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Identification and characterization of a *Neisseria gonorrhoeae* gene encoding a glycolipid-binding adhesin

(bacterial binding/receptors/adhesion/overlay assay)

DURGA K. PARUCHURI*†, H. STEVEN SEIFERT‡, RICHARD S. AJIOKA*, KARL-ANDERS KARLSSON§, AND MAGDALENE SO*

*Department of Molecular Biology, Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, CA 92037; ‡Department of Microbiology, Northwestern Medical School, 303 East Chicago Avenue, Chicago, IL 60611; and §Department of Medical Biochemistry, University of Goteborg, P.O. Box 33031, S-400 33 Goteborg, Sweden

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ABSTRACT **We recently identified a set of mammalian cell receptors for *Neisseria gonorrhoeae* that are glycolipids. These receptors, lactosylceramide [Gal(β1–4)Glc(β1–1)Cer], gangliotriosylceramide [GalNAc(β1–4)Gal(β1–4)Glc(β1–1)Cer], and gangliotetraosylceramide [Gal(β1–3)GalNAc(β1–4)Gal(β1–4)Glc(β1–1)Cer], were shown to be specifically bound by a gonococcal outer membrane protein distinct from pilin and protein II. Here we report the isolation of the gene encoding the gangliotetraosylceramide-binding adhesin from a *N. gonorrhoeae* MS11 gene bank in *Escherichia coli*. Transposon mutagenesis studies in *E. coli* indicate that the adhesin is a protein with a molecular mass of 36,000 Da. The gene encoding the 36-kDa protein is duplicated in MS11 since two transposon insertions were required to abolish expression of the gene in this bacterium. This protein is present on the surface of the gonococcus and is not associated with the pilus.**

The binding of a pathogen to host cells is an important step in most infectious processes. The host cell receptors for several bacterial pathogens have been identified. For example, *Escherichia coli* strains causing urinary tract infections bind to a digalactoside moiety present in glycolipids (1, 2), and type 1 fimbriated *E. coli*, also implicated in urinary tract infections, bind to mannose residues in glycoproteins (3). In *E. coli* strains binding digalactoside, attachment to eukaryotic cells is independent of pilin, the major subunit of the pilus (4). In contrast, attachment of type 1 fimbriated *E. coli* is dependent on piliation (5). Recent work on digalactoside-binding *E. coli* demonstrated the presence of a minor protein at the tip of the pilus that binds to the receptor (6). The mannose-binding adhesin on type 1 fimbriae was shown to be present at the tip and along the sides of the pilus fiber (7, 8). In addition to these pilus-associated adhesins, a non-pilus-associated surface adhesin responsible for mannose-resistant hemagglutination has been reported in uropathogenic *E. coli* KS52 (9).

Pili have been shown to be important in the attachment of *Neisseria gonorrhoeae* to human epithelial cells and erythrocytes (10–12). However, recent *in vitro* studies indicated that both piliated (P^+) and nonpiliated (P^-) gonococci adhered to and were internalized by tissue culture cells with equal efficiency (13). We recently identified a set of glycolipid receptors on epithelial cells that are recognized by both P^+ and P^- *N. gonorrhoeae*. They share lactose as their core sugar. Two of these, lactosylceramide [LacCer; Gal(β1–4)Glc(β1–1)Cer] and gangliotriosylceramide [GgO_3; GalNAc(β1–4)Gal(β1–4)Glc(β1–1)Cer] are present on cultured human endocervical cells. Gonococci bound to GgO_3 and gangliotetraosylceramide [GgO_4; Gal(β1–3)GalNAc(β1–4)Gal(β1–4)Glc(β1–1)Cer] with equal affinities, whereas they bound to LacCer with a lower affinity *in vitro* (14). Binding to these receptors was observed to be independent of pilin and protein II expression.

A detailed characterization of the gonococcal surface adhesin, which recognizes the glycolipid receptor on host cells, is necessary for an understanding of the attachment process. For this purpose, we developed a thin-layer plate overlay assay for isolating the *N. gonorrhoeae* gene encoding the GgO_4-binding adhesin from a pool of *E. coli* recombinants. Expression of this protein in *E. coli* HB101 enables the recombinant bacteria to bind to the glycolipid receptor *in vitro*. Transposon mutagenesis studies indicate that the adhesin is a 36-kDa protein and that the gene encoding the 36-kDa protein is duplicated in MS11. Immunoelectron microscopy studies show that the 36-kDa protein is present on the surface of MS11 and not on the pilus fiber.

