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Structure/Function Analysis of *Neisseria meningitidis* PilW, a Conserved Protein That Plays Multiple Roles in Type IV Pilus Biology▽

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Type IV pili (Tfp) are widespread filamentous bacterial organelles that mediate multiple functions and play a key role in pathogenesis in several important human pathogens, including *Neisseria meningitidis*. Tfp biology remains poorly understood at a molecular level because the roles of the numerous proteins that are involved remain mostly obscure. Guided by the high-resolution crystal structure we recently reported for *N. meningitidis* PilW, a widely conserved protein essential for Tfp biogenesis, we have performed a structure/function analysis by targeting a series of key residues through site-directed mutagenesis and analyzing the corresponding variants using an array of phenotypic assays. Here we show that PilW's involvement in the functionality of Tfp can be genetically uncoupled from its concurrent role in the assembly/stabilization of the secretin channels through which Tfp emerge on the bacterial surface. These findings suggest that PilW is a multifunctional protein.

Throughout the bacterial kingdom, survival is linked to successful colonization of a niche favorable for bacterial growth. This is often mediated by adhesive, surface-exposed, hair-like filaments called pili or fimbriae (40). Of the several types of pili that have been identified so far, the most widespread are type IV pili (Tfp) (33). These organelles continue to generate much research interest because they (i) play a key role in the virulence of important human pathogens (e.g., enteropathogenic *Escherichia coli*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Vibrio cholerae*, *Pseudomonas aeruginosa*) and (ii) display astonishing functional versatility (33). In addition to their role in promoting attachment to a variety of surfaces (a property that they share with other types of pili), Tfp often mediate competence for natural transformation, formation of bacterial aggregates and biofilms, and a flagellum-independent form of locomotion termed twitching motility (3, 26). This functional versatility is attributable to the uncommon capacity of Tfp to be retracted, thereby generating remarkable mechanical force (25, 28).

Tfp are helical polymers composed mainly of one protein generically named pilin. High-resolution structures of pilins from different bacterial species revealed that the conserved, hydrophobic N-terminal domains of these proteins form an extended α-helix representing the primary assembly interface of Tfp and are buried within the interior of the filament (9, 10). Despite the availability of structural models for Tfp and the rather simple composition of these filaments, our understanding of how Tfp are actually assembled remains at best fragmentary. This shortfall is due mainly to the involvement of numerous dedicated proteins in Tfp biogenesis, from 10 in *V. cholerae* to 18 in *P. aeruginosa* (33). Moreover, it has been shown in bacteria expressing type IVa filaments—by far the more widespread subtype—that these Pil proteins (which are highly conserved) act at multiple steps during Tfp biogenesis (4, 46, 47). First, the pilin (PilE in *N. meningitidis* nomenclature, which is used throughout this report) that is synthesized as a preprotein is inserted in the inner membrane by the Sec machinery (1, 13). The leader sequence of the prepilin is then proteolytically cleaved by the dedicated inner membrane prepilin peptidase PilD (41). The processed PilE subunits are thought to be extruded or extracted from the membrane, a process that requires energy provided by the cytoplasmic ATPase PilF (43) and assembled into a helical filament at the inner membrane by a multiprotein machinery consisting at least of PilM, PilN, PilO, and PilP (4). Tfp then emerge on the surface through channels in the outer membrane that are formed by homomultimers of the secretin PilQ (4, 47). True to the dynamic nature of Tfp, pilus extension (assembly) powered by PilF is countered by pilus retraction (disassembly) powered by another cytoplasmic ATPase, PilT (39, 45). Consequently, the remaining proteins involved in Tfp biogenesis (PilC, PilG, PilH, PilI, PilJ, PilK, and PilW) are not involved in pilus assembly *per se* but act individually or collectively after pilus assembly to antagonize pilus retraction. This was shown by the restoration of Tfp in mutants of these genes in which pilus retraction was abolished by a concurrent mutation in *pilT* (4). Moreover, since the filaments restored in these double mutants are often not or only poorly functional (e.g., the *pilWT* mutant is dramatically affected for adhesion to human cells), these proteins also play a role in modulating Tfp-mediated functions (4).

The exact roles played by a majority of the Pil proteins remain unclear, emphasizing the need for further studies. One of the proteins that has been characterized in more detail is the putative outer membrane lipoprotein PilW, which consists of a series of tetratricopeptide repeat (TPR) motifs. TPRs are

