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The Major Outer Membrane Protein of *Haemophilus ducreyi* Consists of Two OmpA Homologs

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The major outer membrane protein (MOMP) of *Haemophilus ducreyi* is an OmpA homolog that migrates on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels as three species with apparent molecular weights ranging from 37,000 to 43,000. Monoclonal antibodies directed against this macromolecule were used to identify recombinant clones containing fragments of the gene encoding this protein. Nucleotide sequence analysis of these fragments confirmed that the MOMP encoded by the intact gene (*momp*) was a member of the OmpA family of outer membrane proteins. Construction of an isogenic *H. ducreyi* mutant unable to express the MOMP led to the discovery of a second outer membrane protein which migrated at the same rate on SDS-PAGE gels as the MOMP. N-terminal amino acid sequence analysis of this second protein revealed that its N terminus was nearly identical to that of the MOMP and also had homology with members of the OmpA family. Nucleotide sequence analysis of the region downstream from the *momp* gene revealed the presence of a partial open reading frame encoding a predicted OmpA-like protein. A modification of anchored PCR technology was used to obtain the nucleotide sequence of this downstream gene which was shown to encode a second OmpA homolog (OmpA2). The N-terminal amino acid sequence of OmpA2 was identical to that of the OmpA-like protein detected in the *momp* mutant. The *H. ducreyi* MOMP and OmpA2 proteins, which comigrated on SDS-PAGE gels and which were encoded by the tandem arranged *momp* and *ompA2* genes, were 72% identical.

Haemophilus ducreyi causes an ulcerogenital disease known as chancroid (2, 51). Interest in this gram-negative coccobacillus has grown due to compelling evidence that chancroid, as well as other genital ulcer diseases, has contributed significantly to the spread of human immunodeficiency virus in the heterosexual population (40). While chancroid is a common cause of genital ulcers in developing countries, until recently it has been relatively rare in the United States. However, following several large outbreaks of chancroid in 1981, the disease has remained endemic in several states (48, 51).

Relatively little is known about the constituents of the *H. ducreyi* outer membrane. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of cell envelope proteins from different *H. ducreyi* strains revealed that a protein with an apparent molecular weight ranging from 37,000 to 43,000 predominated (1, 39, 47). This protein, which contained two epitopes that were present in all *H. ducreyi* strains tested (24, 49), recently was localized to the *H. ducreyi* outer membrane and designated as the major outer membrane protein (MOMP) (49). The purified MOMP from *H. ducreyi* 85-023233 migrated in SDS-PAGE as three species, i.e., a 37- to 39-kDa doublet and a 43-kDa heat-modifiable band, and had an N-terminal amino acid sequence that was 57% identical and 92% similar to that of the OmpA protein of *Escherichia coli* (49).

In *E. coli*, OmpA functions as a mediator in F-dependent conjugation and as a phage and colicin receptor (6, 10). Analyses of *E. coli ompA* mutants in several model systems indicate that OmpA likely plays a role in virulence expression (41, 42, 53). Homologs of OmpA have been described in numerous gram-negative organisms (5, 16, 54). Members of this protein family share a conserved predicted β-barrel structure comprised of eight anti-parallel β-sheets as well as an α-helical motif in the C-terminal portion of these proteins which is thought to interact directly with the peptidoglycan layer (11, 31, 32).

In the present study, we identified and sequenced the gene encoding the MOMP of *H. ducreyi* and confirmed that this protein is a member of the family of OmpA outer membrane proteins. In addition, we constructed an isogenic mutant of *H. ducreyi* lacking the ability to express the MOMP. Characterization of this mutant led to the discovery of a second OmpA homolog in the outer membrane of this sexually transmitted pathogen.

(Part of this research was presented by S. Spinola and T. Hiltke at the 95th General Meeting of the American Society for Microbiology, Washington, D.C., 21 to 25 May 1995.)

MATERIALS AND METHODS

Bacterial strains and culture conditions. The 11 *H. ducreyi* strains used in this study are described in Table 1. *H. ducreyi* 35000 has been extensively characterized (19–21). *H. ducreyi* strains were cultivated at 33°C in an atmosphere of 95% air and 5% CO_2 on chocolate agar supplemented with Isovitalex (CA) (BBL Microbiology Systems, Becton Dickinson, Cockeysville, Md.) as described elsewhere (19, 20). Frozen stocks of all strains were stored at −70°C in fetal bovine serum. Chloramphenicol-resistant mutants of *H. ducreyi* were cultured on CA plates supplemented with chloramphenicol (2 μg/ml) (Sigma Chemical Co., St. Louis, Mo.) *E. coli* XL1-Blue MRF′ (Stratagene, La Jolla, Calif.), XLOLR (Stratagene), Y1090(r⁻) (Promega Biotech, Madison, Wis.), Y1089(r⁻) (Promega), and RR1 (46) were grown at 37°C on Luria-Bertani medium (46) or on NZY medium (46) supplemented with antimicrobials at the following concentrations when appropriate: tetracycline, 12.5 μg/ml; kanamycin, 50 μg/ml; ampicillin, 100 μg/ml; and chloramphenicol, 30 μg/ml. *E. coli* UH203, which does not

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
H. ducreyi strains		
35000	Wild-type strain isolated in Winnipeg, Manitoba, Canada	19
35000.60	*momp* mutant obtained by electroporating 35000 with linearized pJKT636Δ666	This study
1151	Wild-type strain isolated in Gambia	Allan Ronald
025	Wild-type strain isolated in San Diego	This study
041	Wild-type strain isolated in Sweden	Allan Ronald
1145	Wild-type strain isolated in Amsterdam	Allan Ronald
Hd13	Wild-type strain isolated in Singapore	44
STD101	Wild-type strain isolated in Dallas, Tex.	43
Cha-1	Wild-type strain isolated in Dallas, Tex.	43
1352	Wild-type strain isolated in Kenya	Allan Ronald
512	Wild-type strain isolated in Thailand	Allan Ronald
Hd12	Wild-type strain isolated in Korea	38
E. coli strains		
XL1-Blue	Host strain used for phage infections	Stratagene
XLOLR	Host strain used for excising recombinant pBK-CMV phagemids from ZAP Express bacteriophage	Stratagene
RR1	Host strain used for cloning the 3′ half of the *momp* gene	46
Y1090(r^-)	Host strains used for cloning experiments with λgt11	Promega
Y1089($^-$)	Host strain for λgt11 lysogen production	Promega
UH203	*ompA* mutant	34
Plasmids		
pBK-CMV	Phagemid excised from ZAP Express cloning vector	Stratagene
pBluescriptII	Cloning vector, Amp^r	Stratagene
pWKS30	Low-copy-number cloning vector	52
pGEM3Zf($^+$)	Cloning vector with the *lacZ* promoter	Promega
pJKT636	pBK-CMV with a 4.6-kb *H. ducreyi* DNA insert containing the 5′ half of the *momp* gene	This study
pJKT600	pBluescriptII with a 1.5-kb DNA insert containing the 5′ half of the *momp* gene	This study
pJKT666	pBluescriptII containing the 3′ half of the *momp* gene	This study
pMOMP1.2	pGEM3Zf($^+$) containing the *momp* gene fused to *lacZ*	This study
TH100	λgt11 containing the 3′ half of *momp*	This study
E29	Derivative of pRD87 containing the *E. coli ompA* gene	15
pJKT636Δ*cat*	pJKT636 with the 500-bp *Xba*I fragment containing the 5′ half of *momp* deleted and a *cat* cartridge inserted into this deletion site	This study
pJKT636Δ666	pJKT636Δ*cat* with the 1.6-kb *Kpn*I-*Not*I insert from pJKT666 inserted into the *Kpn*I site	This study

