

## **Chapter 3**

### **Targeting of *Pseudomonas aeruginosa* pilin-pseudopilin chimeras to the type II secretion machinery**

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## ABSTRACT

Several proteins involved in type II secretion share homology with components of the type IV piliation apparatus. Among these are proteins that are termed pilins in the Pil system and pseudopilins in case of the type II secretion machinery, both categories being synthesized as precursors with a characteristic short leader peptide. Despite their similarity, pilins and pseudopilins cannot functionally replace each other. Thus, targeting of these subunits to their cognate assembly machinery needs to be efficient, especially when the two pathways co-exist in the same cell, as is the case in the opportunistic pathogen *Pseudomonas aeruginosa*. To assess which part of the pseudopilin XcpT contains such targeting information, we constructed gene fusions in which parts of *xcpT* were substituted by the corresponding part of *pilA*, which encodes the pilus subunit. We found that the leader peptide and the N-terminal 17 amino acid residues of mature XcpT could be functionally replaced by the corresponding part of PilA, which shows that this domain of the protein does not contain information for targeting to the Xcp system. Disruption of the processing site in XcpT or in the functional hybrids resulted in proteins with a strong dominant-negative effect in the wild-type strain on type II secretion, but not on type IV piliation. Production of a similar processing-defective PilA variant inhibited type IV piliation, showing that specific blocking of these systems can be accomplished.

## INTRODUCTION

Gram-negative bacteria use several different pathways for the export of proteins into their extracellular environment. One of these pathways involves the type II secretion system (T2SS) (9). Assembly of the T2SS requires 12-16 different components, generically referred to as Gsp proteins, or, in the case of the main T2SS of *Pseudomonas aeruginosa*, as Xcp proteins. Many of these proteins share sequence similarity with constituents of the type IV piliation system (24). Type IV pili are filamentous appendages with various functions, including adherence to host cells, twitching motility, DNA uptake, and biofilm formation (4). Both type II secretion and type IV piliation require (i) a secretin, probably forming a channel in the outer membrane (3, 21), (ii) a nucleotide-binding protein of the traffic ATPase family, (iii) a multi-spanning integral inner membrane component, (iv) a group of proteins that are termed pilins in case of the Pil system or pseudopilins in case of the T2SS and that are synthesized as precursors with a short positively charged leader peptide, and (v) a dedicated prepilin peptidase that is required for the maturation of pilins and pseudopilins by cleaving off the leader peptide and methylating the new N-terminal residue (31). The prepilin peptidase processing site is characterized by a glycine residue at the -1 position, which is essential for cleavage (29). The processing site is followed by a hydrophobic stretch of approximately 20 amino acid residues, which is considered to be of importance for transport across the inner membrane (1, 12, 30) and for subunit interactions within the pilus (6). The hydrophobic region frequently starts with a phenylalanine at the +1 position, although this residue is not strictly required for proper biogenesis or functionality (29).

The sequence similarity between proteins involved in type IV piliation and type II secretion led to the idea that pseudopilins might assemble into a pilus-like structure (11), which indeed could be shown upon over-expression of the entire system, or of only the major pseudopilin GspG (XcpT) (7, 27, 33). The generation of these long pseudopili was considered to result from unrestricted elongation of a structure that normally would only span the periplasm.

Sequence similarity between pilins and pseudopilins is mainly found in their N termini. However, progressive insights in the three dimensional structures of type IV pilins from several organisms and of the pseudopilin PulG from *Klebsiella oxytoca* revealed that also their overall structures are

substantially conserved (5, 17, 18, 23). Functional conservation is reflected in the interchangeability of type IV pilins between heterologous Pil systems (14, 26, 34). Pseudopilin assembly via the T2SS seems to be rather promiscuous as well, since GspG (XcpT) homologues can be readily exchanged between T2SSs without loss of function (7, 33). Moreover, assembly of pseudopili by the Pil system has been demonstrated (7). However, assembly of pilin subunits into a pilus via the T2SS seems more problematic and has only been shown for *Escherichia coli* K-12 type IV pilin PpdD, which was assembled via the T2SS of *K. oxytoca* (18).

In *P. aeruginosa*, the T2SS and the type IV piliation system are produced simultaneously in the cell. Durand *et al.* (7) have shown that in the absence of the T2SS, the major pseudopilin XcpT can be assembled into a pilus-like structure by the Pil system. However, the pilin subunit cannot functionally replace XcpT and *vice versa*, indicating that under normal conditions targeting of the subunits to the cognate machinery should be efficient.

In this study, hybrid proteins between XcpT and the structural component of the type IV pili, PilA, were constructed to study the targeting of XcpT to the Xcp machinery. Further insight into the targeting process was obtained by blocking the Xcp machinery with processing-defective variants of these chimeras.

