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Mutation of the Conserved Calcium-Binding Motif in *Neisseria gonorrhoeae* PilC1 Impacts Adhesion but Not Piliation

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Neisseria gonorrhoeae* PilC1 is a member of the PilC family of type IV pilus-associated adhesins found in *Neisseria* species and other type IV pilus-producing genera. Previously, a calcium-binding domain was described in the C-terminal domains of PilY1 of *Pseudomonas aeruginosa* and in PilC1 and PilC2 of *Kingella kingae*. Genetic analysis of *N. gonorrhoeae* revealed a similar calcium-binding motif in PilC1. To evaluate the potential significance of this calcium-binding region in *N. gonorrhoeae*, we produced recombinant full-length PilC1 and a PilC1 C-terminal domain fragment. We show that, while alterations of the calcium-binding motif disrupted the ability of PilC1 to bind calcium, they did not grossly affect the secondary structure of the protein. Furthermore, we demonstrate that both full-length wild-type PilC1 and full-length calcium-binding-deficient PilC1 inhibited gonococcal adherence to cultured human cervical epithelial cells, unlike the truncated PilC1 C-terminal domain. Similar to PilC1 in *K. kingae*, but in contrast to the calcium-binding mutant of *P. aeruginosa* PilY1, an equivalent mutation in *N. gonorrhoeae* PilC1 produced normal amounts of pili. However, the *N. gonorrhoeae* PilC1 calcium-binding mutant still had partial defects in gonococcal adhesion to ME180 cells and genetic transformation, which are both essential virulence factors in this human pathogen. Thus, we conclude that calcium binding to PilC1 plays a critical role in pilus function in *N. gonorrhoeae*.

Neisseria gonorrhoeae, a Gram-negative diplococcus, is the causative agent of the sexually transmitted disease gonorrhea. According to the CDC, there were more than 300,000 U.S. cases of gonorrhea reported in 2011 (1); however, estimates are that the number of infections is more likely to be around 870,000 per year (2). Reports of gonococcal antibiotic resistance have led the Centers for Disease Control and Prevention to drop their recommendation of cefixime as a first-line regimen in favor of more complicated combination therapies (3). This makes it increasingly clear that development of novel antimicrobial drugs is a necessity and will require a fundamental understanding of all facets of *N. gonorrhoeae* infection.

It has been a challenge to develop an effective vaccine targeting *N. gonorrhoeae* because of its highly variable surface components. In addition, this pathogen manipulates the host's immune defenses by evading the adaptive immune response and recruiting neutrophils to serve as a protective niche for further colonization (4–7). One mechanism that *N. gonorrhoeae* uses during early colonization is adherence to human cervical epithelial cells prior to the formation of microcolonies on the cell surface (8). Adhesion to epithelial cells appears to be mediated by a tip-associated type IV pilus (T4P) adhesin, PilC, which is also necessary for pilus extension (9). After initial interaction with the cell, the gonococcal pilus undergoes retraction by the action of the ATPase PilT, which positions the bacterium near the cell surface, allowing subsequent interactions necessary for colonization to occur (8).

The 110-kDa *N. gonorrhoeae* PilC protein was first identified as a high-molecular-weight contaminant in highly purified pilus preparations (10). All pathogenic *Neisseria* genomes completed to date contain two similar *pilC* genes, *pilC1* and *pilC2*, with the greatest homology being in the C-terminal (CT) domains (11). PilC1 purified from *N. gonorrhoeae* competitively blocks bacterial adherence to human epithelial cells, lending credence to its important role in mediating adherence (12–14). In *Neisseria meningitidis*, the N-terminal region of PilC1 is responsible for the interaction between the bacteria and the host cell receptor (11). It has been reported that the initial adherence of *N. gonorrhoeae* to human cells is facilitated by the human complement cell receptor CD46 (15, 16); however, there is conflicting evidence that attachment is CD46 independent (12, 17). Preliminary evidence also suggests that CD147 may be the cellular receptor for the meningococcal pilus (18).

The reason for the redundancy of the PilC proteins in the gonococcal genome is unclear, and it remains to be determined whether PilC1 and PilC2 proteins play separate roles during infection. For the purpose of these studies, we will refer to NGO0055 as *pilC1* and NGO1912 as *pilC2* because they are not identified as *pilC1* and *pilC2* in the FA1090 genome. This name assignment was based on the shared synteny of the loci when the completed *Neisseria* genomes are compared. Both *pilC1* and *pilC2* genes can undergo phase variation by slipped-strand mispairing of the ho-

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mopolymeric "G" tract in the sequence encoding the signal peptide, controlling the expression of the corresponding PilC protein (10, 19). This further complicates the study of PilC proteins but may provide a mechanism for selective expression of either PilC1 or PilC2 or both during pathogenesis. Because phase variation of PilC would profoundly affect the phenotypes associated with T4P, we have taken steps in this study to prevent phase variation of both PilC genes.

Type IV pili (T4P) are found in a broad range of Gram-negative bacterial species (see Tables S1 and S2 in the supplemental material) but are best studied in the *Neisseria*, *Pseudomonas*, and *Myxococcus* genera (20). *N. gonorrhoeae* T4P are polymeric structures predominately composed of an 18-kDa pilin protein, PilE (21). In addition to their role during adhesion to host cells, T4P are essential for several other bacterial functions, including DNA uptake (19, 20, 22). Pilin can evade immune recognition by undergoing antigenic variation, a process during which silent *pilS* loci undergo nonreciprocal homologous DNA exchange with the *pilE* locus (6, 23, 24). Antigenic variation of the pilus was perceived to be a complicating factor in our studies of the effects of calcium-binding defects on T4P-related phenotypes. We have therefore taken steps to ensure that all of the gonococcal strains used in this work have the same *pilE* sequence.

The function and biogenesis of the *P. aeruginosa* type IV pili are dependent on PilY1 (*Pseudomonas aeruginosa* PilC ortholog) binding calcium, leading researchers to hypothesize that calcium-bound and -unbound forms correspond to pilus extension and retraction, respectively (25). We have identified a similar calcium-binding domain in the C-terminal region of PilC1 and examined its role in *N. gonorrhoeae*. We describe a new expression and purification method for PilC1 that increased yield. Furthermore, we show that a gonococcal mutant with the D708A change in the *pilC1* gene [*pilC1*(D708A)] expressed a similar amount of pili on the surface to a bacterial strain expressing wild-type (wt) PilC1. Additionally, despite normal expression of pili on the surface, the gonococcal strain expressing the D708A mutation in PilC1 had a significantly reduced ability to adhere to ME180 cells and a partial defect in the ability to undergo DNA transformation. Thus, we conclude that these key pilus functions require PilC to be able to bind calcium.

MATERIALS AND METHODS

Protein constructs, expression, and purification. Standard ligation-independent cloning techniques, as described by Stols et al. (26), were used to construct the expression plasmids encoding the segments of PilC1. The *pilC1* gene encodes a 33-amino-acid signal peptide, which was omitted from all of our expression constructs. All residue numbers are based on the reading frame, excluding the signal peptide. Amplified DNA fragments encoding full-length (FL) PilC1 (residues 1 to 1014) and the C-terminal (CT) region of PilC1 (residues 496 to 1014) were treated and cloned into an empty pMCSG7-Lic-His expression vector. All expression plasmids were verified by sequence analysis.

Expression plasmids were transformed into *Escherichia coli* BL21(DE3) Origami 2 cells (Stratagene). Bacteria were grown in LB medium supplemented with ampicillin, streptomycin, and tetracycline at 37°C with shaking. After the optical density at 600 nm (OD_{600}) reached 0.6, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.2 mM, and bacteria were grown for another 12 h at 16°C with shaking. At the end of the expression period, the bacteria were harvested, pelleted, and stored at −80°C. Pelleted bacteria were thawed on ice and resuspended in lysis buffer (50 mM sodium phosphate [pH 7.6], 500 mM sodium chloride, 25 mM imidazole) supplemented with 0.5 mM EDTA, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), one tablet of a protease inhibitor cocktail (Roche), and 1 μg/ml lysozyme. After 1 h of gentle stirring on ice, cells were sonicated on ice for 1 min, and the lysate was centrifuged at 45,000 × *g* for 90 min at 4°C. By using an ÄKTAxpress fast protein liquid chromatography (FPLC) system (GE HealthCare), protein from the filtered soluble fraction was purified by Ni-nitrilotriacetic acid (NTA) affinity column chromatography. Recombinant His-PilC1 was eluted with buffer containing 50 mM sodium phosphate (pH 7.6), 500 mM sodium chloride, and 500 mM imidazole and further purified by filtration through a Superdex 200 gel column (GE Healthcare) equilibrated with 20 mM Tris-hydrochloride (pH 7.5), 250 mM sodium chloride, 2 mM dithiothreitol, and 5% glycerol. Protein-containing fractions were pooled, concentrated, flash-frozen in liquid nitrogen, and stored at −80°C. The purified protein had a final purity of at least 95%, as determined by SDS-PAGE.

