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Transcellular Passage of *Neisseria gonorrhoeae* Involves Pilus Phase Variation

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Piliated and nonpiliated *Neisseria gonorrhoeae* organisms were added on top of confluent layers of HEC-1-B cells, each maintained on a microporous Transwell-COL membrane. The bacteria released into the lower chamber were characterized with respect to the following virulence determinants: pili, which mediate adherence to target host cells; PilE, the major pilus subunit protein; and PilC, which is involved in pilus biogenesis and adherence. Even if >99% of the added bacteria of *N. gonorrhoeae* MS11 were piliated, bacteria recovered on the other side of the cell layer were predominantly nonpiliated. The recovered clones still expressed unassembled PilE protein, but 50% had lost PilC production. Nonpiliated gonococci, in which the 5′ end of *pilE* had been deleted, were released in reduced numbers, and piliated *recA* bacteria added to the cell layer were not released at all, at time points when piliated *recA*$^+$ clones were found at high numbers in the lower chamber. Our data indicate that bacteria producing unassembled PilE protein are selected for during passage through an epithelial cell layer. The finding that the *pilE* gene sequence was altered in the transmigrants suggests that pilin sequence variation is involved in the transcellular passage of *N. gonorrhoeae*.

Cellular invasion by *Neisseria gonorrhoeae* has previously been shown to occur in cultured cells as well as in organ cultures (18, 27). The fallopian tube organ culture model has been used to study invasion as well as transcytosis and subepithelial release of gonococci (17, 35). Invasion of cultured epithelial cells by *N. gonorrhoeae* occurs slower than observed with other invasive bacteria, such as *Shigella*, *Yersinia*, *Listeria*, and *Salmonella* (3, 4, 7, 12, 29, 32). The capacity of gonococci to invade epithelial cells is enhanced by the expression of certain Opa proteins (14, 16, 28, 34). Invasion of HEC-1-B cells by gonococci has been suggested to be enhanced by preincubation with fixed target cells (2), suggesting an induction of invasion-related functions upon contact with epithelial cells.

The first interaction between bacteria and cells is mediated by pili, composed of the major pilus subunit (PilE), a minor adherence-associated protein (PilC), and possibly other hitherto-unidentified components. The gonococcal pili undergo phase and antigenic variation, which can both be mediated by recombination events between the expressed *pilE* gene and one of the silent *pilS* loci; these lack the 5′ end and promoter region of *pilE* (5, 19, 20, 31). Gonococci can switch between a piliated and a nonpiliated state at frequencies of about 10^{-4} per CFU per generation.

PilE is expressed as a precursor (propilin) that has a seven-amino-acid leader sequence. Additional cleavage can occur after amino acid 39 of the mature pilin (6), generating a soluble 16-kDa pilin truncatation (S-pilin) that is released in the culture supernatant. S-pilin-producing cells form colonies with a nonpiliated colony morphology and revert to a piliated state at high frequency. S-pilin variants often express small amounts of pili. PilC is a 110-kDa protein involved in pilus biogenesis (9, 21–23) and was shown to act as an adhesin at the tip of the pilus (24). Expression of PilC is controlled by frequent frameshift mutations within a poly(G) tract positioned in the signal peptide-coding region (9). Nonpiliated, PilC$^-$ gonococcal derivatives of strain MS11$_{mk}$(P$^+$)-u (piliated) still express PilE but do not assemble the PilE proteins into pili; in addition to frameshift mutations in *pilC*, these nonpiliated derivatives also carry sequence changes in *pilE* relative to the parent. This double genetic event was suggested to be selected for in PilC$^-$ gonococci in order to obtain a PilE sequence degradable to S-pilin at rates sufficient to allow viability of the cells (10).