## MATERIALS AND METHODS

**Strains and growth conditions.** *E. coli* strain DH5 $\alpha$  (16) was used for routine cloning. *P. aeruginosa* strains PAO25 (15), and PAO1 $\Delta$ T (2) are *leu arg* and *xcpT* mutant derivatives, respectively, of strain PAO1. Strain PAK-NP (34) is a mutant derivative of strain PAK (35), carrying a tetracycline-resistance cassette in the *pilA* gene. Strains were grown at 37°C in a modified Luria-Bertani (LB) broth (32). For selection or plasmid maintenance, antibiotics were added in the following concentrations: for *E. coli* ampicillin 50  $\mu$ g/ml, and gentamicin 10  $\mu$ g/ml; for *P. aeruginosa* gentamicin 40  $\mu$ g/ml, tetracycline 40  $\mu$ g/ml, and carbenicillin (300  $\mu$ g/ml). For induction of genes from the *lac* or the *tac* promoter, isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) was added to a concentration of 1 mM.

TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristic*	Source or reference
pCRII-TOPO	Ap <sup>r</sup> ; Km <sup>r</sup> ; TOPO TA cloning vector	Invitrogen
pCRII-PilA	pCRII-TOPO; <i>pilA</i>	This study
pCRII-XcpT	pCRII-TOPO; <i>xcpT</i>	This study
pUC19	Ap <sup>r</sup> ; cloning vector	(37)
pUC-PilA	pUC19; <i>pilA</i>	This study
pUC- XcpT <sub>1pA#2</sub>	pUC19; encoding hybrid XcpT <sub>1pA</sub> with disrupted processing site	This study
pUC- XcpT <sub>17A</sub>	pUC19; <i>xcpT</i> <sub>17A</sub>	This study
pUC- XcpT <sub>65A</sub>	pUC19; <i>xcpT</i> <sub>65A</sub>	This study
pYRC	pBBR1-MCS5; <i>lacI</i>	Chapter 2
pYRC- XcpT <sub>65A</sub>	pYRC; <i>xcpT</i> <sub>65A</sub>	This study
pYRC- XcpT <sub>1pA</sub>	pYRC; <i>xcpT</i> <sub>1pA</sub>	This study
pYRC- XcpT <sub>17A</sub>	pYRC; <i>xcpT</i> <sub>17A</sub>	This study
pYRC- XcpT <sub>65A#2</sub>	pYRC; encoding hybrid XcpT <sub>65A</sub> with disrupted processing site	This study
pYRC- XcpT <sub>1pA#2</sub>	pYRC; encoding hybrid XcpT <sub>1pA</sub> with disrupted processing site	This study
pYRC- XcpT <sub>17A#2</sub>	pYRC; encoding hybrid XcpT <sub>17A</sub> with disrupted processing site	This study
pYRC-PilA <sub>#2</sub>	pYRC; encoding PilA with disrupted processing site	This study
pYRC-PilA	pYRC; <i>pilA</i>	This study
pMMB67HE	Ap <sup>r</sup> ; cloning vector	(13)
pMMB-PilA <sub>#2</sub>	pMMB67HE; encoding PilA with disrupted processing site	This study
pMMB-XcpT <sub>1pA#2</sub>	pMMB67HE; encoding hybrid XcpT <sub>1pA</sub> with disrupted processing site	This study
pAX24	XcpP-Z cluster in pLAFR3	(10)

\*Gm, gentamicin; Ap, ampicillin; Km, kanamycin. The proteins encoded by the *pilA-xcpT* hybrids are schematically depicted in Fig. 1.

**Plasmids and DNA manipulations.** Plasmids used in this study are listed in Table 1. Recombinant DNA methods were performed essentially as described (25). Plasmids were introduced by the CaCl<sub>2</sub> procedure into *E. coli* (25) or by electroporation into *P. aeruginosa* (8). PCRs were performed with the proofreading enzyme *Pwo* DNA polymerase (Roche) and PCR products were cloned into pCRII-TOPO according to manufacturer's protocol. Oligonucleotides used in this study are listed in Table 2. Chromosomal DNA from strain PAO1 was used as the template to amplify *pilA* with the oligonucleotides JAPilAfor and JAPilArev. The resulting product was cloned into pCRII-TOPO resulting in pCRII-PilA.