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widespread protein domains involved in protein-protein interactions, mainly those between TPR-containing proteins and multiprotein complexes (12). It has been found in *N. meningitidis* that PilW, which is involved in the late stages of Tfp biogenesis, also modulates the assembly or stabilization of the secretin channels through which Tfp emerge on the bacterial surface (5). In the absence of PilW, the normally heat- and SDS-resistant PilQ multimers, which remain trapped in the wells of the stacking gel after SDS-PAGE, are no longer detectable by immunoblotting. Instead, only monomeric PilQ is detected. This was later confirmed for the PilW orthologs in *Myxococcus xanthus* and *P. aeruginosa* (22, 32). This defect is specific to *pilW* mutants since PilQ multimers could be detected in the absence of each of the other Pil components (4). These observations raised important questions. Is the role of PilW mainly to assist in the assembly of PilQ multimers and/or to contribute to their exceptional stability? In this case, the phenotypic defects in the absence of PilW could be a consequence of the absence of detectable PilQ multimers. Alternatively, are the absence of PilQ multimers and the absence of functional Tfp in the absence of PilW two unlinked, concurrent properties? In other words, is PilW a bifunctional protein? To address these questions, we have carried out and report here a structure/function analysis of PilW using as a blueprint the high-resolution crystal structure of this protein, which we recently reported (42).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The wild-type (WT) strain of *N. meningitidis* used in this study is a recently sequenced variant of the serogroup C clinical isolate 8013 expressing a highly adhesive pilin variant (31, 37). The *pilW* mutant strain 8013 comes from the NeMeSys archived library of defined transposition mutants and was described previously (5, 15, 37). *N. meningitidis* was grown on GCB agar (Difco) plates containing Kellogg's supplements and, when required, 100 μg/ml kanamycin, 5 μg/ml rifampin, 3 μg/ml erythromycin, and various concentrations of IPTG (isopropyl-β-D-thiogalactopyranoside) (Merck). Plates were incubated at 37°C in a moist atmosphere containing 5% CO_2. *E. coli* DH5α was used for cloning experiments and was grown in liquid or solid Luria-Bertani medium (Difco) containing, when required, 100 μg/ml spectinomycin and 50 μg/ml kanamycin.

In order to complement the *pilW* mutant, we first amplified by PCR the *pilW* gene from strain 8013's genomic DNA using primers *pilW*-IndF and *pilW*-IndR flanked by PacI sites (underlined in Table 1). The resulting PCR fragment was cloned into pCR8/GW/TOPO (Invitrogen), generating pYU41, verified by sequencing and subcloned into pGCC4 restricted with PacI (27). This generated the pYU47 vector, which contains the wild-type *pilW* allele under the transcriptional control of an IPTG-inducible promoter (functional both in *E. coli* and *N. meningitidis*), within a DNA fragment corresponding to a region of the gonococcal genome conserved in *N. meningitidis* (2, 19). Since the *pilW* mutant is nonpiliated and noncompetent, pYU47 was first transformed into the WT strain, where it integrated ectopically by homologous recombination. This strain was subsequently transformed using genomic DNA extracted from a *pilW* mutant.

The same strategy was used to generate mutants expressing *pilW* alleles altered *in vitro* by site-directed mutagenesis. The variant alleles were generated using pYU41 as a template, specific pairs of complementary primers (see below), and a Quickchange site-directed mutagenesis kit (Stratagene) as was done previously for PilX (19). The primer pairs that we used were as follows (mismatched bases generating mutations are underlined in Table 1): C_{20}G#1 and C_{20}G#2, C_{115}G#1 and C_{115}G#2, C_{150}G#1 and C_{150}G#2, N_{108}A#1 and N_{108}A#2, N_{109}A#1 and N_{109}A#2, Y_{137}A#1 and Y_{137}A#2, and N_{146}A#1 and N_{146}A#2. All variant alleles were verified by sequencing before they were subcloned into pGCC4. Protein production upon IPTG induction was verified in *E. coli* whole-cell lysates by Coomassie staining.

The *N. meningitidis* strains generated were stored at −80°C, and all experiments were performed with bacteria from these frozen stocks grown overnight on plates in order to minimize secondary variations. As checked by sequencing, one base change in the WT *pilE* allele was observed in most of the strains, resulting in an $A_{167}T$ substitution. However, this had no impact on Tfp expression or on any of the Tfp-linked properties as determined by the analysis of the *pilW*/$pilW_{WT}$ complemented strain, which also contained such a substitution (Table 2).

TABLE 1. Primers used in this study

Name	Sequence[a]
pilW-IndF	CGCTTAATTAAGGAGTAATTTTATGCCTTTTAAG CCATCCAAAC
pilW-IndR	CGCTTAATTAATTATTGACCGGTGAGGACGG
C_{20}G#1	CTTGCCTTGGGCGCGGGCAGCACTTCCTACC
C_{20}G#2	GGTAGGAAGTGCTGCCGCGCCCAAGGCAAG
C_{115}G#1	CTACGGCTGGTTCCTGGGCGGCAGGCTCAACCG
C_{115}G#2	CGGTTGAGCCTGCCGCCCAGGAACCAGCCGTAG
C_{150}G#1	CCTGAATAAAGGCATAGGCAGCGCAAAACAGGG
C_{150}G#2	CCCTGTTTTGCGCTGCCTATGCCTTTATTCAGG
N_{108}A#1	CGACAGTGCCGAAATCAACGCCAACTACGGCTG GTTCCTG
N_{108}A#2	CAGGAACCAGCCGTAGTTGGCGTTGATTTCGGC ACTGTCG
N_{109}A#1	CAGTGCCGAAATCAACAACGCCTACGGCTGGTTC CTGTGC
N_{109}A#2	GCACAGGAACCAGCCGTAGGCGTTGTTGATTTC GGCACTG
Y_{137}A#1	GCCCTGGCCGACCCCACCGCCCCGACCCCTTATA TTGC
Y_{137}A#2	GCAATATAAGGGGTCGGGGCGGTGGGGTCGGCC AGGGC
N_{146}A#1	CCTTATATTGCCAACCTGGCTAAAGGCATATGCA GCGC
N_{146}A#2	GCGCTGCATATGCCTTTAGCCAGGTTGGCAATAT AAGG

[a] Underlining is defined in Materials and Methods.