TABLE 1 *N. gonorrhoeae* strains used in this study and their respective genotypes (expression phases)

Strain	*pilC1* locus[a,b]	*pilC2* locus[a]	*pilE* locus[c]
MS11 MKC	wt (off)	wt (on)	wt (on)
FA1090 A23	wt (on)	wt (off)	*pilE** (on)
FA7474	Δ*pilC1*	Δ*pilC2*	Δ*pilE*
FA7461	*pilC1* (PL on)	Δ*pilC2*	Δ*pilE*
FA7480	*pilC1* (PL on)	Δ*pilC2*	*pilE** (on)
FA7489	*pilC1* D708A (PL on)	Δ*pilC2*	wt (on)
FA7490	*pilC1* D710A (PL on)	Δ*pilC2*	wt (on)
FA7491	*pilC1* D716A (PL on)	Δ*pilC2*	wt (on)
FA7534	*pilC1* D708A (PL on)	Δ*pilC2*	*pilE** (on)

[a] *pilC1* and *pilC2* deletions remove the entire mature coding sequence.
[b] "PL on" refers to silent alteration of the third base in each codon of the poly(G) tract, preventing phase shifting and locking the expression state in the "on" phase without altering the expressed protein (13).
[c] *pilE** encodes the native PilE found in FA1090 A23.

Calcium-binding assay. Terbium ions were used as a substitute for calcium because of their similar ionic radius and coordination properties. Förster fluorescent resonance energy transfer (FRET) was carried out at 25°C using a SPEX Fluorolog-3 Research T-format spectrofluorometer (Horiba Jobin Yvon, Edison, NJ). The protein sample was excited at 283 nm, and the terbium fluorescence emission was recorded at 543 nm. The assay buffer contained 20 mM Tris-hydrochloride (pH 7.5) and 250 mM sodium chloride. Terbium chloride was added in 0.2-μl increments to cuvettes containing 400 μl of protein at a concentration of 0.2 mg/ml. The error bars represent the standard deviation of three replicates. The data were fitted to a one-site binding model.

CD spectroscopy. Far-UV circular dichroism (CD) spectra of wild-type C-terminal PilC1 and D708A, D710A, and D716A mutants were acquired by using a Chirascan spectropolarimeter (Applied Photophysics). CD spectra were obtained from 260 nm to 205 nm using a 1-mm-path-length cell at 22°C. The assay buffer contained 25 mM HEPES (pH 7.5) and 150 mM NaF. Thermal denaturation was analyzed by monitoring ellipticity changes at 216 nm, while the sample was heated from 10 to 80°C at a constant rate of 1°C every 2 min.

Cell lines. The human cervical epidermal cell line ME180 was grown in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. The cells were grown to approximately 90% confluence in 96-well cell culture plates (Corning) prior to use in the cell adherence assay.

Bacteria. All *N. gonorrhoeae* strains were grown overnight for 18 h on Difco GC medium base (GCB)-agar plates (BD) containing Kellogg's supplement I (27) at 37°C in a humidified 5% CO_2 atmosphere unless otherwise stated. The strains used in this study are described in Table 1. The strain intermediates, plasmids, and primers used to create these strains are described in Tables S3 to S5 in the supplemental material. In general, the changes in the *pilE*, *pilC1*, and *pilC2* loci were introduced sequentially by

using a modification of the method of Johnson and Cannon (28), always maintaining expression of *pilE* and one intact *pilC* gene until the final step. In strains such as FA7474 (Δ*pilE* Δ*pilC1* Δ*pilC2*) in which deletion of either *pilE* or the expressed *pilC* would yield a transformation-incompetent strain, the final strain was obtained by transformation with two cloned DNAs simultaneously to introduce both of the desired mutations. The changes introduced fell into two categories: deletions and alterations of expressed genes. The deletions of *pilC1* and *pilC2* removed the entire mature coding sequence, and the deletion in *pilE* removed 260 bp of unique conserved *pilE* sequence, including its promoter and 105 bp encoding its N terminus. The alterations introduced served three purposes: (i) the incorporation of silent changes to lock the expression phase of *pilC* and create novel restriction sites used in subsequent subcloning efforts, (ii) the alteration of the *pilC1* coding sequence to create the proposed calcium-binding mutants, and (iii) the restoration of a common PilE-coding sequence to make the strains more biologically comparable. Each of these alterations was introduced by a variation of the selectable/counterselectable marker system used previously (28). All of the silent changes were created with PCR primers containing the desired changes. The changes altering the coding sequence of the proposed calcium-binding domain were made by using the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies).

As the final stage of selection, FA7474, FA7480, and FA7534 were screened for Opa-nonexpressing isolates by performing whole-cell immunoblotting assays (29–32). Prior to these isolates' use in any of the biological experiments, each was sequenced to confirm the accuracy of their *pilE* and *pilC1* genes.

Cell adherence assay. Cell adherence assays were performed as previously described (12) by using the human epithelial ME180 cell line. In gonococcal adherence inhibition experiments, ME180 cells were preincubated with different concentrations of various recombinant proteins, followed by a 1-h incubation period with a multiplicity of infection (MOI) of 100 for each gonococcal strain at 37°C and 5% CO_2. The actual number of bacteria placed on the cells was determined by spreading serial dilutions of each strain on GCB agar plates. After 1 h, ME180 cells were washed three times with Hanks' balanced salt solution (HBSS) to remove the nonadherent bacteria and exposed to 1% saponin in GCB for 10 min. This lysate was pipetted vigorously and immediately diluted in GCB broth. CFU were quantified by spreading serial dilutions of bacteria on GCB agar plates. The percentage of gonococcal adherence was determined by dividing the number of bacterial remaining bound by the number of bacteria initially placed on the cells. In assays measuring the inhibition of gonococcal binding by recombinant protein, the percentage of bound bacteria at each concentration of each protein was compared to that of a replicate lacking any inhibitor. The percentage of inhibition was calculated by dividing the reduction in percentage of bacteria bound in the presence of inhibitor by the percentage of bacteria bound by the uninhibited replicate.

Transformation frequency. Using a method adapted from Biswas et al. (33), we grew gonococcal strains FA7474 (Δ*pilC1* Δ*pilC2* Δ*pilE*), FA7480 (PilC1$^+$), and FA7534 [PilC1(D708A)] overnight on GCB agar plates. Bacteria were resuspended in GCB broth supplemented with 5 mM $MgCl_2$ and supplement I (glucose). These suspensions were balanced by OD_{600}, and approximately 10^8 CFU in 100 μl was placed in each well of a 96-well microtiter plate (Falcon) with the indicated amount of plasmid pUNCH977 (either 100 ng/ml or 4 μg/ml). The plasmid pUNCH977 contains a rifampin-resistant allele of gonococcal *rpoB*(D543Y). The plate was incubated for 30 min at 37°C with 5% CO_2, followed by the addition of 1/10 volume of DNase I (Sigma). Approximately 4 or 40 Kunitz units of DNase I was used for those bacteria receiving 100 ng/ml and 4 μg/ml DNA, respectively. This represented more than a 100-fold excess when the incubation was continued for 10 min at 37°C. Thereafter, 10 μl of each mixture was diluted in 90 μl GC broth with supplement I, and the incubation continued for 4.5 h. Each strain was serially diluted and plated in triplicate on GCB plates and GCB plates supplemented with 5 μg/ml rifampin. The frequency of transformants was calculated by dividing the number of rifampin-resistant CFU by the total CFU obtained on GCB plates. The relative frequency was calculated in each experiment by dividing the frequency of transformants of each strain by the frequency of transformants of the positive-control strain (FA7480) so as to normalize all experiments for comparison.

Flow cytometry. The two monoclonal antibodies used to detect surface-exposed epitopes of PilC1 were derived from a BALB/c/J mouse immunized with denatured rPilC1. Monoclonal 4B5 recognized the conserved C-terminal, linear epitope WRExFx, as judged by enzyme-linked immunosorbent assay (ELISA) recognition of an alanine-substituted peptide family (data not shown). This epitope is conserved in most gonococcal and meningococcal PilC proteins. In contrast, the monoclonal antibody 1D5 recognized an N-terminal linear epitope found only in FA1090 PilC1 and only when presented in the context of an assembled pilus. The epitope of 1D5 was YAIImDExn, as determined by ELISA recognition of an alanine-substituted peptide family. (The lowercase letters indicate positions that caused a 30% reduction in peptide recognition when alanine was substituted [data not shown].) All gonococcal strains were grown overnight prior to being resuspended in GCB broth at the same optical density at 600 nm. Approximately 1×10^9 CFU were incubated in 100 μl of primary antibody (used at a dilution of 1:100) in a U-bottom 96-well plate (Corning) for 30 min at room temperature (RT). All samples were washed 3 times by centrifugation at 500 × *g* with GCB broth. Each sample was then incubated with a 1:1,000 dilution of biotin-labeled anti-mouse IgG (Jackson ImmunoResearch) for 30 min at RT prior to washing. Finally, streptavidin-phycoerythrin (PE) (Sigma) was used at a 1:1,000 dilution for an incubation period of 30 min at RT in the dark. After the final wash step, each sample was resuspended in GCB broth prior to fixation with 2% paraformaldehyde for 20 min at RT in the dark. Each sample was then analyzed in an Accuri C6 (BD Biosciences) fluorescence cytometer.

