Supporting Online Material for

Posttranslational Modification of Pili upon Cell Contact Triggers N. meningitidis Dissemination

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Published 11 February 2011, Science 331, 778 (2011)
DOI: 10.1126/science.1200729

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Supporting online material for

**Cell-contact induced posttranslational modification of type IV pilin triggers *Neisseria meningitidis* dissemination**

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Supporting legends
Materials and Methods

Bacterial strains and mutagenesis

All *N. meningitidis* strains described in this study were derived from the recently sequenced 8013 serogroup C strain [http://www.genoscope.cns.fr/agc/nemesys] (1). *N. meningitidis* strains were grown on GCB agar plates (Difco) containing Kellogg’s supplements, in a moist atmosphere containing 5% CO₂ at 37°C. GFP was expressed by introducing the pAM239 plasmid by conjugation (2). The non-adhesive *pilE* and *pilC1* strains are described elsewhere (3, 4). The *pptA* mutant (8013 *pptA:: mini-Himar1*) used in this study was derived from a library of transposition mutants described elsewhere (1, 5). In-frame deletion of the *pptB* gene was introduced into the *N. meningitidis* chromosome by allelic exchange using the spectinomycin cassette from the pT1ΩI plasmid (6). To complement Δ*pptB* mutants, the WT *pptB* ORF was cloned in the pGCC4 vector, adjacent to lacIOP regulatory sequences (7) and introduced into the chromosome by homologous recombination. To generate point mutations in the *pilE* gene we took advantage of the *pilE::kan* transcriptional fusion described elsewhere which allows introduction of the chosen *pilE* allele at the endogenous site under the control of its own promoter (8). Point mutations in the *pilE* gene were introduced with the Quickchange mutagenesis kit (Stratagene) according to the manufacturer’s instructions.

Q-ToF

Pili were prepared as previously described (9). Top down analysis of PilE was performed on a Q-TOF-Premier™- (Waters Corp., Milford, MA, USA). The source temperature was set to 80°C. The capillary and cone voltages were set to 2500 and 40 V.
Q-TOF Premier instrument was operated in wide pass quadrupole mode, for MS experiments, with the TOF data being collected between \(m/z\) 400–2000 with a low collision energy of 10 eV. Argon was used as the collision gas. Scans were collected for 1 s and accumulated to increase the signal/noise ratio. The MS/MS experiments were performed using a variable collision energy (10–30 eV), which was optimized for each precursor ion. Mass Lynx 4.1 was used both for acquisition and data processing. Deconvolution of multiply charged ions into neutral species was performed using MaxEnt1 in the mass range \([10 – 25 \text{ kDa}]\) with a resolution of 0.01 Da/channel. An external calibration in MS was done with clusters of phosphoric acid (0.01M in 50:50 Acetonitrile:H\textsubscript{2}O \text{v:v}). The mass range for the calibration was \(m/z\) 70 - 2000.

**Cell culture**

Cells were grown at 37°C in a humidified incubator under 5% CO\textsubscript{2}. The human endometrial cell line HEC-1B (HTB113) and human intestinal epithelial cell line Caco-2 were purchased from the American Type Culture Collection (Rockville, Md., USA) and maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS; PAA Laboratories). The HEC-1B cell line was selected because it is extensively used in the field of *Neisseria* infections and it was used to demonstrate the induction of the CREN promoters when bacteria are in contact with host cells (10). The Caco-2 cell line is an intestinal cell line that was chosen because it efficiently forms tight junctions and generates transepithelial electrical resistance.

**Bacterial aggregation assay**

Bacteria grown on GCB agar plates were adjusted to OD\textsubscript{600}=0.05 and then incubated for 2 hours at 37°C in pre-warmed RPMI supplemented with 10 % FBS with gentle agitation. The bacterial suspension was concentrated to OD\textsubscript{600}=0.6 or 0.3 by a 1 min centrifugation at 15000 g followed by resuspension in medium containing 0.5 μg/ml of DAPI. Bacterial
suspensions were briefly vortexed and transferred in a glass-bottom 96-well plate (Nunc, Rochester, USA). After 30 min incubation, aggregates were observed microscopically with a 4x lens and size and number determined with the ImageJ software (11). Two images were captured per well, corresponding to the surface of most of the well. Using high magnification images each bacterium was estimated to occupy 4.6 µm³. This value was used to determine the number of bacteria per aggregate based on their volume. Bacterial aggregates smaller than 6 µm in diameter were not considered (about 50 individual bacteria).

