The Meningococcal Minor Pilin PilX Is Responsible for Type IV Pilus Conformational Changes Associated with Signaling to Endothelial Cells

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Neisseria meningitidis crosses the blood-brain barrier (BBB) following the activation of the β 2-adrenergic receptor by the type IV pili (TFP). Two components of the type IV pili recruit the β 2-adrenergic receptor, the major pilin PilE and the minor pilin PilV. Here, we report that a strain deleted of PilX, one of the three minor pilins, is defective in endothelial cell signaling. The signaling role of PilX was abolished when pili were not retractable. Purified PilX was unable to recruit the β 2-adrenergic receptor, thus suggesting that PilX was playing an indirect role in endothelial cell signaling. Considering the recent finding that type IV pili can transition into a new conformation (N. Biais, D. L. Higashi, J. Brujic, M. So, and M. P. Sheetz, Proc. Natl. Acad. Sci. U. S. A. 107:11358–11363, 2010), we hypothesized that PilX was responsible for a structural modification of the fiber and allowed hidden epitopes to be exposed. To confirm this hypothesis, we showed that a monoclonal antibody which recognizes a linear epitope of PilE bound fibers only when bacteria adhered to endothelial cells. On the other hand, this effect was not observed in PilX-deleted pili. A deletion of a region of PilX exposed on the surface of the fiber had phenotypical consequences identical to those of a PilX deletion. These data support a model in which surface-exposed motifs of PilX use forces generated by pilus retraction to promote conformational changes required for TFP-mediated signaling.

Meningeal colonization by *Neisseria meningitidis* is a consequence of bacterial adhesion to brain endothelial cells (17, 27). The initial adhesion of only a few diplococci, followed by bacterial division and growth, leads to the formation of microcolonies on the apical surface of the cells. This process is mediated by type IV pili (TFP) which promote the initial adhesion (14, 15, 25, 29, 32) and the bacterium-bacterium interactions which are required for the formation of bacterial aggregates (19). Subsequent to the formation of these microcolonies, TFP elicit the organization of specific honeycomb-shaped molecular complexes underneath bacterial colonies, referred to as “cortical plaques.” Cortical plaques result from the recruitment of molecular linkers, such as ezrin and moesin, adhesion molecules, membrane receptors, and polymerized cortical actin (9, 18). The formation of the cortical plaque is associated with the recruitment of intercellular junction molecules at the site of bacterium-host cell interaction, thus leading to the depletion of junction proteins at the cell-cell interface, the opening of intercellular junctions, and the subsequent crossing of the blood-brain barrier (BBB) (5). In endothelial cells, the formation of the cortical plaque is due to a direct interaction between TFP and the β 2-adrenergic receptor (4).

Type IV pili are formed of a major subunit, the major pilin PilE. PilE is not only a structural component but is also a signaling protein able to directly interact with the β 2-adrenergic receptor (4). The major pilin subunit is subject to antigenic variation (30). Some pilin variants have been associated with an ability to promote higher adhesiveness than others. The high-adhesive phenotype was linked to the ability of these variants to form bundles of pili and to allow bacterial aggregation, thus increasing interbacterial interactions (25). In addition, three minor pilins, designated PilV, PilX, and ComP, are present in the fiber at low levels (13, 35, 36). These minor pilins are involved in specific functions supported by TFP. ComP is essential for the natural transformation of

the bacteria (36). PilV participates in the induction of signaling, since mutants not producing PilV were defective in endothelial cell signaling (20), and recently PilV has been shown to interact directly with the β 2-adrenergic receptor, as PilE (4). PilX is necessary for the aggregative properties of TFP (12). Bacterium-bacterium interactions due to the aggregative property of PilX are responsible for an increased number of bacteria capable of interacting with cells (13). This property is related to a specific protruding region of the PilX subunit that connects pili from two different bacteria. Another major property of TFP is their ability to retract. Following pilus-mediated adhesion, pili have been shown to retract (28). The cytoplasmic PilT ATPase is responsible for retraction of the fiber. In a *pilT* mutant, PilX is no longer required to form bacterial aggregates; this lead to the hypothesis that the protruding region of PilX links two antiparallel pili and prevents the fibers from sliding upon pilus retraction. In addition to these components, other proteins located in the bacterial membranes or periplasms are important for pilus biogenesis (3). Among these, the PilC proteins play a role which is still enigmatic. Two *pilC* alleles that encode two paralogous proteins, PilC1 and PilC2, have been described (24). PilC-null strains show impaired pilus expression. In addition, PilC1 is required for adhesion, and PilC1[−]

