Invasion of Human Fallopian Tube Epithelium by Escherichia coli Expressing Combinations of a Gonococcal Porin, Opacity-Associated Protein, and Chimeric Lipo-oligosaccharide

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The transepithelial migration of Escherichia coli that expressed all possible combinations of a plasmid-encoded gonococcal porin (Por), opacity-associated protein (Opa), and 3F11 lipo-oligosaccharide (LOS) epitope was investigated. Surface expression of Por mediated selective changes in E. coli antibiotic susceptibility, and coexpression of Opa and the 3F11 LOS epitope mediated bacterial clumping (P < .01). In the human fallopian tube organ-culture model, Opa-producing variants attached up to 44-fold better than control bacteria (P < .01), and Por-producing variants exceeded submucosal invasion of control bacteria by 500-fold (P < .01). Opa and Por each facilitated intracellular invasion 20–40-fold (P < .01). In dual expresser variants, the 3F11 LOS epitope markedly reduced attachment and invasion mediated by Opa or Por. The LOS inhibitory effect was curbed when all 3 factors were expressed, which suggests an additional interaction of the 3 factors at the bacterial surface. Por, Opa, and LOS play important roles in Neisseria gonorrhoeae trafficking across human fallopian tube epithelium.

Three factors implicated in this process include GC opacity-associated (Opa) protein, lipo-oligosaccharide (LOS), and the GC outer-membrane porin (Por).

Opa proteins (Opas) contribute to colony opacity [6] and are thought to facilitate intergonococcal adherence [7, 8]. Each GC strain may encode up to 11 Opas (antigenic variants), which can be expressed alone, in combination, or not at all [9, 10]. Phase variation of Opas occurs at a frequency of per 1 × 10⁻³ per cell per generation [11]. Opas have a relatively conserved structure [12] but have 2 short hypervariable regions, HV1 and HV2 [13, 14]. The interaction of Opa with LOS [7, 8] has suggested that Opas bind to terminal galactosyl (Gal) or N-galactosaminyl residues [8, 15, 16]. Opas mediate nonspecific phagocytosis of gonococci by neutrophils [17, 18] and tight attachment to epithelial cell lines and fallopian tube epithelium [8, 19–21]. Among different cell types, a number of Opa receptors have been identified, including several carcinomaembryonic antigen–like molecules and heparan sulfate proteoglycans [22–26]. Which of these, if any, serve as receptors in the fallopian tube remains unknown. A number of Opa proteins from different GC strains have been cloned and expressed constitutively as PG–like molecules and heparan sulfate proteoglycans [22–26]. Which of these, if any, serve as receptors in the fallopian tube remains unknown. A number of Opa proteins from different GC strains have been cloned and expressed constitutively as PG–like molecules and heparan sulfate proteoglycans [22–26]. Which of these, if any, serve as receptors in the fallopian tube remains unknown. A number of Opa proteins from different GC strains have been cloned and expressed constitutively as PG–like molecules and heparan sulfate proteoglycans [22–26]. Which of these, if any, serve as receptors in the fallopian tube remains unknown. A number of Opa proteins from different GC strains have been cloned and expressed constitutively as PG–like molecules and heparan sulfate proteoglycans [22–26]. Which of these, if any, serve as receptors in the fallopian tube remains unknown. A number of Opa proteins from different GC strains have been cloned and expressed constitutively as PG–like molecules and heparan sulfate proteoglycans [22–26]. Which of these, if any, serve as receptors in the fallopian tube remains unknown. A number of Opa proteins from different GC strains have been cloned and expressed constitutively as PG–like molecules and heparan sulfate proteoglycans [22–26]. Which of these, if any, serve as receptors in the fallopian tube remains unknown. A number of Opa proteins from different GC strains have been cloned and expressed constitutively as PG–like molecules and heparan sulfate proteoglycans [22–26]. Which of these, if any, serve as receptors in the fallopian tube remains unknown. A number of Opa proteins from different GC strains have been cloned and expressed constitutively as PG–like molecules and heparan sulfate proteoglycans [22–26]. Which of these, if any, serve as receptors in the fallopian tube remains unknown. A number of Opa proteins from different GC strains have been cloned and expressed constitutively as PG–like molecules and heparan sulfate proteoglycans [22–26]. Which of these, if any, serve as receptors in the fallopian tube remains unknown. A number of Opa proteins from different GC strains have been cloned and expressed constitutively as PG–like molecules and heparan sulfate proteoglycans [22–26]. Which of these, if any, serve as receptors in the fallopian tube remains unknown. A number of Opa proteins from different GC strains have been cloned and expressed constitutively as PG–like molecules and heparan sulfate proteoglycans [22–26]. Which of these, if any, serve as receptors in the fallopian tube remains unknown. A number of Opa proteins from different GC strains have been cloned and expressed constitutively as PG–like molecules and heparan sulfate proteoglycans [22–26]. Which of these, if any, serve as receptors in the fallopian tube remains unknown. A number of Opa proteins from different GC strains have been cloned and expressed constitutively as PG–like molecules and heparan sulfate proteoglycans [22–26]. Which of these, if any, serve as receptors in the fallopian tube remains unknown.
GC LOS [29, 30]. The presence of poly(G) tracts within the glycosyltransferase gene sequences can allow phase variation to occur via slipped-strand errors in replication, resulting in translational frameshifts. Monoclonal antibody (MAB) 3F11 [31] binds to a 4.5-kD component that is present on many gonococci and on Haemophilus influenzae as well [32]. This antibody also agglutinates human erythrocytes via binding to paragloboside [33]. The terminal tetrasaccharide of paragloboside, lacto-N-neotetraose (Galβ1-4GlcNAcβ1-3Galβ1-4Glc), and the oligosaccharide portion of the 4.5-kD LOS moiety of gonococci have a shared structure [34]. The 3F11 monoclonal recognizes the terminal disaccharide portion of this structure. Throughout this paper, “3F11 LOS” will be used to refer to the LOS recognized by MAB 3F11. In gonococci, 3F11 LOS can be sialylated by GC sialyltransferase in the presence of exogenous cytidine 5′-monophospho-N-acetylneuraminic acid (CMP-NANA) in vitro [35]. In vivo, urethral exudates provide the substrate for sialyltransferase-mediated sialylation [36]. Sialylation converts serum-sensitive gonococci to serum-resistant gonococci [35] and inhibits nonopsonic and opsonic phagocytosis of gonococci by neutrophils [37]. It also prevents binding and bactericidal activity of anti-Por antibodies [38]. 3F11 LOS is able to bind to Opas and to 2 receptors on HepG2 cells in vitro, the asialoglycoprotein receptor and a 70-kD protein termed p70 [8, 21]. It is not known whether 3F11 LOS binds to these receptors at all genital sites in vivo. However, sialylation of GC strains makes them less infective in a human male urethral challenge model [39], perhaps by interfering with the interaction of other GC outer-membrane components with epithelial cells and/or by interfering with LOS interactions with the host. It has been possible to express the 3F11 LOS epitope constitutively in E. coli [32].