SDS-PAGE, antisera, and immunoblotting. *N. meningitidis* whole-cell protein extracts were prepared as described previously (4). *E. coli* whole-cell protein extracts were prepared by resuspending pellets directly in Laemmli sample buffer (Bio-Rad). When necessary, proteins were quantified using a Bio-Rad protein assay as suggested by the manufacturer (Bio-Rad). Separation of the proteins by SDS-PAGE and subsequent blotting to Amersham Hybond ECL membranes (GE Healthcare) were done using standard molecular biology techniques (38). Blocking, incubation with primary/secondary antibodies, and detection using Amersham ECL Plus (GE Healthcare) were done following the manufacturer's instructions. Alternatively, gels were stained using Bio-Safe Coomassie stain (Bio-Rad).

PilW was detected using an affinity-purified rabbit antiserum at a 1/2,000 dilution (5). PilQ was detected using a rabbit antiserum at a 1/50,000 dilution (14). The rabbit antiserum 18P4 at a 1/2,000 dilution was used for PilC1 detection (29). A donkey anti-rabbit horseradish peroxidase (HRP)-linked secondary antibody (GE Healthcare) was used at a dilution of 1/10,000.

Tfp detection and purification. Tfp were stained with the 20D9 monoclonal antibody, which is specific for strain 8013's fibers (35), as previously described (5) and visualized by immunofluorescence (IF) microscopy on a Nikon Eclipse E600 microscope. Tfp were purified from two heavily inoculated agar plates by ammonium sulfate precipitation as previously described (2).

Transformation assay. Natural competence for DNA transformation was quantified as previously described by transforming bacteria using 1 μg genomic DNA extracted from a mutant of strain 8013 spontaneously resistant to rifampin (2). Results are expressed as percentages of recipient cells transformed and are the means ± standard deviations from at least 4 independent experiments.

Aggregation assay. Aggregates forming on the bottom of the wells in a 24-well plate format were visualized after 2 h by phase-contrast microscopy using a Nikon TS100F microscope as previously described (2). Digital images were recorded using a Sony HDR-CX11 high-definition (HD) camcorder.

Twitching motility assay. Twitching motility was assessed as described previously (2) by observing bacteria within aggregates that were attached to the bottom of the wells in 24-well plates in the aggregation assay (see above). When WT aggregates were observed, bacteria exhibited continuous and vigorous jerky movements clearly distinct in amplitude and speed from the Brownian motion exhibited by isolated diplococci. These jerky movements correspond to twitching motility, since the aggregates formed by a *pilT* mutant are completely still (2). Short HD videos were recorded using the camcorder mounted onto the microscope.

TABLE 2. Summary of the Tfp-linked phenotypic properties in PilW variants assessed during this study[a]

Strain genotype	Tfp biology feature[b]					
	Piliation	Aggregation	Twitching motility	Adherence to HUVEC	Competence	PilQ multimers
WT	+	+	+	+	+	+
pilW	−	−	ND	−	−	−
pilW/*pilW*$_{WT}$	+	+	+	+	+	+
pilW/*pilW*(*C20G*)	+/−	+/−	ND	−	+/−	−
pilW/*pilW*(*C20G*)[c]	+/−	+	+	−	+/−	+/−
pilW/*pilW*(*C115G*)	+/−	−	ND	−	+/−	−
pilW/*pilW*(*C150G*)	+/−	−	ND	−	+/−	−
pilW/*pilW*(*N108A*)	+	+	+	+	+	+
pilW/*pilW*(*N109A*)	+	+	+	+	+	+
pilW/*pilW*(*Y137A*)	+	+	+	+	+	+
pilW/*pilW*(*N146A*)	+	+	+	+	+	+
pilW/*pilW*(*N108A*,*Y137A*)	+	+	+	+/−	+	+
pilW/*pilW*(*N108A*,*N146A*)	+	+	+	+	+	+
pilW/*pilW*(*N109A*,*Y137A*)	+	+	+	+/−	+	+
pilW/*pilW*(*N109A*,*N146A*)	+	+	+	+	+	+
pilW/*pilW*(*Y137A*,*N146A*)	+	+	+	+/−	+	+

[a] The WT strain and the *pilW* mutant are included as positive and negative controls, respectively. Experiments were performed in the presence of 12.5 μM IPTG, except where indicated.
[b] Phenotypes are scored as follows: +, WT level; −, *pilW* mutant level; +/−, partial restoration of WT phenotype; ND, not determined.
[c] Experiments were performed in the presence of 100 μM IPTG.

Adherence assay. Adhesion of meningococci to pooled human umbilical vein endothelial cells (HUVEC) (Lonza) was done as described previously (2), with one minor modification. In brief, monolayers of 10^5 cells in 24-well plates were infected with approximately 2×10^7 CFU/ml. Numbers of added bacteria were systematically determined by counting CFU. The infection was continued for 4 h, with the medium being replaced every hour. At the end, several washes were done, and adherent bacteria were recovered by treatment with 1% saponin (wt/vol) for 10 min at 37°C and plated on GCB plates to perform CFU counts. Results are expressed as CFU of adhering bacteria normalized for an inoculum of 10^7 CFU and are the means ± standard deviations from at least 4 independent experiments.

RESULTS

Wild-type *pilW* complements the multiple phenotypic defects observed in a *pilW* mutant. To confirm that the reintroduction of the wild-type *pilW* (*pilW*$_{WT}$) allele is able to complement the multiple phenotypic defects observed in a *pilW* mutant (5), this gene was placed under the control of an IPTG-inducible promoter (27) and integrated ectopically into the genome of the *pilW* mutant, as previously done for the structure/function analysis of the PilX protein (19).

