Chapter 2

Export of the pseudopilin XcpT of the *Pseudomonas aeruginosa* type II secretion system via the SRP/Sec pathway

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ABSTRACT

Type IV pilins and pseudopilins are found in various prokaryotic envelope protein complexes, including type IV pili and type II secretion machineries of Gram-negative bacteria, competence systems of Gram-positive bacteria, and flagella and sugar-binding structures within the archaeal kingdom. The precursors of these proteins have highly conserved N termini, consisting of a short positively charged leader peptide, which is cleaved off by a dedicated peptidase during maturation, and a hydrophobic stretch of approximately 20 amino acid residues. Which pathway is involved in the inner membrane translocation of these proteins is unknown. We used XcpT, the major pseudopilin from the type II secretion machinery of *Pseudomonas aeruginosa*, as a model to study this process. Transport of an XcpT-PhoA hybrid was shown to occur in the absence of other Xcp components in *P. aeruginosa* and in *Escherichia coli*. Experiments with conditional *sec* mutants and reporter-protein fusions showed that this transport process involves the co-translational SRP targeting route and is dependent on a functional Sec translocon.

INTRODUCTION

The type II secretion pathway is widely used by Gram-negative bacteria to secrete proteins into the extracellular environment (18). Release of exoproteins via this pathway occurs in a two-step process, implicating a periplasmic intermediate stage in the secretion pathway. Type II secretion machineries each encompass 12-16 different components (18). Five components show sequence similarity to PilA, the structural subunit of type IV pili, and are therefore named pseudopilins. Their precursors possess a conserved N terminus, which contains a short positively charged leader peptide followed by a highly hydrophobic domain of approximately 20 amino acid residues (20, 47). The leader peptide is removed during export by a specific prepilin peptidase (4, 36). These prepilin peptidases are polytopic inner membrane proteins that cleave off the leader peptide at the cytoplasmic side of the inner membrane and catalyse the methylation of the new N-terminal amino acid residue (usually phenylalanine) of the mature protein (32, 48).

Type IV pilin-like N-terminal sequences are not only found in type IV pili and in the pseudopilin components of type II secretion systems of Gram-negative bacteria, but also in competence systems in Gram-positive bacteria, and in flagella and sugar-binding proteins of archaea (39). In all these systems, the presence of proteins with prepilin-like N termini coincides with the occurrence of accessory proteins, including a prepilin peptidase, an ATPase, and a multispanning transmembrane protein (39).

How pilins and pseudopilins are transported across the cytoplasmic membrane and recruited by their cognate machinery is unknown. Two possible pathways have been proposed: one is via the highly conserved Sec translocon, and the other is via a dedicated machinery implicating the prepilin peptidase, the ATPase and/or the multispanning transmembrane protein mentioned above. The Sec system is generally used for protein transport across the cytoplasmic membrane (13). Two targeting pathways, SRP and SecB, intersect at the Sec translocon (28). SecB interacts with the mature portion of presecretory proteins and, besides targeting of the precursor to the translocase, it prevents their folding. SRP binds co-translationally to hydrophobic sequences in its substrates. The ribosome-nascent chain complex subsequently binds FtsY and is targeted to the Sec translocon. Inner membrane proteins typically depend on a functional SRP pathway, whereas periplasmic and outer membrane proteins predominantly

use the SecB route (11). Proteins targeted to the Sec system carry signal sequences that are characterized by a positively charged N-terminal region, followed by a 10-15 residues long hydrophobic core and a more polar C terminus containing the signal-peptidase cleavage site (53). Passage of the precursor over the inner membrane is followed by cleavage of the signal sequence by the signal peptidase LepB at the periplasmic side of the inner membrane (10). The N termini of the (pseudo)pilin precursors (prepilins) share characteristics with Sec signal sequences, and previous studies have indeed shown that the N-terminal sequences of prepilins from *Pseudomonas aeruginosa* and *Neisseria gonorrhoeae* function as export signals for alkaline phosphatase in *Escherichia coli* (14, 46). However, the N termini of the prepilins are also distinct from Sec signal sequences, since they lack the signal-peptidase cleavage site C-terminally to the hydrophobic domain and are, as mentioned above, processed by a specific prepilin peptidase N-terminally to the hydrophobic segment at the cytoplasmic side of the membrane. These differences and the fact that the pilin-like proteins are always found in concert with other proteins, has led to the proposal that pilins and pseudopilins are exported from the cytoplasm via a dedicated transport route formed by these accessory proteins (9, 18, 35).

To address the question how (pseudo)pilins are translocated across the inner membrane, we used the major pseudopilin XcpT of the *P. aeruginosa* type II secretion system as a model protein. The type II secretion machinery of this organism requires at least 12 components, XcpA and XcpP-Z (20). XcpA functions as the prepilin peptidase and is shared by the Xcp system, the type IV piliation (Pil) system, and the Hxc system, which forms a second type II secretion system dedicated to the export of low-molecular-weight alkaline phosphatases (3). We demonstrate that XcpT translocation can occur independently of the presence of other Xcp components in *P. aeruginosa* and in *E. coli* and that transport is dependent on a functional Sec apparatus. Furthermore, we show that translocation occurs co-translationally via the signal recognition particle (SRP) pathway.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains used in this study are listed in Table 1. The SRP mutant alleles *ffs69* (50) and *ffh87* (51) were introduced in MC1060 by generalized transduction with phage P1 as described (5). After the introduction of construct pJGA03, the transductants

were screened for blue coloring on LB agar plates containing X-gal. Out of six transductants, six and three were scored positive for *ffs69* and *ffh87*, respectively. *P. aeruginosa* strains and *E. coli* strains DH5α, C9, JS7131, MC1060, and the MC1060 derivatives were grown at 37°C, and other *E. coli* strains at 30°C in a modified Luria-Bertani (LB) broth (52), unless otherwise notified. For plasmid maintenance, the following antibiotics were used: for *E. coli* ampicillin 50 μg/ml; kanamycin 25 μg/ml; tetracycline 15 μg/ml; and gentamicin 15 μg/ml; for *P. aeruginosa* gentamicin 40 μg/ml.