Porin-deficient GCs are essential GC proteins, so experimental infections with porin-deficient gonococci have not been possible. Even if these experiments were possible, the rapid variation of attachment factors could lead to erratic results or inappropriate conclusions. A model system in which these factors could be studied alone or in combination without their displaying phase and antigenic variation would greatly enhance the ability to study the role of LOS, Opa, and Por in the pathogenesis of gonococcal infections. This paper describes the development and testing of such a model system in 3 phases: (1) verification of factor surface expression and function in E. coli; (2) construction of a complete model in which several functional putative virulence factors are expressed in all possible combinations; and (3) testing factor function and cooperativity in surrogate model interactions with human fallopian tube epithelium.

Materials and Methods

Organisms and plasmids. Por-producing clones were provided in E. coli strains M16 and DH5α, containing plasmid pUNCH30. Por is expressed from an ampicillin-selectable, pGEM-2–based plasmid (Promega) downstream of its own putative promoter, in an isopropyl-β-D-thiogalactopyranoside–independent manner. It is a P1A porin originally cloned from N. gonorrhoeae strain FA19 [50]. An Opa-producing clone was provided in E. coli strain DH5α, containing plasmid pDS001 (gift of R. Rest, Medical College of Pennsylvania Hahnemann University School of Medicine). This Opa is expressed as a β-lactamase/Opa fusion protein from this ampicillin-selectable, pGEM-3Z–based plasmid (Promega) [15]. It originated from gonococcal strain F62SF, as described by Palmer et al. [27], and is derived from plasmid pLPGC1. This fusion protein corresponds to Opa B from strain F62SF and matches the sequence of this Opa starting at amino acid position 8. The alterations in the amino acid sequence do not affect any surface-exposed regions of the protein but prevent phase variation by removing the variable upstream pentameric repeats [9, 10, 11]. In previous studies, E. coli that expressed this protein were unable to adhere to HeLa 229 or HEC-1B cells in a 1-h assay [27]. In other studies of longer infection duration, this Opa fusion protein mediated invasion of ME-180 cells and HEC-1B cells, but not Chang conjunctival cells [15]. A 3F11 LOS epitope–producing clone was provided in E. coli strain DH5α, containing plasmid pYCLOS5. The genes responsible for assembling the epitope originated from H. influenzae [32]. This 3F11 oligosaccharide epitope is expressed via a 5.5-kb DNA fragment comprising 5 open-reading frames (C–G) of the bositolipid usher cloned into a chloramphenicol-selectable pACYC184-based plasmid [32]. In E. coli, which has only 1 oligosaccharide chain, the 3F11 LOS epitope is formed by the terminal disaccharide of a tetrasaccharide (Galβ1-4GlcNAcβ1-3Galβ1-3GlcNAc) linked to position 7 of the nonreducing terminal branch heptose of the complete E. coli lipopolysaccharide (LPS) core structure. Thus, the terminal 3 sugars are identical in composition and linkage to the GC lacto-N-neotetraose structure, but the proximal β1-4Glc sugar of gonococci has been replaced by β1-3GlcNAc, which is linked differently to the core. These sugars are added sequentially in a segregated fashion after the E. coli core structure is completed [32]. For experiments in which the influence of Por on antibiotic susceptibility was tested, isogenic N. gonorrhoeae strains (original P1A strain FA19) that varied only in their production of Por were used. The P1A producer is designated strain 6564, and the P1B producer is designated strain 6571 [51]. Another GC FA19 variant, 6611, served as a positive control for Western blot assays.
Expression of cloned products. Expression of Opa, Por, and the 3F11 LOS epitope was confirmed via Western immunoblot techniques. For some assays, outer membranes were isolated according to the methods of Sawai et al. [52] before solubilization. Opa was detected with MAb 4B12, which recognizes an epitope common to all Opa proteins (kindly provided by M. Blake, North American Vaccine). Por was detected with monoclonal 4G5 [53], which recognizes an epitope of some Por proteins that is accessible on the surface of gonococci. This conformational epitope is lost in the SDS-PAGE/Western blotting process but can be recovered in the presence of Zwittergent (Sigma) [38]. For detection of the 3F11 LOS epitope, proteinase-K LPS preparations of whole organisms were performed according to the method of Hitchcock and Brown [54]. The LOS/ LPS was electroblotted onto Immobilon P membranes (Bio-Rad) overnight at 30 V, 4°C. MAb 3F11 served as the primary antibody, and goat anti–mouse IgM (62-6822; Zymed) conjugated to alkaline phosphatase replaced the Immobilon secondary antibody (Bio-Rad) that was used for Por or Opa detection.