MATERIALS AND METHODS

Bacterial Strains and Genomic Libraries. P^+ *N. gonorrhoeae* strain MS11 variant A and P^- variant B2 have been described (15). Gonococcal strains were grown on supplemented GCB (Difco) agar plates at 37°C in 5% CO_2/95% air. A genomic library of MS11 B2 was constructed in the vector pHSS6 (16) in *E. coli* HB101 (17).

Antisera. Antiserum against *E. coli* HB101 was raised in rabbits against the whole heat-killed bacteria. Anti-gonococcal outer membrane (OM) antiserum was raised against the whole OM protein prepared from the B2 variant of MS11. One hundred micrograms of protein in 50 mM phosphate buffer (pH 7.5) containing 150 mM NaCl (PBS) was emulsified in complete Freund's adjuvant (Difco) and injected subcutaneously at multiple sites. Antiserum was prepared after two subsequent injections of protein in incomplete Freund's adjuvant. Elution of specific antibodies from nitrocellulose filters was done with 0.1 M glycine hydrochloride (pH 2.6) containing 0.15 M NaCl; the antibodies were immediately neutralized by addition of an equal volume of 1 M Tris·HCl (pH 8.0) (18).

Thin-Layer Chromatogram Overlay Assay. The purification and separation of glycolipids and visualization with anisaldehyde was performed as described (19). After separation of the glycolipids, the thin-layer plates were coated with 0.5% polyisobutylmethacrylate as described (20). Plates were blocked with 3% (wt/vol) bovine serum albumin in PBS for

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Abbreviations: GgO_3, gangliotriosylceramide; GgO_4, gangliotetraosylceramide; P^+ and P^-, piliated and nonpiliated; LacCer, lactosylceramide; OM, outer membrane; Cm^r, chloramphenicol resistant; mTnCm3, minitransposon *3* carrying the chloramphenicol-resistance gene.

†To whom reprint requests should be addressed.

1 hr. For screening the genomic library, recombinant *E. coli* HB101 were grown in Luria broth containing kanamycin (40 μg/ml) to a density of 5×10^8/ml. The culture was divided into 10 aliquots, and each was incubated with glycolipids separated on a thin-layer plate. After a 4-hr incubation at room temperature, unbound recombinant *E. coli* were removed by washing four times (1 min each) with PBS. Recombinants bound to each glycolipid were scraped off the plate with a scalpel, resuspended in Luria broth containing kanamycin, and vortexed gently for 10 sec. These recombinants were grown to 5×10^8/ml, and the screening was repeated two more times. The eluted recombinants after the final enrichment were plated on Luria–Bertani plates containing kanamycin (40 μg/ml).

For staining with the antibody, the plates were blocked with 2% bovine serum albumin in PBS for 2 hr, incubated for 2 hr with bacteria (5×10^8/ml), and washed four times with PBS. The plates were then incubated for 1 hr with the primary antibody (1:400 dilution), washed four times (1 min each) in PBS, and further incubated with mouse anti-rabbit IgG conjugated to alkaline phosphatase (1:1000 dilution). After four more washes, color development was done according to manufacturer's instructions (Promega).

***In Vitro* Analysis of Plasmid-Encoded Proteins.** The proteins encoded by the plasmids were analyzed using a coupled transcription–translation kit (Amersham) according to the manufacturer's instructions.

Western Blot Analysis. OM proteins from recombinant *E. coli* and MS11 were prepared according to published procedures (21, 22). Total cell lysates were prepared by suspending cell pellets directly in 1× sample buffer [0.1 M Tris·HCl, pH 7.5/2% SDS/10% (vol/vol) 2-mercaptoethanol/20% (vol/vol) glycerol] and boiling for 5 min. Samples were centrifuged at 12,000 × *g* for 5 min before being loaded onto gels. Proteins were separated on SDS/13% polyacrylamide gels (23) and transferred to nitrocellulose filters (24). After blocking with 3% nonfat dry milk in PBS for 1 hr, filters were incubated with primary antibody (1:1000) for 3 hr followed by five washes with PBS containing 0.05% Nonidet P-40. Incubation with secondary antibody coupled to alkaline phosphatase continued for 1 hr followed by five more washes with PBS containing Nonidet P-40. The filters were then developed according to manufacturer's instructions.