Preparation of sheared pili. The sheared-pilus preparations were collected according to the protocol of Craig et al. using the modification of Blais et al. (34, 35). Briefly, gonococcal strains FA7474 (Δ*pilC1* Δ*pilC2* Δ*pilE*), FA7480 (PilC1$^+$ Δ*pilC2* PilE$^+$), and FA7534 [Δ*pilC2* PilC1(D708A) PilE$^+$] were grown overnight on GCB agar plates prior to resuspension in 1 ml of cold 50 mM CHES (2-cyclohexylaminoethanesulfonic acid [pH 9.6]). Each suspension was then vortexed for 2 min and then immediately centrifuged at 16,000 × *g* for 10 min at RT. The cell pellets were kept on ice, solubilized in 1% sodium dodecyl sulfate (SDS), and quantitated using a bicinchoninic acid (BCA) assay (Thermo Scientific). Samples were balanced according to the amount of protein in the cell pellet and run on 4 to 12% SDS-PAGE (Invitrogen) and transferred to nitrocellulose. For quantitation of PilE in the sheared-pilus supernatant, Western blots were probed using a rabbit polyclonal antibody (NC809; 1:1,000 dilution) made to the peptide epitope of PilE (KSAVTGYYLNH GIWPADNGA) from FA1090 A23 or a 1:500 dilution of monoclonal antibody 4B5 (PilC). Antibody binding was detected using either horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (dilution, 1:10,000; GE Healthcare) or 1:10,000 HRP-conjugated goat anti-mouse IgG (Sigma) and Western Lightning Plus-ECL enhanced chemiluminescence substrate (PerkinElmer). The amounts of PilE, PilC, and porin in the cell pellet were quantitated similarly, with the porin detected using a 1:500 dilution of monoclonal antibody H5 (against FA1090 PorB). The results were visualized by using the FluorChem E imaging system (ProteinSimple) and quantitated by using Image J software.

Scanning electron microscopy. Each gonococcal strain was grown overnight on GCB agar plates prior to being resuspended in GCB medium with supplement I at a concentration of 5×10^7 CFU/ml. Poly-L-lysine-coated coverslips (Becton Dickenson) were placed in the bottom of a 12-well tissue culture-treated plate (Falcon), to which 2 ml of each suspension was added. The gonococci were allowed to gently settle onto the coverslips for 2 h in an incubator at 37°C with 5% CO_2 prior to one wash with filtered-sterilized phosphate-buffered saline (PBS). Thereafter, the culture was gently fixed with fresh 2.5% glutaraldehyde (Sigma) in filter-sterilized PBS for 24 h. After glutaraldehyde fixation, the coverslips were

processed at the Microscopy Services Laboratory at the University of North Carolina, Chapel Hill. Each coverslip was washed 3 times with filter-sterilized PBS and stained with 1% osmium tetroxide in PBS for 15 min. Coverslips were then washed 3 times with filter-sterilized distilled deionized water (dIH_2O). To ensure optimal staining with osmium tetroxide (Sigma), fresh, filter-sterilized 1% tannic acid (Sigma) in dIH_2O was used to stain the coverslips for 10 min, followed by 3 washes with filter-sterilized dIH_2O. The final stain was fresh, filter-sterilized 1% uranyl acetate and was used to stabilize the pili during scanning electron micoscopy processing. After a 15-min incubation with 1% uranyl acetate, 3 washes were done with filter-sterilized dIH_2O. The coverslips were dehydrated in subsequent incubations in 30%, 50%, 75%, and 90% ethanol and twice with freshly opened 100% ethanol for 10 min each. The coverslips were then dried in a critical point dryer (Samdri 795), mounted, and sputter coated with gold-palladium for 100 s. Samples were visualized in a Zeiss Supra 25 FESEM at magnifications of 1,000×, 25,000×, 75,000×, and 100,000×.

rPilC1 binding to whole cells by ELISA. ME180 cells were grown overnight to approximately 90% confluence, rinsed once in fresh medium, and incubated at 37°C with 5% CO_2 with dilutions of rPilC1 in medium. After 30 min, unbound protein was washed 3 times from cells with PBS before incubation for 30 min with PilC monoclonal antibody 4B5 (which recognizes the C-terminal conserved epitope) in PBS with 1% bovine serum albumin (BSA) added. Unbound antibody was washed away as before and detected with a 1:10,000 dilution of goat anti-mouse IgG-horseradish peroxidase conjugate (Sigma). ELISA reactivity was visualized with the Pierce 1-Step Ultra TMB-ELISA (TMB is 3,3′,5,5′-tetramethylbenzidine) and stopped with 1 M phosphoric acid. Optical densities at 450 nm were corrected for differences in monoclonal antibody recognition of the different protein preparations.

RESULTS

***Neisseria gonorrhoeae* adhesin PilC contains a conserved calcium-binding motif.** Published studies have identified a calcium-binding domain in the *Pseudomonas aeruginosa* protein PilY1, which shares a high degree of homology with the PilC protein from *N. gonorrhoeae* (25). Sequence alignments indicate that *N. gonorrhoeae* PilC1 also has a calcium-binding domain in its C-terminal (CT) domain and that this binding motif is conserved in more than 100 PilC homologs (see Table S1 in the supplemental material), including those of neisserial species, *Kingella kingae*, and other *Betaproteobacteria* (Fig. 1). On the basis of the crystal structure of the CT of *P. aeruginosa* PilY1, Asp-851, Asn-853, Asp-855, and Asp-859 were identified as being Ca-chelating residues (25). Those residues are conserved in the CT of *N. gonorrhoeae* PilC1 and correspond to Asp-708, Asp-710, Asp-712, and Asp-716, respectively, in this protein (Fig. 1). Therefore, we hypothesized that *N. gonorrhoeae* PilC1 function would be impacted by calcium binding.

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                                      708 710 712 716
                             701        \ | |   /        722
PilC1/2  N. gonorrhoeae          LSSPTLVDKDLDGTVDIAYAGD
PilC1/2  N. meningitidis         LSSPTLVDKDLDGTVDIAYAGD
PilC1/2  N. lactamica            LSSPTLVDTDLDGTVDIAYAGD
PilC1    N. polysaccharea        LSSPTLVDTDLDGTVDIAYAGD
PilC1    N. cinerea              LSSPTLVDTDFDGTVDIAYAGD
PilC1    N. subflava             LSTPTLVDTDFDGIVDIAYAGD
PilC1    Kingella kingae         LASPTLLDVNRDGVYDFVFAGD
PilY1    Pseudomonas aeruginosa  LSSPRLADNNSDGVADYAVAGD
                                 *  * * *   **  *   ***
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FIG 1 Partial sequence alignment of calcium-binding motifs in PilC/Y1 orthologues from T4P-containing species. The numbers correspond to positions in mature PilC1 *N. gonorrhoeae* FA1090. Previously, the PilY1 protein from *P. aeruginosa* had been shown to have a set of 3 aspartic acid residues in the C terminus that together with a asparagine and a valine form a calcium-binding domain (25). Here we show that this conserved calcium-binding domain is found in *N. gonorrhoeae* and several other T4P-producing species. Underlined and boldface residues correspond to those predicted to be important to the calcium-binding domain. The predicted amino acid sequence in this region is highly conserved, with only a few conservative replacements across all of the available gonococcal (17 sequences) and meningococcal (80 sequences) PilC sequences. Positions that are 100% conserved are indicated by an asterisk at the bottom of the alignment.

Purification, expression, and function of native FA1090 full-length recombinant PilC1 (FL rPilC1) and the C-terminal domain of PilC1 (CT rPilC1). To identify the fragments of PilC1 proteins that could be expressed as soluble proteins in *E. coli*, we tested a series of PilC1 constructs in several *E. coli* cell lines (data not shown). We found that full-length PilC1 (FL rPilC1) (residues 1 to 1014) and the C-terminal domain (CT rPilC1) (residues 497 to 1014) could be expressed as soluble proteins in *E. coli* BL21(DE3) Origami 2 cells. SDS-PAGE showed that the purity of purified FL rPilC1 and CT rPilC1 was over 95% (Fig. 2A). The expression level of CT rPilC1was considerably higher than that of FL rPilC1. Similarly, the maximum soluble concentration for CT rPilC1 was much higher (>10 mg/ml) than that of FL rPilC1 (0.76 mg/ml). To examine the disulfide bond formation of FL rPilC1 and CT rPilC1, we ran samples of each protein under reducing and nonreducing conditions in an SDS-PAGE (Fig. 2B) (data not shown). As expected, the nonreduced samples ran more slowly than their reduced counterparts, and rPilC1 mirrored the behavior of native PilC1 from gonococcal total membranes, indicating that we had produced proteins containing intramolecular disulfide bonds (Fig. 2B).

