**Neisseria gonorrhoeae** Metalloprotease NGO1686 Is Required for Full Piliation, and Piliation Is Required for Resistance to H$_2$O$_2$- and Neutrophil-Mediated Killing

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**ABSTRACT** The sexually transmitted infection gonorrhea is caused exclusively by the human-specific pathogen *Neisseria gonorrhoeae*. Type IV pili are an essential virulence factor uniformly expressed on clinical gonococcal isolates and are required for several aspects of gonococcal pathogenesis, including adherence to host tissues, autoagglutination, twitching motility, and the uptake of DNA during transformation. Symptomatic gonococcal infection is characterized by the influx of neutrophils or polymorphonuclear leukocytes (PMNs) to the site of infection. PMNs are a key component of gonococcal pathogenesis, mediating the innate immune response through the use of oxidative and nonoxidative killing mechanisms. The M23B family zinc metallopeptidase NGO1686 is required for gonococci to survive oxidative killing by H$_2$O$_2$- and PMN-mediated killing through unknown mechanisms, but the only known target of NGO1686 is peptidoglycan. We report that the effect of NGO1686 on survival after exposure to H$_2$O$_2$ and PMNs is mediated through its role in elaborating pili and that nonpiliated mutants of *N. gonorrhoeae* are less resistant to killing by H$_2$O$_2$, LL-37, and PMNs than the corresponding piliated strains. These findings add to the various virulence-associated functions attributable to gonococcal pili and may explain the selection basis for piliation in clinical isolates of *N. gonorrhoeae*.

**IMPORTANCE** Successful infectious agents need to overcome host defense systems to establish infection. We show that the *Neisseria* pilus, a major virulence factor of this organism, which causes gonorrhea, helps protect the bacterium from two major killing mechanisms used by the host to combat infections. We also show that to express the pilus, an enzyme needs to partially degrade the cell wall of the bacterium.

The human-specific pathogen *Neisseria gonorrhoeae* is the sole causative agent of the sexually transmitted infection gonorrhea. Type IV pili are an essential virulence factor of gonococci (Gc), as only piliated bacteria are isolated from patients with gonorrhea (1). Type IV pili are thin, hair-like appendages that have been implicated in the virulence of both Gram-negative and Gram-positive bacteria, including the closely related species *Neisseria meningitidis*, as well as a variety of other pathogens, including *Legionella pneumophila*, *Vibrio cholerae*, *Pseudomonas aeruginosa* (reviewed in reference 2), and *Clostridium perfringens* (3). Several proteins are required for type IV pilus biogenesis in pathogenic *Neisseria* species; however, only a few of these proteins are absolutely required for pilus assembly (4). For a subset of these pilus biogenesis proteins, type IV pili can be restored when they are inactivated by the pilus motility ATPase PilT (4, 5).

Pilus expression is essential for Gc pathogenesis. Whereas all clinical isolates of Gc are piliated, both nonpiliated and underpiliated variants frequently arise when Gc are grown in *vitro*, suggesting that there is strong selective pressure for pilus expression in the human host (1). Gc pili have a number of established functions that are important for pathogenesis, including adherence to the host’s epithelium, autoagglutination, twitching motility, and uptake of DNA during transformation (6). Since Gc do not express exotoxins or a type II or type III secretion system, and the type IV secretion system is expressed in only some isolates, the pilus can be considered both a fibril organelle and the major secretion system for this pathogen. However, the only known secreted substrate of the type IV secretion system is the pilus itself.

