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

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26 **Abstract**

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

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## 52 **Introduction**

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

68 Type IV pili are formed of a major subunit, the major pilin, PilE. PilE is not only a  
69 structural component but is also a signalling protein able to directly interact with the  $\beta$ 2-  
70 adrenergic receptor (4). The major pilin subunit is subject to antigenic variation (30). Some  
71 pilin variants have been associated with an ability to promote higher adhesiveness than others.  
72 The high adhesive phenotype was linked to the ability of these variants to form bundles of pili  
73 and to allow bacterial aggregation, thus increasing interbacterial interactions (25). In addition,  
74 three minor pilins, designated PilV, PilX and ComP, are present in the fiber at low levels (13,  
75 35, 36). These minor pilins are involved in specific functions supported by tfp. ComP is  
76 essential for the natural transformation of the bacteria (36). PilV, participates in the induction

77 of signalling since mutants not producing PilV were defective in endothelial cell signalling  
78 (20) and recently PilV has been shown to interact directly with the  $\beta$ 2-adrenergic receptor, as  
79 PilE (4). PilX is necessary for the aggregative properties of tfp (12). Bacteria-bacteria  
80 interactions due to the aggregative property of PilX are responsible for an increased number  
81 of bacteria capable of interacting with cells (13). This property is related to a specific  
82 protruding region of the PilX subunit that connects pili from two different bacteria. Another  
83 major property of tfp is their ability to retract. Following pilus-mediated adhesion pili have  
84 been shown to retract (28). The cytoplasmic PilT ATPase is responsible for retraction of the  
85 fiber. In a *pilT* mutant, PilX is no longer required to form bacterial aggregates, this lead to the  
86 hypothesis that the protruding region of PilX links two anti-parallel pili and prevents the  
87 fibers to slide upon pilus retraction. In addition to these components other proteins located in  
88 the bacterial membranes or periplams are important for pilus biogenesis (3). Among these the  
89 PilC proteins play a role which is still enigmatic. Two *pilC* alleles that encode for two  
90 paralogous proteins PilC1 and PilC2 have been described (24). PilC-null strains show  
91 impaired pilus expression. In addition, PilC1 is required for adhesion and PilC1-/PilC2+ strain  
92 are unable to adhere to endothelial cells. On the other hand, PilC2, which is expressed  
93 independently of PilC1 possesses these adhesiveness properties specifically on some cell  
94 types but not on endothelial cells (15, 22-24).

95         It has recently been shown that mechanical force can extend the repertoire of tfp  
96 structures by revealing hidden epitopes of PilE previously buried in the tfp fiber (2, 8).  
97 Indeed, when subject to force, tfp will transition into a new conformation. The new structure  
98 is longer and narrower than resting tfp. Upon release of the force, tfp regain their original  
99 form (2). SM1 monoclonal antibody (mAb) recognizes the conserved epitope EYYLN in the  
100 pilin monomer (31). The SM1 epitope is predicted to be buried in the fiber, and in the resting  
101 unstretched state the SM1 mAb recognizes by electron microscopy only the exposed end of

102 the fiber (2, 7, 10, 26). On the other hand, in the elongated form of the pilus, SM1 mAb binds  
103 to the length of the stretched fiber (2). Thus force-induced stretch exposes residues in the tfp  
104 that are normally hidden. The role *in vivo* of this newly described characteristic of tfp is  
105 unknown.

106         During the course of a systematic analysis of the role of tfp components or tfp  
107 associated proteins in meningococcal signalling, we found that a strain deleted for PilX was  
108 defective in endothelial cells signalling. Unlike the major pilin PilE and the minor pilin PilV,  
109 PilX does not interact directly with the  $\beta$ 2-adrenergic receptor. We subsequently hypothesized  
110 that PilX could be necessary for structural change within the fiber. Based on the labeling of  
111 the fiber by the SM1 mAb, we show that following pilus-mediated adhesion the SM1 epitope  
112 was exposed on the length of the fiber, whereas bacterial aggregation was not sufficient for this  
113 epitope to be exposed. Moreover, the binding of the SM1 mAb in adhesive bacteria along the  
114 pili required PilX. These data suggest that upon bacterial adhesion the fiber was elongated and  
115 that this structural change required PilX. We subsequently propose that the minor pilin PilX  
116 following bacterial adhesion is necessary for the structural modification of the fiber that  
117 allows bacterial cell signalling.

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## 120 **Material and Methods**

### 121 ***Bacterial strains and infection conditions***

122 A pilated and noncapsulated Opa<sup>+</sup> derivative of the serogroup C meningococcal  
123 strain 8013, designated 2C43 siaD<sup>-</sup> Opa<sup>+</sup>, was used throughout the study (5, 25 ). This  
124 noncapsulated derivative was engineered by introduction of *cat* resistance cassette into the  
125 *siaD* gene. All the mutants and derivatives used in this work were isogenic derivatives of  
126 meningococcal C strain 2C43 siaD<sup>-</sup> Opa<sup>+</sup> and are listed in Table 1.

127 *N. meningitidis* strains were grown on GCB agar plates (Difco) containing Kellog's  
128 supplements and supplemented with appropriate antibiotics in a moist atmosphere containing  
129 5% CO<sub>2</sub> at 37°C.

130 On the day of infection, a suspension of the bacteria from an overnight culture on  
131 GCB agar plate was adjusted to OD<sub>600Nm</sub>=0.05 and incubated for 2h at 37°C in a pre-warmed  
132 cell culture medium. Cells were infected with bacteria at a multiplicity of infection (MOI) of  
133 100 for 30 minutes to allow *N. meningitidis* adhesion and then washed with cell culture  
134 medium and maintained in appropriate fresh medium for 1 or 2 hours.

### 135 ***Cell culture***

136 Human Umbilical Vein Endothelial Cells (HUVECs; Promo-cell) were used between  
137 passages 1 and 8 and grown in Endo-SFM supplemented with 10% heat-inactivated fetal  
138 bovine serum (FBS, PAA Laboratories GmbH), 0.5 IU/mL of heparin and 40 mg/mL of  
139 endothelial cell growth supplement (Sigma-Aldrich). Human Embryonic Kidney 293 (HEK-  
140 293) cells were grown in DMEM medium supplemented with 10% Fetal Calf Serum. Cells  
141 were grown at 37°C in a humidified incubator under 5% CO<sub>2</sub>. For immunofluorescence  
142 assays, HUVECs and HEK293 cells were grown on 12-mm diameter glass coverslips coated  
143 with human fibronectin (10 µg/mL, Life-Technologies) or Poly-L-lysine (0,01%, Sigma-  
144 Aldrich), respectively.

145 HEK-293 cells were transfected using Lipofectamine 2000 (Life-Technologies) following  
146 manufacturer instructions.

### 147 *Antibodies and Chemicals*

148 The following antibodies were used for immunofluorescence labeling or enzyme-  
149 linked immunosorbent assay (ELISA): anti-ezrin rabbit polyclonal antibody (generously  
150 provided by Dr. P Mangeat CNRS, UMR5539, Montpellier, France); anti-pilE mouse  
151 monoclonal antibody (clone 20D9) (28); anti-pilE mouse monoclonal antibody (clone SM1)  
152 (generously provided by Dr. Mumtaz Virji). Goat secondary antibodies were used for  
153 immunofluorescence labeling or ELISA: anti-mouse IgG (H+L) coupled to Horse Radish  
154 Peroxidase (Jackson ImmunoResearch Laboratories) and anti-rabbit IgG (H+L) or anti-mouse  
155 IgG (H+L) coupled to Alexa-488 or Alexa-546 (Life-Technologies). 4',6-diamidino-2-  
156 phenylindole (DAPI) was purchased from Life-Technologies. Isopropyl  $\beta$ -D-1-  
157 thiogalactopyranoside (IPTG) was purchased from Promega and used at a concentration of  
158 1mM.