TABLE 1. Strains used in this study

Strain	Relevant characteristics	Reference
E. coli		
DH5α	*thi-1 hsdR17 gyrA96 recA1 endA1 glnV44 relA1 phi80dlacZdelM15 phoA8* λ^-	(26)
C9	Hfr Cav, *relA1 fhuA22 pitA10 spoT1 ompF627 phoR18 creB501*	(23)
MC4100	F^-, Δ*lacU169 araD139 rpsL thi relA*	(6)
MM52	MC4100 *secAts51*	(37)
IQ86	Tn10 *thiA* Δ*lac araD rpsL rpsE relA*	(45)
IQ85	IQ86 *secYts24*	(45)
HPT244	MC4100 Δ*ara714 ffs69 zba-3054*::Tn*10*	(50)
HPT406	MC4100 Δ*ara714 pheA3141*::Tn*10*Kan *ffh87*	(51)
MC1060	Δ(*codB-lacI*)*3 galK16 galE15*(GalS) λ^- *e14*$^-$ *mcrA*$^-$ *relA1 rpsL150(strR) spoT1 mcrB1 hsdR2*	(7)
JS7131	MC1060 *attB::R6Kori* Δ*yidC* P*araBAD-yidC*	(43)
MC1060 *ffs69*	MC1060 *ffs69 zba-3054*::Tn*10*	This study
MC1060 *ffh87*	MC1060 *pheA3141*::Tn*10*Kan *ffh87*	This study
P. aeruginosa		
PAO25	PAO1 *leu arg*	(25)
PAO1ΔT	PAO1 Δ*xcpT*	(3)
DZQ40	PAO1Δ*xcpP-Z*	(2)
PAOΔ*hxc*Δ*pilAC*Δ*R*	Non polar deletion of the entire *hxc* gene cluster, of *pilA* to part of *pilC*, and of the *xcpR* gene	(16)

Plasmids and DNA manipulations. Plasmids used in this study are listed in Table 2. Recombinant DNA methods were performed essentially as described (42), using *E. coli* strain DH5α for routine cloning. Plasmids were introduced by the $CaCl_2$ procedure into *E. coli* (42) or by electroporation into *E. coli* and *P. aeruginosa* (17). PCRs were performed with the proofreading enzyme *Pwo* DNA polymerase (Roche) and PCR products

were cloned into the HincII site of vector pBC18R or into pCRII-TOPO according to manufacturer's protocol. The *lacI* gene was PCR amplified using plasmid pET16b as template and with the primers PB7 (5'-CTCCTTGCATGCACC-3') and PB8 (5'-CCCGCGCCCATGGGAAGG AGCTG-3'), thereby introducing an NcoI restriction site (underlined) downstream of the stop codon. The PCR product was cloned into pBC18R, which resulted in construct pCR-LacI and, subsequently, the SphI-NcoI fragment of pCR-LacI was introduced into the pBBR1-MCS5 vector, resulting in pYRC. The *phoA* gene without promoter and signal sequence-encoding part was PCR amplified from pPHO7 with the primers PB1 (5'-GATCCCCGGGGATCCGACTCTTATACAC-3') and PB2 (5'-CGAAAA TTCACTGTCTAGAGCGGTTTTATTTC-3'). A BamHI site (underlined) was introduced via PB1 upsteam of the fragment encoding signal-sequence-less PhoA and an XbaI site (underlined) via PB2 downstream of the stop codon. The PCR product was cloned into pBC18R, which gave pCR-PhoA. Plasmid pYRC-A was constructed by introduction of the BamHI-XbaI fragment of pCR-PhoA into BamHI-XbaI-digested pYRC. Cosmid pAX24 was used as template to amplify *xcpT* with the oligonucleotides JAXcpTfor02 (5'-CTTCCGATCCTTCGAATCAACCAACTCGTG-3') and JAXcpTrev01 (5'-GCCCGCATGTCGGATCCGTTGTCCCAGTTG-3') and the resulting product was cloned into pCRII-TOPO resulting in pCR-XcpT1-2. Underlined in JAXcpTrev01 is the BamHI site that replaced the stop codon of *xcpT* and that allowed for the construction of a translational fusion between *xcpT* and *phoA*. The PstI-BamHI fragment of pCR-XcpT1-2 was cloned into pYRC-A resulting in construct pJGA01. Plasmid pMPM-K4Ω contains an optimized Shine-Dalgarno sequence upstream of an NcoI site. To clone *xcpT* in the NcoI site, the gene was PCR amplified with primers pJAXcpTfor03 (5'-CGTGGGGTAATCCCATGGATCAGAGC CGC-3') and pJAXcpTrev01. Underlined is the NcoI site that replaces the original GTG start codon. Replacement of the start codon also led to the substitution of the 2nd residue from an asparagine to an aspartic acid in XcpT. The PCR product was cloned into pCRII-TOPO, which resulted in pCR-XcpT1-3. The NcoI-BamHI fragment of pCR-XcpT1-3 and the BamHI-XbaI fragment of pYRC-A were ligated into NcoI-XbaI-digested pMPM-K4Ω resulting in construct pJGA07. To obtain pT7-T, *xcpT* as 850-bp BssH2 fragment from pAX24 was first introduced into SmaI-digested pUC19 resulting in pUX4. Subsequently, the gene was cloned as KpnI-PstI-fragment into KpnI-PstI-digested pSPT19, which resulted in pSX4, and,

finally, the EcoRI-BamHI fragment from pSX4 was ligated into pT7-6. In construct pT7-T, *xcpT* expression is under control of the Φ10 promoter. Construct pGP1.2A is a derivative of pGP1.2 that contains *xcpA* introduced as PstI fragment from pUP2. For construct pJGA03, *lacZ* was PCR amplified from pUR292 with the primers PB3 (5'-CACAGGAAAC AGGATCCACCATGATTACGG-3'), which replaces the start codon by a BamHI site (underlined) and PB4 (5'-GGCTCGAGGTCTAGATTACC CCTGACACC-3') containing an XbaI site (underlined). The resulting product was cloned into pCRII-TOPO. The insert was subsequently excised by BamHI-XbaI digestion and introduced into BamHI-XbaI-digested pJGA01, replacing *phoA* by *lacZ*.

