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The Conserved 18,000-Molecular-Weight Outer Membrane Protein of *Haemophilus ducreyi* Has Homology to PAL

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Haemophilus ducreyi **expresses an 18,000-molecular-weight outer membrane protein that contains a conserved surface-exposed epitope recognized by monoclonal antibody 3B9. Monoclonal antibody 3B9 cross-reacts with proteins of similar molecular weight found in many *Haemophilus* sp. strains, including P6, a candidate vaccine for *Haemophilus influenzae*. The gene encoding the 18,000-molecular-weight outer membrane protein was identified by screening a λgt11 genomic library with 3B9. The coding sequence of the gene was localized to a 471-bp open reading frame, designated *pal* (peptidoglycan-associated lipoprotein). Translation of *pal* predicted a mature polypeptide with a molecular weight of 15,000 that had extensive homology with P6 and *Escherichia coli* PAL. The predicted signal peptide had features characteristic of a prokaryotic lipoprotein, and processing of PAL was sensitive to globomycin in *H. ducreyi*. The sequences encoding mature *H. ducreyi* PAL were subcloned into the vector pRSET B and expressed as a polyhistidine-containing fusion protein that bound 3B9. In Western blot (immunoblot) analysis, serum samples obtained from healthy subjects and patients with chancroid or other genital ulcer diseases contained antibodies to purified PAL. Antibodies that bound to PAL were removed by absorption with a lysate of *Haemophilus* sp. antigens, suggesting that patients with chancroid do not develop an *H. ducreyi*-specific antibody response to PAL.**

Haemophilus ducreyi is the etiologic agent of chancroid, a common genital ulcer disease in developing countries (32, 50). *H. ducreyi* and human immunodeficiency virus (HIV) appear to facilitate the transmission of each other in a process called epidemiologic synergy (1, 49, 51). Genital ulcer disease has an estimated cofactor effect on HIV transmission ranging from 10 to 300 per individual sexual exposure (19). The impact of chancroid on heterosexually acquired HIV infection in certain populations has renewed interest in *H. ducreyi* pathogenesis, host responses, and vaccine development (50).

Immunization with *H. ducreyi* outer membrane envelopes confers partial protection against experimental infection in the temperature-dependent rabbit model (17). Only a few components of *H. ducreyi* outer membrane have been characterized (50). The major outer membrane protein is an OmpA-like protein and contains conserved epitopes that are species specific or shared with other members of the family *Pasteurellaceae* (45). The 28,000-molecular weight (28K) outer membrane protein (OMP) is a unique lipoprotein that contains an *H. ducreyi*-specific surface-exposed epitope (19a). A hemoglobin-binding receptor, HgbA, is regulated by environmental levels of heme and has similarity to TonB-dependent outer membrane receptors (13, 14). *H. ducreyi* lipooligosaccharides immunochemically resemble human glycosphingolipid antigens, can be sialylated, and produce skin abscesses in rabbits (4, 6–8, 30, 41). The role of any of these outer membrane components in the pathogenesis of human disease, host responses, or protective immunity is currently unknown.

We previously reported that *H. ducreyi* expresses an 18K OMP that contains a conserved surface-exposed epitope defined by monoclonal antibody (MAb) 3B9 (44). MAb 3B9 cross-reacts with many proteins of similar molecular weight found in members of the family *Pasteurellaceae* and binds to the 16.6K peptidoglycan-associated lipoprotein (P6 or PAL) of *Haemophilus influenzae*. Patients with chancroid and other genital ulcer diseases frequently have serum antibodies to a protein that comigrates with the 18K OMP. Whether these antibodies resulted from infection with *H. ducreyi* or previous exposure to other species of the family *Pasteurellaceae* is unknown.