### 159 *Analysis of piliation*

160 Analysis of piliation was performed using a whole-cell ELISA, as already described  
161 (12). Briefly, bacteria were grown 2h in RPMI/BSA1% from an  $OD_{600nm}=0.5$  and after  
162 washes were resuspended in PBS at an  $OD_{600nm}=0.1$ . Serial twofold dilutions were coated in  
163 the wells of a microtitre plate. The plates were incubated without covers at 56°C for 1 hour.  
164 Coated plates were washed several times with PBS-0.1% Tween 80 (washing solution), then  
165 the anti-pilE mouse monoclonal antibody 20D9 diluted in washing solution was added to the  
166 plates and incubated 1 h at 37°C. After several washes a peroxidase-linked anti-rabbit IgG  
167 was added to the wells for 1h. Finally, after several washes, the staining was revealed using  
168 TMB (3,3',5,5'-tetramethylbenzidine) substrate and STOP solution following the  
169 manufacturer's instructions (Cell Signalling Technology).



170 ***Immunofluorescence experiments***

171 Infected cells were fixed for 20 min in PBS 4% PFA. For Ezrin immuno-staining only,  
172 cells were permeabilized for 5 min in PBS containing 0.1% Triton X-100. Cells were  
173 incubated with primary antibodies in PBS containing 0.3% BSA or 0.1% Gelatin for 1 hr.  
174 After 3 washes with the same buffer, DAPI was added to Alexa-conjugated secondary  
175 antibodies for 1 hr. After additional washes, coverslips were mounted in Mowiol (Citifluor  
176 Ltd).

177 *N. meningitidis* aggregates were coated on glass coverslips by centrifugation after a 2  
178 hours liquid culture in HUVEC culture medium (3000 rpm, 10 min), fixed using 4% PFA and  
179 processed for immunostaining.

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

183 ***Quantitative Analysis of Ezrin (and other Proteins) Recruitment under Bacterial Colonies***

184 The recruitment efficiency was estimated by determining the proportion of colonies  
185 that efficiently recruit the protein of interest in a “honeycomb shape” just under the colonies.  
186 At least 50 colonies were scrutinized per coverslip. Each experiment was performed in  
187 duplicate and repeated several times (at least 3). Data were examined for significance using  
188 Student’s *t* test.

189 ***Expression and Purification of MBP-Pilin Recombinant Proteins***

190 Recombinant MBP-pilin fusion proteins were produced as described before (4, 13 ).  
191 Briefly, fragments of *pilE*, *pilV*, *comP* and *pilX* genes lacking the region coding for the first  
192 28 amino acid residues were amplified by PCR and subcloned in pMAL-p2X vector (New  
193 England Biolabs). The resulting plasmids, pMAL-pilE, pMAL-pilX, pMAL-pilV and pMAL-  
194 comP contain in frame the *malE* gene, which encodes the maltose-binding protein (MBP),

195 followed by the Factor Xa protease recognition site and the truncated pilin coding regions.  
196 These plasmids were transformed in the PAP5198 E. coli strain (generously provided by Dr.  
197 Olivera Francetic, Institut Pasteur, Paris, France), deleted for the periplasmic enzymes: OmpT  
198 Prt and DegP. The fusion proteins were purified on amylose resin (New England Biolabs).

199 ***Coating of Staphylococcus aureus with MBP-Pilin Fusion Proteins and infection***

200 *S. aureus* ATCC 25923 expressing specific receptors for the Fc domain of IgG  
201 immunoglobulins were used for this experiment as described before (4). Briefly, about  $10^8$   
202 bacteria were incubated with the anti-MBP rabbit polyclonal antibody for 20 min at room  
203 temperature. Bacteria were then centrifuged at 4000 rpm for 5 min, washed with warm LB 3  
204 times, incubated with 2.5  $\mu$ g of each recombinant fusion protein for 20 min at room  
205 temperature. After several washes using DMEM supplemented with 10% Fetal Calf Serum,  
206 bacteria were incubated 30min with HEK293 cells previously transfected with plasmid  
207 encoding the YFP-tagged human  $\beta$ 2-adrenergic receptor ( $\beta$ 2AR-YFP) (1). Cells were then  
208 fixed using 4% PFA in PBS and analyzed by immunofluorescence assay for  $\beta$ 2AR-YFP  
209 recruitment underneath *S. aureus* colonies.

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215 **Results**

216 **The minor pilin PilX is required for type IV pilus-mediated signalling of meningococcal**  
217 **microcolonies to endothelial cells.**

218 In an attempt to identify all pilus components, beside PilE and PilV, involved in  
219 meningococcal signalling, we tested systematically the ability of strains carrying a mutation in  
220 identified tfp components or tfp-associated proteins to signal to endothelial cells. Considering  
221 that some of these mutants are non piliated (Table2) or incapable of pilus-mediated adhesion,  
222 such as the PilC1 defective derivative, all the mutations were introduced into a strain  
223 expressing an Opa protein, a secondary adhesin. However in order to be able to promote  
224 adhesion, an Opa<sup>+</sup> expressing strain has to be non capsulated (33), a non capsulated  
225 background was therefore used by introducing a *siaD* mutation (5). A noncapsulated Opa<sup>+</sup>  
226 strain is indeed able to adhere to eukaryotic cells expressing CEACAMs (Carcinoembryonic  
227 Antigen-related Cell Adhesion Molecules) independently of tfp expression (5, 34). Therefore,  
228 all experiments described in this work were performed in a SiaD<sup>-</sup>, Opa<sup>+</sup> background. As  
229 previously shown, such a piliated strain is not affected in its ability to signal to cells (5).  
230 Results of these experiments are reported Table 2 and Figure 1. As expected, none of the non  
231 piliated mutants (*pilE*, *pilD*, *pilQ*, *pilH*, *pilI*, *pilJ*, and *pilK* mutants (11)) was able to recruit  
232 the cortical plaque, thus confirming that pili are required for meningococcal formation of the  
233 cortical plaque. However this cannot rule out the implication of one of these components in  
234 signalling. The lack of piliation may prevent the evaluation of the role of some components in  
235 signalling independently of their role in pilus biogenesis, such as the major pilin PilE.

236 The adhesive properties of tfp to endothelial cells have been shown to require the  
237 expression of PilC1, an outer membrane protein associated with tfp (24). Indeed, *pilC1*  
238 mutant are piliated if they express PilC2 but are unable to adhere to HUVECs. In a SiaD<sup>-</sup>,  
239 Opa<sup>+</sup> background both *pilC1* or *pilC2* mutant formed microcolonies. Both strains are able to

240 recruit ezrin and actin (Table 2 and Figure 1). The fact that the *pilC1* mutant was capable of  
241 signalling to cells even though unable to promote pilus-mediated adhesion confirms that type  
242 IV pilus mediated-signalling and -adhesion are two independent processes mediated by the  
243 same bacterial attribute (4).

244 Consistent with previous results (20), the *pilV* mutant expressed *tfp*, but recruited the  
245 cortical plaque very poorly (Table1). Surprisingly a *pilX* mutant had a dramatic signalling  
246 defect (Table2 Figure 1 and 2) and was unable to induce cortical plaque recruitment (Figure  
247 2). The introduction into the chromosome of a *pilX* allele under the control of an IPTG  
248 inducible promoter restored the capability to induce signalling upon addition of IPTG (Figure  
249 2), thus excluding that the signalling defect was due to a polar effect of the mutation. The *pilX*  
250 mutant adhered to cells via Opa proteins and, due to the absence of bacteria-bacteria  
251 interactions, formed microcolonies of smaller size than those of the parental strain. It should  
252 be pointed out that none of the microcolonies made by the *pilX* mutant, regardless of their  
253 size, was capable of signaling to endothelial cells. The parental strain expressed a high  
254 adhesive pilin variant, thus being responsible for large colonies adhering to the apical surface  
255 of the endothelial cells. We subsequently repeated this experiment using a derivative  
256 expressing a low adhesive variant, defective in bacterial aggregation (*i.e.* expressing the SA  
257 pilin variant (25)). The SA pilin variant is responsible for few bacteria-bacteria interactions  
258 and hence induced the formation of small microcolonies on the surface of apical cells (25). As  
259 shown Figure 2, adhesive bacteria of this derivative recruited the cortical plaque in a manner  
260 similar to that of the strain expressing a high adhesive pilin variant. Altogether, these results  
261 demonstrate that the lack of signalling observed with the *pilX* mutant is not due to the small  
262 size of the microcolonies interacting with the endothelial cells.