Invasion of epithelial cells is a key virulence mechanism of many pathogenic bacteria. In the fallopian tube organ culture model, gonococci entered the epithelial cells, increased in number inside them, and then invaded the subepithelial tissue (35). The aim of this study was to characterize gonococcal cells released from a layer of epithelial cells. Genetically defined *N. gonorrhoeae* variants and mutants were added on top of layers of HEC-1-B cells, each maintained on a microporous Transwell-COL membrane. The released bacteria were characterized for pilus, PilC, and PilE expression. The transmigrants were also analyzed for *pilE* sequence variation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *N. gonorrhoeae* MS11$_{mk}$(P$^+$) and P$^-$n (nonpiliated) have been described (31). The MS11$_{mk}$(P$^+$) strain sample used in our studies is designated MS11$_{mk}$(P$^+$)-u. The RecA$^-$ mutant VD302 was obtained from M. Koomey (13). Variants 8 (PilC$^-$) and M3 (PilC$^+$) are spontaneous nonpiliated derivatives of MS11$_{mk}$(P$^+$)-u, whereas the piliated variants MS11-8:1 and MS11-3:1 are revertants of two nonpiliated, PilC$^-$ clones (8 and 3) derived from MS11$_{mk}$(P$^+$)-u (10). The construction of single and double mutations in *pilC*, by using the shuttle mutagenesis system developed by Seifert et al. (25, 26), has previously been described (9). Bacteria were grown on GCB agar (Difco) with Kellogg's supplement (11) at 37°C in a 5% CO_2 atmosphere and were restreaked every 18 to 20 h.

Cell lines and growth conditions. ME180 (ATCC HTB33), an epithelioid human cell line from cervical carcinoma, was maintained in McCoy's 5A medium supplemented with 10% fetal calf serum (FCS). The Wong-Kilbourne derivative of Chang conjunctiva (ATCC CCL20.2), an epithelioid human cell line, was grown in medium 199 with Earle's salts supplemented with 10% FCS. The human adenocarcinoma endometrial cell line HEC-1-B (ATCC HTB113) was

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grown in minimal essential medium with Earle's salts (EMEM) supplemented with 10% FCS. All the cell lines were grown at 37°C in a 5% CO_2 atmosphere.

Passage of *N. gonorrhoeae* through a layer of epithelial cells. Epithelial cells were added to microporous Transwell-COL membranes (3.0-μm pore size, 6.5-mm diameter; Costar) at a concentration of 1.5×10^5/well. The cells were maintained on the membranes for 5 to 7 days, during which time the medium was changed every second day. Bacteria were added at a concentration of 8×10^6/well in a volume of 150 μl. Samples were taken from the lower chamber 25 h after infection, and viable counts of released gonococci were taken. At 30 h after addition of bacteria, radioactively labelled mannitol ([^{14}C]mannitol; Amersham Inc.) was added to the upper chamber. Fifty microliters of the basal medium was removed after 2 h of incubation, scintillation fluid was added, and the radioactivity was measured. We concluded that the cell layers were confluent and tight, since only 0.2% of the radioactivity was detected in the lower chamber. The transepithelial resistance was measured with a Millicell-ERS (Millipore, Bedford, Mass.). Invasion assays of $MS11_{mk}(P^+)$-u and the *recA* mutant were performed with cells maintained on Transwell-COL membranes. Bacteria were added on top of the cell layer (8×10^6/well in a volume of 150 μl) and further incubated for 6 h, after which 200 μg of gentamicin per ml was added to both the upper and the lower sides of the filter. Incubation with gentamicin for 2 h in order to kill extracellular gonococci was followed by three washes in EMEM and lysis of the cells in 1% saponin in EMEM for 5 min. Appropriate dilutions were plated onto GCB agar to determine the number of viable bacteria and to examine the colony morphology of released bacteria.

Immunoblots and electrophoresis. Bacterial cell lysates (10 μg) were loaded onto sodium dodecyl sulfate (SDS)–15% polyacrylamide gels for the detection of PilE and onto SDS–10% polyacrylamide gels for the detection of PilC (15). The proteins were transferred from the gel onto nitrocellulose sheets and identified with polyclonal PilC antiserum or pilus antiserum by an immunoblotting protocol described by Towbin et al. (33). The pilus antiserum was raised in rabbits by using highly purified pilus preparations of *N. gonorrhoeae* $MS11_{mk}(P^+)$-u as previously described (9), and the PilC antiserum was generated in a rabbit against gel-purified PilC from *N. gonorrhoeae* $MS11_{mk}(P^+)$-u as previously described (9). All samples were boiled in sample buffer at 100°C for 5 min before electrophoresis. Outer membranes of the different variants were prepared as previously described (8), in order to examine the presence of Opa (PII) proteins. Aliquots of the outer membrane preparations were heated at 100°C for 10 min or at 37°C for 10 min and subsequently separated on SDS–12% polyacrylamide gels and stained with Coomassie brilliant blue.