Shuttle Mutagenesis and DNA Transformation. The mutagenesis procedure developed by Seifert *et al.* (16, 25) uses a defective mini Tn*3* carrying the chloramphenicol-resistance gene (mTnCm3) (17). Transformation of *N. gonorrhoeae* MS11 to generate adhesin-deficient mutants was done as previously described (26).

Southern Hybridization. Chromosomal DNA from wild type as well as chloramphenicol-resistant (Cm^r) gonococcal transformants was prepared according to a published procedure (15). After digestion with *Mlu* I, the DNA was fractionated on 0.7% agarose gels and transferred to nitrocellulose (0.45 μm; Schleicher & Schuell) (15). After blocking, the filters were hybridized at 68°C to appropriate probes labeled by nick-translation in the presence of [α-^{32}P]dNTPs. Filters were washed at high stringency [68°C, 0.1 × SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0) and 0.1% SDS] and analyzed by autoradiography.

Electron Microscopy. Bacteria growth on supplemented GCB agar plates were harvested, resuspended in PBS, and placed on carbon-coated grids. After blocking with 2% bovine serum albumin, grids were incubated with antibodies specific for the 36-kDa protein (eluted from nitrocellulose) for 1 hr, washed with PBS, and reincubated with anti-rabbit IgG conjugated to colloidal gold (5 nm). The grids were washed in PBS and negatively stained with uranyl acetate [1% (wt/vol), pH 4.5] (27).

RESULTS

Identification of GgO_4-Specific Recombinant Clone. The *N. gonorrhoeae* gene encoding the GgO_4-binding adhesin was identified from a pool of *E. coli* recombinants by using the enrichment scheme illustrated in Fig. 1. After separation of the glycolipids (as described in *Materials and Methods*), the thin-layer plates were incubated with an MS11 gene bank in *E. coli* HB101. GgO_4 was chosen as the specific receptor in our assay. Three other glycolipids (globoside, GM1, and GD1a) were used as negative controls. None of the recombinant *E. coli* bound to the control glycolipids, whereas a few recombinants adhered to GgO_4 after three rounds of enrichment. All GgO_4-specific clones contained inserts of two sizes. Approximately two-thirds of the recombinants carried a 5.3-kilobase (kb) insert (p36S; Fig. 2*A*), and the rest had an insert size of ≈11.5 kb (p36L; data not shown). Restriction enzyme analysis of the two inserts revealed the presence of overlapping DNA sequences. The same two classes of recombinants were obtained when the screening was repeated in a separate experiment. *E. coli* HB101 harboring pHSS6 did not bind GgO_4, whereas both classes of recombinants p36S (Fig. 2*B*) and p36L (data not shown) recognized GgO_4 with approximately equal affinities in thin-layer chromatogram overlay assays. We selected the smaller clone, p36S, for further studies.

Characterization of Proteins Encoded by p36S. To determine the number of proteins encoded by the 5.3-kb insert, a coupled transcription–translation system was used (28). The control plasmid vector pHSS6 directed the synthesis of one protein with a molecular mass of 24 kDa, which corresponds to the kanamycin phosphotransferase (Fig. 3*A*, lane 2). In addition to this 24-kDa protein, p36S encoded five other proteins with apparent molecular masses of 36, 35, 22, 16, and 15 kDa (Fig. 3*A*, lane 1). Antiserum raised against OM proteins from P^- *N. gonorrhoeae* (anti-B2 OM antiserum) recognized three proteins with molecular masses of 36, 35, and 22 kDa (Fig. 3*B*, lane 1) from the OM of *E. coli* harboring p36S. This result suggests that one of these three proteins is the receptor-binding adhesin.