TABLE 2. Plasmids used in this study

Plasmid	Relevant characteristic[a]	Source or reference
pAX24	*xcpP-Z* cluster in pLAFR3	(19)
pBBR1MCS-5	Gm^r; cloning vector; P_{lac}	(30)
pBC18R	Ap^r; cloning vector	(8)
pPHO7	Ap^r; *phoA* without ss-encoding part	(24)
pET16b	Ap^r; *lacI*	Novagen
pCR-LacI	pBC18R; *lacI*	This study
pCR-PhoA	pBC18R; *phoA* without ss-encoding part	This study
pYRC	pBBR1MCS-5; *lacI*	This study
pYRC-A	pYRC; *phoA* without ss-encoding part	This study
pCRII-TOPO	Ap^r; Km^r; TOPO TA cloning vector	Invitrogen
pCR-XcpT1-2	pCRII-TOPO; *xcpT*	This study
pJGA01	pYRC; P_{lac}-*xcpT-phoA*	This study
pMPM-K4Ω	Km^r; cloning vector; P*araBAD*	(34)
pCR-XcpT1-3	pCRII-TOPO; *xcpT* (start codon replaced by NcoI site)	This study
pJGA07	pMPM-K4Ω; P_{ara}BAD-*xcpT-phoA*	This study
pUC19	Ap^r; cloning vector	(55)
pUX4	pUC19; *xcpT*	This study
pSPT19	Ap^r; cloning vector	Pharmacia
pSX4	pSPT19; *xcpT*	This study
pT7-6	Ap^r; cloning vector; T7 Φ10 promoter	(49)
pT7-T	pT7-6; PΦ10-*xcpT*	This study
pGP1.2	Km^r; T7 polymerase gene	(49)
pUP2	pUC19; *xcpA*	(12)
pGP1.2A	pGP1.2; *xcpA*	This study
pUR292	Ap^r; cloning vector; promoterless *lacZ*	(41)
pJGA03	pYRC; *xcpT-lacZ*	This study

[a]Gm, gentamicin; Ap, ampicillin; Km, kanamycin; ss, signal sequence

Enzyme assays. Secretion of elastase was analyzed qualitatively on LB plates with a top layer containing 1% elastin (Sigma). After overnight growth, the plates were screened for the presence of halos around the colonies. For alkaline phosphatase activity assays, overnight cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.3 (*E. coli*) or 0.6 (*P. aeruginosa*) in fresh LB and grown for 2.5 (*E. coli*) or 3 h (*P. aeruginosa*). *E. coli* cultures were subsequently split into two and incubated either for another 60 min at 30°C (permissive) or at 42°C (restrictive) to induce the *sec* phenotype of IQ85. Constructs were then induced with 0.01% L-arabinose (Sigma) or 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma), respectively. After 90 min (*E. coli*) or 3 h (*P. aeruginosa*) of growth in the presence of inducer, alkaline phosphatase activity in 1 ml of cell suspension was assayed with the substrate *para*-nitrophenyl phosphate (*p*NPP) (J.T. Baker). Cells were pelleted by centifugation, resuspended in 1 ml 0.9% NaCl and permeabilized by the addition of 20 µl of chloroform and 20 µl of 0.05% sodium dodecyl sulfate (SDS). After vortexing, 420 µl of 0.5 M Tris-HCl (pH 8.0) was added and the suspension was incubated for 10 min at 30°C. The reaction was started by the addition of 50 µl of *p*NPP (30 µg/ml in 0.5 M Tris-HCl, pH 8.0). When significant yellow coloring was observed, the reaction was stopped by the addition of 500 µl 0.5 M NaOH. After centrifugation, released *p*NP was determined by measuring the OD_{420} of the supernatant. Alkaline phosphatase activity in AP units was calculated as OD_{420} x 1000 / reaction time (min) x culture volume (ml) x OD_{600}. Activity of β-galactosidase was analyzed qualitatively on LB plates containing IPTG and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Sigma) as the substrate. For quantitative β-galactosidase activity assays, overnight-grown cells were harvested from LB plates containing the appropriate antibiotic and 0.1% glucose and suspended in 0.9% NaCl. Cells were pelleted by centifugation, resuspended in 950 µl PM2 buffer (40 mM Na_2HPO_4, 26 mM KH_2PO_4, pH 7.0) and permeabelized by the addition of 50 µl chloroform. After the addition of 50 µl *o*-nitrophenyl-β-D- galacto-pyranoside (ONPG) (4 mg/ml in PM2 buffer), the hydrolysis reaction was performed at 30°C for 65 min. The reaction was stopped by the addition of 300 µl of 1 M Na_2CO_3. After centrifugation, released ONP was determined by measuring the OD_{420} of the supernatant. β-galactosidase activity was calculated as OD_{420} x 1000 / reaction time (min) x culture volume (ml) x OD_{600}. In case of strain Js7131, quantitative β-galactosidase activity assays were performed on cells grown in liquid to allow for YidC depletion. Cells

were cultured in LB broth containing 0.2% L-arabinose to an OD_{600} of 0.6. Cells were collected by centrifugation and suspended in LB containing 0.2% L-arabinose or 0.2% glucose. To induce *xcpT-lacZ* expression, 0.5 mM IPTG was added and cultures were grown for an additional 90 minutes. β-galactosidase activities were determined as described above.

SDS-PAGE and immunoblot analysis. Bacterial cells were suspended in SDS-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 using 5% trichloroacetic acid (TCA) and washed with acetone. The amounts of proteins loaded were equivalent to an OD_{600} of 1 of bacterial cells. Samples were heated for 10 min at 95°C and separated on SDS-PAGE gels (8% gels for XcpT-LacZ, 11% gels for XcpT-PhoA, and 14% gels for XcpT). Proteins were stained with Coomassie Brilliant Blue or transferred to nitrocellulose membranes by semidry electroblotting for immunodetection. Primary antisera used were anti-XcpT at 1:1000, and anti-PhoA and anti-LacZ at 1:10.000 dilutions. Either alkaline phosphatase- or peroxidase-conjugated goat anti-rabbit IgG antibodies (Biosource international) were used as secondary antibodies. Detection of the latter was performed with chemiluminescence (Pierce). In the case of pulse-labeled samples, polyacrylamide gels were incubated in Amplify (Amersham) after electrophoresis, vacuum dried and exposed to X-ray films at -80°C.