P6 is receiving intensive study as a vaccine candidate to prevent *H. influenzae* infections (35). P6 and the 18K OMP share several features, including conservation within each species, surface exposure, and antigenic stability. To facilitate studies of the 18K OMP as a vaccine candidate, we report the cloning, the sequence, and restriction fragment length polymorphism analysis of the gene encoding this protein. We also report purification of a recombinant form of the protein and investigation of whether patients with chancroid develop antibody responses to *H. ducreyi*-specific epitopes on the protein.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *H. ducreyi* 85-023233 (New York City), 35000 (Winnipeg), CIP 542 (Hanoi), Hd 183 (Singapore), Hd 188 (Kenya), 82-029362 (California), 84-018676 (Florida), 6644 (Boston), ATCC 33921 (Kenya), and R-1 (Rochester, N.Y.) were described previously (44). The strains were grown on chocolate agar supplemented with 1% IsoVitaleX at 35°C in a 5% CO_2 atmosphere.

Escherichia coli Y1090r-, DH5α, and JM109 (Promega Biotech, Madison, Wis.) were grown on LB agar or broth at 37°C. Where appropriate, *E. coli* strains or transformants were grown on media containing 100 μg of ampicillin per ml.

MAbs and serum. MAb 3B9 was described previously (3, 44). Serum samples obtained from patients with proven chancroid, syphilis, and genital herpes seen in Buffalo or Rochester, N.Y., were described previously (44). Serum samples obtained in Eldoret, Kenya, from two patients who had clinical chancroid and positive serological studies for *H. ducreyi* (38) were kindly provided by Kara Wools, Indiana University, Indianapolis. Normal human serum was obtained from six laboratory workers with no history of genital ulcer disease. Informed consent was obtained from the subjects in accordance with the human experimentation guidelines of the U.S. Department of Health and Human Services and

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the Institutional Review Board of Indiana University-Purdue University, Indianapolis.

SDS-PAGE, Western blot (immunoblot), plaque, and colony blot assays. Whole bacteria or purified protein were solubilized and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 15% acrylamide gels by the method of Laemmli (24) and stained with Coomassie brilliant blue or transferred to nitrocellulose in a Semi-Phor blotter (Hoefer Scientific Instruments, San Francisco, Calif.) (44). Recombinant plaques or colonies of *E. coli* transformants were blotted onto nitrocellulose discs impregnated with isopropylthiogalactopyranoside (IPTG) as previously described (23). Western blots, plaque blots, and colony blots were probed with tissue culture supernatants or serum, protein A peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, Md.), and horseradish peroxidase color developer (Bio-Rad Laboratories, Richmond, Calif.) as described previously (44). In some experiments, serum was absorbed with a soluble antigen mixture prepared from *H. influenzae* G3588, *H. parainfluenzae* G1142, and *H. parahaemolyticus* G4198 (kindly provided by Cheng-Yen Chen, Centers for Disease Control and Prevention, Atlanta, Ga.) as described previously (12, 46). The final dilution of absorbed and unabsorbed serum was 1:200.

Inhibition of processing of the 18K OMP with globomycin. *H. ducreyi* 35000 was grown in broth to mid-log phase. The culture was divided and grown in the presence and absence of 25 μg of globomycin per ml for 1.5 h (21). On the basis of optical density measurements, equal amounts of harvested cells were probed by Western blotting with MAb 3B9.

Cloning. Chromosomal DNA, phage DNA, and plasmid DNA were prepared as described previously (28, 29). *H. ducreyi* 85-023233 was the source of chromosomal DNA used to construct a λgt11 library (5). *E. coli* Y1090r- was transfected with recombinant phage, and plaques were screened with MAb 3B9. Reactive clones were plaque purified twice. An insert was recovered from one recombinant phage by digestion with *Eco*RI, agarose gel electrophoresis, and treatment with Geneclean (Bio 101 Inc., La Jolla, Calif.). The insert was ligated into the *Eco*RI-digested plasmid pGEM-7Zf(+) (Promega Biotech) and transformed into *E. coli* DH5α by electroporation (16). Transformants were screened with MAb 3B9, and a plasmid recovered from one reactive transformant was designated pHD18.