263 The PilT protein is responsible for *tfp* retraction. PilT dependant retraction has been  
264 shown to enhance *Nm* signalling to epithelial cells, probably by a mechano-transduction

265 mechanism (16, 18). Interestingly, on endothelial cells, a retraction deficient isolate was able  
266 to induce the formation of cortical plaques (Table 1 and Figure 1 and 3). Considering that a  
267 *pilT* mutation has been shown to compensate some phenotypical defects in strains carrying a  
268 mutation in tfp components or tfp-associated components (12), a *pilT* mutation was  
269 introduced into the *pilX* mutant. The signalling ability of the resulting strains was then tested.  
270 As shown Figure 1 and 2, the *pilT* mutation partially restored the cortical plaque recruitment  
271 defect of the *pilX* mutant.

272 Altogether, these results clearly confirmed that type IV pili are required for the  
273 formation of the cortical plaque and that the minor pilin PilX was playing a major role in the  
274 recruitment of the cortical plaque but was dispensable in a non retractable strain.

275

#### 276 **PilX does not recruit the $\beta$ 2-adrenergic receptor.**

277 As already mentioned, PilE and PilV are involved in the recruitment of the signalling  
278 receptor, the  $\beta$ 2-adrenergic receptor. Indeed, recombinant PilV and PilE proteins when fused  
279 with the maltose binding protein (MBP) and bound to live staphylococci via anti MBP  
280 antibodies are able to induce the recruitment of the the  $\beta$ 2-adrenergic receptor in a model of  
281 HEK293 cells transfected with both the  $\beta$ -arrestin and the  $\beta$ 2-adrenergic receptor; (see  
282 experimental procedures section and (4)). In order to assess a possible interaction of PilX with  
283 the adrenergic receptor, we used a similar strategy and produced recombinant PilX fused with  
284 the maltose binding protein (MBP). HEK cells overexpressing the  $\beta$ -arrestin and the  $\beta$ 2-  
285 adrenergic receptor tagged with YFP were infected with *Staphylococcus aureus* coated with  
286 anti-MBP antibody and the MBP-Pilin fusion proteins. The ability of these proteins to recruit  
287 the  $\beta$ 2-adrenergic receptor in these transfected HEK293 cells was subsequently tested. Data  
288 are reported in Figure 4. In accordance with our previous work (4), MBP-PilV and MBP-PilE  
289 but not MBP-ComP recruits the  $\beta$ 2-adrenergic receptor. On the other hand, MBP-PilX was

290 unable to recruit the receptor. This result is not consistent with a direct interaction of PilX  
291 with the  $\beta$ 2-adrenergic receptor, thus suggesting that PilX may have an indirect interaction  
292 effect on tfp-induced signalling.

293

294 **Upon bacterial adhesion, PilX is needed to reveal the previously hidden SM1 epitope.**

295 As already mentioned, tfp, when subjected to force, will transition into a new  
296 conformation (2). The new structure is longer and narrower than the original structure. Upon  
297 release of the force, the tfp fiber regains its original form, indicating a reversible transition.  
298 These force-induced conformations expose hidden epitopes, such as those recognized by the  
299 SM1 monoclonal antibody. This epitope is not exposed in fibers of bacteria grown in broth  
300 culture (2, 8). The above data suggesting that PilX does not play a role *via* a direct interaction  
301 with the signalling receptor raised the question of the mechanism by which PilX may enhance  
302 pilus-mediated signalling to endothelial cells. Previous reports have proposed that surface-  
303 exposed motifs in PilX subunits stabilize bacterial aggregates against the disruptive force of  
304 pilus retraction (13). We subsequently postulated that upon bacterial interaction with cells, the  
305 forces generated by pilus retraction may stretch the fiber, thus exposing epitopes previously  
306 buried in the tfp fiber,.

307 The strain used in this study carries the SM1 epitope (31). We subsequently addressed  
308 the availability of this epitope in isogenic  $\Delta pilX$  or  $\Delta pilX/\Delta pilT$  meningococcal strains  
309 adhering or not onto endothelial cells. As already mentioned, since a *pilX* mutant is non  
310 adhesive, all strains used were non capsulated (SiaD-), Opa+ derivatives of strain 2C43. We  
311 first looked at the expression of the SM1 epitope by adhesive bacteria. As shown Figure 5A in  
312 monolayers infected with a strain expressing wild type pili, the SM1 antibody-stained pili  
313 were seen as filamentous structures among bacterial aggregates in the great majority of the  
314 microcolonies. In addition a SM1 staining was seen not only in large microcolonies of

315 adhering bacteria, but also when only a few diplococci were adhering to the cells (Figure 5A,  
316 left panel), thus excluding the possibility that this staining was a consequence of the bacterial  
317 aggregation in large microcolonies. 80 to 90% of the microcolonies on the surface of the cells  
318 are associated with the formation of a cortical plaque, and only bacterial colonies in which a  
319 SM1 staining was observed were associated with the formation of a cortical plaque (Figure  
320 5B). On the other hand, pili of the PilX- derivative were never stained by the SM1  
321 monoclonal antibody (Figure 5A), even though adhesive PilX- bacteria are heavily piliated as  
322 shown in Figure 3 (12). A western blot performed using purified PilX, ruled out the  
323 possibility that the effect observed was due to recognition of the PilX molecule by the SM1  
324 monoclonal antibody (data not shown). A strong SM1 staining of pili was observed in  
325 microcolonies of *pilT*- strains expressing non retractable pili (Figure 5A-5B). However,  
326 consistent with the above data (Figure 2), the SM1 labelling was stronger in the PilT-/PilX+  
327 strain than in the PilT-/PilX- strain. It should be pointed out that a mutation in another minor  
328 pilin such as PilV even though important for meningococcal cell signalling (20) to endothelial  
329 cells did not affect the SM1 binding when bacteria were interacting with the cells (data not  
330 shown). These results clearly showed that the SM1 epitope is exposed in wild type pili upon  
331 bacterial adhesion and not in PilX- strain but are present in non retractable (PilT-) pili  
332 regardless of the presence of PilX.

333 We then assessed the expression of the SM1 epitope in non adhering bacteria grown in  
334 broth. As shown Figure 5C, wild type pili were not stained by SM1 antibody, even though  
335 these bacteria were forming large bacterial aggregates, thus confirming that the exposure of  
336 the SM1 epitope is not a result of the ability of the bacteria to form aggregates. This is  
337 consistent with previous results indicating that the SM1 epitope is hidden when bacteria are  
338 grown in broth (2, 8). On the other hand, in a strain expressing non retractable tfp, a clear  
339 staining of pili by the SM1 monoclonal antibody was observed inside the bacterial aggregates.

340 These data demonstrated that, for broth grown bacteria, the SM1 epitope is not accessible to  
341 the antibody in wild type pili but is exposed in non retractable (PilT-) pili.

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

346

347 **The D-region of PilX is required for pilus-mediated signalling and is responsible for the**  
348 **PilX induced conformational change.**

349 The structure of PilX has previously been reported (6, 13) and shows a classical pilin  
350 structure with a C-terminal region containing two disulfide bonds delineating the D region  
351 which forms a hook exposed on the surface of the fiber (6) (Fig 6A). In a previous work, it  
352 has been shown that this protruding D region is responsible for bacterial aggregation by  
353 enhancing bacterial interaction (13). Considering the above reported results, we aimed at  
354 testing the hypothesis that both the tfp-mediated signalling and the PilX-induced  
355 conformational changes observed upon bacterial interaction with host cells were also linked to  
356 the presence of the hook formed by the D region. We subsequently assessed the ability of a  
357 PilX protein deleted in the D region to signal to cells and to allow the binding of the SM1  
358 monoclonal antibody upon bacterial cell interaction. The strains used for these experiments  
359 were *pilX* derivatives of non capsulated (SiaD-) Opa+ strains, complemented in trans either  
360 with a full length *pilX* allele ( $\Delta pilX::pilX$ ) or a *pilX* allele lacking residues 127 to 138 of the  
361 D-region ( $\Delta pilX::pilX_{127-138}$ ) (Fig 6A). These strains have previously been reported (13) and  
362 this deletion of PilX does not prevent the protein's localisation in the fiber. The data are  
363 reported in Fig 6B and 6C. They clearly show that the ability of tfp to signal to cells required  
364 the presence of the hook formed by the D region of PilX. Moreover, this region is necessary



365 for the pilus conformational change following bacterial adhesion to host cells which allows  
366 the binding of the SM1 antibody to its epitope.