Pulse-labeling experiments. *E. coli* cells carrying plasmid pT7-T in combination with either pGP1.2 or pGP1.2A were grown overnight at 30°C in M9-casamino acids medium [M9 salts (42) supplemented with 1% casamino acids, 0.2% glucose, 1 mM $MgSO_4$ and 0.0001% thiamine]. Overnight cultures were diluted to an OD_{600} of 0.3 in M9 medium supplemented with all amino acids except for methionine and cysteine (all 0.12%, except aspartic acid, glutamic acid, tyrosine, 0.05% and tryptophan 0.01%) and grown for 2.5 h. Then, cells were incubated at 42°C for 60 min to induce the synthesis of T7 RNA polymerase as well as the *sec* phenotype in the case of strains MM52 and IQ85. Cells were pelleted and resuspended in 1/5 of the original volume of the growth medium. Samples were pulse-labeled for 1 min with 3 μCi Redivue L-[^{35}S]methionine (Amersham) at 42°C. Incorporation of label was stopped by placing the samples on ice and by rapidly adding one volume of a solution of 10% TCA.

Immunoprecipitation. After removal of TCA from the pulse-labeled samples by washing with acetone, the pellet was dissolved in SDS

buffer (2% SDS, 50 mM Tris-HCl [pH 8.0], 1 mM EDTA) and incubated for 10 min at 100°C. Subsequently, Triton buffer (2% Triton X-100, 50 mM Tris-HCl [pH 8.0], 0.15 M NaCl) was added and insoluble material was removed by centrifugation. Polyclonal antiserum was added and, after 3 h of incubation at room temperature, protein A CL-4B sepharose beads (Amersham) were added. After incubation for 1 h at room temperature under gentle rocking, immunocomplexes were collected by centrifugation and washed with Triton buffer. Boiling for 10 min in SDS-PAGE sample buffer eluted antigens from the sepharose beads.

Proteinase K-accessibility. Cells carrying plasmid pJGA07 were grown as described in the pulse-labeling experiments. After 1.5 h of growth, arabinose was added to a final concentration of 0.01% to induce XcpT-PhoA expression. After 1 h of induction, cells were incubated for 1 h at 42°C to induce the *sec* phenotype. Pulse-labeling was performed as described above. Directly after the pulse-labeling, an excess of non-radioactive methionine/cysteine was added, and the cells were collected by centrifugation. For spheroplasting, cells were resuspended in ice-cold buffer A (40% [wt/vol] sucrose, 1.5 mM EDTA, 33 mM Tris-HCl [pH 8.0]) and incubated with lysozyme (final concentration 5 μg/ml). After 10 min on ice, incubation was continued at 37°C for 10 min followed by the addition of 10 mM $MgCl_2$. Aliquots of the spheroplast suspension were incubated on ice for 1 h in the presence or absence of proteinase K (final concentration 50 μg/ml). Subsequently, 2 mM phenylmethylsulfonyl fluoride was added to the cell suspension and incubation was continued for 5 min on ice. Proteins were precipitated with 5% TCA and analyzed by SDS-PAGE and autoradiography.

Cell fractionation. Overnight cultures of DH5α carrying pJGA03 were diluted into fresh, pre-warmed LB medium to an OD_{600} of 0.15 and grown for 2 h at 37°C shaking at 200 rpm. Subsequently IPTG was added to a final concentration of 1 mM. After 90 min incubation, the cells were harvested and spheroplasts were prepared as described (38). After pelleting the spheroplasts by centrifugation, the supernatant was kept as periplasmic fraction. The spheroplasts were washed in 0.9% NaCl, resuspended in 2 mM EDTA, 50 mM Tris-HCl (pH 8.5) and frozen at -20°C. After thawing, they were disrupted by sonication and the membranes were collected by centrifugation for 1 h at 150.000 g at 4°C. The supernatant contained the cytoplasmic proteins.

RESULTS

Inner membrane translocation of XcpT-PhoA occurs independently of other Xcp components. To study pseudopilin transport across the inner membrane, the periplasmic reporter enzyme alkaline phosphatase (PhoA) without its signal sequence was C-terminally fused to the complete major pseudopilin XcpT of *P. aeruginosa*. Plasmid pJGA01, carrying the *xcpT-phoA* gene fusion, was introduced in wild–type PAO25 and in the *xcpT* mutant PAO1ΔT, and immunoblot analysis with an antiserum directed against PhoA confirmed production of XcpT-PhoA (Fig. 1A). Some breakdown products migrating at the same position as wild-type PhoA were detected as well. Remarkably, based on halo formation on elastin plates (Fig. 1B) and analysis of extracellular proteins (data not shown), the fusion protein appeared to restore the secretion of the Xcp substrate elastase in the *xcpT* mutant strain, indicating that the PhoA moiety did not interfere with the functionality of the protein.

PAO25 cells carrying pJGA01 displayed alkaline-phosphatase activity (Fig. 2). Since alkaline phosphatase is only active when transported out of the cytoplasm, this result shows that the XcpT-PhoA fusion protein is transported across the inner membrane. Pseudopilin transport has been proposed to occur via a dedicated transport pathway formed by the other components of the secretion machinery. To determine whether other Xcp components indeed were required for inner membrane passage of XcpT-PhoA, pJGA01 was introduced in *P. aeruginosa* strain D40ZQ lacking the *xcp* gene cluster. D40ZQ cells carrying pJGA01 still displayed alkaline phosphatase activity, although the level was somewhat lower than that found in the wild-type strain (Fig. 2). Thus, cytoplasmic membrane passage of XcpT-PhoA can occur independently of the Xcp system. Durand *et al.* (15) have reported that also the Pil system and the Hxc system can function in the assembly of the XcpT protein into a pilus-like structure. To study whether one of these two systems was responsible for the inner membrane transport of XcpT-PhoA in absence of the Xcp apparatus, pJGA01 was introduced in a strain mutated in all three pathways (PAOΔ*hxc*Δ*pilAC*Δ*R*). This strain still showed alkaline phosphatase activity, similar to that of the *xcp* deletion strain (Fig. 2). Hence, inner membrane passage of XcpT-PhoA can occur independently of the Xcp, Hxc and Pil systems.

XcpT-PhoA activity in *E. coli* depends on a functional Sec system. Given the similarity between the N termini of prepilins and Sec

signal sequences, the most likely inner membrane translocation pathway for XcpT-PhoA, since it is not transported via accessory Xcp components, would be via the Sec machinery. To investigate this possibility, XcpT-PhoA was produced in the heterologous host *E. coli*, of which several well-characterized *sec* mutant strains are available. Construct pJGA07, which contains *xcpT-phoA* under control of P*araBAD*, was introduced in the temperature-sensitive *secY* mutant IQ85 and its parental strain IQ86. Expression of the XcpT-PhoA protein was verified by immunoblot analysis with an antiserum against PhoA (results not shown) and inner membrane transport of the fusion protein was studied by measuring alkaline phosphatase activity. At the permissive temperature of 30°C, IQ85 produced similar alkaline phosphatase activity as did the parental strain (Fig. 3). However, at the restrictive temperature of 42°C, PhoA activity was drastically lower in the *secY* mutant as compared with the parental strain. This result shows that XcpT-PhoA is transported over the inner membrane of *E. coli* in a SecY-dependent manner.