**Bacterial adhesion, detachment and transmigration assays**

*Initial adhesion assay.* Experiments using the laminar flow chamber were done essentially as described (2). HEC1B epithelial cells growing on disposable flow chambers were used (Ibidi GmbH, München, Germany). Experiments using the flow chamber were performed in DMEM supplemented with 2% serum and maintained at 37°C. The bacterial culture was diluted to 7.5x10⁷ bacteria/ml and was introduced into the chamber using a syringe pump (Harvard Apparatus). Adhesion of individual bacteria was recorded using a Nikon Eclipse Ti-E/B inverted microscope with a 20x objective and a Hamamatsu ORCA03 CCD camera.

*Adhesion and proliferation in static conditions.* For bacterial adhesion to epithelial cells, 24 well plates were seeded with 10⁵ HEC-1B cells per well and the monolayers were infected with 10⁷ bacteria (MOI=100). After 1h of contact, unbound bacteria were removed by three washes and the infection was continued for 5 h. Adherent bacteria, recovered by scraping the wells, were counted by plating appropriate dilutions on GCB agar plates.

*Bacterial detachment assay.* Epithelial cells were grown in disposable flow chambers. Bacteria grown on GCB agar plates were adjusted to OD₆₀₀=0.02 in prewarmed RPMI medium containing 10% fetal bovine serum and cultivated for 2h at 37°C. Cells were infected with 10⁶ bacteria (MOI=100), adhesion allowed to proceed for 30 min, unbound bacteria removed by three extensive washes and infection continued for 2h in an incubator. Infected
cells were then placed directly in a 0.15 dynes/cm² flow. DMEM supplemented with 10% FBS was maintained at 37°C and introduced into the chamber using a syringe pump. Every hour, samples coming out of the flow chamber were collected, serial dilutions performed and a fraction was plated on GCB agar plates.

**Bacterial transmigration assay.** Caco-2 cells were grown on 12 mm diameter culture plate insert with 3 µm pores (Millipore, Cork, Ireland) for a period of 6 days to reach a trans-epithelial resistance of 600-1000 ohms/cm². The upper compartment was infected at an MOI of 100, infection allowed to proceed for 4 hours, inserts transferred to a new well and bacteria were collected in the lower compartments after 90 min. Results were normalized with passage across well without cells to minimize potential interstrain differences and results presented as a percentage relative to the wild type strain.

**Electron microscopy**

For negative staining transmission electron microscopy, a drop of bacterial suspension in PBS (OD₆₀₀=1) was placed on a Formvar-coated grid for 10 min. Bacteria were fixed for 5 min with 10 mM cacodylate buffer (pH 7.5) containing 2.5% glutaraldehyde. Grids were then washed twice with water and stained for 10 min with 1% phosphotungstic acid, air-dried and viewed using a JEOL JEM-100CX microscope operated at 80 kV.

For scanning electron microscopy, infected cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) 1h at room temperature. Samples were washed three times for 5 min in 0.2 M cacodylate buffer (pH 7.2), fixed for 1 h in 1% (wt/vol) osmium tetroxide in 0.2 M cacodylate buffer (pH 7.2), and then rinsed with distilled water. Samples were dehydrated through a graded series of 25, 50, 75 and 95% ethanol solution (5 min each step). Samples were then dehydrated for 10 min in 100% ethanol followed by critical point drying with CO₂. Dried specimens were sputtered with 10 nm gold palladium, with a GATAN Ion Beam Coater and were examined and photographed with a JEOL JSM 6700F field
emission scanning electron microscope operating at 5 Kv. Images were acquired with the upper SE detector (SEI).

