XCPX CONTROLS BIOGENESIS OF THE PSEUDOMONAS AERUGINOSA XCPT-CONTAINING PSEUDOPILUS

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Running Title: Pseudopilus biogenesis

Pseudomonas aeruginosa is an opportunistic Gram-negative pathogen equipped with multiple secretion systems. The type II secretion machinery (Xcp secreton) is involved in the release of toxins and enzymes. The Xcp secreton is a multi-protein complex and most of its components share homology with proteins involved in type IV pili biogenesis. Among them, the XcpT-X pseudopilins possess characteristics of the major constituent of the type IV pili, the pilin PilA. We have previously shown that XcpT can be assembled in a multifibrillar structure that was called the pseudopilus. By using two different microscopic approaches we show here that the pseudopili are preferentially isolated fibers rather than tight bundles. Moreover, none of the other four pseudopilins are able to form a pseudopilus, suggesting that the assembly of such a structure is a unique property of XcpT. Moreover, we show that five out of the twelve Xcp proteins are not required for pseudopilus biogenesis whereas they are for type II secretion. Interestingly, we showed that one pseudopilin, XcpX, controls the assembly of XcpT into a pseudopilus. Indeed, when the number of XcpX subunits increases, the length of the pseudopilus decreases. Conversely, in the absence of XcpX the pseudopilus length is abnormally long. Our results indicate that XcpT and XcpX directly interact with each other. Furthermore, this interaction induces a clear destabilization of XcpT. The interaction between XcpT and XcpX could be part of the molecular mechanism underlying the dynamic control of pseudopilus elongation, which could be crucial for type II-dependent protein secretion.
maturation of pilins and pseudopilins, namely PilA, PilE, PilV-Y and FimT-U (5,6), or XcpT-X and HxcT-X (4,7-9). The maturation results in the removal of a 6-8 residues leader peptide. Following the maturation event, the pilin PilA is assembled in fibrillar structures called the type IV pili (10).

Additional components of the type II secretion machineries share extensive similarities with proteins involved in the type IV piliation system. Already identified are the secretins PilQ, XcpQ and HxcQ (11), the “traffic ATPases” PilB, XcpR and HxcR (12), and the polytopic inner membrane proteins PilC, XcpS and HxcS. The striking similarities were functionally supported when several groups demonstrated that the pseudopilin of the type II secretion systems could be assembled in multifibrillar structures, called the type II pseudopilus (13-15). Interestingly, beside the major type IV pilin PilA, minor pilins have been described, which did not appear to be part of the fibrillar structure and for which no particular function was ascribed. The Xcp pseudopilins can be divided into one major component, XcpT, and minor components, XcpU-X (9,10). Among the latter, XcpX is atypical (9). Indeed, it is three times longer than XcpT, and it lacks the highly conserved glutamate residue at position +5 in mature pseudopilins and pilins. This residue has been proposed to play a role for the helical assembly of type IV pilins into the pilus structure (16).

Several major issues remain to be understood in the biogenesis of the type II pseudopilus, most importantly is the determination of its function in the secretion process. In this study we discovered several important aspects concerning pseudopilus assembly. We demonstrate that only a sub-set of the Xcp components is specifically dedicated to the assembly of the pseudopilus. We confirmed that among the five pseudopilins, only one, XcpT, appears to have the characteristics that allow the assembly in a fibrillar structure. Finally, we reveal that the atypical pseudopilin XcpX is a key component in controlling the pseudopilus elongation process. We propose that this control can be exerted via a direct interaction between XcpX and XcpT.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions - The strains and plasmids used in this study are described in Tables Ia and Ib, respectively. Strains were grown at 37°C in Luria broth (LB) or on LB agar. The Escherichia coli TG1 and TOP10F' strains were used for standard genetic manipulations. Recombinant plasmids were introduced into P. aeruginosa using the conjugative properties of pRK2013. Transconjugants were selected on Pseudomonas isolation agar (PIA) supplemented with the following antibiotics at the indicated concentrations (in micrograms per milliliter, µg.ml⁻¹): for E. coli, ampicillin, 50; kanamycin, 50; and streptomycin, 50; and for P. aeruginosa, carbenicillin, 300 to 500; and streptomycin, 2,000. The construction of P. aeruginosa deletion mutants was performed as previously described (15).

Construction of the pseudopilin hybrid genes - The primers used for the construction of the pseudopilin-hybrid genes are as follows. To amplify the 5’ region of the xcpT gene using pMTWT as matrix: 5’Rev(-48): 5’ - AGC GGA TAA CAA TTT CAC ACA GGA - 3’ and 3’XCPTnter: 5’- GCC CGA CTG TTG GCG ACG CTG CAA -3’ yielding the amplicon “XcpT-Nter”. To amplify the 3’ region of the xcpUH gene using pET22-U H as matrix: 5’XCPUcter: 5’- CGC CAA CAG TCG GGC TTC ACC CTC ATC GAG CTG – 3’ and 3’OHpET(PstI): 5’- ATT CTG CAG TCA GTG GTG GTG GTG GTG -3’ yielding the amplicon “XcpU-Cter”. To amplify the 3’ region of the xcpVH gene using pET22-V H as matrix: 5’XCPVcter: 5’- CGC CAA CAG TCG GGC TTC ACC CTG CTG GAA GTG – 3’ and 3’OHpET(PstI): 5’- ATT CTG CAG TCA GTG GTG GTG GTG GTG -3’ yielding the amplicon “XcpV-Cter”. To amplify the 3’ region of the xcpWH gene using the pET22-W H as matrix: 5’XCPWcter: 5’- CGC CAA CAG TCG GGC TCC ACC CTG CTC GAG CTG – 3’ and 3’OHpET(PstI): 5’- ATT CTG CAG TCA GTG GTG GTG GTG GTG -3’ yielding the amplicon “XcpW-Cter”.

Bacterial strains

- Pseudomonas aeruginosa
- E. coli

Plasmids

- pMTWT
- pET22-U H
- pET22-V H
- pET22-W H
- pET22-XWTH

Growth conditions

- LB broth
- LB agar

Antibiotics

- Ampicillin
- Kanamycin
- Streptomycin
- Carbenicillin
- Streptomycin

Construction of deletion mutants

- P. aeruginosa

Primers

- 5’Rev(-48)
- 3’XCPTnter
- 5’XCPUcter
- 3’OHpET(PstI)
- 5’XCPVcter
- 3’OHpET(PstI)
- 5’XCPWcter
- 3’OHpET(PstI)
- 5’XCPXcter
- 3’OHpET(PstI)
GTG GTG GTG GTG GTG -3’ yielding the amplicon “XcpX WT-Cter”. To amplify the 3’ region of the xcpXT+5EH gene using pET22-XT+5EH as matrix: 5’XCPXcter: 5’- CGC CAA CAG TCG GGC GTC GCG CTG ATC – 3’ and 3’OHpET(PstI): 5’- ATT CTG CAG TCA GTG GTG GTG GTG GTG -3’ yielding the amplicon “XcpXT+5E-Cter”. The PCR product encoding the N-terminal region of XcpT (XcpT-Nter) was mixed with each of the five PCR products encoding the His6-tagged C-terminus of the various pseudopilins (XcpU H-Cter, XcpV H-Cter, XcpW H-Cter and XcpX WTH-Cter and XcpX T+5EH-Cter). The PCR products were linked by using overlapping PCR and the appropriated external primers, 5’Rev(-48) and 3’OHpET(PstI). The resulting DNA fragments were cloned into the pCR2.1 vector (Invitrogen), and sequenced. These fragments were further digested with appropriated restriction enzymes and sub-cloned into the pMMB190, pMMB67EH or pBBR1MCS2 broad host range vectors.

Plasmid construction for overproduction of the full-length XcpT and XcpX pseudopilins in E. coli - The xcpT gene was amplified using pMTWT as matrix and the primers 5’Rev(-48) : 5’ - AGC GGA TAA CAA TTT CAC ACA GGA - 3’ and 3’ XCPThC (His6): 5’-ATT CAG TGG TGG TGG TGG TGG TTG TCC CAG TTG CCG AT-3’. The resulting gene encodes a C-terminally His-tagged XcpT. The amplicon was first cloned into the pCR2.1 vector (Invitrogen) and sequenced. A BamHI/HindIII 600bp insert was then sub-cloned into the broad-host-range vectors pMMB190 or pBBR1MCS2, yielding pMMB-T and pBBR2-T, respectively.