Electron microscopy. Infected HEC-1-B cells on Transwell-COL membranes were fixed in 2.5% glutaraldehyde for 2 h, washed twice with cacodylate buffer (pH 7.4), incubated with 1% osmium tetroxide in cacodylate buffer for 1 h at 4°C, washed twice, dehydrated in ethanol, and embedded in Epon. Ultrathin sections were obtained with a glass knife, applied to 200-mesh Formvar-coated grids, and stained with uranyl acetate and lead citrate. The grids were examined in a Zeiss EM109 electron microscope.

DNA sequencing. The *pilE* gene from gonococcal variants and mutants was PCR amplified with primers 5′-AAATTTAAGGCCTAATTTGCC-3′ and 5′-TTTCCCCTTTCAATTAGGAGT-3′. Amplified fragments were purified from agarose gels and sequenced with the Taq Dye Cycle sequencing kit (Perkin-Elmer) and the Applied Biosystems model 373A automated DNA sequencer.

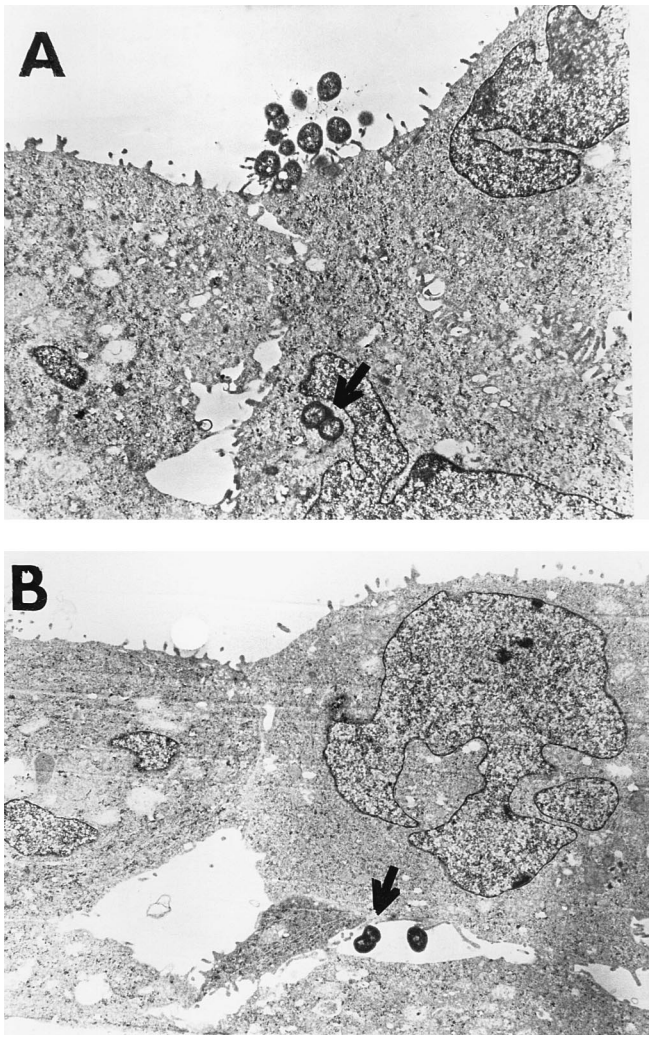

FIG. 1. TEM of ultrathin sections of HEC-1-B epithelial cells maintained on Transwell-COL membranes and infected with *N. gonorrhoeae* MS11-8:1 (piliated, $PilC^+$) for 25 h. The arrows indicate intracellular bacteria (A) and bacteria between two cells (B).