Received 5 April 2012 Returned for modification 4 May 2012

Accepted 29 June 2012

Published ahead of print 9 July 2012

Editor: B. A. McCormick

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doi:10.1128/IAI.00369-12

TABLE 1 Strains used in this study

Strain	Relevant phenotype	Relevant genotype	Antibiotic resistance ^a	Reference(s)
2C43-SO wt	SiaD [−] Opa ⁺ ; expressing pilin SB variant	Δ siaD::cat	Cm	25
2C43-SOE_SAK	Expressing pilin SA variant		Cm, Kn	25
2C43-SOE	PilE [−]	Δ pilE::aphA-3	Cm, Kn	22, 28
2C43-SOX	PilX [−]	Δ pilX::aphA-3	Cm, Kn	12
2C43-SOX/Xi	PilX [−]	Δ pilX::aphA-3	Cm, Kn, Em	
	PilX _{ind}	plac-pilX ^b		13
2C43-SOX/Xi _(Δ127–138)	PilX [−]	Δ pilX::aphA-3	Cm, Kn, Em	13
	PilX _{(Δ127–138)ind}	plac-pilX _(Δ127–138) ^b		
2C43-SOT	PilT [−]	Δ pilT::ermB	Cm, Em	28
2C43-SOXT	PilX [−]	Δ pilX::aphA-3	Cm, Kn, Em	This study
	PilT [−]	Δ pilT::ermB		
2C43-SOV	PilV [−]	Δ pilV::aphA-3	Cm, Kn	20
2C43-SOD	PilD [−]	Δ pilD::aphA-3	Cm, Kn	11
2C43-SOQ	PilQ [−]	Δ pilQ::aphA-3	Cm, Kn	11
2C43-SOT2	PilT2 [−]	Δ pilT2::aphA-3	Cm, Kn	11
2C43-SOU	PilU [−]	Δ pilU::aphA-3	Cm, Kn	11
2C43-SOC1	PilC1 [−]	Δ pilC1::aphA-3	Cm, Kn	22, 28
2C43-SOC2	PilC2 [−]	Δ pilC2::aphA-3	Cm, Kn	11
2C43-SOH	PilH [−]	Δ pilH::aphA-3	Cm, Kn	This study
2C43-SOI	PilI [−]	Δ pilI::aphA-3	Cm, Kn	This study
2C43-SOJ	PilJ [−]	Δ pilJ::aphA-3	Cm, Kn	This study
2C43-SOK	PilK [−]	Δ pilK::aphA-3	Cm, Kn	This study

^a Cm, chloramphenicol (6 μ g · ml^{−1}); Kn, kanamycin (100 μ g · ml^{−1}); Em, erythromycin (2 μ g · ml^{−1}).

^b pilX genes under the control of IPTG-inducible plac promoter (lacI^qOP).

PilC2⁺ strains are unable to adhere to endothelial cells. On the other hand, PilC2, which is expressed independently of PilC1, possesses these adhesiveness properties specifically on some cell types but not on endothelial cells (15, 22–24).

It has recently been shown that mechanical force can extend the repertoire of TFP structures by revealing hidden epitopes of PilE previously buried in the TFP fiber (2, 8). Indeed, when subject to force, TFP will transition into a new conformation. The new structure is longer and narrower than resting TFP. Upon release of the force, TFP regain their original form (2). SM1 monoclonal antibody (MAb) recognizes the conserved epitope EYYLN in the pilin monomer (31). The SM1 epitope is predicted to be buried in the fiber, and in the resting unstretched state the SM1 MAb recognizes by electron microscopy only the exposed end of the fiber (2, 7, 10, 26). On the other hand, in the elongated form of the pilus, SM1 MAb binds to the length of the stretched fiber (2). Thus, force-induced stretch exposes residues in the TFP that are normally hidden. The role *in vivo* of this newly described characteristic of TFP is unknown.

During the course of a systematic analysis of the role of TFP components or TFP-associated proteins in meningococcal signaling, we found that a strain deleted of PilX was defective in endothelial cell signaling. Unlike the major pilin PilE and the minor pilin PilV, PilX does not interact directly with the β 2-adrenergic receptor. We subsequently hypothesized that PilX could be necessary for structural change within the fiber. Based on the labeling of the fiber by the SM1 MAb, we show that following pilus-mediated adhesion, the SM1 epitope was exposed on the length of the fiber, whereas bacterial aggregation was not sufficient for this epitope to be exposed. Moreover, the binding of the SM1 MAb in adhesive bacteria along the pili required PilX. These data suggest that upon bacterial adhesion, the fiber was elongated and that this structural change required PilX. We subsequently propose that

the minor pilin PilX following bacterial adhesion is necessary for the structural modification of the fiber that allows bacterial cell signaling.

MATERIALS AND METHODS

Bacterial strains and infection conditions. A pilated and noncapsulated Opa⁺ derivative of the serogroup C meningococcal strain 8013, designated 2C43 SiaD[−] Opa⁺, was used throughout the study (5, 25). This noncapsulated derivative was engineered by introduction of the cat resistance cassette into the *siaD* gene. All the mutants and derivatives used in this work were isogenic derivatives of meningococcal C strain 2C43 SiaD[−] Opa⁺ and are listed in Table 1.

N. meningitidis strains were grown on gonococcal base (GCB) agar plates (Difco) containing Kellogg's supplements and supplemented with appropriate antibiotics in a moist atmosphere containing 5% CO₂ at 37°C.

On the day of infection, a suspension of the bacteria from an overnight culture on a GCB agar plate was adjusted to an optical density at 600 nm (OD₆₀₀) of 0.05 and incubated for 2 h at 37°C in a prewarmed cell culture medium. Cells were infected with bacteria at a multiplicity of infection (MOI) of 100 for 30 min to allow *N. meningitidis* adhesion and then washed with cell culture medium and maintained in appropriate fresh medium for 1 or 2 h.

Cell culture. Human umbilical vein endothelial cells (HUVECs; PromoCell) were used between passages 1 and 8 and grown in endothelial serum-free medium (Endo-SFM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; PAA Laboratories GmbH), 0.5 IU/ml of heparin, and 40 mg/ml of endothelial cell growth supplement (Sigma-Aldrich). Human embryonic kidney 293 (HEK-293) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Cells were grown at 37°C in a humidified incubator under 5% CO₂. For immunofluorescence assays, HUVECs and HEK-293 cells were grown on 12-mm-diameter glass coverslips coated with human fibronectin (10 μ g/ml; Life Technologies) and poly-L-lysine (0.01%; Sigma-Aldrich), respectively.

HEK-293 cells were transfected using Lipofectamine 2000 (Life Technologies) following the manufacturer's instructions.

