Chapter 5

Species-specific outer membrane targeting of the *Pseudomonas aeruginosa* secretin XcpQ

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ABSTRACT

Many Gram-negative bacteria employ a complex, envelope-spanning type II secretion system (T2SS) for exporting a broad range of proteins across the outer membrane into the extracellular medium. The T2SS of the opportunistic pathogen *Pseudomonas aeruginosa* consists of 12 different Xcp proteins, of which the oligomeric XcpQ protein is thought to form the actual transport channel in the outer membrane. In the present study, we demonstrate that the Xcp proteins assemble into a functional system when expressed in the heterologous bacterial host *Pseudomonas putida*, but not in *Escherichia coli*. In *E. coli*, XcpQ was not targeted properly to the outer membrane, since XcpQ oligomers were found associated with the inner membrane. By exchanging the C terminus of XcpQ by the corresponding segment of PulD, the secretin of the T2SS of *Klebsiella oxytoca*, and co-production of the PulD-specific pilotin PulS, outer membrane targeting could be achieved. In *P. aeruginosa*, production of the XcpQ-PulD hybrid restored secretion in an *xcpQ* mutant strain. However, the stability and functionality of this protein were strictly dependent on the presence of PulS. These results suggest that outer membrane targeting of XcpQ may require a so-far unidentified pilot protein(s), specific for *Pseudomonas* species.

INTRODUCTION

Protein secretion in Gram-negative bacteria relies on a limited number of dedicated transport machineries. Widely spread is the type II secretion pathway (13). Proteins secreted via this pathway are translocated across the cytoplasmic membrane via the Sec translocon or the TAT system (35, 55). Subsequently, the type II secretion system (T2SS) mediates the transport of the folded proteins across the outer membrane (9, 46). The opportunistic pathogen *Pseudomonas aeruginosa* secretes several enzymes and toxins via the T2SS, which is formed by the products of at least 12 *xcp* genes: *xcpA* and *xcpP-Z* (23). Disruption of any of the 12 *xcp* genes leads to the accumulation of the exoproteins in the periplasm (23, 30, 57).

Although the Xcp apparatus functions in the transport of proteins across the outer membrane, most of its constituents are found in or associated with the inner membrane. Only one component, XcpQ, localizes to the outer membrane (23). XcpQ forms oligomers, which supposedly function as the channels through which the Xcp substrates are exported out of the cell (6, 10). The inner membrane protein XcpP is thought to link XcpQ with the other components in the inner membrane (7). XcpY and XcpZ form a stable complex in the inner membrane and the cytoplasmic traffic ATPase XcpR docks to this complex by interacting with XcpY (4, 43). The polytopic integral inner membrane component XcpS also participates in this subcomplex (48). Finally, the Xcp apparatus contains several proteins with N-terminal sequence similarity to PilA, the structural component of type IV pili. These proteins are processed by the dedicated prepilin peptidase XcpA (44).

XcpQ belongs to a large family of proteins called secretins, which participate in the transport of macromolecules across the outer membrane in various systems, including type II and type III protein secretion, filamentous phage release, and type IV pilus assembly (28). Secretins form stable oligomers of 12-14 subunits (5). After their synthesis in the cytoplasm, they are targeted to the Sec machinery for translocation across the inner membrane, which is followed by removal of the N-terminal signal sequence by leader peptidase (47). Later steps in the biogenesis of these proteins are largely unknown. Secretins appear to use the same Omp85-dependent sorting pathway as other integral outer membrane proteins (56). However, secretins are devoid of the C-terminal consensus motif that is typically found in outer membrane proteins (53) and which is recognized by Omp85

(49). Furthermore, their oligomerization severely challenges their periplasmic transfer. Indeed, several secretins require specific chaperones for correct outer membrane localization and protection against proteolytic degradation. Typically, small outer membrane lipoproteins with little sequence conservation are involved in chaperoning the secretins. Such lipoproteins, collectively designated pilotins, have been identified, for example, in the T2SSs of *Klebsiella oxytoca* (PulS) and *Erwina chrysanthemi* (OutS) and they bind their cognate substrates at the extreme C terminus (15, 33, 51). On the other hand, the secretin of the T2SS of *Aeromonas hydrophila* requires the inner membrane ExeAB complex for proper localization (3). Thus, multiple mechanisms appear to exist for secretin assembly in T2SSs.