A second important aspect of gonococcal pathogenesis is the interaction of Gc with polymorphonuclear leukocytes (PMNs). Symptomatic gonorrhea is characterized by the influx of PMNs to the site of infection. The clinical manifestation of this infection is the production of a purulent exudate that consists almost entirely of PMNs with attached and internalized gonococcal cells. PMNs possess both oxidative and nonoxidative mechanisms of killing, yet some Gc found in the purulent exudate remain viable, suggesting that Gc have evolved mechanisms to circumvent killing by PMNs (7, 8). Gc express a plethora of gonococcal proteins that protect against oxidative damage caused by reactive oxygen species (ROS). Some act by detoxifying ROS (9–11), while others
repair damage caused by ROS (12, 13). Although many of these antioxidant defenses have been shown to aid in gonococcal survival within primary human cervical epithelial cells and the mouse genital tract (14, 15), evidence suggests that ROS do not participate in PMN-mediated killing of Gc (16, 17). The nonoxidative components of PMN-mediated killing include degradative enzymes and cationic antimicrobial peptides (18). Gc possess two efflux pump systems which confer resistance to cationic antimicrobial peptides and long-chain fatty acids (19, 20) and aid in colonization of the murine genital tract (21). It is likely that in becoming a successful pathogen, Gc have evolved diverse ways to subvert the killing mechanisms of PMNs to promote infection and transmission (7).

The gonococcal NGO1686 locus, encoding the mpg gene (for M23B metalloprotease active against peptidoglycan), was identified by microarray analysis as being highly upregulated by sublethal levels of H2O2, and mpg mutants are sensitive to H2O2 and nonoxidative PMN-mediated killing (16, 22). Thus far, RecN and NGO1686 are the only gonococcal proteins that have been shown to aid in survival to nonoxidative PMN-mediated killing (16). NGO1686 was characterized as a bifunctional, M23B family, zinc-dependent carboxy- and endopeptidase that hydrolyzes peptidoglycan (PG) side chains (23). Other M23B family metalloproteases have been shown to affect PG cross-linking, contributing to the cellular morphology of Helicobacter pylori (24, 25). Several M23B family proteins have also been shown to cleave septal PG to allow for efficient cell separation (26–29). In contrast, the N. gonorrhoeae mpg mutant (also termed the Δ1686 mutant) shows no defects in cellular morphology but does exhibit an altered colony morphology. Here we provide evidence that NGO1686 is involved in pilus biogenesis and that the mpg mutant colony morphology as well as its corresponding phenotypes of resistance to H2O2 and PMNs are directly mediated through NGO1686’s effect on piliation. This work establishes new roles for the gonococcal pilus in pathogenesis.

RESULTS

The mpg mutant is underpiliated. The mpg mutant strain has a colony morphology different from that of the parent strain, and it can be complemented by supplying mpg at an ectopic locus in the chromosome (22, 23) (Fig. 1 and data not shown). We noted that the colony morphology of the mpg mutant was intermediate between that of the fully piliated (P+) parent strain and spontaneous nonpiliated (P−) pilin variants (Fig. 1). To directly examine the piliation state of the mpg mutant, the mpg mutation was transformed into the FA1090 (RM11.2nv, where “nv” indicates “nonvarying”) genetic background, which expresses a pilin variant that can be detected using immunoelectron microscopy (immuno-EM) (30) and cannot undergo further antigenic variation due to a transposon mutation affecting the guanine quartet sequence required for pilin variation (31, 32). The RM11.2nv mpg mutant also exhibited an altered colony morphology similar to that of the 1-81-S2 mpg strain (data not shown). Enumeration of pilin bundles on each strain revealed that the parent strain FA1090 (RM11.2nv) expressed an average of 3.5 bundles per gonococcal cell (Fig. 2A to C), whereas the corresponding mpg mutant expressed an average of 0.7 bundle per gonococcal cell (Fig. 2D to F). These data demonstrate that the mpg mutant shows decreased pilus expression, which is likely the basis of the altered colony morphology.

The mpg strain is deficient in natural transformation. Gc are naturally competent for DNA transformation at all stages of growth, and the pilus is essential for efficient DNA transformation (33). The number of pili elaborated on the gonococcal cell surface correlates with the transformation efficiency of a given strain (30, 34). To further test whether piliation is affected in the mpg mutant, we compared the transformation efficiencies of the parental strain, the previously described mpg loss-of-function mutant (D1686), and the mutant complemented with the gene expressed from its own promoter at an ectopic locus (Δ1686/1686+ mutant). The mpg mutant showed a statistically significant 3- to 4-fold decrease in transformation efficiency relative to that of the parent strain, and this deficiency was restored in the complemented strain (Fig. 3). These results show that the mpg mutant has decreased transformation competence and suggest that the additional mpg phenotypes of decreased resistance to H2O2- and PMN-mediated killing may also be attributable to decreased piliation.