Transposon Mutagenesis of p36S. To determine which of the three OM proteins encoded in p36S binds GgO_4, shuttle

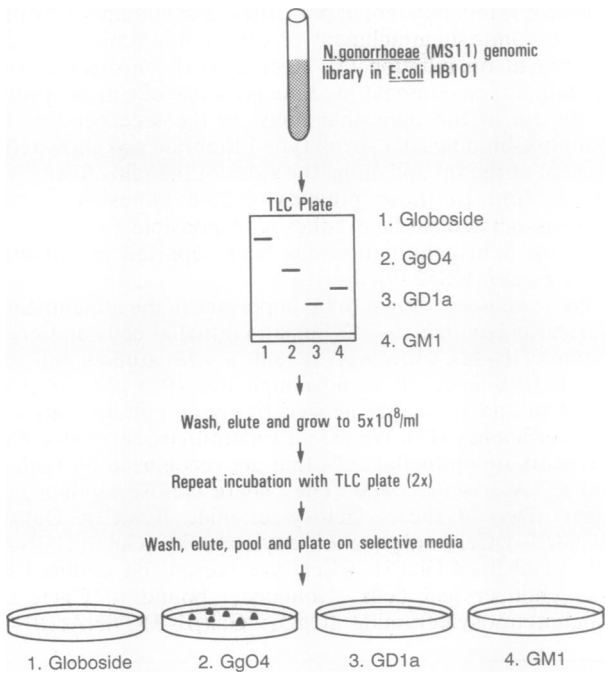

FIG. 1. Screening protocol for obtaining GgO_4-specific clones. GM1, GD1a, and globoside were used as negative controls.

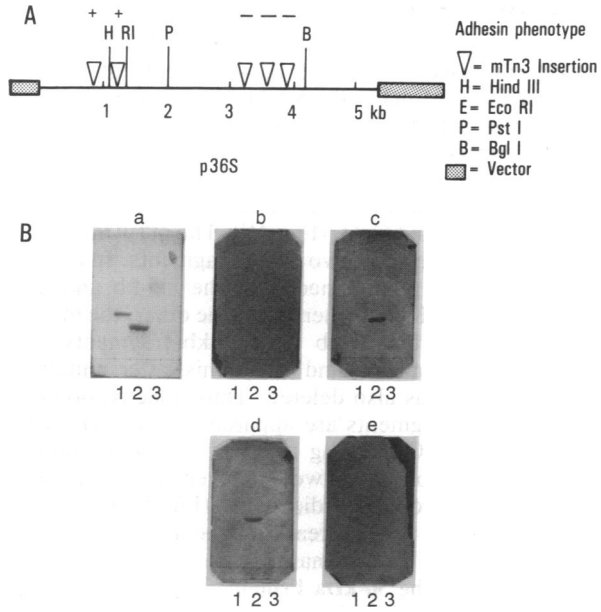

FIG. 2. Transposon mutagenesis of p36S and binding of mutants to GgO_4. (*A*) Genetic and physical map of the plasmid p36S. The stippled bar indicates the vector (pHSS6 DNA) and an open triangle indicates mTnCm3 insertion. + or −, retention or loss of the ability of the recombinants to bind GgO_4. (*B*) Thin-layer chromatogram overlay assays with recombinant *E. coli*. Plate a, anisaldehyde stain represents the glycolipids (1 μg each) separated on each plate. Lanes: 1, globoside; 2, GgO_4; 3, LacCer. Plates b, c, d, and e were incubated with *E. coli* HB101 carrying either vector pHSS6 (plate b), GgO_4-specific plasmid p36S (plate c), p36S with mTnCm3 outside the adhesin gene (p36S-NKO) (plate d), or p36S with mTnCm3 within the adhesin gene (p36S-KO) (plate e). After the incubation with the bacteria, thin-layer chromatogram plates were incubated with antibody against *E. coli*.