In *P. aeruginosa*, no homologues of *pulS*, *outS* or *exeAB* have been identified. Therefore, XcpQ is believed not to require a chaperone for its targeting to the outer membrane (23). However, an alternative possibility is that a putative chaperone has remained undetected so far, due to no or only limited sequence similarity to known secretin chaperones. To determine whether the twelve *xcp* genes are sufficient to assemble into a functional secretion system, we have expressed, in the present study, the *xcp* genes in the heterologous bacterial hosts *Escherichia coli* and *Pseudomonas putida* and report XcpQ assembly defects in *E. coli*.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions. *P. aeruginosa* strains PAO25 (29) and D40ZQ (4) are *leu arg* and Δ*xcpP-Z* mutant derivatives, respectively, of strain PAO1. PAN1 (6) is a mutant derivative of strain PAO25, carrying a gentamicin-resistance cassette in the *xcpQ* gene. Furthermore, *E. coli* strain DH5α (31) and *P. putida* strain WCS358 (27) were used. *P. aeruginosa* and *E. coli* strains were grown at 37°C, and *P. putida* at 30°C in a modified Luria-Bertani (LB) broth (54). For plasmid maintenance, the following antibiotics were used: for *E. coli*: ampicillin 50 µg/ml, chloramphenicol 25 µg/ml, gentamicin 15 µg/ml, and kanamycin 25 µg/ml; for *P. aeruginosa*: gentamicin 40 µg/ml, chloramphenicol 300 µg/ml, and piperacillin 20 µg/ml; for *P. putida* gentamicin 40 µg/ml, and kanamycin 40 µg/ml.

TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristic[*]	Source or reference
pAX24	*xcpP-Z* cluster in pLAFR3	(22)
pCRII-TOPO	Ap^r; Km^r; TOPO TA cloning vector	Invitrogen
pCRII-P	pCRII-TOPO; *xcpP*	This study
pCRII-R	pCRII-TOPO; *xcpR*	Chapter 4
pCRII-201	pCRII-TOPO; *lipA, lif*	This study
pCRII-‘D65	pCRII-TOPO; *‘pulD65*	This study
pCRII-‘Q65	pCRII-TOPO; *‘xcpQ65*	This study
pCRII-‘QD65	pCRII-TOPO; *‘xcpQD65*	This study
pCRII-‘$Q_{\Delta C35}$	pCRII-TOPO; ‘$xcpQ_{\Delta C35}$	This study
pCRII-PulS	pCRII-TOPO; *pulS*	This study
pUC19	Ap^r; cloning vector	(58)
pUP2	PUC19; *xcpA*	(16)
pUAWE-5	PUC19; *xcpPQ*	(17)
pUC-R	PUC19; *xcpR*	This study
pUC-RZ	PUC19; *xcpR-Z*	Chapter 4
pUC-QZ	pUC-RZ; *xcpPQ* behind their own promoter	This study
pUC-QZII	pUC19; *xcpR-Z* and *xcpPQ* all in one operon	This study
pJF29	PUC19; *cbpD*	(24)
pUC-PQD_{65}	PUC19; $xcpPQD_{65}$	This study
pUC-$PQ_{\Delta C35}$	PUC19; $xcpPQ_{\Delta C35}$	This study
pUC-QD_{65}	PUC19; $xcpQD_{65}$	This study
pUC-$Q_{\Delta C35}$	PUC19; $xcpQ_{\Delta C35}$	This study
pUC-$QD_{65}Z$	pUC19; *xcpR-Z* and $xcpPQD_{65}$ in one operon	This study
pUC-$Q_{\Delta C35}Z$	pUC19; *xcpR-Z* and $xcpPQ_{\Delta C35}$ in one operon	This study
pYRC	pBBR1-mcs5; *lacI*; Gm^r; P_{lac}	Chapter 2
pYRC-QZII	pYRC; *xcpR-Z* and *xcpPQ* in one operon	This study
pMMB67HE, EH	Ap^r; cloning vector; P_{tac}	(26)
pB28	pMMB67HE; *xcpQ*	(6)
pMMB67-QD_{65}	pMMB67HE; $xcpQD_{65}$	This study
pMBK201	pMMB67HE; *lipA, lif*	This study
pULF201	PUR6500; *lipA, lif*	This study
pUR6500EH	pMMB67EH::Km^R, Ap^S; P_{tac}	(25)
pULB22	PUR6500; *lasB*	(8)
pMPM-K4Ω	Km^r; cloning vector; P_{araBAD}	(41)
pMPM-K4-22A	pMPM-K4; *lasB, xcpA*	This study
pMPM-K4-29A	pMPM-K4; *cbpD, xcpA*	This study
pMPM-K4-201A	pMPM-K4; *lipA, lif, xcpA*	This study
pCHAP580	pSU19; *pulS*	(15)
pBL7	pBluescript; *lipA, lif*	(20)
pCHAP710	pACYC184; all *pul* genes except *pulA* and *pulB*	(37)
pBBR1-MCS	Cam^R; cloning vector	(39)
pBBR1-PulS	pBBR1-MCS; *pulS*	This study