express OmpA (53), was kindly provided by Jeffrey Weiser, University of Pennsylvania, Philadelphia, Pa. For complementation experiments, *E. coli* strains were grown in M9 minimal medium (46) supplemented with thiamine (10 μg/ml), Casamino Acids (100 μg/ml) (Difco Laboratories, Detroit, Mich.), and 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG; for growth experiments) or on Luria-Bertani medium containing 2 mM IPTG (for antibody binding experiments).

MAbs. Female BALB/c mice were immunized with whole *H. ducreyi* 35000 organisms (10^9 CFU) suspended in Freund's complete adjuvant. One month later, 0.1 ml of phosphate-buffered saline (PBS) containing 10^8 *H. ducreyi* CFU was injected intravenously. Splenocytes were used in a standard hybridoma fusion protocol (45). Culture supernatants from lymphocyte hybridomas were screened for the presence of *H. ducreyi*-directed monoclonal antibodies (MAbs) by means of enzyme-linked immunosorbent assaying and Western blot (immunoblot) analyses with *H. ducreyi* cell envelopes as the source of antigen. A MAb present in culture supernatant from one such hybridoma cell line, 3F12, reacted in Western blot analysis with the MOMP from *H. ducreyi*. Use of the indirect antibody accessibility assay (28) proved that MAb 3F12 reacted with a MOMP epitope exposed on the surfaces of whole cells of *H. ducreyi*. MAbs 9D12 and 2C7 bind to distinct epitopes on the *H. ducreyi* MOMP and have been previously described (24, 49).

Library construction and screening. Two chromosomal libraries were constructed from *H. ducreyi* 35000. Chromosomal DNA from *H. ducreyi* 35000 was partially digested with *Sau*3AI. Fragments ranging from 3 to 8 kb were purified with a sucrose density gradient and ligated into the Lambda ZAP Express vector (Stratagene). XL1-Blue MRF′ cells were infected with the recombinant phage and plaques were screened for reactivity with the MOMP-reactive MAb 3F12. MAb-reactive recombinant bacteriophages were plaque purified, and the pBK-CMV plasmids containing the *H. ducreyi* DNA inserts were excised.

A second genomic library was constructed with 1- to 7-kb fragments from *H. ducreyi* chromosomal DNA that was partially digested with *Apo*I. These fragments were purified from agarose by phenol-chloroform extraction, ligated into λgt11 phage arms, and packaged (Packagene; Promega Biotech). *E. coli* Y1090 (r^-) was infected with the recombinant phages and plaques were screened with MAbs 2C7 and 9D12.

Recombinant DNA techniques. Recombinant DNA techniques used in this study are described in detail elsewhere (46). The chloramphenicol acetyltransferase (*cat*) cartridge utilized in mutant construction was kindly provided by Bruce A. Green, Lederle-Praxis Biologicals, West Henrietta, N.Y. PCR-based amplification of DNA fragments was accomplished by standard techniques (4) except when noted. Selected oligonucleotides used in PCR, for Southern blot analysis, or for nucleotide sequence analysis are listed in Fig. 2.

Nucleotide sequence analysis. Nucleotide sequence analysis of DNA fragments in recombinant plasmids or derived from PCR amplifications was performed with a model 373A automated DNA sequencer (Applied Biosystems, Foster City, Calif.) or by using a Sequenase version 2.0 kit (U.S. Biochemical, Cleveland, Ohio) exactly as described elsewhere (50). In all instances, both strands of the DNA fragments were sequenced in their entirety. DNA sequence information was analyzed through NCBI by using the BLAST network service to search GenBank (3) and by using programs from the University of Wisconsin Genetics Computer Group software sequence analysis package (12). Potential promoter regions were identified by the use of MacTar Search from the MacVector program (Eastman Kodak, Rochester, N.Y.).

N-terminal amino acid sequence analysis. Sarkosyl-insoluble proteins from *H. ducreyi* cell envelope preparations were solubilized in digestion buffer, heated at 100°C for 3 min, and then resolved by SDS-PAGE. The 37- to 39-kDa band from the wild-type strain 35000 and the minor band (that migrated at the same apparent rate as the MOMP) from mutant strain 35000.60 were transferred to a polyvinylidene difluoride membrane by the method of Matsudaira (35) and subjected to N-terminal amino acid sequence analysis as described elsewhere (25).

Expression of recombinant MOMP. The nucleotide sequence encoding the MOMP was amplified from *H. ducreyi* 35000 chromosomal DNA with oligonucleotide primers that contained *Eco*RI or *Bam*HI restriction sites (3f12-40 and 3f12-41 in Fig. 2). The 1.2-kb PCR product was digested with both of these enzymes and cloned into pWKS30, yielding pMOMP1.1. This fragment was excised by digestion with *Xba*I and *Eco*RI and ligated into pGEM3Zf($^+$) so that the *lacZ* promoter could be used to drive expression of this open reading frame (ORF); this final construct was designated pMOMP1.2.

SDS-PAGE, immunoblot, colony blot, and indirect antibody accessibility assay methods. Cell envelopes and Sarkosyl extracts were prepared from *H. ducreyi* strains as described previously (14). The proteins present in these preparations were resolved by SDS-PAGE (33) and either stained with Coomassie blue or transferred to nitrocellulose for Western blot analysis (30). Colony blot radioimmunoassaying was performed as described elsewhere (18). The indirect antibody accessibility assay was performed as described elsewhere (28) with both MAb 3F12 and MAb 10F3. The latter antibody is directed against the CopB outer membrane protein of *Moraxella catarrhalis* and was used as the negative control antibody in these assays.