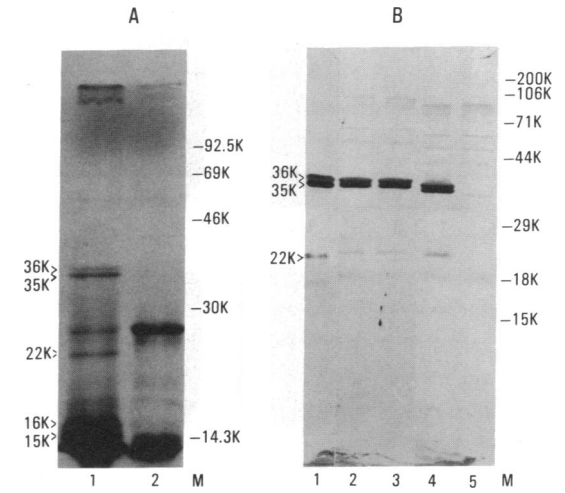

FIG. 3. Analysis of proteins encoded by p36S and transposon mutants. (*A*) SDS/PAGE and autoradiography of [^{35}S]methionine-labeled proteins encoded by the plasmid p36S synthesized *in vitro*. Reactions were programmed with 2.5 μg of plasmid DNA linearized with Cla I. Approximately 100,000 cpm of [^{35}S]methionine-labeled proteins were separated on a 13% polyacrylamide gel and examined by fluorography using EN^{3}HANCE (NEN). M, molecular size markers (in kDa). Lanes: 1, p36S; 2, vector pHSS6. Carats on the left side of the figure represent the molecular mass of the proteins synthesized by the p36S. (*B*) Western blot analysis. Lane 1, OM protein from *E. coli* harboring p36S. Lanes 2–4, total cell lysates of three independent Cm^r transformants of *E. coli* carrying p36S with a mTnCm3 insertion outside the adhesin gene (p36S-NKO). Lane 5, mTnCm3 insertion within the adhesin gene (p36S-KO). M, molecular size markers. Carats on the left side represent the molecular mass of the proteins present in the OM of *E. coli*.

mutagenesis was carried out on p36S in *E. coli*. For this experiment, mTnCm3 (1.6 kb) carrying the chloramphenicol-resistance gene (17) was used. Plasmids that contained mTnCm3 in the 5.3-kb insert were analyzed in Western blots with anti-B2 OM antiserum. Of the 100 transposon mutants tested, 75 expressed the 36-, 35-, and 22-kDa proteins (Fig. 3*B*, lanes 2, 3, and 4), whereas the other 25 failed to express all three proteins (Fig. 3*B*, lane 5). None of the transposon insertions separately inactivated expression of the 36-, 35-, or 22-kDa proteins. One possible explanation for this result is that the genes encoding the three proteins are on one operon and the mTnCm3 insertions exerted a polar effect. Alternatively, the two smaller proteins may have been degradation products of the 36-kDa protein. To distinguish between these two possibilities, antibodies binding to the 22-kDa and 35/36-kDa proteins on nitrocellulose filters were affinity purified from anti-B2 OM antiserum as described in *Materials and Methods* (18). Both the anti-35/36-kDa and anti-22-kDa antibodies recognized all three proteins (36, 35, and 22 kDa) expressed in *E. coli* in a subsequent Western blot and also recognized a single 36-kDa protein from the OMs of MS11 (data not shown). Second, a Western blot of an OM preparation of *E. coli* strain degP$^-$ harboring p36S with anti-B2 OM antiserum showed that only the 36-kDa protein was present (data not shown). The *deg*P gene in *E. coli* encodes a protease that resides in the periplasm and destabilizes proteins that are exported to the membrane. Mutations in this gene were shown to stabilize proteins that are exported (29). These data strongly suggest that the 35- and 22-kDa proteins are degradation products of the 36-kDa protein.

E. coli HB101 carrying mTnCm3 insertions in the 5.3-kb insert were next examined in thin-layer chromatogram overlay assays for their ability to bind GgO_4. Transposon mutants that fail to produce the 36-kDa protein (p36S-KO) could not bind GgO_4 (Fig. 2*B*, plate e) while mutants that still produce the 36-kDa protein (p36S-NKO) were able to bind GgO_4 (Fig. 2*B*, plate d) (Fig. 2*A*). These results demonstrate that the loss of 36-kDa protein expression results in loss of GgO_4-binding ability and strongly suggest that the 36-kDa protein is the GgO_4-binding adhesin.

