Neisseria gonorrhoeae Metalloprotease NGO1686 Is Required for Full Piliation, and Piliation Is Required for Resistance to H_2O_2- and Neutrophil-Mediated Killing

Elizabeth A. Stohl,[a] Erin M. Dale,[b] Alison K. Criss,[b] H. Steven Seifert[a]

Department of Microbiology-Immunology, Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA[a]; Department of Microbiology, Immunology and Cancer Biology, University of Virginia Health Sciences Center, Charlottesville, Virginia, USA[b]

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_2O_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_2O_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_2O_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.

Address correspondence to H. Steven Seifert, h-seifert@northwestern.edu.

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 absent by inactivation of the twitching 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 H_2O_2, and *mpg* mutants are sensitive to H_2O_2 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 H_2O_2 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 (RM11.2$_{nv}$, 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 RM11.2$_{nv}$ *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 (RM11.2$_{nv}$) 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.
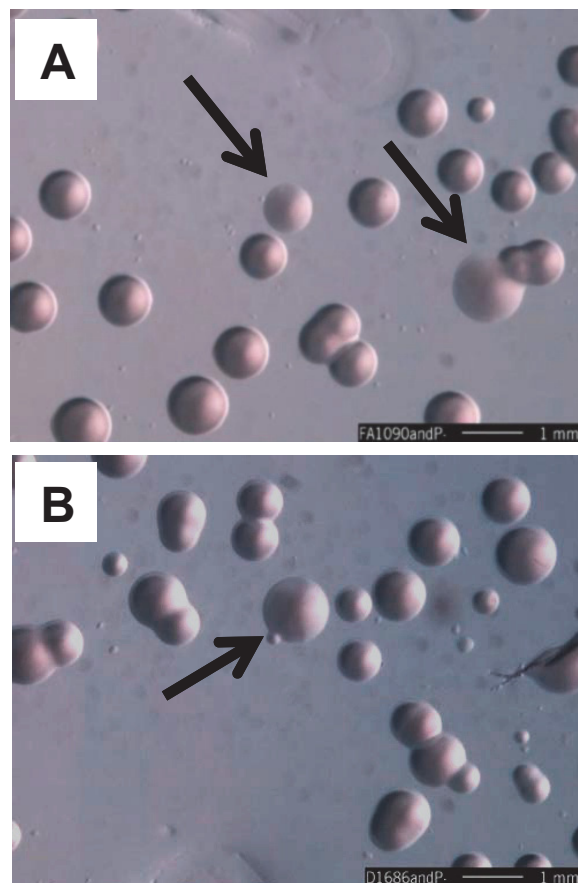

FIG 1 Colony morphologies of strains FA1090, the *mpg* (Δ1686) mutant, and nonpiliated (P^-) derivatives. (A) FA1090; (B) *mpg* mutant. Arrows indicate colonies with P^- morphologies among P^+ colonies. The more P^+ colonies of the *mpg* strain show a colony morphology intermediate between those of FA1090 P^+ and FA1090 P^-. Representative stereomicroscope images of strains after 24 h of growth on solid media (bars, 1 mm). Smaller colonies can appear with both strains for unknown reasons.

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 H_2O_2- and PMN-mediated killing may also be attributable to decreased piliation.

The decreased resistance of the *mpg* mutant to H_2O_2- and PMN-mediated killing is due to decreased piliation. To genetically test whether the *mpg* strain's H_2O_2 sensitivity phenotype was
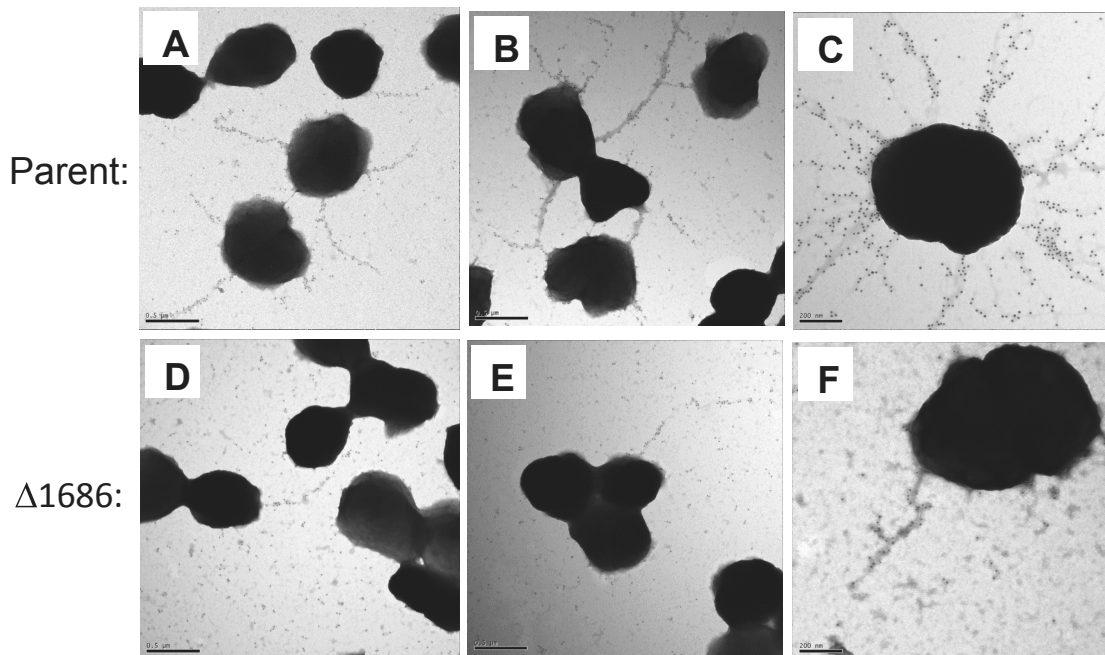
FIG 2 Electron micrographs of immunogold-labeled bundled pili on strains RM11.2$_{nv}$ and RM11.2$_{nv}$ *mpg* (Δ1686). Representative electron micrographs are shown for strain RM11.2$_{nv}$ (parent) and RM11.2$_{nv}$ *mpg* (Δ1686). Bars, 0.5 µm (A, B, D, and E) and 200 nm (C and F). Pili were detected using a rabbit antipeptide polyclonal antiserum directed against the RM11.2 hypervariable pilin sequence and a gold-labeled secondary antibody.