The decreased resistance of the mpg mutant to H2O2- and PMN-mediated killing is due to decreased piliation. To genetically test whether the mpg strain’s H2O2 sensitivity phenotype was...
mediated through pili, we compared the levels of H$_2$O$_2$ resistance conferred by the $\Delta$ pilE, mpg, and $\Delta$ pilE mpg mutations in strain FA1090 (variant 1-81-S2) (Fig. 4A). The $\Delta$ pilE strain was less resistant to H$_2$O$_2$ than the mpg strain, but both the mpg and $\Delta$ pilE strains were less resistant than the parental strain, consistent with the complete lack of piliation in the $\Delta$ pilE strain and the underpiliated phenotype of the mpg strain. There was no further decrease in H$_2$O$_2$ resistance from that shown by the $\Delta$ pilE strain when the two mutations were combined, indicating that the two mutations are in the same epistasis group and are in the same pathway for H$_2$O$_2$ resistance.

To directly test the hypothesis that piliated Gc are more resistant to H$_2$O$_2$ killing than nonpiliated Gc, we utilized a gonococcal strain containing a regulatable pilE gene. The regulatable RM11.2 strain contains the RM11.2 variant pilE sequence under the control of the lac regulatory system, thereby allowing the control of pilE expression and piliation by addition or removal of IPTG (isopropyl-β-D-thiogalactopyranoside) (30). The regulatable RM11.2 strain was ~10-fold more resistant to H$_2$O$_2$ in the presence of IPTG (when wild-type levels of pili were expressed) than in the absence of IPTG (when few or no pili were expressed) (Fig. 4B).

To determine whether the decreased resistance of the mpg mutant to PMN-mediated killing was also mediated through pili, ex vivo PMN survival measurements were performed using freshly isolated human PMNs incubated with the parent strain, the mpg and $\Delta$ pilE single mutants, and the $\Delta$ pilE mpg double mutant. The mpg strain showed the previously reported decreased resistance to PMN-mediated killing (Fig. 4C, and see reference 22). The $\Delta$ pilE strain was less resistant to PMN-mediated killing than the parent or the mpg strain, and the double mutant showed levels of resistance similar to those of the $\Delta$ pilE mutant (Fig. 4C). These results indicate that the basis for decreased resistance to PMN-mediated killing in the mpg strain is also due to decreased levels of piliation and reveal a role for piliation in gonococcal resistance to PMN killing.

Since we previously found that the mpg strain was more sensitive to nonoxidative killing by PMNs (22), we began to explore what nonoxidative-killing mechanisms of PMNs might be responsible for the increased killing of non- and underpiliated Gc. The parent strain, the mpg and $\Delta$ pilE single mutants,
and the ΔpilE mpg double mutant were exposed to the cationic antimicrobial peptide LL-37, which is produced by PMNs. All of the mutant strains showed decreased resistance to human LL-37 (Fig. 4D), implying that at least some of the PMN sensitivity was dependent on the action of this cathelicidin and confirming that pilE and mpg were epistatic and likely in the same pathway.

To confirm a role for piliation in gonococcal resistance to H₂O₂ and PMN-mediated killing, we examined the effect of a pilQ mutation on resistance to H₂O₂ and PMN-mediated killing. PilQ encodes the pore through which the pilus is extruded, and a pilQ mutant has been shown to not elaborate pili (35). The pilQ mutant showed decreased resistance to both H₂O₂ and PMN killing (Fig. 5). A pilC mutant, which is also P⁺, was also less resistant to H₂O₂ than the parent strain (data not shown). Thus far, all the gonococcal mutants which decrease or abolish piliation that we have tested also confer decreased resistance to H₂O₂ (data not shown).