As assessed by immunoblotting, PilW levels in the *pilW*/*pilW*$_{WT}$ strain increase with increasing levels of inducer and are similar to those in the WT strain at approximately 12.5 μM IPTG (Fig. 1). We therefore performed an exhaustive phenotypic analysis of the *pilW*/*pilW*$_{WT}$ strain in the presence of 12.5 μM IPTG using a battery of assays that was recently set up by our group for in-depth analysis of Tfp biology (2). The *pilW*/*pilW*$_{WT}$ strain shows a high level of piliation as assessed by immunofluorescence (IF) microscopy (Fig. 2). In addition, competence for DNA transformation (Fig. 3), formation of bacterial aggregates (Fig. 4), adhesion to human cells (Fig. 5), and twitching motility (Table 2) are all restored to WT levels. Next, we assessed, by immunoblotting, the complementation of a specific phenotypic defect in the *pilW* mutant that is not observed in other nonpiliated mutants, i.e., the absence of PilQ multimers (4, 5). We found that PilQ multimers are restored in the *pilW*/*pilW*$_{WT}$ strain grown in the presence of inducer (Fig. 1). The relative levels of monomeric and multimeric forms of PilQ are similar to those seen in the WT strain and remain constant with increasing levels of PilW expression, suggesting an equilibrium between these two protein species. Taken together, these results confirm that all of the Tfp-linked phenotypic defects that we previously reported in an *N. meningitidis* *pilW* mutant (5) are exclusively due to the inactivation of the *pilW* gene.

Being a lipoprotein is not a strict requirement for PilW's function. Along with its orthologs, PilW possesses a canonical lipobox motif and is predicted to be lipidated via its most N-terminal cysteine residue and membrane anchored. Localization to the outer membrane was shown for *N. meningitidis* PilW and its *P. aeruginosa* ortholog, PilF (5, 22). To test the importance of this cysteine residue (C_{20} in *N. meningitidis*),

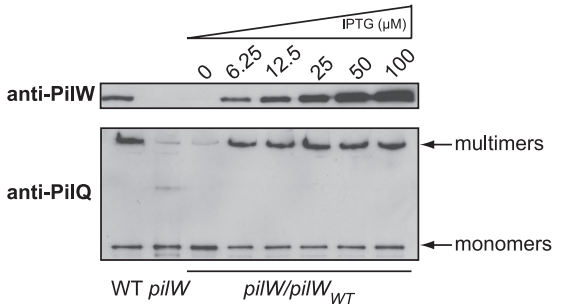

FIG. 1. Immunoblot analysis of PilW (top) and PilQ (bottom) in a *pilW*/*pilW*$_{WT}$ strain in the presence of increasing concentrations of IPTG. This strain is a *pilW* mutant complemented by the ectopic integration of a wild-type *pilW* allele placed under the control of an IPTG-inducible promoter. The WT strain and the *pilW* mutant were included as controls. Equal amounts of whole-cell protein extracts were loaded in each lane. Monomers and multimers of PilQ are indicated by arrows.

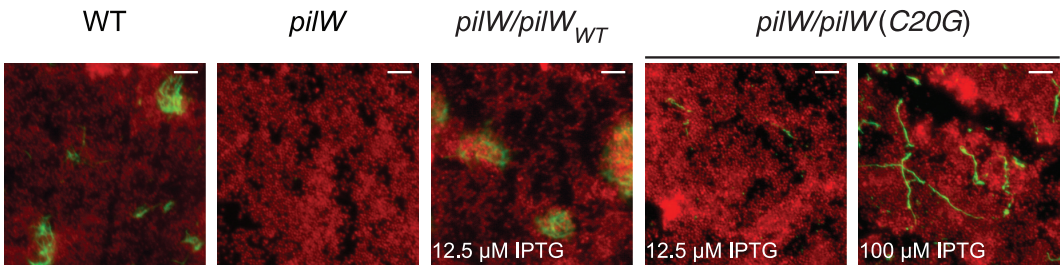

FIG. 2. Piliation as assessed by immunofluorescence microscopy. Tfp (green) were labeled with the monoclonal antibody 20D9, which is specific for *N. meningitidis* 8013 filaments, while the bacteria (red) were stained with ethidium bromide. For the sake of clarity, only a few representative PilW variants constructed in this study are shown. Results for all of the strains are summarized in Table 2. Bar, 5 μm.

we constructed and phenotypically characterized a *pilW/pilW* (*C20G*) strain in which an IPTG-inducible mutant allele of *pilW* encoding a variant where the first cysteine is replaced by a glycine was integrated ectopically in the genome of the *pilW* mutant. Interestingly, the PilW(C20G) protein is undetectable by immunoblotting in the presence of 12.5 μM IPTG (Fig. 6) but could be detected at a higher level (100 μM) of inducer. This was not observed with any of the other PilW variants generated in this study, which all produce PilW protein levels similar to that seen in the WT in the presence of 12.5 μM IPTG (Fig. 6). This suggests that the PilW(C20G) variant is less stable and readily degraded.