DNA sequencing. To localize coding sequences within the pHD18 insert, exonuclease III (Exo III) digestion was performed with the Erase-a-Base system (Promega Biotech). Plasmid vector sequences were digested with *Sac*I to generate an Exo III-resistant 3′ end and with *Sma*I to generate an Exo III-sensitive blunt end. Exo III digestion was performed at 30°C at a rate of 200 bp/min. Deletion products were ligated, transformed into *E. coli* DH5α, and screened for reactivity with MAb 3B9.

Plasmid DNAs were sequenced by the dideoxy-chain termination method (39), using synthetic oligonucleotide primers (Biochemistry Biotechnology Facility, Indiana University School of Medicine, Indianapolis) and a Sequenase version 2.0 kit (United States Biochemical, Cleveland, Ohio). Sequencing reactions were resolved on 8% acrylamide gels with 8 M urea. Dried gels were autoradiographed overnight. Some sequence was obtained with an Applied Biosystems model 373A automated fluorescence sequencer (Biochemistry Biotechnology Facility). Both strands of the insert were sequenced.

PCR and restriction fragment length polymorphism analysis. Genomic DNAs from *H. ducreyi* strains were used as a template for PCR. Coding sequences were amplified by using synthetic primers. Amplification reactions were performed by using *Taq* polymerase in a GeneAmp PCR System 9600 thermal reactor (Perkin-Elmer, Norwalk, Conn.) with reagents supplied by the GeneAmp PCR kit (Perkin-Elmer). Intact amplicons and amplicons digested with *Hae*II or *Rsa*I were electrophoresed on 4% NuSieve agarose (FMC BioProducts, Rockland, Maine) gels and stained with ethidium bromide. *E. coli* JM109 chromosomal DNA and pHD18 DNA served as negative and positive controls for this experiment.

Insertional inactivation of the gene encoding the 18K OMP. Transposon mutagenesis was done in *E. coli* by using the shuttle mutagenesis system of Seifert et al. (42). The pHD18 insert was subcloned into pHSS8 and transformed into *E. coli* RDP146(pTCA). One MAb 3B9-reactive transformant was mated with RDP146 [pOX38::mTn*3*(Cm)], cointegrates were resolved by conjugation with *E. coli* NS2114Sm, and transconjugants were screened for loss of expression with MAb 3B9. The position of the transposon insertion in the plasmid was determined by sequencing.

Attempts to construct an isogenic mutant were done in *H. ducreyi* 35000, which is sensitive to chloramphenicol and transformable by electroporation (16). Plasmid or insert DNA (10 μg) was transformed into *H. ducreyi* 35000 by electroporation (16), using the Cel-Porator electroporation system (Gibco-BRL, Gaithersburg, Md.). Transformants were plated on chocolate agar supplemented with 2 μg of chloramphenicol per ml and screened for loss of MAb 3B9 reactivity in colony or Western blots.

Southern blots. *H. ducreyi* DNA was digested to completion with the appropriate restriction enzyme and electrophoresed on 0.8% agarose gels. Southern blots were probed with either the cloned *H. ducreyi* insert or coding sequences amplified by PCR under high-stringency conditions as described previously (43). Probes were labeled by random priming (NEBlot Phototope kit; New England Biolabs, Beverly, Mass.). Blots were developed with a Phototope detection kit (New England Biolabs).

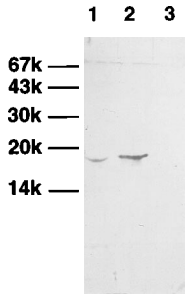

FIG. 1. Western blot of whole cells probed with 3B9. Lane 1, *H. ducreyi* 85-023233; lane 2, *E. coli* DH5α transformed with pHD18; lane 3, *E. coli* DH5α transformed with pGEM 7Zf(+). The relative migration of molecular weight standards is shown on the left.