Construction of Mutants of *N. gonorrhoeae* MS11 That Fail to Produce the 36-kDa Adhesin. Two mTnCm3 insertions into p36S, p36S-KO, which has the transposon in the adhesin gene, and p36S-NKO, which has the transposon outside the gene, were used for DNA transformation in MS11. Transformants with insertion of the mTnCm3 into the homologous flanking region of the chromosome were selected on medium containing chloramphenicol (10 μg/ml). All the Cm^r transformants were piliated, suggesting that pilin expression is not affected by these mTnCm3 insertions. Anti-B2 OM antiserum was next used for Western blot analyses of the Cm^r transformants (data not shown). Surprisingly, of the 50 transformants analyzed, only 2 failed to produce the 36-kDa adhesin. These 2 transformants also failed to bind GgO_4 (Fig. 4*E*). However, the low level of binding of these 36-kDa mutants to GgO_4 may represent nonspecific interaction between the hydrophobic glycolipid and the bacterial cell surface. Alternatively, there may be other surface proteins that recognize this glycolipid with a much lower specificity. All the other 48 p36S-KO Cm^r transformants produced the 36-kDa protein and also bound GgO_4 (Fig. 4*D*). All p36S-NKO Cm^r transformants produced the 36-kDa adhesin and also bound GgO_4 (Fig. 4*C*).

Southern Blot Analysis of Cm^r Transformants. To localize the adhesin gene in the *N. gonorrhoeae* chromosome, Southern analysis of the wild-type and the Cm^r transformants was performed by using the 5.3-kb insert or the chloramphenicol acetyltransferase gene as probes. The 5.3-kb insert, which

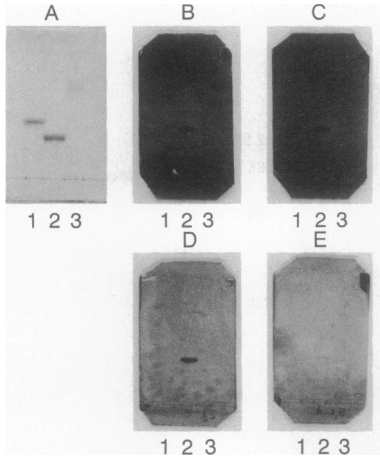

FIG. 4. Thin-layer chromatogram overlay assays with wild-type and Cm^r transformants of *N. gonorrhoeae* MS11. (*A*) Anisaldehyde stain of glycolipids. Lanes 1, 2, and 3 represent 1 μg each of globoside, GgO_4, and LacCer, respectively. (*B*) Wild-type MS11. (*C*) Cm^r transformant obtained with p36S-NKO. (*D* and *E*) Cm^r transformants obtained with p36S-KO. Thin-layer chromatography plates were reacted with anti-B2 OM.

does not contain any *Mlu* I sites, hybridized at high stringency to four different *Mlu* I fragments of ≈22, 10, 8.5, and 2.5 kb in the MS11 genome (Fig. 5*A*, lane 1). The gene encoding the 36-kDa protein has not been mapped in detail. However, a subclone generated by deletion of the 1.2-kb *Eco*RI fragment from the left end of the insert (Fig. 2*A*) still expresses the 36-kDa protein. This subclone also hybridized to all four *Mlu* I fragments (data not shown).

Southern blots of DNA from p36S-NKO Cm^r transformants showed that mTnCm3 had recombined with the 22-kb fragment (Fig. 5, lane 2). In 25 of 50 p36S-KO Cm^r transformants tested, the incoming DNA recombined with the 22-kb