Antibodies and chemicals. The following antibodies were used for immunofluorescence labeling or enzyme-linked immunosorbent assay (ELISA): anti-ezrin rabbit polyclonal antibody (generously provided by P. Mangeat, CNRS, UMR5539, Montpellier, France), anti-PilE mouse monoclonal antibody (clone 20D9) (28), and anti-PilE mouse monoclonal antibody (clone SM1) (generously provided by M. Virji). The following goat secondary antibodies were used for immunofluorescence labeling or ELISA: anti-mouse IgG (H+L) coupled to horseradish peroxidase (Jackson ImmunoResearch Laboratories) and anti-rabbit IgG (H+L) or anti-mouse IgG (H+L) coupled to Alexa-488 or Alexa-546 (Life Technologies). 4',6-Diamidino-2-phenylindole (DAPI) was purchased from Life Technologies. Isopropyl β -D-1-thiogalactopyranoside (IPTG) was purchased from Promega and used at a concentration of 1 mM.

Analysis of piliation. Analysis of piliation was performed using a whole-cell ELISA, as already described (12). Briefly, bacteria were grown 2 h in RPMI-1% bovine serum albumin (BSA) from an OD₆₀₀ of 0.5 and, after washes, were resuspended in PBS at an OD₆₀₀ of 0.1. Serial 2-fold dilutions were coated in the wells of a microtiter plate. The plates were incubated without covers at 56°C for 1 h. Coated plates were washed several times with PBS-0.1% Tween 80 (washing solution), and then the anti-PilE mouse monoclonal antibody 20D9 diluted in washing solution was added to the plates and incubated 1 h at 37°C. After several washes, a peroxidase-linked anti-rabbit IgG was added to the wells for 1 h. Finally, after several washes, the staining was revealed using TMB (3,3',5,5'-tetramethylbenzidine) substrate and stop solution following the manufacturer's instructions (Cell Signaling Technology).

Immunofluorescence experiments. Infected cells were fixed for 20 min in PBS-4% paraformaldehyde (PFA). For ezrin immunostaining only, cells were permeabilized for 5 min in PBS containing 0.1% Triton X-100. Cells were incubated with primary antibodies in PBS containing 0.3% BSA or 0.1% gelatin for 1 h. After 3 washes with the same buffer, DAPI was added to Alexa-conjugated secondary antibodies for 1 h. After additional washes, coverslips were mounted in Mowiol (Citifluor Ltd.).

N. meningitidis aggregates were coated on glass coverslips by centrifugation after a 2-h liquid culture in HUVEC culture medium (3,000 rpm, 10 min), fixed using 4% PFA, and processed for immunostaining.

Image acquisition was performed on an inverted microscope or a laser-scanning confocal microscope (Leica SP5). Confocal images were collected and processed using the Leica Application Suite AF lite software (Leica).

Quantitative analysis of ezrin recruitment (and that of other proteins) under bacterial colonies. The recruitment efficiency was estimated by determining the proportion of colonies that efficiently recruit the protein of interest in a honeycomb shape just under the colonies. At least 50 colonies were scrutinized per coverslip. Each experiment was performed in duplicate and repeated several times (at least 3). Data were examined for significance using Student's *t* test.

Expression and purification of MBP-pilin recombinant proteins. Recombinant MBP-pilin fusion proteins were produced as described before (4, 13). Briefly, fragments of *pilE*, *pilV*, *comP*, and *pilX* genes lacking the region coding for the first 28 amino acid residues were amplified by PCR and subcloned in pMAL-p2X vector (New England Biolabs). The resulting plasmids, pMAL-pilE, pMAL-pilX, pMAL-pilV, and pMAL-comP, contain in frame the *malE* gene, which encodes the maltose-binding protein (MBP), followed by the factor Xa protease recognition site and the truncated pilin coding regions. These plasmids were transformed in the PAP5198 *Escherichia coli* strain (generously provided by O. Francetic, Institut Pasteur, Paris, France), deleted of the periplasmic enzymes OmpT Prt and DegP. The fusion proteins were purified on amylose resin (New England Biolabs).

Coating of *Staphylococcus aureus* with MBP-pilin fusion proteins and infection. *S. aureus* ATCC 25923 strains expressing specific receptors for the Fc domain of IgG immunoglobulins were used for this experiment

TABLE 2 Signaling phenotypes of genes in TFP biology

Mutated gene(s)	Function ^a	Phenotype ^b	
		Piliation	Signalization
<i>pilE</i>	Major pilin	—	—
<i>pilX</i> , <i>pilV</i>	Minor pilins	+	—
<i>pilH</i> , <i>pilI</i> , <i>pilJ</i> , <i>pilK</i>	Pseudopilins	—	—
<i>pilD</i>	Pilin maturation	—	—
<i>pilQ</i>	Secretin	—	—
<i>pilC1</i> , <i>pilC2</i>	Adhesins	+	+
<i>pilT</i> , <i>pilT2</i> , <i>pilU</i>	Pilus retraction	++	+

^a Implication of proteins in TFP biology.

^b Piliation was evaluated according to ELISA techniques: +, pilated, —, nonpilated, or ++, hyperpilated. Signalization was evaluated by counting the proportion of *Neisseria meningitidis* microcolonies able to recruit cortical plaque: —, no cortical plaque recruitment; + cortical plaque recruitment.

as described before (4). Briefly, about 10⁸ bacteria were incubated with the anti-MBP rabbit polyclonal antibody for 20 min at room temperature. Bacteria were then centrifuged at 4,000 rpm for 5 min, washed with warm LB 3 times, and incubated with 2.5 μ g of each recombinant fusion protein for 20 min at room temperature. After several washes using DMEM supplemented with 10% fetal calf serum, bacteria were incubated 30 min with HEK-293 cells previously transfected with plasmid encoding the yellow fluorescent protein (YFP)-tagged human β 2-adrenergic receptor (β 2AR-YFP) (1). Cells were then fixed using 4% PFA in PBS and analyzed by immunofluorescence assay for β 2AR-YFP recruitment underneath *S. aureus* colonies.

