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pilQ Missense Mutations Have Diverse Effects on PilQ Multimer Formation, Piliation, and Pilus Function in *Neisseria gonorrhoeae*▽

R. Allen Helm, Michelle M. Barnhart,† and H. Steven Seifert*

Northwestern University, Feinberg School of Medicine, Department of Microbiology-Immunology, Chicago, Illinois

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Type IV pili are required for virulence in *Neisseria gonorrhoeae*, as they are involved in adherence to host epithelium, twitching motility, and DNA transformation. The outer membrane secretin PilQ forms a homododecameric ring through which the pilus is proposed to be secreted. *pilQ* null mutants are nonpiliated, and thus, all pilus-dependent functions are eliminated. Mutagenesis was performed on the middle one-third of *pilQ*, and mutants with colony morphologies consistent with the colony morphology of nonpiliated or underpiliated bacteria were selected. Nineteen mutants, each with a single amino acid substitution, were isolated and displayed diverse phenotypes in terms of PilQ multimer stability, pilus expression, transformation efficiency, and host cell adherence. The 19 mutants were grouped into five phenotypic classes based on functionality. Four of the five mutant classes fit the current model of pilus functionality, which proposes that a functional pilus assembly apparatus, not necessarily full-length pili, is required for transformation, while high levels of displayed pili are required for adherence. One class, despite having an underpiliated colony morphology, expressed high levels of pili yet adhered poorly, demonstrating that pilus expression is necessary but not sufficient for adherence and indicating that PilQ may be directly involved in host cell adherence. The collection of phenotypes expressed by these mutants suggests that PilQ has an active role in pilus expression and function.

The human-specific pathogen *Neisseria gonorrhoeae*, also referred to as the gonococcus, is the sole causative agent of the sexually transmitted infection gonorrhea. A major virulence factor of *N. gonorrhoeae* is the type IV pilus (Tfp). Tfp are thin polymeric appendages required for efficient virulence in many gram-negative pathogens (11). Generally, Tfp are several micrometers long and 50 to 80 Å wide and consist of thousands of copies of pilin, the major pilus subunit (11). In addition to *N. gonorrhoeae*, Tfp are expressed in many pathogenic bacteria, such as *Neisseria meningitidis* (14), *Pseudomonas aeruginosa* (17), *Vibrio cholerae* (35), *Salmonella enterica* serovar Typhi (43), and enterotoxigenic and enteropathogenic *Escherichia coli* (16, 37).

Tfp expression is essential for *N. gonorrhoeae* pathogenesis, and only piliated bacteria are recovered from gonorrhea patients (36). Tfp are required for a variety of functions related to pathogenesis, including twitching motility, autoagglutination, and adherence to host epithelium (24, 40, 41). Tfp are also involved in high levels of natural DNA transformation in *N. gonorrhoeae* (32).

In *Neisseria*, the major Tfp subunit, pilin, is encoded by *pilE*. After being expressed in the cytosol, premature pilin subunits are transported across the inner membrane and are then cleaved by the prepilin peptidase PilD (15). Fully functional Tfp are dynamic structures that lengthen and retract via pilin polymerization and depolymerization, respectively. Two inner membrane-associated ATPases, PilF and PilT, are thought to antagonistically promote this extension and retraction, with PilF being involved in pilus elongation (15) and PilT being necessary for pilus retraction (26, 40). The portal though which the pilus extends or retracts across the outer membrane (OM) is PilQ (42).

PilQ belongs to a superfamily of OM proteins called secretins that function as large, surface-exposed, homooligomeric rings involved in secretion of intracellular macromolecules across the cellular envelope into the extracellular milieu (3, 38). In addition to Tfp expression, secretins are also required for type II and type III secretion, as reviewed by Bayan et al. (1), and in the secretion of certain filamentous phages (23). It has been reported that in multiple systems in a diverse group of organisms secretin multimers are remarkably resistant to denaturation in the presence of heat and sodium dodecyl sulfate (SDS) (18, 28).

Sequence analyses of secretins from several organisms have revealed that the C-terminal domain is highly conserved across genera, and regions in this domain are predicted to form β-barrel structures, which are hallmarks of OM-spanning regions. Some data suggest that the C-terminal domain is directly involved in multimer assembly (3, 4). Conversely, the N-terminal domain is much less conserved among genera and is proposed to be periplasmic (2). The divergent nature of the N-terminal sequence and the suggested periplasmic localization indicate that the N terminus may be involved in specific interactions required for particular secretin functions.

In *Neisseria*, PilQ is the sole secretin and has been estimated to constitute approximately 10% of the OM mass in *N. gonorrhoeae* (28). Electron microscopy (EM) data suggest that PilQ in *N. meningitidis* exists as a dodecameric doughnut-shaped

* Corresponding author. Mailing address: Department of Microbiology-Immunology, Northwestern University's Feinberg School of Medicine, S213, 303 East Chicago Avenue, Chicago, IL 60611. Phone: (312) 503-9788. Fax: (312) 503-1339. E-mail: h-seifert@northwestern.edu.

† Present address: University of Michigan, Department of Molecular, Cellular, and Developmental Biology, 830 North University, Ann Arbor, MI 48109.

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ring with symmetry suggestive of a tetramer of trimers in the OM (8–10). Far-Western blot data coupled with these EM data strongly suggest that PilQ directly interacts with Tfp and that this interaction stimulates a structural change in PilQ (9). In other organisms, EM analyses of secretins involved in filamentous phage secretion (29) and type II secretion in *Klebsiella* (5) revealed a similar general surface-exposed, multimeric ring structure in the OM. The variations among the secretins studied in these examples include variations in the nature and symmetry of a plug domain that is proposed to occlude the secretin pore, as well as the symmetry of the secretins (1).

In *Neisseria*, *pilQ* null mutants are nonpiliated and not competent for DNA transformation (13); however, the distinct phenotypes of two independently isolated mutants with spontaneous point mutations in *pilQ* in *N. gonorrhoeae* have suggested that PilQ can be involved in physiological events in addition to secretion of the pilus. The *pilQ1* mutation, which results in an F563L substitution, allows *N. gonorrhoeae* to grow with hemoglobin as the sole source of iron when normal transport pathways are blocked, and the mutant is hypersensitive to the toxic effects of free heme (6). The *pilQ1* strain is also more sensitive to certain antimicrobial compounds, suggesting that this mutation allows increased transport of large molecules through the PilQ complex. The *pilQ1* mutant is able to express pili and undergoes transformation, albeit slightly less efficiently than the parent strain. Conversely, the *pilQ2* mutation results in a E666K substitution, which appears to be a loss-of-function mutation (44). The *pilQ2* mutant has a nonpiliated (P^-) colony morphology and displays no pili, yet it still undergoes DNA transformation at a reduced frequency. These findings suggest that PilQ may participate directly in pilus-dependent processes and demonstrate that mutants with missense mutations can provide insight into PilQ function.