[*] Ap, ampicillin; Cam, chloramphenicol; Gm, gentamicin; Km, kanamycin

DNA manipulations. Plasmids used in this study are listed in Table 1. Recombinant DNA methods were performed essentially as described (50), using *E. coli* strain DH5α for routine cloning. Plasmids were introduced with the $CaCl_2$ procedure (50) into *E. coli* or by electroporation into *P. aeruginosa* and *P. putida* (21). PCRs were performed with the proofreading *Pwo* DNA polymerase (Roche) and PCR products were cloned into pCRII-TOPO according to the manufacturer's protocol. Oligonucleotides used in this study are listed in Table 2.

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5'→ 3')*	Restriction site
JAXcpP01for	CGGCCAGTCAATCGATTTGATAGAAGTAGG	ClaI
JAXcpP02rev	CGTACGAAAAGCTTGAAGGGGCAAACAGGG	HindIII
BK101	CCGCTCGAGATGACCAGGAGAACTGCATATGAAGAA	XhoI
BK104	CATGGATCCCGGTCAGCGCTGCTCGGCCTG	BamHI
D65for	TATCTGGCCAACGGTGATCCGCGACCGC	MscI
D65rev	ACGAGCTCAGCGGCGGGGGTCATAGATTG	SacI
Q65for	CGACCGAGCAACATCCTCATCC	
Q65rev	AGGCCTCAGGAAGACCATCAGGTTG	StuI
Qtrunc_rev	AGAGCTCTCTTAGACCCGGATGTCGCTG	SacI
pulSfor	GAGCTCAGATTTTCTGATGACTACGG	SacI
pulSrev	GAGCTCCGATTGAGGAGAGTCCGCAG	SacI

* Restriction sites are underlined

The *xcpR* gene on a HindIII-XbaI fragment of pCRII-R was ligated into HindIII-XbaI-digested pUC19, which resulted in pUC-R. The SacI-XbaI fragment of pUAWE-5 with the *xcpPQ* genes was inserted into SacI-XbaI-digested pUC-RZ behind the *xcpR-Z* operon, resulting in construct pUC-QZ. With the oligonucleotides JAXcpP01for and JAXcpP02rev *xcpP* was amplified from pAX24. The product was introduced into pCRII-TOPO, resulting in pCRII-P. The XbaI-SbfI product of pCRII-P was ligated into XbaI-SbfI-digested pUC-QZ, resulting in pUC-QZII, which carries the two *xcp* operons in tandem behind the *lac* promoter. The HindIII-XbaI fragment of pUC-QZII was ligated into the broad-host-range vector pYRC digested with the same enzymes, resulting in pYRC-QZII. Plasmid pMPM-K4-29A was constructed by ligation of *cbpD* on an SphI-EcoRI fragment of pJF29 and *xcpA* on an SphI-PstI fragment from pUP2 into EcoRI-PstI-digested pMPM-K4Ω. Plasmid pMPM-K4-22A was constructed by ligation of *lasB* on an SphI-EcoRI fragment of pULB22 and *xcpA* on a SphI-PstI fragment