RESULTS

The minor pilin PilX is required for type IV pilus-mediated signaling of meningococcal microcolonies to endothelial cells. In an attempt to identify all pilus components, besides PilE and PilV, involved in meningococcal signaling, we tested systematically the ability of strains carrying a mutation in identified TFP components or TFP-associated proteins to signal to endothelial cells. Considering that some of these mutants are nonpilated (Table 2) or incapable of pilus-mediated adhesion, such as the PilC1-defective derivative, all the mutations were introduced into a strain expressing an Opa protein, a secondary adhesin. However, in order to be able to promote adhesion, an Opa⁺-expressing strain has to be noncapsulated (33); a noncapsulated background was therefore used by introducing an *siaD* mutation (5). A noncapsulated Opa⁺ strain is indeed able to adhere to eukaryotic cells expressing carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) independently of TFP expression (5, 34). Therefore, all experiments described in this work were performed in an SiaD[−] Opa⁺ background. As previously shown, such a pilated strain is not affected in its ability to signal to cells (5). Results of these experiments are reported in Table 2 and Fig. 1. As expected, none of the nonpilated mutants (*pilE*, *pilD*, *pilQ*, *pilH*, *pilI*, *pilJ*, and *pilK* mutants [11]) was able to recruit the cortical plaque, thus confirming that pili are required for meningococcal formation of the cortical plaque. However, this cannot rule out the implication of one of these components in signaling. The lack of piliation may prevent the evaluation of the role of some components in signaling independently of their role in pilus biogenesis, such as the major pilin PilE.

The adhesive properties of TFP to endothelial cells have been shown to require the expression of PilC1, an outer membrane protein associated with TFP (24). Indeed, *pilC1* mutants are pili-

FIG 1 The *Neisseria meningitidis* *pilX* mutant strain is defective in ezrin recruitment. HUVECs were infected with a pilated noncapsulated strain of *Neisseria meningitidis* (wt) and its *pilE*, *pilX*, *pilC1*, *pilT*, and *pilX pilT* derivatives. The formation of the cortical plaque was assessed by the recruitment of ezrin. Ezrin was immunostained (in green), and DNA was stained using DAPI (in blue). N, nucleus; arrowheads, microcolonies. Scale bars = 5 μ M. All bacteria are Opa⁺ and noncapsulated.

ated if they express PilC2 but are unable to adhere to HUVECs. In an SiaD[−] Opa⁺ background, both *pilC1* or *pilC2* mutants formed microcolonies. Both strains are able to recruit ezrin and actin (Table 2 and Fig. 1). The fact that the *pilC1* mutant was capable of signaling to cells even though unable to promote pilus-mediated adhesion confirms that type IV pilus-mediated signaling and adhesion are two independent processes mediated by the same bacterial attribute (4).

Consistent with previous results (20), the *pilV* mutant ex-

FIG 2 *pilT* mutation partially restores the signaling defect of the *pilX* mutant. HUVECs were infected with a pilated noncapsulated strain of *Neisseria meningitidis* (wt), its *pilE*, *pilX*, *pilT*, and *pilX pilT* derivatives, and a strain expressing the pilin variant SA. The strain $\Delta pilX::pilX$ is a *PilX* mutant which has been complemented in *trans* with a *pilX* allele under the control of an IPTG-inducible promoter. It should be pointed out that this strain promotes even in the absence of IPTG a low level of ezrin recruitment due to a low activity of the promoter in the absence of IPTG. Ezrin recruitment was revealed by immunostaining. The ezrin recruitment index was estimated by determining the proportion of colonies that efficiently recruit ezrin under the colonies and expressed as normalized mean values ($\pm$ standard errors of the means [SEM]) from three independent experiments in duplicate. a, Student's *t* test ($P < 0.001$) shows statistical significance when the ezrin recruitment was compared to that of the strain expressing wild-type *pili*.

pressed TFP but recruited the cortical plaque very poorly (Table 1). Surprisingly, a *pilX* mutant had a dramatic signaling defect (Table 2 and Fig. 1 and 2) and was unable to induce cortical plaque recruitment (Fig. 2). The introduction into the chromosome of a *pilX* allele under the control of an IPTG-inducible promoter restored the capability to induce signaling upon addition of IPTG (Fig. 2), thus excluding that the signaling defect was due to a polar effect of the mutation. The *pilX* mutant adhered to cells via Opa proteins and, due to the absence of bacterium-bacterium interactions, formed microcolonies of smaller size than those of the parental strain. It should be pointed out that none of the microcolonies made by the *pilX* mutant, regardless of their size, was capable of signaling to endothelial cells. The parental strain expressed a high adhesive pilin variant, thus being responsible for large colonies adhering to the apical surface of the endothelial cells. We subsequently repeated this experiment using a derivative expressing a low-adhesive variant, defective in bacterial aggregation (i.e., expressing the SA pilin variant) (25). The SA pilin variant is responsible for few bacterium-bacterium interactions and hence induced the formation of small microcolonies on the surface of apical cells (25). As shown in Fig. 2, adhesive bacteria of this derivative recruited the cortical plaque in a manner similar to that of the strain expressing a high-adhesive pilin variant. All together, these results demonstrate that the lack of signaling observed with the *pilX* mutant is not due to the small size of the microcolonies interacting with the endothelial cells.

