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The Major Outer Membrane Protein of *Haemophilus ducreyi* Is a Member of the OmpA Family of Proteins

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***Haemophilus ducreyi* contains a major outer membrane protein (MOMP) whose apparent molecular weight is 39,000 to 42,000 for all strains tested. Two monoclonal antibodies (MAbs), designated 9D12 and 2C7, bound to the MOMP for all strains of *H. ducreyi* tested. As reported previously, MAb 9D12 was *H. ducreyi* specific (E. J. Hansen and T. A. Loftus, Infect. Immun. 44:196–198, 1984). MAb 2C7 bound to all members of the family *Pasteurellaceae* tested, suggesting that the MAbs bound to distinct epitopes on the MOMP. The MOMP was purified by extraction of whole cells with Zwittergent and ion-exchange chromatography. A peak eluted from a cation-exchange column contained three bands. All three species bound both MAbs, and the fraction yielded a single N-terminal amino acid sequence, suggesting that the bands represented different conformations of the MOMP. The MOMP was heat modifiable, contained two cysteine residues, and was cationic at pH 8.0, features not usually associated with classical porin proteins. The N-terminal amino acid sequence and total amino acid content of the MOMP were homologous to the OmpA proteins of members of the family *Enterobacteriaceae* and the OmpA-like protein of *Actinobacillus actinomycetemcomitans*. An OmpA-specific polyclonal serum bound to the MOMP, and MAb 2C7 bound to *Haemophilus influenzae* protein 5, an OmpA-like protein, indicating that the MOMP was antigenically related to OmpA. These data indicated that the most abundant protein in the outer membrane of *H. ducreyi* was not a classical porin and belonged to the OmpA family of proteins.**

Haemophilus ducreyi, the etiologic agent of chancroid, is a major cause of genital ulcer disease in developing countries. In areas of Africa and Southeast Asia, chancroid accounts for approximately 10 to 40% of sexually transmitted disease clinic visits (20). Genital ulcer diseases, including chancroid, are independent risk factors for human immunodeficiency virus (HIV) seropositivity in populations of areas where both infections are endemic (14, 30, 36). Chancroid may enhance HIV transmission by facilitating shedding of the virus from HIV-seropositive patients, by disrupting epithelial barriers, or by recruiting activated T cells and monocytes to the viral portal of entry (15). Patients who are coinfected with chancroid and HIV often do not respond to antibiotic therapy for *H. ducreyi* (14). A cycle in which chancroid enhances HIV transmission and HIV infection potentially increases the spread of *H. ducreyi* has emerged (14). Understanding chancroid pathogenesis may be important in efforts to interrupt the mutual enhancement of transmission by *H. ducreyi* and HIV.

There is little information available on bacterial components that are important in the pathogenesis of chancroid (20). The major outer membrane proteins (MOMPs) of gram-negative nonenteric bacteria are usually classical porins that bear important immunogenic determinants (10, 24). Previous studies have shown that all *H. ducreyi* strains contain a MOMP that migrates (depending on gel conditions) with an apparent molecular weight of 39,000 to 42,000 and may contain conserved antigenic determinants (1, 28, 32, 33, 41). In this study, we examine the physical and antigenic properties of the MOMP of *H. ducreyi*.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. The *H. ducreyi* strains used in this study were described previously (37). Strain 85-023233 was isolated during an outbreak of chancroid in New York, New York, in 1985; strain 35000 was isolated in Winnipeg, Canada, in 1975. The majority of the remaining strains were isolated from chancroid outbreaks in North America; six of the strains originated from Africa or the Far East. The identities of the strains were confirmed by colonial morphology, Gram stain, requirement for X factor but not V factor, oxidase positivity, catalase negativity, and the inability to ferment dextrose, lactose, and sucrose. Other *Haemophilus* and *Actinobacillus* sp. strains were obtained from our collection in the Division of Infectious Disease at State University of New York at Buffalo. All strains were grown on chocolate agar supplemented with 1% IsoVitaleX at 35°C in a 5% CO_2 atmosphere.

Isolation of outer membranes. Whole membranes were prepared from French pressure cell lysates of *H. ducreyi* and separated into inner and outer membrane fractions by Sarkosyl extraction as described previously (39).