Southern blot analysis. *H. ducreyi* chromosomal DNA was digested to completion with restriction enzymes, electrophoresed in 0.7 to 1% (wt/vol) agarose gels, and transferred to a nitrocellulose support (46). Oligonucleotide labeling and hybridizations were done with the Renaissance kit (New England Nuclear, Boston, Mass.) per the manufacturer's instructions. Southern blot hybridization temperatures were 10°C below the calculated melting temperature for each oligonucleotide probe.

Mutant construction. Isogenic mutants of *H. ducreyi* were constructed by using the electroporation-based technique described by Hansen and colleagues (23). All constructs used for electroporation were purified by using CsCl density gradient centrifugation and then linearized by digestion with a restriction enzyme that cut in the polylinker site.

Nucleotide accession number. The nucleotide sequences of the *momp* and *ompA2* genes were deposited at GenBank and assigned the accession number U60646.

RESULTS

Cloning of the gene encoding the *H. ducreyi* MOMP. The *H. ducreyi* MOMP-reactive MAb 3F12 was used to screen 72,000 recombinant bacteriophages from an *H. ducreyi* 35000 genomic library. Twelve MAb-reactive phages were purified and the pBK-CMV phagemid in each was excised. A conserved 1.5-kb *Eco*RI fragment was subcloned into pBluescript II to obtain the recombinant plasmid pJKT600 (Fig. 1 and Table 1). Nucleotide sequence analysis revealed the presence of an incomplete ORF encoding a 19-kDa polypeptide. Searches of the relevant data bases indicated that this truncated polypeptide was similar to the N-terminal half of outer membrane proteins belonging to the OmpA family. The N-terminal region of this predicted protein matched 11 of 14 amino acids from the N-terminal sequence of the MOMP purified from *H. ducreyi* 85-023233 (49).

Two different approaches were used to obtain the 3′ half of the gene encoding the MOMP. The oligonucleotide 3f12-HIND (Fig. 2), corresponding to the sequence immediately downstream from the *Hin*dIII site present in this partial ORF, bound to a 6 kb-*Hin*dIII fragment from *H. ducreyi* 35000 chromosomal DNA in Southern blot analysis (data not shown). Therefore, 5- to 7-kb *Hin*dIII fragments of *H. ducreyi* chromosomal DNA were ligated into the *Hin*dIII site of pBluescript II, and the ligation reaction mixture was transformed into *E. coli* RR1. Screening of the transformants with the 3f12-HIND oligonucleotide identified a single clone, designated pJKT666 (Fig. 1), that hybridized this probe. Interestingly, pJKT666 contained only a 1.6-kb DNA insert, likely as the result of a spontaneous deletion. Nucleotide sequence analysis revealed an 88-bp overlap with the insert from pJKT600; this overlap contained the *Hin*dIII site present in pJKT600. The remainder of the ORF encoding the C-terminal half of MOMP was contained in this 1.6-kb insert. PCR amplification was performed with oligonucleotide primers from the 5′ and 3′ ends of this ORF (3f12-3 and 3f12-22, respectively, in Fig. 2) together with strain 35000 chromosomal DNA to verify that this gene, designated *momp*, represented a contiguous nucleotide sequence in the *H. ducreyi* chromosome.

Concurrently, an immunologic approach was used to obtain the 3′ half of the gene encoding the MOMP. MAbs 2C7 and 9D12 (which bind to distinct epitopes on the MOMP) were used to screen a *H. ducreyi* genomic library constructed in λgt11. Screening of approximately 14,000 plaques yielded a single recombinant bacteriophage, designated TH100, which formed plaques that bound these MAbs and which contained a 1.2-kb insert of *H. ducreyi* chromosomal DNA (Fig. 1). Nucleotide sequence analysis revealed that this clone contained the 3′ half of the *momp* gene fused with *lacZ*. When this bacteriophage was used to lysogenize *E. coli* Y1089(r^-), the lysogen expressed a 140-kDa fusion protein that bound MAbs 9D12 and 2C7 (data not shown).

Features of the *H. ducreyi momp* gene and its protein product. A putative promoter region (nucleotides 839 to 869) was identified immediately in front of the *momp* ORF (Fig. 3). Putative −35 and −10 regions as well as a possible ribosomal binding site are underlined in Fig. 3. Inverted repeat sequences (nucleotides 990 to 995 and 1002 to 1007 and 1016 to 1021 and 1023 to 1028 in Fig. 3) which could form stem-loop structures were noted in the 5′ upstream untranslated region of the *momp* gene. Stem-loop structures similar to these have been shown to be responsible for the prolonged half-life of *ompA* mRNA in *E. coli* (13, 26). A possible stem-loop structure was identified downstream (nucleotides 2386 to 2392 and 2410 to 2416) of the termination codon for the *momp* gene.

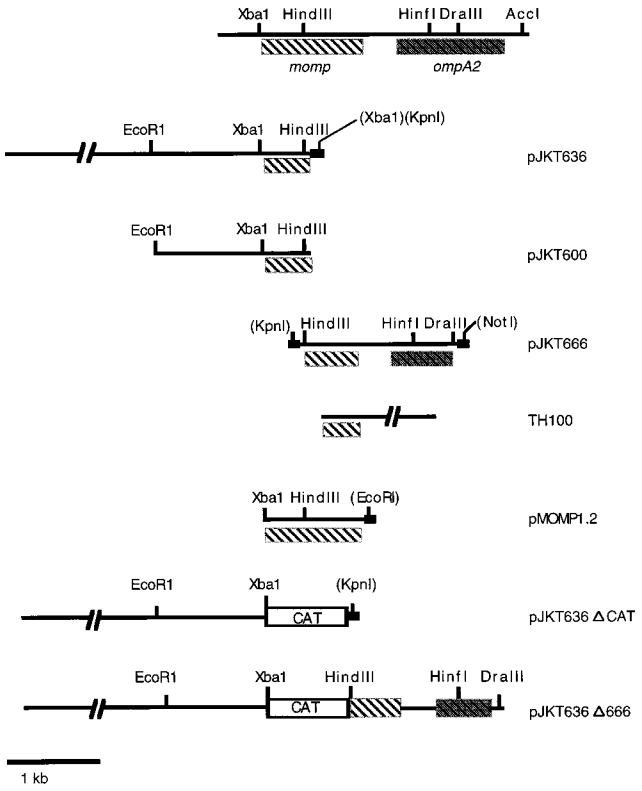

FIG. 1. Partial restriction map of the chromosomal locus containing the *momp* and *ompA2* genes of *H. ducreyi* 35000 and of DNA inserts in recombinant clones used in this study. Restriction sites shown in parentheses represent vector DNA. Detailed descriptions of these inserts are provided in Table 1.