367       Altogether these data demonstrate that the ability of meningococcal tfp to signal to  
368 host cells correlate with changes in the fiber structure induced by the exposed D region of the  
369 PilX protein.

370

371

372 **Discussion**

373           Type IV pili are essential for meningococcal pathogenesis. In invasive capsulated  
374 bacteria, they promote bacterial adhesion to endothelial cells and signalling. The latter is a  
375 consequence of the interaction of pili components with the N-terminal portion of the  $\beta$ 2-  
376 adrenergic receptor. Two components of the bacterial pili have been implicated as interacting  
377 with the extra cellular N-terminal portion of the  $\beta$ 2-adrenergic receptor, the major pilin PilE  
378 and a minor pilin component PilV (4). How these two components interact with the receptor  
379 remain to be understood, especially whether they act cooperatively or independently is  
380 unknown.

381           In this work a third component of the pili has been identified as playing a role in pilus  
382 mediated signalling, the minor pilin PilX. This component has previously been identified as  
383 required for efficient bacterial adhesion and aggregation. However PilX could not be  
384 identified as an adhesin and it is believed that the bacteria-bacteria interactions due to the  
385 aggregative property of PilX are responsible for the increase number of bacteria capable of  
386 interacting with cells (13). This property is related to a specific protruding region of the PilX  
387 subunit that is believed to be able to connect pili from two different bacteria upon pilus  
388 retraction preventing them to slide. Here, we identify PilX as a pilus component required for  
389 tfp-mediated signalling. As for adhesion and aggregation, this effect of PilX is abolished in a  
390 PilT deficient strain. Unlike the major pilin PilE and the minor pilin PilV, purified PilX does  
391 not promote recruitment of the  $\beta$ 2-adrenergic receptor, thus making it unlikely that the  
392 function of PilX in signalling is via a direct interaction with the signalling receptor. We  
393 subsequently hypothesized that PilX may be responsible for conformational changes which  
394 allow some pilus epitopes to be exposed. Our data clearly demonstrate that the SM1 epitope  
395 expressed by wild type pili is not accessible to monoclonal antibodies in bacteria grown in  
396 liquid cultures whereas it is clearly labelled once bacteria interact with cells. These results are

397 consistent with a recent report showing that neisserial tfp can undergo a reversible force-  
398 induced transition into a longer and narrower structure than the resting state, and that the  
399 labelling of the SM1 epitope along the length of the pili is made possible by this elongation of  
400 the fiber (2). Altogether our data suggest that upon meningococcal-cell interaction pili are  
401 elongated, and that PilX is required for this structural change. In addition this function of PilX  
402 is supported by the D region of the molecule that forms a hook exposed at the surface of the  
403 fiber. This conformational change is not due to bacterial aggregation, since bacterial  
404 aggregates grown in broth are not labelled by the SM1 epitope (Fig 5C) and SM1 labelling is  
405 observed when only a few diplococci adhere to cells (Fig 5A). These results suggest that the  
406 force necessary for this conformational change is coming from pilus retraction following  
407 bacterial adhesion. PilX, via its protruding region, by preventing pili sliding, may be  
408 responsible for the elongation of the fiber upon interaction with cells, thus exposing  
409 previously hidden epitopes in the pili. The fact that the SM1 epitope is spontaneously exposed  
410 in the fiber of PilT- pili suggests that the structure of non retractable pili is not that of the  
411 resting pilus but that of an elongated one. This hypothesis is consistent with a previous report  
412 showing that pili tend to retract spontaneously (21) and may explain why non retractable pili  
413 are able to signal to cells in the absence of PilX.

414         The absence of recruitment of the  $\beta$ 2-adrenergic receptor by purified PilX, the fact  
415 that strain expressing non retractable pili do not require PilX bacterial cell signalling, and the  
416 association between the ability of the fibers to be stained by the SM1 epitope and to signal to  
417 cells support the hypothesis that the epitopes responsible for bacterial signalling are capable  
418 of interacting with the  $\beta$ 2-adrenergic receptor only in specific conformations of the quaternary  
419 structure of the fiber.

420

421

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429

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548  
549

550 **Figure Legends**

551

552 **Figure 1.** *Neisseria meningitidis pilX* mutant strain is defective in ezrin recruitment. HUVEC  
553 cells were infected with a piliated non-capsulated strain of *Neisseria meningitidis* (wt) and its  
554 *pilE*, *pilX*, *pilC1*, *pilT* and *pilX/pilT* derivatives. The formation of the cortical plaque was  
555 assessed by the recruitment of ezrin. Ezrin was immunostained (in green) and DNA was  
556 stained using dapi (in blue). Nucleus (N); Microcolonies (Arrowhead). Scale bars=5μM. All  
557 bacteria are Opa<sup>+</sup> and noncapsulated.

558

559 **Figure 2.** *pilT* mutation partially restores signalling defect of the *pilX* mutant. HUVEC cells  
560 were infected with a piliated non-capsulated strain of *Neisseria meningitidis* (wt), its *pilE*,  
561 *pilX*, *pilT* and *pilX/pilT* derivatives and a strain expressing the pilin variant SA. The strain  
562  $\Delta pilX::pilX$  is a PilX mutant which has been complemented in trans with a *pilX* allele under  
563 the control of an IPTG inducible promoter. It should be pointed out that this strain promotes  
564 even in the absence of IPTG a low level of ezrin recruitment due to a low activity of the  
565 promoter in the absence of IPTG. Ezrin recruitment was revealed by immunostaining. The  
566 ezrin recruitment index was estimated by determining the proportion of colonies that  
567 efficiently recruit ezrin under the colonies and expressed as normalized mean values ( $\pm$ SEM)  
568 of three independent experiments in duplicate. <sup>a</sup> student *t* test ( $p < 0,001$ ) shows statistical  
569 significance when the ezrin recruitment was compared to that of the strain expressing wild  
570 type pili

571

572 **Figure 3.** Adhesive bacteria of a PilX derivative are heavily piliated. HUVEC cells were  
573 infected with a piliated non-capsulated strain of *Neisseria meningitidis* (wt) and its *pilT* and  
574 *pilX* derivatives. Type IV pili and ezrin were visualized by immunofluorescence using the



575 anti-pilE (clone 20D9) mouse monoclonal antibody and the anti-ezrin polyclonal antibody (in  
576 green and red, respectively). DNA was stained using dapi (in blue). Microcolonies  
577 (Arrowhead).

578

579 **Figure 4.** PilX is not able to recruit the  $\beta$ 2 adrenergic receptor. HEK cells over expressing the  
580  $\beta$ 2 -adrenergic receptor tagged with YFP were infected with *Staphylococcus aureus* coated  
581 with anti-MBP antibody and MBP-Pilin fusion proteins as described in the materials and  
582 methods sections. Receptor recruitment was counted in all cells in contact with bacterial  
583 aggregates and expressed as normalized mean values ( $\pm$ SEM) of three independent  
584 experiments in duplicate. <sup>a</sup> student t test ( $p < 0,001$ ).

