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Characterization of a Novel Lipoprotein Expressed by *Haemophilus ducreyi*

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Pooled sera from patients with chancroid contain antibodies to a *Haemophilus ducreyi* antigen with an approximate molecular weight of 28,000 (28K). Rabbit polyclonal serum that reacts to a 28K protein can be used to detect *H. ducreyi* in clinical samples. A monoclonal antibody, designated 5C9, bound to a 28K outer membrane protein and to 35 of 35 *H. ducreyi* isolates with diverse geographic origins and did not bind to many species of the families *Pasteurellaceae*, *Neisseriaceae*, and *Enterobacteriaceae* or to *Corynebacterium* and *Candida* species strains. A 5C9-reactive phage was recovered from a genomic library, and the gene encoding the 28K protein was localized to a 626-bp open reading frame, designated *hlp*, for *H. ducreyi* lipoprotein. Translation of *hlp* predicted a 23K polypeptide that contained a lipoprotein processing site. *Escherichia coli* transformed with a plasmid containing *hlp* expressed a novel, membrane-associated protein that could be labeled with [^{3}H]palmitic acid. In *H. ducreyi*, processing of Hlp was inhibited by globomycin. Database searches found no homologies to *hlp* or to the predicted Hlp amino acid sequence. Restriction enzyme analysis indicated that *hlp* was conserved among *H. ducreyi* isolates. Serum samples from patients with chancroid and other genital ulcer diseases and from normal subjects contained antibodies that bound to purified, recombinant Hlp. Although monoclonal antibody 5C9 recognizes a species-specific epitope of a unique *H. ducreyi* lipoprotein, the presence of serum antibodies to Hlp may not indicate previous infection with *H. ducreyi*.

Chancroid, caused by the gram-negative coccobacillus *Haemophilus ducreyi*, is a major sexually transmitted disease in developing countries and occurs sporadically in the United States (5, 33). Chancroid is a risk factor for human immunodeficiency virus transmission and seroconversion in areas where both infections are endemic (19, 27, 31). The association between chancroid and human immunodeficiency virus transmission has renewed interest in *H. ducreyi* pathogenesis, host responses, and the development of specific diagnostic reagents (33).

Identification of immunodominant antigens of *H. ducreyi* may facilitate the development of reagents to diagnose chancroid. However, antibodies that bind to *H. ducreyi* frequently cross-react with many species of the family *Pasteurellaceae* (1, 7, 24, 25). Absorption of serum samples with lysates of several *Haemophilus* sp. strains or other bacterial species may remove the cross-reactive antibodies and render serum *H. ducreyi* specific. For example, pooled chancroid patient sera, absorbed with a *Haemophilus* sp. strain lysate, bind to an *H. ducreyi* antigen with an apparent molecular weight of 28,000 or 28,500 (28K or 28.5K), depending on the strain tested (24). A pool of absorbed sera obtained from people with no history of chancroid does not bind to the 28K or 28.5K antigen, suggesting that the presence of anti-28K or anti-28.5K antibodies represents a specific immune response to *H. ducreyi* (24). Animals experimentally infected with *H. ducreyi* develop an antibody response to *H. ducreyi* antigens in the 26K to 29K size range (13, 23, 32). Rabbit polyclonal serum raised to *H. ducreyi* antigens and absorbed with a mixture of *Haemophilus* sp., *Corynebacterium* sp., *Escherichia coli*, and *Candida albicans* detects *H. ducreyi* in clinical specimens obtained from patients with culture-proven chancroid and does not react with lesion material of patients who have other genital ulcer diseases (25). In Western blot (immunoblot) analyses, the absorbed rabbit antiserum binds to a 28K or 28.5K antigen in each of 450 isolates of *H. ducreyi* tested (25). A monoclonal antibody (MAb) also binds to either the 28K or 28.5K antigen in all 450 isolates, indicating that these proteins are antigenically related (8, 25). Taken together, the data suggest that the 28K and 28.5K proteins may be useful as a serodiagnostic reagent for chancroid or as a target antigen for detection of *H. ducreyi*.

The 28K or 28.5K membrane protein of *H. ducreyi* has not been characterized on a molecular level (33). A gene encoding a 28K *H. ducreyi* antigen was isolated from an EMBL3 library; however, neither the gene nor its product was characterized (30). In this study, we report the cloning and sequence characterization of a gene encoding a 28K outer membrane protein (OMP) of *H. ducreyi*. We also examine the antigenic and physical properties of the 28K OMP and the prevalence of antibodies to this protein in sera from patients with chancroid and other genital ulcer diseases and from healthy subjects.