Presence and functionality of cloned products at the bacterial surface. Presence of the 4G5 P.1A epitope on the surface of intact E. coli–bearing pUNCH30 was verified via whole-cell dot blot. Intact bacteria were spotted onto nitrocellulose paper via the Bio-Dot Microfiltration Apparatus (Bio-Rad). Overnight incubation with Zwittergent was omitted, because the bacteria were not disrupted by physicochemical means, leaving the protein in its native state on the surface of intact bacteria. Processing with primary and secondary antibodies was identical to the Western blot procedure. The P.1A-producing GC strain (6564) served as a positive control. E. coli strains DHSa(pGEM-2) and M16(pGEM-2) were negative controls.

The influence of PI or Por production on the relative susceptibility to a variety of antibiotics was tested. E-test MIC determinations (AB BIODISK) were performed on Por-producing E. coli, the vector-bearing negative controls, and isogenic gonococcal mutants producing either P1A or P1B [55]. The only modification to usual E-test methods was the inclusion of ampicillin in all E. coli groups, to maintain plasmid selection.

The ability of the Opa fusion protein to facilitate bacterial clumping through interactions with the 3F11 LOS epitope was evaluated via computerized image analysis in 2 ways. In the first experimental design, E. coli producing only the Opa fusion protein were mixed with E. coli producing only the 3F11 LOS epitope, to test the association of bacteria producing the 2 products separately. Control groups included mixtures of E. coli–bearing vectors alone, E. coli expressing one factor mixed with E. coli bearing the vector of the other factor, and suspensions of E. coli producing only one of the factors. Suspensions of plate-grown organisms in Luria broth were adjusted to identical optical densities, and equal volumes were mixed. The suspensions were incubated overnight at 4°C and then were vortexed for equal amounts of time. The suspensions were serially diluted, and a drop of each dilution was placed on a glass slide. Slides were examined under oil with a Zeiss Axioskop equipped with Nomarski differential interference contrast (DIC) optics (Carl Zeiss). Contrast was adjusted to give a dark background, with the organisms appearing as bright spots. From these slides identical dilutions that gave good separation of individual organisms or organism clumps were chosen for image analysis [56]. Microscope fields were randomly selected, and digital images were acquired with a computer-controlled charge-coupled device camera (Dage MTI). For each experimental group, the sizes of ≥2000 organism clumps were measured with computer software (Image 1; Universal Imaging). As described elsewhere [56], the software was calibrated for measurement in square micrometers with a stage micrometer, and organisms were distinguished from the dark background for measurement by using a grayscale threshold that was held constant for all groups. All computer-controlled image acquisition and analysis was carried out on a personal computer (Pentium Pro 100 MHz; Vaytek), running the Windows 95 operating system (Microsoft).

In the second experimental design, for evaluating the interaction of Opa with the 3F11 LOS epitope, E. coli producing 2 gonococcal products simultaneously were tested. By use of electroporation, DHSa(pYCLOS5)3F11, was transformed with plasmids isolated from DHSa(pUNCH30)P.1A, or from DHSa(pDS001)bns, via alkaline lysis [57]. The transformants were plated on LB agar containing both ampicillin (35 μg/mL) and chloramphenicol (25 μg/mL). Plasmid profiles of cotransformants and controls were determined by agarose gel electrophoresis, and production of gonococcal products was verified by Western blot, as described above. The experimental groups included DHSa(pYCLOS5)3F11, DHSa(pDS001)bns, DHSa(pDS001, pYCLOS5)bns, and DHSa(pUNCH30, pYCLOS5)3F11. The phenotypes of these variants are 3F11 LOS alone, Opa fusion protein alone, both the 3F11 LOS epitope and Opa fusion protein, and both Por and the 3F11 LOS epitope, respectively. Organism suspensions were grown overnight at 37°C in Luria broth containing the appropriate antibiotic selection, were adjusted to an optical density of 0.4, with all samples vortexed for the same time duration, and were serially diluted. Dilutions were processed and were analyzed by computerized image analysis, as described above.