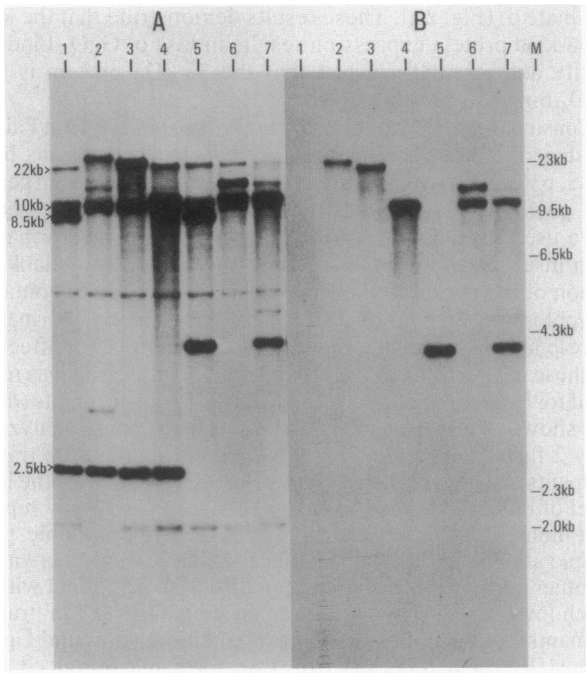

FIG. 5. Southern blot analysis of the DNA isolated from the wild-type and Cm^r MS11 transformants. (*A*) Southern blot probed with the 5.3-kb insert from p36S. (*B*) A similar blot probed with the chloramphenicol acetyltransferase gene. Lanes: 1, wild-type MS11; 2, Cm^r transformant (p36S-NKO); 3–7, DNAs from Cm^r transformants (p36S-KO).

fragment (Fig. 5*B*, lane 3). These Cm^r transformants still expressed the 36-kDa protein and bound GgO_4 in a thin-layer chromatogram overlay assay (Fig. 4*C*). Eight of the transformants had mTnCm3 in the 8.5-kb fragment, while 15 had mTnCm3 inserted into the 2.5-kb fragment (Fig. 5, lanes 4 and 5). While all three classes of transformants still expressed the 36-kDa protein and bound GgO_4, the fourth class of the Cm^r transformants (2 transformants) failed to express this protein and also failed to bind GgO_4 (Fig. 4*E*). The mTnCm3 in these mutants had inserted into two *Mlu* I fragments. In one case, the mTnCm3 had recombined with the 8.5-kb and 2.5-kb fragments (p36S-DKO1), whereas, in the other, the mTnCm3 had inserted into the 10-kb and 8.5-kb fragments (p36S-DKO2) (Fig. 5*A*, lanes 6 and 7). In this latter mutant, the 2.5-kb fragment was also deleted. Thus, inactivation of the 8.5- and 2.5-kb fragments are apparently required for inactivation of the GgO_4-binding adhesin. In a few other Cm^r gonococcal transformants, we observed amplification and deletion of some cross-hybridizing loci (Fig. 5, lanes 2, 3, and 6), suggesting that genetic rearrangements may have taken place during DNA transformation.

Localization of the 36-kDa Protein. Immunogold electron microscopy of a P$^+$ MS11 strain with affinity-purified antibodies to the 36-kDa protein, obtained as described in *Materials and Methods*, revealed the presence of this protein on the surface of the gonococcus. Pili are clearly present on the bacterium, but no antibody binding could be detected on these structures (Fig. 6). In contrast, preimmune serum did not recognize any proteins on the surface of the gonococcus (data not shown). These data strongly suggest the presence of 36-kDa adhesin on the surface of MS11 but that is not associated with the pilus.

DISCUSSION

In a previous report, we identified a set of glycolipids of epithelial origin that are bound by both P$^+$ and P$^-$ *N. gonorrhoeae* and showed that the bacterial ligand is a surface protein. These glycolipids, LacCer, GgO_3, and GgO_4, share lactose as the core sugar. *N. gonorrhoeae* bound GgO_3 and GgO_4 with equal affinity *in vitro*, whereas they bound LacCer weakly. GgO_3 and LacCer were shown to be present on human endocervical cells in culture and may therefore play important roles in gonococcal attachment to this anatomical site. In order to understand the interaction of gonococci with host epithelial cells, we have identified the gonococcal gene encoding the GgO_4-binding adhesin from an *E. coli* gene bank. We have used GgO_4 for developing our assay because it is available in large quantities in pure form. In two separate experiments, this method yielded identical clones that bound only GgO_4 and not to control glycolipids. That a specific set of *E. coli* recombinants were able to bind the glycolipid *in vitro* indicates that the gonococcal adhesin is presented properly on the bacterial surface. Thus, this procedure may be useful for identifying other bacterial adhesin genes.