**Modeling of the N. meningitidis pilin and pilus structure**

Due to the high sequence identity (77 %) the sequences of *N. gonorrhoeae* and *N. meningitidis* pilins could be simply aligned by eye. The basis of the modeling was the *N. gonorrhoeae* pilin structure as deposited in the model of the pilus (PDB code 2HIL). Missing backbone and side chains were added and optimized for packing within the context of the pilus, in a multi-stage procedure that we implemented in the program CNS (12). We assumed that the overall helical parameters of the pilus are the same for *N. gonorrhoeae* and *N. meningitidis* (rise 10.5 Å, angle 105.5). The symmetry of the pilus was enforced throughout the modeling of the pilus using the NCS STRICT command in CNS. In this way, only one single protomer is modeled explicitly, while all the neighbors are treated as images that are created on the fly to calculate the non-bonded interactions. 20 neighbors of the pilin were included in the calculation. We used a modified version of the CHARMM19 force field for all modeling.

The first stage is a quick optimization of the geometry and the packing, with a simplified non-bonded interaction (repulsive Van der Waals only). During this stage, positional restraints were used on those residues that were strictly identical to the residues in the *N. meningitidis* pilin. The second stage is a refinement in vacuo, using adapted non-bonded parameters (a distance-dependent dielectric, a switching function between 2 and 9 Å, and a non-bonded cut-off of 10 Å). The third stage is a short refinement in water, similar to the one used in NMR structure determination (13). We used a water layer of 10 Å thickness and a non-bonded cutoff of 12 Å. During this stage, the harmonic positional restraints were slowly switched off. During all three stages, the initial structures were maintained in a flexible and adaptive way using log-harmonic distance restraints and automated weighting (14).
The CHARMM19 force field was extended for the serine modifications (15). Topology and parameter files for these modifications were obtained with the help of the PRODRG2 server (16). The atom types were as far as possible mapped onto those of the CHARMM19 force field, or, if not possible, onto those of the CHARMM11 force field (for example, for the glycerophosphate group).

Bundles of pili were generated as symmetric antiparallel tetramers by randomly varying the distance, the rotation angle around the long axis of a pilus, and the crossing angle between pili. The energetic analysis was performed with the ACE generalized Born model implemented in CNS for symmetric systems (17). The binding energy was estimated as the difference between the electrostatic, van der Waals and generalized Born contributions to the total energy calculated in the complex and in an isolated pilus. We used 6 for the internal dielectric and 80 for the external dielectric.

2D gel electrophoresis

The isoelectric point of the major pilin subunit in different conditions was determined by 2D gel electrophoresis followed by immunoblot and detection of PilE with specific antiserum. Infection of an epithelial monolayer growing in a 6-well plate was initiated for a period of 30 min at an MOI of 400, cells were washed, infection was allowed to proceed for 2-4 hours as indicated, rinsed with PBS and loading buffer added directly in the wells (8 M urea, 2 M thiourea, 4% (w/v) CHAPS). All samples were treated with 2D Clean-Up kit (GE Healthcare) according to the manufacturer’s instructions and the resultant dry pellets were resuspended in loading buffer. Two-dimensional gel electrophoresis was performed as described previously (18) and proteins were blotted onto nitrocellulose membrane by standard western blotting procedures (19). The PilE protein was detected with a polyclonal antiserum directed against the PilE protein (diluted 1/1000), followed by horseradish peroxydase-linked
anti-IgG (Jackson Immunoresearch Laboratories, diluted 1/10000) and ECL Plus luminescence kit (Amersham Biosciences).

**Supporting figures:**

*Figure S1: Pilin modification with PG, structure and gene involved.*

**A.** Chemical representation of the modification of a serine with phosphoglycerol. **B.** Type IV pili were purified from a mutant in the *pptA*, a gene responsible for the transfer of PE and PC onto *N. gonorrhoeae* pilin and the molecular mass of the major pilin subunit was determined by mass spectrometry. **C.** Phylogenetic analysis of members of COG1368. To generate the phylogenetic tree, multiple alignments were done using the Muscle software package (20) and the phylogeny was done with the PhyML software (21). We took advantage of the on-line integrated tools found at the following address: http://www.phylogeny.fr/ (22). To generate and edit the tree we used the iTol (interactive tree of life) on-line tool (23). The genes were colored according to their association with one of the four groups that appear from this analysis. Genes with known function are indicated in a darker color and their function is indicated. Red dots indicate the genes in the COG that present significant homology with PptB and its counterparts in the different *N. meningitidis* strains. Asterisks indicate the genes reported to be regulated at the transcriptional level by changes in the environment.