from pUP2 into pMPM-K4Ω. A DNA fragment containing the *lipA* and *lif* genes was PCR amplified with the primers BK101 and BK104 and pBL7 as template. The product was cloned into pCRII-TOPO resulting in pCRII-201. The *lipA* and *lif* genes were excised from this construct by XhoI-BamHI digestion and the fragment was ligated into XhoI-BamHI-digested pMMB67HE resulting in construct pMBK201. Construct pULF201 contains *lipA* and *lif* on SacI-DrdI and KpnI-DrdI fragments of pMBK201 ligated into KpnI-SacI-restricted pUR6500HE. Construct pMPM-K4-201A was obtained by the insertion of *lipA* and *lif* as an XbaI-SacI fragment from pMBK201 and *xcpA* as a SacI-HindIII fragment from pUP2 into XbaI-HindIII-digested pMPM-K4Ω. For the construction of the $xcpQD_{65}$ hybrid, part of *pulD*, encoding the C-terminal 65 amino acid residues of the protein, was amplified from pCHAP710 with the oligonucleotides D65for and D65rev. The PCR product was inserted into pCRII-TOPO resulting in pCRII-'D65. Part of *xcpQ*, encoding amino acid residues 319-603 of the protein, was amplified from pAX24 with primers Q65for and Q65rev. The PCR product was ligated into pCRII-TOPO resulting in construct pCRII-'Q65. The MscI-SacI fragment of pCRII-'D65 was inserted into StuI-SacI-digested pCRII-'Q65, which resulted in pCRII-'QD65. This construct was digested and *xcp'QD65* on an EcoRV-SacI fragment was inserted into EcoRV-SacI-digested pUAWE-5, which resulted in pUC-PQD_{65}. To place $xcpQD_{65}$ under control of the *lac* promoter, *xcpPQ'* on a HindIII-EcoRV fragment from pUC-PQD_{65} was replaced by the HindIII-EcoRV fragment of pB28, which resulted in pUC-QD_{65}. The HindIII-SacI fragment of pUC-QD_{65} was introduced in HindIII-SacI-digested pMMB67HE resulting in pMMB67-QD_{65}. Digestion of pUC-QD_{65} with SbfI-SacI followed by ligation into SbfI-SacI-digested pUC-QZII resulted in pUC-QD_{65}Z. To construct the gene encoding $XcpQ_{\Delta C35}$, part of *xcpQ*, encoding amino acid residues 319-623 of the protein, was amplified from pAX24 with the primers Q65for and Qtrunc_rev. The PCR product was ligated into pCRII-TOPO resulting in pCRII-'$Q_{\Delta C35}$. The EcoRV-SacI fragment of pCRII-'$Q_{\Delta C35}$ was ligated into EcoRV-SacI-digested pUAWE-5 resulting in pUC-$PQ_{\Delta C35}$. To remove *xcpP* and upstream DNA, and to place $xcpQ_{\Delta C35}$ under control of the *lac* promoter, the HindIII-EcoRV fragment of pB28 was introduced into HindIII-EcoRV-digested pUC-$PQ_{\Delta C35}$, which resulted in pUC-$Q_{\Delta C35}$. The SbfI-SacI fragment of pUC-$PQ_{\Delta C35}$ was inserted into SbfI-SacI-digested pUC-QZII, resulting in pUC-$Q_{\Delta C35}$Z. With the primers pulSfor and pulSrev *pulS* was amplified from pCHAP710 and the product

was introduced into pCRII-TOPO, resulting in pCRII-PulS. The HindIII-XbaI fragment of pCRII-PulS was subsequently ligated into HindIII-XbaI-digested pBBR1MCS, resulting in pBBR-PulS.

Enzyme assay. Secretion of elastase was analyzed qualitatively on LB plates with a top layer containing 1% elastin (Sigma). After overnight growth, the plates were examined for the presence of clear haloes around the colonies.

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 using 5% trichloracetic acid (TCA; Sigma) and washed with acetone before resuspending them in SDS-PAGE sample buffer. Samples were boiled for 10 min unless otherwise indicated and proteins were separated on acrylamide gels. For immunodetection, proteins were transferred to nitrocellulose membranes by semi-dry electroblotting. Primary antisera used were anti-XcpQ (6) at 1:3000, anti-CbpD (24) at 1:10.000, anti-LipA (40) at 1:1000, anti-PspA (36) at 1:1000, anti-XcpS (2) at 1:1000, anti-XcpT (19) at 1:1000, anti-XcpY (43) at 1:5000, and anti-XcpZ (43) at 1:500 dilutions. Alkaline phosphatase-conjugated goat anti-rabbit IgG antiserum (Biosource international) was used as secondary antibody unless stated otherwise. Detection was performed by staining with 5-bromo-4-chloro-3-indolylphosphate (Sigma) and nitroblue tetrazolium (Sigma). Alternatively, where indicated, horse radish peroxidase-conjugated goat anti-rabbit IgG antiserum (Biosource international) was used as secondary antibody. Detection was performed by chemiluminescence (Pierce).