RESULTS

Establishment of a model system for passage of *N. gonorrhoeae* through an epithelial cell layer. In this study we examined three different human epithelial cell lines, HEC-1-B (endometrial adenocarcinoma), ME180 (cervical), and Chang conjunctiva, for their abilities to form tight layers on Transwell-COL membranes with 3-μm pores. Under such growth conditions cells of epithelioid cell lines may form confluent layers impermeable to ions and macromolecules. Piliated *N. gonorrhoeae* $MS11_{mk}(P^+)$-u organisms were added on top of an epithelial cell layer maintained on the microporous Transwell membranes at 8×10^6 bacteria/well. Release of gonococci into the lower medium compartment started 15 to 20 h after addition of gonococci to the upper chamber, and the amount recovered increased over several hours. In order to test the integrity of the monolayer, [^{14}C]mannitol was added to the cells 30 h after the addition of bacteria, at which time the gonococci were efficiently released, and liquid samples were removed from the lower medium after two additional hours of incubation. Less than 0.2% of the radioactive mannitol had reached the lower medium during this time (data not shown), suggesting that the cell layer was tight. Intracellular gonococci were visualized by electron microscopy of thin sections of the cell layers, but gonococci were also embedded at the junction between two epithelial cells (Fig. 1). Cells of all three lines grown on the membrane formed several layers. The HEC-1-B cell line, grown on the filters, had a more columnar appearance and tight interaction between cells according to transmission electron microscopy (TEM) of thin cryosections and was therefore used for further studies. Further, the HEC-1-B cell line maintained a low but constant transepithelial resistance for more than 48 h after infection (data not shown), which suggests that the cell layer remained intact.

Piliated *N. gonorrhoeae* organisms released from a layer of epithelial cells are predominantly nonpiliated. We examined a number of genetically defined gonococcal variants of $MS11_{mk}(P^+)$-u for their ability to pass through a layer of HEC-1-B cells. None of the bacterial variants produced Opa, as detected by Coomassie blue staining of SDS-polyacrylamide gels. Bacteria started to be released at 15 h after addition to the upper chamber, and the number of released bacteria increased slightly over time; however, after 60 h the cell layers were sometimes disrupted, resulting in a sudden entry of a large number of bacteria into the lower chamber. For the

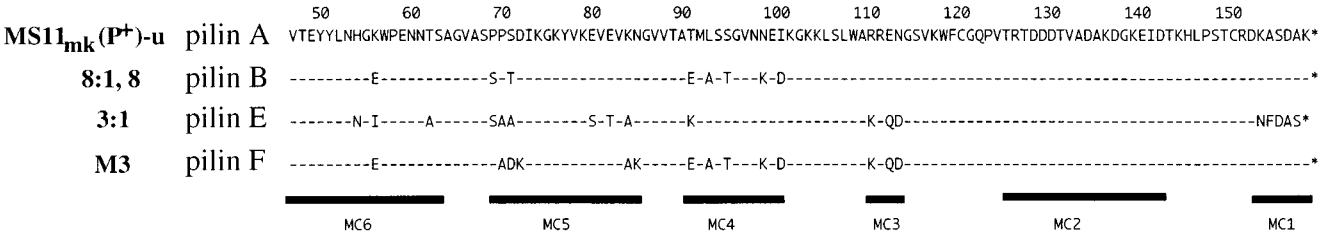

FIG. 2. Deduced amino acid sequence of *pilE* of *N. gonorrhoeae* $MS11_{mk}(P^+)$-u derivatives. The *pilE* coding region was PCR amplified and sequenced directly. The six variable minicassettes are marked MC6 to MC1. Amino acids that differ from the $MS11_{mk}(P^+)$-u parental pilin (pilin A) are shown. Identical stretches of amino acids are indicated with dashed lines. Pilin A is expressed by the parent *N. gonorrhoeae* strain, $MS11_{mk}(P^+)$-u, and pilin B is produced by the nonpiliated, $PilC^-$ derivative 8 of $MS11_{mk}(P^+)$-u and its piliated, $PilC^+$ revertant MS11-8:1. Pilin E is expressed by the second-generation piliated, $PilC^+$ variant MS11-3:1. Pilin F is expressed by the nonpiliated, $PilC^+$ derivative M3. Numbering above the sequences starts at position 1 of the mature PilE protein.