The xcpXWT and xcpXT+5E alleles were amplified using pSB28 (XcpXWT) and pRV20 (XcpXT+5E) as matrix, respectively, and the primers 5’AF024* (SD): 5’-ACT GGA TCC TAA GGG AA GCG GAA TGA GG- 3’ and 3’FLAG1 (FLAG1): 5’-TTT TCA CTT GTC ATC GTC GTC CTT GTA GTC TAA CTT GTC ATC TTA GAA TGA GG- 3’ and 3’FLAG2 (FLAG) : 5’-TAA TCA CTT GTC GTC ATC CTT GTA GTC TAA CTT GTC ATC TTA GAA TGA GG- 3’. “SD” indicates that we have slightly modified the corresponding Shine-Dalgarno sequence upstream of the xcpX gene to closely resemble the consensus. The amplicons were cloned into the pCR2.1 vector (Invitrogen) and sequenced. 1000 bp BamHI/HindIII inserts were sub-cloned into the pET22b plasmid in frame with the sequence encoding a His6-tag, but out-of-frame with the pelB sequence, yielding the plasmids pET-XWT and pET-XT+5E. In these plasmids the genes are expressed under the control of the T7 promoter.

flag-tagged derivatives of xcpX were also constructed. In a first PCR step, the xcpXWT and xcpXT+5E alleles were amplified using pSB28 (XcpXWT) or pRV20 (XcpXT+5E) as matrices, respectively, and the primers 5’AF024* (SD): 5’-ACT GGA TCC TAA GGG AA GCG GAA TGA GG- 3’ and 3’FLAG1 (FLAG1): 5’-TTT TCA CTT GTC ATC GTC GTC CTT GTA GTC TAA CTT GTC ATC TTA GAA TGA GG- 3’. The PCR products were cloned into the pCR2.1 vector (Invitrogen) and sequenced. 1200 bp EcoRI inserts were sub-cloned into the pET22b plasmid out-of-frame with both the pelB sequence and the DNA region encoding the His6-tag, yielding the plasmids pET-XWF and pET-XT+5EF. In these plasmids the genes are expressed under the control of the T7 promoter.

Preparation of pseudopili - A P. aeruginosa PAK mutant strain with mutations in the pilA and fliC genes was used as genetic background to prepare pseudopili samples free from type IV pili and flagella. The plasmid pMTWT was introduced in this strain by mobilization to allow overproduction of XcpT. The PAKpilA/fliC mutant containing pMTWT was streaked on large square plates containing LB agar supplemented with 300 µg.ml⁻¹ of carbenicillin and 2mM of IPTG. After overnight incubation at 37°C, bacterial colonies were scraped off from 8 plates and resuspended in 10 mM Tris-HCl/pH7.5 buffer containing 150 mM KCl. After homogenization, the cells were pelleted by low speed centrifugation (6,000 x g) and the pseudopili-enriched supernatant was collected.
The pseudopili were further pelleted by high speed centrifugation (158,000 x g). The resulting pseudopili pellet was resuspended in a small volume of 10 mM Tris-HCl/pH7.5 buffer containing 150 mM KCl. These preparations were directly observed by classical TEM or cryo-EM.

**TEM and immunogold labelling** - The negative-staining procedure and TEM observation were performed as previously described (15).

**Cryo-electron microscopy** - A 5 μl droplet of the pseudopili preparation was placed on a carbon-coated copper grid (Plano GmbH, Wetzlar, Germany). The biological sample was embedded in vitreous ice by plunge freezing the grids into liquid ethane (T = -186 °C) as described by Dubochet et al. (17). The grid was then transferred into a tilt cryo-holder (Gatan Inc., Pleasanton, California) cooled with liquid nitrogen (T = -196°C) and inserted into the microscope. 2-D images were recorded using an FEI CM200 transmission electron microscope (TEM) operating at 160 kV. The instrument was equipped with a field emission gun (FEG). The defocus level was about 2 μm. The pixel size at the specimen level was 0.3 nm.

**Image processing** - Cryo-electron micrographs were treated with Eman software package (18). EMAN: semi automated software for high-resolution single-particle reconstructions (18). Different filaments were boxed (64*64 pixels) and rotated using Boxer. No CTF correction was performed. The boxed filaments were then grouped into self-similar classes and good classes were selected for modeling.

**Immunofluorescence microscopy** - Bacteria were grown in a similar manner as the one previously described for shearing experiments (15). After ON incubation at 37°C, bacteria were scraped from the plate with a toothpick and resuspended into PBS buffer. All further incubations were performed at room temperature. 25 μl of this suspension were deposited onto a glass slide. After 5 min, bacteria were fixed for 10 min by adding 100 μl of paraformaldehyde (1%) directly onto the bacterial drop. After 3 washing steps with PBS, the preparations were saturated with BSA 5% in PBS for 10 min. The glass slides were washed three times with PBS and incubated with a 1:400 dilution of the primary antibodies against XcpT (rabbit) or against the His tag (mouse) in PBS/BSA 0.5% for 1 h. After three washing steps, the preparations were incubated with a 1:400 dilution of the Fluorescein/anti-rabbit (Vector) or Fluorescein/anti-mouse (Vector) conjugates, for 1 h. After three washing steps, the preparations were covered by a drop of Vectashield mounting medium (Vector) and with a small glass slide. Samples were observed on a Zeiss Axio microscope.

**Shearing of pseudopili** - The method to purify cell surface associated pseudopili was previously described (15).

**Biofilm formation assay on glass slides** – The methodology used is adapted from (19). Bacterial strains were grown in 5 ml M63 minimal medium supplemented with 0.2% glucose, 0.5% Casamino acids, 1mM MgCl₂ and 2mM IPTG, in 50 ml Corning tube containing a semi-immersed glass cover slide. After a short period of incubation of 1 h, at 30°C without shaking, the slide was removed and rinsed. The attached bacterial cells were visualized by phase-contrast microscopy using an Axiovert 200M microscope (magnification X 100). Images were captured with a Hamamatsu type Orca ER camera.
of the sulfobetaine detergent SB12 (n-dodecyl-n,n-dimethyl-3-ammonio-1-propanesulfonate, Sigma) and Complete EDTA-free 1:100. Solubilization was performed at room temperature for 1 h with gentle shaking. Insoluble material was removed by centrifugation at 100,000 x g for 30 min. The detergent-soluble supernatants containing extracted membrane proteins were saved and stored at -80°C with 10% glycerol.

Proteinase K sensitivity assay – Detergent solubilized proteins were used for this assay. Equal volumes of extracts enriched in XcpT, XcpXWT, and XcpXT+5EF were mixed and pre-incubated at 4°C for 10 min. 1.2 μg of proteinase K was added and the samples were incubated at 37°C for selected times. The protease activity was stopped by adding 10 mM PMSF at 4°C during 10 min. Samples were solubilized in Laemmli SDS-buffer and heated for 7 min at 95°C.

Cloning and expression of the soluble periplasmic domains of the Xcp pseudopilins - The DNA fragment encoding the N-terminal truncated xcpT and xcpX genes from position +25 and +23 relative to the leader peptide cleavage site, respectively, were amplified by standard PCR. Primers used for amplification were 5’TpD1(BamHI;His6) 5’-ATA GGA TCC ACA CCA CCA CCA CCA CCA CAT GAG CCG TCC CGA CCA G-3’ and 3’TpD2(HindIII) 5’-ATA AAG CTT ATC AGT TGT CCC AGT TGC CGA TGT CGG GTG CTG TGT C-3’ for xcpT; 5’XpC1(BamHI;His6) 5’-ATA GGA TCC A CAC CAC CAC CAC CCG CAG CAG TGG GCG ATA-3’ and 3’XpC2(HindIII) 5’ ATA AAG CTT A TCA TCG CTC GTC CTT CTT CCA ATC GTC GCC GCC GTG 3’ for xcpX. The PCR reaction introduced a region encoding an N-terminal His6 tag together with BamHI/HindIII cloning sites. PCR products were first sub-cloned into the pCR2.1 vector (Invitrogen) and sequenced. BamHI/HindIII DNA fragments were then generated and sub-cloned into the pET22b (Novagen). The cloning created an in frame fusion of the xcp genes with the pelB region encoding the N-terminal signal sequence. In this way the recombinant protein could be produced in the periplasm. The resulting plasmids were called pET-Tp25-148NH and pET-Xp23-333NH. Each gene fusion was expressed from the T7 promoter of the pET22b vector in E. coli BL21(DE3) host cells. The recombinant proteins were called: XcpTp25-148NH and XcpXp23-333NH, where “p” stands for periplasmic, “NH” for N-terminal His6 tag and the numbers correspond to the amino acid position in the primary sequence of the protein starting from the first residue after the leader-peptide-cleavage site (F+1 for XcpT or V+1 for XcpX). Recombinant BL21 strain carrying the plasmids pET-TpNH and pET-XpNH were grown in ZYP–5052 Auto-inducing medium developed by Studier (BNL; Rich medium containing Yeast extract; Tryptone; Phosphate buffered; 0.05% glucose, 0.5% glycerol and 0.2% lactose). After 4 days of growth, the periplasmic fractions containing the soluble recombinant proteins were obtained from osmotically shocked bacteria, and dialyzed against 50 mM Sodium Phosphate (pH 8) buffer overnight at 4°C in dialysis tubing (SIGMA-ALDRICH).

Affinity purification of the pseudopilin soluble domains - Dialyzed periplasmic fractions were applied on a 5 ml Hi-Trap chelating column (Pharmacia), loaded with Nickel, operated by an AKTA PRIME liquid chromatography system (Amersham Biosciences). After equilibration of the column with buffer A (50 mM Sodium Phosphate pH 8 and 500 mM NaCl) supplemented with 10 mM imidazole, the periplasmic fractions were applied to the column. After several washing steps with buffer A supplemented with 20 to 50 mM imidazole, His6-tagged proteins were eluted with buffer A gradually supplemented with 50 to 500 mM imidazole. Xcp proteins-containing eluted fractions were pooled and dialyzed overnight at 4°C against 50 mM Sodium Phosphate (pH 8) and 150 mM NaCl. After dialysis proteins were concentrated using Centricon devices (AMICON; Biomax-5) with a cut-off size of 5kDa. The concentrations reached were 10.4 mg.ml⁻¹ for XcpTp25-148NH and 2.82 mg.ml⁻¹ for XcpXp23-333NH, as evaluated by UV spectra and BRADFORD colorimetric measurement. Purity, before and after concentration, was checked by analysis on 15% SDS-PAGE and Coomassie Blue staining.