Previous studies showed that FL rPilC1, purified from *N. gonorrhoeae*, is capable of blocking the adherence of *N. gonorrhoeae* to human cells by competing with pilus-associated native PilC1 (14). Thus, we examined the impact of the recombinant proteins FL rPilC1 and CT rPilC1 on the adherence of *N. gonorrhoeae* strain FA1090 to the human cell line ME180. FL rPilC1 inhibited the adherence in a dose-dependent manner with a 50% inhibitory concentration (IC_{50}) of approximately 10 nM (Fig. 2C); this is similar to the concentrations observed with native gonococcus-expressed PilC (12). Our results also showed that CT rPilC1 did not affect the adherence of *N. gonorrhoeae* to ME180 cells (Fig. 2B), consistent with the observation that the N-terminal portion of neisserial PilC was associated with adherence (11). In accordance with a published report using natively purified protein (12), we found that heat-inactivated FL rPilC1 was unable to inhibit gonococcal binding (data not shown). Taken together, our results suggest that the recombinant FL rPilC1 obtained in this study is likely well folded and able to mimic *in vivo*-expressed PilC1.

Confirmation of the calcium-binding domain using C-terminal domain proteins. As we were able to successfully produce FL rPilC1 and CT rPilC proteins, we then sought to make recombinant PilC1 proteins with single substitutions of each aspartic acid in the calcium-binding domain (Table 1). The resulting D708A, D710A, and D716A proteins were purified to >95% purity (data not shown). Using the fluorescent calcium analog terbium, we showed that CT rPilC1 bound calcium with a dissociation constant (K_d) of 0.9 ± 0.2 μM, similar to those reported for *P. aeruginosa* PilY1 and *K. kingae* PilC1 (25, 36, 37) (Fig. 3A). Moreover, all three of the tested changes to the aspartic acid residues of the putative calcium-binding domain (D708A, D710A, and D716A) eliminated the ability of PilC1 to bind calcium (Fig. 3A).

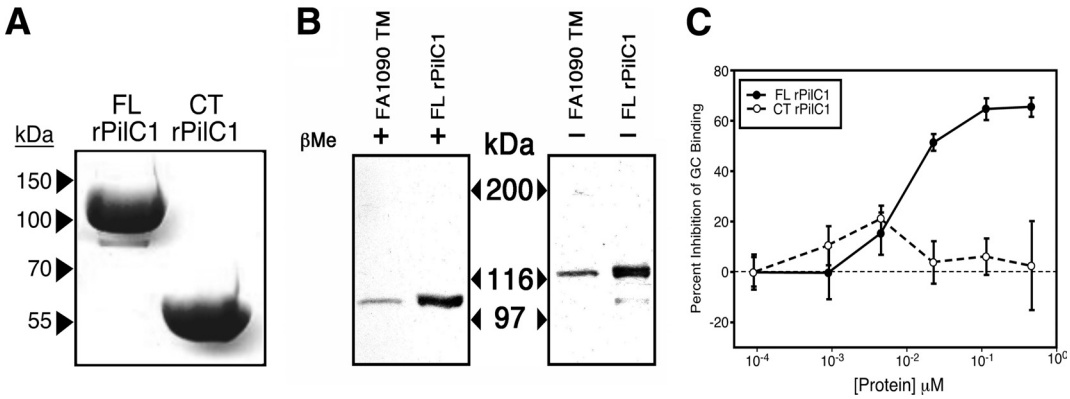

FIG 2 Recombinant FA1090 PilC1 purified as a soluble protein from *E. coli* retains properties of native PilC1. (A) Coomassie-stained gel with 5 μg of purified FA1090 rPilC1 full-length (FL; residues 1 to 1014) and C-terminal (CT; residues 497 to 1014) domain as soluble proteins from *E. coli* Origami 2 estimated to be 95% pure. (B) Western blot showing that PilC1 is reduction modifiable when expressed in *E. coli* Origami 2, as it is in *Neisseria gonorrhoeae*. Lanes 1 and 3 were loaded with 35 μg of FA1090 total membranes (TM), while lanes 2 and 4 were loaded with 1 μg of FL rPilC1 purified from *E. coli*. Lanes 1 and 2 were boiled in the presence of the reducing agent 5% β-mercaptoethanol (βMe), while lanes 3 and 4 were boiled in the absence of reducing agent. Shown beside each gel are the relative migration positions of protein molecular mass markers. (C) Purified full-length PilC1 protein inhibits the adherence of *Neisseria gonorrhoeae* gonococci (GC) to human epithelial cells in a dose-dependent manner. ME180 cells were preincubated with increasing concentrations of PilC1 proteins for 30 min before being washed and incubated with *N. gonorrhoeae* strain FA1090 in the cell adherence assay. The assay was performed in triplicate on 3 days. The error bars represent the standard error of the normalized fraction of adherent *N. gonorrhoeae*.

To rule out the possibility that those aspartic acid-to-alanine substitutions significantly disrupted the native structure of PilC, we examined the secondary structure of each protein by far-UV circular dichroism (CD) spectroscopy. No significant change in CD profiles was observed between PilC calcium-binding domain mutants and wt PilC. This indicated that the mutations did not significantly disrupt the structure of the protein (Fig. 3B).

We also investigated the thermostability of the proteins carrying the calcium-binding domain alterations compared to that of calcium-bound (and chelated) wt CT rPilC1. We found no significant difference in the melting temperature (T_m) between all forms of CT rPilC1 (Fig. 3C). These results further confirm that mutations to the PilC1 calcium-binding domain did not change the proteins' structure or stability. Thus, we have confirmed both that PilC1 binds calcium and that the residues D708, D710, and D716 are important to this binding.

Biological assays to determine the effect of a calcium-binding defect on the function of PilC1. To examine the role calcium plays in the function of PilC1 *in vivo*, we engineered several derivatives of *N. gonorrhoeae* strain FA1090 containing mutations altering the residues shown above to abrogate calcium binding. These strains contained silent alterations of the G-string encoding the signal peptide to prevent phase variation of their *pilC1* gene and deletions of their *pilC2* genes so that it could not phase vary to an "on" phase (Table 1). Western blot analysis of whole-cell lysates showed that FA7489 [PilC1(D708A)] had wt-level expression, while for unclear reasons, FA7490 [PilC1(D710A)] and FA7491 [PilC1(D716A)] had reduced PilC1 expression (Fig. 3D). While these strains had some normal antigenic variation in their *pilE* loci, all of the individual isolates of a given mutation expressed similar levels of PilC1 (data not shown). As a result, only the *pilC1*(D708A) calcium-binding mutant was examined further. The actual strain used was FA7534, which not only has the *pilC1*(D708A) mutation, but is also PilE matched to our phase-locked wt surrogate, FA7480, which was used in the remaining studies.

Calcium-binding domain mutation affects gonococcal adhesion to cervical epithelial cells. Initial gonococcal adhesion to cervical epithelial cells involves both PilC and PilE (14). To observe whether a mutation in the calcium-binding domain affects the ability of the gonococcus to adhere to target cells, we used an adherence assay similar to that used previously (12). The *N. gonorrhoeae* strain expressing PilC1(D708A) (FA7534) exhibited significantly reduced adherence to human ME180 cells compared with the strain expressing wt PilC1, FA7480, but more than the strain lacking pili and PilC1, FA7474 (Fig. 4A). We conclude that disruption of the ability of PilC1 to bind calcium partially disrupts the ability of *N. gonorrhoeae* cells to bind to human ME180 cells.

D708A mutation reduces transformation frequency in *N. gonorrhoeae*. Competence for DNA uptake is another important pilus-dependent biological function (19, 38). To determine if the mutation in the calcium-binding domain of PilC1 affected this function, we examined transformation frequency. As expected, the nonpiliated strain (Δ*pilC1* Δ*pilE*) had undetectable levels of transformation (frequency of less than 9×10^{-6} transformants/CFU), while the piliated strain (PilC1$^+$) had a transformation frequency of 3×10^{-3} transformants/CFU when exposed to 4 μg/ml transforming DNA and 6×10^{-4} transformants/CFU when exposed to 100 ng/ml transforming DNA (Fig. 4B). The calcium-binding-deficient PilC1(D708A) strain had significantly less competence at higher levels of DNA than the piliated (PilC1$^+$) strain, but it had equivalent competence at lower levels of DNA (Fig. 4B). Therefore, the loss of the calcium-binding domain in PilC1 appears to have a subtle effect on PilC's contribution to DNA uptake.

