioimmunoassay (28).

Southern blot analysis. Purified chromosomal DNA preparations from wild-type *H. ducreyi* 35000 and its isogenic *znuA* mutant 35000.901 were digested to completion with *Spe*I, subjected to electrophoresis in a 0.7% (wt/vol) agarose gel, transferred to nitrocellulose paper, and probed via Southern blot analysis as previously described (54). A 1-kb DNA fragment obtained by *Spe*I digestion of pJKT933 (see Fig. 2) was used as a probe for the *znuA* gene, and the 0.85-kb *kan2* cartridge was used as a probe for this antibiotic resistance cartridge. This same *znuA* DNA probe was used to screen *Spe*I digests of chromosomal DNA from 10 other *H. ducreyi* isolates (strains 1352, 1153, 511, Hd12, 78226, 6V, BG411, Cha-1, 041, and 1145).

Complementation of the *znuA* mutation in *H. ducreyi* and the *pzp1* mutation in NTHI 6564. A 1.9-kb DNA fragment containing the *H. ducreyi znuA* gene (see Fig. 2) was amplified from strain 35000 chromosomal DNA by using *Pfu* DNA polymerase and the oligonucleotide primers P3 (5′-TGGACCTTGTGTAATC GTGAG-3′) and P4 (5′-TACGATCGGGTGGATCACCCGAATATCG-3′); the underlined sequence indicates a *Pvu*I site. This 1.9-kb PCR product was digested with both *Pvu*I and *Xho*I to produce a 1.8-kb fragment containing the *znuA* gene and flanking *H. ducreyi* DNA sequences (see Fig. 2). Next, the 6.2-kb vector pCW170 was digested with both *Pvu*I and *Xho*I; this digestion removed a 0.4-kb portion of the kanamycin resistance gene. The *znuA*-containing fragment was ligated into the 5.8-kb *Pvu*I-*Xho*I fragment of pCW170. The ligation mixture was used to transform *E. coli* DH5α; transformants were selected by the ability to grow on LB agar containing chloramphenicol and screened for lack of growth on LB agar containing kanamycin. Transformants were screened for possession of the *H. ducreyi znuA* gene by PCR by using primers P1 and P4 and for reactivity with MAb 3F1 in a colony blot radioimmunoassay. Plasmid pDL6-2, obtained from one of these transformants, was purified utilizing the Wizard Plus Miniprep system. This plasmid was used to electroporate both the *H. ducreyi znuA* mutant 35000.901 and the NTHI 6564 *pzp1* mutant.

Preparation of cell fractions for ZnuA localization studies. Whole-cell lysates of *H. ducreyi* were prepared from CA-Zn plate-grown cells as described earlier (47). *H. ducreyi* cells grown in the same manner were used for the preparation of cell envelopes and their Sarkosyl-insoluble extracts as described previously (22). To prepare periplasmic fractions from *H. ducreyi* strains, the osmotic shock method described by Hultgren and coworkers (38) was modified slightly. Bacterial growth from 20 CA-Zn plates was scraped into 10 ml of cold phosphate-buffered saline (PBS) and subjected to centrifugation at 8,000 × *g* for 10 min. The wet weight of the pellet was determined, and the cells were resuspended to a final concentration of 25% (wt/vol) in cold, filter-sterilized 20 mM Tris-HCl (pH 8.0) containing 20% (wt/vol) sucrose (Sigma). To this suspension, held at 4°C, was added 0.1 M pH 8.0 EDTA (200 μl per g of cells) and lysozyme (600 μg per g of cells). After a 40-min incubation on ice, 0.5 M $MgCl_2$ (160 μl per g cells) was added, and the suspension was shaken gently. Spheroplasts were pelleted by centrifugation at 23,000 × *g* for 20 min (4°C), and the resultant supernatant fluid was centrifuged at 200,000 × *g* for 90 min (4°C). The final supernatant fluid represented the periplasmic fraction and was shown to contain periplasmic contents by Western blot analysis with polyclonal antiserum raised against the *H. ducreyi* periplasmic Cu-Zn superoxide dismutase (55) as the primary antibody.

Concentration of bacterial culture supernatant fluids. *H. ducreyi* strains were grown in the Columbia broth-based medium containing 100 μM $ZnCl_2$ for 24 h. The culture was centrifuged at 8,000 × *g* for 10 min (4°C), and the supernatant fluid was filter sterilized by using Acrodisc syringe filters (0.2-μm pore size) (Gelman Sciences, Ann Arbor, Mich.). The supernatant fluid was centrifuged at 215,000 × *g* for 90 min (4°C) prior to being concentrated 40-fold by using a Centriprep-10 centrifugal concentrator (Amicon, Inc., Beverly, Mass.).

Colony blot radioimmunoassay, SDS-PAGE, and Western blot (immunoblot) methods. These procedures were accomplished as described previously (28, 35).