Cell fractionation. Inner and outer membranes of *E. coli* were separated as described (11). In the case of *P. aeruginosa* and *P. putida*, the membranes were separated as described by Hancock and Nikaido (32) with three modifications: (i) Dithiothreitol was added after disruption of the cells to a final concentration of 2 mM and was present throughout the remaining part of the procedure, (ii) isolation of the total membrane fractions on a 70% sucrose cushion was omitted, and (iii) sucrose gradient centrifugation was carried out in a Beckman SW28 rotor at 25,000 rpm for 16 h. NADH-oxidase activity was determined as described (45) and the presence of the outer membrane porins in the different fractions was evaluated by SDS-PAGE. Cell envelope preparations of *E. coli* and *P. aeruginosa* were

isolated from ultrasonically disrupted cells (6, 11) and analyzed on 8% acrylamide gels.

RESULTS

The *P. aeruginosa* Xcp system is not functional in *E. coli*. The T2SSs of *K. oxytoca* and *E. chrysanthemi* have been successfully reconstituted in *E. coli* (14, 34). We wanted to determine whether the twelve *xcp* genes of the more distantly related *P. aeruginosa* could assemble into a functional system in this host as well. The Xcp system of *P. aeruginosa* is encoded by two divergently transcribed operons, *xcpR-Z* and *xcpPQ*, which are under quorum-sensing control (12). The twelfth gene, encoding the prepilin peptidase XcpA, is located elsewhere on the chromosome in a cluster of genes involved in type IV pilus biogenesis. To obtain sufficient expression in *E. coli*, the two operons were cloned in tandem resulting in construct pYRC-QZII, which contains the *xcpRSTUVWXYZPQ* genes under the control of P_{lac}. When this construct was introduced into a *P. aeruginosa* strain lacking the *xcp* gene cluster, i.e. strain D40ZQ, secretion of the Xcp-dependent substrate elastase was restored as evidenced by halo formation on elastin-containing agar plates (results not shown). Production of the Xcp proteins in *E. coli* strain DH5α from pYRC-QZII was confirmed by immunoblot analysis with the available antisera (anti-XcpQ, S, T, Y and Z). When induced by the addition of IPTG to a final concentration of 0.1 mM, the production levels of these Xcp components were similar to those in wild-type *P. aeruginosa* (results not shown). To determine whether the Xcp proteins assembled into a functional system, a second construct containing *xcpA* as well as a gene for an Xcp-dependent substrate under control of the P*araBAD* was introduced into DH5α already containing pYRC-QZII. Three different substrates were tested, i.e. chitin-binding protein D (CbpD), elastase (LasB) and lipase (LipA). The substrates accumulated inside the cells and were not detected in the extracellular medium, as shown for CbpD in Fig. 1A. By using various concentrations of IPTG and arabinose, the stoichiometry of substrate and Xcp system was varied. However, Xcp-dependent secretion of the substrates was never observed (data not shown).