Protein purification. Isolation of the MOMP from strain 85-023233 was accomplished through a modification of the method that was used by Lytton and Blake to purify *Neis-*

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seria gonorrhoeae protein III (17). All manipulations were done at 4°C or on ice in the presence of 1 mM phenylmethylsulfonyl fluoride. An overnight growth of bacteria was scraped from 40 agar plates (100 mm in diameter) and washed in 0.9% saline. The harvested bacteria were solubilized in a buffer containing 1 ml of 1 M sodium acetate, pH 4, and 6 ml of 5% Zwittergent 3-14 (Calbiochem, La Jolla, Calif.) in 0.5 M $CaCl_2$ (per gram of bacterial wet weight). Two volumes of absolute ethanol were added, and insoluble material and nucleic acids were removed by centrifugation at 17,000 × *g* for 10 min. The supernatant was incubated overnight with absolute ethanol (final concentration, 80%), and the precipitate was collected by centrifugation at 17,000 × *g* for 10 min.

The pellet was extracted with 10 ml of 50 mM Tris-HCl–10 mM EDTA–5% Zwittergent 3-14 (pH 8.0) (5% buffer Z). The Zwittergent extract was applied to an anion-exchange column that was linked in tandem to a cation-exchange column. The anion-exchange column was DEAE–Sepharose CL-6B (Pharmacia Fine Chemicals, Piscataway, N.J.) packed in a 10-ml syringe; the cation-exchange column was carboxymethyl–Sepharose CL-6B (Pharmacia Fine Chemicals) packed in a 35-ml syringe. Both columns were equilibrated with 0.1% buffer Z (containing 0.1% Zwittergent 3-14) prior to loading the sample. The columns were washed with 0.1% buffer Z until the A_{280} fell to baseline. The columns were disconnected and eluted separately with a 0.0 to 0.5 M sodium chloride gradient in 0.1% buffer Z. One-milliliter fractions were collected, monitored by A_{280}, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing the MOMP were pooled, precipitated with absolute ethanol, and washed several times with absolute ethanol prior to solubilization.

Amino acid analysis and N-terminal amino acid sequence. The precipitated MOMP was solubilized in 90% formic acid and diluted with an equal volume of water. Amino acid composition was determined by Audree Fowler (University of California, Los Angeles, Protein Microsequencing Facility), using the Pico-Tag system after hydrolysis in 6 N HCl. Methionine and cysteine content was obtained after performic oxidation of the sample. The N-terminal amino acid sequence was determined by Edman degradation using a Porton PI 2090E sequencer.

Cyanogen bromide cleavage of the MOMP. Cyanogen bromide cleavage of the MOMP was performed exactly as described by Murphy and Bartos (23).

MAbs and polyclonal sera. Monoclonal antibody (MAb) 2C7 was the product of a fusion that resulted from immunizing mice with whole cells of a *Haemophilus influenzae* biogroup aegyptius Brazilian purpuric fever case strain and was provided by Alan Lesse of the Buffalo Veterans Administration Medical Center (16a). MAb 9D12, raised against *H. ducreyi* 35000, was described previously and was kindly supplied by Eric Hansen of the University of Texas Health Science Center at Dallas (12). Normal rabbit serum and antiserum raised against the OmpA protein of *Escherichia coli* K-12 was provided by Mark Wilson of the State University of New York at Buffalo (44).

SDS-PAGE and Western blot (immunoblot) assays. Whole bacteria, membrane fractions, and protein preparations were subjected to SDS-PAGE by a modification (39) of the method of Laemmli (16) as described previously. Proteins were solubilized in Laemmli sample buffer containing 5% 2-mercaptoethanol at room temperature or 100°C for 5 min (39). In some experiments, 2-mercaptoethanol was either omitted from the sample buffer or increased to a final concentration of 10%, or proteins were treated with proteinase K (25 mg/ml) prior to solubilization. Three gel systems, containing 10% acrylamide, 15% acrylamide, or a 4 to 30% linear acrylamide gradient, were used. The gels were stained with Coomassie brilliant blue or silver or transferred to nitrocellulose as described previously (2, 39). Western blots were probed with tissue culture supernatants, protein A-peroxidase (Zymed Laboratories, Burlingame, Calif.), and horseradish peroxidase color developer (Bio-Rad Laboratories, Richmond, Calif.) as described previously (38).