mediated through pili, we compared the levels of H$_2$O$_2$ resistance conferred by the Δ*pilE*, *mpg*, and Δ*pilE mpg* mutations in strain FA1090 (variant 1-81-S2) (Fig. 4A). The Δ*pilE* strain was less resistant to H$_2$O$_2$ than the *mpg* strain, but both the *mpg* and Δ*pilE* strains were less resistant than the parental strain, consistent with the complete lack of piliation in the Δ*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 Δ*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 Δ*pilE* single mutants, and the Δ*pilE mpg* double mutant. The *mpg* strain showed the previously reported decreased resistance to PMN-mediated killing (Fig. 4C, and see reference 22). The Δ*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 Δ*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 Δ*pilE* single mutants,
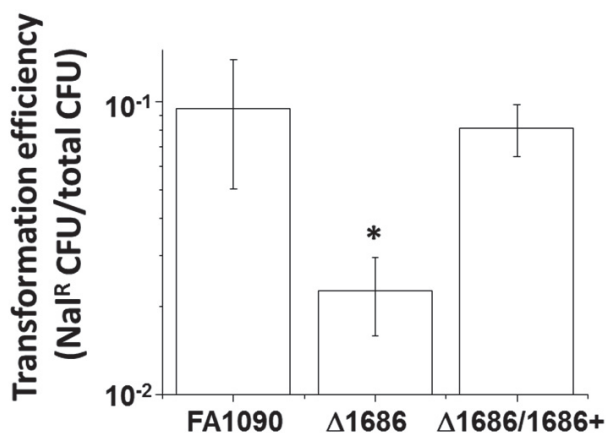