Chemical in vitro cross-linking - Purified periplasmic soluble domains (PD), XcpTp25-148NH and XcpXp23-333NH, were used for in vitro cross-linking.
chemical cross-linking, 5 μM of each protein was mixed and diluted in 35 μl of 50 mM Sodium Phosphate (pH 8) and 150 mM NaCl. After 20 min of incubation at room temperature 4.3 μl of freshly prepared para-formaldehyde (1% final) were added to the mix. Incubation was continued at room temperature for 20 min, after which the reaction was blocked by adding 14.3 μl of 4-fold concentrated SDS-βME buffer. 20 μl of each mix was directly analyzed on a 4-15% gradient SDS-PAGE (BIORAD). The gel was either directly stained with Coomassie Blue or immunoblotted with the Penta-His horseradish peroxidase (HRP) conjugate mouse IgG1 (Qiagen) following the instructions of the kit. The membranes were developed by chemiluminescence (PIERCE). Interesting bands revealed in the first dimension and stained by Coomassie Blue were cut out from the 4-15% acrylamide gel, boiled or not for 20 min in plastic films immersed in a water bath, and placed at the bottom of large wells prepared in a 12% and 1.5 mm-thick SDS-containing gels. After electrophoresis the proteins were immunoblotted on nitrocellulose membranes, probed with the Penta-His horseradish peroxidase (HRP) conjugate mouse IgG1 (Qiagen) and revealed by chemiluminescence using the Super Signal kit (PIERCE).

**Western blot quantification** - Densitometric quantification of bands on western blot were carried out using the Scion image 1.62c software (for MacOS).

**RESULTS**

*Cryo-EM analysis of the pseudopilus* - We have previously shown (19) that the *P. aeruginosa* type II protein secretion system is able to assemble its major pseudopilin XcpT into a multifibrillar structure that we called the pseudopilus. Pseudopili preparations were obtained using a *P. aeruginosa* mutant strain defective for both type IV pili and flagella (PAKpilA/fliC), in order to avoid any confusion between these extracellular appendages and the pseudopili. The broad-host range plasmid (pMTWT) was mobilized in this strain to overexpress the *xcpT* gene under control of the IPTG inducible *tac* promoter and pseudopili isolated as indicated in Materials and Methods. Briefly, bacteria were streaked on plates, grown overnight, scraped off and resuspended in Tris buffer (see Materials and Methods). The bacterial cells were separated from the pseudopili-containing supernatant by centrifugation. The pelleted pseudopili were resuspended in a small volume of the same Tris buffer and directly used for electron microscopy observations. The observation of the pseudopili samples using classical negative staining and high-resolution transmission electron microscopy (TEM) confirmed our previous data (19) and the formation of multifibrillar structures organized in bundles (Fig. 1, panels A-C). It is known that negative staining used in TEM can modify the appearance of biological structures, especially the thickness and aggregation state (20-22). In order to investigate whether it could be the case with pseudopili, we used the same preparations for analysis with cryo-electron microscopy (cryo-EM). Images acquired by cryo-EM (Fig. 1, panels D-F) revealed pseudopili that appear predominantly as single filaments with a maximum thickness of about 6 nm (Fig. 1F). Even though the preparations showed some disordered aggregates when the fibers are close together (Fig. 1D), these are unrelated to the organized bundles seen previously (Fig. 1B and 1C). In addition, the pseudopili appeared to be less straight and to have more curvatures. From these observations we propose that the pseudopili assembled by the Xcp secreton of *P. aeruginosa* are single filaments, and that bundling might be due to the methodology used for pseudopili preparation and observation.

We used the cryo-electron micrographs as starting material to perform initial Single Particle Analysis (SPA) and obtain a model for the pseudopilus filament architecture (Fig. 2). Using 65 images (Fig. 2A), 323 pieces of filament were boxed (Matrix view Fig. 2B) and grouped into 16 classes. Six of these classes were selected as “good”, representing 125 original boxed images (Fig. 2C). Based on these images, a model was constructed. Upon visualization with Chimera, and using a particular angle, a helical structure could clearly be predicted (Fig. 2D). This model is supporting the proposed helical assembly of
the pseudopilus filament that could be inferred from our previous studies (15) and by other related works (14,23). The Xcp-U-X pseudopilins do not assemble into the pseudopilus—In addition to XcpT, four other pseudopilins (XcpU-X) are found within the Xcp secreton, which share the same conserved N-terminal characteristics as XcpT and the pilin PilA. We investigated whether these pseudopilins were, like XcpT or PilA, able to assemble in a pilus-like structure. The corresponding genes encoding the C-terminal His-tagged pseudopilins XcpT, XcpU, XcpV, XcpW, and XcpX, were cloned in the broad host range vector pMMB190 under control of the tac promoter (see Materials and Methods). The plasmids were mobilized into P. aeruginosa strain PAO1, and the genes were expressed upon IPTG induction. In contrast to the recombinant PAO1 strain that produced high amount of XcpT proteins, only small amounts of XcpU, XcpV, XcpW or XcpX proteins were found to be produced (data not shown). XcpT is naturally the most abundant pseudopilin in the cell (8). We hypothesized that the 5’ region of xcpT gene, encompassing the Shine Dalgarno (SD) sequence, is at least partially involved in the high production level of XcpT. We thus engineered gene fusions between the 5’ end of the xcpT gene and the 3’ end of the four other pseudopilin genes. The 5’ end of the xcpT gene, up to the codon corresponding to the conserved glycine residue (Fig. 3), was fused with the 3’ end of each of the four other pseudopilin genes, starting at codon +1 after the conserved glycine residue (Fig. 3). The resulting hybrid proteins contain the N-terminal leader sequence of XcpT, up to the prepeptidase cleavage site, followed by the C-terminal mature domain of either one of the pseudopilins XcpU, XcpV, XcpW and XcpX at which a His6 tag was added. The proteins were named XcpU\textsubscript{H}, XcpV\textsubscript{H}, XcpW\textsubscript{H}, and XcpX\textsubscript{H} where (h) stands for hybrid and (H) for His\textsubscript{6} tag. Finally, the gene fusions were cloned in a broad-host range vector, pMMB190, to allow expression in P. aeruginosa. The different constructions were mobilized in the PAO1 wild type strain, and production of the pseudopilins was detected by western blotting using antibodies directed against the His\textsubscript{6} tag (Fig. 4A). The amount of proteins produced is variable but XcpU\textsubscript{H} and XcpW\textsubscript{H} level are similar to those obtained with XcpT\textsubscript{H} (Fig. 4A, lanes 1, 2, and 4). We further tested whether in these strains, pseudopilin-containing XcpU\textsubscript{H}, XcpV\textsubscript{H}, XcpW\textsubscript{H}, or XcpX\textsubscript{H} pseudopilins could be observed. The different recombinant strains were analyzed by shearing and TEM observation as previously described. The results, summarized in figure 4B, show that in contrast to XcpT\textsubscript{H} none of the four other pseudopilins (XcpU\textsubscript{H}, XcpV\textsubscript{H}, XcpW\textsubscript{H}, or XcpX\textsubscript{H}) were either released by shearing or assembled in a detectable pseudopilus structure. We concluded that in the conditions we used, none of the pseudopilins but XcpT are assembled into a pseudopilus. Moreover, when the untagged version of XcpT (pMTWT) was assembled into a pseudopilus in a strain that also produced either one of the XcpU\textsubscript{H}, XcpV\textsubscript{H}, XcpW\textsubscript{H}, or XcpX\textsubscript{H} pseudopilins, none of these recombinant proteins were detectable in the XcpT-containing pseudopilus using immunogold-labeling and antibodies directed against the His\textsubscript{6} tag (data not shown). Overall, these observations suggested that minor pseudopilins such as XcpU-X are not part of an extracellular pseudopilus structure. It should be noted, that the plasmids producing the XcpU\textsubscript{H}, XcpV\textsubscript{H}, XcpW\textsubscript{H}, or XcpX\textsubscript{H} recombinant pseudopilin allowed restoration of the Xcp-dependent protein secretion in the respective mutant strains deleted for the xcpU, xcpV, xcpW or xcpX genes (Fig. 4B), indicating that these proteins are fully functional. Xcp components required for type II pseudopilus assembly—We further investigated whether all known Xcp components are required for assembly of the type II pseudopilus. As we have previously shown (15), three pathways exist in P. aeruginosa that promote exposure of XcpT at the cell surface, including the type II secretons Xcp and Hxc, and the type IV pili assembly system, Pil. In order to study the pseudopilus assembly process via the sole Xcp secreton, we constructed a PAO1 genetic background in which the hxcR and pilQ genes were deleted (PAOΔhxcRΔpilQ = PAORQ). In this context, the Xcp machinery is the only system available to handle XcpT assembly. We confirmed that this mutant was affected both in Hxc-dependent secretion and type IV piliation, but presented a wild type level of Xcp-dependent secretion.
corresponding by introducing a plasmid which carried the corresponding xcp gene, as seen by the halo formed around colonies grown on skim-milk plate (data not shown). We further introduced the plasmid pMTWT in these 12 mutants. The bacteria were grown in conditions that induce pseudopilus formation (see Materials and Methods). XcpT extracellular exposition was analyzed by shearing, and pseudopilus assembly was checked by TEM. The results are summarized in Table II and in all cases both techniques correlate. If each of the Xcp complexes is required for efficient secretion, it appeared not to be the case for pseudopilus assembly. Indeed, the mutants strains lacking either xcpP or -Q or -R or -T or -U or -W or -X genes were still able to assemble pseudopilus. It should be noted that the xcpP mutant formed much less pseudopilus (Table II and data not shown). In contrast the mutant strains lacking either the xcpA or -R or -S or -V or -Y genes no longer exposed XcpT on the cell surface nor assembled a pseudopilus. We concluded that among the 12 Xcp proteins required for the secretion process, only a restricted number of secreton components are essential for pseudopilus assembly. The absence of requirement for XcpQ in pseudopilus biogenesis was unexpected. It is however very likely that the XqhA secretin replaces XcpQ for pseudopilus assembly as it does for Xcp-dependent secretion (24). Moreover, in the P. aeruginosa genome (25) the xqhA gene (PA1868) is organized in tandem with another gene, PA1867. We found that the product of this gene shares homology with the XcpP protein (46% similar). We suggest that the PA1867 gene product could replace XcpP during pseudopilus assembly.