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5' → 3')	Restriction site
JAPilAfor	<u>AAGCTTAGTTTCCTTGATCGTGGCG</u>	HindIII
JAPilArev	<u>GAGCTCTACCGACTGAGCTAATCCG</u>	SacI
pUC19for	TCAGTGAGCGAGGAAGCGGAAGA	
pilA_rev09#2	CGATCAAGT <u>CTAGAC</u> CTTTTTGAGC	XbaI
pilA_rev10#2	GAATGGC <u>ATCTAGAG</u> CCAGGATAC	XbaI
JAXcpTshrev01	<u>CGAGCTCTACGCTGATGATGACCATCACC</u>	SacI
JAXcpTfor02	CTTCCGATCCTTCGAATCAACCAACTCGT G	
JAXcpT_for11	TCGCCAACAT <u>CTAGACTT</u> CACCCTGATCG AA	XbaI
JAXcpT_for12	ATCCTCGGCAT <u>TCTAGAC</u> GCCCTGGTGGT	XbaI
SDM_A9T11#3for	GAAAGCTCAAAAAGGCTTCACCCTGATCG	
SDM_A9T11#3rev	CGATCAGGGTGAAGCCTTTTTGAGCTTTC	
SDM_A10T12#3for	GGCACCACCAGGGCGGCCAGGATACCGA TG	
SDM_A10T12#3rev	CATCGGTATCCTGGCCGCCCTGGTGGTGC C	
SDM_A9T11for	GAAAGCTCAAAAAGGCTCTAGATTACCT TGATCGAAC	
SDM_A9T11rev	GTTTCGATCAAGGTAAATCTAGAGCCTTTT TGAGCTTTC	
JAPilAfor01	GATATTA <u>AAGCTT</u> GGTAAGTGCTTGTTGAG G	HindIII
JAPilArev02	TTCGGTCGCTCTAG <u>AAGCAGTAGTACC</u>	XbaI
JAXcpT_for4	CATGTACTCTAGAGACA <u>ACTTCGCCTATC</u> CG	XbaI
JAPilAfor23	CATGAAAGCTCA <u>TCTAGACTT</u> TACC	XbaI

The HindIII-SacI fragment of pCRII-PilA was introduced into HindIII-SacI-digested pUC19 as well as in similarly digested pYRC, resulting in pUC-PilA and pYRC-PilA, respectively. The pUC-PilA construct was used as template to amplify *pilA* fragments with pUC19for as the forward primer and either pilA\_rev09#2 or pilA\_rev10#2 as the reverse primer to generate the *pilA* parts of the *pilA-xcpT* hybrid genes *xcpT<sub>1pA</sub>*, and of *xcpT<sub>17A</sub>*, respectively (Fig. 1). For the construction of *xcpT<sub>65A</sub>*, a fragment of the *pilA* gene was amplified with the oligonucleotides JAPilAfor01 and JAPilArev02. The resulting products were cloned into pCRII-TOPO generating pCRII-A9, pCRII-A10#3, and pCRII-A2. Cosmid pAX24 was used as the template to amplify *xcpT* with the oligonucleotides JAXcpTshrev01 and JAXcpTfor02 and the resulting product was cloned

into pCRII-TOPO, resulting in pCRII-XcpT. This construct was used as a template to amplify *xcpT* fragments with JAXcpTshrev01 as the reverse primer and JAXcpT\_for11, JAXcpT\_for12, or JAXcpT\_for4 as the forward primer to generate the *xcpT* parts of the hybrid genes *xcpT<sub>lpA</sub>*, of *xcpT<sub>17A</sub>*, or of *xcpT<sub>65A</sub>*, respectively. The resulting PCR fragments were introduced into pCRII-TOPO, resulting in pCRII-T11, pCRII-T12, or pCRII-T4, respectively. The HindIII-XbaI fragment of pCRII-A9 and the XbaI-SacI fragment of pCRII-T11 were ligated into HindIII-SacI-digested pUC19 resulting in pUC-XcpT<sub>lpA#2</sub>. The HindIII-XbaI fragment of pCRII-A10#3 and the XbaI-SacI fragment of pCRII-T12 were ligated into HindIII-SacI-digested pUC19 resulting in pUC-A10#3. The HindIII-XbaI fragment of pCRII-A2 and the XbaI-SacI fragment of pCRII-T4 were ligated into HindIII-SacI-digested pYRC yielding pYRC-XcpT<sub>65A</sub> and in HindIII-SacI-digested pUC19 yielding in pUC-XcpT<sub>65A</sub>. The HindIII-SacI fragment of pUC-XcpT<sub>lpA#2</sub> was cloned into HindIII-SacI-digested pYRC, resulting in pYRC-XcpT<sub>lpA#2</sub> and into HindIII-SacI-digested pMMB67HE, resulting in pMMB-XcpT<sub>lpA#2</sub>. To remove the XbaI site from the insert of pUC-XcpT<sub>lpA#2</sub>, the QuikChange site-directed mutagenesis kit (Stratagene) was used with the complementary oligonucleotides SDM\_A9T11#3for and SDM\_A9T11#3rev and pUC-XcpT<sub>lpA#2</sub> as template DNA. After nucleotide sequencing to verify removal of the XbaI site, the HindIII-SacI insert was ligated into HindIII-SacI-digested pYRC, resulting in pYRC-XcpT<sub>lpA</sub>. Similarly, the XbaI site was removed from the insert of pUC-A10#3 by site-directed mutagenesis using the complementary oligonucleotides SDM\_A10T12#3for and SDM\_A10T12#3rev and pUC-A10#3 as template DNA. The HindIII-SacI fragment of the resulting construct, pUC-XcpT<sub>17A</sub>, was ligated into HindIII-SacI-digested pYCR, which resulted in pYRC-XcpT<sub>17A</sub>. An XbaI site was introduced between the nucleotides that encode the processing site of XcpT<sub>17A</sub> and XcpT<sub>65A</sub> by site-directed mutagenesis with the complementary oligonucleotides SDM\_A9T11for and SDM\_A9T11rev, and pUC-XcpT<sub>17A</sub> and pUC-XcpT<sub>65A</sub>, respectively, as template DNA. After nucleotide sequencing to verify the introduction of the XbaI site, the HindIII-SacI fragments were ligated into HindIII-SacI-digested pYCR, yielding pYRC-XcpT<sub>17A#2</sub> and pYRC-XcpT<sub>65A#2</sub>, respectively. With the primers JAPilAfor23 and JAPilArev and chromosomal DNA from strain PAO1 as the template, part of the *pilA* gene was amplified. After cloning of this PCR fragment into pCRII-TOPO, the XbaI-SacI fragment was cloned together with the HindIII-XbaI fragment of