D708A mutation does not impact PilC1 localization to pili in *N. gonorrhoeae*. We next investigated the ability of the PilC1(D708A) mutant to localize to the surface of *N. gonorrhoeae* by flow cytometry. The monoclonal antibody 4B5 recognized all surface-exposed PilC, while the monoclonal antibody 1D5 only recognized PilC1 when it was expressed in the context of an intact pilus. We attribute this behavior to an interaction between PilC1

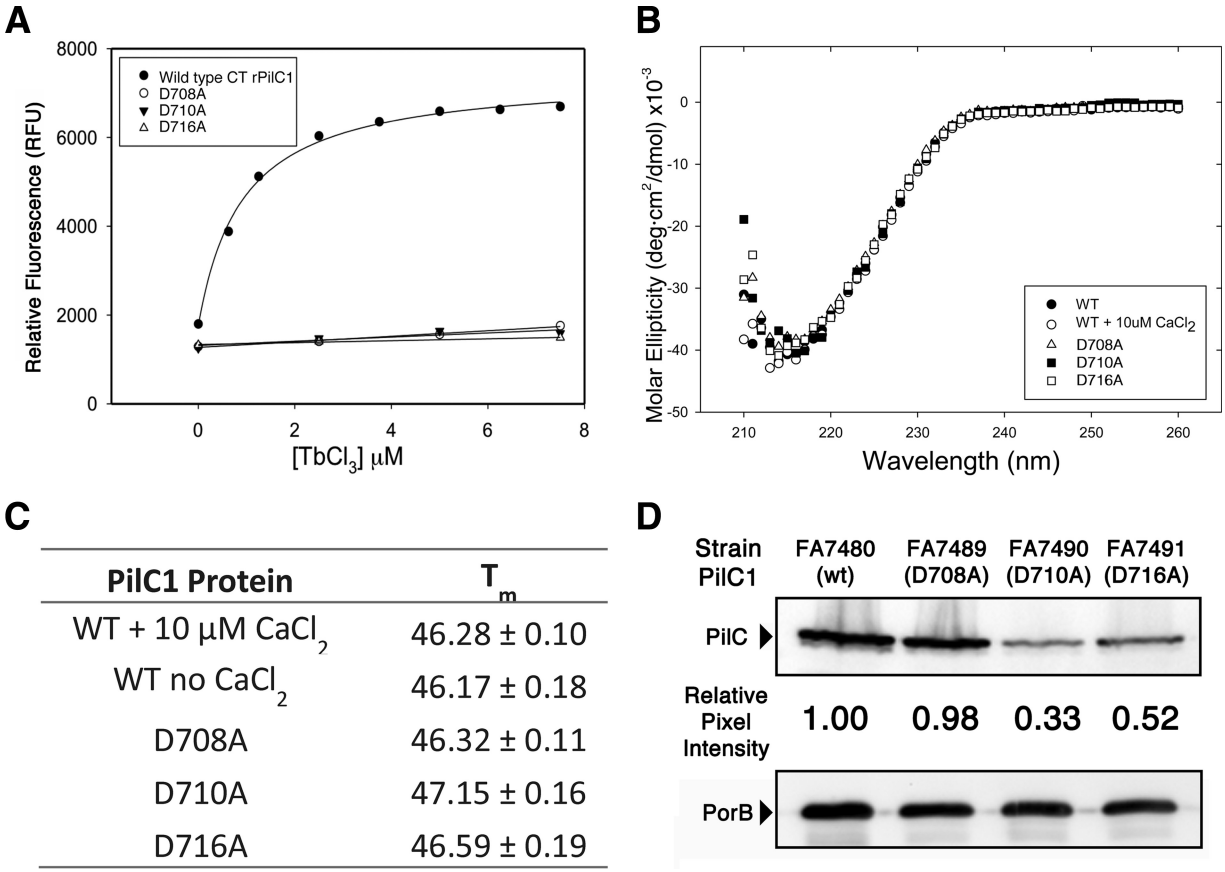

FIG 3 Mutations affecting the conserved aspartic acid residues in the C-terminal calcium-binding domain of PilC1 abrogate binding of the calcium analogue terbium (Tb) without grossly altering secondary structure. (A) Different CT rPilC1 constructs were titrated with increasing concentrations of $TbCl_3$. The solid line is a computer fit of the data using the one-site binding model. Solid circles show the terbium fluorescence of a 3.4 μM solution of native soluble CT rPilC1at increasing concentrations of $TbCl_3$. The open circles, closed inverted triangles, and open triangles indicate the fluorescence of similar protein solutions, each with one of the conserved aspartic acids altered. RFU, relative fluorescence units. (B) Far-UV circular dichroism spectra of CT PilC1 proteins (wt and calcium-binding mutant), recorded in the absence or presence of $CaCl_2$. (C) The change of ellipticity at 216 nm due to the increase of temperature was recorded. The melting temperature (T_m) indicates the temperature at which the protein lost half of its ellipticity at 216 nm. (D) Mutant PilC1 proteins were not equally expressed in *Neisseria gonorrhoeae*. Shown are two Western blots of gonococcal whole-cell lysates probed for PilC and PorB, balanced by culture density. The isolates used are representative of strains expressing PilC1 with the different calcium-binding mutations (D708A, D710A, and D716A). The samples were equally loaded, as judged by the Ponceau S stain of the blot, and Western blot signal was quantitated using ImageJ, corrected for minor variations in loading based on PorB signal and expressed relative to a strain expressing wt PilC1 (FA7480). Since only the level of D708A mutant was similar to that of the wt PilC1 protein, only the D708A mutant was used in the remaining studies.

and the pilus exposing the otherwise hidden N-terminal 1D5 epitope. The nonpiliated *N. gonorrhoeae* strain FA7474 ($PilC1^-$ $PilE^-$) was used as a negative control, and the strains FA7480 ($PilC1^+$ $PilE^+$) and FA7461 ($PilC1^+$ $PilE^-$) were used as positive controls. Our results show that the amount of PilC1(D708A) on the bacterial surface was not significantly reduced relative to wt PilC1 (Fig. 5A), suggesting that the D708A mutation to the calcium-binding domain of PilC1 did not significantly interfere with localization of PilC1 on the bacterial surface. In addition, the amount of PilC1 associated with pilin was not affected by its ability to bind calcium, as demonstrated by the binding of antibody 1D5 to both the calcium-binding mutant FA7534(D708A) and the wt (Fig. 5A).

The calcium-binding domain mutation does not significantly affect the amount of shearable pili on the bacterial surface. To ascertain whether the inability of PilC1 to bind calcium might affect its ability to mediate pilus biogenesis, we looked at the amount of pili present on the bacterial cell surface. We grew each of the strains for 18 h prior to resuspending them in CHES buffer, followed by vigorous vortexing and centrifugation. The samples were balanced by a BCA protein assay of whole-cell lysate, and samples representing similar numbers of bacteria were evaluated by Western blot analysis. The blots were probed with the indicated antibodies (Fig. 5B). To further normalize the amount of shear preparation between strains, the amount of PilE in the shear preparation is presented as a ratio to the amount of PorB signal from matched samples. The amount of PilE in the PilC1(D708A) mutant, FA7534, was not detectably different from that of the wt PilC1 gonococcal strain, FA7480, based on statistical analysis of densitometry data. This indicates that, although the ability of the pili in the D708A mutant to adhere and mediate transformation at high levels of DNA is significantly impaired, this does not appear to be due to a lack of pili present on the surface of the bacterium.

Scanning electron microscopy of the calcium-binding D708A mutant shows pili on the bacterial surface. To confirm the results of the shear pilin preparations, we examined our strains

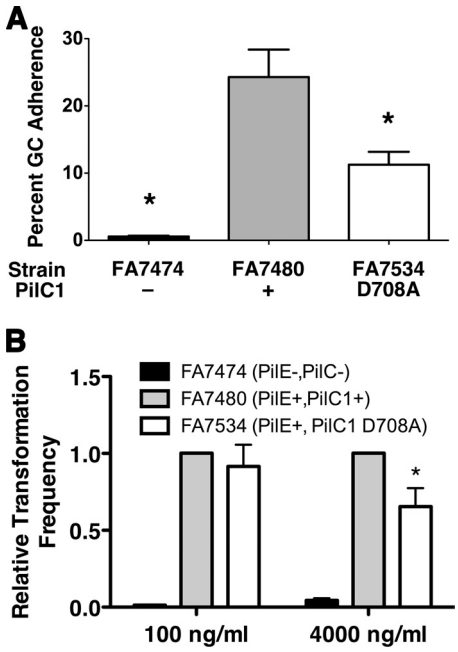

FIG 4 Gonococcal mutant expressing calcium-binding-deficient PilC1 is less adherent to ME180 cells and less competent for DNA transformation under some conditions. (A) The FA1090 derivative expressing the PilC1(D708A) calcium-binding mutation was significantly less adherent than the wild type to ME180 cells in a viable count adherence assay ($P < 0.01$). Error bars indicate the standard errors of the mean (SEM) of triplicate determinations from 6 days. Asterisks indicate a significant difference compared to the wild-type PilC1-expressing strain. (B) Relative transformation frequency of strains FA7474, FA7480, and FA7534 after receiving 100 ng/ml or 4000 ng/ml of transforming DNA (normalized to FA7480). FA7534 was only significantly different from FA7480 when exposed to 4000 ng/ml transforming DNA (denoted by * [$P < 0.05$]). The error bars represent the SEM of 4 independent determinations. Statistical significance was determined by one-way analysis of variance (ANOVA) with Tukey's posttest, and P values of <0.05 were considered significant.

using scanning electron microscopy. As can be seen in Fig. 5C, FA1090-derived strains are much less piliated than MS11 (including the parental strain FA1090a23 [data not shown]). This made it very difficult to evaluate piliation by both the shear pilin preparation experiments and scanning electron microscopy, as there is very little piliation in our positive control FA7480 (wt PilC1 PilE$^+$). However, we did see some pili in the PilC1(D708A) strain, FA7534, similar to our positive control FA7480 strain and more than our negative control, FA7474 (Δ*pilC1* Δ*pilE*). The low levels of piliation in the FA1090 derivatives make it difficult to distinguish between true T4P and artifacts, such as extended membrane blebs. We can, however, say that any residual fiberlike structures in FA7474 do not contain PilE, since it is deleted in this strain, as demonstrated in Fig. 5B. We also considered the possibility that the residual fiberlike structures were DNA but consider this unlikely, since they persisted even after treatment with Benzonase nuclease (Novogen) (data not shown).