In order to be sure that there is no overlap between homologous components belonging to one of the three distinct systems (Xcp, Hxc and Pil), we constructed a mutant in which the entire hxc cluster and the pilA-C cluster have been deleted. This mutant was called PAOΔhxcΔpilA-C. In this genetic background we constructed single deletions of the xcpP, xcpR, xcpU, or xcpV gene. These mutants were analyzed for pseudopilus assembly and behave similarly as the mutants constructed in the PAOΔhRΔpXcpQ genetic background. Indeed, pseudopilus assembly was observed for the PAOΔhxcΔpilA-CΔxcpU and PAOΔhxcΔpilA-CΔxcpR and PAOΔhxcΔpilA-CΔxcpV mutants (data not shown), confirming that XcpR and XcpV are essential components for the pseudopilus biogenesis whereas XcpP and XcpU are not. 

XcpV is the only minor pseudopilin required for XcpT incorporation into the pseudopilus. - In spite of being involved in pseudopilus ultrastructure, the minor pseudopilins XcpU-X could be involved in a subtle control of its biogenesis. In our systematic analysis of the involvement of xcp genes in pseudopilus biogenesis we demonstrated that among the four minor pseudopilin genes, only the xcpV gene is essential for pseudopilus assembly (Table II). Indeed, the analysis of the mutant PAOΔhRΔpXcpV overproducing XcpT did not allow the observation of any pseudopilus by TEM (data not shown). Moreover, shearing the cells also did not allow any release of XcpT in the supernatant (Fig. 5A, lane 4). Conversely, our TEM results demonstrated that XcpU, XcpW and XcpX are not essential for pseudopilus assembly (Table II) and the shearing analysis revealed that XcpT could be recovered in the supernatant fraction (Fig. 5A, lanes 3, 5 and 6 respectively) of the corresponding mutants. We concluded that XcpV is the only minor pseudopilin required for the assembly of XcpT into pseudopilus.

XcpX controls the pseudopilus assembly. - Strikingly, the amount of XcpT recovered in the supernatant fraction is much more abundant with a xcpX mutant (Fig. 5A, compare lanes 1 and 6), which suggests a deregulation in the XcpT extrusion process and an abnormal elongation of the pseudopilus. Observation using immunofluorescence microscopy confirmed that the higher level of XcpT corresponded to the assembly of numerous (Fig. 5C compare panels 1-3 to panels 4-8) and longer (compare panels 1-3 to panel 9) pseudopilus as compared to the parental strain. If the lack of XcpX allows an overassembly of XcpT into pseudopilus, we reasoned that overproduction of XcpX could interfere with pseudopilus assembly. The xcpX gene was thus cloned into a broad host range
plasmid (pBBR2-X\textsuperscript{hWTH}) and introduced in a strain that contains the pMTWT. We checked pseudopilus formation by shearing analysis and TEM. As it is shown in figure 5B, the shearing analysis indicated that the release of XcpT into the extracellular medium was strongly decreased (62%) upon XcpX overproduction (Fig. 5B, lane 2). These results were confirmed by TEM observation, which showed that the decreased extracellular exposition of XcpT was associated with a significant decrease in the number of assembled pseudopili (data not shown). We thus concluded that the amount of XcpX produced could strongly contribute to the control of XcpT incorporation into pseudopili.

XcpX-dependent number of pseudopili correlates with bacterial attachment properties - We have previously demonstrated that pseudopilus assembly increased the adherence and biofilm formation capacities of \textit{P. aeruginosa} (15). We thus tested whether the increased number of pseudopili with the \textit{xcpX} mutant was concomitant with increased adhesive properties. Bacteria were allowed to adhere on glass slides for 1.5 h (see Materials and Methods). In these conditions, XcpT-overproducing PAO\textsuperscript{\textit{hxcR}\textit{ pilQ}} strain had a tendency to form bacterial clusters whereas the non-overproducing strain was found attached as single bacterium (Fig. 6, compare panels A and D). Such phenotype could not be seen with an \textit{xcpR} mutant derivative overproducing XcpT (Fig. 6, panel E) confirming that the microcolony formation phenotype is related to the Xcp-dependent biogenesis of the pseudopilus. Interestingly, when XcpT was overproduced in a \textit{xcpX} mutant derivative (Fig. 6, panel F) all the bacteria appeared to be organized in microcolonies, with only few isolated cells as compared to what was observed with a PAO\textit{ΔhxcRΔpilQ} strain (Fig. 6, compare panels D and F). Overall, by looking at pseudopilus-related functions such as adherence and biofilm formation, we could confirm that the lack of XcpX results in an increase in pseudopilus assembly.

The E+5 residue of XcpX is important for XcpX function - The XcpX pseudopilin is atypical because of a larger size and the lack of the highly conserved glutamate residue at position +5 after the prepilin peptidase cleavage site. It has been proposed (16) that this residue may help the interaction between pilin subunits during type IV pilus assembly. We investigated whether introduction of a glutamate at position +5 may influence the function of XcpX. To test this hypothesis we used a \textit{xcpX} allele mutation in which, at the corresponding +5 position, the threonine codon was changed for a glutamate codon (9). As for the other pseudopilins, the region encoding the leader peptide of XcpX was replaced with the 5’ end of the \textit{xcpT} gene whereas the addition of a C-terminal His\textsubscript{6} tag was engineered to yield a recombinant protein called XcpX\textsuperscript{hT+5Eh}. The mutated allele was cloned into the pBBR1MCS2 broad host-range vector and mobilized in the PAO1 strain-containing pMTWT. Co-overproduction of XcpT along with XcpX\textsuperscript{hT+5Eh} resulted in a much weaker exposition of XcpT. Indeed, upon pseudopili shearing a decrease of about 95% in the amount of recovered XcpT was observed as compared to the PAO1 strain that overproduced XcpT alone (Fig. 5B, compare lanes 1 and 3). This reduction in the exposition of XcpT is more drastic in this case as compared to what was observed with the wild type XcpX\textsuperscript{hWTH} (Fig. 5B, compare lanes 2 and 3). Moreover, with a strain that co-overproduces XcpX\textsuperscript{hT+5Eh} and XcpT, we could not observe any single pseudopilus using TEM (data not shown). We concluded that the presence of a glutamate at position +5 in XcpX might alter the function of this pseudopilin and strengthen the negative control exerted by XcpX on XcpT extrusion and further pseudopilus assembly.

XcpX increases proteinase K sensitivity of XcpT - The observation that strains overproducing both XcpT and XcpX had a severe defect in pseudopilus assembly suggested that XcpX interferes with XcpT assembly. Interestingly, we observed by immunoblotting, with whole cell extracts, that the level of detectable XcpT is strongly decreased when XcpT and XcpX are co-overproduced in the \textit{E. coli} strain BL21. In these conditions, XcpT could only be detected in the cells after 6 h of induction, whereas it is readily detected after 2 h when XcpT is produced alone (Fig. 7, panel A, compare lanes 1-3 and lanes 4-6). Moreover, this time-delay for reaching a detectable level of XcpT is even longer when co-producing the mutant form
XcpX-TSE (Fig. 7, panel A, compare lanes 4-6 and lanes 7-9). In contrast, the level of XcpX production was not strongly affected by the co-expression of XcpT (Fig. 7, panel B), and more importantly this level is strictly identical when considering either XcpX or the XcpX-TSE derivative (Fig. 7, panel B, compare lanes 4-6 and 7-9). These observations suggested that XcpX could have a destabilizing effect towards XcpT. In order to check this possibility we assessed the proteinase K sensitivity of XcpT produced in these different conditions. Upon overproduction in E.coli, both proteins were extracted from the inner membrane and solubilized with the zwitterionic sulfobetaine detergent, SB12. The extracts were used in a proteinase K sensitivity assay. As shown in figure 7 (panel C), XcpT is degraded by proteinase K in a time-dependent manner, but is still detectable after 60 min of proteolysis, with a rate of degradation of 73% (Fig. 7, panel C, lane 3). However, the proteinase K digestion kinetic of XcpT is strongly increased when XcpX is present (Fig. 7, panel C, compare lanes 1-3 and lanes 4-6). In these conditions 60% of the XcpT protein is degraded after 10 min (Fig. 7, panel C, lane 5), whereas XcpX forms dimers upon para-formaldehyde cross-linking (see Materials and Methods). The results presented in the figure 8 show that XcpT forms dimers upon para-formaldehyde cross-linking, the band pattern (Fig. 8, panel A, lane 5) appears as a combination of cross-linked bands of each individual protein. However, at least one additional band is detected (arrow head with black circle) with an apparent molecular weight corresponding to a heterodimer between XcpT and XcpX (14 + 37 = 51 kDa). To confirm this, we cut this band out, together with control bands, boilded them or not, and allowed to migrate through a second SDS-PAGE. After immunoblotting with an antibody directed against the His tag (Fig. 8, panel B), we analyzed the composition of each bands. The band corresponding to the putative heterodimer migrates just above the 43 kDa molecular weight marker and releases the two XcpT and XcpX monomers (lane 4). This result was confirmed by mass spectroscopy analysis (data not shown). Here, we thus demonstrated a direct interaction between XcpT and XcpX.