The goal of this study was to generate a library of *pilQ* missense mutants of *N. gonorrhoeae* to further understand the role of secretins in general and of PilQ in particular in terms of formation of stable multimers, pilus secretion, and pilus-dependent functions. To accomplish this goal, a method for performing error-prone mutagenesis of the *pilQ* chromosomal locus was devised. Upon mutagenesis of the middle one-third of *pilQ*, mutants with a P^- colony morphology were isolated in order to identify mutations involved in pilus function. For each P^- mutant isolated, Western blot analysis was performed to ascertain the effect of the mutation on PilQ monomer expression and heat- and SDS-resistant complex formation. Mutants expressing a nontruncated PilQ monomer were sequenced and analyzed to determine the effects of the mutations on pilus expression and pilus-dependent events. These missense mutants varied greatly in their ability to form stable PilQ complexes and to perform pilus-related functions. These mutants were grouped into five phenotypic classes based on PilQ functionality in terms of the ability to secrete pili, the ability to transform DNA, and the ability to adhere to host epithelial cells. Class 1 mutants were completely nonfunctional, and this class was a null class; class 2 mutants were minimally functional; class 3 mutants were moderately functional; class 4 mutants were differentially functional; and the single class 5 mutant was highly functional.

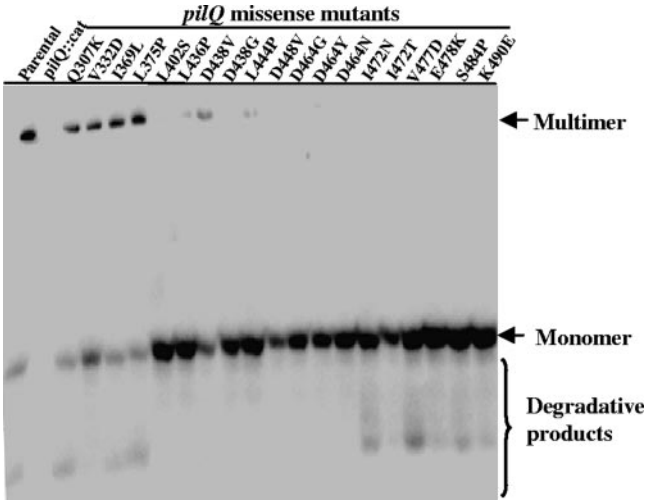

FIG. 1. Representative PilQ Western blot of all 19 missense mutants with single amino acid substitutions along with the parent strain and the *pilQ*::*cat* null mutant. The null mutant does not show any PilQ reactivity. The parent strain contains three regions of PilQ reactivity: (i) the multimer complex, which is too large to enter the gel; (ii) the monomer (approximately 80 kDa); and (iii) smaller immunoreactive degradative products.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Missense mutants and the *pilQ* null mutant were generated using the RM11.2 pilin variant of FA1090 (21) in a *recA6* background in the absence of isopropyl-β-D-thiogalactopyranoside (IPTG) to prevent pilin phase variation (31). The only time that these strains were grown on IPTG was during transformations, which were followed by *pilE* DNA sequence analysis to ensure that pilin antigenic variation did not occur. The *pilQ* null mutant used here, *pilQ*::*cat*, has been described previously (22). Briefly, a portion of the FA1090 *pilQ* gene was removed and replaced with a chloramphenicol resistance gene (*cat*), generating a null mutant that has no PilQ function and no PilQ immunoreactivity in SDS-polyacrylamide gel electrophoresis (PAGE) Western blots (Fig. 1).

To check for growth defects in *pilQ pilT* double mutants, the 19 *pilQ* missense mutations and the *pilQ*::*cat* null mutation were transformed into an FA1090 background with IPTG-inducible *pilT* (40) in which pilin antigenic variation was eliminated due to a *cis*-acting transposon inserted upstream of *pilE* (P^{+NV}) that has been described previously (12, 30).

All bacterial strains were grown on GCB plates with Kellogg's supplements I and II (19) at 37°C in a 5% CO_2 humidified atmosphere. Erythromycin was used in GCB at a concentration of 0.5 μg/ml, chloramphenicol was used in GCB at a concentration of 1 μg/ml, and nalidixic acid was used in transformation assays at a concentration of 1 μg/ml.

Construction of pMB9. A 2,557-bp DNA fragment from FA1090, starting 55 bp downstream of the *pilQ* coding start site and ending 438 bp downstream of the *pilQ* stop codon, was amplified using primers PilQSacIITop (5′-TTTCAGACG GCATCCGCGGGAAACATTACAGAC-3′) and PilQDS2Bot (5′-GGCGGAA ATCCGAACACGTCC-3′), which replaced both a G at position 70 and an A at position 72 with C to generate a SacII site between positions 68 and 73 relative to the start site of *pilQ* (Fig. 2A). This fragment was cloned into the pSMART ampicillin-resistant vector using the CloneSmart Blunt cloning system (Lucigen). The erythromycin resistance (Erm^r) gene from pJD1145 (25) was amplified using primers ErmUpTopBamHI (5′-GGATCCGCCGTCTGAAGTTTGACAGCTT ATCATCGCGTGC-3′) and ErmBot (5′-CACAAAAAATAGGTACACGAAA AC-3′), resulting in a BamHI restriction site immediately upstream of the start of the Erm^r gene, and the fragment was inserted into the naturally occurring NdeI restriction site 17 bp downstream of the *pilQ* stop codon by blunt end cloning (Fig. 2A).

PCR mutagenesis of *pilQ* region 2. PCR was used to amplify and mutagenize *pilQ* region 2 using pMB9 as the template (Fig. 2A) in the presence of 5 mM $MgCl_2$ and 1.25 U *Taq* DNA polymerase (Promega). Primer PilQXhoITop (5′-CCTGCCGAAAAAACAAAACCTCGAG-3′), which anneals to DNA 834 to 858 bp downstream of the *pilQ* start site, and primer PilQBsmIBot (5′-CAC

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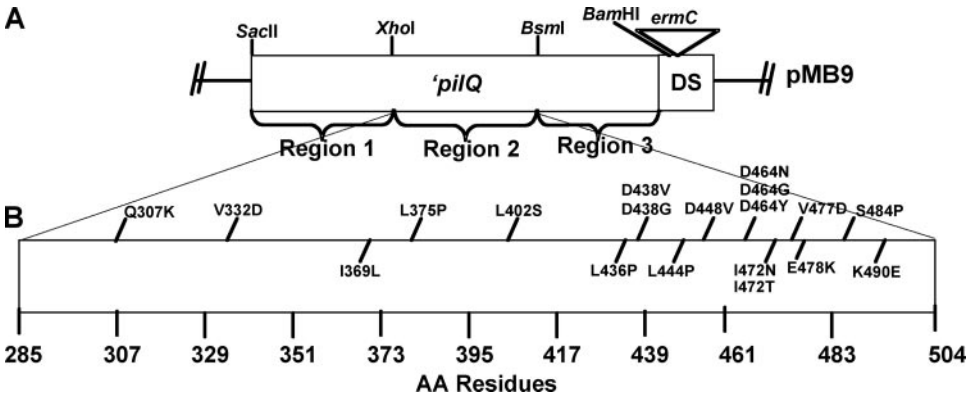

FIG. 2. (A.) Map of the *pilQ* mutagenesis vector pMB9. The *pilQ* gene, minus the first 54 bp of the coding sequence (*'pilQ*) along with 438 bp downstream of the *pilQ* stop site (DS), was cloned into a pSMART vector. An Erm^r cassette (*ermC*) was cloned into an NdeI site 17 bp downstream of the *pilQ* stop codon, leaving 421 bp of homology downstream of *ermC*. Region 2 begins 854 bp downstream of the *pilQ* coding start site (corresponding to amino acid 285) and ends 1,511 bp downstream of the *pilQ* coding start site (corresponding to amino acid 504), spanning a total of 657 bp (or 220 residues). (B) Map showing the locations of the 19 single amino acid substitutions in gonococcal PilQ that result in P$^-$ colony morphology. The mutants were divided into five phenotypic classes, as shown in Table 1.