Virulence testing. The relative virulence of the *H. ducreyi* strains used in this study was determined by using the temperature-dependent rabbit model for experimental chancroid (50). The mean scores of the lesions resulting from injection of 10^5 and 10^4 CFU were analyzed statistically as described earlier (6). After lesion scoring on day 7, the rabbits were euthanized and the lesions resulting from injection of an inoculum containing 10^5 CFU were excised; the pustular material from these lesions was cultured on CA-Zn plates.

Nucleotide accession number. The nucleotide sequence of the *H. ducreyi* 35000 *znuA* gene was assigned GenBank accession number AF141971.

RESULTS

Identification and cloning of the *H. ducreyi znuA* gene. Polyclonal rabbit antiserum to *H. ducreyi* 35000 was used in Western blot analysis to probe supernatant fluid from cocultures of *H. ducreyi* 35000 and human foreskin fibroblasts embedded in a matrix of collagen (26, 36a). Among the immunoreactive antigens detected in these preliminary experiments was a 32-kDa molecule (data not shown). An MAb (3F1) reactive with this 32-kDa molecule also bound this protein in concentrated culture supernatant fluids of *H. ducreyi* alone (Fig. 1A, lane 1). The use of MAb 3F1 to screen a *H. ducreyi* genomic library yielded the recombinant plasmid pJKT913 which contained a 3.7-kb *H. ducreyi* DNA insert that expressed this MAb 3F1-reactive antigen. This *H. ducreyi* DNA insert together with a small amount of flanking vector DNA was subcloned into pBluescript II to form pJKT933 (Fig. 2). Nucleotide sequence analysis revealed the presence of two complete and two incomplete ORFs (Fig. 2). The first complete ORF (ORF 2) encoded a predicted protein that had homology to a putative oxidoreductase in *E. coli* (9), whereas the other complete ORF encoded a predicted protein that was 43% identical to the *E. coli* ZnuA (YebL) zinc-binding protein (48) and 54% identical to the *H. influenzae* (NTHI 6564) periplasmic zinc-binding protein Pzp1 (39). One of the incomplete open reading frames (ORFs) (ORF 1) encoded a partial protein with homology to the hypothetical *H. influenzae* protein HI0318 (23), and the other (ORF 3) encoded a partial protein with similarity to the *H. influenzae* ribose-5-phosphate isomerase A (23).

Features of the *H. ducreyi* 35000 *znuA* gene and its protein product. A putative promoter region immediately upstream of the *znuA* ORF contained predicted −35 and −10 regions, as well as a potential ribosomal binding site. The *H. ducreyi znuA* ORF contained 930 nucleotides encoding a predicted protein with a calculated molecular weight of 34,289. The existence of a signal peptide was confirmed by N-terminal amino acid sequence analysis of the MAb 3F1-reactive protein band obtained from *H. ducreyi* 35000 cells. The N-terminal amino acid sequence (DVLTSIKPLGFIANAITDGV) derived from this approach matched the predicted N-terminal amino acid sequence of the mature protein exactly. The calculated molecular weight of the mature protein was 32,198. This mature protein also contained the central histidine-rich region as well as the two cysteine residues in the C-terminal region characteristic of the NTHI 6564 Pzp1 protein (39, 40).

Detection and characterization of the *znuA* gene in *H. ducreyi* clinical isolates. Southern blot analysis of *Spe*I-digested chromosomal DNA from 11 geographically diverse *H. ducreyi* clinical isolates (including strain 35000) was performed with a