FIG 3 Transformation efficiencies of the indicated strains to nalidixic acid resistance (Nal^r) by plasmid pSY6. Error bars represent the standard errors of the means of results from at least 4 experiments. *, $P < 0.02$, relative to values for FA1090 and the Δ1686 1686+ mutant (D1686/1686+), by Student's two-tailed *t* test.
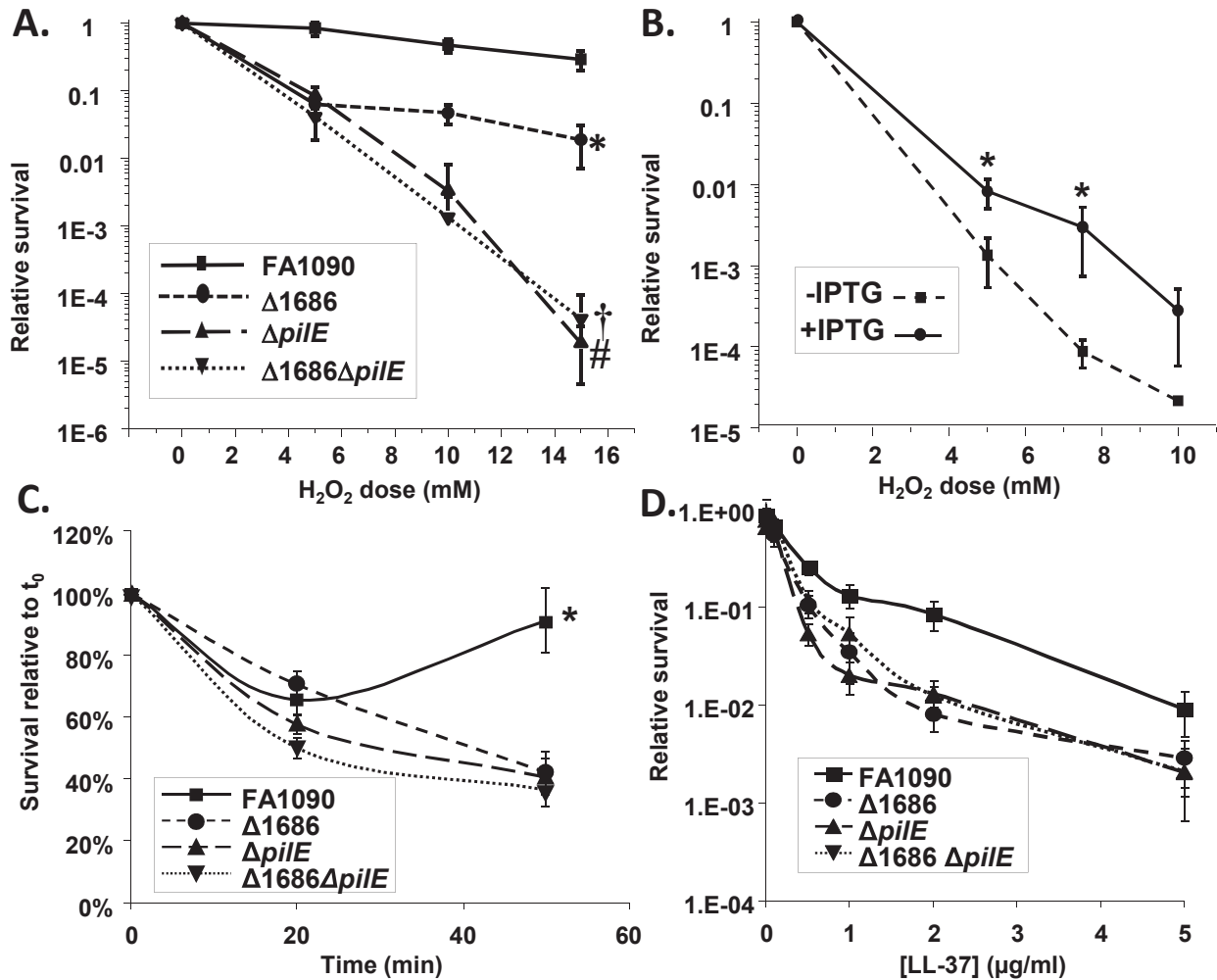
FIG 4 Effect of piliation on H_2O_2 and PMN-mediated resistance. (A and B) Dose-response curves of the H_2O_2 resistance of the indicated gonococcal strains after 15 min of treatment (A) and of regulatable *N. gonorrhoeae* strain RM11.2 either expressing pili (+IPTG) or not expressing pili (−IPTG) (B). (A) Error bars represent the standard errors of the means from at least 4 experiments. *, $P < 0.02$, in comparisons with all strains at 5, 10, and 15 mM H_2O_2; #, $P < 7.8E-4$, in comparisons with the *mpg* (Δ1686) mutant and FA1090 at 10 and 15 mM; †, $P > 0.37$, in a comparison with the Δ*pilE* strain at all doses by Student's two-tailed *t* test. (B) Error bars represent the standard errors of the means of results from at least 3 experiments. *, $P < 0.04$, relative to values for regulatable strain RM11.2 (without IPTG) at the indicated doses by Student's two-tailed *t* test. (C) PMN-mediated survival of the indicated strains over time relative to that at time zero (t_0). At 50 min, P was <0.005 for the Δ1686 mutant versus the wild type, the *pilE* strain versus the wild type, and the double mutant versus the wild type. (D) LL-37 resistance. The indicated strains were exposed to increasing concentrations of LL-37. Bacterial survival after 45 min is expressed as survival relative to that of the inoculum. Statistically significant survival differences were obtained for FA1090 versus the Δ1686 mutant at 0.5, 1, and 2 μg/ml LL-37 and for FA1090 versus the Δ*pilE* mutant at 0.5 and 1 μg/ml LL-37 ($P < 0.05$, Student's two-tailed *t* test).

and the Δ*pilE 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 H_2O_2 and PMN-mediated killing, we examined the effect of a *pilQ* mutation on resistance to H_2O_2 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 H_2O_2 and PMN killing (Fig. 5). A *pilC* mutant, which is also P−, was also less resistant to H_2O_2 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 H_2O_2 (data not shown).