remaining experiments, we studied the bacteria released into the lower chamber 25 h after addition of 8×10^6 bacteria/well. All released bacteria had a transparent appearance under the light microscope and showed no visible changes in opacity colony phenotype after intracellular passage. Further, 20 colonies were examined for the presence of Opa proteins by SDS-polyacrylamide gel electrophoresis of outer membrane preparations. None of these colonies produced detectable levels of Opa. Three piliated, $PilC^+$ variants containing different *pilE* gene sequences from each other were used: $MS11_{mk}(P^+)$-u (pilin A), MS11-3:1 (pilin E), and MS11-8:1 (pilin B) (Fig. 2). After 25 h, all three piliated variants were released into the lower chamber; however, variant 8:1 was recovered at higher numbers than $MS11_{mk}(P^+)$-u and MS11-3:1 (Fig. 3). All the piliated variants were recovered predominantly as nonpiliated clones after passage through the HEC-1-B cells (Fig. 3). Between 75 and 90% of the colonies were characterized as nonpiliated, as evidenced by colony morphology. We also examined 20 nonpiliated colonies by TEM, and all expressed zero to two pili/bacterium, which is typical of nonpiliated, revertible clones. As a control, all gonococcal variants were grown in cell culture medium with or without fixed cells and maintained their colony morphology for at least 48 h.

Two nonpiliated, revertible variants of $MS11_{mk}(P^+)$-u, i.e., M3 ($PilC^+$; expresses pilin F) and 8 ($PilC^-$; produces pilin B), were recovered at levels similar to those of the piliated variants MS11-3:1 and MS11-8:1. PilC mutants of $MS11_{mk}(P^+)$-u, constructed by insertional inactivation by a minitransposon derivative of Tn*3* expressing a Cm^r gene (mTnCm) (26), have been described (9). The mutants D1 ($PilC1^+$ *pilC2*::mTnCm) and D2 (*pilC1*::mTnCm $PilC2^+$), both producing pilin A, were released at nearly identical amounts from the cell layer. P^-n derivatives, in which the 5′ end and promoter region of *pilE* had been deleted, were released at much-reduced numbers, independent of whether PilC was produced or not (Fig. 3). The P^-n bacteria continued to be released at much-reduced rates for at least 72 h.

Piliated *recA* mutants are not released from the HEC-1-B cell layer. Phase variation of pili is frequently caused by recombination events between silent and expressed *pil* gene copies, a process which is dependent on the *recA* gene product. Piliated *recA* gonococcal mutants undergo pilus phase variation at a 100-fold lower frequency than the wild type (13). Since piliated variants were released from the HEC-1-B cell layer as mostly nonpiliated clones, we expected the piliated *recA* mutant to be released at reduced rates. When the piliated *recA* mutant VD302 was added to the epithelial cell layer, no bacteria were released into the medium underneath the epithelial cells at 25 h after addition (Fig. 3). These data strongly suggest that the ability to switch to a nonpiliated phenotype at high frequency is required for the efficient passage of Opa^- gonococci through the epithelial cell layer. The piliated *recA* mutant and the wild type are capable of invading HEC-1-B cells at similar rates (data not shown). Thus, piliated bacteria enter HEC-1-B cells without first converting to nonpiliated bacteria.

Characterization and analysis of bacteria released from epithelial cells. Piliated *N. gonorrhoeae* $MS11_{mk}(P^+)$-u organisms were recovered as 90% nonpiliated clones from the lower medium compartment 25 h after addition of bacteria to the cell layer. Twenty of the clones were restreaked onto GCB plates and carefully examined for phase switches from piliated to nonpiliated. All the nonpiliated variants were still capable of