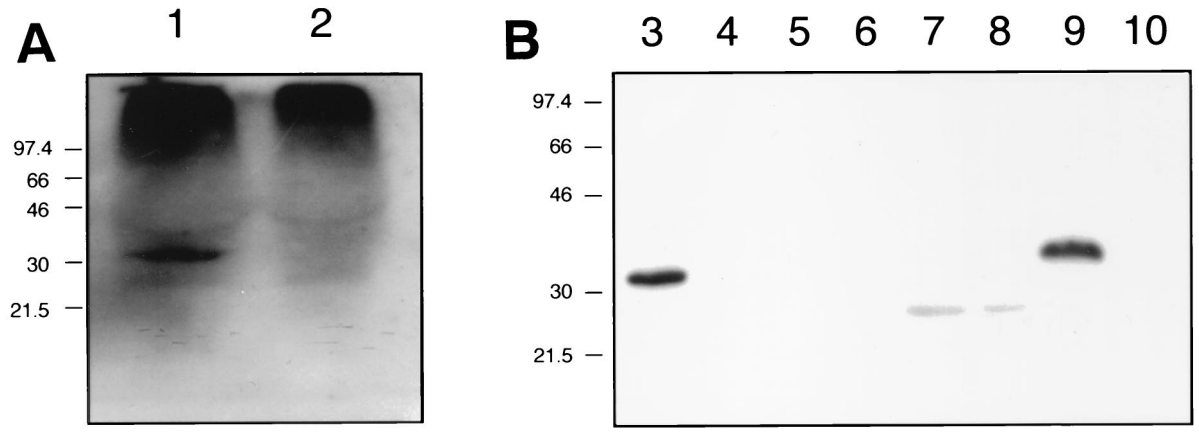

FIG. 1. Western blot-based detection of ZnuA in culture supernatant fluid and subcellular fractions of *H. ducreyi*. The *H. ducreyi* ZnuA-reactive MAb 3F1 was used as the primary antibody. (A) Concentrated culture supernatant fluid from wild-type 35000 (lane 1) and the *znuA* mutant 35000.901 (lane 2). (B) Subcellular fractions from wild-type 35000 (lanes 3, 5, 7, and 9) and the *znuA* mutant 35000.901 (lanes 4, 6, 8, and 10). Lanes 3 and 4, whole-cell lysate; lanes 5 and 6, total cell envelopes; lanes 7 and 8, Sarkosyl-insoluble proteins from cell envelopes; lanes 9 and 10, periplasmic fraction. The aberrant migration of the ZnuA protein in the periplasmic fraction in lane 9 was caused by the presence of a high concentration of sucrose derived from the preparation of the periplasmic fraction; when this sample was diluted 1:4 in PBS, the ZnuA protein migrated at the same rate as the ZnuA protein seen in lane 3.

1-kb *znuA*-containing DNA fragment derived from pJKT933 by *Spe*I digestion (Fig. 2). All of these isolates possessed a 1-kb *Spe*I fragment that bound this *znuA* probe (data not shown). Nucleotide sequence analysis of the *znuA* gene from five *H. ducreyi* strains (RO18, Cha-1, A77, 1151, and 512) revealed that the encoded ZnuA proteins had almost complete identity with the ZnuA protein of strain 35000, the only variation being seen within the histidine-rich central area (Fig. 3).

Construction of an isogenic *H. ducreyi znuA* mutant. The inactivated *H. ducreyi* 35000 *znuA* gene containing the *kan2* cartridge in pDL5-3 was used to electroporate *H. ducreyi* 35000. Five kanamycin-resistant *H. ducreyi* transformants were tested initially by PCR (with primers P1 and P2) to detect allelic exchange. All five yielded a single 2.3-kb PCR product which was 0.85 kb larger than the PCR product from the wild-type strain; this finding was consistent with the presence