pCRII-A9 into HindIII-SacI-digested pYRC resulting in pYRC-PilA<sub>#2</sub>. The *pilA*<sub>#2</sub> gene was subsequently inserted into HindIII-SacI-digested pMMB67HE resulting in pMMB-PilA<sub>#2</sub>.

**Enzyme assays.** Secretion of elastase was analyzed qualitatively on plates with a top layer containing 1% elastin (Sigma). For quantitative analysis, the colorimetric elastin-Congo red assay (20) was used. Briefly, 250 µl of culture supernatant of cells grown overnight in the presence of IPTG were incubated for 2 h at 37°C with 500 µl of 10 mg/ml elastin-Congo red (Sigma) dissolved in assay buffer (45 mM Tris-HCl, 1.5 mM CaCl<sub>2</sub> pH 7.2). The reaction was stopped by the addition of 500 µl of 0.7 M NaH<sub>2</sub>PO<sub>4</sub> pH 6.0. After removal of elastin-Congo red by centrifugation, absorbance was measured at 495 nm.

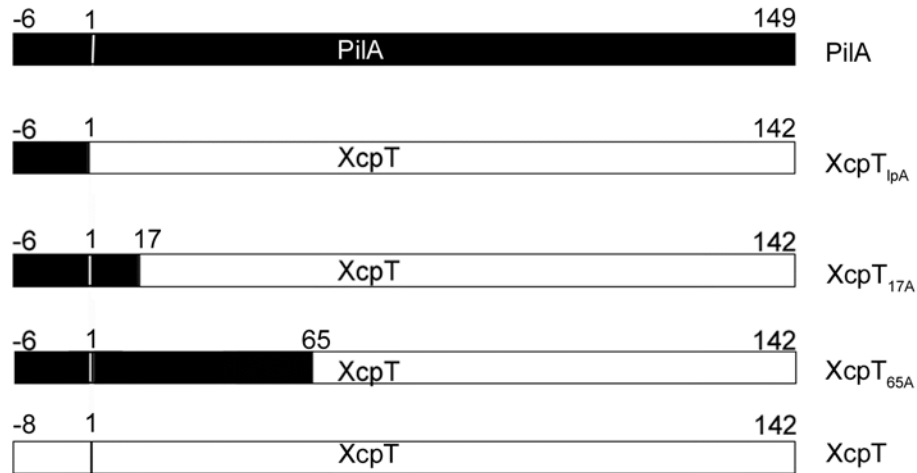
**SDS-PAGE and immunodetection.** Bacterial cells were suspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue, 0.1 M Tris-HCl pH 6.8). Extracellular proteins were precipitated from culture supernatants by the addition of trichloroacetic acid to a final concentration of 5% (wt/vol) and, after washing in acetone, proteins were dissolved in SDS-PAGE sample buffer, boiled and analyzed by SDS-PAGE. Whole cell lysates were heated for 10 min at 95°C and proteins were separated on gels containing 14% acrylamide. The proteins were transferred onto nitrocellulose membranes by semidry electroblotting. Immunoblots were incubated with polyclonal antisera against XcpT (1:1000) (7), or against PilA (1:10.000) (28). Alkaline phosphatase-conjugated goat anti-rabbit IgG antiserum (Biosource international) was used as secondary antiserum, unless stated otherwise. Detection was performed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

**Phage sensitivity.** To test the sensitivity of bacterial strains to bacteriophage PO4, a high-titer suspension of the phage was streaked on LB agar plates and, subsequently, bacteria were cross-streaked through the phage suspension. After incubation at 37°C, clear lysis zones indicated sensitivity.