To ensure that a defect in calcium binding did not have a direct effect on PilC1's ability to mediate adherence, we confirmed the adherence function of FL rPilC1(D708A) protein in both the PilC1 binding ELISA and a gonococcal adherence inhibition assay. FL rPilC1(D708A) actually bound better to ME180 cells than did the FL wild-type protein, as measured by whole-cell ELISA (Fig. 6A). One of the disadvantages of this approach to measuring PilC binding to epithelial cells is that it is a measurement of total binding—productive binding and nonspecific binding. Subtle differences in the quality of the two protein preparations may explain the increased binding of the protein associated with reduced bacterial adherence. When FL rPilC1(D708A) was used to inhibit the adherence of bacteria to epithelial cells, it showed similar inhibition to FL rPilC1-wt (Fig. 6B), suggesting that the recombinant PilC1(D708A) used in this study is likely well folded and able to compete with *in vivo*-expressed PilC1 in adherence to epithelial cells. More importantly, the mutation in the calcium-binding domain of PilC1 does not interfere with the ability of FL rPilC1 to bind to its receptor on ME180 cells and inhibit gonococcal binding.

DISCUSSION

N. gonorrhoeae is well established as a sexually transmitted human pathogen and the cause of numerous inflammatory diseases, such as pharyngitis, endocarditis, and pelvic inflammatory disease (39, 40). During infection, *N. gonorrhoeae* interacts with numerous cells types, including epithelial cells. The initial adhesion events of gonococcal infection are believed to involve gonococcal PilC and PilE. PilE has been proposed to interact with CD147 on endothelial cells during adherence of *N. meningitidis* (18), although much remains to be elucidated about gonococcal adherence (12, 13, 16). PilC was originally proposed as an adhesin located at the tip of the pilus (13) and is also thought to act as an antagonist of the pilus retraction protein PilT (9). The delicate balance between retraction and antagonist forces may allow PilC to initially interact with its host-specific receptor, followed by the gonococcus being repositioned closer to the epithelial cell due to retraction.

Recently, another PilC family member, PilY1 in *P. aeruginosa*, was found to have a calcium-binding domain that reduced the amount of type IV pili found on the surface of the bacterium (25). PilC from *N. gonorrhoeae* also has this conserved domain, which has been found in other PilC family members as well (Fig. 1; see Table S1 in the supplemental material) (37). The focus of this study was to observe whether the calcium-binding domain in the *N. gonorrhoeae* T4P adhesion protein, PilC, also affected piliation in the same manner as that previously seen in *P. aeruginosa*.

First, we defined a new expression and purification method for recombinant full-length and C-terminal PilC1 (Fig. 2A). We confirmed that the *E. coli*-expressed FL rPilC1 had a reduction mobility shift similar to that of the gonococcus-expressed PilC1 (Fig. 2B), indicating that it probably formed some of the same disulfide bonds as the natively expressed protein when expressed in an *E. coli* strain (Origami 2) containing the *trxB* and *gor* mutations to enhance disulfide bond formation (41). More importantly, this FL rPilC1 protein retained the ability to competitively inhibit gonococcal binding, similar to the previously published gonococcus-expressed PilC protein (Fig. 2C) (12). This argues that the N-terminal region, which is predicted to have a much less ordered structure than the C-terminal region and has been implicated in epithelial cell binding (11), retains a functionally relevant amount of its native structure. The secondary structure of the C-terminal region was sufficiently intact to bind calcium (Fig. 3A) and remained unchanged even in the versions of the protein altered to no longer bind calcium (Fig. 3B and C). Using these recombinant proteins, we confirmed that the adhesion region of PilC1 was N-terminal domain dependent and that purified FL PilC1 and