The gonococcal insert identified by this procedure encodes proteins of 36, 16, and 15-kDa. Shuttle mutagenesis of the insert in *E. coli* indicated that the 36-kDa protein bound GgO_4. Southern analysis of gonococcal chromosomal DNA using the insert as probe revealed the presence of at least four strongly hybridizing *Mlu* I bands of ≈22, 10, 8.5, and 2.5 kb. Homologous recombination with the transposon-marked copy of the adhesin gene occurred in all of these fragments. At this time, it cannot be concluded that these bands represent multiple copies of the gene encoding the 36-kDa adhesin. However, our transposon mutagenesis data strongly suggest the presence of at least two copies of this gene. Since inactivation of two loci was necessary to block expression of the 36-kDa protein as well as the binding ability to GgO_4, it appears that both genes encode a functional protein.

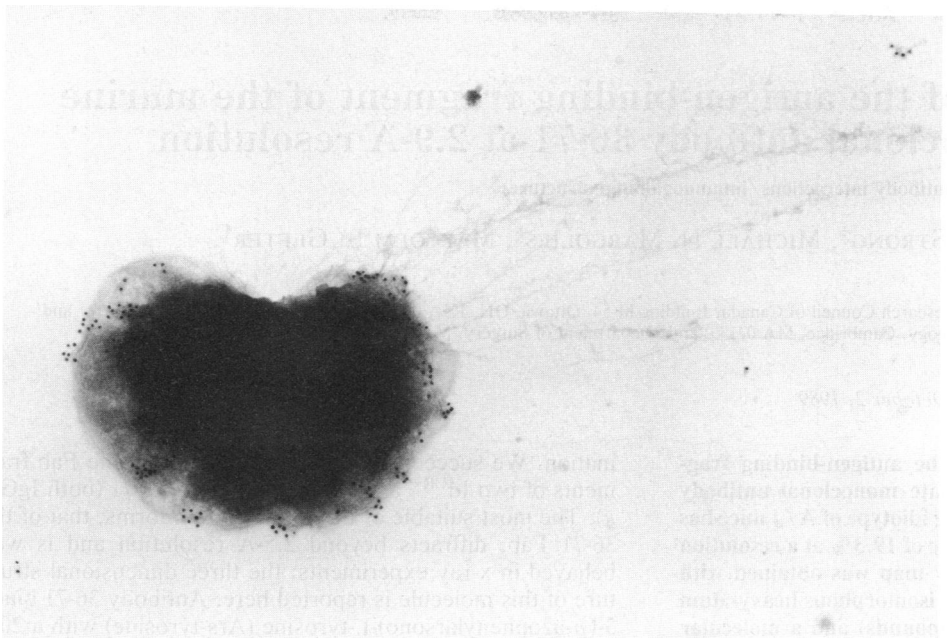

FIG. 6. Immunoelectron microscopy of *N. gonorrhoeae* MS11 incubated with antibodies against the 36-kDa protein. The dark granules are electron-dense gold particles, seen present on the surface of MS11. (×26,000.)

Transformation of gonococci with mTnCm3-mutated inserts resulted in a low frequency of rearrangements of the hybridizing DNA. These rearrangements are unlikely to be due to the mTnCm3 element, since this transposon has been used to generate hybrid gonococcal porin genes without difficulty (30). Rather, these rearrangements may reflect the presence of repetitive elements in or near the adhesin gene. We have previously observed such rearrangements in the *pilS1* locus when it recombined with an incoming pilin gene containing mTnCm3 (26).

Previous studies have underscored the importance of the gonococcal pilus in attachment and establishment of infection (10, 12, 31). To date, the molecular mechanism by which the pilus mediates these processes is unknown. Our immunoelectron microscopy data indicate that the 36-kDa protein is surface located but is not in association with the pilus. The gonococcal mutants of MS11 that do not express 36-kDa protein are piliated and exhibit hemagglutination of human erythrocytes. This strongly suggests that the surface localized GgO_4-binding adhesin is distinct from the pilus associated factor responsible for binding human erythrocytes. These results also suggest that gonococcal attachment to host cells is a multi-faceted event, involving the pilus and other surface proteins.

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