Construction of model. Plasmid isolation, restriction endonuclease digestion, and electroporation were performed via standard techniques [57]. The general approach involved subcloning the blaclopa gene from pDS001 to pUNCH30. An EcoRI/BamHI fragment from pDS001 bearing the blaclopa insert was gel purified, and the restriction enzyme–produced recesses were filled in with Klenow fragment and deoxyribonucleotide triphosphates (New England Biolabs), to produce blunt ends. The blunt-ended fragment was ligated to linearized calf intestinal phosphatase–treated pUNCH30 at its unique SmaI site. The ligation product, pINVOMP.P1A, was electroporated into E. coli DH-1, and transformants were selected with ampicillin. Likewise, the blaclopa insert was blunt ligated into vector pGEM-2, resulting in the recombinant plasmid pINVOP.A, and was electroporated into E. coli DH-1. Each of the plasmids encoding none, one, or both of the outer-membrane proteins was isolated and was electroporated into recipient strain DHSa(pACYC184)cont, or DHSa(pYCLOS5)3F11. Cotransformants were selected with ampicillin and chloramphenicol. This cotransformation strategy was used with various combinations of vector plasmids and plasmids bearing 1 or 2 factors, to complete construction of a model in which the 3 factors were encoded in every combination. Plasmid profiles of recombinants were documented with agarose gel electrophoresis. Growth curves for model strains were done twice, to document similar growth characteristics. Expression of recombinant factors in each model variant was documented by Western blot, as described above.

Experimental infections. Experimental infections of human fallopian tube organ cultures were performed, as described elsewhere.
Results

Presence and functionality of factors at bacterial surface. To document the presence of GC P1A at the surface of recombinant bacteria, P1A-expressing E. coli whole cells were probed with MAb 4G5. Bactericidal MAb 4G5 binds to surface-exposed loop 1 of P1A in intact gonococci [38] and was able to bind to E. coli expressing P1A from pUNCH30 (figure 1). The antibody recognized the gonococcal control and both porin-expressing E. coli strains. This indicates that P1A can be found in the outer membrane when it is expressed in E. coli with its extracellular domain (loop 1) accessible to antibody binding, similar to the native state in gonococci.

In addition to its presence at the bacterial surface, P1A affects bacterial sensitivity to antibiotics. E-test MICs of E. coli producing P1A were compared with the corresponding E. coli controls possessing only the plasmid vectors. P1A had no effect on susceptibility to imipenem. Sensitivity to ciprofloxacin and aztreonam also was unaffected (data not shown). P1A production moderately decreased the MIC of cefotaxime and cefturoxime. P1A production by E. coli was associated with a markedly decreased vancomycin MIC (table 1). To ascertain the influence of P1A on vancomycin susceptibility in a gonococcal background, we determined the vancomycin MICs of isogenic gonococcal strains that varied only in their production of P1 or Por. The P1A-producing variant 6564 had a MIC of 8 μg/mL, compared with a MIC of 48 μg/mL for the P1B-producing variant 6571. These data indicate that P1A production in E. coli mediates selective changes in antibiotic susceptibility consistent with enhanced permeability to some antibiotics, due to its porin function. Markedly lower MICs of vancomycin result when P1A of strain FA19 is expressed in gonococci or E. coli.

Gonococcal Opa and the 3F11 LOS epitope also are functional at the surface of E. coli. Because the 3F11 LOS epitope was expressed only in DH5α, the following experiments were not performed on strain M16. In random microscopic fields, the sizes of organism clumps were measured with computerized image analysis, to see if Opa and 3F11 LOS mediate interbacterial adherence in an E. coli background. When E. coli producing single gonococcal factors were mixed in suspension (strategy 1 in Materials and Methods), only the mixture of E. coli producing the 3F11 LOS epitope with E. coli producing β-lactamase/Opa fusion proteins showed significant clumping.

![Figure 1](image-url) Whole-cell dot blot detection of porin surface exposure in Neisseria gonorrhoeae and recombinant Escherichia coli probed with anti-porin 1A (P1A; monoclonal 4G5). Left to right: gonococcal (GC) 6564 (P1A variant), GC 6571 (P1B variant), E. coli (pUNCH30), and E. coli (pGEM-2). Note that the antibody binds only to P1A expressers GC 6564 and E. coli (pUNCH30).
Epitopes were made, compared with either alone ( ).

Organism clumps was larger when both Opa and the 3F11 LOS epitope ( ). These data indicate Production of both P.1A and the 3F11 LOS epitope failed to single factor. The clumping assay results for 1 or 2 gonococcal factors simultaneously (strategy 2 in Methods) are shown in figure 2. The mean size of the Opa was originally cloned (figure 4, lane 1). The clumping of E. coli producing none of these factors was indistinguishable from mixtures of strains that produced a single factor. The clumping assay results for E. coli producing 1 or 2 gonococcal factors simultaneously (strategy 2 in Materials and Methods) are shown in figure 2. The mean size of organism clumps was larger when both Opa and the 3F11 LOS epitope failed to single factor. The clumping assay results for E. coli producing 1 or 2 gonococcal factors simultaneously (strategy 2 in Materials and Methods) are shown in figure 2. The mean size of organism clumps was larger when both Opa and the 3F11 LOS epitope had a more mucoid character than did the Opa-only phenotype; and DH5α, Opa plus LOS phenotype; and DH5α, LOS-only phenotype; and DH5α(pUNCH30, pYCLOS5), Opa and porin 1A (P1A) phenotype. Note significantly larger organism clumps in the group producing both Opa and the 3F11 LOS epitope. Error bars, SDs.

**Table 1.** E-test MICs (µg/mL) of selected antibiotics for Escherichia coli M16 or DH5α porin 1A producers (pUNCH30) vs. nonproducers (pGEM-2).