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MATERIALS AND METHODS

Bacteria and culture conditions. The 35 isolates of *H. ducreyi* and all other *Haemophilus*, *Neisseria*, *Actinobacillus*, and *Salmonella* sp. strains have been described previously (29). *C. albicans* ATCC 14053 and *Corynebacterium aquaticus* ATCC 14665 were obtained from Janet Reynolds, Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis. All strains were grown on chocolate agar with 1% IsoVitaleX at 35°C in a 5% CO_2 atmosphere as described previously (29). In some experiments, *H. ducreyi* was grown in broth consisting of brain heart infusion, 0.1% soluble starch, 50 μg of hemin per ml, and 1% IsoVitaleX at 34°C. *E. coli* DH5α, JM109, and Y1090r$^-$

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(Promega Biotech, Madison, Wis.) were grown in Luria-Bertani broth or agar at 37°C (17). Where appropriate, the media were supplemented with ampicillin (50 μg/ml).

Isolation of outer membranes. *H. ducreyi* outer membranes were prepared by Sarkosyl extraction of a French press lysate as described previously (29).

MAbs and sera. BALB/c mice were immunized intraperitoneally with a mixture of *H. ducreyi* CIP542, 85-023233, and 35000 suspended in phosphate-buffered saline, pH 7.1 (PBS). Hybridomas were established from splenic lymphocytes as described previously (29). Hybridomas that produced antibodies that recognized *H. ducreyi* were purified twice by limiting dilution.

Normal human sera obtained from subjects who had no history of chancroid, and sera from patients with proven chancroid, syphilis, or genital herpes, have been described elsewhere (28, 29).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blot, and colony blot assays. Whole cells, outer membranes, or purified proteins were solubilized in sample buffer, and the proteins were separated on 12.5 or 15% linear or 4 to 30% gradient polyacrylamide gels by the method of Laemmli (16). Gels were stained with Coomassie brilliant blue, or the proteins were transferred to nitrocellulose in a Semi-Phor blotter (Hoefer Scientific Instruments, San Francisco, Calif.). For colony blots, organisms were suspended in PBS, applied to nitrocellulose, and dried for 30 min. Western blots and colony blots were probed overnight with MAb 5C9 or serum diluted in 2% nonfat dry milk in PBS. In some experiments, sera were absorbed with a lysate containing *H. influenzae*, *H. parainfluenzae*, and *H. parahaemolyticus* (kindly provided by Cheng-Yen Chen, Centers for Disease Control and Prevention, Atlanta, Ga.) as described previously (7). In all experiments, the final serum concentration was 1:200. Bound antibody was detected with protein A or goat anti-human immunoglobulin G(γ) coupled to horseradish peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) and horseradish peroxidase color development reagent (Bio-Rad Laboratories, Richmond, Calif.).

Immunoelectron microscopy. *H. ducreyi* cells were collected from a chocolate agar plate and suspended in distilled water. The suspension was placed onto Formvar-coated copper grids and dried. The cells were probed with either MAb 5C9 or Sp2/0-Ag14 tissue culture supernatant, washed, and stained with protein A coupled to 10-nm gold spheres (Sigma Chemical, St. Louis, Mo.). Cells were examined with a Philips CM10 electron microscope (Philips Electronics, Eindhoven, The Netherlands) at 60 kV. The distribution of cells that bound various numbers of gold particles were statistically compared in a nonparametric (Mann-Whitney *U*) test as described previously (18).

Absorption of MAb 5C9 with intact bacteria. Absorption of MAb 5C9 was performed according to a modification of a method described previously (6). Briefly, MAb 5C9 was diluted in PBS and incubated with either intact *H. ducreyi* 85-023233 or *E. coli* y1090r$^-$ for 1 h. To monitor cell viability, colony counts were performed before and after incubation. Bacteria were removed by centrifugation, and the supernatant was used to probe an *H. ducreyi* lysate in Western blotting.

DNA manipulation. The genomic λgt11 library constructed from *H. ducreyi* 85-023233 was described previously (4). Isolation of bacteriophage and plasmid DNA, restriction enzyme digestion, and ligation of plasmid DNA were performed according to standard protocols (17). *E. coli* strains were transformed by electroporation in a Cell-Porator equipped with a Voltage Booster (BRL Life Technologies, Grand Island, N.Y.) according to the manufacturer's instructions. Plasmids pGEM3Zf(+) and pGEM7Zf(+) were obtained from Promega Biotech. Plasmid pHD24, which encodes the major pilin subunit of *H. ducreyi*, was described previously (4).