Consistent with these observations, very few pilus filaments could be detected in the *pilW/pilW*(*C20G*) strain by IF microscopy in the presence of 12.5 μM IPTG (Fig. 2), and consequently this strain behaves mostly like a nonpiliated *pilW* mutant (Table 2) when assayed for aggregation (although very few, small aggregates can be detected, as seen in Fig. 4), adhesion to human cells (Fig. 5), and formation of PilQ multimers (Fig. 6). In contrast, competence for DNA transformation was not abolished since the frequency of transformation in the *pilW/pilW*(*C20G*) strain was ~29-fold higher than in the *pilW* mutant (Fig. 3). Assays performed at a higher level of induction clearly demonstrate that the PilW(C20G) variant is functional since it is capable of partially overcoming some of the phenotypic defects seen in the *pilW* mutant. In the presence of 100 μM IPTG, which results in levels of PilW(C20G) protein detectable by immunoblotting (Fig. 6), the *pilW/pilW*(*C20G*) strain is more piliated than when grown in the presence of 12.5 μM IPTG (Fig. 2) and forms bacterial aggregates similar to those in the WT strain (Fig. 4), and a small amount of PilQ multimers can be seen (Fig. 6).

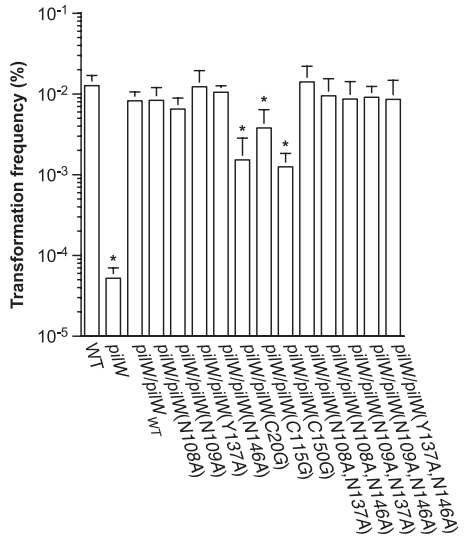

FIG. 3. Quantification of competence for DNA transformation in PilW variants constructed in this study. The WT strain and the *pilW* mutant were included as positive and negative controls, respectively. Results are expressed as percentages of recipient cells transformed and are the means ± standard deviations from 4 to 6 independent experiments. Results statistically significantly different from the WT strain ($P \leq 0.01$), as assessed by two-tailed Student's *t* test, are indicated by an asterisk.

These results are in favor of the notion that correct localization of PilW at the outer membrane via a lipid anchor greatly enhances its stability and therefore its functional efficiency but that it is not essential for the function of this protein.

The disulfide bond in PilW is crucial for its function. Recently, crystal structures have been determined for PilW (42) and its ortholog in *P. aeruginosa*, PilF (21, 22), revealing that despite notable differences at the sequence level these proteins are remarkably similar and consist mainly of 6 TPR motifs that fold into a superhelix (Fig. 7A). In PilW and most of its orthologs, the two halves of the TPR superhelix are interconnected by a disulfide bond between Cys_{115} and Cys_{150} (Fig. 7B), which suggests an important conformational and hence functional role. To probe the functional significance of this disulfide bond, we generated *pilW/pilW*(*C115G*) and *pilW/pilW*(*C150G*) strains in which each cysteine was individually replaced with a glycine residue. PilW(C115G) and PilW(C150G) variants are produced at levels indistinguishable from that of the WT protein in the presence of 12.5 μM IPTG (Fig. 6).

Critically, these variants are among the most dramatically affected for piliation and Tfp-linked functions of all those generated and tested during this study. Indeed, the *pilW/pilW*(*C115G*) and *pilW/pilW*(*C150G*) strains behave mainly like the nonpiliated *pilW* mutant (Table 2). Although very few pilus filaments can be seen, indicating residual PilW functionality in Tfp biogenesis, the *pilW/pilW*(*C115G*) and *pilW/pilW*(*C150G*) strains are not capable of aggregation, adhesion to human cells (Fig. 5), or formation of PilQ multimers (Fig. 6). As for the PilW(C20G) protein described above, and consistent with the expression of few pilus filaments, the only Tfp-linked property that is not abolished in the *pilW/pilW*(*C115G*) and *pilW/pilW*(*C150G*) strains is competence for DNA transformation (Fig. 3). Collectively, these results confirm the importance of

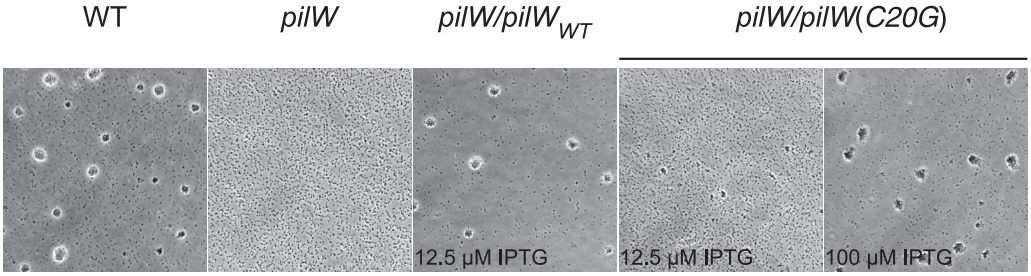

FIG. 4. Aggregation as assessed by phase-contrast microscopy in PilW variants constructed in this study. The WT strain and the *pilW* mutant were included as positive and negative controls, respectively. Aggregates were observed after 2 h of growth under static conditions (×240 magnification). For the sake of clarity, only a few representative PilW variants constructed in this study are shown. Results for all of the strains are summarized in Table 2.

the disulfide bond for PilW function in *N. meningitidis*, most likely by maintaining a tertiary structure compatible with its role in Tfp biology.