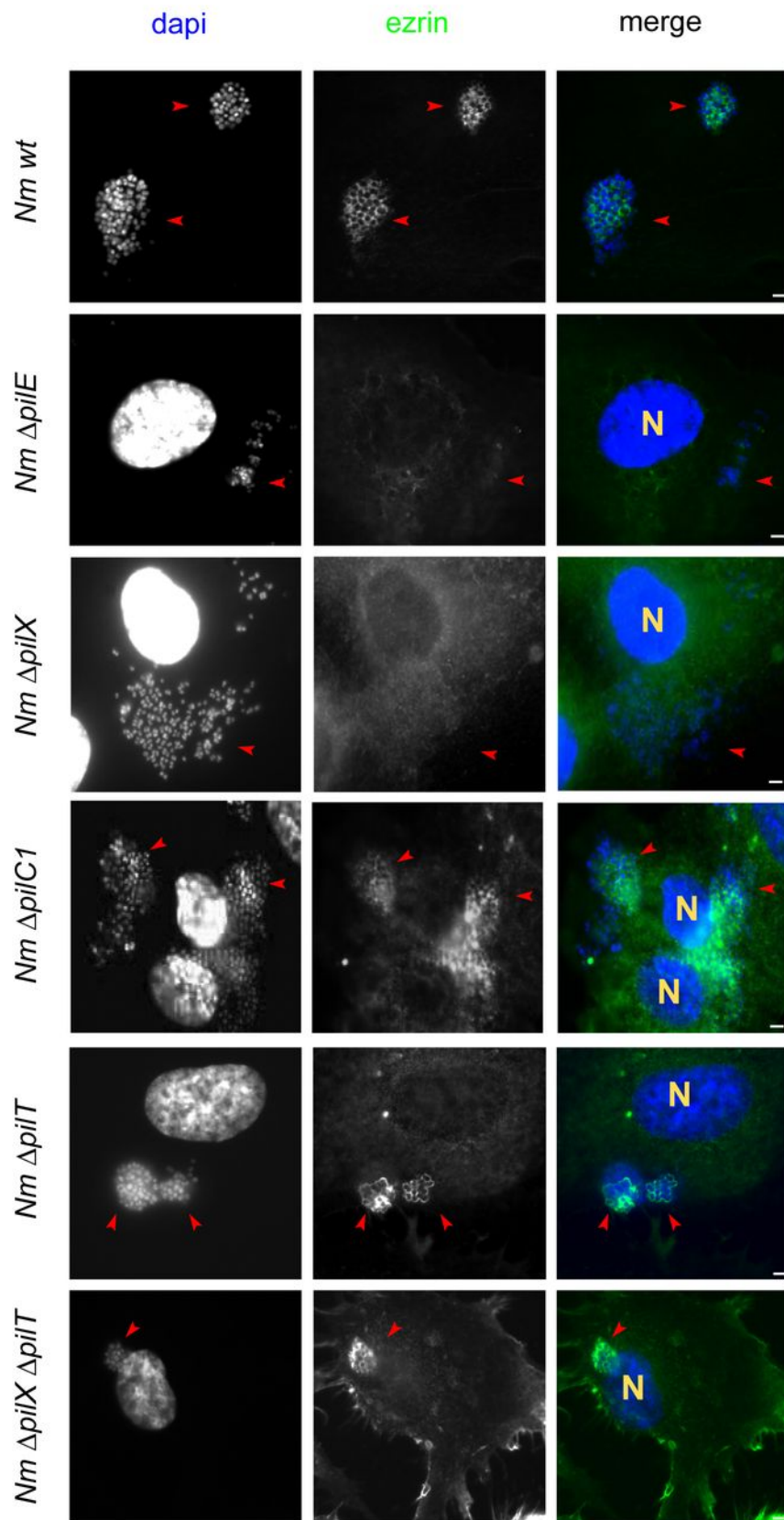
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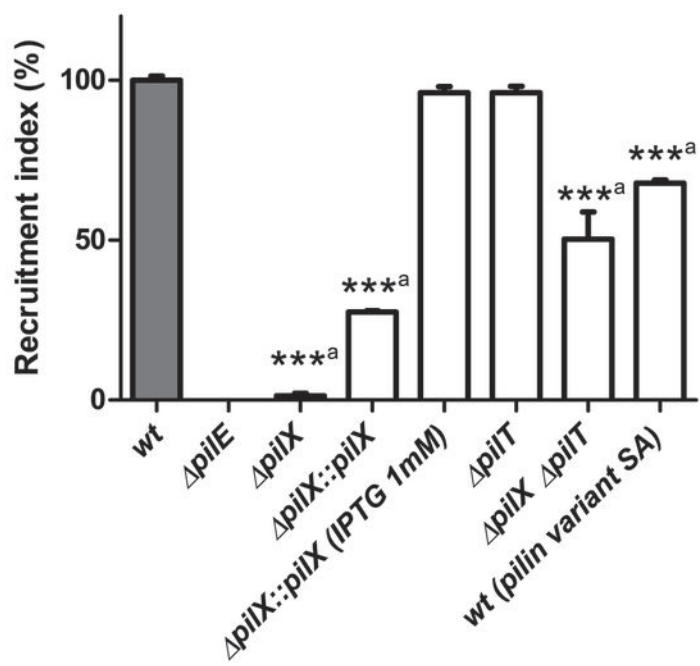
586 **Figure 5.** SM1 epitope is exposed upon bacterial adhesion to endothelial cells or in a *pilT*  
587 background. HUVEC cells were infected with a piliated non-capsulated strain of *Neisseria*  
588 *meningitidis* (wt) and its *pilT* and *pilX* and *pilX/T* derivatives. **(A)** SM1 epitope is visualized  
589 by immunofluorescence using the anti-SM1 monoclonal antibody (in green). DNA was  
590 stained using dapi (in blue). SM1 stained pili (Arrowhead). **(B)** HUVEC cells were infected  
591 with a piliated non-encapsulated strain of *Neisseria meningitidis* (wt) and its *pilT* derivative.  
592 SM1 epitope and ezrin were immunostained (in green and in red, respectively). DNA was  
593 stained using dapi. SM1 stained pili (Red arrowhead). Microcolonies without SM1 stained pili  
594 (White arrowhead). (A, B, C) Nuclei (N); Scale bars=10 $\mu$ M **(C)** *Neisseria meningitidis*  
595 aggregates were obtained after a 2h liquid culture in HUVEC culture medium. SM1 epitope is  
596 visualized by immunofluorescence using the anti-SM1 monoclonal antibody (in green). DNA  
597 was stained using dapi (in blue). Non specific staining (\*). SM1 stained pili (Arrowhead).