The *PilT* protein is responsible for TFP retraction. *PilT*-dependent retraction has been shown to enhance *N. meningitidis* signaling to epithelial cells, probably by a mechano-transduction mechanism (16, 18). Interestingly, on endothelial cells, a retraction-deficient isolate was able to induce the formation of cortical plaques (Table 1 and

FIG 3 Adhesive bacteria of a PilX derivative are heavily piliated. HUVECs were infected with a piliated noncapsulated strain of *Neisseria meningitidis* (wt) and its *pilT* and *pilX* derivatives. Type IV pili and ezrin were visualized by immunofluorescence using the anti-PilE (clone 20D9) mouse monoclonal antibody and the anti-ezrin polyclonal antibody (in green and red, respectively). DNA was stained using DAPI (in blue). Arrowhead, microcolonies.

Fig. 1 and 3). Considering that a *pilT* mutation has been shown to compensate some phenotypical defects in strains carrying a mutation in TFP components or TFP-associated components (12), a *pilT* mutation was introduced into the *pilX* mutant. The signaling ability of the resulting strains was then tested. As shown in Fig. 1 and 2, the *pilT* mutation partially restored the cortical plaque recruitment defect of the *pilX* mutant.

All together, these results clearly confirmed that type IV pili are required for the formation of the cortical plaque and that the minor pilin PilX was playing a major role in the recruitment of the cortical plaque but was dispensable in a nonretractable strain.

PilX does not recruit the β 2-adrenergic receptor. As already mentioned, PilE and PilV are involved in the recruitment of the signaling receptor, the β 2-adrenergic receptor. Indeed, recombinant PilV and PilE proteins, when fused with the maltose binding protein (MBP) and bound to live staphylococci via anti-MBP antibodies, are able to induce the recruitment of the β 2-adrenergic receptor in a model of HEK-293 cells transfected with both the β -arrestin and the β 2-adrenergic receptor (see Materials and Methods and reference 4). In order to assess a possible interaction of PilX with the adrenergic receptor, we used a similar strategy and produced recombinant PilX fused with the MBP. HEK cells overexpressing the β -arrestin and the β 2-adrenergic receptor tagged with YFP were infected with *Staphylococcus aureus* coated with anti-MBP antibody and the MBP-pilin fusion proteins. The ability of these proteins to recruit the β 2-adrenergic receptor in these transfected HEK-293 cells was subsequently tested. Data are reported in Fig. 4. In accordance with our previous work (4), MBP-PilV and MBP-PilE, but not MBP-ComP, recruit the β 2-adrenergic receptor. On the other hand, MBP-PilX was unable to recruit the receptor. This result is not consistent with a direct interaction of PilX with the β 2-adrenergic receptor, thus suggesting that PilX may have an indirect interaction effect on TFP-induced signaling.

Upon bacterial adhesion, PilX is needed to reveal the previously hidden SM1 epitope. As already mentioned, TFP, when subjected to force, will transition into a new conformation (2). The new structure is longer and narrower than the original structure. Upon release of the force, the TFP fiber regains its original form, indicating a reversible transition. These force-induced conformations expose hidden epitopes, such as those recognized by the SM1 monoclonal antibody. This epitope is not exposed in fibers of bacteria grown in broth culture (2, 8). The above-described data suggesting that PilX does not play a role via a direct interaction with the signaling receptor raised the question of the

FIG 4 PilX is not able to recruit the β 2-adrenergic receptor. HEK cells overexpressing the β 2-adrenergic receptor tagged with YFP were infected with *Staphylococcus aureus* coated with anti-MBP antibody and MBP-pilin fusion proteins as described in Materials and Methods. Receptor recruitment was counted in all cells in contact with bacterial aggregates and expressed as normalized mean values ($\pm$ SEM) from three independent experiments in duplicate. a, $P < 0.001$ (Student's *t* test).

mechanism by which PilX may enhance pilus-mediated signaling to endothelial cells. Previous reports have proposed that surface-exposed motifs in PilX subunits stabilize bacterial aggregates against the disruptive force of pilus retraction (13). We subsequently postulated that upon bacterial interaction with cells, the forces generated by pilus retraction may stretch the fiber, thus exposing epitopes previously buried in the TFP fiber.

The strain used in this study carries the SM1 epitope (31). We subsequently addressed the availability of this epitope in isogenic $\Delta pilX$ or $\Delta pilX \Delta pilT$ meningococcal strains adhering or not onto endothelial cells. As already mentioned, since a *pilX* mutant is nonadhesive, all strains used were nonencapsulated (*SiaD*[−]), *Opa*⁺ derivatives of strain 2C43. We first looked at the expression of the SM1 epitope by adhesive bacteria. As shown in Fig. 5A in monolayers infected with a strain expressing wild-type pili, the SM1 antibody-stained pili were seen as filamentous structures among bacterial aggregates in the great majority of the microcolonies. In addition, SM1 staining was seen not only in large microcolonies of adhering bacteria but also when only a few diplococci were adhering to the cells (Fig. 5A, left), thus excluding the possibility that this staining was a consequence of the bacterial aggregation in large microcolonies. Eighty to ninety percent of the microcolonies on the surface of the cells are associated with the formation of a cortical plaque, and only bacterial colonies in which an SM1 staining was observed were associated with the formation of a cortical plaque (Fig. 5B). On the other hand, pili of the *PilX*[−] derivative were never stained by the SM1 monoclonal antibody (Fig. 5A), even though adhesive *PilX*[−] bacteria are heavily piliated, as shown in Fig. 3 (12). A Western blot analysis, performed using purified PilX, ruled out the possibility that the effect observed was due to recognition of the PilX molecule by the SM1 monoclonal antibody (data not shown). A strong SM1 staining of pili was observed in microcolonies of *pilT*[−] strains expressing nonretractable pili (Fig. 5A and B). However, consistent with the above-described data (Fig. 2), the SM1 labeling was stronger in the *PilT*[−] *PilX*⁺ strain than in the *PilT*[−] *PilX*[−] strain. It should be pointed out that a mutation in another minor pilin, such as PilV, even though important for meningococcal cell signaling (20) to endothelial cells, did not affect the SM1 binding when bacteria were interacting with the cells (data not shown). These results clearly showed that the SM1 epitope is exposed in wild-type pili upon bacterial adhesion and not in *PilX*[−] strains but are present in nonretractable (*PilT*[−]) pili regardless of the presence of PilX.