A constellation of highly conserved residues in the central groove of PilW specifically perturb Tfp-mediated adhesion. A structure-based amino acid sequence alignment of PilW orthologs identified four invariant residues (N108, N109, Y137, and N146) not part of the TPR motifs and therefore unlikely to be critical for the folding of the protein but rather of functional importance (22, 42). When mapped onto PilW's crystal structure, these residues cluster in the concave region of the TPR superhelix that is stabilized by the disulfide bond (Fig. 7B). Variants in which these residues were first individually changed to alanines were constructed to test their significance for PilW functionality.

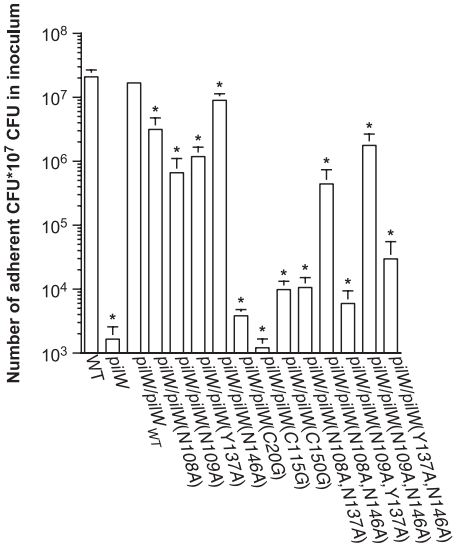

FIG. 5. Quantification of adhesion to human umbilical vein endothelial cells (HUVEC) of PilW variants constructed in this study. The WT strain and the *pilW* mutant were included as positive and negative controls, respectively. After a 30-min contact, during which standardized numbers of bacteria were incubated with standardized numbers of cells, nonadherent bacteria were removed by replacing the medium. After a further 4 h of incubation (the medium being replaced every hour), cells were washed, and adherent bacteria were recovered and plated. Results are expressed as CFU of adhering bacteria normalized for an inoculum of 10^7 CFU and are the means ± standard deviations from 4 to 6 independent experiments. Results statistically significantly different from the WT strain ($P \leq 0.01$), as assessed by two-tailed Student's *t* test, are indicated by an asterisk.

The *pilW*/*pilW*(*N108A*), *pilW*/*pilW*(*N109A*), *pilW*/*pilW*(*Y137A*), and *pilW*/*pilW*(*N146A*) strains are mainly indistinguishable from the WT strain (Table 2) when assayed for piliation, competence, aggregation, twitching motility, and formation of PilQ multimers. However, a statistically significant reduction in adhesion was observed for these strains compared to the WT (as assessed by two-tailed Student's *t* test), especially for the *pilW*/*pilW*(*Y137A*) and *pilW*/*pilW*(*N109A*) strains, which exhibit 17- to 30-fold reductions, respectively. Although significant, it should be noted that these differences are minor compared to that for the nonpiliated *pilW* mutant, which exhibits a 10^4-fold reduction in adhesion (Fig. 5). These results show that none of the above-mentioned residues is absolutely essential for PilW's functionality when substituted individually. This prompted us to generate a series of strains in which two of the residues were simultaneously replaced by alanines [*pilW*/*pilW*(*N108A*,*Y137A*), *pilW*/*pilW*(*N108A*,*N146A*), *pilW*/*pilW*(*N109A*,*Y137A*), *pilW*/*pilW*(*N109A*,*N146A*), and *pilW*/*pilW*(*Y137A*,*N146A*) strains]. Phenotypic characterization of these strains led to interesting results. Although production and stability of these variants are indistinguishable from those of $PilW_{WT}$ and these strains behave mostly like the WT strain (Table 2), including in their ability to form PilQ multimers, some of them are dramatically impaired in their ability to adhere to human cells. The double mutants in which the Y_{137} residue was mutated [*pilW*/*pilW*(*N108A*,*Y137A*), *pilW*/*pilW*(*N109A*,*Y137A*), and *pilW*/*pilW*(*Y137A*,*N146A*) strains] are almost as affected for adhesion as the nonpiliated mutant, with ~10^3-fold reductions in the number of adherent CFU (Fig. 5). We have tested and ruled out (data not shown) that these adhesion defects could be due to (i) antigenic variation of the pilin (31) and (ii) lack of expression (30) or impaired localization in purified pilus fractions (44) of the PilC1 protein, which is key for Tfp-mediated adhesion to human cells.

These results demonstrate that PilW's roles in PilQ multimerization, piliation, and modulation of Tfp-linked properties can be genetically uncoupled since it is possible to design *pilW* site-directed mutants that are piliated and still able to promote PilQ multimerization but are dramatically affected for Tfp-mediated adhesion to human cells. Hence, PilW is likely to be a multifunctional protein.