## RESULTS

**Complementation of the secretion defect in an *xcpT* mutant by PilA-XcpT fusions.** To obtain insight in the targeting of the pseudopilin

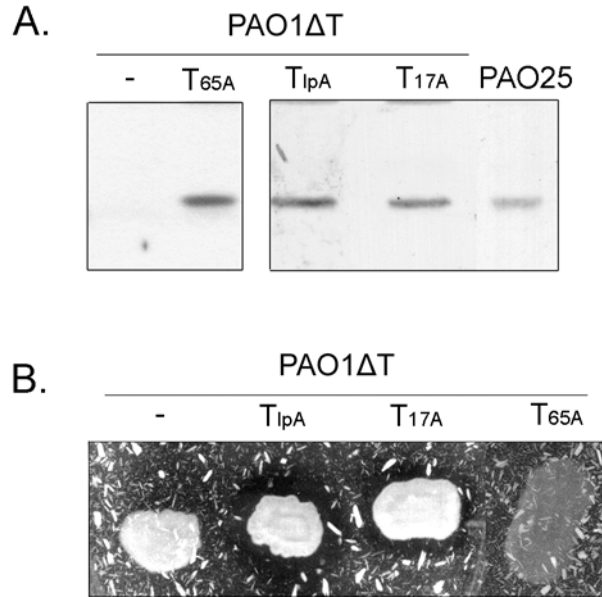
XcpT to the Xcp machinery, hybrids of *pilA* and *xcpT* were constructed and introduced into the broad-host-range vector pYRC. The leader peptide alone, the leader peptide including the hydrophobic domain, or the first 65 amino acid residues of XcpT were replaced by the corresponding segments of PilA. These chimeras are referred to as XcpT<sub>lpA</sub>, XcpT<sub>17A</sub>, and XcpT<sub>65A</sub>, respectively. A schematic representation of the PilA-XcpT hybrid proteins encoded by the gene fusions is depicted in Fig. 1.



**FIG.1. Schematic representation of PilA-XcpT hybrids. PilA sequences are depicted in black and XcpT sequences in white. Numbers indicate the amino acid residues relative to the prepilin peptidase cleavage site. The nomenclature of the hybrids is shown at the right.**

The *pilA-xcpT* constructs were introduced into the non-polar *xcpT* mutant PAO1ΔT. Production of the three hybrid proteins was confirmed by immunoblot analysis with antiserum raised against XcpT (Fig. 2A). However, detection of fusion XcpT<sub>65A</sub> required considerably longer exposure times than that of hybrids XcpT<sub>lpA</sub> and XcpT<sub>17A</sub>. Whether this fusion was more prone to proteolytic degradation or whether it was recognized less well by the XcpT-specific antiserum due to the loss of epitopes is not known. Functionality of the hybrid proteins was examined on elastin-containing plates where clear halos are visible around colonies of secretion-proficient strains. Like the production wild-type XcpT (results not

shown), the production of the fusions XcpT<sub>IpA</sub> and XcpT<sub>17A</sub> restored secretion in PAO1ΔT (Fig. 2B). In contrast, XcpT<sub>65A</sub> was not functional (Fig. 2B). These results show that the leader peptide and the N-terminal hydrophobic segment of 17 amino acid residues of mature XcpT can be substituted by those of PilA without loss of functionality. However, replacing the first 65 amino acid residues resulted in a non-functional protein.

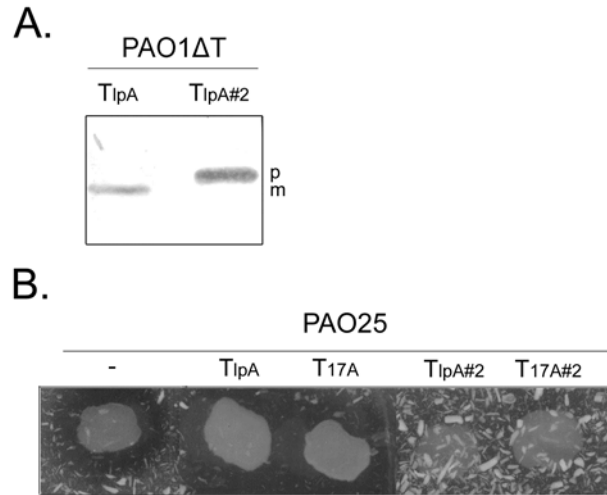


**FIG. 2. Production and functionality of PilA-XcpT hybrids. (A)** Immunoblot analysis of whole cell lysates of the *P. aeruginosa* xcpT mutant PAO1ΔT containing the empty vector (-), or constructs pYRC-XcpT<sub>65A</sub> (T<sub>65A</sub>), pYRC-XcpT<sub>IpA</sub> (T<sub>IpA</sub>), or pYRC-XcpT<sub>17A</sub> (T<sub>17A</sub>) grown overnight in the presence of IPTG. A whole cell lysate of PAO25 was included as a reference. Immunodetection was performed with XcpT-specific antiserum, peroxidase-conjugated goat anti-rabbit IgG antiserum and chemiluminescence (Pierce). Exposure time for the left panel was 30 min and for the right panel 4 min. **(B)** The same strains were grown on LB agar containing 1% elastin. Secretion of elastase is visualized by clearance of elastin from the plate around the colonies.