GCCCCAGCCGAATGCGCTCG-3′), which anneals to DNA 1,508 to 1,530 bp downstream of the *pilQ* start site, were each used at a concentration 50 μM. The deoxynucleotides dATP, dCTP, dGTP, and dTTP were each used at a concentration of 0.2 mM. The PCR consisted of 30 cycles of melting at 94°C for 1 min, annealing at 62°C for 1 min, and elongation at 72°C for 5 min.

The mutagenized PCR products were gel purified and cloned back into the pMB9 vector from which *pilQ* region 2 had previously been removed by digestion with BsmI and XhoI (Fig. 2A). The new plasmid pool, with various region 2 *pilQ* mutations, was transformed into *N. gonorrhoeae* strain RM11.2, the parental strain, which does not permit plasmid replication. Since the plasmid is unable to replicate in *Neisseria* and there is no homology between *N. gonorrhoeae* and the plasmid vector, selection for transformants on erythromycin ensured that the Erm^r cassette had recombined onto the bacterial chromosome along with some part of the mutant *pilQ* locus and the *pilQ* downstream region.

Sequence analysis. DNA sequencing was performed commercially (SeqWright, Houston, TX). The primers used for sequencing the *pilQ* region 2 mutants were designed to anneal to DNA flanking the outer edges of region 2, so any mutations at the extreme 5′ or 3′ end of the region could be detected (Fig. 2). Primer PilQ757Top (5′-CAGCTGATTATCACAACAACCGGC-3′) anneals to DNA 757 to 780 bp downstream of the *pilQ* start site, and primer PilQ1566Bot (5′-C CGGCAGGTTGATTTTGGTTTGG-3′) anneals to DNA 1,566 to 1,588 bp downstream of the *pilQ* start site. DNA sequence analysis of *pilE* was performed as previously described (34).

SDS-PAGE and Western blotting. Whole-cell lysates were prepared as described previously (44). Samples were run on SDS-polyacrylamide gels (10%), separated by electrophoresis, and electrotransferred to a polyvinylidene difluoride membrane in 10 mM CAPS (*N*-cyclohexyl-3-aminopropanesulfonic acid) buffer with 10% methanol at pH 11 for 1.5 h. Upon transfer, the membrane was blocked with 5% dry milk in Tris-buffered saline with 0.5% Tween for 1 h and then incubated with a 1:100,000 dilution of a polyclonal anti-PilQ antibody (graciously provided by C. E. Wilde, Indiana University School of Medicine) for 1 h at room temperature. The membrane was washed three times with Tris-buffered saline with 0.5% Tween and then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Chemicon International, Temecula, CA) for 1 h and washed three times. Blots were developed with the ECL-Plus Western detection reagent (Amersham Biosciences) used according to the manufacturer's instructions.

Transformation assays. Transformation assays were performed as previously described, with slight modifications (44). Strains were grown for 18 h on GCB plates and resuspended in liquid GCB to an optical density at 600 nm of approximately 0.2. Then 20 μl of a bacterial resuspension was added to 200 μl of liquid GCB containing supplements, 5 mM $MgSO_4$, 1 mM IPTG, and 10 ng of plasmid pSY6, which carries a *gyrB* mutation (33) that confers nalidixic acid resistance (Nal^r) in *N. gonorrhoeae*. Transformation mixtures were incubated at 37°C for 15 min. The reaction mixtures were then treated with 1 U RQ1 DNase (Promega), incubated at 37°C for 5 min, and added to 2 ml of liquid GCB plus supplements. After 5 h of incubation at 37°C in the presence of 5% CO_2, the reaction mixtures were 10-fold serially diluted and spotted onto GCB plates in the presence and absence of 1 μg/ml nalidixic acid. The efficiencies reported below were determined by dividing the number of CFU/ml recovered on nalidixic acid-containing by the total number of CFU/ml recovered on nonselective plates.

Adherence assays. ME180 human endocervical epithelial cells (ATCC HTB 33) were maintained in RPMI 1640 medium without L-glutamine (Mediatech, Inc) supplemented with 5% fetal bovine serum (RPMI-FBS) containing penicillin (100 U/ml), streptomycin (100 mg/ml), and amphotericin B (2.5 μg/ml) at 37°C in the presence of 5% CO_2. All adherence assays were performed in the absence of antibiotics and amphotericin B.

Standard CFU adherence assays were performed as described previously, with slight modifications (21). Briefly, 1 ml of a culture containing 5×10^5 ME180 cells/ml was added to each well of a 24-well culture dish (Corning) and incubated for 24 h prior to the assay. *N. gonorrhoeae* was cultured on GCB plates for 18 h and was gathered with Dacron swabs, suspended in RPMI-FBS, and diluted to obtain a concentration of approximately 1×10^7 CFU/ml. One milliliter of a bacterial suspension was incubated in each well for 1 h. Monolayers were washed three times with phosphate-buffered saline (PBS) and incubated with 1% saponin for 10 min. Two milliliters of RPMI-FBS was then added to each well, the monolayers were disrupted by pipetting, and 10-fold serial dilutions were prepared and plated on GCB plates. The results were determined by dividing the number of CFU/ml recovered by total number of CFU/ml initially placed in each well.

Immunofluorescence microscopy for detection of surface-exposed PilQ. All *N. gonorrhoeae* strains were labeled with the fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) (Molecular Probes-Invitrogen), which enters a bacterial cell and irreversibly binds to proteins associated with the membrane. After 18 h of growth on solid medium, approximately 10^9 *N. gonorrhoeae* cells were swabbed into 1 ml of 5 mM $MgSO_4$ in PBS (MPBS), centrifuged at $3{,}000 \times g$ for 5 min, and resuspended in 1 ml of a solution containing 0.2 mg/ml CFDA-SE in MPBS prepared according to the manufacturer's instructions. Bacteria were incubated in the presence of CFDA-SE for 20 min at 37°C, washed by centrifugation at $3{,}000 \times g$ for 5 min, and resuspended in 1 ml MPBS. Resuspended bacteria were placed on top of a glass coverslip and incubated at room temperature for 45 min. The coverslip was washed once with MPBS, and the bacteria were fixed by adding 1 ml of 4% paraformaldehyde in PBS for 15 min and then washed three times with PBS and blocked with 10% goat serum in PBS for 45 min at room temperature.