We then assessed the expression of the SM1 epitope in nonadhering bacteria grown in broth. As shown in Fig. 5C, wild-type pili were not stained by SM1 antibody, even though these bacteria were forming large bacterial aggregates, thus confirming that the exposure of the SM1 epitope is not a result of the ability of the bacteria to form aggregates. This is consistent with previous results indicating that the SM1 epitope is hidden when bacteria are

grown in broth (2, 8). On the other hand, in a strain expressing nonretractable TFP, a clear staining of pili by the SM1 monoclonal antibody was observed inside the bacterial aggregates. These data demonstrated that, for broth-grown bacteria, the SM1 epitope is not accessible to the antibody in wild-type pili but is exposed in nonretractable (*PilT*[−]) pili.

All together, these data demonstrate that in wild-type pili, upon bacterial adhesion onto cells, the minor pilin PilX allows the expression of hidden epitopes, such as the SM1 epitope, which are not exposed on pili in liquid culture growth, thus demonstrating that upon bacterial adhesion, conformational changes occur in the fiber.

The D region of PilX is required for pilus-mediated signaling and is responsible for the PilX-induced conformational change.

The structure of PilX has previously been reported (6, 13) and shows a classical pilin structure with a C-terminal region containing two disulfide bonds delineating the D region, which forms a hook exposed on the surface of the fiber (6) (Fig. 6A). In a previous work, it has been shown that this protruding D region is responsible for bacterial aggregation by enhancing bacterial interaction (13). Considering the above-reported results, we aimed at testing the hypothesis that both the TFP-mediated signaling and the PilX-induced conformational changes observed upon bacterial interaction with host cells were also linked to the presence of the hook formed by the D region. We subsequently assessed the ability of a PilX protein deleted in the D region to signal to cells and to allow the binding of the SM1 monoclonal antibody upon bacterial cell interaction. The strains used for these experiments were *pilX* derivatives of nonencapsulated (*SiaD*[−]) *Opa*⁺ strains, complemented in *trans* either with a full-length *pilX* allele ($\Delta pilX::pilX$) or a *pilX* allele lacking residues 127 to 138 of the D region ($\Delta pilX::pilX_{127-138}$) (Fig. 6A). These strains have previously been reported (13), and this deletion of PilX does not prevent the protein's localization in the fiber. The data are reported in Fig. 6B and C. They clearly show that the ability of TFP to signal to cells required the presence of the hook formed by the D region of PilX. Moreover, this region is necessary for the pilus conformational change following bacterial adhesion to host cells, which allows the binding of the SM1 antibody to its epitope.

All together, these data demonstrate that the ability of meningococcal TFP to signal to host cells correlates with changes in the fiber structure induced by the exposed D region of the PilX protein.

DISCUSSION

Type IV pili are essential for meningococcal pathogenesis. In invasive capsulated bacteria, they promote bacterial adhesion to endothelial cells and signaling. The latter is a consequence of the interaction of pili components with the N-terminal portion of the β_2 -adrenergic receptor. Two components of the bacterial pili have been implicated as interacting with the extracellular N-terminal portion of the β_2 -adrenergic receptor, the major pilin PilE,

FIG 5 SM1 epitope is exposed upon bacterial adhesion to endothelial cells or in a *pilT* background. HUVECs were infected with a piliated nonencapsulated strain of *Neisseria meningitidis* (wt) and its *pilT* and *pilX* and *pilX pilT* derivatives. (A) SM1 epitope is visualized by immunofluorescence using the anti-SM1 monoclonal antibody (in green). DNA was stained using DAPI (in blue). Arrowhead, SM1-stained pili. (B) HUVECs were infected with a piliated nonencapsulated strain of *Neisseria meningitidis* (wt) and its *pilT* derivative. SM1 epitope and ezrin were immunostained (in green and in red, respectively). DNA was stained using DAPI. Red arrowheads, SM1-stained pili; white arrowheads, microcolonies without SM1-stained pili. (A, B, C) N, nuclei; scale bars = 10 μ M. (C) *Neisseria meningitidis* aggregates were obtained after a 2-h liquid culture in HUVEC culture medium. SM1 epitope is visualized by immunofluorescence using the anti-SM1 monoclonal antibody (in green). DNA was stained using DAPI (in blue). *, nonspecific staining; arrowheads, SM-stained pili.

FIG 6 The D region of PilX is required for adhesion-induced pili conformational change. (A) Sequence of the mature PilX protein from *N. meningitidis* strain 8013. The D region delimited by a disulfide bond is highlighted in orange. The deleted surface-exposed hydrophobic residues of the D region are indicated by the black line (residues 127 to 138). Arrows, β sheets; black waves, α helix. (B and C) HUVECs were infected with a piliated nonencapsulated (SiaD⁻) Opa⁺ strain of *Neisseria meningitidis* expressing wild-type (wt) pili or a *pilX* derivative (Δ *pilX*) or a *pilX* derivative complemented either with a full-length *pilX* allele (Δ *pilX*::*pilX*) or a *pilX* allele lacking residues 127 to 138 of the D region (Δ *pilX*::*pilX*₁₂₇₋₁₃₈). SM1 epitope and ezrin were visualized by immunofluorescence. (B) The ezrin recruitment index (clear bars) and SM1 staining index (hatched bars) were estimated by determining the proportion of colonies that efficiently recruited ezrin under the colonies (or showed a positive SM1 staining) and are expressed as normalized mean values ($\pm$ SEM) from three independent experiments in duplicate. a , $P < 0.001$ (Student's *t* test). (C) SM1 epitope and ezrin were immunostained (in green and red, respectively). DNA was stained using DAPI. Scale bars = 10 μ M.

and a minor pilin component PilV (4). How these two components interact with the receptor remains to be understood, especially whether they act cooperatively or independently.