***Pseudomonas aeruginosa* type IV pili do not confer resistance to H_2O_2 killing.** We next considered whether H_2O_2 resistance is a trait conferred by all type IV pili. We measured the H_2O_2 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 H_2O_2 resistance on *P. aeruginosa* (Fig. 6). These results indicate that H_2O_2 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.
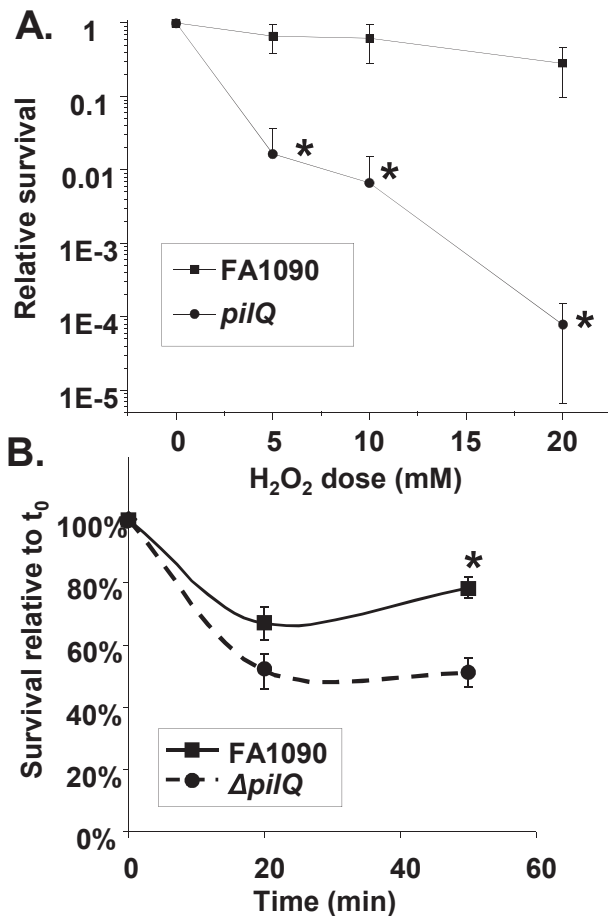
FIG 5 Resistance of *pilQ* mutant strains to H_2O_2 and PMN-mediated killing. (A) Dose-response curve of H_2O_2 resistance after 15 min of treatment of the *pilQ* mutant strain. *, $P < 0.02$. (B) Resistance of the *pilQ* mutant strain to PMN-mediated killing. *, $P < 0.0005$ (Student's two-tailed *t* test).
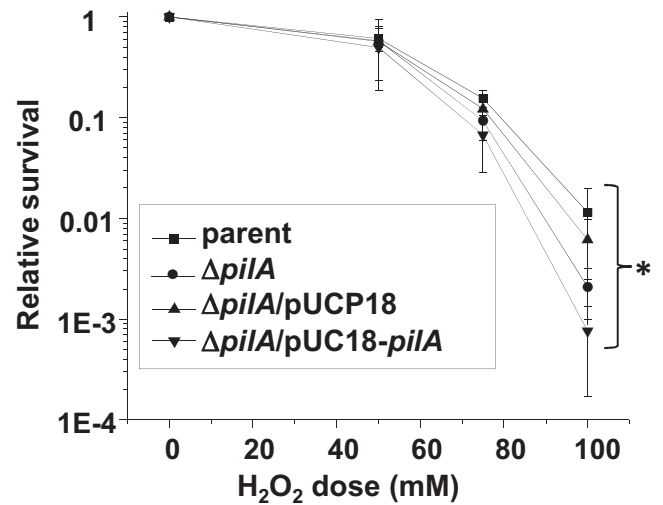

FIG 6 Relative survival of *P. aeruginosa* strain 15692, the isogenic *pilA* mutant, an empty vector control, and the *pilA* complemented strain. Error bars represent the standard errors of the means of results from at least 5 experiments. *, $P > 0.21$, between all strains at all doses.

Resistance to H_2O_2 requires active pili. To determine whether functional pili or merely the presence of pili affects resistance to H_2O_2 killing, we measured the effect of *pilT* mutation on H_2O_2 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 H_2O_2 than the parent strain (Fig. 7A), showing that active pili are necessary to mediate resistance to H_2O_2 killing. We also purified pili from a distinct gonococcal strain, strain MS11, and added them to MS11 Δ*pilE* bacteria to determine whether the pili were able to confer protection on the Δ*pilE* strain. The addition of purified pili did not confer H_2O_2 resistance to the MS11 Δ*pilE* strain (Fig. 7B). These data indicate that active pili are required to mediate resistance to H_2O_2 and that the absence of pili in both strain FA1090 and strain MS11 leads to decreased H_2O_2 resistance.

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 piliation (4, 5, 38). We therefore introduced an IPTG-regulatable *pilT* (reg *pilT*) construct (37) into the FA1090 (RM11.2$_{nv}$) *mpg* mutant background

and used immunogold transmission EM (immunogold-TEM) to analyze piliation. In the presence of IPTG, strain RM11.2$_{nv}$ *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 RM11.2$_{nv}$ *mpg* (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 RM11.2$_{nv}$ (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 H_2O_2 resistance of the RM11.2$_{nv}$ *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 H_2O_2. The strain showed the same level of H_2O_2 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 H_2O_2.