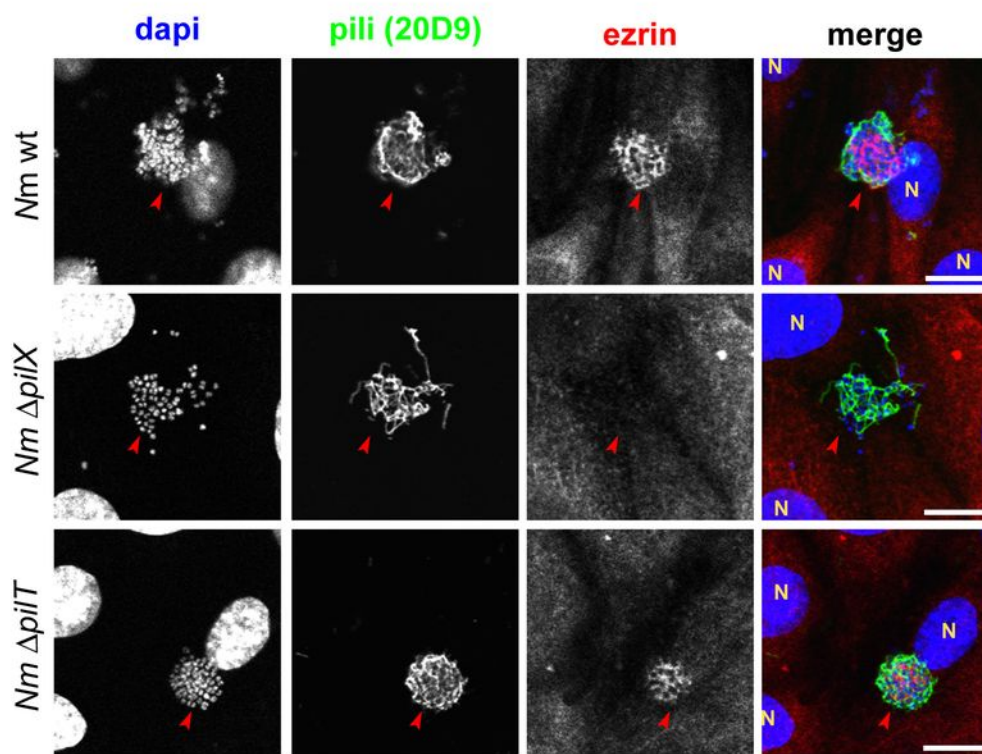
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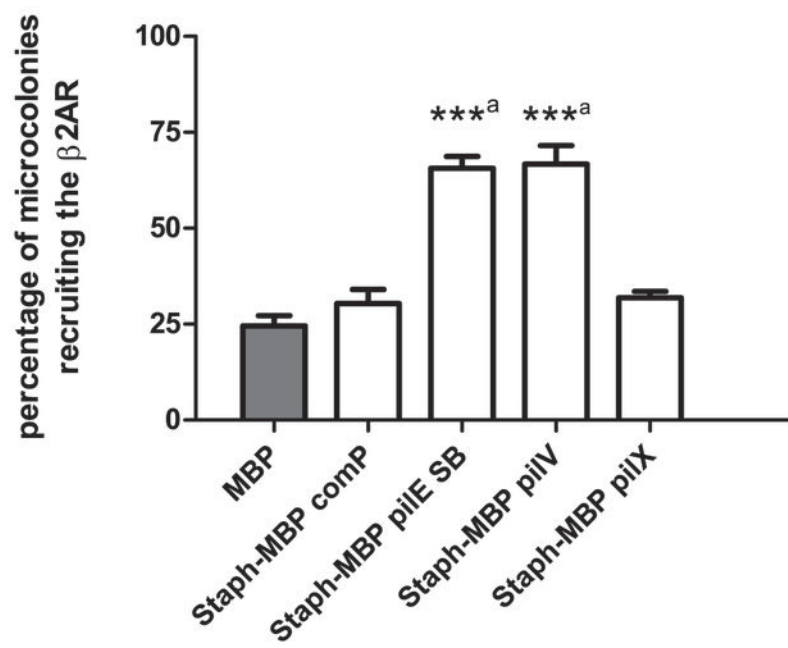
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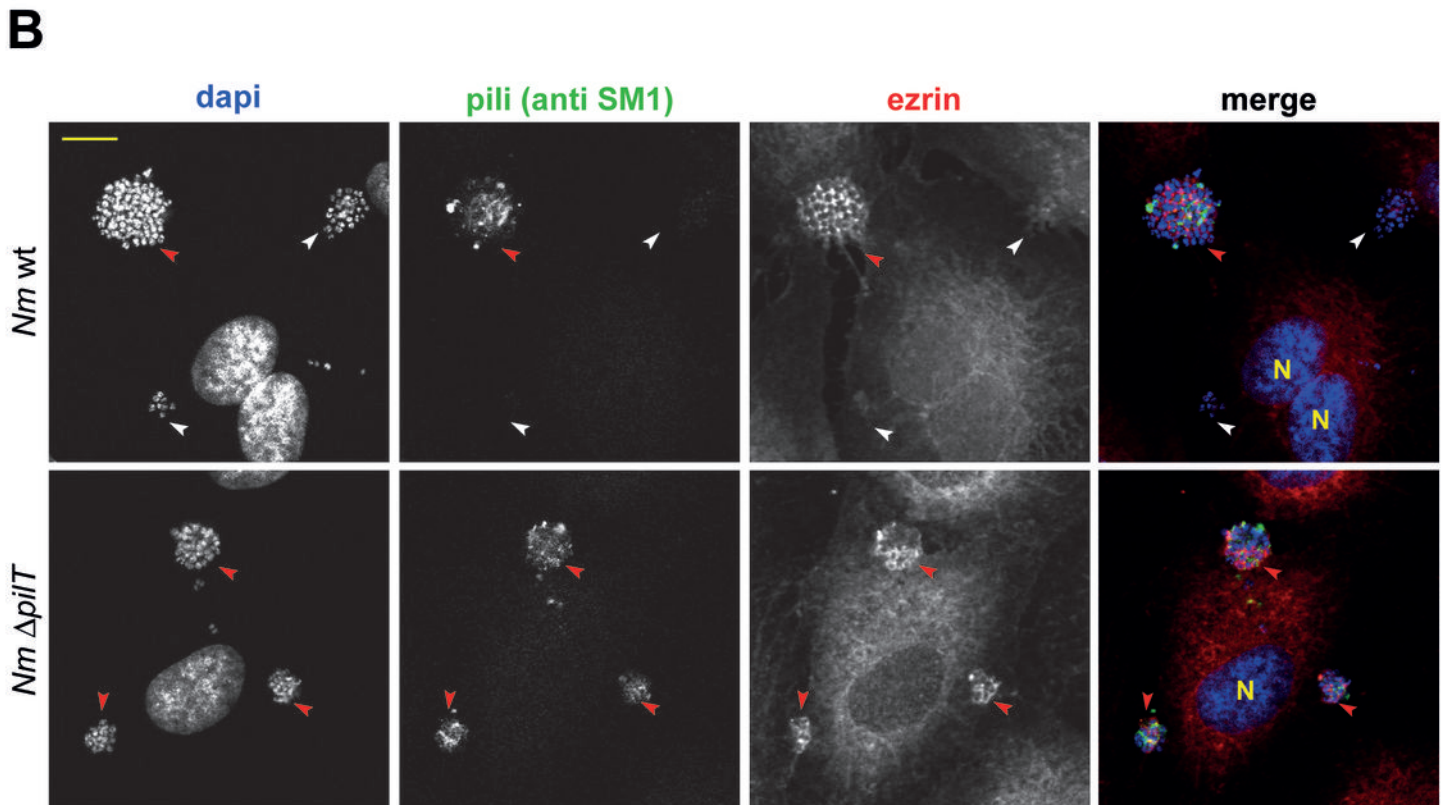
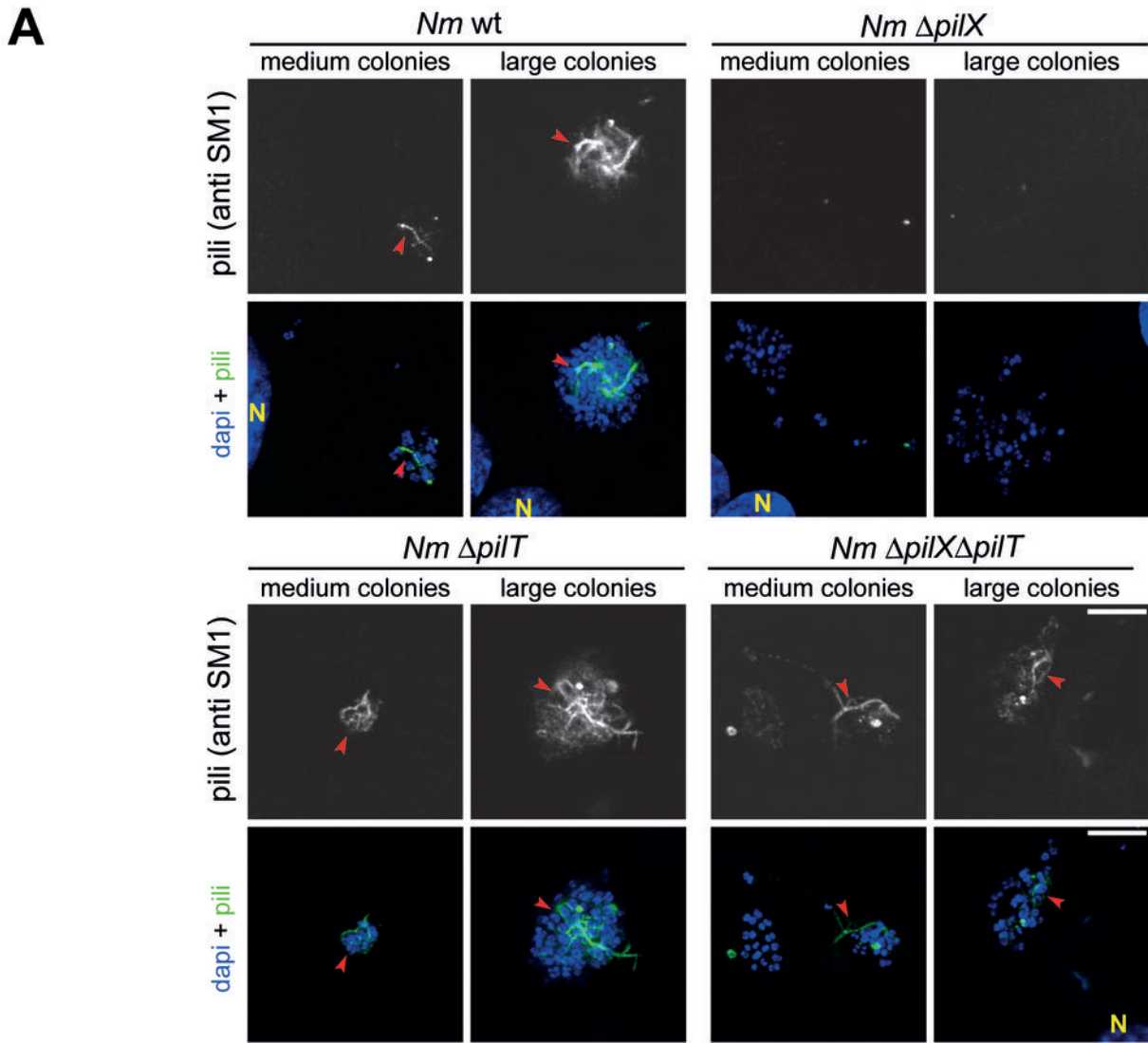
600 **Figure 6.** The D region of PilX is required for adhesion induced pili conformational change.  
601 **(A)** Sequence of the mature PilX protein from *N. meningitidis* strain 8013. The D-region  
602 delimited by a disulfide bond is highlighted in orange. The deleted surface-exposed  
603 hydrophobic residues of the D-region are indicated by the black line (Residues 127 to 138).  $\beta$   
604 sheets (Arrows).  $\alpha$  helix (black waves).  
605 **(B C)** HUVEC cells were infected with a piliated non-encapsulated (SiaD-) Opa+ strain of  
606 *Neisseria meningitidis* expressing wild type pili (wt) or a *pilX* derivative ( $\Delta pilX$ ) or a *pilX*  
607 derivative complemented either with a full length *pilX* allele ( $\Delta pilX::pilX$ ) or a *pilX* allele  
608 lacking residues 127 to 138 of the D-region ( $\Delta pilX::pilX_{127-138}$ ). SM1 epitope and ezrin were  
609 visualized by immunofluorescence. **(B)** The ezrin recruitment index (clear bars) and SM1  
610 staining index (hatched bars) were estimated by determining the proportion of colonies that  
611 efficiently recruited ezrin under the colonies (or showed a positive SM1 staining) and are  
612 expressed as normalized mean values ( $\pm$ SEM) of three independent experiments in duplicate.  
613 <sup>a</sup> student *t* test ( $p < 0,001$ ). **(C)** SM1 epitope and ezrin were immunostained (in green and red,  
614 respectively). DNA was stained using dapi. Scale bars=10 $\mu$ M.  
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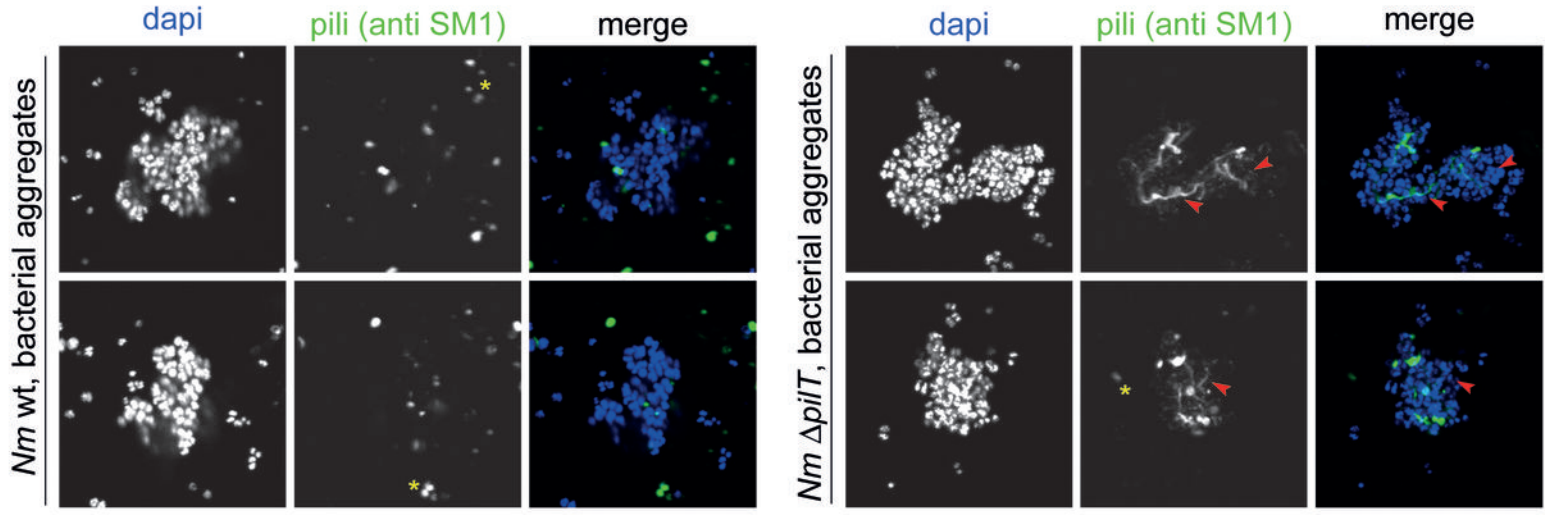