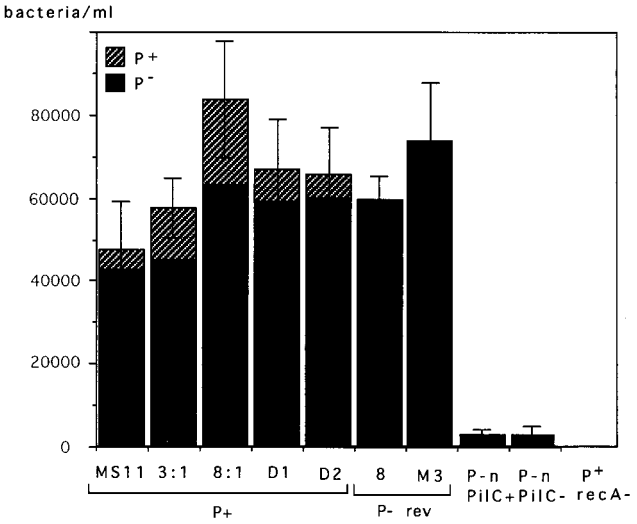

FIG. 3. *N. gonorrhoeae* isogenic variants were added to a HEC-1-B cell layer maintained on Transwell-COL membranes. Bacteria released into the lower chamber at 25 h after infection were spread onto GCB plates. Shown are the numbers of colonies obtained. The piliation phenotypes of the recovered bacteria, as evidenced by colony morphology, are indicated by black (nonpiliated [P^-]) or hatched (piliated [P^+]) bars. *N. gonorrhoeae* $MS11_{mk}(P^+)$-u, MS11-8:1, and MS11-3:1 are piliated variants carrying pilins A, B, and E, respectively. Variants 8 and M3 are nonpiliated derivatives of $MS11_{mk}(P^+)$-u. M3 is $PilC^+$ and expresses pilin F; variant 8 is $PilC^-$ and produces pilin B. D1 (piliated; $PilC1^+$ *pilC2*::mTnCm) and D2 (piliated; $PilC2^+$ *pilC1*::mTnCm) are *pilC* mutants of $MS11_{mk}(P^+)$-u which both produce pilin A. The P^-n derivatives had deletions of the *pilE* gene. The P^-n $PilC^-$ variant is a nonrevertible variant of a PilC1-PilC2 double knockout. The piliated *recA* mutant has been described (13). Shown are the averages of three independent experiments. As determined by an unpaired two-tailed *t* test, the difference in number of released bacteria between variants MS11-3:1 and MS11-8:1 was statistically significant ($P = 0.014$). The differences between MS11 and MS11-3:1 and between variant 8 and M3 were not significant ($P = 0.13$ and $P = 0.10$, respectively). rev, revertible.

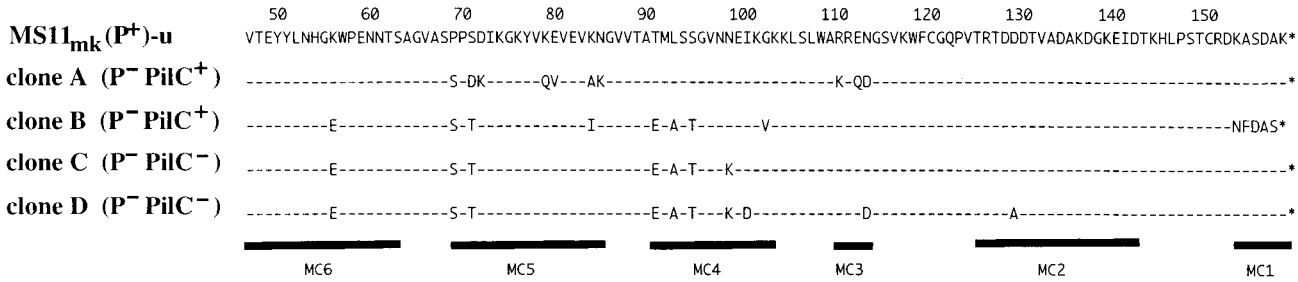

FIG. 4. Deduced amino acid sequence of the *pilE* gene of *N. gonorrhoeae* MS11$_{mk}$(P$^+$)-u and four nonpiliated, PilE-expressing variants released from a HEC-1-B cell layer. The *pilE* coding region was PCR amplified and sequenced directly. Dashes indicate amino acids identical to those of MS11$_{mk}$(P$^+$)-u. The six variable minicassettes of *pilE* are marked MC6 to MC1. Numbering above the sequences starts at position 1 of the mature PilE protein.

reverting to a piliated phenotype. The nonpiliated clones were tested in immunoblots for expression of PilC and PilE with polyclonal anti-PilC serum and anti-PilE serum, respectively, and the results were compared to those for 50 nonpiliated clones obtained from piliated MS11$_{mk}$(P$^+$)-u on GCB agar plates. PilC expression was 50 and 62% for clones released from HEC-1-B cells and clones obtained from MS11$_{mk}$(P$^+$)-u on GCB agar plates, respectively. PilE expression was 100 and 48% for clones released from HEC-1-B cells and clones obtained from MS11$_{mk}$(P$^+$)-u on GCB agar plates, respectively. The nonpiliated phenotype in the clones which still produced PilC may depend on an alteration in *pilE* that generated an assembly-deficient pilin (6, 31). Taken together, our data argue that nonpiliated, pilin-producing gonococcal clones are selected for during the passage through a layer of HEC-1-B cells.