*Figure S2: Relationship between pptB transcription level and pilin modification with PG*

The WT and *pptBind* strains were cultivated in the presence of different concentrations of IPTG, pili purified and analyzed by mass spectrometry. Occupancy of serine 93 with PG in
the \( \text{pptB}^{\text{ind}} \) strain was plotted as the relative to the total amount of pilin based on the analysis of spectra from three samples for each point (circles). The experiment was done in parallel for the wild type strain (squares). Error bars denote mean of three experiments +/- SEM.

**Figure S3: The isoelectric point of the pilin becomes more acidic during growth on the cellular surface.**

**A,** A total bacterial lysate was analyzed by 2D gel electrophoresis, transferred onto a nitrocellulose membrane and proteins were visualized using Ponceau S. **B,** The isoelectric point of PilE from a WT strain was compared when bacteria are proliferating in suspension prior to host cell infection (0) with bacteria proliferating on host cells for a period of 2 hours (2h) and 4 hours (4h). As a control, cells were infected with the \( \text{pptB}^{\text{ind}} \) strain in the absence of IPTG and with the \( \text{pptB}^{\text{ind}}\text{pilES93A} \) for 4 hours. In each case the same amount of protein was loaded. In the case of the time point 0 the bacterial lysate was mixed with a cellular lysate prepared separately. Open arrowheads indicate the position of the more basic forms, full arrowhead indicates the main form of the pilin and the arrows indicate the acidic forms that accumulated after contact with host cells. The corresponding area analyzed by western blot on panel B is indicated as a rectangle on the Ponceau S stain on panel A.

**Figure S4: Impact of serine 93 modification with PG on pilus electrostatic surface**

**A,** An explicit model of a twentimer was generated by repeated duplication, translation and rotation of the primary pilin and represented using the Pymol software. The pilin backbone is in green and the phosphoglycerol on serine 93 in space filling model. **B, C,** Modeling of electrostatic surface distribution indicates introduction of a diffuse negative charge around serine 93 when PG is present. Electrostatic calculations were done with APBS (24), with an
interior dielectric of 4 and an exterior dielectric of 80, a cube length of 300 Å and grid dimensions of 129 * 129 * 129 points. Defaults were used for all other parameters. The electrostatic potential was mapped onto the surface with -5 and 5 kT as extreme values for red and blue. The surface of the pilus was displayed with VMD (25). B, Electrostatic surface around serine 93. Blue indicates positive charge and red negative. A positively charged cavity is surrounded by an oval C, Electrostatic surface around serine 93 when modified with phosphoglycerol, the cavity surrounded by the oval has lost its positive charge and is physically filled by the modification.

**Figure S5: Impact of PG modification on the amount of pili and their ability to form bundles**

The amount of pili expressed by different bacteria was determined on whole bacteria preparations by ELISA assay essentially as previously (26). A, The wild type strain (WT) was compared to the pptB deletion strain (ΔpptB) and the non-piliated pilE mutant (pilE). Results are presented relative to the wild type strain. Statistical analyses were done with Student’s t test. n.s. not significant. B, The pptBind strain in the presence of inducer, 0.1 mM of IPTG. Results are presented relative to the non-induced conditions. C, Ability of different strains to form pili bundles was determined. Bacteria displaying pili were classified in two categories: (i) with individual fibers only; (ii) with bundles (including bacteria with bundles and individual fibers). One hundred bacteria were scored per strain and condition. Three independent experiments each done in triplicate were performed, error bars denote mean of three experiments +/- SEM.

**Figure S6: Aggregation**
A, The graph represents the level of aggregation as a function of the level of pilin modification with PG. This plot was obtained by combining: (i) the ability of the \( pptB_{\text{ind}} \) strain to form aggregates as a function of IPTG concentration (Fig. 3f) and; (ii) the level of modification of pilin with PG also as a function of IPTG concentration in the same strain (Fig. S2). B, To demonstrate that the effect of serine 93 modification was mostly due to introduction of a charge at this site, serine 93 was mutated into different amino acids with different properties: negative charge, aspartic acid (S93D) and glutamic acid (S93E); bulky, tyrosine (S93Y). Three independent experiments each done in triplicate were performed, error bars denote mean of three experiments +/- SEM. Statistical analyses were done with Student’s \( t \) test. n.s. not significant, *\( p < 0.05 \). C, The level of insertion of the minor pilin PilX into pili was determined. Pili were purified from the indicated strains, the amount of the major pilin was normalized in the different samples and the amount of PilX was determined by western blot. D, Impact of PilX mutation on pilin modification with PG as determined by mass spectrometry. PilE isolated from the \( pilX \) mutant displays the same pattern as the wild type strain.