Pseudomonas aeruginosa type IV pili do not confer resistance to H₂O₂ killing. We next considered whether H₂O₂ resistance is a trait conferred by all type IV pili. We measured the H₂O₂ resistance of P. aeruginosa strain 15692, an isogenic pilA mutant (pilA encodes the pilin subunit), the complemented strain containing the pilA gene in vector pUCP18, and the empty vector (36). The observed small differences are not consistent with pili conferring H₂O₂ resistance on P. aeruginosa (Fig. 6). These results indicate that H₂O₂ resistance is not a phenotype shared among type IV pili from all bacterial species, although it is still possible that other type IV-pilus-expressing species share this property with Gc.
Resistance to H₂O₂ requires active pili. To determine whether functional pili or merely the presence of pili affects resistance to H₂O₂ killing, we measured the effect of pilT mutation on H₂O₂ resistance. pilT encodes the ATPase that mediates the retraction of the pilus. Although pilT mutants are piliated, they no longer exhibit twitching motility and are defective in DNA uptake (37). An FA1090 pilT mutant was less resistant to H₂O₂ than the parent strain (Fig. 7A), showing that active pili are necessary to mediate resistance to H₂O₂ killing. We also purified pili from a distinct strain (Fig. 7A), showing that active pili are necessary to mediate resistance to H₂O₂. The strain showed the same level of resistance to H₂O₂ in the presence and absence of IPTG (Fig. 8C). These results in combination with those obtained with the pilQ mutant strongly support the role of active pili in mediating resistance to H₂O₂.

NGO1686 is involved in pilus biogenesis. The underpiliated phenotype of the mpg mutant was similar to phenotypes described for pathogenic Neisseria strains with mutations in pilus assembly genes. For a subset of these mutants, concomitant mutation of the retraction ATPase gene, pilT, restores pilation (4, 5, 38). We therefore introduced an IPTG-regulatable pilT (reg pilT) construct (37) into the FA1090 (RM11.2nv) mpg mutant background and used immunogold transmission EM (immunogold-TEM) to analyze piliation. In the presence of IPTG, strain RM11.2nv mpg reg pilT expressed an average of 0.6 pilus bundle per gonococcal cell (Fig. 8B), a number of bundles similar to that of strain RM11.2nv pilQ (Fig. 2D to F). However, in the absence of IPTG, the strain expressed an average of 3.3 pilus bundles per gonococcal cell (Fig. 8A), a number of bundles nearly equivalent to that of strain RM11.2nv (Fig. 2A to C). By analogy with Neisseria pilus mutants having similar pilT-dependent phenotypes, this suggested a role for NGO1686 in pilus biogenesis.

We next measured the H₂O₂ resistance of the RM11.2nv mpg reg pilT strain in the presence and absence of IPTG. This allowed us to modulate PilT and pilus expression for the purpose of determining whether the pili elaborated in the absence of PilT could mediate resistance to H₂O₂. The strain showed the same level of H₂O₂ resistance in the presence and absence of IPTG (Fig. 8C). These results in combination with those obtained with the pilT mutant strongly support the role of active pili in mediating resistance to H₂O₂.

DISCUSSION

We have determined that the three previously described phenotypes conferred by the PG-targeted metalloprotease NGO1686—colony morphology, resistance to H₂O₂, and PMN-mediated killing—are all mediated through NGO1686’s effect on piliation. This discovery identifies a novel activity required for gonococcal type IV pilus biogenesis and also reveals two new pathogenesis-related phenotypes for the pilus.

NGO1686 is an M23B metalloprotease that exhibits dual peptidoglycanase activities in vitro. Mutation of several M23B active sites in NGO1686 results in loss of enzymatic activity as well as decreased pilation (23). Although there are many published examples of PG-hydrolyzing enzymes that facilitate the emergence of macromolecular structures on the cell surface (reviewed in reference 39), there is only one PG-hydrolyzing enzyme that has previously been shown to play a role in type IV pilus biogenesis. The PleA protein of Caulobacter crescentus is required for assembly of both the flagellum and pili (40).

FIG 5 Resistance of pilQ mutant strains to H₂O₂ and PMN-mediated killing. (A) Dose-response curve of H₂O₂ resistance after 15 min of treatment of the pilQ mutant strain. *, P < 0.02. (B) Resistance of the pilQ mutant strain to PMN-mediated killing. *, P < 0.0005 (Student’s two-tailed t test).