Surface-exposed PilQ was visualized using PilQ antiserum whose cross-reactive antibodies had been removed by whole-cell adsorption with a *pilQ* null mutant. To adsorb the antiserum, approximately 10^{11} RM11.2 *pilQ*::*cat* cells grown overnight on solid medium were swabbed into 3 ml liquid GCB, and 30 μl of antiserum was added and incubated at 4°C on a moving rotor for 18 h. Following incubation, the adsorbed antiserum was clarified by centrifugation and sterilized by passage through a 0.2-μm syringe filter. One hundred microliters of the cleaned antiserum was added to fixed bacteria on the coverslip and incubated at room temperature in a humidified chamber for 1 h. After incubation, the coverslip was washed three times with PBS, and 100 μl of Alexa Fluor 647 goat anti-rabbit antiserum (Invitrogen) diluted 1:200 in PBS was added and incubated

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at room temperature in a humid chamber for 1 h. After incubation the coverslip was washed three times with PBS and once in distilled deionized water. The coverslip was covered with 10 μl Fluoromount (Southern Biotechnology, Birmingham, AL) containing 2.5 mg/ml propyl gallate (ICN Biomedicals, Inc., Costa Mesa, CA) to prevent photobleaching and mounted on a slide. Samples were examined with a Leica DMIRE2 microscope. CFDA-SE-labeled *N. gonorrhoeae* was visualized using a Leica green fluorescent protein filter, PilQ was indirectly visualized using a CY5 filter, and the images were overlaid. Images were analyzed using the Openlab software (Agilent Technologies, Palo Alto, CA).

Immunoelectron microscopy for detection of pilus expression. Immunogold transmission EM (TEM) with an antipilin antibody was performed as described previously (6). Values were obtained by dividing the number of pilus bundles observed by the total number of bacterial cells observed. For each mutant, at least two grids were observed and at least 200 cells were analyzed. To be classified as a pilus bundle, a structure had to display filamentous striations and be labeled with gold particles (Fig. 3). No unbundled pili were detected.

RESULTS

Generation, isolation, and initial characterization of PilQ region 2 missense mutants. *N. gonorrhoeae* strain FA1090 PilQ is a 723-amino-acid protein. To increase the probability of obtaining single point mutations, the *pilQ* gene was divided into three regions, each bordered by unique restriction enzyme sites (Fig. 2A). Protein sequence alignments of PilQ from *N. gonorrhoeae* with PilQ from *N. meningitidis*, *Pseudomonas syringae*, *P. aeruginosa*, and *V. cholerae* and with a more distantly related secretin involved in type II secretion in *Psychrobacter cryohalolentis* suggested that regions 1 and 2 fall in the generally well-conserved C-terminal domain of PilQ (data not shown).

Plasmid pMB9 carrying the entire *N. gonorrhoeae pilQ* gene minus the first 54 bp of the coding sequence was constructed (Fig. 2A). Region 2 of *pilQ*, which is flanked by naturally occurring XhoI and BsmI restriction sites, encodes amino acid residues 258 through 504 and was mutated via error-prone PCR in the presence of 5 mM $MgCl_2$. A pool of mutated region 2 plasmids was isolated, and the pool of mutated inserts was cloned between the XhoI and BsmI sites of pMB9 to reconstitute *pilQ* with mutations throughout region 2. The pool of mutated pMB9 plasmids was transformed into *N. gonorrhoeae* strain FA1090 pilus variant RM11.2 *recA6*, a highly piliated strain (21), with selection for the Erm^r cassette inserted into the downstream flanking sequences of *pilQ*.

Following mutagenesis, approximately 2,000 Erm^r mutant colonies were isolated and screened to identify those with a nonpiliated (P^-) colony morphology, as described previously (21). A total of 204 P^- Erm^r colonies were identified, and chromosomal DNA was isolated from each colony and used to retransform the RM11.2 parent strain to confirm that Erm^r was linked to the P^- colony morphology (transformational backcross). Approximately 97% of the backcrossed mutants (a total of 197 mutants) exhibited erythromycin resistance and the P^- colony morphology, indicating that the mutation or mutations causing the P^- colony morphology were linked to the *pilQ* locus. PilQ Western blot analysis of these 197 mutants revealed that 138 mutants had no detectable full-length PilQ monomer (data not shown). DNA sequence analysis of *pilQ* of 20 randomly selected mutants with no detectable monomer showed that 19 of these mutants had nonsense mutations and one had three substitutions (E409K, V327I, and R475H). Mutants that lacked a stable PilQ monomer were not tested further.

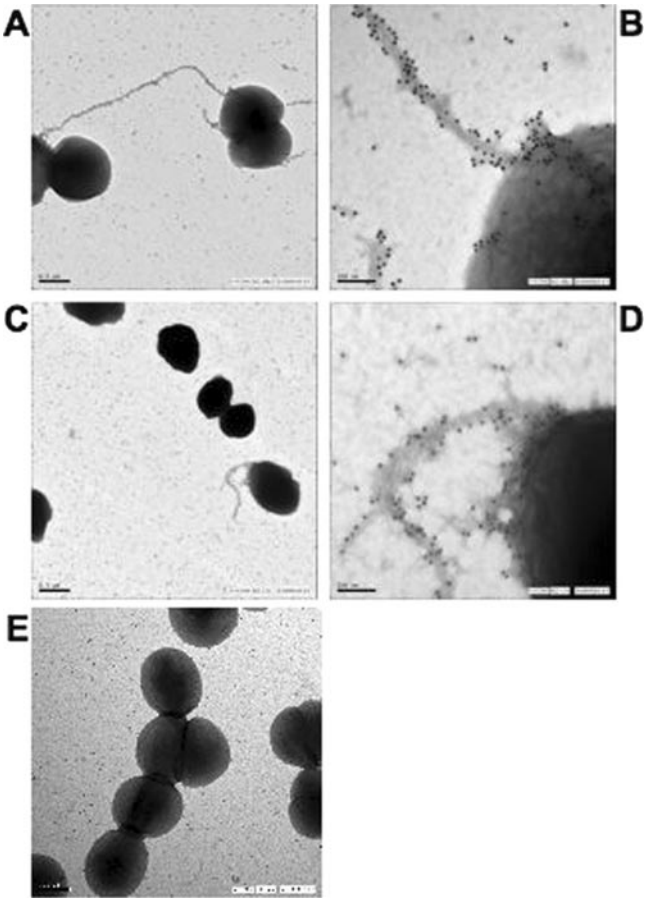

FIG. 3. Representative immunogold TEM for detection of pili. (A) *pilQ* I369L mutant, which expresses pili at a high level (41%). Pili are present in medium-size and large bundles. Similar levels of expressed pili were seen in the parental strain (35%) and the Q307K (27%) and L375P (17%) mutants (not shown). (B) Magnification of a large pilus bundle in panel A. To confirm that the structures were pilus bundles, we visualized individual pili (striations in bundles) and observed gold particles (small black spheres). (C) *pilQ* D438G mutant, which expresses pili at a low level (2.0%). Similar levels of expressed pili were seen in D438V (2.6%), E478K (2.4%), and K490E (0.91%) (not shown). The slight decrease in the size of gonococci in this image is not indicative of all the gonococci observed in this sample. The size of the *N. gonorrhoeae* strains analyzed by TEM varied from approximately 0.25 to 0.75 μm. (D) Magnification of the pilus bundle in panel C. Striations and gold particles are evident. (E) *pilQ*::*cat* null mutant. No pili were detected in 200 bacteria. The following *pilQ* missense mutants had no detectable pili: V332D, L402S, L436P, L444P, D448V, D464G, D464Y, D464N, I472N, I472T, V477D, and S484P (not shown). (A, C, and E) Bar = 0.5 μm. (B and D) Bar =100 nm.