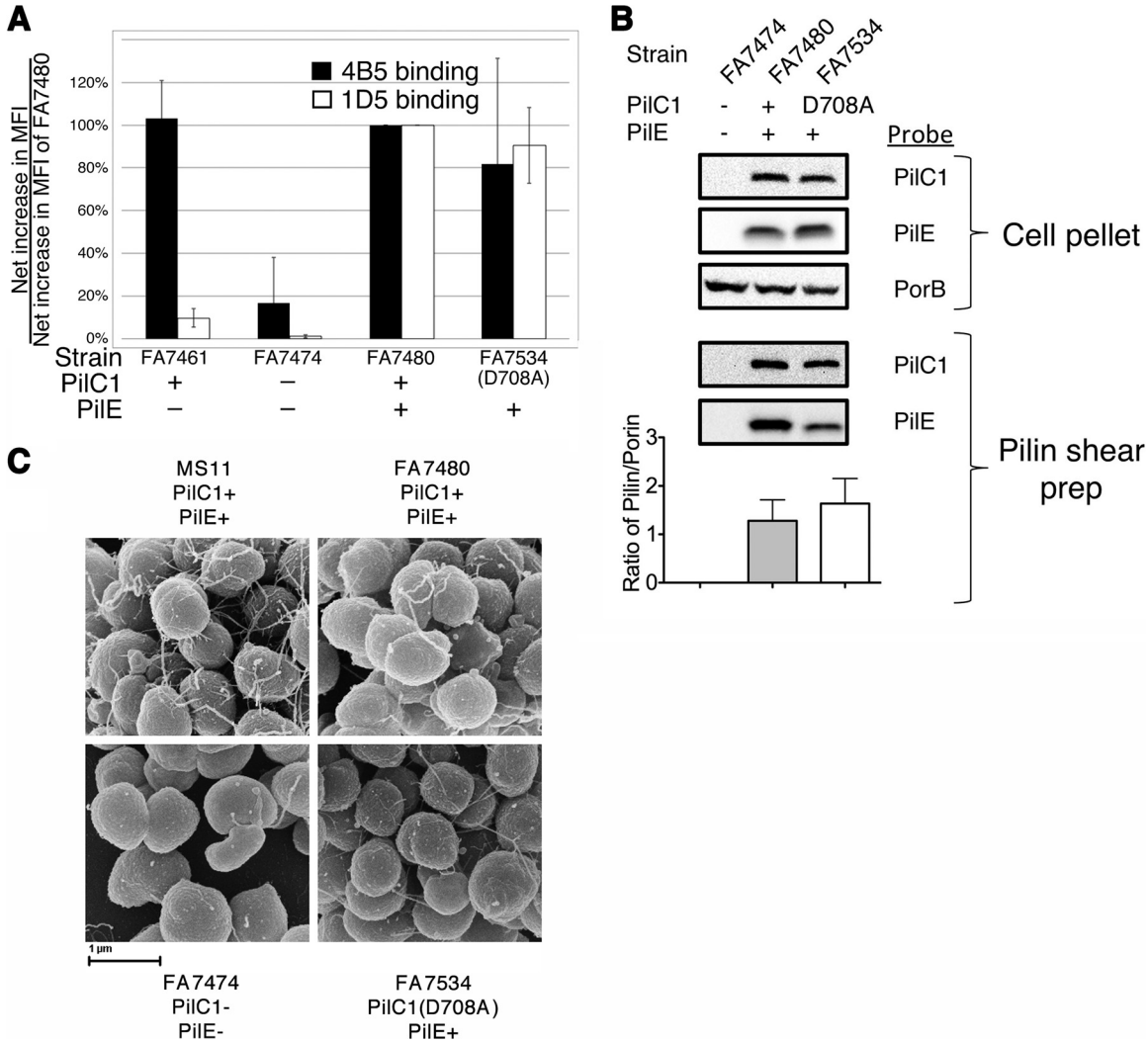

FIG 5 A gonococcal mutant expressing calcium-binding-deficient PilC1 expresses pili that are indistinguishable from those of the wild type. (A) The surface exposure of calcium-binding mutant PilC1(D708A) was similar to that of the wild type. Binding of two different monoclonal antibodies to whole bacteria was measured by flow cytometry. Monoclonal antibody 4B5 bound to all surface-exposed PilC1, regardless of the expression of PilE, while monoclonal 1D5 only bound to PilC1 when presented in the context of an intact pilus. (The negative control is FA7461.) The amount of PilC1(D708A) on the surface of FA7534 was not statistically different from that of the wild type based on the binding of either of these monoclonal antibodies. Error bars represent the standard deviations (SD) of 5 independent determinations. (B) Western blots of whole-cell lysates and pilin shear preparations showed that gonococcus strains expressing calcium-binding-deficient PilC1 produced comparable amounts of pili to the wild type. FA7474, FA7480, and FA7534 are shown as probed with antibodies recognizing PilC1, PilE, or PorB. The samples were balanced by total protein in cell pellets. Shown in the graph is the ratio of Western signal of PilE-probed shear preparation to PorB-probed whole-cell lysate from bacteria grown on four different days. (C) Scanning electron microscopy of MS11 and FA1090-derived strains expressing either wt PilC1, PilC1(D708A), or no PilC1. Each panel depicts a representative grouping of bacteria. MS11 is shown in the upper left panel, the nonpiliated control FA7474 ($PilC1^-$ $PilE^-$) is shown in the lower left panel, the wild-type surrogate FA7480 ($PilC1^+$ $PilE^+$) is shown in the upper right panel, and the calcium-binding mutant FA7534 ($PilE^+$) [PilC1(D708A)] is shown in the lower right panel. The scale bar in the lower left corner represents 1 μm. Statistical significance was determined by one-way ANOVA with Tukey's posttest, and P values of <0.05 were considered significant.

PilC1(D708A), but not CT PilC1, proteins could be used as competitive inhibitors of *N. gonorrhoeae* binding to ME180 cells (Fig. 2C and 6B). Having characterized the calcium-binding domain by making changes at residues D708, D710, and D716 (Fig. 1 and 3A), we found that, while all of these residues are important for binding calcium, only one, D708A, was expressed at wt levels in *N. gonorrhoeae* (Fig. 3D). This mutant was used in the remainder of this study. We are at present unsure of why there was unequal expression of the different mutant PilC1 proteins. Perhaps this is indicative of necessary interactions of other neisserial proteins with PilC1 during trafficking of PilC1 to the surface of the bacterium or otherwise affecting the *in vivo* stability of PilC1. Finally, the method defined here to successfully express and purify both recombinant full-length and C-terminal PilC1 could prove beneficial in determining the specific PilC1 N-terminal domain residues responsible for adhesion in future studies.

While previous studies found that elimination of the ability of PilY1 (the *P. aeruginosa* PilC homolog) to bind calcium abolished both adhesion and twitching by preventing surface piliation in *P. aeruginosa* (25, 36), the attenuated adhesion observed in *K. kingae* (37) and this study for *N. gonorrhoeae* was not dependent on preventing the surface expression of pili. We found that while the

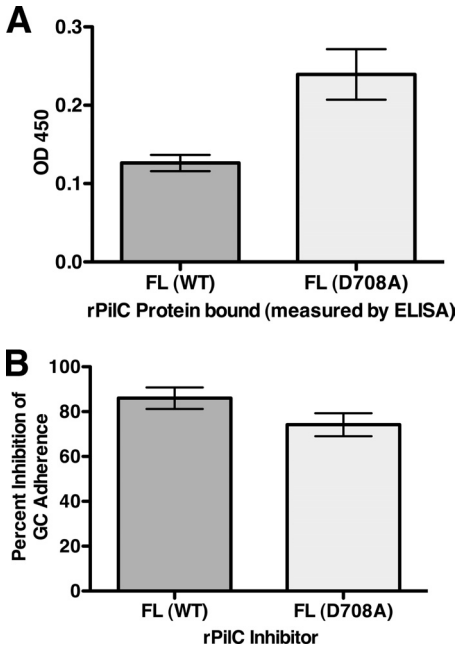

FIG 6 Purified PilC1(D708A) binds to ME180 epithelial cells. ME180 cells were incubated with 54 nM FL wild-type or rPilC1(D708A) mutant proteins before being subjected to whole-cell PilC ELISA or incubated with *N. gonorrhoeae* strain FA1090 in the bacterial adherence assay. The ELISA (A) was performed in triplicate on three separate days. The error bars represent the standard errors of the means. Significantly more of the rPilC1(D708A) protein bound to the cells by one-way ANOVA ($P < 0.05$), even after correction for differences in ELISA reactivity. The adherence inhibition assay (B) was performed in triplicate on five separate days. The error bars represent the standard errors of the normalized fraction of *N. gonorrhoeae* adherence. There was no statistical difference between the inhibition by the wt and that of the mutant protein by one-way ANOVA ($P > 0.05$) at any of several tested concentrations (data not shown).

gonococcal calcium-binding-deficient PilC1 mutant was reduced in its ability to adhere to epithelial cells, this defect could not be attributed to a lack of piliation. Scanning electron microscopy and a sheared pilin preparation showed that there were normal levels of pili present on the D708A gonococcal surface (Fig. 5B and C). The defect in calcium binding could be affecting the localization or presentation of PilC; however, flow cytometric staining of PilC1 also indicated normal levels of PilC1 on the surface of the D708A-expressing gonococcal strain (Fig. 5A). The calcium-binding defect could have altered the ability of the pili to properly present the N-terminal PilC adhesion domain, but PilC's exposure was not altered even when a monoclonal antibody recognizing an N-terminal epitope was used.

In addition to a defect in adherence capability, the PilC(D708A) mutant was reduced in its transformability but only at larger amounts of transforming DNA (Fig. 4B). One potential reason for these partially altered phenotypes in the face of apparently equal piliation is that our measure of piliation was insufficient to measure a small effect on the level of piliation. This explanation is inconsistent with the magnitude of the biological defects (40 to 50%), assuming a linear relationship between either of the defects and the amount of pili produced. Also at odds with this explanation is evidence shown by Long et al. that even low levels of pilin expression allow for substantial DNA transformation competence (42).

An alternative explanation is that the quality of the pili being produced is changed in the PilC1(D708A)-producing strain. If the PilC1(D708A)-expressing strain produced fewer pili but they were longer than those produced by the wt parent, it might explain the observed biological effects while still yielding a similar amount of shearable PilE. Fewer, longer pili in the mutant might make them more prone to breakage and lead to the observed reduction in adherence. Fewer pili during transformation might lead to a partial defect in frequency only when nearly all of the pili are saturated with bound DNA, while the effect of fewer pili was negligible when the DNA was in limiting supply.

Another explanation might be that the altered PilC1 led to a change in the bundle-forming behavior of the pili, although bundle formation is normally associated with the posttranslational modification state of pili. Bundle-forming behavior of pili is known to affect both interbacterial interactions as well as bacterial adherence in *N. meningitidis* (43). We did not measure the posttranslational modifications of PilE of the strains used in these studies, but since they are closely related, they are presumed to be similar. Since it was very difficult to detect pili by scanning electron microscopy in these strains, it is certainly possible that a difference in the number of pili, their length, or their bundle-forming behavior could easily have gone unnoticed. Future studies might include additional electron microscopy to observe the exact piliation state of bacteria expressing PilC(D708A) as well as where the protein is located on the bacterial surface. It may be necessary to extend the genetics developed in FA1090 into one or more other strains based on the limited amount of pili expressed by the parental strain.

T4P are used for a variety of cellular processes, including motility, DNA uptake, and adhesion-mediated infection (19, 35). Here, we elucidate the importance of calcium-binding domains in the PilC family of proteins. Although the mechanism by which these calcium-binding domains control adhesion and some pilin function is unclear, we extend the calcium-bound and -unbound model for the PilC family of proteins in the control of pilus function.

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REFERENCES

1. **Centers for Disease Control and Prevention.** 2013. CDC Grand Rounds: the growing threat of multidrug-resistant gonorrhea. MMWR Morb. Mortal. Wkly. Rep. **62:**103–106.
2. **Satterwhite CL, Torrone E, Meites E, Dunne EF, Mahajan R, Ocfemia MC, Su J, Xu F, Weinstock H.** 2013. Sexually transmitted infections among US women and men: prevalence and incidence estimates, 2008. Sex. Transm. Dis. **40:**187–193.
3. **Centers for Disease Control and Prevention.** 2012. Update to CDC's sexually transmitted diseases treatment guidelines, 2010: oral cephalospo-