DNA sequencing. Both strands of the entire insert of pHD28.5 were sequenced by the dideoxy-chain termination method, using synthetic oligonucleotide primers (Biochemistry Biotechnology Facility, Indiana University School of Medicine, Indianapolis) and the Sequenase version 2.0 sequencing kit (United States Biochemical, Cleveland, Ohio) (26).

[^{3}H]palmitic acid labeling and globomycin inhibition. Labeling of *E. coli* proteins was performed according to a modification of the method of Woods et al. (34). *E. coli* JM109 transformed with pHD28.5, pHD24, or pGEM3Zf(+) was grown in M9 minimal medium supplemented with 2% Casamino Acids at 37°C to early log phase. Two hundred microcuries of [9, 10(n)-^{3}H]palmitic acid (specific activity, 54 Ci/mmol; Amersham Corporation, Arlington Heights, Ill.) was added, and the cultures were grown for approximately 3 h. The cells were pelleted by centrifugation, suspended in PBS, and lysed by sonication and rapid freeze-thawing. Cellular debris was pelleted at 2,000 × g for 10 min at 4°C, and the supernatant was collected. The membrane fraction was obtained by centrifugation of the supernatant at 25,000 × g for 3 h at 4°C. Membrane proteins were solubilized, separated by SDS-PAGE, transferred to nitrocellulose, and prepared for autoradiography with En^{3}Hance spray (NEN Research Products, Boston, Mass.). The blots were autoradiographed for 8 days at −70°C.

The MIC of globomycin (a kind gift from Janne Cannon, University of North Carolina, Chapel Hill) for *H. ducreyi* was determined by the broth dilution method (12). *H. ducreyi* 35000 was grown overnight in broth. The bacteria were diluted in fresh medium, and globomycin was added at early log phase to a final concentration of 25 μg/ml. Incubation of the culture was continued for another 2 h. The cells were harvested and analyzed by Western blotting with MAb 5C9.

PCR and restriction fragment length polymorphism analysis. The open reading frame (ORF) encoding the 28K OMP was amplified from *H. ducreyi* genomic DNA with synthetic primers and the reagents provided in the GeneAmp kit (Perkin-Elmer, Branchburg, N.J.). Reactions were performed in a GeneAmp PCR System 9600 thermocycler (Perkin-Elmer). Thirty cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min were used. Amplicons were purified by phenol-chloroform extraction and subjected to restriction digests with *Pst*I and *Hae*II. Amplicons and digestion products were electrophoresed in 1.8% agarose gels (FMC BioProducts, Rockland, Maine) and visualized by ethidium bromide staining.

TABLE 1. Binding of MAb 5C9 to various species of bacteria

Species	No. positive/ no. tested
Haemophilus ducreyi	35/35
H. influenzae	0/9
Haemophilus sp.	0/9
Actinobacillus actinomycetemcomitans	0/2
Neisseria sp.	0/4
Salmonella minnesota	0/2
Escherichia coli	0/2
Corynebacterium aquaticus	0/1
Candida albicans	0/1

Fusion protein expression and purification. The Xpress System kit (Invitrogen Corporation, San Diego, Calif.) was used to express and purify a polyhistidine-containing 28K OMP fusion protein. The ORF encoding the 28K OMP was amplified from *H. ducreyi* 35000 genomic DNA by using synthetic primers containing *Eco*RI sites located 5′ to the coding sequences, digested with *Eco*RI, and ligated into *Eco*RI-digested pRSET B. Following transformation of the recombinant plasmid into *E. coli* JM109, fusion protein expression was induced by infection with T7 polymerase-expressing M13 phage (Xpress system). Recombinant protein was purified from a cleared lysate by using ProBond Columns and the native buffer system provided in the kit. Fractions were analyzed by SDS-PAGE and Western blotting, and those that bound to MAb 5C9 were pooled. Fractions were dialyzed overnight against PBS in 10 10K-cutoff Slidalyzers (Pierce, Rockford, Ill.) and concentrated with a Centricon-10 microconcentrator (Amicon Corp., Beverly, Mass.).

Nucleotide sequence accession number. The sequence of *hlp* will appear under GenBank accession number U57817.