Purification of the recombinant 18K OMP. The sequences encoding the mature 18K OMP were amplified from pHD18 by using synthetic primers that contained *Eco*RI sites 5′ to the coding sequences. The PCR product was digested with *Eco*RI, ligated into *Eco*RI-digested pRSET B (Xpress system; Invitrogen, San Diego, Calif.), and transformed into *E. coli* JM109. Transformants were induced to express fusion proteins with M13/T7 phage according to the manufacturer's instructions and screened with MAb 3B9. Recombinant protein was purified by using ProBond columns (Xpress System) according to the manufacturer's instructions. The protein was eluted from the column by using native pH elution buffer (Xpress System). Fractions of 1 ml were collected and analyzed by SDS-PAGE, and Western blots were probed with 3B9. Fractions containing purified protein were pooled, dialyzed with phosphate-buffered saline, and concentrated with a Centricon-10 microconcentrator (Amicon Corp., Beverly, Mass.).

Nucleotide sequence accession number. The sequence shown in Fig. 2 was submitted to GenBank and assigned accession number U42466.

RESULTS

The 18K OMP has characteristics of a lipoprotein. A chromosomal library was constructed from *H. ducreyi* 85-023233 in phage λgt11. Of 10,000 plaques screened, 6 bound MAb 3B9. A 2.8-kb insert was purified from one phage, ligated into pGEM-7Zf(+), and designated pHD18. *E. coli* DH5α transformed with pHD18 expressed an 18K protein that bound 3B9 in the absence of IPTG induction (Fig. 1). The pHD18 insert contained no *Xba*I or *Ava*I sites and one *Hin*dIII site. In Southern blots, the insert hybridized to a 4.1-kb *Xba*I fragment, an 11-kb *Ava*I fragment, and 4.3- and 6.4-kb *Hin*dIII fragments of *H. ducreyi* 85-023233 and 35000 chromosomal DNA and did not hybridize to *E. coli* DNA (data not shown).

A nested set of deletions was made in the insert by Exo III digestion, and *E. coli* transformants were screened for loss of reactivity to MAb 3B9. The gene encoding the 3B9-reactive protein was localized to a 471-bp open reading frame designated *pal* (Fig. 2). The open reading frame was preceded by a typical Shine-Dalgarno sequence and −10 and −35 promoter sequences. The predicted N-terminal amino acid sequence had features of a typical lipoprotein signal peptide and signal peptidase II processing site (Val-Leu-Thr-Ala-Cys) (18, 37). The predicted molecular weight of the mature polypeptide was 15,000, which corresponded to the apparent molecular weight of the protein determined by SDS-PAGE. A region of GC-rich dyad symmetry surrounded by runs of d(AT) was found distal to the translational terminator and probably represents an independent transcriptional terminator (20).

To test the hypothesis that the 18K OMP was subject to processing by signal peptidase II, *H. ducreyi* 35000 was grown in broth in the presence and absence of globomycin. Treatment of cells with globomycin caused the 18K OMP to migrate as a doublet, with the appearance of a novel band of approximately 20K. The data suggest that a precursor protein had accumu-