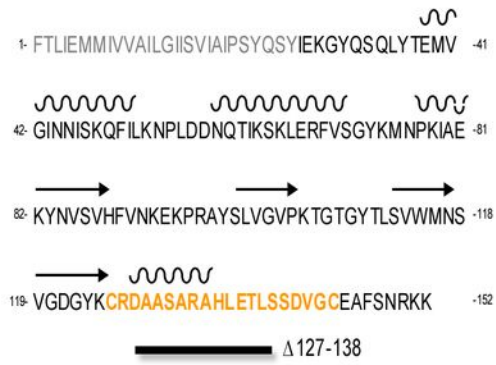
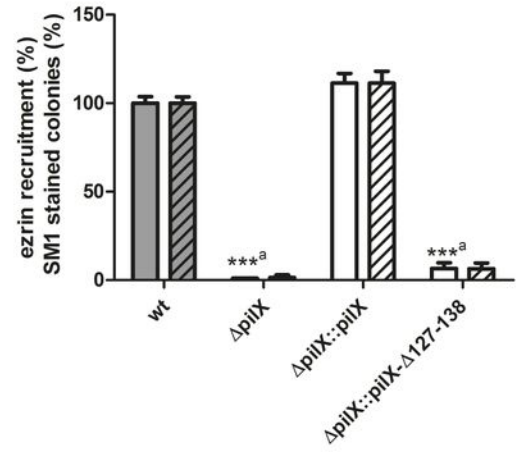