RESULTS

Binding of MAb 5C9 to *H. ducreyi*. In colony blots, MAb 5C9 bound to all 35 *H. ducreyi* strains tested but did not bind to *C. albicans*, *C. aquaticus*, or any other members of the families *Pasteurellaceae*, *Neisseriaceae*, and *Enterobacteriaceae* (Table 1). In Western blot analysis, MAb 5C9 bound to a 28K or 28.5K protein in 10 isolates with diverse geographic origins (Fig. 1). MAb 5C9 also bound a 28K protein in a Sarkosyl-insoluble fraction of *H. ducreyi* (Fig. 1). These data suggested

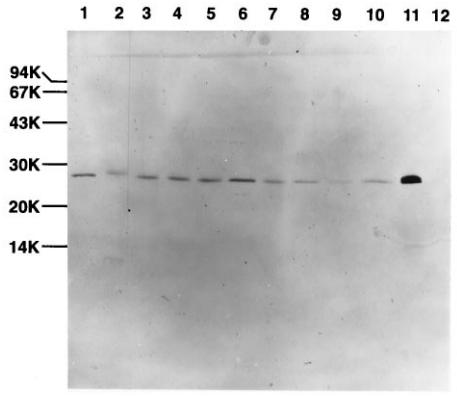

FIG. 1. Western blot of whole cell preparations and OMPs probed with MAb 5C9. Lanes 1 to 10, *H. ducreyi* strains; lane 11, OMP preparation; lane 12, *E. coli* JM109. A 28.5K protein was expressed by the Kenyan strain 33921 (lane 2). The relative migration of molecular markers is shown on the left.

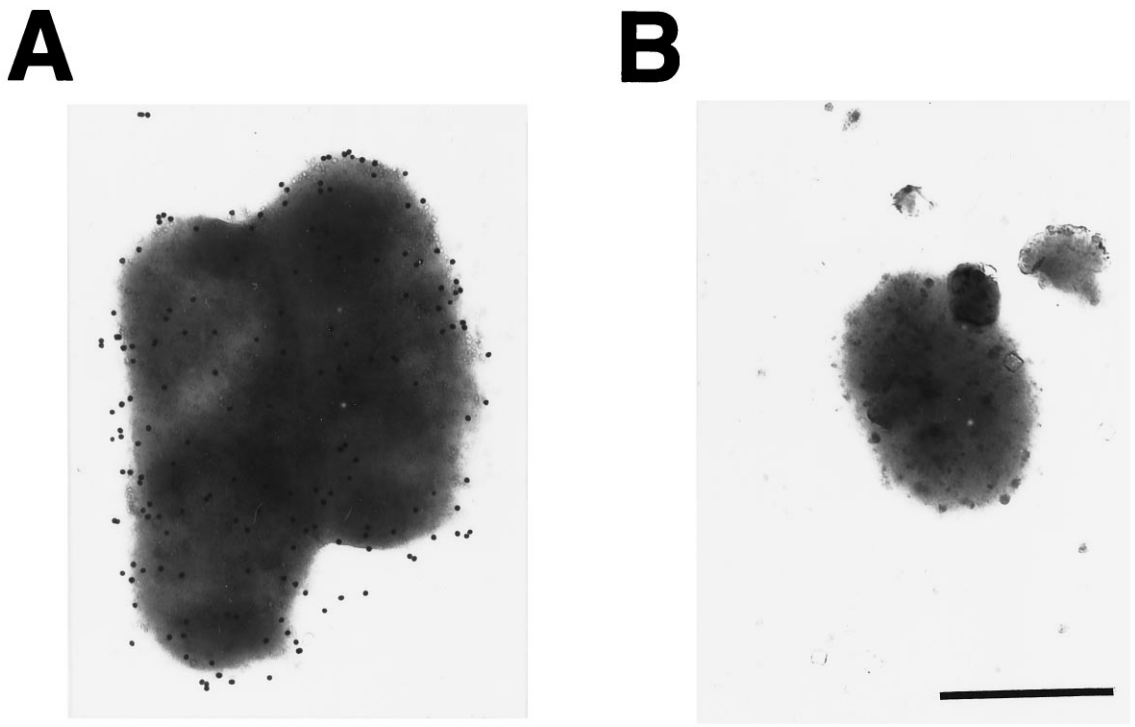

FIG. 2. Electron micrographs of *H. ducreyi* 35000 whole cells probed with MAb 5C9 (A) or Sp2/0-Ag14 (B) tissue culture supernatants and goat anti-mouse immunoglobulin G coupled to 10-nm gold spheres. Magnification, ×30,000; bar, 0.5 μm.

that MAb 5C9 bound to a species-specific epitope and that the 28K protein was localized to the outer membrane.