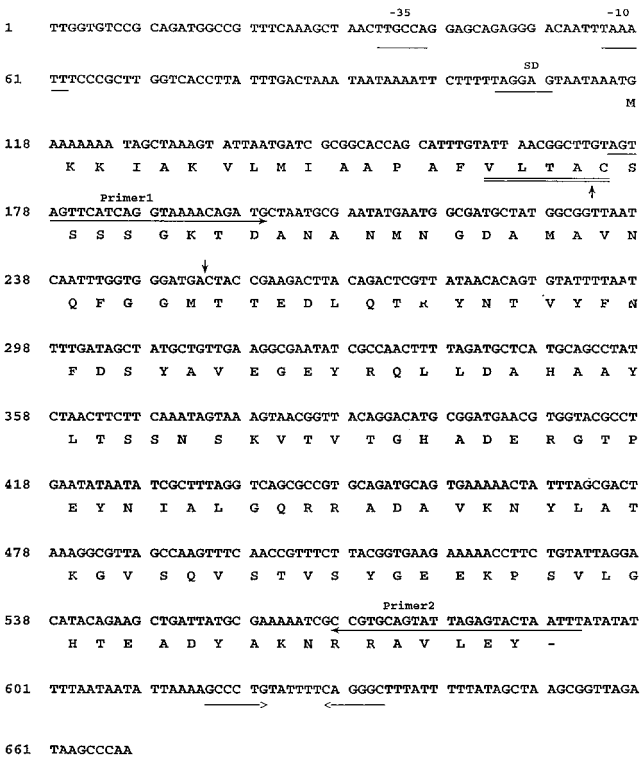

FIG. 2. Nucleotide sequence of *H. ducreyi pal* and predicted amino acid sequence of the PAL protein. Putative -35, -10, and Shine-Dalgarno (SD) regions and sequences corresponding to primers 1 and 2 and the transcription terminator (> <) are underlined. The lipoprotein processing site with the site of cleavage (↑) is double underlined. ↓ denotes insertion of mTn*3*(Cm) in the coding sequence.

lated in the globomycin-treated cells and that *H. ducreyi* has an enzyme similar to *E. coli* signal peptidase II (Fig. 3).

Homologies of *H. ducreyi* PAL. Search of the SWISSPROT 30 database showed extensive homology of *H. ducreyi* PAL to *H. influenzae* P6 (62% identity and 71% similarity) and to *E. coli* PAL (57% identity and 66% similarity) (Fig. 4).

MAb 3B9 binds to both P6 and *H. ducreyi* PAL (44). Peptide mapping studies of P6 suggest that 3B9 binds to a discontinuous epitope localized to the peptide ^{87}GNTDERGT^{94} and the dipeptide ^{147}RR^{148} (3). Exo III digestion of the pHD18 insert led to the loss of 3B9 binding activity after sequences encoding amino acids 141 to 157 of PAL were deleted (data not shown), suggesting that the dipeptide ^{151}RR^{152} was in part responsible for 3B9 binding to *H. ducreyi* PAL.

Conservation of *pal* in *H. ducreyi*. We previously reported that MAb 3B9 bound to an 18K protein in all of 35 *H. ducreyi*

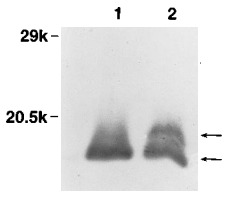

FIG. 3. Western blot of whole cells of *H. ducreyi* 35000 probed with MAb 3B9. The cells were grown to mid-log phase and then grown in the absence (lane 1) and presence (lane 2) of globomycin. The arrows designate mature PAL and the PAL precursor protein. The relative migration of molecular weight standards is shown on the left.

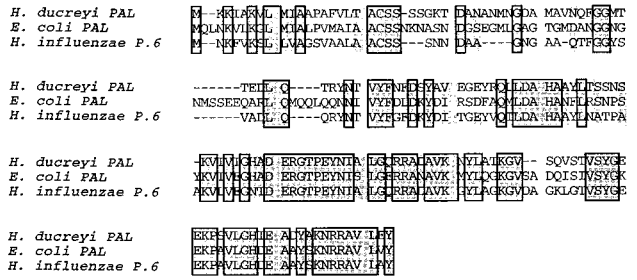

FIG. 4. Alignment of *H. ducreyi* PAL with *E. coli* PAL and *H. influenzae* P6, using the GeneWorks program. Identical residues are boxed, and similar residues are shaded.

strains tested (44). To examine whether *pal* coding sequences were conserved in *H. ducreyi*, genomic DNAs from 10 *H. ducreyi* strains with diverse geographic origins and from *E. coli* DH5α and pHD18 DNA were amplified by PCR using primers 1 and 2 (Fig. 2). Amplicons of the expected size (420 bp) were obtained from pHD18 and from all *H. ducreyi* strains. Digestion of the amplicons with enzymes (*Hae*II and *Rsa*I) that cut once in the *pal* coding sequence yielded two identical fragments for all strains except a Kenyan isolate, whose PCR products were repeatedly resistant to digestion with *Hae*II (Fig. 5). Thus, there was little heterogeneity in the restriction fragments encoding *pal.*