DNA sequence analysis of *pilQ* was performed for all 59 mutants with a full-length PilQ monomer to identify the amino acid substitutions that resulted in the P^- colony morphology. Of these 59 *pilQ* mutants, 2 had four amino acid substitutions, 8 had three substitutions, 13 had two substitutions, and 36 had single substitutions. Since identification of individual residues involved in PilQ structure and function was the aim of this study, the 36 mutants with single residue changes were the focus of this work. Some of these mutants had redundant amino acid substitutions, so there was a total of 19 unique amino acid substitutions (Fig. 2B), and further analysis was

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TABLE 1. Phenotypes of 19 *pilQ* missense mutants, the parental strain, and the *pilQ* null mutant

Strain	Mutation	Multimer intensity[a]	Transformation efficiency[b]	Adherence[c]	Piliation (%)[d]	Growth defect in *pilT* background[e]
Parental	None	++	+++	++	35	No
Null	*pilQ*::*cat*	−	−	−	<0.5	Yes
Class 1						
M2-64	L436P	+/−	−	−	<0.4	Yes
M2-66	L444P	+/−	−	−	<0.4	Yes
M2-67	D448V	+/−	−	−	<0.4	Yes
M2-100	D464G	+/−	−	−	<0.4	Yes
M2-128	D464Y	+/−	−	−	<0.3	Yes
M2-132	D464N	+/−	−	−	<0.4	Yes
M2-117	I472N	−	−	−	<0.4	Yes
M1-188	I472T	−	−	−	<0.3	Yes
M2-157	V477D	−	−	−	<0.4	Yes
Class 2						
M2-164	V332D	+	+	−	<0.4	Yes
M2-76	L402S	+/−	+	−	<0.4	Yes
M2-259	S484P	−	+	−	<0.5	Yes
Class 3						
M2-38	D438V	+	++	+	2.6	No
M2-116	D438G	+/−	+	−	2.0	No
M2-178	E478K	−	+	−	2.4	No
M2-187	K490E	−	+	−	0.91	No
Class 4				−		
M2-4	I369L	+	++	−	41	No
M2-181	L375P	+	++	−	17	No
Class 5						
M2-17	Q307K	+	++	++	27	No

[a] PilQ multimer intensity as detected by SDS-PAGE Western blot analysis based on a minimum of four tests for each mutant (Fig. 1). ++, multimer band was consistently similar to that of the parental strain; +, multimer band was consistently detectable but the intensity was diminished compared to that of the parent strain; +/−, faint multimer band was observed on some blots but not on others; −, no multimer band was detected.

[b] See Fig. 5. +++, wild-type level of transformation; ++, high but decreased level of transformation efficiency compared to that of the parental strain; +, transformation was detectable but the transformation efficiency was greatly diminished; −, transformation efficiency was below the limit of detection.

[c] Efficiency of adherence to ME180 cells (Fig. 6). ++, adherence efficiency similar to that of the parental strain; +, intermediate level of adherence efficiency, significantly less than that of the parental strain yet higher than that of the PilQ null mutant; −, adherence efficiency not distinct from that of the PilQ null mutant.

[d] Piliation status as detected by immunogold TEM (Fig. 3). The values were determined as follows: number of pilus bundles detected/total number of gonococci observed × 100%.

[e] A mutation was transformed into an IPTG-regulatable *pilT* background described previously (40), and the presence of a growth defect during the down-regulation of *pilT* was assessed.

performed with these mutants. Collectively, these 19 *pilQ* mutants had substitutions in 15 residues. The most N-terminal substitution was Q307K, and the most C-terminal substitution was K490E. At 12 residues there was a single substitution (Q307K, V332D, I369L, L375P, L402S, L436P, L444P, D448V, V477D, E478K, S484P, and K490E), at two residues there were two different substitutions (D438Vand D438G and I472N and I472T), and at one residue there were three substitutions (D464G, D464Y, and D464N) (Fig. 2B and Table 1).

A Western blot analysis of PilQ expression and multimer stability in these 19 mutants was performed, but for mutants with a detectable multimer form the amounts in experimental repeats were variable (data not shown). A representative blot is shown in Fig. 1, and a summary of the results for at least four different blots is reported below and shown in Table 1. The four mutants with the most N-terminal mutations (Q307K, V332D, I369L, and L375P) consistently showed the same reactive bands as the parent strain, with the stable PilQ monomer at approximately 80 kDa, detectable yet diminished levels of an SDS-resistant multimer in the well too large to enter the gel, and immunoreactive degradative products that migrated faster than the PilQ monomer migrated (Fig. 1). One mutant (D438V) consistently produced detectable multimer and monomer bands, but the levels of degradative products were greatly diminished relative to those of the parent strain (Fig. 1). Six mutants (I472N, I472T, V477D, E478K, S484P, and K490E) produced monomer bands that were consistently more intense than those of the parent strain, and multimer bands were undetectable. These six mutants had immunoreactive degradative products similar to those of the parent strain (Fig. 1). The remaining eight mutants (L402S, L436P, D438G, L444P, D448V, D464G, D464Y, and D464N) produced PilQ monomer bands that were consistently more intense than those of the parent strain, yet they produced multimer bands that were either very faint or undetectable (the detectability varied from blot to blot). These eight mutants also had greatly diminished or undetectable levels of immunoreactive degradative products (Fig. 1). These results demonstrate that while all 19