Surface exposure of the MAb 5C9 epitope. In immunoelectron microscopy, *H. ducreyi* 35000 cells exhibited variable binding of gold spheres when probed with MAb 5C9 (Fig. 2). Little to no binding was observed when Sp2/0-Ag14 was used as a probe (Fig. 2). The number of gold spheres that bound to each of 100 *H. ducreyi* 85-023233 cells after incubation with MAb 5C9 was statistically compared with the number obtained with the Sp2/0-Ag14 control (29). When probed with MAb 5C9, 3 cells had heavy (≥20 spheres), 12 cells had moderate (6 to 19 spheres), and 85 cells had low (0 to 5 spheres) binding. The 100 cells that were probed with Sp2/0-Ag14 bound no spheres or one sphere. The level of MAb 5C9 surface labeling was significantly greater than that of the Sp2/0-Ag14 control (Mann-Whitney *U* test; $P = 0.0001$). To confirm that the 5C9 epitope was surface exposed, MAb 5C9 was incubated with intact bacteria and then used to probe *H. ducreyi* in Western blot analysis. Absorption with *H. ducreyi* abolished the reactivity of MAb 5C9, while absorption with *E. coli* had no effect on MAb 5C9 binding (data not shown). Colony counts showed no loss of bacterial viability during the absorption procedure, indicating that MAb 5C9 was not binding to an epitope exposed upon lysis of the bacteria. Thus, the 5C9 epitope was surface exposed by two criteria.

Cloning and sequence analysis of the gene encoding the 28K OMP. Approximately 3,000 plaques of an *H. ducreyi* λgt11 genomic library were screened with MAb 5C9, and 9 positive plaques were identified. A 4.1-kb insert from one of the positive phages was ligated into pGEM7Zf(+) and designated pHD28. A 1.3-kb *Hin*dIII-*Cla*I fragment was subcloned from pHD28 into pGEM3Zf(+) to generate pHD28.5. *E. coli* DH5α transformed with pHD28 or pHD28.5 expressed a 28K protein and a protein of approximately 18K that bound to MAb 5C9 (Fig. 3).

Although pHD28.5 expressed two proteins that bound 5C9, the sequence of the 1,314-bp insert of pHD28.5 contained a single complete 626-bp ORF (Fig. 4). The ORF was preceded by −10 and −35 regions and a ribosome binding site and was followed by an imperfect inverted repeat, indicative of a factor-independent termination signal (14). The predicted polypeptide encoded by the ORF was approximately 23K and contained a putative leader peptide with a signal peptidase II cleavage site (21). The data suggested that the 28K OMP was a lipoprotein, and the gene was designated *hlp*, for *H. ducreyi* lipoprotein. The 18K species most likely was encoded by *hlp* and probably represents a breakdown product. Some lipoproteins migrate aberrantly in SDS-PAGE (35), and this may account for the discrepancy between the predicted molecular weight of the mature protein (21K) and its apparent molecular weight in SDS-PAGE (28K).

[^{3}H]palmitate labeling and globomycin sensitivity of Hlp processing. To test the hypothesis that Hlp was a lipoprotein, *E. coli* transformed with pHD28.5 or control plasmids was grown in the presence of [^{3}H]palmitate. Compared to *E. coli*

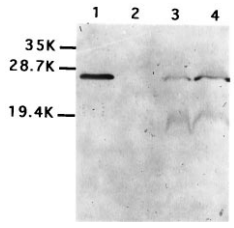

FIG. 3. Western blot of whole cells probed with MAb 5C9. Lane 1, *H. ducreyi* 35000; lane 2, *E. coli* DH5α transformed with pGEM3Zf(+); lane 3, *E. coli* DH5α transformed with pHD28; lane 4, *E. coli* DH5α transformed with pHD28.5. The relative migration of molecular markers is shown on the left.