**Production of processing-defective variants of the chimeras interferes with secretion.** In *P. aeruginosa*, PilA and the pseudopilins are both processed by the same dedicated prepilin peptidase XcpA/PilD (22). We hypothesized that subunits with a defect in processing may specifically

block their cognate pathway, and that blocking of either T2SS-mediated secretion or piliation could be used as an indicator for efficient targeting to either one of the corresponding machineries. Therefore, genes were constructed encoding processing-defective PilA-XcpT chimeras. The variant proteins contain two amino acid residues (leucine and aspartic acid) inserted in between the -1 glycine and the +1 phenylalanine and are indicated in the nomenclature by the addition of #2. Maturation of the wild-type PilA or the original PilA-XcpT hybrids results in the removal of six amino acid residues from the N terminus, which can be visualized as a small mobility shift on acrylamide gels. Expression of the altered proteins in a *P. aeruginosa xcpT* mutant PAO1ΔT resulted in the production of forms with a slightly lower electrophoretic mobility (shown for hybrid XcpT<sub>1pA</sub> in Fig. 3A), which appeared similar in both wild-type and an *xcpA* mutant strain (data not shown), consistent with a processing defect. As expected, production of the processing-defective hybrids XcpT<sub>1pA#2</sub> or XcpT<sub>17A#2</sub> did not complement the secretion defect of the *xcpT* mutant (results not shown). Interestingly, upon production of these proteins in wild-type *P. aeruginosa* PAO25 strong interference with secretion was observed (Fig. 3B). This dominant-negative phenotype was even observed when the expression of the hybrid genes was repressed in the absence of IPTG and by the addition of glucose to the growth medium, showing that even small amounts of unprocessed subunit precursors obstructed the secretion process. Production of mutant fusion XcpT<sub>65A#2</sub> did not have such a dominant-negative effect on elastase secretion. However, production of this protein could not be detected (results not shown).

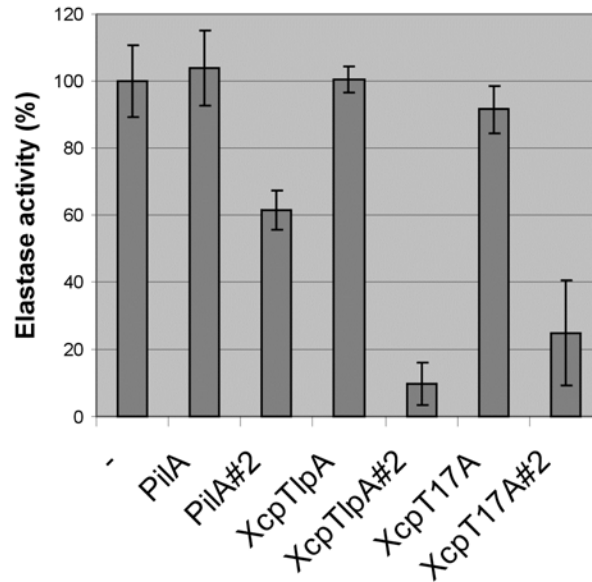
To determine whether specific targeting to the Xcp machinery was required to block secretion, a defective processing site was also introduced into the PilA protein. Production of this protein was confirmed in a *pilA* mutant by immunoblotting (results not shown). Based on halo formation on elastin-containing plates, production of PilA<sub>#2</sub> somewhat reduced elastase secretion in the wild-type strain PAO25 (results not shown), but not to the same extent as did the XcpT<sub>1pA#2</sub> and XcpT<sub>17A#2</sub> proteins. Quantification of the elastase activity in culture supernatants with an elastin-Congo red assay showed that production of PilA<sub>#2</sub> in strain PAO25 reduced elastase secretion by 40%, while XcpT<sub>1pA#2</sub> or XcpT<sub>17A#2</sub> reduced elastase secretion by 80-90% (Fig. 4). These results are consistent with the notion that XcpT<sub>1pA#2</sub> and XcpT<sub>17A#2</sub> are more efficiently targeted to the Xcp secretion machinery than PilA<sub>#2</sub>.