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>M16 (pUNCH30)</th>
<th>M16 (pGEM-2)</th>
<th>M16 (pUNCH30)</th>
<th>M16 (pGEM-2)</th>
</tr>
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<tbody>
<tr>
<td>Cefotaxime</td>
<td>0.032</td>
<td>0.094</td>
<td>&lt;0.016</td>
<td>0.032</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>3.0</td>
<td>8.0</td>
<td>0.5</td>
<td>4.0</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>32.0</td>
<td>&gt;256</td>
<td>4.0</td>
<td>&gt;256</td>
</tr>
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</table>

(mean clump size, 3.49 µm² vs 2.636 µm² in the controls; P < .001). The clumping of E. coli producing none of these factors was indistinguishable from mixtures of strains that produced a single factor. The clumping assay results for E. coli producing 1 or 2 gonococcal factors simultaneously (strategy 2 in Materials and Methods) are shown in figure 2. The mean size of organism clumps was larger when both Opa and the 3F11 LOS epitope were made, compared with either alone (P < .000008).

Production of both P1A and the 3F11 LOS epitope failed to induce clumping, in comparison with the strain that made Opa and the 3F11 LOS epitope (P < .000008). These data indicate a significant interbacterial physical interaction related to the production of Opa and the 3F11 LOS epitope.

**Model construction and factor expression.** To create an E. coli variant that expressed all 3 gonococcal factors simultaneously, the β-lac/opa fusion protein sequence was subcloned into pUNCH30P1A, yielding pINVOAP1A-Opa. Once pINVOAP1A-Opa was constructed and was electroporated into E. coli DH5α bearing pYCLOS5 [p11], a cotransformant encoding all 3 factors was generated. HindIII endonuclease digestion of plasmid preparations from the variant encoding all 3 factors resulted in the resolution of 3 bands by agarose gel electrophoresis. One 5.2-kb band resulted from the single HindIII cleavage site in pINVOAP1A-Opa. Two additional bands of 4.2 and 5.7 kb corresponded to DNA fragments resulting from cleavage of the 2 restriction sites in pYCLOS5 [p11] (data not shown). Expression of each of the 3 factors was verified by Western blot analysis. MAb 4G5, which recognizes P1A, identified an immunoreactive band in outer-membrane proteins isolated from P1A-expressing gonococci or E. coli (figure 3). Note that the GC control produced significantly more of the porin protein than did the E. coli model strains. Within the E. coli model strains, the amount of the porin produced was similar. Production of the β-lactamase Opa fusion protein by E. coli was verified by probing Western blots with MAb 4B12, which recognizes a conserved epitope of Opa proteins (figure 4). MAb 4B12 also reacted with an outer-membrane protein preparation of the GC control strain, which is not the strain from which the Opa was originally cloned (figure 4, lane 1). This strain made a different Opa that migrates at a lower apparent molecular weight than do the E. coli model variants. The amount of Opa fusion protein produced was noticeably less for the variant that produced both Por and Opa (figure 4, lane 2) than for other strains in the model, including the strain that encoded all 3 factors (figure 4, lane 5). The Opa fusion protein retained the heat-modifiable character of the native protein—that is, proteins solubilized at 100°C migrated at an apparent molecular weight higher than that of proteins solubilized at 37°C (data not shown). Epitope expression by 3F11 LOS was verified by probing blots with MAb 3F11 (figure 5). The molecular weight of the 3F11 reactive band was slightly higher in E. coli variants than in the gonococcal control strain, presumably because of differences in the acceptor molecules for the 3F11 LOS epitope in the 2 hosts [32]. Silver-stained LPS preparations of E. coli DH5α showed a single band of rough LPS in control E. coli (~4 kDa) and 2 bands in the 3F11 LOS producers: the “native” LPS band and a predominance of a band of slightly higher molecular weight (~5 kDa). No additional intermediate bands were seen. Incubation with CMP-NANA in vitro showed no evidence of sialylation (data not shown).

**Model growth characteristics.** Strains that produced both Opa and the 3F11 LOS epitope had a more mucoid character when grown on plates than did other model strains and the control strain, which expressed none of the factors. To verify similar growth rates among the various E. coli model strains, bacterial growth was monitored over time (figure 6). For the sake of clarity, not all of the strains are shown. The growth plots showed that model strains did not reach the same density as the control strain, but growth rates ran roughly parallel. This was consistent between replicate curves. Among the model strains that produced ≥1 factor, colony-forming units per milliliter after 24 h of growth ranged from 6.6 x 10⁷ to 2.0 x 10⁷, a 3-fold difference. This difference appeared to be related to variability in determinations of colony-forming units, as one...
model strain did not grow consistently better than the others in replicate growth curves. Thus, model strains had comparable growth among themselves but did not grow to the same density as the control strain that expressed none of the factors.

**Experimental infections.** To assess the role of these GC virulence factors in the pathogenesis of female upper genital tract infection, human fallopian tube organ cultures were infected with the experimental model strains. Measurement of bacterial attachment to and invasion of the epithelium was performed on microscopic sections of infected tissue with computerized image analysis. Data trends were reproducible and were statistically significant when analyzed for each experiment (i.e., different patient tissue sources). To simplify the data presentation, measurements from all the experiments were pooled. This represented analysis of 1488 images (figure 7). In general, the 3F11 LOS epitope was unable to promote meaningful interaction with the epithelium, compared with the control. However, both Opa and Por were effective at promoting bacterial interaction with the epithelium when produced singly.