In this work, a third component of the pili has been identified as playing a role in pilus-mediated signaling, the minor pilin PilX. This component has previously been identified as required for efficient bacterial adhesion and aggregation. However, PilX could not be identified as an adhesin, and it is believed that the bacterium-bacterium interactions due to the aggregative property of PilX are responsible for the increase number of bacteria capable of interacting with cells (13). This property is related to a specific protruding region of the PilX subunit that is believed to be able to connect pili from two different bacteria upon pilus retraction, preventing them from sliding. Here, we identify PilX as a pilus component required for TFP-mediated signaling. As for adhesion and aggregation, this effect of PilX is abolished in a PilT-deficient strain. Unlike the major pilin PilE and the minor pilin PilV, purified PilX does not promote recruitment of the β 2-adrenergic receptor, thus making it unlikely that the function of PilX in signaling is via a direct interaction with the signaling receptor. We subsequently hypothesized that PilX may be responsible for conformational changes which allow some pilus epitopes to be exposed. Our data clearly demonstrate that the SM1 epitope expressed by wild-type pili is not accessible to monoclonal antibodies in bacteria grown in liquid cultures, whereas it is clearly labeled once bacteria interact with cells. These results are consistent with a recent report showing that neisserial TFP can undergo a reversible force-induced transition into a longer and narrower structure than the resting state and that the labeling of the SM1 epitope along the length of the pili is made possible by this elongation of the fiber (2). All together, our data suggest that, upon meningococcus-cell interaction, pili are elongated and that PilX is required for this structural change. In addition, this function of PilX is supported by the D region of the molecule that forms a hook exposed at the surface of the fiber. This conformational change is not due to bacterial aggregation, since bacterial aggregates grown in broth are not labeled by the SM1 epitope (Fig. 5C) and SM1 labeling is observed when only a few diplococci adhere to cells (Fig. 5A). These results suggest that the force necessary for this conformational change is coming from pilus retraction following bacterial adhesion. PilX, via its protruding region, by preventing pili sliding, may be responsible for the elongation of the fiber upon interaction with cells, thus exposing previously hidden epitopes in the pili. The fact that the SM1 epitope is spontaneously exposed in the fiber of PilT[−] pili suggests that the structure of nonretractable pili is not that of the resting pilus but that of an elongated one. This hypothesis is consistent with a previous report showing that pili tend to retract spontaneously (21) and may explain why nonretractable pili are able to signal to cells in the absence of PilX.

The absence of recruitment of the β 2-adrenergic receptor by purified PilX, the fact that strains expressing nonretractable pili do not require PilX bacterial cell signaling, and the association between the ability of the fibers to be stained by the SM1 epitope and to signal to cells support the hypothesis that the epitopes responsible for bacterial signaling are capable of interacting with the β 2-adrenergic receptor only in specific conformations of the quaternary structure of the fiber.

ACKNOWLEDGMENTS

We thank Colin Tinsley for a careful reading of the manuscript. We thank Mumtaz Virji for sending us the monoclonal antibody directed against the SM1 epitope. We thank M. Garfa-Traore and N. Goudin of the Necker Institute imaging facility for their technical support.

We thank the Imagine Foundation for the Leica SP5 microscope funding. The laboratory of X.N. is supported by INSERM, Université Paris Descartes, a grant from "La Fondation pour la Recherche Médicale," and ANR grant number ANR-AAP-2009-06-26.