The calculated molecular weight of the protein encoded by the *momp* gene was 44,169. The presence of a leader peptide with a signal peptidase I cleavage site was confirmed by N-terminal amino acid sequence analysis of the MOMP from *H. ducreyi* 35000. The N-terminal amino acid sequence (APDA NTFYLGAKAG) derived from the major 37- to 39-kDa band (isolated by SDS-PAGE) matched the predicted protein sequence exactly (Fig. 3). The calculated molecular weight of the mature MOMP was 42,248. This protein was 56% identical to the *H. influenzae* type b P5 outer membrane protein (37) and 46% identical to *E. coli* OmpA (7) (Fig. 4) and contained the conserved ($NX_2LSX_2RAX_2VX_3L$) α-helical motif proposed as a fingerprint for OmpA-related proteins (11, 32).

Complementation of an *E. coli ompA* mutant with MOMP. The OmpA-deficient *E. coli* mutant strain UH203 grows poorly at 42°C in minimal medium but shows normal growth at 30°C (27). To examine whether the product of the *H. ducreyi momp* gene could enhance the ability of the *E. coli ompA* mutant to grow at 42°C, the growth rates of *E. coli* UH203 containing pMOMP1.2 with the *H. ducreyi momp* gene, the pGEM3Zf($^+$) vector, or a plasmid (E29) containing a wild-type *E. coli ompA* gene were compared (Fig. 5). All three recombinant strains grew equally well at 30°C (Fig. 5A). When grown at 42°C, *E. coli* UH203(pMOMP1.2) grew better than *E. coli* UH203 containing only the vector but clearly did not grow as well as this *ompA* mutant containing E29 (Fig. 5B). These data suggested that the protein encoded by the *H. ducreyi momp* gene partially complemented the growth defect of this *E. coli ompA* mutant.

MAb 3F12 has been shown to be directed against an epitope of MOMP that is exposed on the surface of *H. ducreyi* cells. In

primer designation	sequence
3f12-3	CCAATTATCTCCTATTGC
3f12-7	AGATGGCTATCTAGAGG
3f12-HIND	CCGAGCTATGAAATTCTGCCTAACTTAGACGTATATGGTAAAGTTGGTATGGG
3f12-22	GCCTGCGAACACACC
3f12-30a	GCGGATACTTTTTATGTGGGTG
3f12-33A	GCACGTTAAAGACGAAAC
3F12-40	AGCGGGATCCGGAACAAGAGTGTGTTGTTATTATG
3F12-41	ACACGAATTCAATGACCGATTACATTGTTACTTCT
OmpA2-4	AGACATACAGCACACGGTG
OmpA2-5	AACACCTGTAGCAGAGCCAG
OmpA2-9	GCGATAAATCACTCTTAACCCGAC
OmpA2-12	AGAGCAACCGATAATAATGAAG

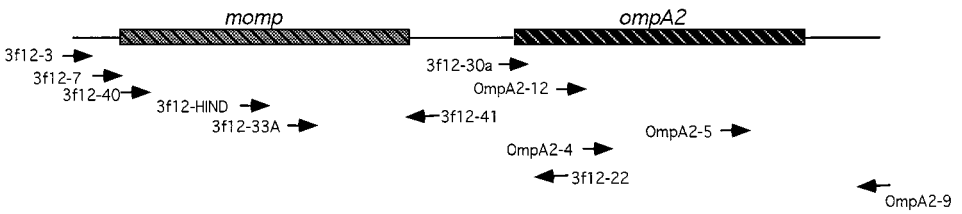

FIG. 2. Nucleotide sequences and relative positions of oligonucleotides used in this study for PCR amplification and Southern blot analysis.

an effort to determine whether the recombinant MOMP had been inserted properly into the outer membrane of *E. coli* UH203(pMOMP1.2), MAb 3F12 was used to probe whole cells of *E. coli* UH203(pMOMP1.2) and *E. coli* UH203 (pGEM3Zf+) in the indirect antibody accessibility assay (28). MAb 3F12 was readily bound by cells of *E. coli* UH203 (pMOMP1.2) but was not bound by the *E. coli* strain containing only the plasmid vector (data not shown). These results indicated that at least some of the MOMP in the former strain had been inserted into the outer membrane such that the MAb 3F12-reactive epitope was exposed on the bacterial cell surface.

Construction of an isogenic *H. ducreyi* mutant lacking the ability to express the MOMP. The plasmid pJKT636, an excision construct containing a 4.6-kb *H. ducreyi* DNA insert (Fig. 1) and originally derived from the *H. ducreyi* genomic library described above, was digested with *Xba*I. Agarose gel electrophoresis was used to separate the 500-bp *Xba*I fragment, containing the 5′ half of the *momp* gene, from the remainder of the plasmid. A *cat* cartridge was ligated into this deletion site to obtain the plasmid pJKT636Δ*cat* (Fig. 1). This plasmid was linearized by digestion with *Kpn*I. Then, the *H. ducreyi* DNA insert in pJKT666 (Fig. 1) was excised by digestion with *Not*I and *Kpn*I and isolated by gel electrophoresis. This 1.6-kb *Not*I-*Kpn*I fragment was ligated into the *Kpn*I site in pJKT636Δ*cat* to provide contiguous flanking *H. ducreyi* DNA downstream of the *cat* cartridge, for the purpose of enhancing allelic exchange. This final construct was designated pJKT636Δ666 (Fig. 1 and Table 1).

The plasmid pJKT636Δ666, containing a 500-bp deletion in the *momp* gene, was purified in CsCl, linearized with the restriction enzyme *Sac*I, and used to electroporate *H. ducreyi* 35000. Two chloramphenicol-resistant mutants that were unreactive with MAb 3F12 were obtained and one of these, designated 35000.60, was chosen for further study. Proper allelic exchange, involving insertion of the *cat* cartridge into the *momp* gene of this mutant strain, was verified by PCR-based analysis (data not shown).

SDS-PAGE and Western blot analysis were used to characterize the outer membrane proteins expressed by the wild-type (Fig. 6A) and the chloramphenicol-resistant mutant (Fig. 6B). It should be noted that, in the SDS-PAGE system used in this study, the MOMP doublet band consisting of the 37-kDa protein and the 39-kDa protein described by Spinola and colleagues (49) migrated as a single entity (Fig. 6A, closed arrow). The 43-kDa form of the MOMP (49) was also visible (Fig. 6A, open arrow). The chloramphenicol-resistant mutant (Fig. 6, panel 1, lane B, closed arrow) expressed a minor protein band that migrated at the same rate as the major 37- to 39-kDa band in the wild-type strain (Fig. 6, panel 1, lane A, closed arrow). This mutant also expressed an increased level, relative to the wild-type strain (Fig. 6, panel 1, lane A, open arrow) of the protein band which migrated with an apparent molecular weight of 43,000 (Fig. 6, panel 1, lane B, open arrow).