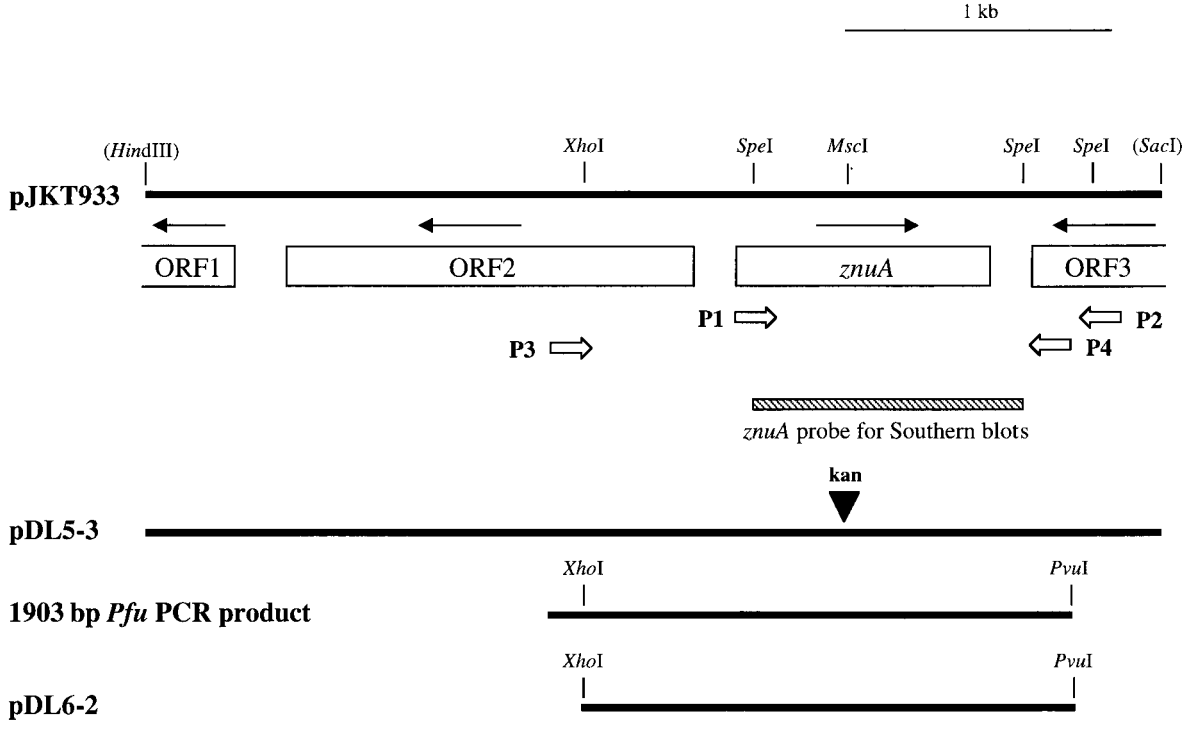

FIG. 2. Partial restriction map of the *H. ducreyi* 35000 chromosomal DNA insert in pJKT933 and related plasmids. The two complete ORFs and two incomplete ORFs in this insert are indicated together with arrows designating the proposed direction of transcription. Restriction sites in parentheses indicate vector cloning sites. The open arrows indicate various oligonucleotide primers used in PCR. The cross-hatched bar beneath the *znuA* gene indicates the 1-kb probe used for Southern blot analysis in Fig. 4. The *kan2* cartridge was ligated into the *Msc*I site of pJKT933 to construct pDL5-3. Plasmid pDL6-2 is a modified pCW170 vector (see Materials and Methods) containing the 1.8-kb *Xho*I- and *Pvu*I-digested PCR product with the *znuA* gene and flanking DNA.

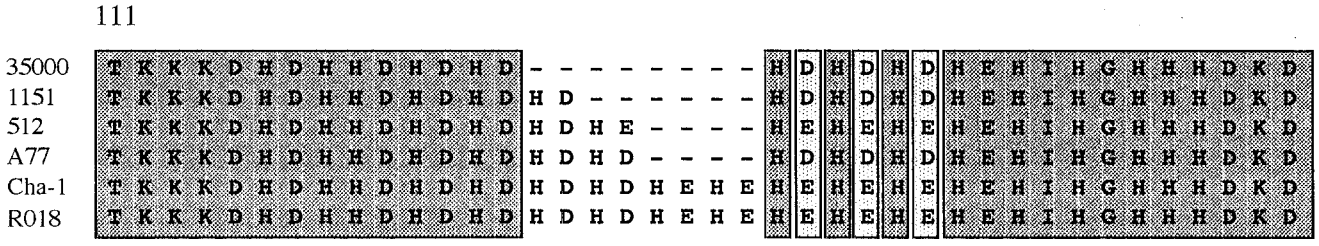

FIG. 3. Comparison of the histidine-rich regions from the ZnuA proteins of six *H. ducreyi* strains using the CLUSTAL-W Alignment program in MacVector version 6. Dark shading indicates residues that are identical. Light shading indicates residues that are similar. The amino acid sequences begin with residue 111 in each strain. The upstream and downstream amino acid sequences of all six proteins (not shown) were identical.

of the *kan2* cartridge in the *znuA* gene (data not shown). In addition, all five transformants failed to react with the *H. ducreyi* ZnuA-reactive MAb 3F1 in a colony blot assay (data not shown). One of these transformants was selected for further study and designated strain 35000.901. Western blot analysis indicated that strain 35000.901 did not express detectable ZnuA in concentrated culture supernatant fluid (Fig. 1A, lane 2).

Southern blot analysis confirmed the occurrence of the desired allelic exchange in strain 35000.901 (Fig. 4). When chromosomal DNA from wild-type parent strain 35000 was probed with the *znuA* probe described above (Fig. 2), a 1-kb *Spe*I fragment hybridized with this probe (Fig. 4, lane 1). This same probe hybridized with a 1.85-kb *Spe*I fragment of chromosomal DNA from strain 35000.901 (Fig. 4, lane 2). A 1.85-kb fragment from strain 35000.901 also hybridized with a *kan2* cartridge-based probe (Fig. 4, lane 4). Chromosomal DNA from the wild-type parent strain 35000 failed to hybridize with the *kan2* probe (Fig. 4, lane 3).

In vitro growth characteristics of the isogenic *H. ducreyi znuA* mutant. The *znuA* mutant strain 35000.901 grew more slowly than the wild-type strain 35000 in broth culture (Fig. 5A). The addition of $ZnCl_2$ to a final concentration of 100 μM almost completely corrected the growth deficiency of strain 35000.901 in vitro. This growth deficiency was not reversed by the addition of $ZnCl_2$ to a final concentration of 5 μM or by 100 μM concentrations of $FeCl_3$, $MnCl_2$, $CuSO_4$, $NiCl_2$, or $MgCl_2$ (data not shown), a result which suggested that the growth defect in strain 35000.901 was zinc specific.