DISCUSSION

We have determined that the three previously described phenotypes conferred by the PG-targeted metalloprotease NGO1686—colony morphology, resistance to H_2O_2, 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 piliation (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).
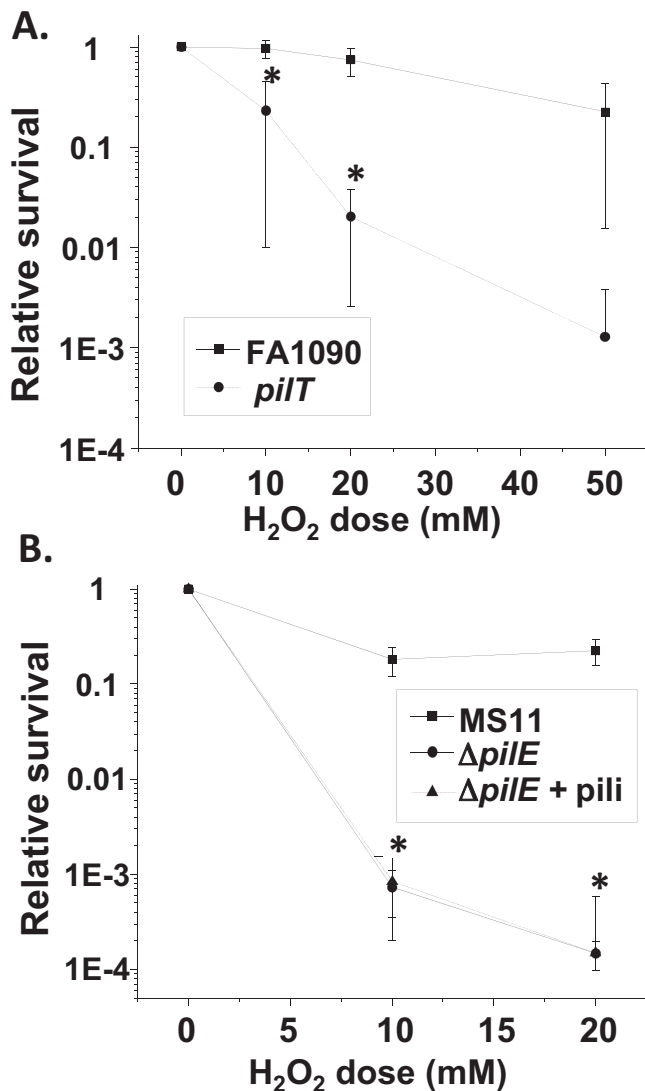
FIG 7 Effect of active pili on H_2O_2 resistance. (A) Dose-response curve of H_2O_2 resistance after 15 min of treatment of strains FA1090 and FA1090 *pilT*. Error bars represent the standard errors of the means of results of 4 to 8 experiments. *, $P < 0.05$, between strains at the indicated doses. (B) H_2O_2 resistance of *N. gonorrhoeae* strain MS11 and the isogenic Δ*pilE* mutant with and without purified pili added back. Error bars represent the standard errors of the means of results from 2 experiments. *, $P > 0.89$, between the Δ*pilE* strain and the Δ*pilE* strain with pili added at both doses.

The enzymatic activity of PleA has not been verified experimentally, but it is predicted to encode a lytic transglycosylase, and mutation of the proposed active site recapitulates the *pleA* mutant phenotype (40). Whereas lytic transglycosylases cleave the PG sugar backbone, NGO1686 cleaves PG side chains. NGO1686 is the first carboxypeptidase/endopeptidase demonstrated to function in type IV pilus biogenesis. Results from a tBLASTn search of available bacterial entries revealed that all of the genomes containing NGO1686 homologues (defined as having >50% amino acid similarity over >66% of the protein) also contained genes coding for either type IV pili or flagellar-biogenesis proteins. These data suggest that NGO1686 homologues may also have a role in pilus biogenesis in other species.

The exact role that NGO1686 plays in pilus biogenesis is un-

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 pili from neighboring bacteria (PilX) (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 piliation 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 H_2O_2- and PMN-mediated killing. This observation is even more surprising given that H_2O_2 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 H_2O_2 associated with them. Our observations that only "active" pili (from *pilT*-expressing strains) and not exogenously added pili have a role in H_2O_2 resistance show that inert pili do not function to provide H_2O_2 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 Δ*pilE* 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 H_2O_2, PMN, and LL-37, it is possible that there is a common physiological change in piliated *N. gonorrhoeae* that renders the organism more resistant to killing by both H_2O_2 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 H_2O_2 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 piliated 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 H_2O_2- and PMN-mediated killing enriches for piliated gonococci in the purulent exudate, where PMNs are in excess. Since pili are
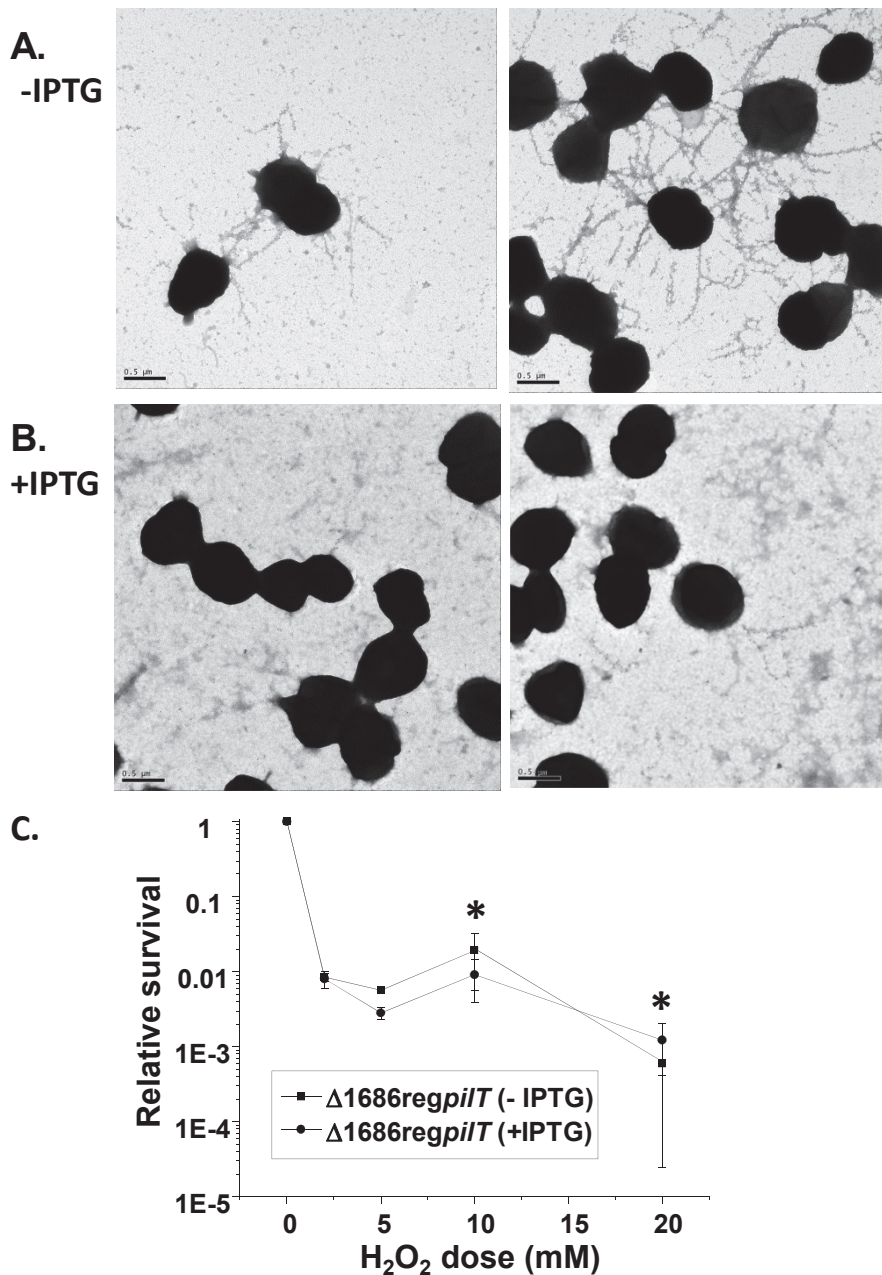
FIG 8 Effect of *pilT* mutation on the RM11.2$_{nv}$ *mpg* (Δ1686) piliation state and H_2O_2 resistance. (A and B) Representative immunoelectron micrographs of immunogold-labeled pilus bundles on strain RM11.2$_{nv}$ *mpg* with regulatable *pilT* grown on solid medium in the presence (B) or absence (A) of IPTG and lifted onto Formvar-coated grids. (C) H_2O_2 resistance of the strain in the presence and absence of IPTG. Cells were treated with the indicated doses of H_2O_2 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. *, $P > 0.48$.