rins no longer a recommended treatment for gonococcal infections. MMWR Morb. Mortal. Wkly. Rep. **61:**590–594.
4. **Chen A, Seifert H.** 2011. *Neisseria gonorrhoeae*-mediated inhibition of apoptotic signalling in polymorphonuclear leukocytes. Infect. Immun. **79:**4447–4458.
5. **Johnson M, Criss A.** 2011. Resistance of *Neisseria gonorrhoeae* to neutrophils. Front. Microbiol. **2:**77. doi:10.3389/fmicb.2011.00077.
6. **Seifert H, Ajioka R, Marchal C, Sparling P, So M.** 1988. DNA transformation leads to pilin antigenic variation in *Neisseria gonorrhoeae.* Nature **336:**392–395.
7. **Zhu W, Ventevogel M, Knilans K, Anderson J, Oldach L, McKinnon K, Hobbs M, Sempowski G, Duncan J.** 2012. *Neisseria gonorrhoeae* suppresses dendritic cell-induced, antigen-dependent CD4 T cell proliferation. PLoS One **7:**e41260. doi:10.1371/journal.pone.0041260.
8. **Merz A, So M, Sheetz M.** 2000. Pilus retraction powers bacterial twitching motility. Nature **407:**98–102.
9. **Morand P, Bille E, Morelle S, Eugène E, Beretti J-L, Wolfgang M, Meyer T, Koomey M, Nassif X.** 2004. Type IV pilus retraction in pathogenic *Neisseria* is regulated by the PilC proteins. EMBO J. **23:**2009–2017.
10. **Jonsson A, Nyberg G, Normark S.** 1991. Phase variation of gonococcal pili by frameshift mutation in *pilC*, a novel gene for pilus assembly. EMBO J. **10:**477–488.
11. **Morand P, Tattevin P, Eugene E, Beretti J, Nassif X.** 2001. The adhesive property of the type IV pilus-associated component PilC1 of pathogenic *Neisseria* is supported by the conformational structure of the N-terminal part of the molecule. Mol. Microbiol. **40:**846–856.
12. **Kirchner M, Heuer D, Meyer T.** 2005. CD46-independent binding of neisserial type IV pili and the major pilus adhesin, PilC, to human epithelial cells. Infect. Immun. **73:**3072–3082.
13. **Rudel T, Scheurerpflug I, Meyer T.** 1995. *Neisseria* PilC protein identified as type-4 pilus tip-located adhesin. Nature **373:**357–359.
14. **Scheuerpflug I, Rudel T, Ryll R, Pandit J, Meyer T.** 1999. Roles of PilC and PilE proteins in pilus-mediated adherence of *Neisseria gonorrhoeae* and *Neisseria meningitidis* to human erythrocytes and endothelial and epithelial cells. Infect. Immun. **67:**834–843.
15. **Källström H, Blackmer Gill D, Albiger B, Liszewski M, Atkinson J, Jonsson A.** 2001. Attachment of *Neisseria gonorrhoeae* to the cellular pilus receptor CD46: identification of domains important for bacterial adherence. Cell. Microbiol. **3:**133–143.
16. **Källström H, Liszewski M, Atkinson J, Jonsson A.** 1997. Membrane cofactor protein (MCP or CD46) is a cellular pilus receptor for pathogenic *Neisseria.* Mol. Microbiol. **25:**639–647.
17. **Tobiason DM, Seifert HS.** 2001. Inverse relationship between pilus-mediated gonococcal adherence and surface expression of the pilus receptor, CD46. Microbiology **147:**2333–2340.
18. **Bernard S, Simpson N, Federici C, Laran-Chich MP, Chretiem F, Join-Lambert O, Coureuil M, Niedergang F, Marullo S, Courand PO, Nassif X, Bourdoulous S.** 2012. CD147 is a cellular receptor for pilus-mediated adhesion of meningococci to vascular endothelia, p 130. *In* Frosch M, Rudel T, Vogel U (ed), XVIIIth International Pathogenic *Neissera* Conference. Conventus Congressmanagement & Marketing GmbH, Würzburg, Germany.
19. **Rudel T, Facius D, Barten R, Scheuerpflug I, Nonnenmacher E, Meyer T.** 1995. Role of pili and the phase-variable PilC protein in natural competence for transformation of *Neisseria gonorrhoeae.* Proc. Natl. Acad. Sci. U. S. A. **92:**7986–7990.
20. **Mattick J.** 2002. Type IV pili and twitching motility. Annu. Rev. Microbiol. **56:**289–314.
21. **Parge H, Forest K, Hickey M, Christensen D, Getzoff E, Tainer J.** 1995. Structure of the fibre-forming protein pilin at 2.6 A resolution. Nature **378:**32–38.
22. **Rudel T, van Putten J, Gibbs C, Haas R, Meyer T.** 1992. Interaction of two variable proteins (PilE and PilC) required for pilus-mediated adherence of *Neisseria gonorrhoeae* to human epithelial cells. Mol. Microbiol. **6:**3439–3450.
23. **Cahoon L, Seifert H.** 2011. Focusing homologous recombination: pilin antigenic variation in the pathogenic *Neisseria.* Mol. Microbiol. **81:**1136–1143.
24. **Haas R, Meyer T.** 1986. The repertoire of silent pilus genes in *Neisseria gonorrhoeae*: evidence for gene conversion. Cell **44:**107–115.
25. **Orans J, Johnson M, Coggan K, Sperlazza J, Heiniger R, Wolfgang M, Redinbo M.** 2010. Crystal structure analysis reveals *Pseudomonas* PilY1 as an essential calcium-dependent regulator of bacterial surface motility. Proc. Natl. Acad. Sci. U. S. A. **107:**1065–1070.
26. **Stols L, Gu M, Dieckman L, Raffen R, Collart F, Donnelly M.** 2002. A new vector for high-throughput, ligation-independent cloning encoding a tobacco etch virus protease cleavage site. Protein Expr. Purif. **25:**8–15.
27. **Kellogg D, Cohen I, Norins L, Schroeter A, Reising G.** 1968. *Neisseria gonorrhoeae.* II. Colonial variation and pathogenicity during 35 months in vitro. J. Bacteriol. **96:**596–605.
28. **Johnston D, Cannon J.** 1999. Construction of mutant strains of *Neisseria gonorrhoeae* lacking new antibiotic resistance markers using a two gene cassette with positive and negative selection. Gene **236:**179–184.
29. **Jerse AE, Cohen MS, Drown PM, Whicker LG, Isbey SF, Seifert HS, Cannon JG.** 1994. Multiple gonococcal opacity proteins are expressed during experimental urethral infection in the male. J. Exp. Med. **179:**911–920.
30. **Barritt DS, Schwalbe RS, Klapper DG, Cannon JG.** 1987. Antigenic and structural differences among six proteins II expressed by a single strain of Neisseria gonorrhoeae. Infect. Immun. **55:**2026–2031.
31. **Black WJ, Schwalbe RS, Nachamkin I, Cannon JG.** 1984. Characterization of Neisseria gonorrhoeae protein II phase variation by use of monoclonal antibodies. Infect. Immun. **45:**453–457.
32. **Sugasawara RJ, Cannon JG, Black WJ, Nachamkin I, Sweet RL, Brooks GF.** 1983. Inhibition of Neisseria gonorrhoeae attachment to HeLa cells with monoclonal antibody directed against a protein II. Infect. Immun. **42:**980–985.
33. **Biswas G, Lacks S, Sparling P.** 1989. Transformation-deficient mutants of piliated *Neisseria gonorrhoeae.* J. Bacteriol. **171:**657–664.
34. **Biais N, Higashi D, Brujic J, So M, Sheetz M.** 2010. Force-dependent polymorphism in type IV pili reveals hidden epitopes. Proc. Natl. Acad. Sci. U. S. A. **107:**11358–11363.
35. **Craig L, Pique ME, Tainer JA.** 2004. Type IV pilus structure and bacterial pathogenicity. Nat. Rev. Microbiol. **2:**363–378.
36. **Johnson M, Garrett C, Bond J, Coggan K, Wolfgang M, Redinbo M.** 2011. *Pseudomonas aeruginosa* PilY1 binds integrin in an RGD- and calcium-dependent manner. PLoS One **6:**e29629. doi:10.1371/journal.pone.0029629.
37. **Porsch EA, Johnson MD, Broadnax AD, Garrett CK, Redinbo MR, St. Geme JW, III.** 2013. Calcium binding properties of the *Kingella kingae* PilC1 and PilC2 proteins have differential effects on type IV pilus-mediated adherence and twitching motility. J. Bacteriol. **195:**886–895.
38. **Strom M, Lory S.** 1993. Structure-function and biogenesis of the type IV pili. Annu. Rev. Microbiol. **47:**565–596.
39. **Rice P, Schachter J.** 1991. Pathogenesis of pelvic inflammatory disease. What are the questions? JAMA **266:**2587–2593.
40. **Thompson E, Brantley D.** 1996. Gonoccocal endocarditis. J. Natl. Med. Assoc. **88:**353–356.
41. **Bessette PH, Aslund F, Beckwith J, Georgiou G.** 1999. Efficient folding of proteins with multiple disulfide bonds in the *Escherichia coli* cytoplasm. Proc. Natl. Acad. Sci. U. S. A. **96:**13703–13708.
42. **Long C, Tobiason D, Lazio M, Kline K, Seifert H.** 2003. Low-level pilin expression allows for substantial DNA transformation competence in *Neisseria gonorrhoeae.* Infect. Immun. **71:**6279–6291.
43. **Marceau M, Forest K, Béretti J, Tainer J, Nassif X.** 1998. Consequences of the loss of O-linked glycosylation of meningococcal type IV pilin on piliation and pilus-mediated adhesion. Mol. Microbiol. **27:**705–715.