**FIG. 3. Negative dominance of processing-defective fusion proteins.** (A) Whole cell lysates of the *xcpT* mutant *PAO1ΔT* producing PilA-XcpT hybrid from pYRC-XcpT<sub>lpA</sub> (*T<sub>lpA</sub>*), or its variant with the disrupted processing site from pYRC-XcpT<sub>lpA#2</sub> (*T<sub>lpA#2</sub>*) were analyzed by SDS-PAGE, followed by Western blotting. Immunodetection was carried out with XcpT-specific antiserum. The positions of mature XcpT and its precursor on the immunoblot are indicated with m and p, respectively. (B) Wild-type *P. aeruginosa* strain *PAO25* containing the empty vector (-), or producing PilA-XcpT hybrids from pYRC-XcpT<sub>lpA</sub> (*T<sub>lpA</sub>*) and pYRC-XcpT<sub>17A</sub> (*T<sub>17A</sub>*), or the variants disrupted for processing from pYRC-XcpT<sub>lpA#2</sub> (*T<sub>lpA#2</sub>*) and pYRC-XcpT<sub>17A#2</sub> (*T<sub>17A#2</sub>*) were grown on LB agar containing 1% elastin. Secretion of elastase is visualized by clearance of elastin from the plate around the colonies.

**Defective processing of pilin subunits affects piliation.** To test whether production of the chimeric proteins and the processing-defective variants, PilA<sub>#2</sub>, XcpT<sub>lpA#2</sub>, XcpT<sub>17A#2</sub> and XcpT<sub>65A#2</sub> affected type IV piliation, *P. aeruginosa* strain PAK producing the different proteins from plasmids was tested for sensitivity to phage PO4. PO4 is a PAK-specific phage, which infects the cells by interacting with the type IV pili and loss of piliation results in phage resistance (36). Production of the PAO1 pilin subunit PilA in PAK does not interfere with PO4 sensitivity (34). Phage-sensitivity was assayed by streaking the phage on a LB agar plate and cross streaking of the bacterial strains. When no IPTG was added to the growth medium, neither the original, nor their processing-defective variants affected phage sensitivity (results not shown). In the presence of IPTG, however,

production of PilA<sub>#2</sub> resulted in phage resistance, indicating that the processing-defective pilin interferes with type IV piliation (Fig. 5A).

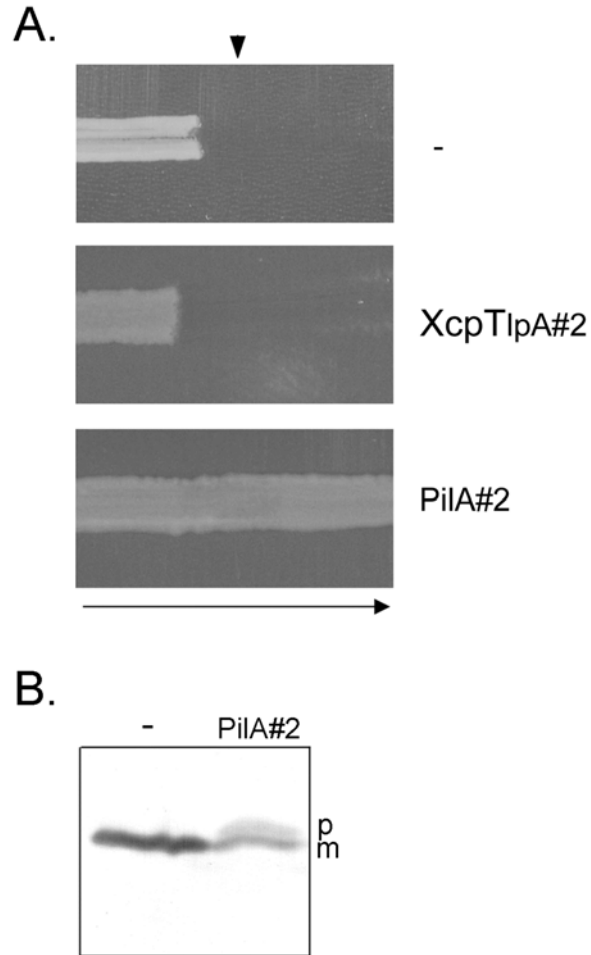


**FIG. 4. Quantitative analysis of the extracellular elastase activity.** Wild-type *P. aeruginosa* PAO25 producing from the pYRC derivatives PilA, hybrid XcpT<sub>lpA</sub>, hybrid XcpT<sub>17A</sub>, or their non-processed variants (PilA<sub>#2</sub>, T<sub>lpA#2</sub>, and T<sub>17A#2</sub>) from the pYRC-based plasmids were grown overnight in the presence of IPTG. Extracellular elastase activity was determined with the elastin-Congo red assay. The activity of the wild-type strain PAO25 with the empty vector (-) was set at 100%. Bars represent the averages of three independent experiments and standard deviations are indicated.

Immunoblotting showed substantial accumulation of the unprocessed pilin subunit within the cells (Fig. 5B). Phage sensitivity was not affected when processing-defective hybrid proteins were produced (shown for XcpT<sub>lpA#2</sub> in Fig. 5A).