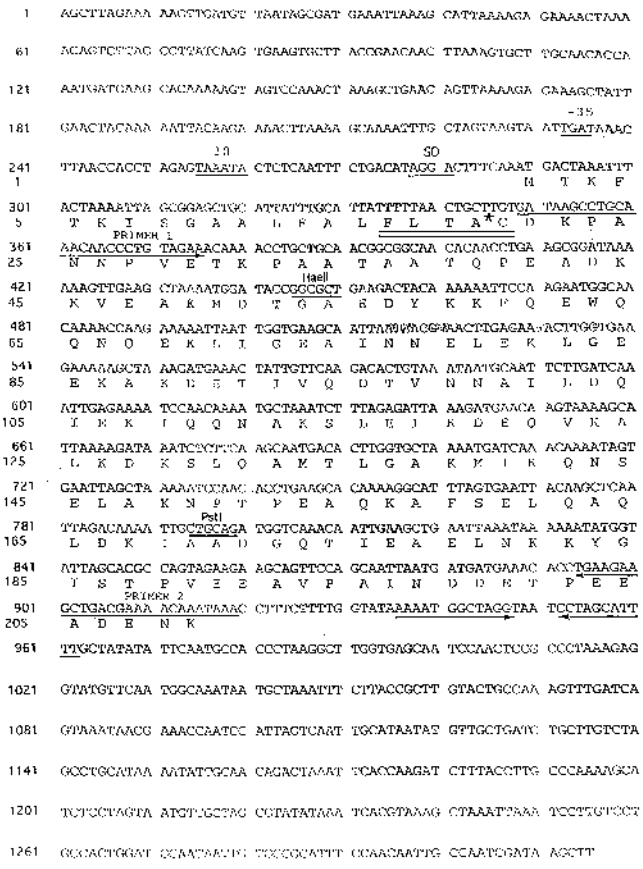

FIG. 4. Nucleotide sequence of the insert of pHD28.5 and predicted amino acid sequence of Hlp. Putative −35, −10, and Shine Dalgarno (SD) regions and sequences of the 5′ and 3′ primers are underlined. The lipoprotein processing site is double underlined. Cleavage at the site indicated by the asterisk would result in the cysteine occupying the +1 amino acid position of the mature protein. The positions of *Hae*II and *Pst*I sites are indicated.

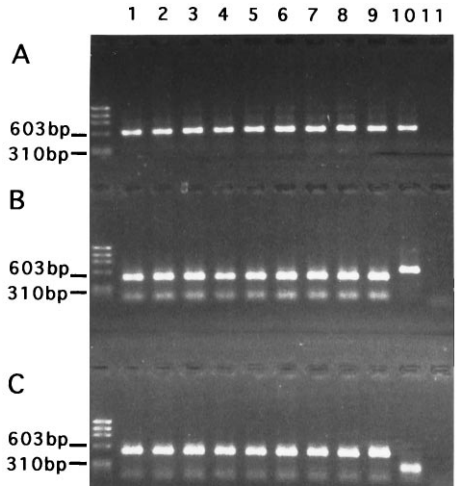

FIG. 6. Agarose gel electrophoresis of PCR products stained with ethidium bromide. DNAs were amplified with the 5′ and 3′ ORF primers (A) and digested with *Pst*I (B) and *Hae*II (C). Amplicons were obtained from 10 *H. ducreyi* strains (lanes 1 to 10) and *E. coli* JM109 (lane 11). Several independent PCR products obtained from the Kenyan strain 33921 (lane 10) were resistant to *Pst*I digestion and had a distinct *Hae*II digestion pattern. Relative migration of DNA standards is shown at the left.

containing pGEM3Zf(+) and pHD24, *E. coli* cells containing pHD28.5 expressed a novel 28K labeled protein (Fig. 5). To confirm that Hlp was processed by signal peptidase II-like enzyme, *H. ducreyi* 35000 was grown in the presence and absence of globomycin. The MIC of globomycin for *H. ducreyi* was 25 μg/ml. Treatment with globomycin resulted in the appearance of a 30K protein that bound to MAb 5C9 in Western

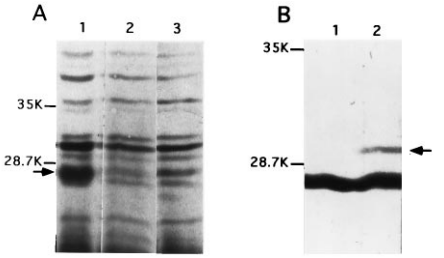

FIG. 5. (A) Autoradiograph of [^{3}H]palmitate-labeled proteins separated by SDS-PAGE from membrane preparations of *E. coli* JM109 transformed with the following plasmids: lane 1, pHD28.5; lane 2, pGEM3Zf(+); lane 3, pHD24. The unique labeled protein in cells containing pHD28.5 is indicated by the arrow. (B) Western blot of whole cells of *H. ducreyi* 35000 probed with MAb 5C9. The cells were grown in the absence (lane 1) and presence (lane 2) of globomycin. A shift in the migration of Hlp is indicated by the arrow. The relative migration of molecular markers is shown on the left.

blot analysis (Fig. 5). The 30K band probably represented unprocessed Hlp (15), and its detection confirmed the presence of a signal peptidase II-like enzyme in *H. ducreyi* (28).