**DISCUSSION**

The results presented here describe the assembly by the type II secreton of *P. aeruginosa* (Xcp) of the major pseudopilin XcpT into a fiber-like structure that we called type II pseudopilus (15). We have addressed several questions such as which are the genes required for the assembly process and what are the functions fulfilled by the minor pseudopilins. We demonstrated that one of these minor pseudopilins, XcpX, controls the length and number of assembled pseudopili and we presented data that begins to explain...
the molecular mechanism underlying this control. Our results can be directly compared to analogous studies performed in *E. coli* with the reconstituted Pul secreton of *Klebsiella oxytoca* (13,14,23), revealing similarities but also significant differences.

*The natural shape of the P. aeruginosa pseudopilus:* We have previously shown that the pseudopilus assembled by the Xcp secreton in *P. aeruginosa* appeared as a multifibrillar structure that did form bundles of individual fibers (15). The Pul secreton of *K. oxytoca* also assembles the pseudopilin PulG into similar bundles of filaments (13,14). However, while obtaining new images for ultra-structural studies of the pseudopili by using cryo-electron microscopy (cryo-EM), we observed that most of the XcpT-containing pseudopili appeared as single filament, much thinner than those previously observed (Fig. 1). The discrepancy between current and previous observations may be related to the techniques used for preparing the samples for observation. Indeed, it is well known that negative staining induces artefacts that could modify the shape of biological materials such as pili (22). More recently, Resch *et al.* (26) observed the actin cytoskeleton both by negative staining and cryo-EM techniques. They highlighted the particular susceptibility of actin filament networks to distortion during preparation for TEM. They concluded that the cryo-EM method offers the least possibility of creating artefacts in filament organization. We thus propose that the XcpT-containing pseudopilin of *P. aeruginosa* are single filaments about 6 nm thick, which stick together under certain circumstances. This fits well with the model of the type II secreton (2) where individual fiber would be extruded by one single secretin complex located in the outer membrane (internal diameter: 9 nm). Upon assembly of a long extracellular pseudopilus through the secretin complex, secretion of type II substrates is blocked as we previously described (15). Moreover, Lee *et al.* (27) have recently shown that the *Xanthomonas campestris* XcpT-homolog, XpsG, interacts directly with the XpsD secretin further suggesting that the GspG-pseudopilus and the GspD-secretin are in contact at some stages during the secretion process. Studies using the reconstituted Pul secreton in *E. coli* revealed no interference of the PulG-pilus assembly on pullulanase secretion (13,14). Such differences could be explained if one imagines that in the latter case only some secretions are dedicated to the assembly of the pilus whereas a substantial number of secretions are free of pseudopilus and available for secretion of the substrate. Since in this study the complete set of Pul secreton components was overproduced it might have increased the number of secretion sites available, allowing both functions, secretion and pilus assembly, to be fulfilled at the same time. By contrast in our study, we overproduced only the major pseudopilin XcpT in the homologous host *P. aeruginosa*. 

*xcp genes involved in the assembly of the XcpT-pseudopilus in P. aeruginosa:* The twelve known Xcp components are essential for the secretion process. In this study we demonstrated that some Xcp proteins are not required for pseudopilus assembly. Pseudopilus assembly could be seen as a subfunction of the secreton requiring fewer components than the secretion process. Namely, XcpU; -W; -X and -Z appeared non-essential for pseudopilus assembly (Table II). In a comparable study Sauvonnet *et al.* (13) have demonstrated that the minor pseudopilins PulH, -J and –K from *K. oxytoca*, respectively homologous to XcpU, -W and -X, were also dispensable for the assembly of PulG, the XcpT homolog, into a pilus-like structure. This might suggest that their role could take place at a later stage, after pseudopilus assembly, or that their absence does not result in a black or white phenotype with respect to pseudopilus assembly.

In contrast to the work reported about the Pul secreton (13), we observed that XcpZ, the PulM homolog, is not essential for the assembly of XcpT into pseudopilus (Table II). XcpZ is a bitopic inner membrane protein (28), which interacts with XcpY (29,30). Such an interaction results in a mutual stabilization between XcpY and XcpZ. Moreover, it has been demonstrated that membrane association of the putative ATPase XcpR required the interaction with the N-terminal domain of XcpY (31). In the absence of XcpZ we thus might expect a lower level of XcpY within the cell and consequently a partial mislocalization of XcpR. Since XcpZ is dispensable for pseudopilus assembly, whereas XcpR and...
XcpY are not (Table II), this suggests that a lower level of XcpY and XcpR is sufficient to support pseudopilus assembly but not protein secretion. Because XcpZ is essential for protein secretion, it is thus likely that the function of this component is not only to stabilize XcpY, but it might also be related to exoprotein recognition for example. The observation that, in the absence of XcpQ, normal amount of pseudopili could still be seen at the bacterial cell surface was intriguing (Table II and Fig. 5). Indeed, with type IV pili from *N. gonorrhoeae*, it was shown that the filament crosses the outer membrane via the pore formed by PilQ (32). Because PilQ and XcpQ are both members of the secretin family (33), we anticipated that pseudopilus extrusion needed the XcpQ homomultimeric pore. Martinez *et al.* (24) have previously reported that, in *P. aeruginosa*, the secretin XqhA can replace XcpQ in the secretion process, and we suggest that it also replaces XcpQ for pseudopilus assembly.

**Function of the minor pseudopilins in pseudopilus assembly** - We demonstrated that none of the minor pseudopilins, XcpU-X, is able to form a pseudopilus such as XcpT does. Moreover it seems that these proteins are not part of the extracellular pseudopilus (Fig. 4). Similarly, Vignon *et al.* (14) have shown that the purified PulG-pili did not contain any of the four other minor pseudopilins, PulH-K. We demonstrated that XcpV is the only minor pseudopilin required for pseudopilus assembly. Likewise, PulI, an XcpV homolog of the Pul secreton, appeared to be essential for the assembly of the PulG-pili (14). One could imagine that XcpV acts at an early stage during fiber assembly, such as the initiation step, and is found at the base of the pseudopilus, in connection with the inner membrane components of the Xcp secreton.

Another minor pseudopilin, XcpX, seems to play a crucial function, albeit not essential, in pseudopilus assembly. Indeed, our results showed that, in the absence of XcpX, pseudopilus assembly still occurs but the number and length of pseudopili increased markedly (Table II and Figure 5). Reciprocally, overproduction of XcpX interfered with- or abolished pseudopili formation (Fig. 5). We hypothesized that XcpX could control pseudopilus assembly, by limiting the incorporation of XcpT, and began to investigate the molecular mechanism involved in this control. Firstly, we demonstrated that XcpX can be cross-linked with XcpT (Fig. 8). Secondly, we showed that co-overproduction of XcpX with XcpT diminished the amount of XcpT normally recovered (Fig. 7, panel A). Thirdly, it appeared that XcpT was more sensitive to proteinase K digestion, in an *in vitro* assay, when incubated together with XcpX (Fig. 7, panel C). These observations suggest that XcpX interacts with XcpT and that interaction induces conformational changes that destabilize XcpT and increase its sensitivity to proteolytic degradation. Remarkably, the effects observed with the wild-type XcpX are strengthened when using the mutant protein XcpX_T+5E (Fig. 5, panel B and Fig. 7, panels A-C). We suggest that XcpX_T+5E interacts more strongly with XcpT than XcpX, increasing the conformational changes and the destabilization of this protein.