The released bacteria have altered *pilE* sequences. The findings that the piliated *recA* mutant did not pass through the cell layer and that the nonpiliated revertible variants were still able to be released from the HEC-1-B cells suggested that pilin structural variation may be involved. To determine whether the *pilE* sequence in the released bacteria was altered, we PCR amplified and sequenced *pilE* of four nonpiliated clones released from the HEC-1-B cells. In all four transmigrants the *pilE* sequence differed from the sequence of the added MS11$_{mk}$(P$^+$)-u (Fig. 4). The *pilE* gene was altered in both PilC$^+$ and PilC$^-$ clones. These data suggest that transcellular passage of *N. gonorrhoeae* involves sequence variation of PilE.

DISCUSSION

This work demonstrates a selective advantage for nonpiliated, PilE-producing *N. gonorrhoeae* variants during the passage through an epithelial cell layer maintained on Transwell-COL membranes. TEM confirmed that gonococci invaded the cells. However, a population of the bacteria was found in between the cells. This finding is in agreement with the human fallopian tube model developed by Ward et al. (35), which showed gonococci within epithelial cells as well as embedded at the junction between two cells. Stephens et al. (30) described a human nasopharyngeal organ culture model for *Neisseria meningitidis*. Meningococci were found in phagocytic vacuoles in the apical portion of these cells, and bacteria were observed in the subepithelial space at 18 to 24 h after infection, suggesting that meningococci had crossed the mucosal surface. Recently, a model system for passage of *N. meningitidis* through a layer of host cells was reported (1). This model incorporated the human cervical epithelial cell line HEC-1-B and the human microvascular endothelial cell line HMEC-1. Bacteria were observed inside the cells and in the junction between two cells.

In this study, piliated gonococci applied to the upper side of a HEC-1-B cell layer were released into the lower medium as mostly nonpiliated phase variants. The recovered nonpiliated bacteria still expressed the PilE protein, but half of the clones had turned off PilC. Thus, nonpiliated, pilin-producing phase variants are released when piliated *N. gonorrhoeae* organisms are added to a layer of HEC-1-B epithelial cells. The advantage for nonpiliated, PilE$^+$ clones may be due to differences in growth rates between piliated and nonpiliated clones or due to signals from the eucaryotic cells inducing the loss of pili expression. Birkness et al. (1) reported that piliated and nonpiliated *N. meningitidis* FAM18 organisms did not differ in their abilities to pass through the cell layers. It is, however, not known whether the nonpiliated variants used in their studies still expressed or were capable of expressing the PilE protein.

Under the growth conditions prevailing on GCB plates, more than 50% of the nonpiliated derivatives came out like P$^-$n, i.e., did not express PilE due to deletions in the *pilE* locus (10), which is in contrast to what was found for nonpiliated bacteria released from the HEC-1-B layer, which all expressed PilE. These data argue that the production of unassembled PilE protein may play a role in the passage of *N. gonorrhoeae* through a layer of HEC-1-B cells. Nonpiliated, PilE-producing gonococci express a PilE protein that can be proteolytically cleaved after amino acid 39 to a shortened S-pilin and subsequently be released from the gonococci. We have obtained results suggesting that the switch from piliated PilC$^+$ to nonpiliated PilC$^-$ gonococcal cells selects for changes in the PilE protein, allowing it to be processed to S-pilin (10). Consequently, we argue that structural diversity of PilE is enhanced by a selective process. Our findings point to the need for a nonpiliated phase during gonococcal infection to provide selection for PilE alterations. The data given in this paper suggest that a nonpiliated phase is selected for during passage across a layer of epithelial cells. We found that the *pilE* gene sequence was altered in the transmigrants, suggesting that the bacteria released provide a pool of clones which carry structurally altered pili that may have different antigenic and binding properties.

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