Western blot analysis indicated that both the 37- to 39-kDa and 43-kDa forms of the MOMP of the wild-type strain (Fig. 6, panel 2, lane A, closed and open arrows, respectively) bound the MOMP-reactive MAb 3F12, with the 43-kDa band reacting very weakly. In contrast, no proteins from the mutant strain 35000.60 (Fig. 6, panel 2, lane B) reacted with MAb 3F12. When probed with MAb 9D12, the wild-type strain (Fig. 6, panel 3, lane A) exhibited both a 37- to 39-kDa band (closed arrow) and a 43-kDa band (open arrow) that bound this MAb. This pattern of reactivity of MAb 9D12 with *H. ducreyi* outer membrane antigens was previously described with a different wild-type *H. ducreyi* strain (49). Interestingly, the mutant strain 35000.60 (Fig. 6, panel 3, lane B) also expressed a 37- to 39-kDa band and a 43-kDa band reactive with MAb 9D12, although the relative intensities of these immunoreactive bands were reversed relative to that observed with the wild-type strain.

N-terminal amino acid sequence analysis. The minor 37- to 39-kDa protein present in outer membranes of the mutant strain 35000.60 (Fig. 6, panel 1, lane B, closed arrow) was excised from polyacrylamide gels, eluted, and subjected to Edman degradation. The N-terminal amino acid sequence of this

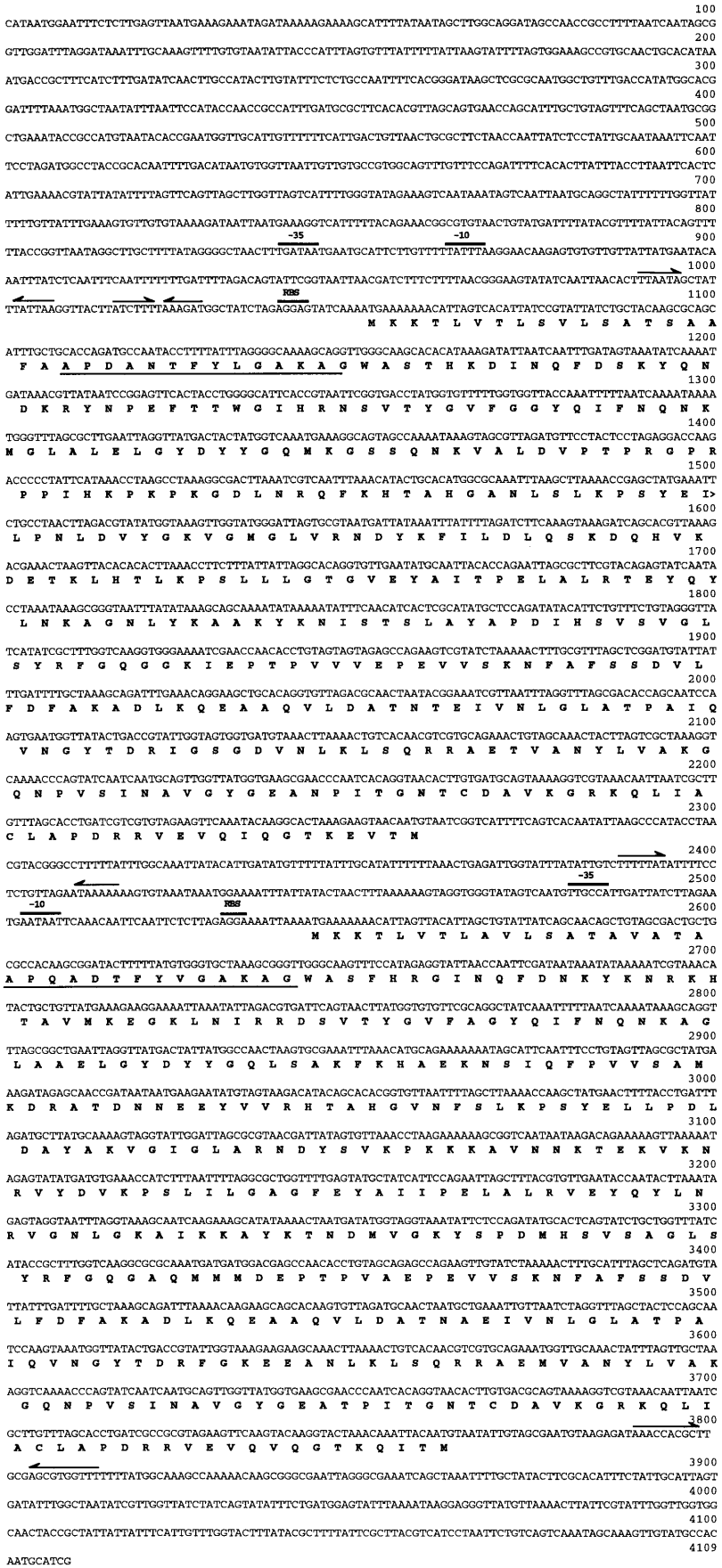

FIG. 3. Nucleotide sequence of the *H. ducreyi* strain 35000 *momp* and *ompA2* genes, together with the deduced amino acid sequences of the MOMP and OmpA2 proteins, respectively, encoded by these genes. Putative −35 and −10 regions are underlined, as are the putative ribosomal binding sites (RBS). The N-terminal amino acid sequence of the mature form of each protein is underlined. Inverted nucleotide repeats are indicated by opposing arrows.