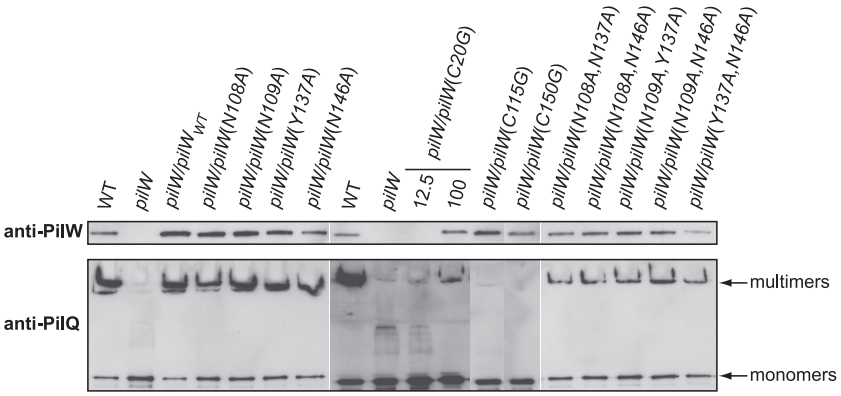

FIG. 6. Immunoblot analysis of PilW production/stability (top) and PilQ multimerization (bottom) in PilW variants constructed in this study. Strains were grown overnight on plates containing 12.5 μM IPTG, unless otherwise indicated. Whole-cell lysates were prepared and quantified, and equal amounts of total protein were loaded in each lane. The WT strain and the *pilW* mutant were included as controls. Monomers and multimers of PilQ are indicated by arrows.

DISCUSSION

Tfp biogenesis is a complex process involving the coordinated actions of multiple proteins, e.g., 15 in the meningococcus (PilC1/PilC2, PilD, PilE, PilF, PilG, PilH, PilI, PilJ, PilK, PilM, PilN, PilO, PilP, PilQ, and PilW) (4). The exact roles of most of these proteins are still to be understood on a molecular level. This is in part due to the fact that atomic structures of very few of these proteins have been solved so far and even fewer have undergone structure-guided functional analysis. The recently reported structure of PilW and its PilF ortholog (21, 22, 42) prompted us to perform a structure/function analysis of this protein, which plays multiple roles in Tfp biology (5).

PilW and its orthologs are putative lipoproteins that localize to the outer membrane in species as diverse as *N. meningitidis*, *P. aeruginosa*, and *M. xanthus* (5, 22, 36). As shown in *N. meningitidis*, *pilW* mutants are not piliated. However, poorly functional Tfp could be restored on the surface of a *pilWT* mutant in which pilus retraction has been abolished by a concurrent mutation in *pilT* (5). This demonstrated that PilW is one of the many Pil proteins that act after pilus assembly to antagonize PilT-mediated pilus retraction and to promote Tfp functionality (4). In addition, PilW is important for the assembly and/or stability of PilQ channels through which Tfp emerge on the bacterial surface, since in its absence PilQ multimers are no longer detectable by immunoblotting (4, 22, 32). The latter observation has led to speculation that PilW might be a pilotin or pilot protein like the well-characterized outer membrane lipoprotein PulS, involved in the main terminal branch of the general secretory pathway from *Klebsiella oxytoca*. PulS binds to its cognate secretin PulD (11) and ensures its proper targeting to the outer membrane (17), thereby protecting it from proteolysis and/or premature multimerization in the inner membrane (16).

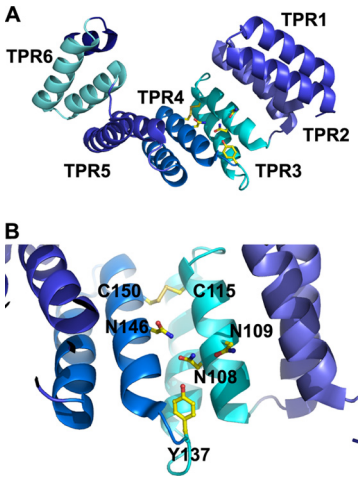

FIG. 7. (A) Three-dimensional structure of PilW (Protein Data Bank identifier 2vq2) in ribbon form. (B) Zoom on the residues targeted during this study, except Cys_{20} (not present this structure, which encompasses residues 34 to 250 out of 253). These residues cluster in the concave region of the TPR superhelix, between TPR3 and TPR4, that is stabilized by a disulfide bond between Cys_{115} and Cys_{150}. This figure was generated with PyMol (http://www.pymol.org).

The construction and functional characterization of a PilW variant in which the cysteine ending the canonical lipobox was mutated, making it a periplasmic protein as predicted *in silico* (20), add another line of evidence that PilW is not a pilot protein. Unlike PulS, whose mislocalization to the periplasm (18) or inner membrane (7) prevents insertion of the secretin multimers into the outer membrane and leads to a loss-of-function mutant, the PilW(C20G) variant is partly functional despite its strongly impaired stability (a feature that sets this variant apart from all of the others that were constructed and analyzed in this study). The *pilW*/*pilW*(*C20G*) strain remains capable of assembling surface-exposed Tfp that are partly functional. Even at a low level of inducer, when no PilW(C20G) is detectable using our anti-PilW antibody, few Tfp and small aggregates can be seen, and the *pilW*/*pilW*(*C20G*) strain is only slightly less competent (~8-fold decrease) than the WT strain. This is consistent with the finding in *N. gonorrhoeae* that very few pili can significantly increase transformation efficiency over that of a nonpiliated mutant (24). At a higher level of IPTG, when PilW(C20G) is produced to a WT level, more Tfp are seen, large round aggregates and twitching motility are restored, and some PilQ multimers can be detected. These re-