Failure to construct a *pal* mutant. We attempted to construct a *pal* mutant in *H. ducreyi* by allele exchange. The *pal* open reading frame was insertionally inactivated in *E. coli* by shuttle mutagenesis. Sequence analysis of one plasmid [pHD18*pal*:mTn*3*(Cm)] showed that the mTn*3*(Cm) had inserted 138 bp downstream from the transcriptional start of *pal* (Fig. 2). Circular plasmid, linearized plasmid, and the *H. ducreyi* insert were transformed into *H. ducreyi* 35000 by electroporation (16). Chloramphenicol-resistant transformants were obtained with circular (n = 323) or linearized plasmid (n =

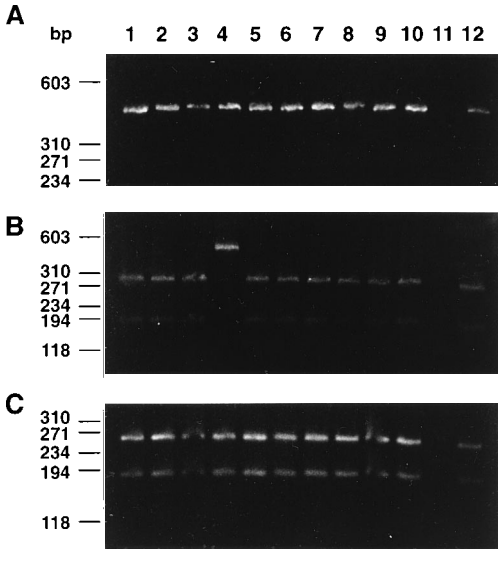

FIG. 5. Agarose gel electrophoresis of PCR products stained with ethidium bromide. DNAs were amplified with primers 1 and 2 (A) and digested with *Hae*II (B) and *Rsa*I (C). Amplicons were obtained from 10 *H. ducreyi* strains (lanes 1 to 10), *E. coli* JM109 (lane 11), and pHD18 (lane 12). Several independent PCR products obtained from the Kenyan strain ATCC 33921 (lane 4) were resistant to digestion with *Hae*II.

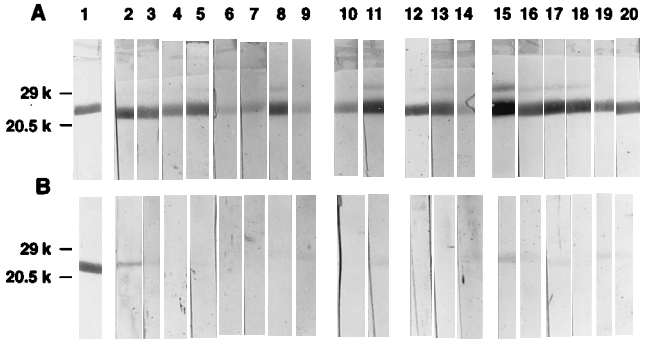

FIG. 6. (A) Western blot of purified recombinant PAL probed with MAb 3B9 or with human sera. Lanes were probed as follows: 1, with 3B9; 2 to 9, with sera from patients with chancroid; 10 and 11, with sera from patients with genital herpes; 12 to 14, with sera from patients with syphilis; 15 to 20, with sera from volunteers with no history of genital ulcer disease. (B) The sera were absorbed with a lysate of soluble antigen mixture prepared from *H. influenzae*, *H. parainfluenzae*, and *H. parahaemolyticus*. Note that absorption removed most of the antibody reactivity to the recombinant protein. The relative migration of the molecular mass markers is shown at left.

193) and with the insert ($n = 28$). However, all transformants bound MAb 3B9 in colony or Western blots. PCR analysis and Southern blotting showed that single-crossover events had taken place in all of the 15 transformants analyzed, so that each transformant contained a copy of the parental allele and mutant allele (data not shown). The single-crossover events that occurred with linear or insert DNA were probably due to the presence of undigested plasmid in these preparations.