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, 0.68 mM glutamine, 0.45 mM cocarboxylase) and II [1.23 mM Fe(NO$_3$)$_3$] (44) at 37°C in 5% CO_2 or in Gc liquid (GCBL) medium (1.5% proteose peptone no. 3 [Difco], 0.4% K_2HPO_4, 0.1% KH_2PO_4, 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 [OD$_{600}$] ≅ 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).

Construction of strains. All RM11.2 (45) strains with the subscript "nv" (indicating "nonvarying") carry a Tn*3* mutation upstream of the *pilE* gene that abrogates antigenic pilin variation (32). RM11.2$_{nv}$ *mpg* 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^r transformants were screened for the characteristic *pilT* colony morphology and were verified to contain the mutant *mpg* allele by selection on GCB-Erm^r and PCR of the *mpg* mutant. To create strains MS11 Δ*pilE* and FA1090 *mpg* Δ*pilE*, genomic DNA from strain FA1090 Δ*pilE*, which was created by transforming genomic DNA from strain RM11.2 *recA6* Δ*pilE* (47), was used to transform strains MS11 and FA1090 *mpg*. Colonies exhibiting a nonpiliated colony morphology were screened for full deletion of the *pilE* gene by PCR using primers OPAEJ and PILRBS.

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

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

Killing assays. Gc were grown as described above to mid-log phase, and H_2O_2 killing assays were performed as described previously (22).

Adherent, interleukin 8 (IL-8)-treated, primary human PMNs were collected from consenting healthy human 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 $0.1\times$ GCBL with 20 mM HEPES, pH 7.4. Bacterial survival at each concentration was calculated as the CFU enumerated after 45 min divided by the number of CFU present in the input. Results are the averages ± standard errors of the means from 3 to 7 replicates per strain.