Processing and inner membrane transport of XcpT requires a functional Sec system. To study the inner membrane translocation of native XcpT in *E. coli*, pulse-labeling experiments were performed. In these experiments, processing of XcpT by the prepilin peptidase XcpA was used as an indicator of inner membrane transport. Processing of XcpT results in the removal of eight residues from the N terminus and can be visualized by a small mobility shift on acrylamide gels. To allow for simultaneous induction of the *sec* phenotype and XcpT expression, the *xcpT* gene was introduced in pT7-6 under the control of the T7 promoter. The resulting construct, pT7-T, was combined either with helper plasmid pGP1.2, which contains the gene for T7 RNA polymerase under the control of the λ P_L promoter and the *cI857* gene encoding a temperature-sensitive λ repressor, or with pGP1.2A, which additionally contains *xcpA*. These constructs were introduced into the temperature-sensitive *secA* and *secY* mutant strains MM52 and IQ85, respectively, and their cognate parental strains. To induce the synthesis of T7 RNA polymerase and the Sec phenotype, cells were shifted to 42ºC for 1 h before pulse-labeling was performed. In these experiments, processed XcpT was only detected upon co-expression of XcpA (data not shown). In the XcpA-expressing parental strains MC4100 and IQ86, processing of the pseudopilin XcpT was nearly complete after 1 min pulse-labeling (Fig. 4). However, maturation of XcpT was clearly

reduced in the *secA* and *secY* mutants, which shows that processing requires a functional Sec translocase.

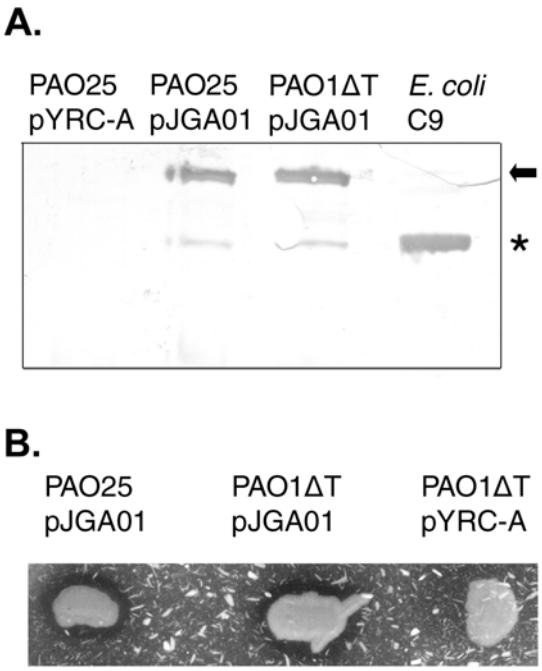

FIG. 1. Production and functionality of XcpT-PhoA fusion protein. (A) Immunoblot analysis of whole cell extracts of *P. aeruginosa* strain PAO25 and an *xcpT* mutant PAO1ΔT both expressing XcpT-PhoA from pJGA01, or containing the fusion vector pYRC-A. Immunodetection was carried out with PhoA-specific antibodies. The position of XcpT-PhoA is indicated by an arrow. Total cell lysate of *E. coli* strain C9 constitutively producing PhoA (indicated by an asterisk) was included for reference. (B) PAO25 and PAO1ΔT expressing XcpT-PhoA from pJGA01 or containing the vector pYRC-A were grown on LB agar containing 1% elastin. Secretion of elastase is visualized by clearance of elastin around the colonies.

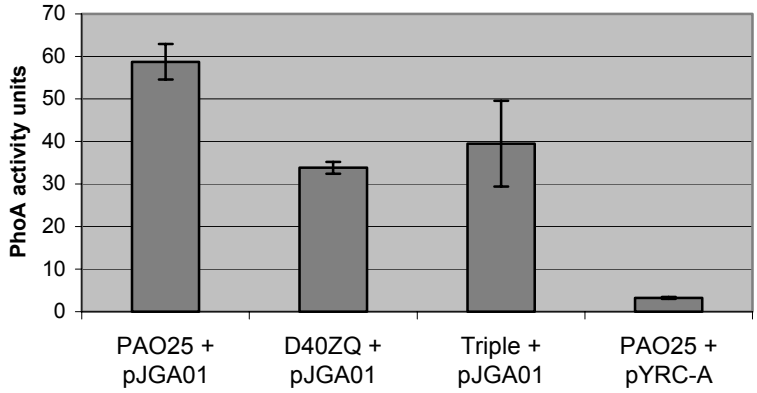

FIG. 2. Enzyme activity of XcpT-PhoA in *P. aeruginosa*. Alkaline phosphatase activities were measured in *P. aeruginosa* strains expressing XcpT-PhoA from pJGA01, or containing the vector pYRC-A. The strains used were the wild-type strain PAO25, strain D40ZQ, which lacks the *xcp* cluster, and strain PAOΔ*hxc*Δ*pilAC*Δ*R* (triple), which lacks *hxcP-Z, pilA-C*, and *xcpR*. Bars represent the averages of three independent assays and standard deviations are indicated.

The results above suggest that the Sec translocase is required for transport of XcpT and that transport is a prerequisite for processing to occur. Lack of transport in the *sec* mutants was further evaluated in proteinase K-accessibility experiments on spheroplasted cells. Since XcpT is intrinsically very resistant to the protease (results not shown), the XcpT-PhoA fusion protein was employed in these experiments. When the fusion protein was expressed from pJGA07 in the parental strains MC4100 and IQ86, it was almost completely degraded and thus accessible for proteinase K after spheroplasting of the cells (Fig. 5A). In these experiments, proteinase K treatment did not yield the stable PhoA moiety, likely because kinetics were not fast enough to allow PhoA to obtain its mature conformation. When produced under the restrictive conditions, the XcpT-PhoA protein was completely protected from the protease in spheroplasts of strain IQ85, showing that the protein remains at the cytoplasmic side of the membrane in the absence of a functional Sec system. In these cells, also the precursor of a well-known Sec substrate, OmpA, was found to accumulate in a form inaccessible to proteinase K after spheroplasting (Fig. 5B). In spheroplasts of MM52 cells grown at the restrictive temperature, XcpT-PhoA was also protected against the proteinase K treatment, although not completely (Fig.