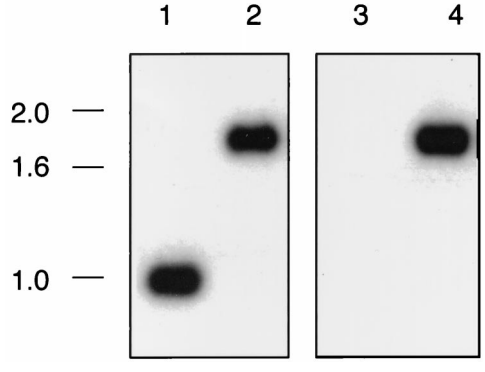

FIG. 4. Southern blot analysis of chromosomal DNA preparations from *H. ducreyi* wild-type and mutant strains. Chromosomal DNAs were digested with *Spe*I, resolved by agarose gel electrophoresis, and probed with either a 1-kb *Spe*I fragment derived from the *H. ducreyi znuA* gene (lanes 1 and 2) or with the *kan2* cartridge (lanes 3 and 4). Lanes 1 and 3, strain 35000; lanes 2 and 4, *znuA* mutant 35000.901. Size markers (in kilobases) are on the left side of the figure.

Complementation of the *znuA* mutation in *H. ducreyi*. To eliminate the possibility that an undetected secondary mutation was responsible for the altered growth phenotype of the *H. ducreyi znuA* mutant, complementation analysis was performed. The wild-type *znuA* gene was amplified by PCR and cloned into pCW170 to yield the recombinant plasmid pDL6-2 (Fig. 2). Both the pCW170 vector and pDL6-2 were introduced into the *H. ducreyi znuA* mutant by electroporation. Southern blot analysis indicated that there had been no rearrangement between the mutated *znuA* gene in the chromosome and the vector-borne wild-type *znuA* gene in the recombinant strain 35000.901(pDL6-2) (data not shown). The presence of the wild-type *H. ducreyi znuA* gene in *trans* in strain 35000.901 (pDL6-2) resulted in restoration of wild-type growth characteristics (Fig. 5B). In contrast, the presence of the vector alone in strain 35000.901(pCW170) did not correct the growth phenotype of the *znuA* mutant (Fig. 5B).

Complementation of the *H. influenzae pzp1* mutant with pDL6-2. The *pzp1* (*znuA*) mutant of NTHI 6564 (39) was electroporated with pDL6-2 and with the pCW170 vector. Numerous transformants grew within 24 h on BHIs-Zn plates containing chloramphenicol, and six transformants from each electroporation were further characterized. All six *pzp1* mutants containing pDL6-2 expressed *H. ducreyi* ZnuA, as evidenced by their reactivity with the *H. ducreyi* ZnuA-reactive MAb 3F1 (data not shown). The presence of the *H. ducreyi znuA* gene in these six transformants was confirmed by PCR with the oligonucleotide primers P1 and P4 (data not shown). The six *pzp1* mutants containing pCW170 were not reactive with MAb 3F1 and did not yield a PCR product with primers P1 and P4 (data not shown). One recombinant strain from each group of six was selected for in vitro growth studies. The presence of the wild-type *H. ducreyi znuA* gene in *trans* in the NTHI 6564 *pzp1* mutant resulted in restoration of the ability to grow in broth without additional zinc supplementation (Fig. 5C). In contrast, the presence of the pCW170 vector alone in the NTHI 6564 *pzp1* mutant did not correct the growth deficiency of this strain (Fig. 5C).

Localization of ZnuA in *H. ducreyi*. To localize ZnuA in *H. ducreyi*, whole-cell lysates, total cell envelopes, Sarkosyl-insoluble cell envelope material, and periplasmic extracts were prepared from cells of the wild-type strain 35000 and of its isogenic *znuA* mutant 35000.901 grown on CA plates supplemented with $ZnCl_2$ at 100 μM. In addition, concentrated (40-fold) supernatant fluids were prepared from 24-h broth cultures of both strains grown in the presence of 100 μM $ZnCl_2$. Western blot analysis with the *H. ducreyi* ZnuA-reactive MAb 3F1 detected ZnuA in both the whole-cell lysate and periplasmic extract of the wild-type strain (Fig. 1B, lanes 3 and 9, respectively) but not in the same preparations from the *znuA* mutant (Fig. 1B, lanes 4 and 10). ZnuA was not detectable in