REFERENCES

1. **Swanson J, Robbins K, Barrera O, Corwin D, Boslego J, Ciak J, Blake M, Koomey JM.** 1987. Gonococcal pilin variants in experimental gonorrhea. J. Exp. Med. **165**:1344–1357.
2. **Pelicic V.** 2008. Type IV pili: e pluribus unum? Mol. Microbiol. **68**:827–837.
3. **Varga JJ, Therit B, Melville SB.** 2008. Type IV pili and the CcpA protein are needed for maximal biofilm formation by the gram-positive anaerobic pathogen *Clostridium perfringens*. Infect. Immun. **76**:4944–4951.
4. **Winther-Larsen HC, Wolfgang M, Dunham S, van Putten JP, Dorward D, Løvold C, Aas FE, Koomey M.** 2005. A conserved set of pilin-like molecules controls type IV pilus dynamics and organelle-associated functions in *Neisseria gonorrhoeae*. Mol. Microbiol. **56**:903–917.
5. **Carbonnelle E, Helaine S, Nassif X, Pelicic V.** 2006. A systematic genetic analysis in *Neisseria meningitidis* defines the Pil proteins required for assembly, functionality, stabilization and export of type IV pili. Mol. Microbiol. **61**:1510–1522.
6. **Tønjum T, Koomey M.** 1997. The pilus colonization factor of pathogenic neisserial species: organelle biogenesis and structure/function relationships—a review. Gene **192**:155–163.
7. **Criss AK, Seifert HS.** 2012. A bacterial siren song: intimate interactions between *Neisseria* and neutrophils. Nat. Rev. Microbiol. **10**:178–190.
8. **Johnson MB, Criss AK.** 2011. Resistance of *Neisseria gonorrhoeae* to neutrophils. Front. Microbiol. **2**:77.
9. **Soler-García AA, Jerse AE.** 2004. A *Neisseria gonorrhoeae* catalase mutant is more sensitive to hydrogen peroxide and paraquat, an inducer of toxic oxygen radicals. Microb. Pathog. **37**:55–63.
10. **Tseng HJ, Srikhanta Y, McEwan AG, Jennings MP.** 2001. Accumulation of manganese in *Neisseria gonorrhoeae* correlates with resistance to oxidative killing by superoxide anion and is independent of superoxide dismutase activity. Mol. Microbiol. **40**:1175–1186.
11. **Turner S, Reid E, Smith H, Cole J.** 2003. A novel cytochrome *c* peroxidase from *Neisseria gonorrhoeae*: a lipoprotein from a gram-negative bacterium. Biochem. J. **373**:865–873.
12. **Skaar EP, Tobiason DM, Quick J, Judd RC, Weissbach H, Etienne F, Brot N, Seifert HS.** 2002. The outer membrane localization of the *Neisseria gonorrhoeae* MsrA/B is involved in survival against reactive oxygen species. Proc. Natl. Acad. Sci. U. S. A. **99**:10108–10113.
13. **Stohl EA, Seifert HS.** 2006. *Neisseria gonorrhoeae* DNA recombination and repair enzymes protect against oxidative damage caused by hydrogen peroxide. J. Bacteriol. **188**:7645–7651.
14. **Wu H, Soler-García AA, Jerse AE.** 2009. A strain-specific catalase mutation and mutation of the metal-binding transporter gene *mntC* attenuate *Neisseria gonorrhoeae* in vivo but not by increasing susceptibility to oxidative killing by phagocytes. Infect. Immun. **77**:1091–1102.
15. **Wu HJ, Seib KL, Edwards JL, Apicella MA, McEwan AG, Jennings MP.** 2005. Azurin of pathogenic *Neisseria* spp. is involved in defense against hydrogen peroxide and survival within cervical epithelial cells. Infect. Immun. **73**:8444–8448.
16. **Criss AK, Katz BZ, Seifert HS.** 2009. Resistance of *Neisseria gonorrhoeae* to non-oxidative killing by adherent human polymorphonuclear leucocytes. Cell. Microbiol. **11**:1074–1087.
17. **Seib KL, Simons MP, Wu HJ, McEwan AG, Nauseef WM, Apicella MA, Jennings MP.** 2005. Investigation of oxidative stress defenses of *Neisseria gonorrhoeae* by using a human polymorphonuclear leukocyte survival assay. Infect. Immun. **73**:5269–5272.
18. **Levy O.** 2004. Antimicrobial proteins and peptides: anti-infective molecules of mammalian leukocytes. J. Leukoc. Biol. **76**:909–925.
19. **Lee EH, Shafer WM.** 1999. The *farAB*-encoded efflux pump mediates resistance of gonococci to long-chained antibacterial fatty acids. Mol. Microbiol. **33**:839–845.
20. **Shafer WM, Qu X, Waring AJ, Lehrer RI.** 1998. Modulation of *Neisseria gonorrhoeae* susceptibility to vertebrate antibacterial peptides due to a member of the resistance/nodulation/division efflux pump family. Proc. Natl. Acad. Sci. U. S. A. **95**:1829–1833.
21. **Jerse AE, Sharma ND, Simms AN, Crow ET, Snyder LA, Shafer WM.** 2003. A gonococcal efflux pump system enhances bacterial survival in a female mouse model of genital tract infection. Infect. Immun. **71**:5576–5582.
22. **Stohl EA, Criss AK, Seifert HS.** 2005. The transcriptome response of *Neisseria gonorrhoeae* to hydrogen peroxide reveals genes with previously uncharacterized roles in oxidative damage protection. Mol. Microbiol. **58**:520–532.
23. **Stohl EA, Chan YA, Hackett KT, Kohler PL, Dillard JP, Seifert HS.** 2012. *Neisseria gonorrhoeae* virulence factor NG1686 is a bifunctional M23B family metallopeptidase that influences resistance to hydrogen peroxide and colony morphology. J. Biol. Chem. **287**:11222–11233.
24. **Boneca IG.** 2005. The role of peptidoglycan in pathogenesis. Curr. Opin. Microbiol. **8**:46–53.
25. **Sycuro LK, Pincus Z, Gutierrez KD, Biboy J, Stern CA, Vollmer W, Salama NR.** 2010. Peptidoglycan crosslinking relaxation promotes *Helicobacter pylori*'s helical shape and stomach colonization. Cell **141**:822–833.
26. **Goley ED, Comolli LR, Fero KE, Downing KH, Shapiro L.** 2010. DipM links peptidoglycan remodelling to outer membrane organization in *Caulobacter*. Mol. Microbiol. **77**:56–73.
27. **Möll A, Schlimpert S, Briegel A, Jensen GJ, Thanbichler M.** 2010. DipM, a new factor required for peptidoglycan remodelling during cell division in *Caulobacter crescentus*. Mol. Microbiol. **77**:90–107.
28. **Poggio S, Takacs CN, Vollmer W, Jacobs-Wagner C.** 2010. A protein critical for cell constriction in the gram-negative bacterium *Caulobacter crescentus* localizes at the division site through its peptidoglycan-binding LysM domains. Mol. Microbiol. **77**:74–89.