5A). In these cells, also some mature OmpA was detected, indicating that the SecA phenotype was not complete. As expected, the proteinase K treatment resulted in the degradation of the mature form only of OmpA (Fig. 5B).

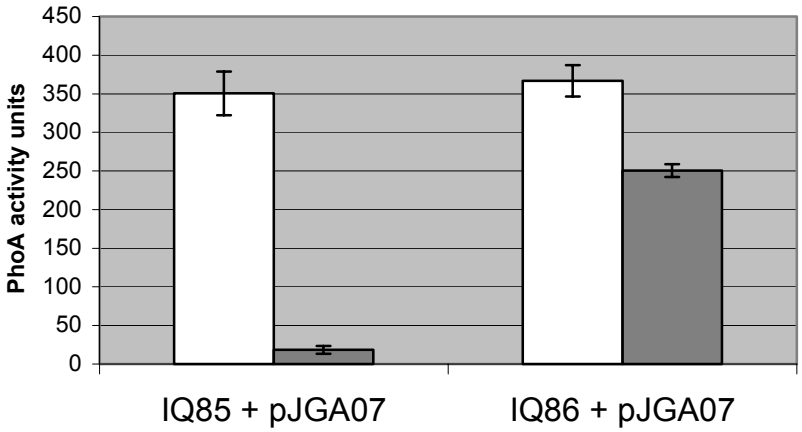

FIG. 3. Enzyme activity of XcpT-PhoA is dependent on SecY in *E. coli*. Alkaline phosphatase activities were measured in the temperature-sensitive *secY* mutant strain IQ85 and its parent IQ86, both expressing XcpT-PhoA from pJGA07. Activities were measured in cultures incubated for 2.5 h at 30°C (white bars) or at 42°C (gray bars). Bars represent the averages of three independent assays and standard deviations are indicated.

Co-translational transport of XcpT-LacZ. Transport of XcpT across the inner membrane is largely affected in the temperature-sensitive *secY* mutant and, albeit to a lesser extent, also in the *secA* mutant. The cytoplasmic protein β-galactosidase (LacZ) can be used as a reporter to distinguish between the SRP and the SecB pathways (5), which both intersect at the Sec translocon. Cells expressing LacZ fused to a SecB substrate display a Lac^+ phenotype because of the cytoplasmic accumulation of β-galactosidase. They are also sensitive to induction of the expression of the fusion protein because of jamming of the Sec translocon due to rapid folding of the LacZ moiety of the fusion protein. In contrast, when LacZ is fused to an SRP substrate, cells are Lac^- because β-galactosidase is exported. Since export is co-translational, the enzyme cannot fold in the cytoplasm and no jamming of the translocon occurs. To some extent, the cells can still be sensitive to induction of the expression of the fusion

protein, because of the formation of toxic aggregates of LacZ in the periplasm (5).

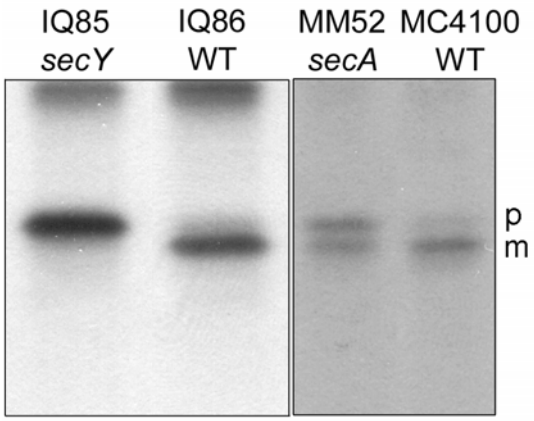

FIG. 4. Inhibitory effect of *sec* mutations on XcpT processing. XcpT was expressed from plasmid pT7-T in the *E. coli* strains MC4100 and IQ86 and their isogenic temperature-sensitive *secA* and *secY* mutants MM52 and IQ85, respectively, both co-expressing XcpA from pGP1.2A. After pulse-labeling at 42°C and immunoprecipitation with anti-XcpT, samples were analyzed by SDS-PAGE, followed by autoradiography. The location on the autoradiogram of mature XcpT and its precursor are indicated by m and p, respectively.

To make use of this system, plasmid pJGA03 was constructed, encoding an XcpT-LacZ fusion protein. Production of this chimaeric protein from pJGA03 in DH5α resulted in a Lac^- phenotype as indicated by the white colony phenotype on plates containing the β-galactosidase substrate X-gal (Fig. 6A), even after two days of incubation. Immunoblot analysis with antisera against LacZ and XcpT revealed that the fusion protein was produced (Fig. 6B), although the majority was detectable as a smaller degradation product only recognized by the LacZ antibodies. The degradation product was not always observed (see Fig. 7B) and probably reflects degradation during sample preparation. However, the appearance of the degradation product allowed us to determine the localization of XcpT-LacZ. Cell fractionation showed that the fusion protein was associated with the membranes (Fig. 6B), whereas the degradation product corresponding to the LacZ part was found mostly in the periplasmic fraction, showing that the fusion protein is transported over the inner membrane. DH5α cells carrying pJGA03 were not sensitive to induction of the expression of the chimeric

gene with IPTG. The efficient export of the XcpT-LacZ fusion protein across the inner membrane without jamming the Sec pathway is consistent with co-translational targeting via the SRP pathway.