**How does fiber elongation is controlled in other extracellular-appendage assembly machineries?** - The CS5 pili produced by the enterotoxigenic *E. coli* (ETEC) involves a chaperone-usher type assembly system. CsfA is the major subunit that composes mostly the extracellular pilus and requires the specific chaperone CsfB, while CsfD and CsfE are minor subunits interacting with the chaperone CsfF (34). Pilus biogenesis is thought to be initiated by the binding of the CsfD-CsfF complex to the outer membrane usher CsfC, resulting in the translocation of CsfD across the outer membrane and leaving CsfC in an assembly-competent state. Pilus elongation proceeds via incorporation of multiple copy of the CsfA major subunit, and sometimes incorporation of fewer copy of CsfD, adding flexibility to the fiber. Duthy *et al.* (34) also demonstrated that pilus termination relies on the interaction of the CsfE-CsfF complex with the usher CsfC. Indeed, the cseF mutant assembled pili three times longer than those of the wild-type strain. Moreover, production of CsfE in the cseF mutant cannot restore the wild-type pili length. The authors concluded that CsfE did control pilus length but was not rate-limiting. Furthermore, the cseF mutant exhibited the same alteration in pilus length as the cseF mutant, but in this case the default was...
fully rescued by expression of CsfF, showing that the chaperone CsfF was rate-limiting for the control of pilus length. Duthy et al. (34) proposed a model in which the CsfE-CsfF complex is targeted to CsfC, resulting in the irreversible association of CsfE with CsfC, and preventing further polymerisation of the pilus. This mechanism has been originally described for the P pili and type I pili expressed by uropathogenic E. coli (35,36). In the XcpT-pseudopilus assembly, the lack of XcpX or its overproduction led respectively to an increase of the pseudopilus assembly (number and length) or to a total abolition. It thus suggests that XcpX does have a rate-limiting function in the regulation of pseudopilus biogenesis and might also indicate that XcpX operates alone.

Based on these observations we proposed a model in which XcpX could play a central role in controlling the assembly of XcpT into pseudopilus. In the case of P. aeruginosa or N. gonorrhoeae type IV pili, which are long and filamentous fibers, two antagonistic “traffic warden” ATPases are required for pilus function. One, PilB/PilF is involved in the assembly of the major pilin PilA/PilE into pili, and the other, PilT, is required for disassembly of the filament (32,37). The extracellular XcpT-containing pseudopilus is only observed upon XcpT overproduction. The physiological size of the pseudopilus in the type II secreton might thus not exceed the dimension of the periplasmic space (20 nm = 5 x repeat units = 10 x XcpT; (15)). It has been proposed that the pseudopilus must retract to allow protein secretion (2,15). It is thus reasonable to suggest that the energetic cost to assemble and disassemble the pseudopilus might not be as high as for type IV pili. In support of this hypothesis type II secretion systems are known to use one single “traffic warden” ATPase, GspE. Either GspE might support both pseudopilus assembly and disassembly or GspE might only be required for assembly whereas disassembly is achieved via another mechanism. XcpX is a minor and non-abundant pseudopilin as compared to XcpT. We have shown that XcpX and XcpT interact and that the XcpT/XcpX ratio appeared to be crucial for pseudopilus elongation. In physiological conditions, the frequency of contact between XcpX and XcpT is low and XcpT-XcpT interactions might be favored to allow pseudopilus growth. The non-frequent XcpX interaction with an XcpT molecule already incorporated in the pseudopilus will stop its growth. This hypothesis is based on the type IV pili model proposed by Parge and collaborators (16). They suggested a registration function for the glutamate residue at position +5 in pilins and pseudopilins (E+5). According to the pilus structure, a salt bridge is formed between the negative charge (E+5 side chain) exposed on the surface of a pilin subunit already assembled into the fiber and the positive charge exposed at the N-terminus of an incoming subunit. This bond could stabilize subunit-subunit interaction and further drive pilus assembly. The lack of the E+5 residue in XcpX might block further incorporation of incoming XcpT subunits. In our experiments, by overproducing XcpX we have increased the frequency at which it interacts with the XcpT-containing pseudopilus, and thus interfere with its elongation. Furthermore, by introducing an E+5 residue in XcpX we have observed a complete block of pseudopilus assembly that could be caused by the integration of the mutant XcpXT+5E at the base of the pseudopilus. Such an association could be stabilized by the salt-bridge, leading to an irreversible blockage of the assembly. Moreover, we propose that XcpX incorporation could arrest pseudopilus elongation as well as triggering fiber disassembly. Indeed, we demonstrated that XcpX can interact with XcpT and that this contact destabilizes XcpT. Given these observations, we suggest that XcpX could induce disassembly of the pseudopilus by triggering conformational changes in XcpT that increase its protease sensitivity and accelerate its degradation. Such an instability and proteolytic degradation of the pseudopilin subunits might be an alternative to the PilT powered disassembly of type IV pili. As previously mentioned, pseudopilus disassembly might not require a high energetic driving force, as it is the case for type IV pili (37), but could rely on the arrest of subunits incorporation and their subsequent degradation. Whether a specific protease plays a role in this process should be investigated.
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FOOTNOTES

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The abbreviations used are: ON, over night; LP, leader peptide; HD, hydrophobic domain; PD, periplasmic domain; SD, Shine Dalgarno; Nter, N-terminal; Cter, C-terminal; h, hybrid; H6, hexahistidine tag; F, flag tag; WT, wild type; Xcp, extracellular protein deficient; Gsp, general secretory pathway; Hxc, homologous to Xcp; XqhA, XcpQ homolog A; PCR, polymerase chain reaction; TEM, transmission electron microscopy; Cryo-EM, cryo-electron microscopy; IM, immunofluorescence microscopy; SPA, single particle analysis; 2-D, two-dimensional; HRP, horseradish peroxidase; SB12, n-dodecyl-n,n-dimethyl-3-ammonio-1-propanesulfonate; IPTG, isopropyl-β-D-thiogalactopyranoside; pF, paraformaldehyde; βME, β-mercapto-ethanol.

FIGURE LEGENDS

Fig. 1: Electron micrographs of pseudopili. A-C, Transmission Electron Micrographs of pseudopili preparations spotted on EM grids that were further immuno-labeled with an antibody directed against XcpT (A, x30.000) or simply negatively stained (B, x140.000 and C). In each case the scale bar is indicated. D-F, Cryo-Electron Micrographs of the same pseudopili preparations. Gold markers were added to facilitate further image processing and give a reference size (12 nm Ø). In panel E the scale bar (top right) represent 100 nm and the end magnification is x 46.035. In panels D and F, enlargements of the initial image (E) are shown.

Fig. 2: A model for pseudopilus structure. A. Selection of pseudopili fragments from cryo-electron micrographs, which were then straightened and centered. B. Collection of the 323 images that was used as the starting matrix for modeling. C. Enlargement showing one pseudopilus fragment after

15
processing. D, Proposed model after sorting and class selection. On the right, the same model is presented after removal of background noise.

**Fig. 3:** Pseudopilin hybrids construction. A, Linear representation of the five Xcp pseudopilins showing the conserved N-terminal region which is composed of the leader peptide (LP, blue), the residues between which lies the cleavage site for XcpA/PilD and the hydrophobic domain (HD, red). The variable C-terminal periplasmic domain is shown in black (PD). B, Schematic representation of the hybrids. The 5′ part of the xcpT gene, encompassing the corresponding Shine Dalgarno (SD) sequence, the start codon and the sequence coding for the LP domain, is fused to the 3′ end of the four other pseudopilin genes, starting with the codon of the first residue after the cleavage site for XcpA/PilD (F/V) and containing the HD and PD domains. A DNA sequence encoding a hexahistidine tag (His6) was fused to the 3′ end of each hybrid genes. The precursor protein expresses from this hybrid gene is composed of the leader peptide of XcpT, which is cleaved off by the preprotein peptidase XcpA/PilD, to yield the wild type sequence of the mature pseudopilins XcpU-X with a His6-tag at the C terminus.

**Fig. 4:** Capacity of minor pseudopilins to form a pseudopilus. A, Expression level of the pseudopilin hybrids. SDS PAGE was performed on a 15% acrylamide gel. Each lane was loaded with an equivalent amount of cellular proteins obtained from PAO1 strains overproducing XcpT (lane 1), XcpUh (lane 2), XcpVh (lane 3), XcpWh (lane 4) or XcpXh (lane 5). Western blot probed with an antibody directed against the His6 tag. B, Assembly of minor pseudopilins into a pseudopilus. Summary of the results obtained by shearing and TEM analysis of pseudopilus assembly by PAO1 overproducing the various pseudopilins. The amount of pseudopilin produced has been checked by western blot analysis on total cell extract and varies from low (+/-) to high (+++/+). Production of the tagged Xcp pseudopilins complemented the respective deletion mutant as it is indicated in the third column (+). The fourth column indicates whether the various pseudopilins are able (+) or not (-) to assemble into a pseudopilus.

**Fig. 5:** Function of minor pseudopilins in pseudopilus assembly. XcpX controls the number and the length of pseudopili assembled. A, Release of XcpT by shearing of various *P. aeruginosa* strains. Bacteria were grown on plates supplemented with 2 mM of IPTG to induce the overproduction of XcpT and thus pseudopilus assembly (see Materials and Methods). The sheared fractions (SF) were separated on a 15% polyacrylamide gel and the proteins were blotted onto a nitrocellulose membrane. The membranes were probed with a primary antibody directed against XcpT and proteins revealed by chemiluminescence using the Super-Signal West Pico kit (PIERCE). 0.5 equivalent OD600 units were loaded for each extract. Each mutant is derived from the parental strain PAOΔhxcRΔpilQ (=PAORQ), which is further deleted for one of the five genes encoding Xcp pseudopilins (XcpT-X). The corresponding cellular fractions (not shown) were analyzed and indicated that comparable amount of XcpT was produced from these various strains. B, Shearing experiments revealing extracellular exposition of XcpT in various *P. aeruginosa* strains. These strains were grown in presence of 2 mM IPTG to induce the co-overproduction of XcpT and XcpX. PAO1 wild type strain carrying pMTWT/pBBR1MCS2 (T, lane 1), pMTWT/pBBR2-Xh (TX, lane 2), pMTWT/pBBR2-XhT+5EH (TXΕ+5, lane 3). The sheared fractions (SF) are presented. The amount of XcpT released in the supernatant after shearing is expressed in percentage relative to the amount found in lane 1. Equivalent amount of proteins were loaded in each lane. C, Immunofluorescence microscopy using the antibody directed against XcpT as primary antibody for pseudopili labelling. The strains were grown in the conditions described in A. XcpT was overproduced in the parental strain PAOΔhxcRΔpilQ (PAORQ, panels 1-3) and the derivative strain deleted of xcpX (panels 4-9).