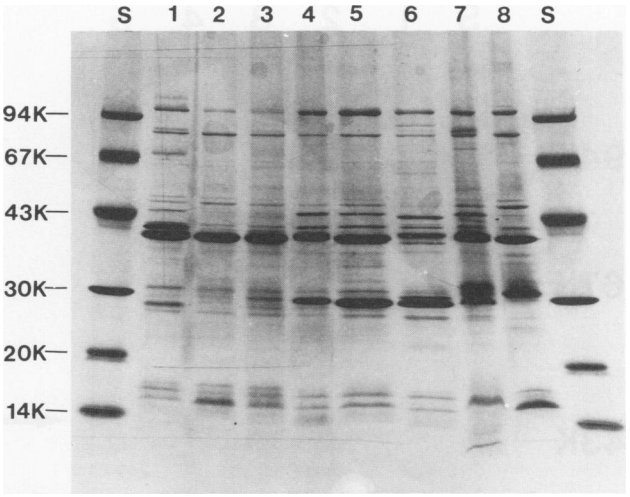

FIG. 1. SDS-PAGE (4 to 30% gradient) of Sarkosyl-extracted OMPs from eight strains of *H. ducreyi*. Lanes S contain molecular weight standards. All strains contain a MOMP with an apparent molecular weight of 39,000 to 40,000.

Immunodot assay. Colonies of bacteria were scraped from plates, sonicated in phosphate-buffered saline or solubilized in Laemmli sample buffer, applied to nitrocellulose, and probed with 2C7, 9D12, or Sp2/0 tissue culture supernatants; protein A-peroxidase; and horseradish peroxidase color developer as described previously (38).

RESULTS

Outer membrane proteins (OMPs) were prepared by Sarkosyl extraction and analyzed in a 4 to 30% gradient gel. As reported previously (28, 41), strains of *H. ducreyi* contained a MOMP that migrated with an apparent molecular weight of 39,000 to 40,000 (Fig. 1).

Purification of the MOMP. Colonies of *H. ducreyi* 85-023233 were scraped from agar plates and solubilized in a buffer containing 5% Zwittergent 3-14. Nucleic acids were removed by ethanol precipitation, and proteins were precipitated and extracted in 5% buffer Z. The Zwittergent extract was enriched for the MOMP and lipopolysaccharides (LPS), and the extract contained a 43K species when solubilized at 100°C (Fig. 2). The extract was applied to anion- and cation-exchange columns linked in tandem, and a single peak containing the MOMP was eluted from the cation-exchange column with a salt gradient. The fraction contained a 37K-39K doublet and the 43K species regardless of solubilization conditions (Fig. 2). Silver stain analysis showed that the MOMP fraction did not contain LPS, suggesting that the heat modifiability of the protein was due to its association with LPS.