FIG 6 Relative survival of P. aeruginosa strain 15692, the isogenic pilA mutant, an empty vector control, and the pilA complemented strain. Error bars represent the standard errors of the means of results from at least 5 experiments. *, P > 0.21, between all strains at all doses.
clear. Piliation of the mpg mutant strain was restored in a pilT mutant background, where PilT-mediated pilus retraction was abolished. Thus, NGO1686 must have a direct or indirect role in preventing pilus retraction. Similar effects have been observed with numerous other Neisseria pilus biogenesis proteins (4, 5, 41, 42). It has been proposed that each of these proteins somehow antagonizes retraction of the pilus, perhaps by forming a prefilament that promotes pilin polymerization (PilC/H-PilL) (4) or by interacting with the pilus from neighboring bacteria (PilIX) (41), but no mechanism for preventing retraction has been demonstrated. Since NGO1686 has peptidoglycanase activities (23), it is possible that NGO1686 specifically cleaves PG as part of the pilus apparatus assembly process, thus removing a barrier that would otherwise prevent the elaboration of pili on the cell surface. However, if this were the case, we would expect the mpg mutant to express no pili on its cell surface rather than simply to exhibit a large decrease in the numbers of pili. Moreover, if PG were a complete barrier, a Δ1686 mpg pilT mutant would show a growth defect similar to that of a pilQ pilT mutant (43). Therefore, it is unlikely that NGO1686’s role in pilus elaboration is simply through restructuring the cell wall to allow passage of pili. It is feasible that the pili produced in the mpg mutant are using a different pathway through the PG to exit the cell, but we think it is unlikely that this putative alternative pathway for pilus extrusion would allow for full piliation when PilT was inactivated. Therefore, we do not favor the model where the sole role of NGO1686 in pilation is to remove a barrier to assembly; rather, we think that it remodels the PG to allow an antiretraction complex to form.

The most surprising observation made in these studies was that the ability of Gc to elaborate pili is important for conferring resistance to both H₂O₂- and PMN-mediated killing. This observation is even more surprising given that H₂O₂ and PMN kill by disparate mechanisms and that PMN-mediated killing is likely to be mediated in part by LL-37. Our data imply that pili do not simply have antioxidant properties that directly detoxify H₂O₂ associated with them. Our observations that only “active” pili (from pilT-expressing strains) and not exogenously added pili have a role in H₂O₂ resistance show that inert pili do not function to provide H₂O₂ resistance. We have confirmed that there is no change in catalase activity or in intracellular iron, magnesium, or manganese levels in either the mpg or ΔpilE mutant (data not shown), suggesting that these molecules are not differentially altered in the mutants. Although it is certainly plausible that there are distinct mechanisms by which piliated Gc maintain resistance to H₂O₂, PMN, and LL-37, it is possible that there is a common physiological change in piliated Neisseria gonorrhoeae that renders the organism more resistant to killing by both H₂O₂ and PMNs.

The gonococcal type IV pilus is the linchpin of gonococcal pathogenesis. Several decades of work have catalogued the myriad functions of this organelle, and our current work has revealed two new pilus-dependent phenotypes: resistance to H₂O₂ and PMN-mediated killing. These findings may alter the conclusion of the role of the pilus in cell interactions if differential survival of the bacteria are not taken into account. The observation that pilus expression is required for resistance may also explain the strong selection for pilated clinical isolates. Most clinical isolates are taken from the purulent exudate that is characteristic of symptomatic gonorrhea. The ability of the pilus to confer resistance to H₂O₂- and PMN-mediated killing enriches for pilated gonococci in the purulent exudate, where PMNs are in excess. Since pili are
required for full colonization and the establishment of disease, the linkage of resistance to piliation provides a unique mechanism for maximizing the infectious potential of this strict human pathogen. This report also raises the possibility that underpiliated or nonpiliated Gc variants have unrealized roles in the pathogenesis of gonorrhea.