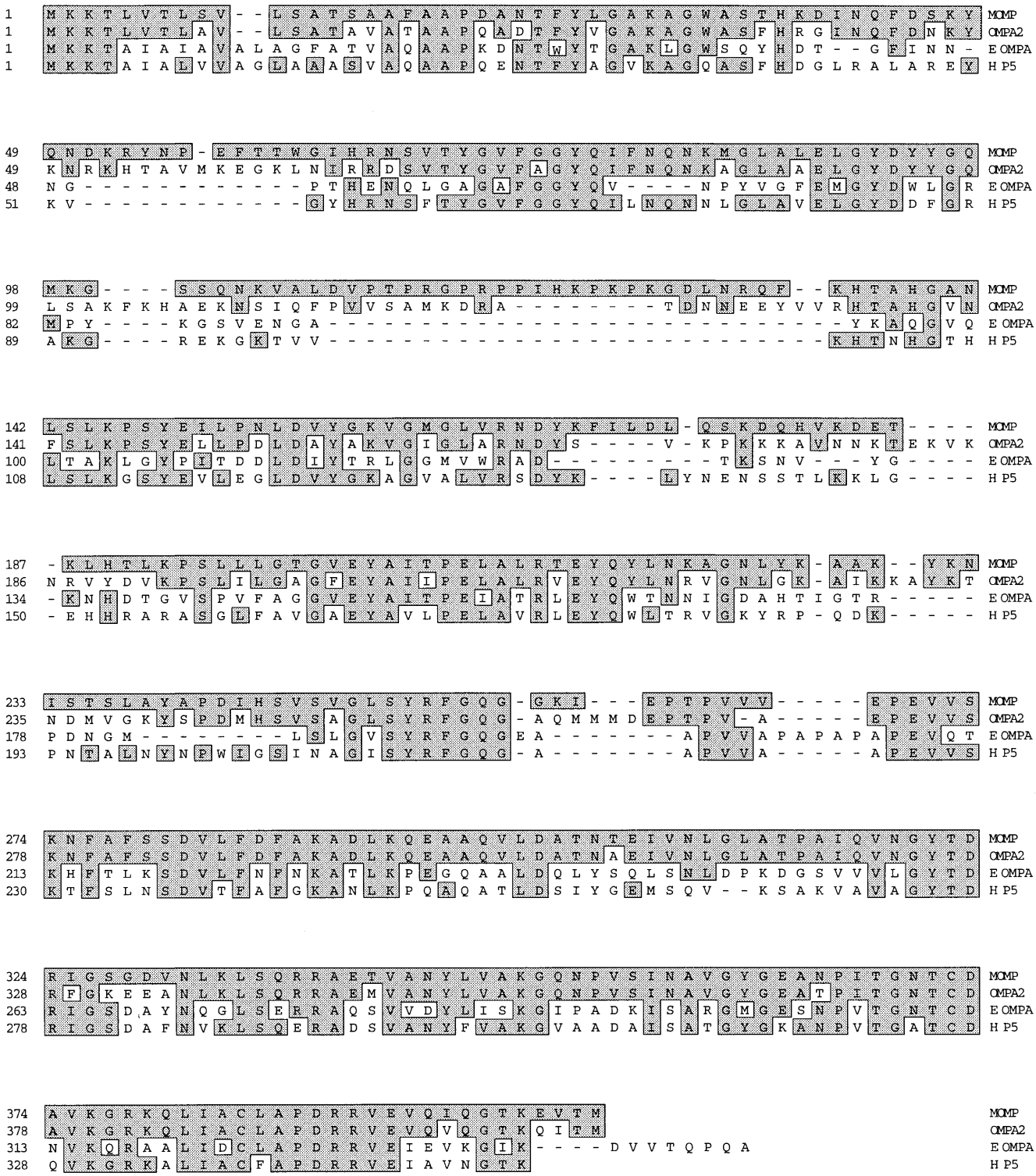

FIG. 4. Comparison of the amino acid sequences of *H. ducreyi* MOMP, *H. ducreyi* OmpA2, *E. coli* (E) OmpA, and *H. influenzae* type b (H) P5. Shading indicates residues that are identical between MOMP and one or more of the other three proteins.

minor protein, APQADTFYVGAKAG, was distinct from that of the MOMP (i.e., APDANTFYLGAKAG) and shared homology with N-terminal sequences from the OmpA family. We tentatively designated this minor 37- to 39-kDa protein from the mutant strain OmpA2. Interestingly, the N-terminal amino acid sequence of this minor 44-kDa protein matched 14 of 14 amino acids of the N-terminal sequence for the *H. ducreyi* MOMP published by Spinola and colleagues (49), indicating that the OmpA homolog purified in this previous study was likely OmpA2.

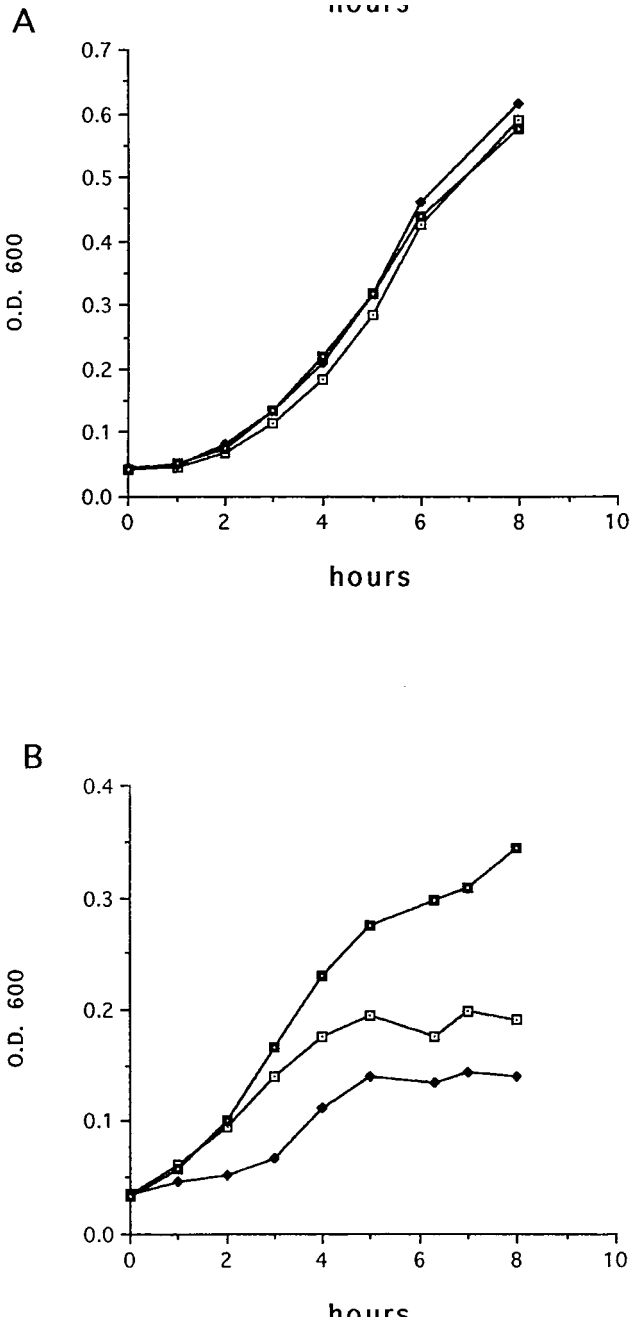

FIG. 5. Effect of the *H. ducreyi* MOMP on the growth of an *E. coli ompA* mutant. The *E. coli ompA* mutant strain UH203 containing the plasmid pMOMP1.2 bearing the *H. ducreyi momp* gene (open squares), the plasmid vector pGEM3Zf(+) (closed diamonds), or the plasmid E29 bearing an *E. coli ompA* gene (closed squares) was grown in minimal medium at 30°C (A) and 42°C (B). O.D. 600, optical density at 600 nm.