Prevalence of antibodies to *H. ducreyi* PAL in patients with genital ulcer diseases and normal subjects. We had previously reported that sera from patients with chancroid and other genital ulcer diseases and from healthy subjects contained antibodies to a protein that comigrated with the 18K OMP (44). To confirm that the antibody reactivity was to *H. ducreyi* PAL, sequences encoding mature PAL were amplified by PCR and cloned into the expression vector pRSET B. The recombinant plasmid, pRSET B HD18, produced a polyhistidine-containing fusion protein with an apparent molecular weight of 24,000 that bound 3B9. The recombinant protein was purified by chromatography with a nickel-containing column (data not shown) and probed in Western blots with sera obtained from patients with genital ulcers at the time of presentation and normal human serum. All sera obtained from seven patients with chancroid, two patients with genital herpes, three patients with syphilis, and six healthy subjects contained antibodies to recombinant PAL (Fig. 6). Absorption of the serum samples with a lysate of *H. influenzae*, *H. parainfluenzae*, and *H. parahaemolyticus* removed most of the antibody reactivity to the recombinant protein (Fig. 6). Thus, patients with chancroid had serum antibodies of the immunoglobulin G class that bound to PAL, but these antibodies did not recognize *H. ducreyi*-specific epitopes.

DISCUSSION

H. ducreyi and all members of the *Pasteurellaceae* tested except *Haemophilus parainfluenzae* share a conserved, surface-exposed epitope defined by MAb 3B9 on OMPs that have similar apparent molecular weights (3, 44). Using 3B9 as a probe, we isolated the gene encoding the 18K OMP from an *H. ducreyi* library. The predicted amino acid sequence of the gene product had sequence homology with the peptidoglycan-associated lipoproteins, *H. influenzae* P6 and *E. coli* PAL. Although P6 was once thought to be unique to *H. influenzae*, other members of the *Pasteurellaceae*, including *Pasteurella multocida* and *Haemophilus somnus*, contain P6-like proteins or genes (22, 47). Taken together, these data suggest that P6-like proteins are conserved among the *Pasteurellaceae.*

The predicted N-terminal amino acid sequence of *H. ducreyi* PAL had features typical of a lipoprotein signal peptide and a signal peptidase II processing site (18, 37). Treatment with globomycin inhibited processing of the PAL protein in *H. ducreyi*. Although we did not label the protein with radioactive fatty acids, these data suggested that *H. ducreyi* has a signal peptidase II-like enzyme and that *H. ducreyi* PAL is likely to be a lipoprotein and therefore have a blocked N terminus (21, 37). We previously reported that the N-terminal amino acid sequence of an 18K band purified from an outer membrane fraction of *H. ducreyi* by preparative SDS-PAGE had no homology to the predicted sequence of mature P6 (44). The sequence obtained from the 18K band was not that of *H. ducreyi* PAL and probably represents an OMP that comigrated with PAL (data not shown). Our previous conclusion that *H. ducreyi* PAL and P6 had unrelated primary structures was in error.

We previously reported that MAb 3B9 binds to all *H. ducreyi* strains tested (44). Restriction fragment length polymorphism analysis of *pal* coding sequences showed that *pal* was highly conserved in *H. ducreyi* strains. Although *H. ducreyi* strains may be differentiated by ribotyping and OMP pattern analysis (36, 40, 48), members of the species form a homogeneous DNA hybridization group (9), and there is no genetic diversity among strains in multilocus enzyme analysis (31a). The conservation of *pal* could reflect a lack of genetic diversity within *H. ducreyi*. However, the gene encoding P6 is highly conserved in *H. influenzae*, a species that is genetically and phenotypically diverse (15, 33). Thus, the conservation of PAL most likely reflects an important role for the protein in maintaining the structure of the organism.

We were unable to construct a *pal* mutant in *H. ducreyi* by allele exchange, a technique that we and others have used successfully to construct mutations in several *H. ducreyi* genes (5, 14, 16). Attempts to construct a mutation in the gene encoding P6 have also been unsuccessful, suggesting that expression of P6 may be essential for bacterial survival (18). Although attempts to place a transposon in *E. coli pal* were unsuccessful (10), mutations in *E. coli excC*, made by chemical mutagenesis, eventually were proven to be in *pal* (25–27). Our inability to obtain a double-crossover event suggests that expression of PAL may be essential for *H. ducreyi* survival. However, we cannot exclude the possibility that the reagents used in this study were insufficient to achieve allele exchange.