Homologies and conservation of *hlp*. A BLAST algorithm search (3) of the PDB, Swiss-Prot, PIR, and GenBank databases found no homologies to either *hlp* or the predicted Hlp amino acid sequence. No homologies to *hlp* were found in the *H. influenzae* Rd genome (9).

Genomic DNA from 10 *H. ducreyi* isolates with diverse geographic origins and from *E. coli* JM109 were amplified by PCR with primers 1 and 2 (Fig. 4). Amplicons of the expected size were obtained from all 10 of the *H. ducreyi* isolates. Nine of the ten amplicons yielded identical restriction fragments when digested with either *Pst*I or *Hae*II (Fig. 6). One amplicon, generated from the Kenyan isolate 33921, was repeatedly resistant to *Pst*I digestion and had a unique *Hae*II digestion pattern (Fig. 6, lane 10). This isolate also expressed the 28.5K form of Hlp (Fig. 1, lane 2). The data suggested that the divergence in *hlp* among strains of *H. ducreyi* correlated with the expression of the 28K or 28.5K form of Hlp.

Presence of antibodies to Hlp in human sera. A recombinant form of mature Hlp was purified and analyzed by Western blotting with human sera. Sera from eight patients with chancroid, two patients with genital herpes, three patients with syphilis, and six healthy volunteers with no history of chancroid contained antibodies to recombinant Hlp (Fig. 7). Although patients with chancroid generally had stronger reactivity to recombinant Hlp than patients with other genital ulcer diseases or normal subjects, sera from individuals without a history of chancroid sometimes had strong reactivity (Fig. 7). Anti-Hlp antibodies were not completely absorbed from any of the sera by a *Haemophilus* sp. strain lysate (data not shown). These data indicated that the presence of antibodies to Hlp in serum may not correlate with a history of *H. ducreyi* infection.

DISCUSSION

All isolates of *H. ducreyi* tested expressed a 28K or 28.5K OMP that contained an epitope defined by MAb 5C9. MAb

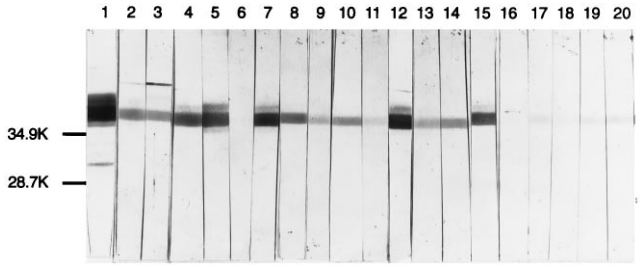

FIG. 7. Western blot of purified recombinant Hlp probed with MAb 5C9 or with human sera. Lane 1 was probed with MAb 5C9; lanes 2 to 9 were probed with sera from patients with chancroid; lanes 10 and 11 were probed with sera from patients with genital herpes; lanes 12 to 14 were probed with sera from patients with syphilis; lanes 15 to 20 were probed with sera from volunteers with no history of chancroid. The relative migration of molecular markers is shown on the left.

5C9 did not bind to any other species tested. The gene encoding the 28K OMP, designated *hlp*, was isolated from an *H. ducreyi* genomic library. BLAST and *H. influenzae* Rd database searches found no homologies to *hlp* or the predicted Hlp amino acid sequence. Taken together, these data suggest that MAb 5C9 binds to an epitope that was species specific and that Hlp is conserved among *H. ducreyi* isolates.

Immunoelectron microscopy indicated that MAb 5C9 bound variably to a surface-exposed epitope. Antibody binding may have been affected by degradation of Hlp, phase variation of Hlp or other bacterial surface components, or fixation of cells to the hydrophobic surface by drying. Absorption experiments confirmed that the MAb 5C9 epitope was surface exposed. Although the cause of the variable binding of MAb 5C9 in immunoelectron microscopy is unclear, similar results have been described for other surface-exposed epitopes in *H. ducreyi* and other organisms (18, 29).