In quantitative studies, more Opa-producing bacteria were found on the surface of the epithelium in comparison with nonproducers. In fact, the mean attachment of the single Opa producer exceeded the control value by ~45-fold. The differences between the attachment values of the single Opa producer and all the other groups were statistically significant ($P < .000029$), except for the Opa plus Por variant and the Opa plus Por plus LOS variant ($P = .392$ and $P = .434$, respectively).

Opa was also effective at promoting internalization of bacteria, as it exceeded control levels by 37-fold. The differences in the intracellular values for the single Opa producer did not differ significantly from the Opa plus Por dual expresser ($P = .233$), the Por expresser ($P = .767$), or the triple expresser ($P = .937$). Thus, the reduced level of Opa expression in the Opa plus Por dual expresser was not sufficient to impede attachment and intracellular penetration. The mean subepithelial measurement value for the single Opa expresser exceeded the control by 3-fold, but this did not quite reach statistical significance ($P = .0635$). The mean subepithelial values of the single Opa expresser significantly exceeded those of the single and dual LOS-expressing variants and of the triple expresser ($P < .0002$) but were significantly less than those of the Por group and the Opa plus Por group ($P < .000005$).

Preliminary qualitative evaluations of the interactions of Opa-producing bacteria with the fallopian tube were performed via electron microscopy. The *E. coli* Opa producers showed a preference for nonciliated cells. The degree of attachment was similar to what one observes with GC strains, but, in general, the degree of invasion was somewhat less. Opa-expressing *E. coli* resided in membrane-bound vacuoles analogous to intracellular GC morphology (data not shown). In general, Opa-producing bacteria were adept at attaching to the epithelium and entering the cells, but relatively few had achieved a subepithelial location by the end of the experiment.

By contrast, Por-producing bacteria were relatively more inter-
Figure 5. Outer-membrane proteins from *Escherichia coli* DH5α strains producing a 3F11 lipo-oligosaccharide (LOS) epitope, compared with *Neisseria gonorrhoeae* 6611 probed with monoclonal antibody 3F11, as shown by Western blot. Lanes: 1, Gonococcal positive control; 2, DH5α(pUNCH30, pYCLOS5), variant expresses porin 1A (P1A) and 3F11 LOS; 3, DH5α(pINVOAP, pYCLOS5), variant expresses P1A, opacity-associated fusion protein (Opa), and 3F11 LOS; 4, DH5α(pGEM-2, pACYC184), negative control; 5, DH5α(pINVOA, pYCLOS5), variant expresses Opa and 3F11 LOS; and 6, DH5α(pGEM-2, pYCLOS5), variant expresses 3F11 LOS.

Figure 6. Growth curves of model *Escherichia coli* DH5α strains expressing porin (Por), Por plus opacity-associated fusion protein (Opa), and Por plus Opa plus the 3F11 lipo-oligosaccharide (LOS) epitope, compared with *E. coli* expressing none of the factors.

Discussion

Experimental infections that simulate the host/pathogen interaction at an epithelial surface have been established in a variety of models. Because the gonococcus has a narrow host spectrum, an inexpensive representative animal model has been lacking, although an estradiol-treated murine intravaginal model was recently described [62]. Despite its utility, certain human factors important for GC pathogenesis, such as carcinoembryonic antigens, CD46, and human transferrin, are naturally absent in the animal model. The pathogenesis experiments in this study centered on the human fallopian tube model of infection, because the model uses a natural human target tissue of the gonococcus that is affected by the infection in a devastating way. This study addresses an important barrier to understanding GC pathogenesis in the human fallopian tube model. That barrier is the ability to study putative GC virulence factors in isolation from one another. Naturally, the phase and antigenic variation of a combination of factors—such as pili, LOS, Opa proteins, and a variety of uncharacterized putative adhesins detected by the GC genome sequencing project—could exert a large effect on experimental results, proportional to the length of time over which the experiment takes place. One might...
expect these effects to be greatest in the models that have a longer time course, that is, the urethral challenge and FTOC models. Because Opa, Por, and the 3F11 LOS epitope had been expressed previously in *E. coli*, an *E. coli* model that expressed these virulence factors in combinations became a natural candidate for study in the FTOC.

The work reported in this paper has confirmed that GC Por can be expressed in *E. coli* DH5α and *E. coli* M16 from its own promoter and that it is exposed on the bacterial surface. Initial attempts at direct cloning of complete GC porin genes in *E. coli* were unsuccessful due to presumed toxic effects of Por production [48, 49]. Despite this, Elkins et al. [50] were able to show expression in *E. coli* DH5α and M16 and in several *Salmonella typhimurium* strains. Similarly, dot blot analysis indicated that Por was surface exposed in *E. coli*, although the data were not shown in the publication. The signal peptide sequence of this clone contains requisite sequence features to be processed by *E. coli* leader peptidase [63].

The present study extends earlier findings by reporting a functional role for Por in the outer membrane of *E. coli*. The production of Por in *E. coli* resulted in selective changes in organism sensitivity to antibiotics, most notably vancomycin. Likewise, influence on vancomycin sensitivity was demonstrated in a GC background by using isogenic GC mutants that varied only in their production of Por type, P1A versus P1B. Earlier work had not documented functionality of Por production in *E. coli*, but a study by Carbonetti et al. [51] examined the effect of different Por types on antibiotic sensitivity in isogenic GC mutants. That study found that P1A, P1B, and hybrid porins affected the sensitivity to penicillin and tetracycline.
to a small degree, due presumably to porin effects on outer-membrane permeability. This is consistent with the modest effects of Por production on the MIC of E. coli strains to β-lactam antibiotics in the current study. The prior study did not examine effects on vancomycin MICs.