REFERENCES

- Angers S, et al. 2000. Detection of beta 2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). *Proc. Natl. Acad. Sci. U. S. A.* 97:3684–3689.
- Biais N, Higashi DL, Brujic J, So M, Sheetz MP. 2010. Force-dependent polymorphism in type IV pili reveals hidden epitopes. *Proc. Natl. Acad. Sci. U. S. A.* 107:11358–11363.
- Carbonnelle E, et al. 2009. Meningococcal interactions with the host. *Vaccine* 27(Suppl 2):B78–B89.
- Coureuil M, et al. 2010. Meningococcus hijacks a beta2-adrenoceptor/beta-arrestin pathway to cross brain microvasculature endothelium. *Cell* 143:1149–1160.
- Coureuil M, et al. 2009. Meningococcal type IV pili recruit the polarity complex to cross the brain endothelium. *Science* 325:83–87.
- Craig L, Pique ME, Tainer JA. 2004. Type IV pilus structure and bacterial pathogenicity. *Nat. Rev. Microbiol.* 2:363–378.
- Craig L, et al. 2003. Type IV pilin structure and assembly: X-ray and EM analyses of *Vibrio cholerae* toxin-coregulated pilus and *Pseudomonas aeruginosa* PAK pilin. *Mol. Cell* 11:1139–1150.
- Craig L, et al. 2006. Type IV pilus structure by cryo-electron microscopy and crystallography: implications for pilus assembly and functions. *Mol. Cell* 23:651–662.
- Doulet N, et al. 2006. Neisseria meningitidis infection of human endothelial cells interferes with leukocyte transmigration by preventing the formation of endothelial docking structures. *J. Cell Biol.* 173:627–637.
- Forest KT, Tainer JA. 1997. Type-4 pilus-structure: outside to inside and top to bottom—a minireview. *Gene* 192:165–169.
- Geoffroy MC, Floquet S, Metais A, Nassif X, Pelicic V. 2003. Large-scale analysis of the meningococcus genome by gene disruption: resistance to complement-mediated lysis. *Genome Res.* 13:391–398.
- Helaïne S, et al. 2005. PilX, a pilus-associated protein essential for bacterial aggregation, is a key to pilus-facilitated attachment of *Neisseria meningitidis* to human cells. *Mol. Microbiol.* 55:65–77.
- Helaïne 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.
- Kallstrom H, Liszewski MK, Atkinson JP, Jonsson AB. 1997. Membrane cofactor protein (MCP or CD46) is a cellular pilus receptor for pathogenic *Neisseria*. *Mol. Microbiol.* 25:639–647.
- Kirchner M, Heuer D, Meyer TF. 2005. CD46-independent binding of neisserial type IV pili and the major pilus adhesin, PilC, to human epithelial cells. *Infect. Immun.* 73:3072–3082.
- Lee SW, et al. 2005. PilT is required for PI(3,4,5)P3-mediated crosstalk between *Neisseria gonorrhoeae* and epithelial cells. *Cell Microbiol.* 7:1271–1284.
- Mairey E, et al. 2006. Cerebral microcirculation shear stress levels determine *Neisseria meningitidis* attachment sites along the blood-brain barrier. *J. Exp. Med.* 203:1939–1950.
- Merz AJ, Enns CA, So M. 1999. Type IV pili of pathogenic *Neisseriae* elicit cortical plaque formation in epithelial cells. *Mol. Microbiol.* 32:1316–1332.
- Merz AJ, So M. 2000. Interactions of pathogenic *Neisseriae* with epithelial cell membranes. *Annu. Rev. Cell Dev. Biol.* 16:423–457.
- Mikaty G, et al. 2009. Extracellular bacterial pathogen induces host cell surface reorganization to resist shear stress. *PLoS Pathog.* 5:e1000314. doi:10.1371/journal.ppat.1000314.
- Morand PC, et al. 2004. Type IV pilus retraction in pathogenic *Neisseria* is regulated by the PilC proteins. *EMBO J.* 23:2009–2017.
- Morand PC, Drab M, Rajalingam K, Nassif X, Meyer TF. 2009. *Neisseria meningitidis* differentially controls host cell motility through PilC1

- and PilC2 components of type IV pili. PLoS One 4:e6834. doi:10.1371/journal.pone.0006834.
23. Morand PC, Tattévin P, Eugene E, Beretti JL, Nassif X. 2001. The adhesive property of the type IV pilus-associated component PilC1 of pathogenic *Neisseria* is supported by the conformational structure of the N-terminal part of the molecule. Mol. Microbiol. 40:846–856.
 24. Nassif X, et al. 1994. Roles of pilin and PilC in adhesion of *Neisseria meningitidis* to human epithelial and endothelial cells. Proc. Natl. Acad. Sci. U. S. A. 91:3769–3773.
 25. Nassif X, et al. 1993. Antigenic variation of pilin regulates adhesion of *Neisseria meningitidis* to human epithelial cells. Mol. Microbiol. 8:719–725.
 26. Parge HE, et al. 1995. Structure of the fibre-forming protein pilin at 2.6 Å resolution. Nature 378:32–38.
 27. Pron B, et al. 1997. Interaction of *Neisseria meningitidis* with the components of the blood-brain barrier correlates with an increased expression of PilC. J. Infect. Dis. 176:1285–1292.
 28. Pujol C, Eugene E, Marceau M, Nassif X. 1999. The meningococcal PilT protein is required for induction of intimate attachment to epithelial cells following pilus-mediated adhesion. Proc. Natl. Acad. Sci. U. S. A. 96:4017–4022.
 29. Rudel T, Scheurerpflug I, Meyer TF. 1995. *Neisseria* PilC protein identified as type-4 pilus tip-located adhesin. Nature 373:357–359.
 30. Seifert HS, So M. 1988. Genetic mechanisms of bacterial antigenic variation. Microbiol. Rev. 52:327–336.
 31. Virji M, Heckels JE, Potts WJ, Hart CA, Saunders JR. 1989. Identification of epitopes recognized by monoclonal antibodies SM1 and SM2 which react with all pili of *Neisseria gonorrhoeae* but which differentiate between two structural classes of pili expressed by *Neisseria meningitidis* and the distribution of their encoding sequences in the genomes of *Neisseria* spp. J. Gen. Microbiol. 135:3239–3251.
 32. Virji M, et al. 1991. The role of pili in the interactions of pathogenic *Neisseria* with cultured human endothelial cells. Mol. Microbiol. 5:1831–1841.
 33. Virji M, Makepeace K, Ferguson DJ, Achtman M, Moxon ER. 1993. Meningococcal Opa and Opc proteins: their role in colonization and invasion of human epithelial and endothelial cells. Mol. Microbiol. 10:499–510.
 34. Virji M, Watt SM, Barker S, Makepeace K, Doyonnas R. 1996. The N-domain of the human CD66a adhesion molecule is a target for Opa proteins of *Neisseria meningitidis* and *Neisseria gonorrhoeae*. Mol. Microbiol. 22:929–939.
 35. Winther-Larsen HC, et al. 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.
 36. Wolfgang M, van Putten JP, Hayes SF, Koomey M. 1999. The *comP* locus of *Neisseria gonorrhoeae* encodes a type IV prepilin that is dispensable for pilus biogenesis but essential for natural transformation. Mol. Microbiol. 31:1345–1357.