**Fig. 6:** Pseudopilus-dependent adherence and biofilm formation. Adherence assay on glass slide with bacteria overproducing XcpT (see Materials and Methods). Each mutant is derived from the same parental strain PAOΔhxcRΔpilQ (=PAORQ, panels A and D) and is further deleted for xcpR (panels B
and E) or xcpX (panels C and F). The various strains carry either the empty vector pMMB190 (pMMB; panels A-C) or the xcpT-containing plasmid pMTWT (pxcpT; panels D-F). The biofilms were allowed to form during 1 h 20' at 30°C (see the text for details).

Fig. 7: XcpX-dependent instability of XcpT. A-B, XcpX decreases the amount of XcpT produced. Western blot analysis of cellular extracts obtained from E. coli strain BL21(DE3) bearing the pBBR2-TWT (xcpT=TWT) and the pET22b empty vector (Ø) (panel A, lanes 1-3), the pET-XWT (xcpXWT=TXWT) and the pBBR1MCS2 empty vector (panel B, lanes 1-3), the pBBR2-TWT and pET-XWT (panels A and B, lanes 4-6), the pBBR2-TWT and pET-XT+5E (xcpXT+5E=TX+5E) (panels A and B, lanes 7-9). The nitrocellulose membranes were revealed by (A) the primary antibody directed against XcpT or (B) the primary antibody directed against the His6-tag. Each lane is loaded with material coming from the same amount of bacteria: 0.1 U of OD600. Samples were taken at various times after induction with 1 mM IPTG, 2 h (lanes 1, 4 and 7), 4 h (lanes 2, 5 and 8) and 6 h (lanes 3, 6 and 9). C, XcpX increases XcpT instability. In vitro proteinase K sensitivity assay. Detergent solubilized extracts containing XcpT and XcpX were mixed and then incubated with proteinase K (see Materials and Methods). At different time (0, 10 and 60 min), the proteolysis was stopped and the samples were loaded on a 12% SDS-PAGE and immunoblotted. Nitrocellulose membranes were revealed with the primary antibody directed against the His6-tag. The following mixtures are shown, XcpT alone (lanes 1-3), XcpT together with XcpX WT (lanes 4-6), XcpT together with XcpXT+5E (lanes 7-9). The amount of proteins degraded is expressed in percentage relative to time 0 (Panel C, bottom). In panel C, flag-tagged forms of XcpX were used (XWTF and XT+5EF).

Fig. 8: Direct interaction between XcpT and XcpX. In vitro chemical cross-linking. Affinity purified soluble periplasmic domains (PD) of XcpTpNH (N-ter His6) and XcpXpNH were mixed and cross-linked (+) or not (-) with paraformaldehyde (see Materials and Methods). A, The proteins were analyzed on a 4-15% gradient SDS-PAGE (BIORAD), followed by immunoblotting using a primary antibody directed against the His6 tag. The following mixtures are shown: XcpTpNH alone (lanes 1-2); XcpXpNH alone (lanes 3-4); XcpTpNH together with XcpXpNH (lanes 5-6). B, Bands of interest (dashed circle in panel A) were cut out from the first gel and allowed to migrate in a 12% SDS-PAGE. Immunoblotting was performed using a primary antibody directed against the His6 tag. The following bands were analyzed: XcpTpNH (lane 1); XcpXpNH (lane 2); XcpTpNH + XcpXpNH (lanes 3-4). The bands were unboiled (UB, lane 3) or boiled (B, lane 4). The position of individual proteins (Tp, Xp) and homodimers (XpX2, TpX2) or heterodimers (Tp + Xp) is indicated on the right side of each gel by arrows.
## Table Ia: Strains used in this study

| Strain     | Genotype | Source or Reference |
|------------|----------|---------------------|
| **P. aeruginosa** |          |                     |
| PAO1       | Prototrophe, chl-2 | B Holloway (15) |
| PAOΔhRΔpQ  | Non polar deletions of hxcR and pilQ genes in PAO1 | This study |
| PAOΔhRΔpQΔA | Non polar deletion of the xcpA gene in PAOΔhRΔpQ | This study |
| PAOΔhRΔpQΔP | Non polar deletion of the xcpP gene in PAOΔhRΔpQ | This study |
| PAOΔhRΔpQΔQ | Non polar deletion of the xcpQ gene in PAOΔhRΔpQ | This study |
| PAOΔhRΔpQΔR | Non polar deletion of the xcpR gene in PAOΔhRΔpQ | This study |
| PAOΔhRΔpQΔS | Non polar deletion of the xcpS gene in PAOΔhRΔpQ | This study |
| PAOΔhRΔpQΔT | Non polar deletion of the xcpT gene in PAOΔhRΔpQ | This study |
| PAOΔhRΔpQΔU | Non polar deletion of the xcpU gene in PAOΔhRΔpQ | This study |
| PAOΔhRΔpQΔW | Non polar deletion of the xcpW gene in PAOΔhRΔpQ | This study |
| PAOΔhRΔpQΔX | Non polar deletion of the xcpX gene in PAOΔhRΔpQ | This study |
| PAOΔhRΔpQΔY | Non polar deletion of the xcpY gene in PAOΔhRΔpQ | This study |
| PAOΔhRΔpQΔZ | Non polar deletion of the xcpZ gene in PAOΔhRΔpQ | This study |
| PAOΔhxcΔpilAC’ | Non polar deletions of the entire hxc gene cluster and from pilA to part of the pilC genes in PAO1 | This study |
| PAOΔhxcΔpilAC’ΔP | Non polar deletion of the xcpP gene in PAOΔhxcΔpilAC’ | This study |
| PAOΔhxcΔpilAC’ΔR | Non polar deletion of the xcpR gene in PAOΔhxcΔpilAC’ | This study |
| PAOΔhxcΔpilAC’ΔU | Non polar deletion of the xcpU gene in PAOΔhxcΔpilAC’ | This study |
| PAOΔhxcΔpilAC’ΔV | Non polar deletion of the xcpV gene in PAOΔhxcΔpilAC’ | This study |
| PAKpilA/fliC | PAKpilA::TeR::fliC::GmR | (38) |
| **E Coli** |          |                     |
| TG1        | SupEΔ(lac-proAB)tyhi hsdRΔ5 (F’-traD36 rpoA::lacIΔZAM15) | (39) |
| BL21(DE3)  | hsdS gal (l cts857 ind1 Sam7 nin5 lacUV5-T7 gène1) | Invitrogen |
| CC118pir   | Δ ara-leu araD ΔlacX74 galE galK phoA20 thi-1 rpsE rpoB argE(Am) recA1 RIR(2,pir) | (40) |