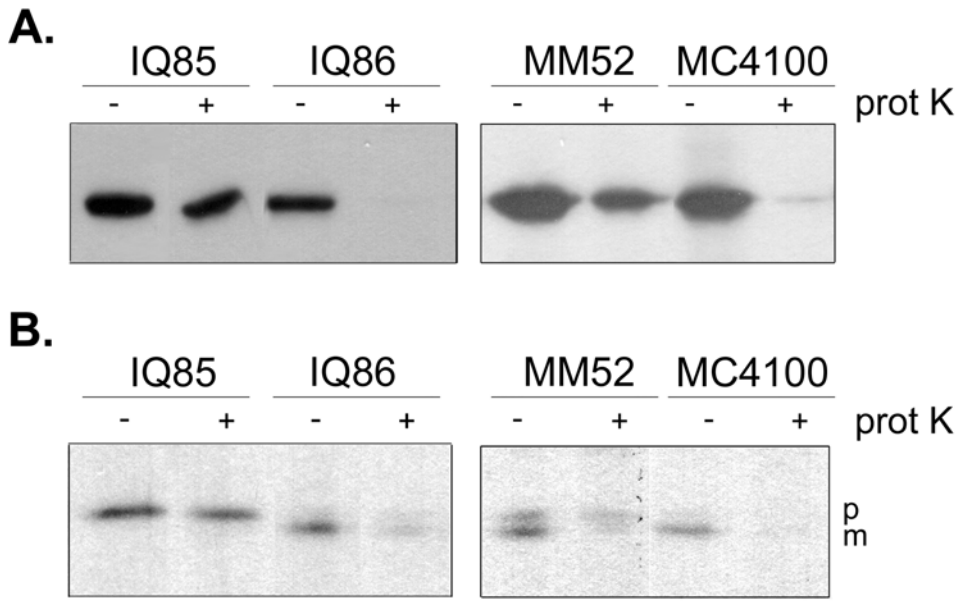

FIG. 5. Proteinase K accessibility of XcpT-PhoA and OmpA in spheroplasts. (A) Cultures of temperature-sensitive *secA* and *secY* mutants MM52 and IQ85, respectively, and their parental strains MC4100 and IQ86 were induced for XcpT-PhoA expression from pJGA07 and incubated at the restrictive temperature. Cells were subsequently pulse-labeled with [^{35}S]methionine, converted to spheroplasts and, where indicated, treated with proteinase K (prot K). After immunoprecipitation with anti-PhoA serum, samples were analyzed by SDS-PAGE, followed by autoradiography. Because of the large size of XcpT-PhoA (~65 kDa), removal of the short XcpT leader peptide during processing is not visible as a mobility shift. (B) The same samples were also used for immunoprecipitation with anti-OmpA serum. Samples were analyzed by SDS-PAGE and autoradiography. The location on the autoradiogram of mature OmpA and its precursor are indicated by m and p, respectively.

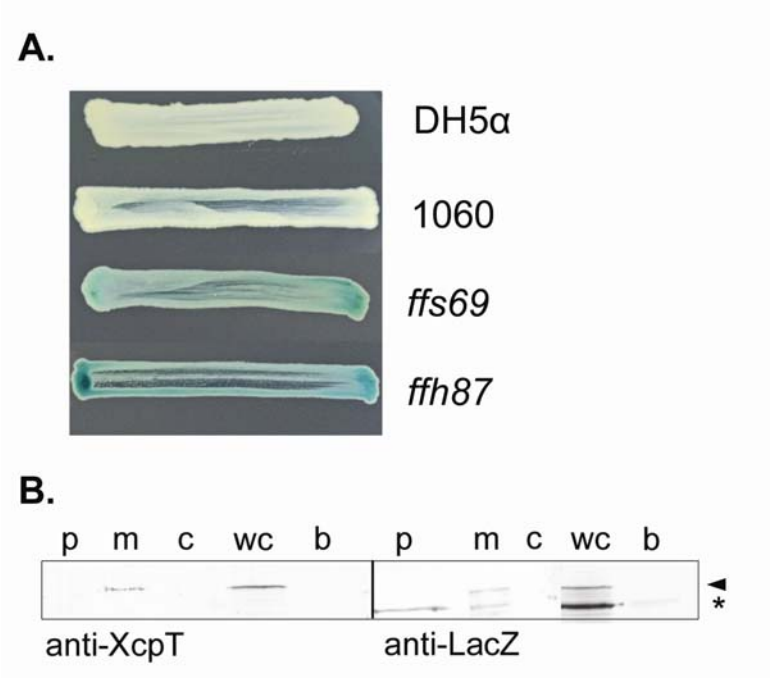

FIG. 6. XcpT-LacZ production and localization in *E. coli*. (A) *E. coli* strains DH5α, MC1060 and the MC1060 derivatives with the alleles *ffs69* and *ffh87*, respectively, producing XcpT-LacZ from pJGA03 were grown overnight on LB plates with X-gal. White colonies correspond to a Lac$^-$ phenotype; blue colonies to a Lac$^+$ phenotype. (B) DH5α cells carrying pJGA03 were induced for the expression of XcpT-LacZ, and whole cell extracts (wc) or cell fractions were analyzed by SDS-PAGE and Western blotting. Immunodetection was performed with antibodies against LacZ and XcpT. The positions of XcpT-LacZ and of LacZ are indicated with an arrowhead and an asterisk, respectively. p, periplasmic fraction; m, membrane fraction; c, cytoplasmic fraction; wc, whole cell extract; b, whole cell extract of plasmid-less DH5α cells; the weak band detected by the anti-LacZ serum in this extract likely corresponds to the inactive LacZdelM15 protein encoded from the chromosome (31).

To further substantiate this conclusion, pJGA03 was introduced into MC1060 and its derivatives containing the SRP mutant alleles *ffs69* (50) and *ffh87* (51), which confer mild defects on SRP-dependent secretion. The strains with the SRP mutant alleles formed light blue colonies on X-gal-containing plates, while the parental strain remained white (Fig. 6A), showing that the SRP mutations interfere with efficient translocation of XcpT-LacZ to the periplasm. This effect was quantified by measuring β-

galactosidase activity. As shown in Fig. 7A, the strains with the mutant alleles displayed a five-fold increase in β-galactosidase activity compared to the wild-type strain, whereas the production of XcpT-LacZ was the same in all the strains (Fig. 7B). These results underscore the SRP dependency of XcpT targeting to the Sec translocon. To test the effect of YidC on translocation of XcpT-LacZ, pJGA03 was introduced into strain Js7131, which carries a ParaBAD-yidC operon. No effects on β-galactosidase activities were measured upon removal of arabinose, which results in YidC depletion (43) (data not shown).

A.

Strain	Miller units	Normalized[a]
MC1060	0,42 (0,03)	100
MC1060 *ffs69*	1,92 (0,03)	457
MC1060 *ffh87*	2,01 (0,06)	479

B.