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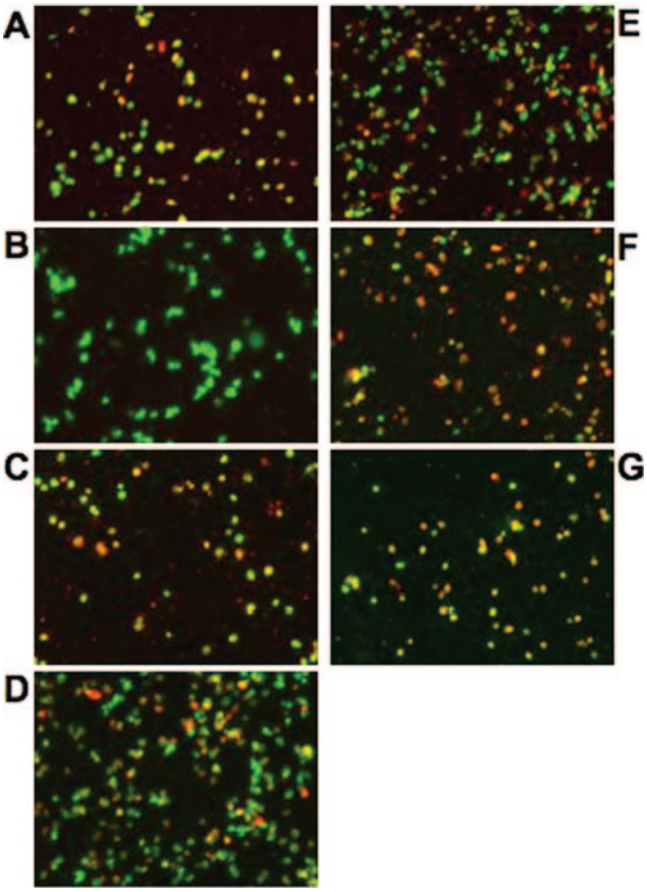

FIG. 4. Detection of surface-exposed PilQ by immunofluorescence. All 19 *pilQ* missense mutants were checked for surface-exposed PilQ and compared to the parent strain and the null mutant. All gonococci fluoresced green due to CFDA-SE treatment. PilQ reactivity is indicated by red. (A) Parent strain. Note that some cells are completely covered with PilQ reactivity, other cells have no PilQ reactivity, and some cells have intermediate levels of PilQ reactivity. This phenotype, in which various amounts and different patterns of PilQ reactivity are observed on individual cells of a single strain, was seen in all the *pilQ* missense mutants examined in this study. (B) *pilQ*::*cat* mutant. No PilQ reactivity was detected. (C to G) Representative *pilQ* missense mutants belonging to the five phenotypic classes. (C) D464N, a class 1 null mutant. (D) S484P, a class 2 minimally functional mutant. (E) E478K, a class 3 moderately functional mutant. (F) I369L, a class 4 differentially functional mutant. (G) Q307K, the class 5 highly functional mutant.

pilQ missense mutants expressed full-length PilQ monomer, they varied in terms of their degradative products and the formation of SDS-resistant PilQ multimers.

PilQ is surface exposed in missense mutants. Since all of the *pilQ* missense mutants expressed the full-length monomer subunit and some of them expressed detectable levels of an SDS-resistant multimer, immunofluorescence microscopy was performed with all 19 mutants to determine whether the mutant PilQ was surface exposed. All *pilQ* missense mutants had detectable levels of surface-exposed PilQ, while the *pilQ*::*cat* null mutant showed no PilQ reactivity (Fig. 4). It is noteworthy that for the parent strain, as well as for all 19 missense mutants, some cells had no PilQ reactivity, some cells were totally covered with PilQ reactivity, and some cells had punctate areas of PilQ reactivity (Fig. 4). These results demonstrate that the diminished pilus expression and altered pilus-related events observed for the missense mutants were not due to interference with the ability of PilQ to localize to the OM or to be surface exposed.

***pilQ* missense mutants secrete various levels of pili.** Immunogold TEM was used to determine the ability of each of the 19 P^- *pilQ* missense mutants to express pili. It has been shown previously that pili secreted by *N. gonorrhoeae* variant RM11.2 are normally observed in medium-size bundles by TEM (21). Because of this pilus bundling and the variability that can occur with TEM analysis, precise quantification of piliation is not possible. However, the number of observed pili and pilus bundles per bacterial cell can be determined, and a relative level of piliation can be assigned based on the observations. TEM analysis of the parent strain revealed 35 detectable pilus bundles for every 100 cells observed (Table 1 and Fig. 3). Therefore, 35% is level of piliation for the parent strain. As expected, no pili (either individually or in bundles) were detected in the *pilQ* null mutant when 200 cells were observed; therefore, the observed level of piliation for the *pilQ* null mutant is <0.05% (Table 1 and Fig. 3). Consistent with the P^- colony morphology, 12 mutants (V332D, L402S, L436P, L444P, L448V, D464G, D464Y, D464N, I472N, I472T, V477D, and S484P) expressed no detectable pili or pilus bundles, as shown in Fig. 3E. Interestingly, seven mutants did express detectable levels of pili, as shown in Fig. 3A to D. All pili observed were gathered in pilus bundles whose sizes were similar to the size observed for the parent strain (Table 1 and Fig. 3). Four mutants (D438V, D438G, E478K, and K490E) had greatly reduced levels of piliation compared to the parent strain, with values ranging from 0.91 to 2.6% (Fig. 3C and D). These results show that missense mutations in *pilQ* can block pilus secretion, as previously determined for the *pilQ2* allele (44). Of the seven mutants in which pili were detected, three (Q307K, I369L, and L375P) surprisingly displayed levels of piliation similar to that of the parent strain (range 17 to 41%) (Fig. 3A and B). These results demonstrate that a P^- colony morphology can result from *pilQ* mutants that still express parental and near-parental levels of pili. The fact that all pili observed in these mutants (even mutants with very low levels of pilus expression), which had identical *pilE* sequences, were bundled like those in the parental strain supports previous findings indicating that the *pilE* sequence, and not PilQ, determines pilus bundle formation (21).

***pilQ* missense mutants with no detectable pili have growth defects in a *pilT* background.** *N. gonorrhoeae pilQ* null mutants with a regulatable *pilT* background have severe growth defects when PilT is depleted compared to the growth of a *pilT* or *pilQ* single mutant (42). To determine whether the *pilQ* missense mutations used in this study had a similar effect in a *pilT* mutant background, all 19 *pilQ* missense mutations were transformed into a strain with an IPTG-inducible *pilT* gene (40). All of the *pilQ* mutants with no detectable pili as determined by TEM had a growth defect when PilT was not expressed, and this defect was rescued in the presence of IPTG (Table 1 and data not shown). Conversely, all of the strains with detectable levels of pili, even very low levels, grew like the parental strain when PilT was depleted (Table 1). This finding shows that *pilQ* missense mutants with no detectable pilus secretion react to

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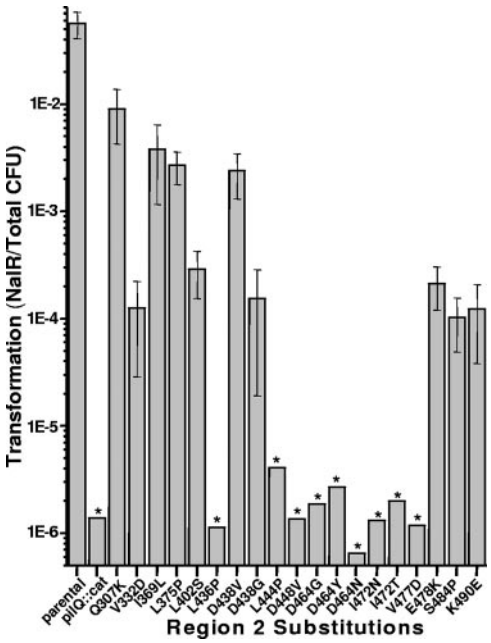

FIG. 5. Transformation efficiencies of *pilQ* mutants. Data for all 19 *pilQ* missense mutants, the parental strain, and *pilQ* null mutant are shown. The values were obtained by dividing the number of Nal^r CFU by the total number of CFU. An asterisk indicates that the transformation efficiency was below the limit of detection. The transformation efficiencies of all mutants were statistically different from that of the parental strain ($P < 0.01$, as determined by Student's *t* test). A statistical analysis of the *pilQ* null mutant was not performed since all values for this mutant were below the limit of detection.