29. **Uehara T, Dinh T, Bernhardt TG.** 2009. LytM-domain factors are required for daughter cell separation and rapid ampicillin-induced lysis in *Escherichia coli*. J. Bacteriol. **191**:5094–5107.

30. **Long CD, Tobiason DM, Lazio MP, Kline KA, Seifert HS.** 2003. Low-level pilin expression allows for substantial DNA transformation competence in *Neisseria gonorrhoeae*. Infect. Immun. **71**:6279–6291.

31. **Cahoon LA, Seifert HS.** 2009. An alternative DNA structure is necessary for pilin antigenic variation in *Neisseria gonorrhoeae*. Science **325**:764–767.

32. **Sechman EV, Rohrer MS, Seifert HS.** 2005. A genetic screen identifies genes and sites involved in pilin antigenic variation in *Neisseria gonorrhoeae*. Mol. Microbiol. **57**:468–483.

33. **Sparling PF.** 1966. Genetic transformation of *Neisseria gonorrhoeae* to streptomycin resistance. J. Bacteriol. **92**:1364–1371.

34. **Long CD, Hayes SF, van Putten JP, Harvey HA, Apicella MA, Seifert HS.** 2001. Modulation of gonococcal piliation by regulatable transcription of *pilE*. J. Bacteriol. **183**:1600–1609.

35. **Drake SL, Koomey M.** 1995. The product of the *pilQ* gene is essential for the biogenesis of type IV pili in *Neisseria gonorrhoeae*. Mol. Microbiol. **18**:975–986.

36. **Shrout JD, Chopp DL, Just CL, Hentzer M, Givskov M, Parsek MR.** 2006. The impact of quorum sensing and swarming motility on *Pseudomonas aeruginosa* biofilm formation is nutritionally conditional. Mol. Microbiol. **62**:1264–1277.

37. **Wolfgang M, Lauer P, Park HS, Brossay L, Hébert J, Koomey M.** 1998. PilT mutations lead to simultaneous defects in competence for natural transformation and twitching motility in piliated *Neisseria gonorrhoeae*. Mol. Microbiol. **29**:321–330.

38. **Carbonnelle E, Hélaine S, Prouvensier L, Nassif X, Pelicic V.** 2005. Type IV pilus biogenesis in *Neisseria meningitidis*: PilW is involved in a step occurring after pilus assembly, essential for fibre stability and function. Mol. Microbiol. **55**:54–64.

39. **Scheurwater EM, Burrows LL.** 2011. Maintaining network security: how macromolecular structures cross the peptidoglycan layer. FEMS Microbiol. Lett. **318**:1–9.

40. **Viollier PH, Shapiro L.** 2003. A lytic transglycosylase homologue, PleA, is required for the assembly of pili and the flagellum at the *Caulobacter crescentus* cell pole. Mol. Microbiol. **49**:331–345.

41. **Helaine S, Dyer DH, Nassif X, Pelicic V, Forest KT.** 2007. 3D structure/function analysis of PilX reveals how minor pilins can modulate the virulence properties of type IV pili. Proc. Natl. Acad. Sci. U. S. A. **104**:15888–15893.

42. **Winther-Larsen HC, Hegge FT, Wolfgang M, Hayes SF, van Putten JP, Koomey M.** 2001. *Neisseria gonorrhoeae* PilV, a type IV pilus-associated protein essential to human epithelial cell adherence. Proc. Natl. Acad. Sci. U. S. A. **98**:15276–15281.

43. **Wolfgang M, van Putten JP, Hayes SF, Dorward D, Koomey M.** 2000. Components and dynamics of fiber formation define a ubiquitous biogenesis pathway for bacterial pili. EMBO J. **19**:6408–6418.

44. **Kellogg DS, Jr, Peacock WL, Deacon WE, Brown L, Pirkle CI.** 1963. *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. J. Bacteriol. **85**:1274–1279.

45. **Long CD, Madraswala RN, Seifert HS.** 1998. Comparisons between colony phase variation of *Neisseria gonorrhoeae* FA1090 and pilus, pilin, and S-pilin expression. Infect. Immun. **66**:1918–1927.

46. **Stein DC.** 1991. Transformation of *Neisseria gonorrhoeae*: physical requirements of the transforming DNA. Can. J. Microbiol. **37**:345–349.

47. **Chen CJ, Tobiason DM, Thomas CE, Shafer WM, Seifert HS, Sparling PF.** 2004. A mutant form of the *Neisseria gonorrhoeae* pilus secretin protein PilQ allows increased entry of heme and antimicrobial compounds. J. Bacteriol. **186**:730–739.