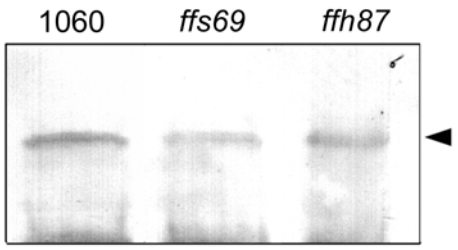

FIG. 7. XcpT-LacZ production in the presence of mutant SRP alleles. (A) β-galactosidase activity (in Miller units) of cells grown on LB plates. The activity values represent the mean of four independent experiments, with standard deviation indicated in brackets. [a]Values of β-galactosidase activity normalized against the wild-type strain in percentage. (B) Immunoblot analysis of whole cell lysates of *E. coli* strains MC1060 and and the MC1060 derivatives with the alleles *ffs69* and *ffh87*, respectively, producing XcpT-LacZ from pJGA03. Immunodetection was carried out with LacZ-specific antibodies. The position of XcpT-LacZ on the immunoblot is indicated by an arrowhead.

DISCUSSION

Sequences resembling the N terminus of prepilins are frequently detected in proteins from Gram-negative and -positive bacteria, as well as from archaea (39). Although these sequences mediate transport across the inner membrane, the pathway involved in this process was largely unknown. To study this transport process, alkaline phosphatase was fused to XcpT, the major pseudopilin of the type II secretion machinery of *P. aeruginosa,* and alkaline phosphatase activity was used to monitor transport. Export of XcpT-PhoA did occur in a *P. aeruginosa* strain lacking functional Xcp, Hxc, and Pil systems as well as in *E. coli*. These findings argue against the hypothesis that accessory Xcp components or their homologues form a dedicated inner membrane transport system for the pseudopilins. This idea was based on the observation that proteins with prepilin-like N termini are always found in concert with an ATPase (XcpR in *P. aeruginosa*), an integral inner membrane protein (XcpS), and a prepilin peptidase. In support of this hypothesis, Chung and Dubnau (9) reported that the prepilin peptidase ComC of the *Bacillus subtilis* competence system is required for translocation of the pilin-like protein ComGC. Moreover, Kagami *et al.* (27) showed that a conditional mutation in XcpT could be suppressed by a secondary mutation in the cytoplasmic ATPase XcpR, indicating that these proteins interact during assembly and/or functioning of the machinery.

However, our conclusion that the pseudopilin XcpT is not transported via accessory Xcp proteins is consistent with the observation that a mutant of the archaeon *Sulfolobus solfataricus*, in which the genes encoding the accessory ATPase (homologue of XcpR) and the integral inner membrane component (homologue of XcpS) are deleted, is not affected in its ability to insert sugar-binding proteins with prepilin-like N termini into the membrane (B. Zolghadr, personal communication). Moreover, although pilins and pseudopilins are unable to functionally replace each other, it is possible to exchange their leader peptides and hydrophobic domains without loss of function (29). Even in *P. aeruginosa*, where the Pil and Xcp systems function side by side, the N termini of PilA and XcpT could be exchanged without affecting function (our unpublished results). Thus, apparently, the N termini do not contain the information for targeting of the (pseudo)pilins to the cognate machinery, which is in agreement with export of these proteins via a general route. An explanation for the afore mentioned results described by Kagami *et al. (27)* and Chung and Dubnau (9) might be offered by the

possibility that the accessory proteins, although not required for the transport of the pseudopilins across the inner membrane, are required for their assembly into a pilus-like structure (16, 44). In the case of ComGC, translocation was assayed in NaOH-solubility studies and it is conceivable that this method distinguishes ComGC assembled in a pilus-like structure from membrane-embedded ComGC rather than showing translocation.

Since the N termini of (pseudo)pilins to a certain degree resemble Sec signal sequences, we reasoned that inner membrane translocation of XcpT would occur via the Sec machinery. The involvement of the Sec translocon was studied in the heterologous host *E. coli*. In a temperature-sensitive *secY* mutant, translocation of XcpT-PhoA was blocked at the restrictive temperature, indicating that indeed the Sec pathway is involved. The requirement for SecA and SecY in XcpT transport could also be demonstrated in pulse-labeling experiments. Both XcpT processing and protease accessibility were affected in the *secA* and *secY* mutants at the restrictive temperature. Maturation of XcpT in these experiments was strictly dependent on the presence of the *P. aeruginosa* prepilin peptidase XcpA. Nonetheless, immunoblot analysis with anti-XcpT serum of extracts of overnight grown *E. coli* cells expressing XcpT showed partial processing (unpublished observation). Apparently, previously described *E. coli* prepilin peptidase homologues (22, 54) are functional, but their ability to process XcpT was not sufficiently efficient to be detected in the short pulse-labeling experiments. These experiments showed that processing of the pseudopilin occurs after transport, despite the fact that the prepilin peptidase has its active centre at the cytoplasmic side of the inner membrane (32). Apparently, membrane insertion of XcpT is required to expose its processing site accurately to the XcpA peptidase.

In order to distinguish between targeting via the SecB or the SRP route, XcpT was fused to the reporter LacZ, which was previously shown to be a useful reporter for this purpose (5). This fusion protein was efficiently transported to the periplasm without jamming the Sec translocon, consistent with co-translational transport via the SRP pathway. Moreover, efficient inner membrane translocation of the XcpT-LacZ fusion was dependent on fully functional Ffh and Ffs, but not on YidC. Taken together, our results demonstrate that XcpT is targeted to the Sec system via the SRP route, which is common for inner membrane proteins. The precise requirements for SRP signals are still not completely clear, but hydrophobicity and secondary structure have been shown to be important determinants (1, 5,

33). Indeed, the N termini of prepilins are highly hydrophobic and contain an extended N-terminal α-helix. Since pseudopilins have a high tendency to associate (40), co-translational translocation may be important to circumvent premature interactions.

This report shows for the first time the Sec and SRP dependency of export of a pseudopilin. Given the strong conservation of their N-terminal sequences, it is likely that also other pilins and pseudopilins use the SRP/Sec pathway. Indeed, similar conclusions were reached by Francetic *et al*. in the accompanying paper on the export of the pseudopilin PulG of the type II secretion system of *Klebsiella oxytoca* (21). We propose that, after translocation, the (pseudo)pilins laterally leave the Sec translocon, after which they become recruited by their cognate machinery. The polytopic inner membrane component will subsequently act as a platform for the assembly of the subunits into a pilus (-like) structure, using the energy provided by the ATPase.

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