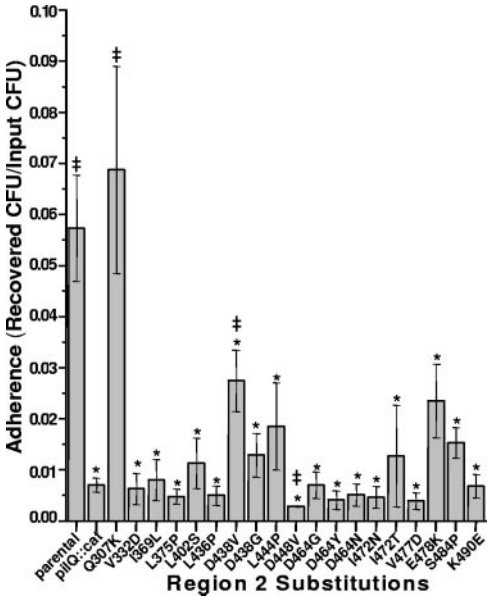

FIG. 6. Adherence of *pilQ* mutants to ME180 cells. Data for all 19 *pilQ* missense mutants, the parental strain, and the *pilQ* null mutant are shown. The values were obtained by dividing the number of CFU recovered after 1 h of incubation with ME180 cells by the number of CFU initially added. An asterisk indicates that the *P* value is <0.02 for a comparison with the parental strain. A double dagger indicates that the *P* value is <0.03 for a comparison with the *pilQ*::*cat* mutant. The adherence of only one mutant, Q307K, was statistically similar to the adherence of the parental strain. The levels of adherence of the D438V and D444V mutants were statistically different from the levels of adherence of both the parent strain and the null mutant; these mutants had intermediate and ultralow adherence efficiencies, respectively. The other 16 missense mutants had adherence levels that were statistically similar to that of the null mutant.

PilT depletion like the *pilQ* null mutant reacts. Furthermore, we concluded that with this collection of *pilQ* missense mutants, any detectable pilus secretion is sufficient to rescue *pilQ pilT* double mutants from such growth defects (42).

***pilQ* missense mutants have a wide range of transformation efficiencies.** The DNA transformation efficiency was determined for all 19 missense mutants, along with the wild-type parent strain and the *pilQ*::*cat* null mutant. As seen previously with *N. gonorrhoeae* (44), the difference between the level of acquisition of a Nal^r marker by a wild-type strain and the level of acquisition of a Nal^r marker by a *pilQ* null mutant was several orders of magnitude (Fig. 5). For four mutants (Q307K, I369L, L375P, and D438V) the transformation levels approached that of the parental strain, and all but one of these four mutants (D438V) secreted levels of pili that were near wild-type levels (Fig. 5 and Table 1). For six mutants (V332D, L402S, D438G, E487K, S484P, and K490E) the transformation levels were diminished yet detectable; three of these mutants expressed reduced levels of pili (D438G, E478K, and K490E), while the other three displayed no detectable pili (V332D, L402S, and S484P) (Fig. 5 and Table 1). For the remaining nine mutants (L436P, L444P, D448V, D464G, D464Y, D464N, I472N, I472T, and V477D) the transformation efficiencies were below the detection limit, similar to the transformation efficiency of the *pilQ* null mutant. None of these nine mutants expressed pili (Fig. 5 and Table 1). It is notable that all but one amino acid substitution that abolished transformation were in a single region spanning residues 444 to 477 (Fig. 5B). While all *pilQ* mutants with detectable pili were able to undergo transformation, not all transformable *pilQ* mutants expressed pili, an observation made previously (22, 44). This observation supports the current hypothesis that transformation is not dependent upon the presence of fully formed pili as much as it is dependent upon the presence of functional pilus assembly apparatuses and suggests that the role of PilQ in the formation of the pilus assembly apparatus is less stringent for transformation than for expression of the pilus.

***pilQ* missense mutants show different levels of adherence to human endocervical epithelial cells.** The ability of all missense mutants to adhere to ME180 cells was also tested. There was an approximately eightfold difference between the adherence efficiency of the parental strain and the adherence efficiency of the *pilQ* null mutant (Fig. 6). Eighteen of the 19 missense mutants had greatly diminished adherence efficiencies compared to that of the parent strain. One mutant (D438V) had an intermediate phenotype, and its adherence efficiency was significantly lower than that of the parent strain yet statistically higher than that of the *pilQ* null mutant (Fig. 6). Only one missense mutant (Q307K) had an adherence level similar to that of the parental strain. This mutant also exhibited high levels of DNA transformation and displayed secreted pili like the parent strain. These adherence data show that *N. gonorrhoeae* mutants with no pili or with low levels of detectable pili

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are deficient in adherence to host epithelium, confirming well-established previous results showing that expression of pili is necessary to mediate adherence (20). However, the fact that two of these missense mutants (I369L and L375P) adhered like the *pilQ* null mutant yet displayed a level of piliation similar to that of the parent strain shows that pilus secretion is not sufficient to mediate adherence and that the PilQ requirement for adherence is not solely at the level of pilus secretion.

DISCUSSION

In this directed genetic screen we generated 19 unique missense mutants, each of which had a P^- colony morphology. The P^- mutants had a variety of pilus-associated phenotypes, which allowed assignment to five phenotypic classes based on pilus secretion, transformation efficiency, and epithelial cell adherence. PilQ multimer stability in SDS-PAGE and PilQ surface exposure were also examined to determine whether PilQ processing or transport to the OM were affected by the mutations or involved in the effects on piliation and pilus functions observed.

There are nine class 1 mutants, which have no detectable pili, exhibit no detectable transformation, exhibit no pilus-mediated adherence, and thus are classified as *pilQ* null mutants (Fig. 2B and Table 1). The substitutions in this class are L436P, L444P, D448V, D464G, D464Y, D464N, I472N, I472T, and V477D. None of these substitutions is conservative. There are two leucine-to-proline substitutions, which is noteworthy because the introduction of proline residues is known to disrupt β-strands, which are important for proper functioning of OM-spanning proteins (7). It is also noteworthy that three mutants with substitution of the aspartic acid at residue 464 were isolated and all have similar phenotypes. Additionally, two mutants with substitution of the isoleucine at residue 472 were isolated and have similar phenotypes (Table 1). No class 1 mutant consistently produced SDS-stable PilQ multimers, even though the mutants displayed surface-exposed PilQ (Fig. 1 and 4). All of the class 1 substitutions are clustered in the C-terminal domain of region 2, which is hypothesized to be involved in PilQ multimer formation (3, 4); hence, the most probable explanation for the class 1 mutant phenotype is that PilQ is transported to the outer membrane but do not form stable dodecamers and thus do not form a functional pilus apparatus.