To test whether the three species copurified by ion-

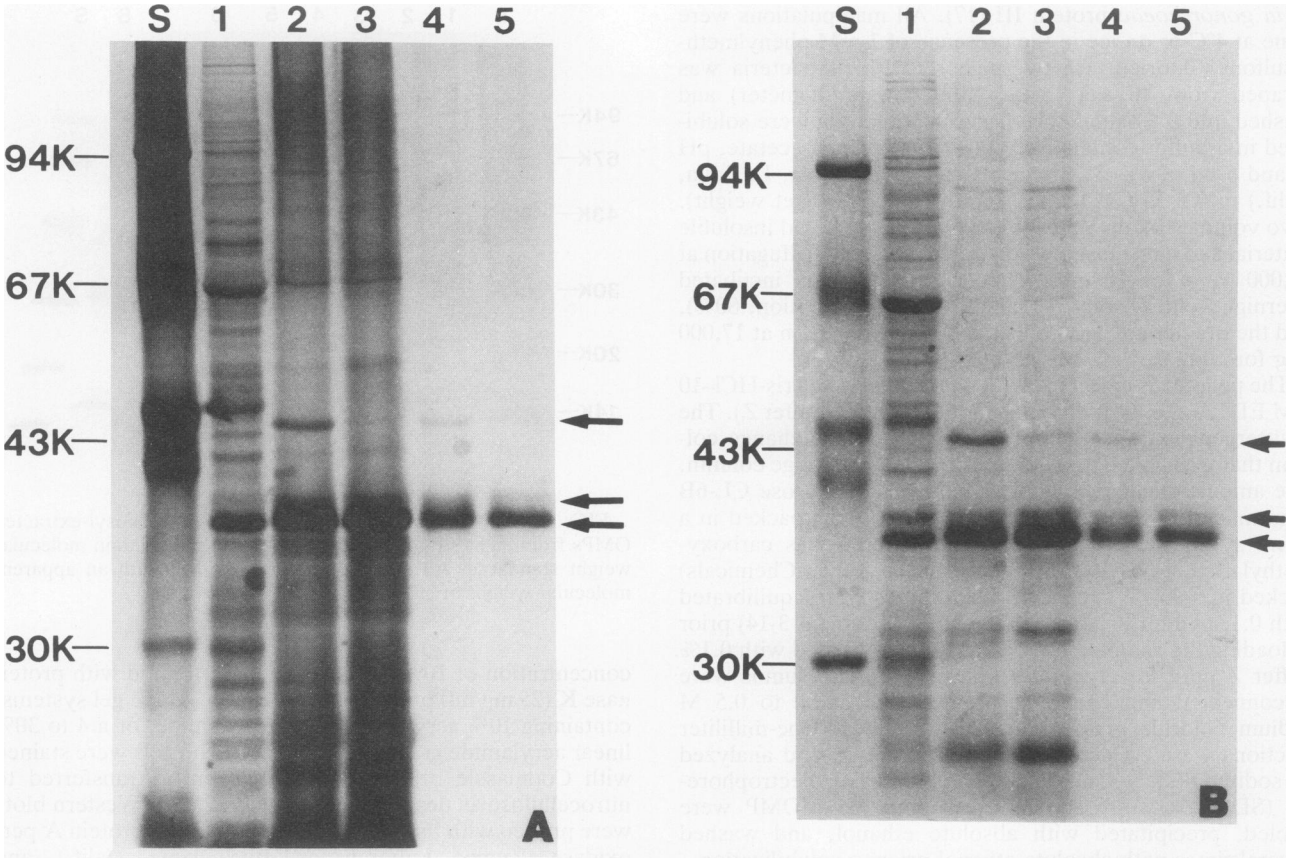

FIG. 2. SDS-PAGE (10% acrylamide gel) of whole cells, Zwittergent extracts, and the cation-exchange column fraction containing the MOMP prepared from *H. ducreyi* 85-023233 and stained with silver (A) or Coomassie brilliant blue (B). Lanes S, molecular weight standards; lanes 1, whole cells; lanes 2 and 3, Zwittergent extracts; lanes 4 and 5, the MOMP fraction. For lanes 1, 2, and 4, the preparations were solubilized at 100°C; for lanes 3 and 5, they were solubilized at room temperature. Note that the MOMP fraction contains three species, as shown by the arrows.

exchange chromatography represented different proteins or were different conformations of a single protein, we determined the N-terminal amino acid sequence of the MOMP fraction and probed the fraction with MAbs that bind to distinct epitopes on the MOMP (see below). The MOMP fraction contained a single N-terminal amino acid sequence and no minor sequences. Both MAbs bound to the 37K-39K doublet and to the 43K species when outer membranes were solubilized at 100°C in the presence and absence of 2-mercaptoethanol (Fig. 3 and data not shown). Both MAbs bound to the three bands in the MOMP fraction, regardless of solubilization conditions. The data suggested that the three species in the MOMP fraction represented different conformations of a single protein and may account for the range of apparent molecular weights (39,000 to 42,000) previously assigned to the MOMP (1, 28, 32, 33, 41).

Binding of the MAbs 2C7 and 9D12 to the MOMP. MAbs 2C7 and 9D12 bound to 35 of 35 *H. ducreyi* strains tested in immunodot assays. In Western blots, both MAbs bound to protease-sensitive epitopes on the MOMP (Fig. 3 and data not shown). As reported previously by Hansen and Loftus (12), MAb 9D12 bound only to *H. ducreyi*. However, MAb 2C7 bound to all members of the family *Pasteurellaceae* tested, including strains of *H. influenzae*, *H. influenzae* biogroup aegyptius, *Haemophilus haemolyticus*, *Haemophilus aphrophilus*, *Haemophilus parahaemolyticus*, *Haemophilus parainfluenzae*, *Haemophilus paraphrohaemolyticus*, *Haemophilus paraphrophilus*, *Haemophilus segnis*, and *Actinobacillus actinomycetemcomitans*. The data suggested that the MAbs bound to distinct epitopes on the MOMP.