*GmR, TeR and RIR represent antibiotic resistant cassettes to gentamicin, tetracyclcin and rifampicin, respectively.*
| Plasmid | Genotype | Source or Reference |
|--------|----------|---------------------|
| pMMB190 | Ap<sup>R</sup>, pMMB67EH, tac promoter, lacZα | (41) |
| pMMB67EH | Ap<sup>R</sup>, RSF replicon (IncQ), tac promoter | (42) |
| pBBR1MCS<sub>2</sub> | Broad-host-range vector Kmr<sup>R</sup> lacZα, mob<sup>+</sup>, tac and T7 promoters in opposite direction | (43) |
| pET22b | pelB, T7lac, Ap<sup>R</sup>, origin = pBR322, C-ter his6 tag | Novagen |
| pRK2013 | ColE1, Tra<sup>+</sup> Mob<sup>+</sup>, lacZα, mob<sup>+</sup>, tac and T7 promoters in opposite direction | (44) |
| pKNG101 | Sm<sup>R</sup>, oriR6K, oriTRK2, mobRK2, sacBR<sup>+</sup> (suicide vector) | (45) |
| pET-XWT<sub>F</sub> | EcoRI 1200bp DNA fragment carrying the xcpX<sub>WT</sub> gene with tandem Flag tags at the C-ter, cloned into the pET22b (out of frame the pelB sequence and the his tag C-ter) | This study |
| pET-XT<sub>T+</sub>5EF | EcoRI 1200bp DNA fragment carrying the xcpX<sub>T+</sub>5E gene with tandem Flag tags at the C-ter, cloned into the pET22b (out of frame the pelB sequence and the his tag C-ter) | This study |
| pET-XWT<sub>H</sub> | BamHI/HindIII 1000bp DNA fragment carrying the xcpX<sub>WT</sub> gene cloned into the pET22b, in frame with the C-ter his6 tag | This study |
| pET-XT<sub>T+</sub>5EH | BamHI/HindIII 1000bp DNA fragment carrying the xcpX<sub>T+</sub>5E gene cloned into the pET22b, in frame with the C-ter his6 tag | This study |
| pET-T<sub>H</sub> | EcoRI/SalI 500bp DNA fragment carrying the xcpT gene cloned into the pET22b, in frame with the C-ter his6 tag | This study |
| pET-T<sub>p25-148NH</sub> | BamHI/HindIII 500bp DNA fragment coding for the soluble domain of XcpT with a N-ter his6 tag, fused to the PelB leader peptide, cloned into the pET22b | This study |
| pET-Xp<sub>p23-333NH</sub> | BamHI/HindIII 1000bp DNA fragment coding for the soluble domain of XcpX with a N-ter his6 tag, fused to the PelB leader peptide, cloned into the pET22b | This study |
| pMTWT | EcoRI 860bp DNA fragment carrying the xcpT gene cloned into the pMMB190, under the tac promoter | (15) |
| pMMB-T<sub>H</sub> | BamHI/HindIII 600bp DNA fragment carrying the xcpT(his6) gene cloned into the pMMB190 | This study |
| pMMB-U<sub>H</sub> | EcoRI/PstI 700bp DNA fragment carrying the xcpU(his6) gene cloned into the pMMB190 | This study |
| pMMB67-V<sub>H</sub> | BamHI/XbaI 650bp DNA fragment carrying the xcpV(his6) gene cloned into the pMMB67EH | This study |
| pMMB67-W<sub>H</sub> | BamHI/XbaI 950bp DNA fragment carrying the xcpW(his6) gene cloned into the pMMB67EH | This study |
| pMMB-X<sub>H</sub> | EcoRI 1000bp DNA fragment carrying the xcpX<sub>H</sub> gene cloned into the pMMB190 | This study |
| pMMB-XWT<sub>T+</sub>5E<sub>H</sub> | EcoRI 1000bp DNA fragment carrying the xcpX<sub>T+</sub>5E(his6) gene cloned into the pMMB190 | This study |
| pBBR2-U<sub>H</sub> | BamHI/XbaI 700bp DNA fragment carrying the xcpU(his6) gene cloned into the pBBRMC<sub>S</sub>2, tac | This study |
| pBBR2-V<sub>H</sub> | BamHI/XbaI 650bp DNA fragment carrying the xcpV(his6) gene cloned into the pBBRMC<sub>S</sub>2, tac | This study |
| pBBR2-W<sub>H</sub> | BamHI/XbaI 950bp DNA fragment carrying the xcpW(his6) gene cloned into the pBBRMC<sub>S</sub>2, tac | This study |
| pBBR2-X<sub>H</sub> | EcoRI 1000bp DNA fragment carrying the xcpX<sub>H</sub> gene cloned into the pBBRMC<sub>S</sub>2, tac | This study |
| pBBR2-XWT<sub>T+</sub>5E<sub>H</sub> | EcoRI 1000bp DNA fragment carrying the xcpX<sub>T+</sub>5E(his6) gene cloned into the pBBRMC<sub>S</sub>2, tac | This study |
| pBBR2-TWT | EcoRI 860bp DNA fragment from the pMTWT carrying the xcpT gene cloned into the pBBRMC<sub>S</sub>2, under the T7 promoter | This study |
| pBBR2-T<sub>H</sub> | EcoRI 600bp DNA fragment from the pMMB-T<sub>H</sub> carrying the xcpT(his6) gene cloned into the pBBRMC<sub>S</sub>2, under the T7 promoter | This study |
| pKN - | Fragment Carried | Description |
|-------|------------------|-------------|
| pKN-ΔA | BamHI/ApaI 1000 bp fragment carrying pilC'-coaE' | This study |
| pKN-ΔP | BamHI/ApaI 1000 bp fragment carrying xcpR'Q' | This study |
| pKN-ΔR | BamHI/ApaI 1000 bp fragment carrying xcpP'S' | This study |
| pKN-ΔS | BamHI/XbaI 1000 bp fragment carrying xcpR'T' | This study |
| pKN-ΔT | BamHI/ApaI 1000 bp fragment carrying xcpS'U' | This study |
| pKN-ΔV | BamHI/ApaI 1000 bp fragment carrying xcpU'W' | This study |
| pKN-ΔX | BamHI/ApaI 1000 bp fragment carrying xcpW'Y' | This study |
| pKN-ΔY | BamHI/ApaI 1000 bp fragment carrying xcpV'X' | This study |
| pKN-ΔZ | BamHI/ApaI 1000 bp fragment carrying xcpY'Int | G. Ball |
| pKN-Δhxc | BamHI/XbaI 1000 bp fragment carrying hx12-34 | This study |
| pKN-ΔpilAC' | BamHI/XbaI 1000 bp fragment carrying pilAC' | This study |

A Ap^R, Km^R and Sm^R represent antibiotic resistant cassettes to ampicillin, kanamycin and streptomycin, respectively.

This study
Table II: Xcp components essential for pseudopilus assembly

| Protein family /Cellular localization\(^a\) | Deleted \(xcp\) gene\(^b\) | Type II pseudopilus assembly\(^c\) |
|-------------------------------------------|--------------------------|----------------------------------|
| Bitopic (IM)                              | P                        | +/-                              |
| Secretin (OM)                             | Q                        | + *                              |
| Traffic ATPase (IM ass)                   | R                        | -                                |
| Polytopic (IM)                            | S                        | -                                |
|                                           | T                        | NA                               |
|                                           | U                        | +                                |
| Pseudopilins (IM)                         | V                        | -                                |
|                                           | W                        | +                                |
|                                           | X                        | +++                              |
| Bitopic (IM)                              | Y                        | -                                |
| Bitopic (IM)                              | Z                        | +                                |
| Prepilin peptidase (IM)                   | A                        | -                                |

\(^a\) IM = inner membrane, OM = outer membrane, IM ass = IM associated.

\(^b\) Each \(xcp\) gene is deleted in the strain PAO\(\Delta xcR\)\(\Delta pilQ\) is indicated.

\(^c\) The ability of the various mutant to assemble (+) or not (-) a pseudopilus is indicated. For the \(\Delta xcpT\) mutant, NA (not applicable) means that the deletion of the chromosomal \(xcpT\) gene is compensated by the overproduction of the XcpT pseudopilin. A semi-quantitative analysis of pseudopilus assembly by IMF has given the evaluations showing that pseudopilus assembly varies between: (+) parental level; (-) no pseudopili; (+++) high level and (+/-) low level. The star (*) indicates that the lack of XcpQ could be compensated by another secretin, XqhA, during the assembly of the pseudopilus (see the text for details).
Figure 1
Figure 2

A

B

D

C

10 nm  10 nm
Figure 3

A

XcpA  C
XcpT  LP  PD  C
XcpU  PD  C
XcpV  C
XcpW  C
XcpX  C

Conserved domain  Variable domain

PROTEIN

B

pMIE190

5' region of the xcpT gene
WT pseudopilin genes (xcpU-X) deleted from their 5' region.

DNA Hybrid gene

Precursor protein  Mature pseudopilin
### Figure 4

#### A

![Image of gel blot with bands labeled XpH, WpH, UpH, TpH, VpH](image)

#### B

| Pseudopilins overproduced | Amount | Complementation of the Δxcp mutant | Pseudopilus assembly |
|---------------------------|--------|-----------------------------------|----------------------|
| XcpT                      | +++    | +                                 | +                    |
| XcpT<sub>H</sub>          | +++    | +                                 | +                    |
| XcpU<sub>H</sub>          | +++    | +                                 | -                    |
| XcpV<sub>H</sub>          | +/-    | +                                 | -                    |
| XcpW<sub>H</sub>          | +++    | +                                 | -                    |
| XcpX<sub>H</sub>          | +      | +                                 | -                    |
Figure 5

A

PAORQ \( \Delta T \) \( \Delta U \) \( \Delta V \) \( \Delta W \) \( \Delta X \)

B

\( XcpT \)

C

100% 38% 5%

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Figure 6

PAORQ/pMMB

-ΔxcpR/pMMB

-ΔxcpX/pMMB

PAORQ/pxcpT

-ΔxcpR/pxcpT

-ΔxcpX/pxcpT
Figure 7

A

|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Twt/Ø |   |   |   | 2 | 4 | 6 |   |   |   |
| Twt/XwT_H |   |   |   | 2 | 4 | 6 |   |   |   |
| Twt/Xt+8/H |   |   |   | 2 | 4 | 6 |   |   |   |

< XcpTwT

B

|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Ø/XwT_H |   |   |   | 2 | 4 | 6 |   |   |   |
| Twt/XwT_H |   |   |   | 2 | 4 | 6 |   |   |   |
| Twt/Xt+8/H |   |   |   | 2 | 4 | 6 |   |   |   |

< XcpXh

C

|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| T_H |   |   |   | 0'  | 10'  | 60'  |   |   |   |
| T_H + XwT |   |   |   | 0'  | 10'  | 60'  |   |   |   |
| T_H + Xt+8 |   |   |   | 0'  | 10'  | 60'  |   |   |   |

< XcpTH

*Degradation: 0 60 73 60 100 82 100
Figure 8
XCPX controls biogenesis of the Pseudomonas aeruginosa XCPT-containing pseudopilus
Éric Durand, Gérard Michel, Romé Voulhoux, Julia Kürner, Alain Bernadac and Alain Filloux

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