The predicted leader peptide of Hlp contained a consensus signal peptidase II processing site. When grown in the presence of [^{3}H]palmitate, *E. coli* containing *hlp* expressed a novel, labeled 28K protein. In *H. ducreyi*, Hlp processing was sensitive to the signal peptidase II inhibitor globomycin. These data indicate that Hlp is a lipoprotein and that *H. ducreyi* possesses an enzyme similar to *E. coli* signal peptidase II, as we have reported previously (28).

A feature of the predicted amino acid sequence of processed Hlp is the presence of aspartate and lysine residues at the +2 and +3 positions (Fig. 4). A +2 aspartate leads to the sorting of lipoproteins to the inner membrane in *E. coli* (20, 36). However, site-specific mutations in the leader peptide of the Braun lipoprotein show that sorting of lipoproteins is also influenced by the identity of the amino acid in the +3 position (10). For example, constructs containing +2 aspartate, +3 serine localize to the inner membrane while proteins containing a +2 aspartate, +3 lysine are sorted to both the inner and outer membrane (10). Alternatively, Hlp may be analogous the *Klebsiella pneumoniae* lipoprotein pullulanase, which also contains a +2 aspartate (22). Pullulanase is sorted to the inner membrane, where it engages specific secretion factors that mediate the transport of the protein through the outer membrane to the cell surface (20, 21). Whether the sorting of lipoproteins in *H. ducreyi* is similar to that of *E. coli* or *K. pneumoniae* is unknown. Our data indicate that Hlp is located in the outer membrane in that Hlp is Sarkosyl insoluble and the MAb 5C9 epitope is surface exposed. However, we did not test whether Hlp is also present in the inner membrane.

The strain-dependent size of Hlp of 28K or 28.5K correlated directly with restriction enzyme analysis of *hlp*, suggesting that there are at least two alleles of *hlp* among *H. ducreyi* isolates. The characteristics of Hlp are very similar to those attributed to the 28K to 28.5K antigen described by Roggen et al. (24, 25). Their data suggest that patients with chancroid develop antibodies to the 28K or 28.5K protein. Studies in animal models support an immunodominant role for *H. ducreyi* antigens in the 28K size range. Rabbits and macaques experimentally infected with *H. ducreyi* develop a strong antibody response to antigens in the 26K to 29K range (11, 23, 32). Hobbs et al. reported that experimentally infected pigs develop antibodies to an antigen that comigrates with a MAb 5C9-reactive protein (13). These data led us to hypothesize that Hlp could induce an *H. ducreyi*-specific antibody response during human infection and that Hlp could be used as a reagent for the serodiagnosis of chancroid. However, sera from patients with chancroid, syphilis, and genital herpes and healthy volunteers contained antibodies to recombinant Hlp. Some sera with strong reactivity to Hlp were from individuals with no history of chancroid. Absorption of the sera with a lysate of *H. influenzae*, *H. parainfluenzae*, and *H. parahaemolyticus* did not completely remove the anti-Hlp antibodies from the sera of both chancroid patients and controls. Although we cannot exclude the possibility that chancroid patient sera contain antibodies to *H. ducreyi*-specific epitopes on Hlp, the data presented here suggest that anti-Hlp antibodies do not arise necessarily from infection with *H. ducreyi* and that Hlp may not be a useful reagent for the serodiagnosis of chancroid.

Normal human serum contains antibodies that cross-react with *H. ducreyi* (1, 2, 28, 29). Cross-reacting antibodies to *H. ducreyi* are believed to arise in response to colonization or infection of the host by other microorganisms (28). Anti-Hlp antibodies in individuals with no history of chancroid probably arise in a similar manner, indicating the existence of an organism(s) that expresses an antigen(s) that is antigenically similar to Hlp. These organisms need to be identified and included in absorbents if Hlp is to be used as the target in diagnostic procedures that require serum.

In summary, we have described an *H. ducreyi* outer membrane lipoprotein that contains a species-specific epitope. We conclude that despite the observation that Hlp has no homology to the sequences in present databases, Hlp-like protein(s) may exist in other organisms and could reduce the specificity and sensitivity of Hlp-based diagnostic reagents for chancroid. Future studies will include the construction and evaluation of an Hlp deletion mutant and evaluation of the biological effect of anti-Hlp antibodies on *H. ducreyi*.

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