There are three class 2 mutants, which do not secrete pili, exhibit low yet detectable levels of transformation, adhere like the *pilQ* null mutant, and are classified as minimally functional mutants. The class 2 substitutions span nearly the length of region 2 (V332D, L402S, and S484P) (Fig. 2B and Table 1). Like the substitutions in the class 1 mutants, none of these substitutions are conservative, and the PilQ protein is surface exposed (Fig. 4). One mutant (V332D) has a high level of a detectable SDS-stable multimer, one mutant (L402S) has a greatly diminished level of an SDS-stable multimer compared to the parent strain, and one mutant (S484P) has no detectable SDS-stable multimer (Fig. 1). By isolating transformable mutants with no detectable pili yet finding no mutants with pili that do not transform, we confirmed previous work that suggested that the pilus assembly apparatus can still mediate transformation even without extended pili (22, 44). We predict that the class 2 mutant proteins form a minimally functional secretin structure and that these mutant proteins either allow transformation of a small subset of cells or form a partially functional assembly apparatus in all cells. Regardless of which possibility is correct, the class 2 mutant PilQ proteins do not complete a functional pilus assembly apparatus and thus do not secrete pili; thus, the proteins do not facilitate adherence.

There are four class 3 mutants, which display detectable yet reduced levels of pili, have intermediate levels of transformation, exhibit little or no adherence, and therefore are classified as moderately functional mutants. The amino acid substitutions in the class 3 mutants are D438V, D438G, E478K, and K490E (Fig. 2B and Table 1). The D438V substitution results in a higher transformation efficiency and statistically higher level of adherence than D438G, although both transformation and adherence are still significantly reduced compared to the transformation and adherence of the parent strain. Only one class 3 mutant (D438V) produces consistently detectable levels of an SDS-resistant multimer, although the level is lower than that of the parent strain (Fig. 1).

It is likely that the class 3 mutants form an assembly apparatus that is functional to the point of allowing transformation and some level of pilus expression, yet not sufficient for proper adherence. The decreased adherence may be the result of a structural defect in the pili secreted by the mutant PilQ; alternatively, the reduced number pili secreted by these *pilQ* mutants may be insufficient for pilus-mediated adherence. The fact that the moderately functional class 3 substitutions are clustered in the same C-terminal domain of region 2 as the substitutions of the class 1 null mutants suggests that either the residues affected in class 3 mutants are not as critical as those in class 1 mutants for the formation of a functional apparatus or that the amino acid substitutions in class 3 mutants retain partial functionality compared to the class 1 amino acid substitutions.

There are two class 4 mutants, which display high levels of pili and high, albeit less than parental, levels of transformation, yet adhere to ME180 cells with an efficiency that is similar to that of the *pilQ* null mutant. The two mutants have similar high levels of an SDS-resistant multimer (Fig. 1). Because of these opposite effects on transformation and piliation relative to adherence efficiency, these mutants are classified as differentially functional mutants. The substitutions are I369L and L375P (Fig. 2B and Table 1). Although the two mutations result in similar phenotypes, one substitution is highly conserved (I369L), while the other is nonconservative (L375P). The I369L mutant displays secreted pili at a level that is approximately twofold higher than the level observed for the L375 mutant (41 and 17%, respectively); however, both mutants are more similar to the parent strain (35%) than to the mutant with the next highest level of pilus secretion (D438V [2.6%]). These data confirm previous studies demonstrating that *Neisseria* pilus expression is necessary, but not sufficient, for adherence (27, 39). However, this is the first time that PilQ has been directly implicated in adherence.

There are two hypotheses that explain why these differentially functional mutants express high levels of pili but exhibit low levels of adherence. The first hypothesis is that the mutant PilQ secretes pili that are not functional for mediating adherence, which suggests that the isoleucine at residue 369 and the

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leucine at residue 375 are somehow involved in the secretion of adherence-facilitating pili. The second hypothesis is that there is no defect in the pili secreted by the mutant PilQ; instead, PilQ plays a direct role in adherence, and the mutations disrupt this role. Either way, these phenotypes suggest that the two residues substituted in class 4 mutants are involved in interactions between PilQ and another component required for adherence to host epithelium, but we cannot differentiate between the two hypotheses at this time.

Class 5 consists of a single mutant with the substitution Q307K (Fig. 2B and Table 1). This mutant is classified as a functional mutant and is similar to the parental strain in terms of piliation and adherence. Although this mutant transforms better than any of the *pilQ* mutants examined in this study, the transformation efficiency is still significantly lower than that of the parent strain. By isolating mutants that display high levels of pili yet still have a P^- colony morphology, we demonstrated that factors in addition to parental levels of pilus expression are involved in colony morphology.

It is likely that this *pilQ* mutant forms a pilus assembly apparatus that is not as stable as the parent apparatus, yet is functionally adequate to secrete pili, allowing parental levels of adherence and near-parental levels of transformation. The data also suggest that the glutamine at residue 307, the most N-terminal residue substituted in this study, is involved in some aspect of *Neisseria* physiology that affects colony morphology, since the Q307K mutant still has a nonpiliated colony morphology compared to the parental strain. As stated above, the differences in piliation, pilus function, and colony morphology are not the result of pilin antigenic variation, as the *pilE* sequence of all missense mutants was shown to be identical to that of the parent strain.

In sum, we identified 15 PilQ residues in *N. gonorrhoeae* that, when replaced, alter colony morphology. The replacements have diverse effects on PilQ multimer stability, secretion of pili, DNA transformation, and adherence to host epithelium. Since PilQ is surface exposed in all of our missense mutants, the altered function is not a result of defective transport to the OM. The fact that mutants with no stable PilQ multimer still had surface-exposed PilQ indicates that formation of SDS-stable multimers is not required for PilQ mobilization to the OM. Furthermore, we demonstrated that the oligomer status of PilQ as determined by SDS-PAGE does not necessarily predict the biological activity of a given mutant, a concept that has been suggested previously for *N. gonorrhoeae* (22, 44), although not based on a collection of *pilQ* mutants of this size.

The observations made using a collection of *pilQ* missense mutants in this study clearly indicate that gonococcal PilQ may be directly involved in pilus-dependent functions and is not simply a passive pore in the OM. Whether this is because the pilus that is expressed from the mutant secretin is altered via direct interactions between the mature pilus and PilQ, which has been suggested previously (9), or because PilQ directly interacts with other components involved in transformation and adherence has not been determined yet. Based on the results of this study, additional mutagenesis of the other two regions of *pilQ* and additional analysis of some of the region 2 mutants obtained are warranted in order to further understand the role of PilQ in the biology of the Tfp and possibly the pathogenesis of *N. gonorrhoeae*.

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