E. coli mutants that do not express OmpA or PAL have abnormal shapes or leak periplasmic enzymes (26, 27, 52). Thus, PAL and OmpA-like proteins are thought to have a role in stabilizing the outer membrane of gram-negative bacteria (11, 26, 52). PAL proteins are linked to peptidoglycan through noncovalent forces (31). These proteins and the MotB protein of *Bacillus subtilis* have extended homology and have three perfectly conserved arginine residues in their C-terminal regions (11). The MotB protein anchors the flagellar motor to peptidoglycan, and two of the conserved arginine residues are required for MotB function (2). These arginines may interact with free carboxyl groups in peptidoglycan and anchor MotB, OmpA, and PAL to the cell wall (11), but this hypothesis has not been experimentally tested. *H. ducreyi* PAL contains the three conserved arginine residues at positions 111, 151, and 152 but does not copurify with peptidoglycan under conditions in which P6 remains peptidoglycan associated (44). These data

suggest that any association between *H. ducreyi* PAL and peptidoglycan is relatively weak.

MAb 3B9 binds to *H. ducreyi* PAL and *H. influenzae* P6 (3, 44). The MAb also binds to *E. coli* OmpA under nonreducing conditions (3). P6 mapping studies suggest that 3B9 binds to a conformational epitope composed of two discontinuous regions (^{87}GNTDERGT^{94} and ^{147}RR^{148}) (3). The C terminus of OmpA contains two similar sequences (^{259}GYTDRIGS^{266} and ^{328}RR^{329}) that may be brought into the correct conformation via a disulfide bridge and form the epitope (3). Although 3B9 does not bind *E. coli* PAL, the putative 3B9 binding regions of *H. ducreyi* PAL (^{93}GHADERGT^{100} and ^{151}RR^{152}) and *E. coli* PAL (^{107}GHADERGT^{114} and ^{167}RR^{168}) are identical. The amino acid sequences and the spacings between the two regions are similar in *H. influenzae* (53 residues), *H. ducreyi* (51 residues), and *E. coli* (53 residues). Exo III digestion of *H. ducreyi pal* confirmed that ^{151}RR^{152} was in part responsible for 3B9 binding to the protein. However, we cannot explain why 3B9 does not bind to *E. coli* PAL.

Sera obtained from all patients with chancroid or genital ulcer diseases and from healthy subjects contained antibodies that bound to recombinant *H. ducreyi* PAL. Antibodies to PAL were removed by absorption with a lysate of other *Haemophilus* sp. strains, indicating that patients with chancroid did not develop antibody responses to PAL that were *H. ducreyi* specific. These observations were consistent with our previous findings that most human sera tested contain antibodies to a band that comigrated with the 18K OMP and that these sera competitively inhibited the binding of 3B9 to *H. ducreyi* (44). Thus, the antibodies that bind to PAL in patients with chancroid are likely to have resulted from previous exposure to the cross-reacting epitopes found in proteins of the *Pasteurellaceae* or *E. coli.*

H. influenzae P6 is a vaccine candidate because it is genetically and phenotypically conserved, antigenically stable, and surface exposed and binds antibodies found in normal human serum that are bactericidal (34, 35). Although *H. ducreyi* PAL has many of the features of P6, four of four normal human sera that contained antibodies to recombinant PAL (Fig. 6) did not have bactericidal activity against *H. ducreyi* 35000 in concentrations as high as 50% (data not shown). At this time, we cannot assign a functional role to human antibodies that react to *H. ducreyi* PAL. Studies that address whether serum antibodies from patients with chancroid or antiserum raised to recombinant PAL are bactericidal for *H. ducreyi* are in progress in our laboratory.

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