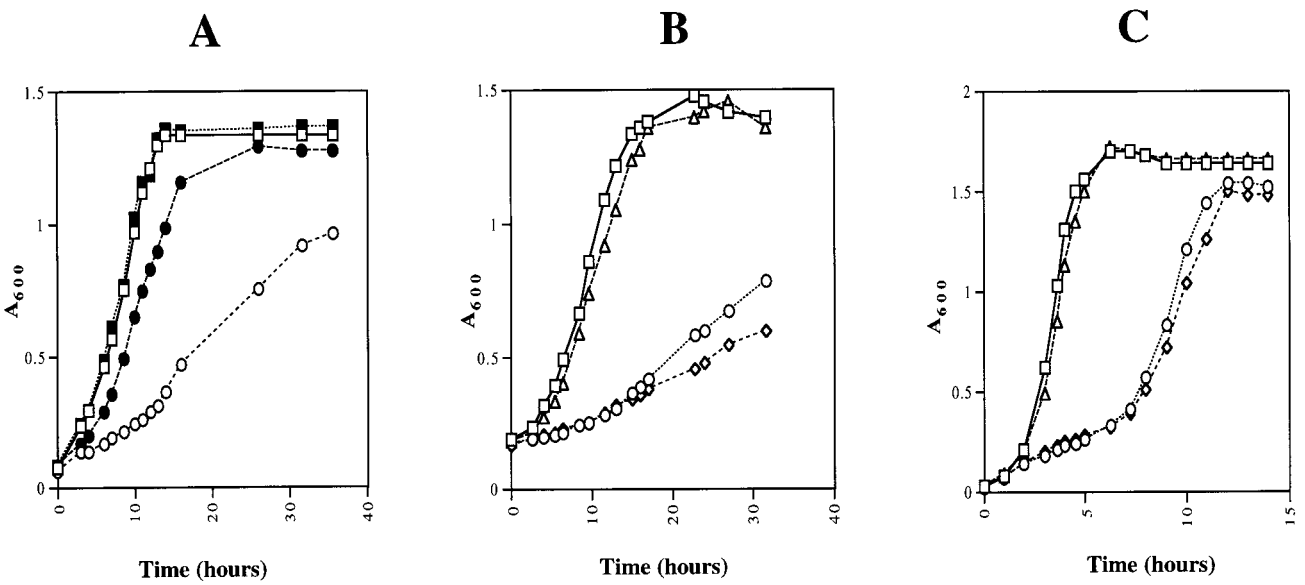

FIG. 5. Growth of wild-type, mutant, and recombinant strains of *H. ducreyi* and *H. influenzae* in vitro. These data are from representative experiments. (A) *H. ducreyi* strains grown overnight on CA-Zn plates were inoculated into broth with (solid symbols) and without (open symbols) 100 μM $ZnCl_2$, and growth was monitored for 36 h. Wild-type strain 35000 is indicated by squares, and *znuA* mutant 35000.901 is indicated by circles. (B) *H. ducreyi* strains grown overnight on CA-Zn plates were inoculated into broth without supplemental zinc, and growth was monitored for 32 h. Symbols: squares, wild-type *H. ducreyi* 35000; circles, *H. ducreyi znuA* mutant 35000.901; diamonds, *H. ducreyi znuA* mutant 35000.901(pCW170); triangles, *H. ducreyi znuA* mutant 35000.901(pDL6-2). (C) *H. influenzae* strains grown overnight on BHIs plates containing 100 μM $ZnCl_2$ were inoculated into BHIs broth without supplemental zinc, and growth was monitored for 14 h. Symbols: squares, wild-type NTHI 6564; circles, NTHI 6564 *pzp1* mutant; diamonds, NTHI 6564 *pzp1* mutant containing pCW170; triangles, NTHI 6564 *pzp1* mutant containing pDL6-2.

either the total cell envelope preparation or Sarkosyl-insoluble material of both the wild-type strain (Fig. 1B, lanes 5 and 7) and the mutant strain (Fig. 1B, lanes 6 and 8). A small amount of ZnuA was detected in the wild-type culture supernatant fluid (Fig. 1A, lane 1) but not in culture supernatant fluid from the mutant (Fig. 1A, lane 2).

Virulence testing of the *H. ducreyi znuA* mutant. Four *H. ducreyi* strains were tested in the temperature-dependent rabbit model (Table 2): wild-type strain 35000, the *znuA* mutant 35000.901, strain 35000.901(pCW170), and strain 35000.901 (pDL6-2). In the first of two experiments (Table 2, experiment A), statistical analysis showed that the *znuA* mutant was less

TABLE 2. Lesion formation by wild-type, *znuA* mutant, and complemented *znuA* mutant strains of *H. ducreyi* in the temperature-dependent rabbit model[a]