To localize the epitopes defined by the MAbs, cyanogen bromide cleavage products of the MOMP were probed with 2C7 and 9D12 in Western blots. The total amino acid composition of the MOMP predicted that cyanogen bromide cleavage should yield four peptide fragments. Cyanogen bromide cleavage yielded two major peptide fragments with apparent molecular weights of 16,000 and 28,000 and two minor fragments with molecular weights of 30,000 and 31,000, which probably represented partial cleavage products of the protein. MAb 2C7 bound to the 30K and 31K bands, while MAb 9D12 bound to none of the cleavage products (data not shown). Thus, the MAbs bound to distinct epitopes.

Relationship of the MOMP to the OmpA proteins. The N-terminal amino acid sequence of the MOMP was compared with published sequences in the National Biomedical Research Foundation protein data base and was homologous to the OmpA proteins of *E. coli*, *Salmonella typhimurium*, and *Shigella dysenteriae* and the heat-modifiable OMP of *A. actinomycetemcomitans* (Fig. 4). The total amino acid composition of the MOMP had a high percentage of dicarboxylic (26%) and basic (14%) amino acid residues and was similar to that of *E. coli* OmpA and the heat-modifiable OMP of *A. actinomycetemcomitans* (5, 44).

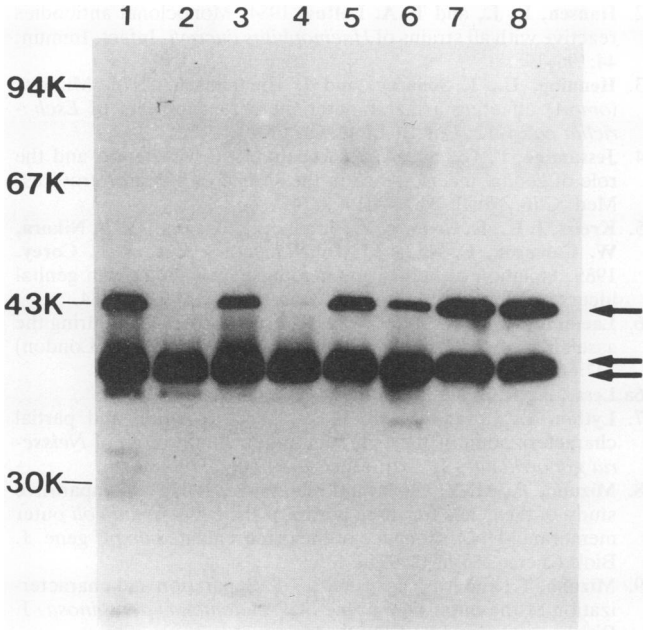

FIG. 3. Western blot (10% acrylamide gel) of Sarkosyl-extracted OMPs and the fraction containing the MOMP prepared from *H. ducreyi* 85-023233 and probed with 2C7. Lanes 1 to 4, OMPs; lanes 5 to 8, the MOMP fraction. Samples in the odd-numbered lanes were solubilized at 100°C; in even-numbered lanes, they were solubilized at room temperature. For samples in lanes 3, 4, 7, and 8, 2-mercaptoethanol was omitted from the solubilization buffer. Note that 2C7 binds to three bands, as shown by the arrows.

To confirm that the MOMP was a member of the OmpA family of proteins, we probed the MOMP with a rabbit polyclonal serum raised against *E. coli* K-12 OmpA. In Western blots, the rabbit polyclonal serum bound to the purified MOMP, while normal rabbit serum exhibited no binding (data not shown). MAb 2C7 bound to protein 5 of *H. influenzae* and the heat-modifiable OMP of *A. actinomycetemcomitans* (16a, 43a), which are antigenically related to OmpA (42, 44). Thus, the MOMP of *H. ducreyi* was structurally and antigenically related to OmpA and OmpA proteins found in members of *Pasteurellaceae*.