## DISCUSSION

Since type IV piliation and the T2SS co-exist in *P. aeruginosa*, efficient targeting of the constituents to their cognate apparatus is required. However, from previous studies it is clear that the two systems can interfere when out of balance. For example, when overproduced in the absence of the T2SS, XcpT can be assembled into a pilus-like structure by the Pil system



**FIG. 5. Phage PO4 sensitivity and production of processing-defective PilA. (A)** Wild-type *P. aeruginosa* strain PAK containing the empty vector (-), or producing hybrid XcpT<sub>IpA#2</sub> or PilA<sub>#2</sub> from the pMMB67HE-derived plasmids were cross-streaked against phage PO4 on LB agar plates containing 1 mM IPTG and grown overnight. The arrowhead indicates the position of the phage suspension. The arrow shows the direction of inoculation of the bacterial strains. Phage sensitivity is visualized by the absence of bacterial growth after contact with the phage suspension. **(B)** Whole cell lysates of wild-type *P. aeruginosa* containing the empty vector (-) or producing processing-defective PilA from pMMB-PilA<sub>#2</sub> (PilA#2) were analyzed by SDS-PAGE, followed by Western blotting. Immunodetection was carried out with PilA-specific antiserum. The positions of mature PilA and its precursor on the immunoblot are indicated with m and p, respectively.

(7). Moreover, Lu *et al.* (19) reported that disruption of the *pilA* gene resulted in a significant delay in protein secretion in *P. aeruginosa*, which may be explained by targeting of pseudopilins to the Pil system when the natural substrate, PilA, is not available. Since cross talk between the two systems does occur, it is conceivable that correct targeting of pilins and pseudopilins to their cognate machineries relies on subtle differences in affinity and on an accurate stoichiometry. The presence of both systems makes *P. aeruginosa* a good model organism to identify regions within (pseudo)pilins that determine recognition by their cognate assembly machinery.

By using chimeric PilA-XcpT proteins, we found that the leader peptide and the N-terminal 17 residues of the mature pseudopilin XcpT could functionally be replaced by the corresponding part of pilin PilA. This result is in agreement with findings of Köhler *et al.* (18) who showed that the leader peptide and the N-terminal 17 amino acid residues of the pseudopilin PulG can be substituted by the corresponding segments of the pilins PpdD or PilE without loss of function. In addition, we observed that production of low amounts of processing-defective variants of the two functional chimeras completely blocked the secretion of elastase by the wild-type *P. aeruginosa* strain. Apparently, these fusions are still efficiently recognized by the Xcp system, indicating that the information needed for targeting of the (pseudo)pilins to the appropriate machinery is contained in another part of the protein. The third chimeric protein, in which the N-terminal 65 amino acid residues of mature XcpT were replaced by those of PilA, was non-functional and its processing-defective variant did not interfere with elastase secretion. Possibly, this protein is not targeted to the Xcp machinery, which would indicate that the segment C-terminally to the hydrophobic domain is important for recruitment by the Xcp machinery. Together with the hydrophobic domain, this segment forms an extended  $\alpha$ -helix in PilA, and these long helices of the subunits form the central core of the assembled pilus structure (6). Thus, efficient packing of subunits is dependent on this region, and may be required for targeting as well. Unfortunately, XcpT<sub>65A</sub> was considerably less well detected than the functional hybrids, which makes it difficult to draw conclusions from the experiments with this chimeric protein.

In wild-type *P. aeruginosa*, low amounts of XcpT<sub>1pA#2</sub> and XcpT<sub>17A#2</sub> were sufficient to obstruct secretion of elastase. The production of these proteins did not interfere with the processing of PilA and, *vice*



*versa*, accumulation of unprocessed PilA did not interfere with the processing of XcpT (results not shown). Hence, this phenotype cannot be explained by titration of prepilin peptidase. More likely, incorporation of processing-defective subunits blocks elongation of the pseudopilus. Interestingly, also production of processing-defective PilA in the wild-type strain PAO25 reduced elastase secretion to some extent, suggesting a low affinity of this protein for the Xcp machinery.

Consistent with the negative dominance of processing-defective XcpT variants, production of PilA<sub>#2</sub> interfered with the type IV piliation. Such a phenotype was not observed when any of the processing-defective PilA-XcpT hybrids was produced. IPTG induction was necessary to obstruct the Pil system, whereas the T2SS could be blocked even in the absence of IPTG and the presence of glucose. This difference may relate to a higher prevalence of PilA in the cell. An alternative explanation is provided by the fact that the type IV piliation apparatus contains not only an assembly ATPase, PilB, but also two retraction ATPases, PilU and PilT (35, 36). One of these proteins may remove aberrant subunits from the Pil system.

This study shows that the leader peptide and the hydrophobic region of XcpT are not required for the targeting of this pseudopilin to the Xcp system and, thus, that the targeting information is contained within another part of the protein. In addition, production of processing-defective variants of functional PilA-XcpT chimeras specifically obstructed the Xcp secretion system, whereas processing-defective PilA specifically blocked the Pil system. The latter findings can be used as valuable tools for the further identification of the targeting domains in the pseudopilins and pilins.

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