Identification of a gene encoding a second OmpA homolog in *H. ducreyi*. Nucleotide sequence analysis of the region downstream from the *momp* gene in pJKT666 revealed the presence of an incomplete ORF, truncated by the vector's multicloning site; this partial ORF encoded a predicted product that had homology with the OmpA protein from *E. coli* (data not shown). To obtain the nucleotide sequence of the complete ORF, we utilized a modification of the anchored PCR strategy. In this technique, which is similar to that described recently by Gray-Owen and colleagues (17), DNA fragments (2 to 4 kb in size) derived from a partial *Sau*3AI digestion of the *H. ducreyi* chromosome were ligated into the *Bam*HI site in pBluescript II. The ligation reaction was precipitated and subjected to PCR amplification with an oligonucleotide primer (3f12-30a in Fig. 2) derived from the DNA immediately downstream from the *momp* gene and a T7 oligonucleotide primer for the vector. By this technique, a 500-bp PCR product was obtained and sequenced. With a new oligonucleotide primer (OmpA2-4 in Fig. 2) derived from this new sequence information together with the T7 primer, the PCR amplification of the ligation reaction mixture was repeated. A third and final amplification with the oligonucleotide primer OmpA2-5 (Fig. 2) and the T7 primer allowed a total of 2 kb of additional DNA to be sequenced without subcloning of the desired sequence into a vector. This 2-kb fragment was amplified from the chromosome of *H. ducreyi* 35000 by PCR, and both strands were sequenced in their entirety.

Utilization of this approach resulted in the identification of a 1.2-kb ORF located 285 bp downstream from the termination codon of the *momp* gene (Fig. 1 and 3). This ORF encoded a predicted protein of 44,698 Da. A putative promoter region (nucleotides 2480 to 2508) was identified, including possible −10 and −35 regions and a putative ribosomal binding site (Fig. 3). A stem-loop structure was identified downstream from the termination codon of this ORF (Fig. 3).

The N-terminal amino acid sequence of the predicted protein encoded by this ORF matched exactly the N-terminal sequence of the minor 37- to 39-kDa protein (OmpA2) from the *momp* mutant 35000.60 and is underlined in Fig. 3. The mature form of OmpA2 had a calculated molecular weight of 42,827. OmpA2 shared 43% identity with *E. coli* OmpA and 53% identity with the *H. influenzae* P5 protein (Fig. 4). Additionally, OmpA2 was 72% identical to MOMP (Fig. 4). Similarly, the *ompA2* gene was 74% identical to the *H. ducreyi momp* gene. Located 206 bp downstream from the termination codon for *ompA2* was an incomplete ORF encoding a predicted protein that shared homology with the 1-acyl-*sn*-glycerol-3-phosphate acyltransferase of *E. coli* (8).

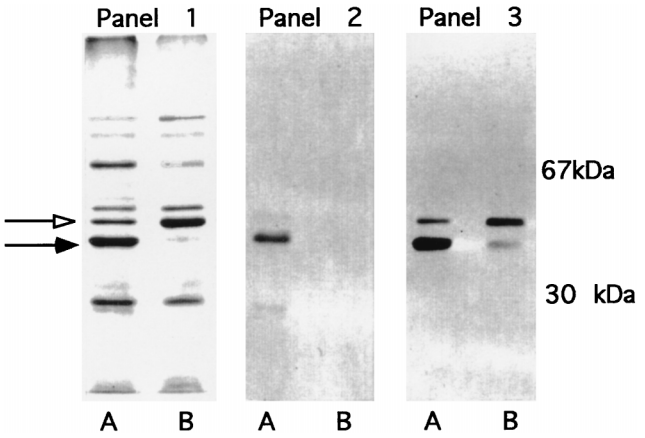

FIG. 6. Proteins present in outer membranes from wild-type and mutant *H. ducreyi* strains. Proteins in Sarkosyl extracts from cell envelopes of the wild-type strain 35000 (lane A) and the *momp* mutant strain 35000.60 (lane B) were resolved by SDS-PAGE and stained with Coomassie blue (panel 1) or were transferred to nitrocellulose and probed with MAb 3F12 (panel 2) and MAb 9D12 (panel 3) in Western blot analysis. Closed arrow, position of the 37- to 39-kDa band; open arrow, position of the 43-kDa band. Molecular weight position markers are given on the right.

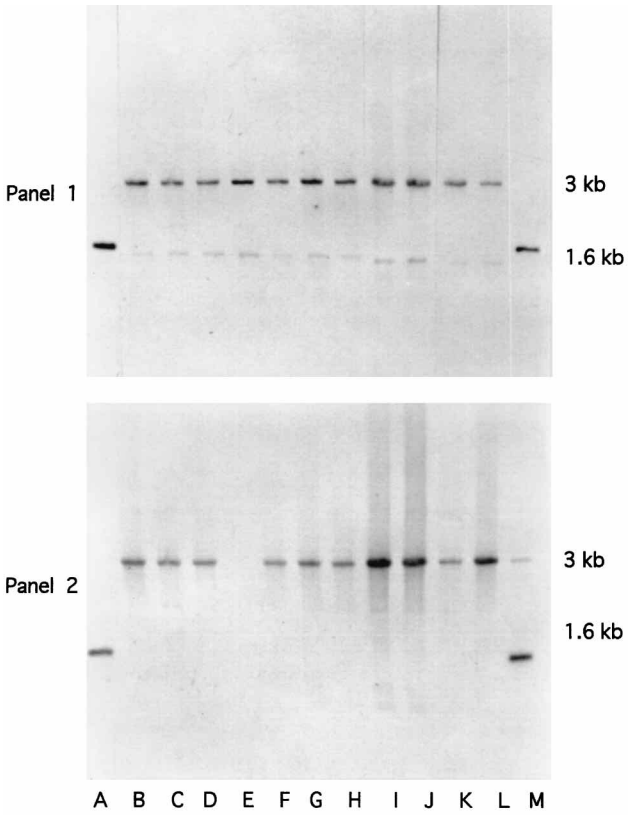

FIG. 7. Southern blot analysis of PCR products derived from wild-type *H. ducreyi* strains. Oligonucleotide primers (3f12-7 and OmpA2-9) were used to amplify a 3.1-kb fragment from chromosomal DNAs from 11 wild-type *H. ducreyi* strains; these PCR products were probed in Southern blot analysis with oligonucleotides specific for either *momp* (panel 1) or *ompA2* (panel 2). Lanes: B, 35000; C, 025; D, 1145; E, 041; F, Hd13; G, STD101; H, Cha-1; I, 1352; J, 512; K, Hd12; L, 1151. The 3.1-kb PCR products from strain 35000 (lane A) and strain 1151 (lane M) were also digested with *Hin*fI to test the specificity of these oligonucleotide probes. Size position markers (in kilobases) are shown on the right.