**Figure S7: Detachment and invasion**

A, Detachment of bacteria from aggregates proliferating on the cellular surface after 7h. Results from three independent experiments each done in duplicate were pooled on this graph for the 7h time point. B, Caco-2 cells were grown on 12 mm diameter culture plate inserts with 3 µm pores (Millipore, Cork, Ireland) for a period of 6 days to reach a trans-epithelial resistance of 600-1000 ohms/cm\(^2\). Samples from the top and bottom compartments of the inserts were collected at the different time points and the results are indicated as a ratio of the amount of bacteria that transmigrated relative to the amount of bacteria on the top of the insert. Filled circles correspond to the wild type strain, open circles to the \( \Delta pptB \) strain and
squares to the non-piliated \textit{pilE} strain. C, Impact of PG modification on the invasion process. Invasion of Caco-2 cells by \textit{N. meningitidis} was tested using a gentamicin resistance assay. The number of gentamicin resistant colony-forming units were normalized by the number of total cell associated bacteria. Results are presented relative to the wild type strain. Three independent experiments each done in triplicate were performed, error bars denote mean of three experiments +/- SEM. Statistical analyses were done with Student’s \textit{t} test, \(*p < 0.05.\)

\textbf{Figure S8: Model of epithelium colonization}

Proposed sequence of events taking place during tissue colonization by \textit{Neisseria meningitidis}: \textit{adhesion}, individual bacteria in suspension attach to the cellular surface, transcription of the \textit{pptB} gene is induced to reach full expression after 2-4 hours; \textit{proliferation}, bacteria multiply in tight aggregates involving bacteria/cell and bacteria/bacteria contacts, pilin is increasingly modified with PG; \textit{detachment}, pilin modification with PG disengages the interaction between pili thus allowing the detachment of individual bacteria from the surface of the microcolony; \textit{propagation}, detached bacteria in suspension can colonize new sites in the same host or in another host. The level of \textit{pptB} goes back to basal level and the level of pilin phosphorylation decreases back to initial levels allowing a new cycle to take place and; \textit{dissemination}, bacteria disengaging from the microcolony but remaining in contact with host cells are in a position to cross the epithelium and disseminate throughout the body.

\textbf{Supporting references:}

1. C. Rusniok \textit{et al.}, \textit{Genome Biol} 10, R110 (Oct 9, 2009).


Figure S1

**A**

\[
\text{NH} - \text{C} - \text{CH}_2 - \text{OH} \\
\text{O}=\text{C} \\
\downarrow +154 \text{ Da} \\
\text{NH} - \text{C} - \text{CH}_2 - \text{O} - \text{P} - \text{O} - \text{CH}_2 - \text{CH} - \text{CH}_2 \\
\text{O}=\text{C}
\]

**B**

![Mass spectrum of ΔpptA](image)

![Osmoregulated Periplasmic glucans modification with PG](image)

**C**

![Diagram showing PG lipoteichoic acid synthesis and Type IV Pilin modification](image)
Figure S2
Figure S6

A

B

C

D

PilE

PilX

WT    ∆pilB  ∆pilX

Bacteria in aggregates (%) vs. Occupancy of S93 with PG (%)

WT  pilE  S93D  S93E  S93Y

Bacteria in aggregates (%)

Occupancy of S93 with PG (%)

WT  ∆pilX

PilE

PilX

Mass (Da)

17491.0 (+1PG)

17644.0 (+2PG)

Figure S6
Figure S7

A. Bacterial detachment (%) for different strains: 2C43, pilE, ΔpptB, S93A.

B. Percentage of bacteria passed through the monolayer over time (in hours) for WT, ΔpptB, and S93A.

C. Invasion (%) for WT, ΔpptB, and S93A.
- Propagation

- Adhesion
- Proliferation
- Detachment

Transcriptional induction of the pptB gene

Loss of aggregative properties

Pilin modification with glycerophosphate

- Dissemination

Figure S8