Strain	Inoculum size	Mean lesion score (SD) at:			*P* value[b]
		Day 2	Day 4	Day 7	
Expt A					
35000 (wild-type)	10^5	4.00 (0)	4.00 (0)	4.00 (0)	
35000.901 (*znuA* mutant)	10^5	3.25 (0.46)	2.75 (0.46)	2.75 (0.46)	0.0001[c]
35000.901(pDL6-2)	10^5	4.00 (0)	4.00 (0)	4.00 (0)	0.1334
35000	10^4	3.13 (0.35)	3.13 (0.35)	3.63 (0.52)	
35000.901	10^4	3.00 (0)	2.13 (0.64)	2.00 (0.93)	
35000.901(pDL6-2)	10^4	3.38 (0.52)	3.50 (0.54)	3.88 (0.35)	
Expt B					
35000	10^5	3.88 (0.35)	4.00 (0)	4.00 (0)	
35000.901(pCW170)	10^5	3.13 (0.35)	2.75 (0.71)	3.13 (0.35)	0.0002
35000.901(pDL6-2)	10^5	3.38 (0.52)	3.88 (0.35)	4.00 (0)	0.0366
35000	10^4	3.13 (0.35)	3.38 (0.52)	3.50 (0.54)	
35000.901(pCW170)	10^4	3.00 (0)	2.63 (0.52)	2.25 (0.46)	
35000.901(pDL6-2)	10^4	3.00 (0.54)	3.00 (0.54)	3.13 (0.64)	

[a] Eight rabbits were used in each experiment.
[b] *P* value calculated for the difference between wild-type and test strain lesion scores. *P* values were calculated by using the lesion scores from both inoculum sizes and from all 3 days.
[c] The complemented *znuA* mutant 35000.901(pDL6-2) was significantly more virulent than both the *znuA* mutant containing the vector pCW170 ($P = 0.0061$) and the *znuA* mutant alone ($P = 0.0001$).

virulent than both the wild-type parent strain ($P = 0.0001$) and the complemented mutant ($P = 0.0001$). There was no difference between the wild-type strain 35000 and the complemented mutant strain 35000.901(pDL6-2) ($P = 0.1334$). Viable *H. ducreyi* organisms were recovered at the same frequency (i.e., eight of eight) from lesions produced by both the wild-type strain 35000 and the complemented mutant 35000.901 (pDL6-2). In contrast, viable *H. ducreyi* organisms were recovered from only one of eight lesions produced by the *znuA* mutant 35000.901.

In the second experiment (Table 2, experiment B), the *znuA* mutant containing the pCW170 vector was less virulent than both the wild-type strain ($P = 0.0002$) and the complemented mutant ($P = 0.0061$). The difference in lesion scores between the wild-type strain 35000 and the complemented mutant strain 35000.901(pDL6-2) just achieved significance ($P = 0.0366$). Viable *H. ducreyi* organisms were recovered from seven of eight lesions produced by the wild-type strain 35000 and from five of eight lesions resulting from inoculation with the complemented mutant 35000.901(pDL6-2). In contrast, no viable *H. ducreyi* organisms were recovered from the eight lesions produced by the *znuA* mutant 35000.901 containing the plasmid vector.

DISCUSSION

In the present study, a putative zinc transport protein (ZnuA) was identified in *H. ducreyi* which had homology to both the Pzp1 protein of *H. influenzae* (54% identity) and the ZnuA (YebL) protein of *E. coli* (43% identity). The gene (*znuA*) encoding this *H. ducreyi* protein appears to be well conserved among strains of this pathogen. Lu et al. (39) demonstrated that the *H. influenzae* Pzp1 protein functions as a periplasmic zinc binding protein based on results of protein localization studies, direct binding of ^{65}Zn to recombinant Pzp1, neutron activation analysis, and atomic absorption spectroscopy. The observation that the *H. ducreyi* ZnuA protein could complement the in vitro growth defect of the NTHI 6564 *pzp1* mutant suggests that the two proteins have identical functions in zinc transport. The localization of ZnuA to the periplasmic compartment in *H. ducreyi* is also compatible with its postulated role as the binding component of a zinc transport system. Whereas *H. influenzae* Pzp1 was undetectable in culture supernatant fluid (39), *H. ducreyi* ZnuA was detected in a Western blot analysis of a concentrated 24-h culture supernatant fluid (Fig. 1A). This finding probably reflects leakage of ZnuA into the medium from dying bacteria, although we have not formally excluded the less likely possibility of active secretion of this protein into the culture medium.

Inactivation of the *znuA* gene in *H. ducreyi* 35000 resulted in the isogenic mutant 35000.901, which exhibited decreased growth in broth compared to the wild-type parent strain (Fig. 5A). This growth defect of the mutant was overcome by complementation with the wild-type *znuA* gene in *trans* (Fig. 5B) or by the addition of $ZnCl_2$ to the broth at a final concentration of 100 μM (Fig. 5A). In addition, the *znuA* mutant exhibited significantly reduced virulence in the temperature-dependent rabbit model for experimental chancroid (Table 2). It must be noted that provision of the wild-type *H. ducreyi znuA* gene in *trans* in the *H. ducreyi znuA* mutant restored virulence in the animal model.