sults are consistent with those recently reported in *P. aeruginosa* for the similarly constructed nonlipidated PilF(C18G) mutant of the ortholog of PilW, which is also at least partly functional, since the corresponding strain exhibits Tfp-mediated twitching motility and bacteriophage susceptibility (22). However, there are notable differences between the two studies for reasons that are unclear at this time. In *P. aeruginosa*, mislocalization of PilF to the periplasm does not seem to affect the stability of this protein or the multimerization of PilQ, and it only very moderately affects the localization of PilQ multimers into the outer membrane (22). Nevertheless, our functional analysis, together with previous findings, such as the fact that the structure of PilW is strikingly different from that of the *bona fide* MxiM pilotin in the type III secretion system of *Shigella flexneri* (23), strongly suggests that PilW is not a pilotin. However, this does not preclude that PilW might interact with PilQ and thereby stabilize the secretin multimers, but we have so far been unsuccessful at demonstrating this (data not shown).

The other variants of PilW that were constructed and tested in this study all focused on the deep groove in the TPR superhelix, which was predicted to be crucial for the function of this protein possibly by acting as a binding pocket for another protein (21, 22, 42), most likely the secretin. The first mutants targeted two cysteines (C_{115} and C_{150}) that form a disulfide bond which was speculated to stabilize the TPR superhelix (42). This disulfide bond is a general feature in most of the PilW orthologs, with the notable exception of PilF and Tgl from *P. aeruginosa* and *M. xanthus*, respectively, in which these cysteines are replaced by residues with hydrophobic side chains (42) Our functional analysis confirms that the disulfide bond is critical for PilW's function. Although the PilW(C115G) and PilW(C150G) proteins are perfectly stable as judged by immunoblotting, which shows that the disulfide bond is not necessary for the stability of this protein, they exhibit only residual functionality. Both *pilW*/*pilW*(*C115G*) and *pilW*/*pilW*(*C150G*) strains display very few surface-exposed Tfp and are significantly more competent than the *pilW* mutant, but they do not form aggregates, adhere to human cells, or promote PilQ multimerization. The simplest explanation we envisage is that the disulfide bond in PilW acts to maintain tertiary integrity important for function and that mutation of either cysteine results in a conformational change that is incompatible with function. It is possible that the residues with hydrophobic side chains that replace cysteines in PilF and Tgl could play a similar role, but this remains to be tested.

The analysis of the remaining mutants, which targeted almost universally conserved residues in PilW orthologs (including PilF and Tgl) in the deep groove of the TPR superhelix, led to another important finding. As noted above, it was previously shown in *N. meningitidis* that although a *pilWT* double mutant is perfectly piliated, its aggregative and adhesive abilities are dramatically impaired (5). The fact that this double mutant contained a mutation in the *pilT* gene, which plays an important role in almost every aspect of Tfp biology (2), and the intriguing absence of PilQ multimers despite restoration of surface-exposed pilus filaments (5) made it difficult to disentangle whether the dramatic phenotypic defects that were observed were (i) merely a consequence of the absence of detectable PilQ multimers and/or absence of pilus retraction or (ii) due to the possibility that PilW has a dual role in PilQ multimer formation or stabilization and Tfp-mediated adhesion and aggregation. The construction and analysis of several variants during this study suggest that the second scenario is the most likely one. First, the PilW(C20G), PilW(C115G), and PilW(C150G) variants confirm that surface-exposed Tfp, although much diminished, can be seen in the absence of detectable PilQ multimers. Since Tfp cannot be surface exposed in the absence of PilQ (4, 47), it is likely that in these PilW variants as well as in the *pilWT* double mutant, PilQ multimers are present in the outer membrane but susceptible to heat and SDS and therefore dissociated and undetectable by immunoblotting. Second, the PilW(N108A,Y137A), PilW(N109A,Y137A), and PilW (Y137A,N146A) variants show, for the first time, that Tfp-mediated functions can be affected (i.e., the corresponding strains are almost as affected for adhesion as a nonpiliated *pilW* mutant) in the absence of any detectable effect on PilQ multimerization. In other words, the two phenotypes can be genetically uncoupled. This defect in adhesion is specific, as none of the other Tfp-linked functions (aggregation, competence, and twitching motility) was found to be affected. Taken together, these results prompt us to conclude that besides its role in PilQ multimer stabilization or formation PilW plays an additional role in Tfp functionality. Given that the N108, N109, Y137, and N146 residues that were mutated are invariant in PilW orthologs, we propose that this multifunctionality might be a shared property of these proteins. Since the adhesion defects that were observed are not due to antigenic variation of the pilin or lack of expression of the PilC1 protein or its impaired copurification with Tfp, properties that are known to modulate adhesion to human cells in *N. meningitidis* (30, 31), at this time we can only speculate about the underlying mechanism(s). Does PilW modulate Tfp composition or fine three-dimensional structure of the filaments in order to promote the synthesis of functional Tfp? Is this modulation direct or indirect through possible interferences with secretin channels that have been shown to interact directly with Tfp (8)?

In conclusion, by performing a detailed structure-guided functional analysis of a widely conserved Tfp biogenesis factor, this study sheds some light on the intricate biology of pilus filaments that play a key role in pathogenesis in several important human pathogens (26, 33). Moreover, by highlighting the functional importance of the putative protein-binding pocket in PilW, our findings suggest that this protein, due to its sequence conservation and accessible localization, might be a suitable target for the development of new antivirulence drugs, similar to pilicides (6, 34), with a wide spectrum of action.

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