In contrast to the initial cloning attempts for Opa and Por, expression of additional surface sugars in the form of GC LOS epitopes in E. coli has not caused toxicity issues. Eventually, multiple genes encoding the 3F11 LOS epitope common to GC and Haemophilus species were cloned and were expressed in E. coli [32]. Although the genes that assemble the 3F11 LOS epitope were derived from H. influenzae, the tetrasaccharide structure differs from the GC version only in the proximal sugar linking the oligosaccharide to the E. coli LPS structure. The disaccharide recognized by the 3F11 monoclonal is identical. We detected some native LPS and a predominance of chimeric disaccharide recognized by the 3F11 monoclonal is identical. We detected some native LPS and a predominance of chimeric disaccharide recognized by the 3F11 monoclonal is identical.

Porat et al. [21] showed that radiolabeled GC LOS bearing the 3F11 epitope bound to intact Opa-producing gonococci and to purified Opa protein. The results in the present paper are the first to demonstrate interbacterial clumping mediated by the production of these 2 factors in E. coli. Clumping occurred when each factor was produced separately and the 2 bacterial phenotypes were mixed together in suspension. It also occurred when both factors were expressed in the same bacterium. The control groups indicated that the clumping was not mediated by changes induced by the vectors or by the 3F11 LOS epitope’s clumping with gonococcal outer-membrane proteins in general. This suggests that 3F11 LOS-Opa interactions are of sufficient magnitude to mediate intergonococcal adherence of Opa-positive GC phenotypes.

These initial results laid the groundwork for additional fallopian tube studies by showing that 3 putative virulence factors expressed in the outer membrane of the foreign E. coli host performed some of the functions seen in the native GC background. This indicated that the multiple virulence factor expression model could be a valid means to dissect the function of Por, Opa, and the 3F11 LOS epitope in conjunction with the human fallopian tube model.

Of the 3 factors studied singly in the FTOC model, Opa facilitated the greatest E. coli attachment to epithelial cells. The preferences for nonpiliated fallopian tube epithelial cells and intracellular residence within membrane-bound vacuoles that were observed are similar to past observations of gonococci [4]. Dekker et al. [20] studied the effect of different Opa protein expressions on the attachment of a nonpiliated GC strain, F-62-SF, to fallopian tube epithelium. In qualitative analyses, the morphology of attachment (clumping) and the degree of mucosal damage varied according to the Opa being expressed.

Each Opa variant expressed the 3F11 LOS epitope and had identical LOS profiles on SDS-PAGE gels of our model strains. However, Phillips et al. [32] used matrix-assisted laser desorption ionization, electrospray ionization, and tandem mass spectrometry techniques, along with composition and linkage analyses, to clearly define the structure of chimeric LPS produced in E. coli by the same 5 genes used by our model. Although some unmodified E. coli rough LPS was detected in chimeric variants, there was a lack of intermediate chimeric structures, suggesting that addition of the chimeric sugars proceeded rapidly to a defined end point. Addition of the H. influenzae chimeric structures was segregated, so that those sugars were only added to complete E. coli LPS, rather than interdigitated with rough LPS synthesis [32]. Sialylation, a potential confounding factor that can vary and interfere with invasion and interbacterial clumping of gonococci, did not occur in E. coli used for this study, presumably because it lacked the necessary sialyltransferase.

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aminoglycans, fibronectin, and integrin receptors, which added to the complexity of Opa interactions with host cells [72]. Thus, variable presence of several factors in immortalized cell line models may account for some disparities in the literature. The ability of heparan sulfate proteoglycans and/or specific CEACAMs to facilitate GC invasion of fallopian tube epithelium is currently unknown, as is the receptor specificity of the Opa investigated in this work. However, one can conclude that this particular Opa can facilitate attachment to and invasion of the fallopian tube in the absence of pili, LOS, other gonococcal factors, or serum. It is not known if other Opas can perform a similar function.

The involvement of Por in the GC invasion process has been suspected on the basis of epidemiologic evidence that associated P.1A strains more commonly with patients who presented with disseminated gonococcal disease [73]. This was strengthened by early physicochemical studies that demonstrated the ability of P.1A porins to translocate into lipid bilayers at 10-fold the rate of P.1B porins [44]. More recently the porin function has been shown to regulate apoptosis as well [74, 75]. In allelic replacement studies, P.1A was found to confer invasion of gonococci into Chang epithelial cells in the absence of Opa proteins under conditions of a low phosphate level [76]. When the P.1B porin of GC MS11 (PorB_{ab}) was replaced by porB of N. lactamica, GC uptake by Chang conjunctival cells was reduced. An 11–amino acid deletion of the first surface-exposed loop of the porin also reduced invasion [77]. In the present report, a P.1A porin from GC strain FA19 facilitated marked subepithelial accumulation of E. coli that expressed the protein. Given the wide variety of porin-modulated cellular functions, including inhibition of phagocyte functions [46, 47], porin-induced changes in the local milieu around the bacteria likely facilitated bacterial survival within, and/or transport through, the epithelium.