**C**

2h, liquid culture





**A****B****C**

Bacteria adhering onto HUVECs

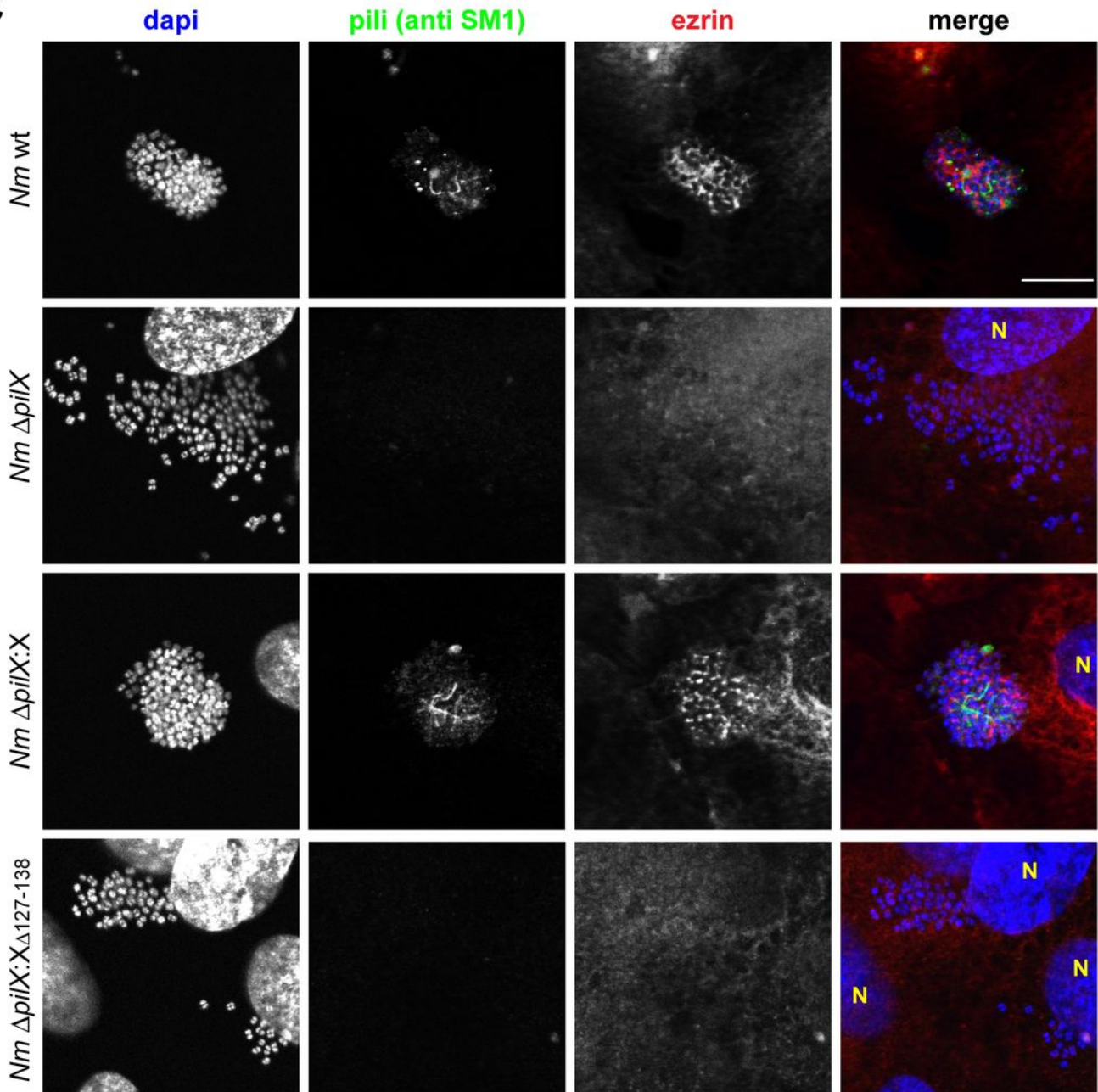


Table 1. Strains used in this study

Strain	Relevant Phenotype	Relevant Genotype	Antibiotic resistance <sup>a</sup>	Ref.
2C43-SO wt	SiaD <sup>-</sup> ; Opa <sup>+</sup> Expressing pilin SB variant	<i>ΔsiaD::cat</i>	Cm	25
2C43-SOE_SAK	Expressing pilin SA variant	-	Kn	25
2C43-SOE	PilE <sup>-</sup>	<i>ΔpilE::aphA-3</i>	Cm, Kn	22; 28
2C43-SOX	PilX <sup>-</sup>	<i>ΔpilX::aphA-3</i>	Cm, Kn	12
2C43-SOX/Xi	PilX <sup>-</sup> PilX <sub>ind</sub>	<i>ΔpilX::aphA-3</i> <i>plac-pilX<sup>b</sup></i>	Cm, Kn, Em	13
2C43-SOX/Xi <sub>(Δ127-138)</sub>	PilX <sup>-</sup> PilX <sub>(Δ127-138)ind</sub>	<i>ΔpilX::aphA-3</i> <i>plac-pilX<sub>(Δ127-138)</sub><sup>b</sup></i>	Cm, Kn, Em	13
2C43-SOT	PilT <sup>-</sup>	<i>ΔpilT::ermB</i>	Cm, Em	28
2C43-SOXT	PilX <sup>-</sup> PilT <sup>-</sup>	<i>ΔpilX::aphA-3</i> <i>ΔpilT::ermB</i>	Cm, Kn, Em	This study
2C43-SOV	PilV <sup>-</sup>	<i>ΔpilV::aphA-3</i>	Cm, Kn	20
2C43-SOD	PilD <sup>-</sup>	<i>ΔpilD::aphA-3</i>	Cm, Kn	11
2C43-SOQ	PilQ <sup>-</sup>	<i>ΔpilQ::aphA-3</i>	Cm, Kn	11
2C43-SOT2	PilT2 <sup>-</sup>	<i>ΔpilT2::aphA-3</i>	Cm, Kn	11
2C43-SOU	PilU <sup>-</sup>	<i>ΔpilU::aphA-3</i>	Cm, Kn	11
2C43-SOC1	PilC1 <sup>-</sup>	<i>ΔpilC1::aphA-3</i>	Cm, Kn	22; 28
2C43-SOC2	PilC2 <sup>-</sup>	<i>ΔpilC2::aphA-3</i>	Cm, Kn	11
2C43-SOH	PilH <sup>-</sup>	<i>ΔpilH::aphA-3</i>	Cm, Kn	This study
2C43-SOI	PilI <sup>-</sup>	<i>ΔpilI::aphA-3</i>	Cm, Kn	This study
2C43-SOJ	PilJ <sup>-</sup>	<i>ΔpilJ::aphA-3</i>	Cm, Kn	This study
2C43-SOK	PilK <sup>-</sup>	<i>ΔpilK::aphA-3</i>	Cm, Kn	This study

<sup>a</sup> Cm: Chloramphenicol (6 μg.mL<sup>-1</sup>), Kn: Kanamycin (100 μg.mL<sup>-1</sup>), Em: Erythromycin (2 μg.mL<sup>-1</sup>)

<sup>b</sup> pilX genes under control of IPTG inducible plac promoter (lacI<sup>q</sup>OP)

**Table 2.** Signalling phenotypes of genes in in Tfp biology

Gene <sup>a</sup>	Function <sup>b</sup>	Phenotypes <sup>c</sup>	
		Piliation	Signalization
<i>pilE</i>	Major pilin	-	-
<i>pilX, pilV</i>	Minor pilins	+	-
<i>pilH, pilI, pilJ, pilK</i>	Pseudopilins	-	-
<i>pilD</i>	Pilin maturation	-	-
<i>pilQ</i>	Secretin	-	-
<i>pilC1, pilC2</i>	Adhesins	+	+
<i>pilT, pilT2, pilU</i>	Pilus retraction	++	+

<sup>a</sup> mutated gene

<sup>b</sup> implication of proteins in Tfp biology

<sup>c</sup> piliation was evaluated according ELISA techniques, Signalization was evaluated by counting the proportion of *Neisseria meningitidis* microcolonies able to recruit cortical plaque