Although the *znuA* mutant clearly produced lower lesion scores than did the wild-type strain in the rabbit model, interpretation of these virulence data is not straightforward. We have no information on the physiological concentration ranges of zinc in the skin of male New Zealand White rabbits but assume that it is very low since the normal plasma zinc concentration in this species is approximately 23 μM (36). The decreased virulence observed with the *znuA* mutant may therefore simply reflect limited in vivo growth of the mutant compared to that of the wild-type parent strain or complemented mutant. This hypothesis is substantiated by the observation that the in vitro growth defect of the *znuA* mutant is almost completely abolished by adding zinc chloride to a concentration of 100 μM or by complementing the *znuA* chromosomal mutation with a plasmid expressing *H. ducreyi* ZnuA. However, we cannot eliminate the possibility that the *znuA* mutation may affect virulence indirectly, either by affecting regulation of other virulence factors or because zinc is an essential cofactor for one or more virulence-related proteins.

The *H. ducreyi znuA* gene belongs to the recently described family of proteins involved in metal cation binding and subsequent transport into the bacterial cell (18). The MntABC Mn^{2+} transport system of the cyanobacterium *Synechocystis* sp. strain 6803 was the first member identified in which *mntC* encodes for a Mn^{2+} binding protein (5). A putative ABC-type zinc permease complex (Adc) and a putative ABC-type manganese permease complex (Psa) were subsequently identified in *S. pneumoniae* (18). The most recent addition to this family is the ZnuABC transport system of *E. coli* in which ZnuA performs the role of the zinc-binding protein (48). The *znuA*, *znuB*, and *znuC* genes correspond to the *yebL*, *yebI*, and *yebM* genes identified in the *E. coli* K-12 genome sequencing project (9). The Pzp1 zinc-binding protein of *H. influenzae* also belongs to this family but, interestingly, both in this species and in *H. ducreyi* adjacent genes encoding for hydrophobic membrane proteins or ATP-binding proteins that would be typical in an ABC transporter operon appear to be absent. It is possible that genes with these functions could be located at another site(s) on their respective chromosomes. In support of this hypothesis, the predicted protein products of ORFs HI0407 and HI0408 identified in the *H. influenzae* Rd genome (23) have 60 and 48% identity to the ZnuB (YebI) and ZnuC (YebM) proteins of *E. coli*, respectively.

Both ZnuA of *H. ducreyi* and ZnuA of *E. coli* have two conserved cysteine residues in their C-terminal region, making it likely that they have a C-terminal disulfide-bonded domain similar to that in Pzp1 of *H. influenzae* (39, 40). In support of this, the *H. ducreyi* ZnuA protein migrates more slowly in SDS-PAGE when in the presence of 2-mercaptoethanol than it does under nonreducing conditions (data not shown). These cysteines are not present in the pneumococcal AdcA protein (18). The amino acids histidine (H), aspartic acid (D), and glutamic acid (E) appear to be characteristic for zinc-binding proteins (18); all four proteins also possess a central histidine- and acidic amino acid-rich region, which is most pronounced in Pzp1 of *H. influenzae* (39). In contrast, the putative manganese-binding proteins MntC of *Synechocystis* sp. strain 6803 (5) and PsaA of *S. pneumoniae* (18) both lack this histidine- and acidic amino acid-rich central region. The ZnuA proteins from five other *H. ducreyi* isolates showed variation in amino acid sequence only in this histidine-rich area (Fig. 3). In eukaryotes, a metal binding motif $(HX)_3$ has been proposed as the metal binding site of the high-affinity zinc transporter (Zrt1) of *Saccharomyces cerevisiae* (64) and of two of the four putative zinc transporter proteins described in *Arabidopsis thaliana* (21, 27). These proteins form part of the 15-member Zrt- and Irt-related protein (ZIP) family of eukaryotic proteins reviewed by Eng et al. (21) which includes functionally uncharacterized homologues in *Caenorhabditis elegans*, *Mus musculus*, and *Homo sapiens*.

Preliminary experiments using the *H. ducreyi* ZnuA-reactive

MAb 3F1 to probe lysates of *H. ducreyi* 35000 cells grown in broths containing increasing zinc concentrations have failed to provide evidence for regulation of ZnuA protein expression, at least in vitro (data not shown). This is in contrast to the situation in *E. coli*, in which the first regulatory protein (i.e., Zur) to affect zinc uptake was described (48). In vivo-expressed genes with similarity to the *E. coli zur* gene have been identified in *Vibrio cholerae* (*iviXI*) (12) and *Pseudomonas aeruginosa* (*np20*) (61) and support the hypothesis that zinc could regulate expression of one or more gene products important for virulence in the mammalian host. Whether *H. ducreyi* possesses a homologous *zur* gene that is expressed solely in the in vivo environment remains to be determined.

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