Previously, computerized image analysis measurements of bacterial invasion in the fallopian tube model ignored bacteria in the subepithelial regions of the tissue [56]. This region was purposely avoided, because one could not ascertain if subepithelial bacteria had arrived in that location by traversing through epithelial cells or, alternatively, by entering the area from the cut edge of sectioned tissue. The latter explanation, if true, should have resulted in many or all experimental groups in the current study showing similar degrees of subepithelial invasion, overall. This was not the case, as porin-producing E. coli in the absence of 3F11 LOS consistently attained high numbers in subepithelial locations, compared with other groups. Had this region not been measured, the impact of Por on GC interaction with fallopian tube epithelium would have been markedly underestimated. As it stands, Por production alone contributed the greatest to invasion, even when considering both intracellular and subepithelial compartments. However, this does not mean that Por is a more important invasion factor for gonococci overall, since it is quite possible that untested Opas or other GC factors might supercede the advantage shown by this FA19 porin. Moreover, the relative expression of Por and Opa in E. coli is less than what is seen in wild-type gonococci. For example, a higher density of Opa expression could result in a higher local recruitment of Opa receptors on the epithelial cell surface, perhaps greatly facilitating bacterial internalization. Given that the E. coli control variant did not adhere on its own, one must also consider the possibility that Por possesses some adhesin properties that allowed it to initiate efficient transepithelial passage of the organisms. This can only be inferred, since very few adherent bacteria were observed when Por was expressed as a single factor. The P.1A porin and Opa tested in this work appeared to facilitate invasion via separate, possibly competitive pathways, since there was not an additive or synergistic effect on intracellular and subepithelial invasion when both Opa and Por were expressed simultaneously.

Purified 3F11 LOS can bind to HepG2 cells [8, 21]. Porat et al. [7, 8] identified 2 receptors for the 3F11 LOS epitope on these cells. One of these is a 70-kDa protein, and the second is the asialoglycoprotein receptor. The former protein has antigenic similarity to GC Opa proteins, since MAb 4B12 binds to it also. The asialoglycoprotein receptor has been detected on human urethral epithelium and human sperm [61, 78]. It was reasonable to hypothesize that the 3F11 LOS might also mediate interactions with fallopian tube cells. Two studies have examined the ability of several genetically constrained LOS structures to alter adherence to and invasion of epithelial cells [79, 80]. The first of these, which used GC F62 (Opa^{+}), found that variants expressing the lacto-N-neotetraose LOS could promote gonococcal invasion into ME180 cells better than variants with more truncated LOS structures, despite equivalent levels of attachment [79]. The second study, using GC strain MS11, found no difference in attachment and invasion of ME 180 cells until the entire ω-chain was lost by the elimination of the proximal glucose, although the majority of comparisons were made with Opa+ organisms. In an Opa- variant that expressed the 3F11 LOS with an additional GalNAc on the end, invasion was greater than in the variant lacking the proximal glucose [80]. The first study did not examine variants in which this proximal sugar had been ablated. Despite a lack of directly comparable variants, both studies indicated a role for the ω-oligosaccharide chain in the invasion process. The failure in the present study of E. coli expressing the 3F11 LOS epitope to invade more efficiently could indicate that the fallopian tube lacks the appropriate receptor(s). Perhaps the difference in the way that this epitope is presented in E. coli (see Materials and Methods) might account for an inability of the 3F11 LOS to promote attachment. However, the clumping experiments suggest that the conformation is adequate for the 3F11 LOS epitope to interact with Opa on the surface of E. coli. Taken together, these studies point out a certain degree of unappreciated complexity to the interaction of LOS with host cells and other bacterial components that remains to be sorted out.

One of the most interesting findings in this study was the ability of the 3F11 LOS structure to inhibit the interaction of Opa- or Por-producing E. coli with the fallopian tube. LOS dual producers were very similar to control bacteria. Although
these results have cast some doubt on the role of the 3F11 LOS epitope in fallopian tube infections, subject to the caveats mentioned above, they have strengthened the results that support the attachment- and invasion-promoting functions of Opa and Por. Given the inhibitory effects of the 3F11 LOS epitope in the LOS dual expressers, one would have expected the marked inhibitory effect to carry over to the triple expresser, that is, less attachment and invasion than the Opa plus Por dual expresser. Surprisingly, this was not the case. Although subepithelial invasion was significantly reduced, the mean attachment and intracellular values of the triple expresser were about half that seen with Opa alone. These latter differences were not statistically significant. Overall, the triple expresser greatly exceeded the interaction of the control bacteria, in contrast to the dual expressers that produced LOS. Conformational hindrance by a lengthened LPS molecule is an attractive simple explanation for the 3F11 LOS epitope inhibition seen in dual expressers. To explain the moderation of this inhibitory effect in the triple expresser, one must hypothesize a conformational change in the molecules brought on by the juxtaposition of all 3 factors in the bacterial outer membrane, since the levels of Por or Opa expression were similar in the dual LOS expressers and the triple expresser.

These data are the first definitive evidence in a natural human target tissue that Por, Opa, and the 3F11 LOS epitope each have individual profound effects on bacterial trafficking across the normally protective epithelial barrier and influence the effects of one another when they are expressed in combination. From a subepithelial location in the upper genital tract, the bacteria wreak havoc with the female human host in the form of severe, complicated infection, often leading to infertility and a high risk of ectopic pregnancy.

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References


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