Conservation of the tandem *momp* and *ompA2* genes. To investigate whether the *momp* and *ompA2* genes existed in tandem in other *H. ducreyi* strains, chromosomal DNAs from 11 strains of this pathogen were prepared. When PCR primers (3f12-7 and OmpA2-9 in Fig. 2) flanking these two ORFs were used together with these chromosomal DNA preparations in PCR amplification, a 3.1-kb fragment was the major product obtained with each strain. Oligonucleotide probes specific for each ORF (3f12-33a for *momp* and OmpA2-12 for *ompA2*) were used in Southern blot analysis of these PCR products. The 3.1-kb PCR product from all 11 strains bound the *momp*-specific oligonucleotide (Fig. 7, panel 1, lanes B to L). The 3.1-kb PCR products from 10 of 11 strains hybridized with the *ompA2*-specific probe (Fig. 7, panel 2, lanes B to D and F to L); only the PCR product from strain 041 (Fig. 7, panel 2, lane E) failed to bind this probe. Preliminary nucleotide sequence analysis of the PCR product from strain 041 indicated that this *H. ducreyi* strain did possess an *ompA2* gene (data not shown); the observed lack of hybridization with the oligonucleotide probe was likely due to nucleotide sequence differences.

To verify the specificities of these oligonucleotide probes, the 3.1-kb PCR products derived from the strain 35000 and strain 1151 chromosomal DNA preparations were digested with *Hin*fI, which cut this fragment asymmetrically to yield a 1.7-kb fragment containing the *momp* gene and a 1.4-kb fragment bearing the *ompA2* gene. Oligonucleotide 3f12-33A, specific for *momp*, reacted with a 1.7-kb band from both PCR products (Fig. 7, panel 1, lanes A and M), while oligonucleotide OmpA2-12, specific for *ompA2*, reacted with a 1.4-kb band (Fig. 7, panel 2, lanes A and M), thus confirming the specificity of each gene probe.

DISCUSSION

The present study demonstrates that the *H. ducreyi* MOMP is actually comprised of two OmpA homologs encoded by the tandem genes *momp* and *ompA2*. The fact that many, if not all, gram-negative bacteria express an OmpA homolog suggests a basic role for this protein that would be common to all species (11). In *E. coli*, OmpA provides stability to the outer membrane, possibly through an interaction with peptidoglycan, and this function may account for the poor growth of *E. coli ompA* mutants at 42°C (34). The fact that expression of the *H. ducreyi* protein encoded by the *momp* gene partially complemented the growth deficiency of an *E. coli ompA* mutant indicates that there is some degree of functional homology between MOMP and OmpA from *E. coli.*

Analysis of the *H. ducreyi* genes encoding these two OmpA homologs was performed with strain 35000. It had been previously reported that the MOMP purified by cation exchange chromatography from *H. ducreyi* 85-023233 migrated as three species in SDS-PAGE (49). The N-terminal amino acid sequence of this purified protein was identical to that of OmpA2. In contrast, the major 37- to 39-kDa band purified from Sarkosyl-insoluble cell envelope proteins of *H. ducreyi* 85-023233 by SDS-PAGE had an N-terminal amino acid sequence that was identical to that of the MOMP (data not shown). Thus, while strain 85-023233 expressed both MOMP and OmpA2, the latter protein appears to have preferentially bound to the cation exchange resin used in the purification procedure (49).

The demonstrated ability of OmpA proteins to migrate heterogeneously in SDS-PAGE (22, 29, 36) and the lack of an antibody specific for OmpA2 precluded definitive determination of the relative abundance of MOMP and OmpA2 in the outer membrane of the wild-type *H. ducreyi* strain 35000. However, when the 37- to 39-kDa band present in Sarkosyl-insoluble material from cell envelopes of strain 35000 was subjected to N-terminal amino acid sequence analysis, quantitative measurements suggested that MOMP is approximately four to five times more abundant than OmpA2 in this strain (data not shown).

An isogenic *momp* mutant of *H. ducreyi* 35000, constructed by allelic exchange, expressed a minor protein that migrated as a 37- to 39-kDa band and had increased expression, relative to the wild-type parent strain, of a 43-kDa protein (Fig. 6). Both of these bands of the mutant strain bound MAb 9D12 (Fig. 6) and MAb 2C7 (data not shown). At this time, it cannot be determined whether this increase in the relative amount of the 43-kDa band of the mutant represents a compensatory increase in the amount of OmpA2, an increase in expression of another protein, or both.

Southern blot and PCR analyses of the genes encoding MOMP and OmpA2 indicate that they exist in tandem in all strains examined. The high degree of identity shared by these two genes suggests that they likely arose from a duplication event. The two genes, separated by 284 nucleotides, appear to be transcribed independently (data not shown). An examination of the two putative promoter regions reveals one interesting difference. Two inverted repeats are located in the region between the putative ribosomal binding site and −10 se-

quences upstream from the *momp* gene; these repeats are structurally similar to the inverted repeats present in an analogous region upstream from the *E. coli ompA* gene (13, 26). In *E. coli*, these inverted repeats are responsible for the prolonged half-life of *ompA* mRNA (13, 26). Similar inverted repeat structures were not noted in the putative promoter region of the *ompA2* gene. Whether the presence of these inverted repeats near the *momp* gene or the lack of these repeats in the same region near the *ompA2* gene plays a role in mRNA stability awaits further study.

At the protein level, MOMP and OmpA2 are 72% identical, with the most striking conservation being present in the C-terminal region as seen in previous comparisons among OmpA homologs of other gram-negative organisms (11). Whether the differences between the primary amino acid sequences of MOMP and OmpA2 reflect functional differences between these proteins remains to be determined. The presence of two OmpA homologs in *Aeromonas salmonicida* was recently described, indicating that this occurrence, while unusual in gram-negative organisms, is not unique to *H. ducreyi* (9).

The identification of the genes encoding these two OmpA homologs should permit construction of isogenic *H. ducreyi* mutants lacking OmpA2 or both MOMP and OmpA2. The eventual availability of these mutants will allow investigation of the relative contributions of MOMP and OmpA2 to the physiology of *H. ducreyi*. Given the multifunctional nature of OmpA as well as its newly described roles in virulence expression in other gram-negative organisms (42, 53), it will be interesting to determine whether these two OmpA homologs in *H. ducreyi* play a role in the pathogenesis of chancroid.

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