DISCUSSION

The MOMP of *H. ducreyi* was purified by extraction of cells with Zwittergent and ion-exchange chromatography. The MOMP was cationic at pH 8.0, contained two cysteine residues, and was heat modifiable. The most abundant OMPs of gram-negative nonenteric organisms are usually classical porin proteins that have acidic pIs and lack cysteine residues and heat modifiability (9, 10, 18, 21, 27). Thus, the outer membrane of *H. ducreyi* is somewhat unusual in that a classical porin is not the most abundant protein in the outer membrane.

```
E. coli OmpA                    APKDNTWYTGAKLG
                                ||..:|:|.||| |
H. ducreyi MOMP                 APQADTFYVGAKAG
                                ||||:|||.|||||
A. actinomycetemcomitans OMP    APQANTFYAGAKA
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FIG. 4. Comparison of the N-terminal amino acid sequences of the OmpA protein of *E. coli*, the MOMP of *H. ducreyi*, and the heat-modifiable OMP of *A. actinomycetemcomitans*. Identical residues are indicated by a vertical line, similar residues are indicated by two dots, and less similar residues are indicated by a single dot.

The MOMP had an amino acid composition and N-terminal amino acid sequence similar to those of OmpA proteins, which are major components of bacterial outer membranes and are conserved in many gram-negative species. Members of this protein family include *H. influenzae* protein 5, the heat-modifiable OMP of *A. actinomycetemcomitans*, *Pseudomonas aeruginosa* protein F, and *N. gonorrhoeae* protein III (3, 8, 11, 22, 42, 44). The apparent molecular weight of the MOMP was somewhat higher than that reported for most OmpA proteins but was similar to that of protein F, which migrates at 37,000 to 42,000 in its unmodified and heat-modified forms (3).

The migration of the MOMP as three species was dependent on gel and solubilization conditions and is consistent with the observation that proteins in the OmpA family migrate heterogeneously in SDS-PAGE (11, 13, 19). The heterogeneous migration of OmpA proteins may be due to heat modifiability, reduction modifiability, or conformational changes that occur as the protein is processed from its precursor into mature form (6–8, 11, 17). When associated with the membrane, the MOMP was heat modifiable but not reduction modifiable, and the heat modifiability of the MOMP was lost when it was purified from lipooligosaccharide. Similarly, the heat modifiability of OmpA is due to interactions between protein and LPS (35). Most OmpA proteins are completely denatured by heating, but prolonged boiling (60 min) of the purified MOMP or membrane preparations in sample buffer did not entirely convert the MOMP to the 43K species. In general, the physical characteristics of the MOMP were similar to those of other members of the OmpA family.

The MOMP contained epitopes present in some OmpA proteins, and MAbs 2C7 and 9D12 bound to distinct epitopes on the MOMP. As reported previously, MAb 9D12 was *H. ducreyi* specific (12). MAb 2C7 bound to *H. influenzae* protein 5 and the heat-modifiable OMP of *A. actinomycetemcomitans*. The conservation of the 2C7 epitope may account for some of the observed immunologic cross-reactivity between *H. ducreyi* and other *Haemophilus* species (1, 33, 34). Although an OmpA polyclonal serum bound to the MOMP, two MAbs (3C9 and 7B7) and an affinity-purified polyclonal serum directed to gonoccocal protein III did not recognize the MOMP (data not shown). The antigenic differences among these OmpA-like proteins may reflect sequence polymorphisms in regions of the proteins likely to be exposed on the cell surface (4).

The biologic function of OmpA proteins and whether the OmpA proteins constitute a novel class of porin proteins are controversial (26, 40, 45). Preliminary studies indicate that the MOMP does not form ion-permeable channels similar to those of the classical porins *E. coli* OmpF and gonococcal protein I in planar lipid membranes composed of phosphatidylethanolamine and phosphatidylcholine (3a). *H. ducreyi* is susceptible to expanded-spectrum cephalosporins and amoxicillin-clavulanic acid (20), and these β-lactam antibiotics usually permeate the outer membrane through porin channels (25). Thus, the outer membrane of *H. ducreyi* probably contains porin proteins other than the MOMP. The acquisition of polymixin resistance by *H. ducreyi* has been associated with the loss of a 47K OMP that may have a porin function (29).

The abundance of an antigenically conserved OmpA-like

protein in the outer membrane of *H. ducreyi* may point toward the MOMP playing a role in serum resistance of this organism. Recently, Weiser and Gotschlich showed that an *E. coli* K-1 mutant lacking OmpA was more sensitive to the bactericidal effect of normal human serum than was its wild-type parent (43). Expression of OmpA may stabilize the bacterial outer membrane and confer serum resistance on the organism. Alternatively, OmpA may bind antibodies that block serum bactericidal activity, as has been described for gonococcal protein III (31). Infection with *H. ducreyi* does not elicit a protective immune response in humans, and clinical isolates of *H. ducreyi* are resistant to the bactericidal effects of normal human serum (29). Future studies will be directed towards examining whether antibodies that bind to the MOMP block serum bactericidal activity against *H. ducreyi*.

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