**MATERIALS AND METHODS**

**Bacterial strains and media.** All gonococcal strains were derivatives of FA1090 or MS11. Gonococcal strains were grown at 37°C on Gc medium base (GCB; Difco) plus Kellogg supplement I (22.2 mM glucose, 0.45 mM cocarboxylase) and II [1.23 mM Fe(NO3)3] (44) at 37°C in 5% CO2 or in Gc liquid (GCBL) medium (1.5% proteose peptone no. 3 [Difco], 0.4% K2HPO4, 0.1% KH2PO4, 0.1% NaCl). Liquid-grown gonococcal strains additionally contained Kellogg supplement I and II and 0.042% sodium bicarbonate and were grown to exponential phase as previously described (22). Cultures were then grown at 37°C to mid-log phase (optical density at 600 nm [OD600] ≈ 0.5). The following antibiotics (Sigma) were used at the concentrations for *Escherichia coli*: kanamycin (Kan), 40 μg/ml; erythromycin (Erm), 275 μg/ml; and chloramphenicol (Cam), 25 μg/ml. For Gc, the concentrations were 0.75 μg/ml Erm, 40 μg/ml Kan, and 0.2 μg/ml tetracycline (Tet).

![A. -IPTG](image1.png) ![B. +IPTG](image2.png) ![C.](image3.png)

**FIG 8** Effect of *pilT* mutation on the RM11.2<sub>mpg</sub> piliation state and H<sub>2</sub>O<sub>2</sub> resistance. (A and B) Representative immunoelectron micrographs of immunogold-labeled pilus bundles on strain RM11.2<sub>mpg</sub> with regulatable *pilT* grown on solid medium in the presence (B) or absence (A) of IPTG and lifted onto Formvar-coated grids. (C) H<sub>2</sub>O<sub>2</sub> resistance of the strain in the presence and absence of IPTG. Cells were treated with the indicated doses of H<sub>2</sub>O<sub>2</sub> for 15 min, and the relative survival at each dose was calculated. Error bars represent the standard errors of the means of results from 2 to 8 experiments. *, P > 0.48.
Construction of strains. All RM11.2 (45) strains with the subscript “nv” (indicating “nonvarying”) carry a Tn3 mutation upstream of the pilE gene that abrogates antigenic pilin variation (32). RM11.2_nv mpp was created by transforming RM11.2_nv with plasmid construct pBLUNT/1686Erm and selecting for colonies on GCB-Erm using spot transformation (46). The altered colony morphology as well as PCR confirmed replacement with the mutated allele. This strain was subsequently transformed with genomic DNA containing the IPTG-regulatable pilT allele (37) and plated on GCB-Tet medium to select for transformants. Tet’ transformants were screened for the characteristic pilT colony morphology and were verified to contain the mutant mpp allele by selection on GCB-Erm and PCR of the mpp gene. To create strains MS11 ΔpilE and FA1090 mpp ΔpilE, genomic DNA from strain FA1090 ΔpilE, which was created by transforming genomic DNA from strain RM11.2 recA6 ΔpilE (47), was used to transform strains MS11 and FA1090 mpp. Colonies exhibiting a nonpiliated colony morphology were screened for full deletion of the pilE gene by PCR using primers OPAEI and PLRBS.

Pilus detection. Immuno electron microscopy was performed as described previously (30). Grids were viewed using a FEI JEM-1220 transmission electron microscope.

Pilus purification. Pil were purified using the method described in reference 37.

Killing assays. Gc were grown as described above to mid-log phase, and H2O2 killing assays were performed as described previously (22).

Adherent, interleukin 8 (IL-8)-treated, primary human PMNs were collected from consenting healthy volunteers, according to a protocol approved by the University of Virginia Institutional Review Board for Health Sciences Research. Bacterial survival after exposure to PMNs was determined from cell lysates at the time points indicated in the figures by colony counting as described in reference 22.

Mid-logarithmic-phase Gc were exposed to increasing concentrations of LL-37 (synthesized by Jan Pohl, Centers for Disease Control and Prevention) dissolved in 0.01% acetic acid as described previously (20) and in reference 22.

Immunoelectron microscopy was performed as described in reference 7.

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