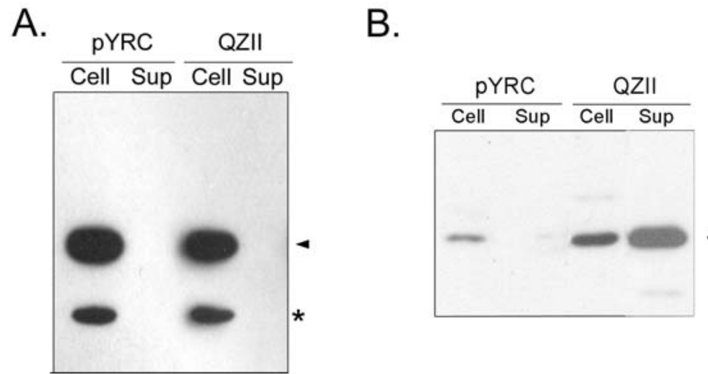

FIG. 1. Functionality of the Xcp system produced in *E. coli* DH5α and in *P. putida* WCS358. Extracellular proteins (sup) and whole cell lysates (cell) were analyzed by SDS-PAGE, followed by Western blotting using CbpD- or LipA-specific antibodies. Extracellular proteins were loaded in 5 times excess compared to cell lysates. Peroxidase-conjugated antiserum and chemiluminescence (Pierce) was used for immunodetection. (A) DH5α producing CbpD and XcpA from pMPM-K4-29A and containing either the pYRC-QZII (QZII) encoding the Xcp system or the empty vector (pYRC). Overnight grown cultures were diluted 1:10 into fresh medium and grown for 3 h. Subsequently, production of Xcp proteins was induced by the addition of 0.1 mM IPTG, and that of CbpD and XcpA by adding 1% L-arabinose. The position of CbpD is indicated with an arrowhead, and that of a degradation product with an asterisk. (B) *P. putida* WCS358 producing LipA and Lif from pULF201 and containing either the pYRC-QZII (QZII) or the empty vector (pYRC). Production of Xcp proteins and of lipase and foldase was induced by the addition of 1 mM IPTG. An arrowhead indicates the position of lipase.

We also tested the possibility to reconstitute the *P. aeruginosa* Xcp system in *P. putida*, which is more closely related to *P. aeruginosa* than is *E. coli*. Therefore, pYRC-QZII and a second plasmid, pULF201, encoding *P. aeruginosa* lipase (LipA) and its cognate foldase (Lif) were introduced into *P. putida* strain WCS358. It was not considered necessary to introduce the *P. aeruginosa xcpA* gene, since *P. putida* has been shown to produce a functional prepilin peptidase that is able to process *P. aeruginosa* XcpT (18). Immunoblot analysis showed that considerable amounts of lipase were secreted into the extracellular medium, whereas in the absence of the *P. aeruginosa* Xcp system, lipase was detected only inside the cells (Fig. 1B). The total amount of lipase was considerably lower in the strain lacking the *P. aeruginosa* Xcp system, indicating that intracellular lipase is prone to proteolytic degradation. These results show that the *P. aeruginosa* Xcp system can be functionally expressed in *P. putida*.

XcpQ oligomers localize in the inner membrane in *E. coli*. To identify the reason for the non-functionality of the Xcp system in *E. coli*, we first studied oligomerization and localization of the secretin XcpQ. XcpQ oligomers are stable in SDS-PAGE sample buffer and only dissociate upon boiling (6). Non-boiled cell lysates of *E. coli* producing XcpQ from pYRC-QZII were compared with those of wild-type *P. aeruginosa* overproducing the Xcp system from pAX24. As shown in Fig. 2A, XcpQ oligomers were detected in both samples. Thus, oligomerization of XcpQ appears unaffected in *E. coli*. Subsequently, the localization of the XcpQ oligomers produced from pYRC-QZII in *P. aeruginosa* D40ZQ *, P. putida* WCS358, and *E. coli* DH5α, was determined by separating inner and outer membranes with sucrose gradient centrifugation. Whereas in *P. aeruginosa* and *P. putida*, the XcpQ oligomers were mainly found in the fractions that contained markers for the outer membrane, the majority of the XcpQ oligomers co-localized with the inner membrane marker NADH-oxidase in *E. coli* (Fig. 2B). Hence, XcpQ is not properly targeted to the outer membrane in *E. coli*.

Mislocalization of secretins often results in the induction of the synthesis of phage shock response protein A (PspA) (1, 15, 33, 38). However, PspA production was found to be below detection levels in DH5α cells induced for the expression of *xcpQ* from pYRC-QZII by the presence of 0.1 mM IPTG (data not shown).

PulS-dependent outer membrane localization of an XcpQ-PulD hybrid in *E. coli*. Studies by Shevchik *et al.* (52) and by Burghout *et al.* (11) showed that C-terminally truncated derivatives of the secretins OutD and YscC were still functional. In contrast to the intact proteins, these truncates were targeted to the outer membrane in the absence of their dedicated pilotins. To investigate whether the removal of the C terminus of XcpQ may facilitate its outer membrane targeting in *E. coli*, a truncated XcpQ lacking the C-terminal 35 amino acid residues was produced in *E. coli* from pUC-$Q_{\Delta C35}$Z (Fig. 3A). The mutant protein formed oligomers, which, however, fractionated with markers for the inner membrane in sucrose gradients (results not shown).

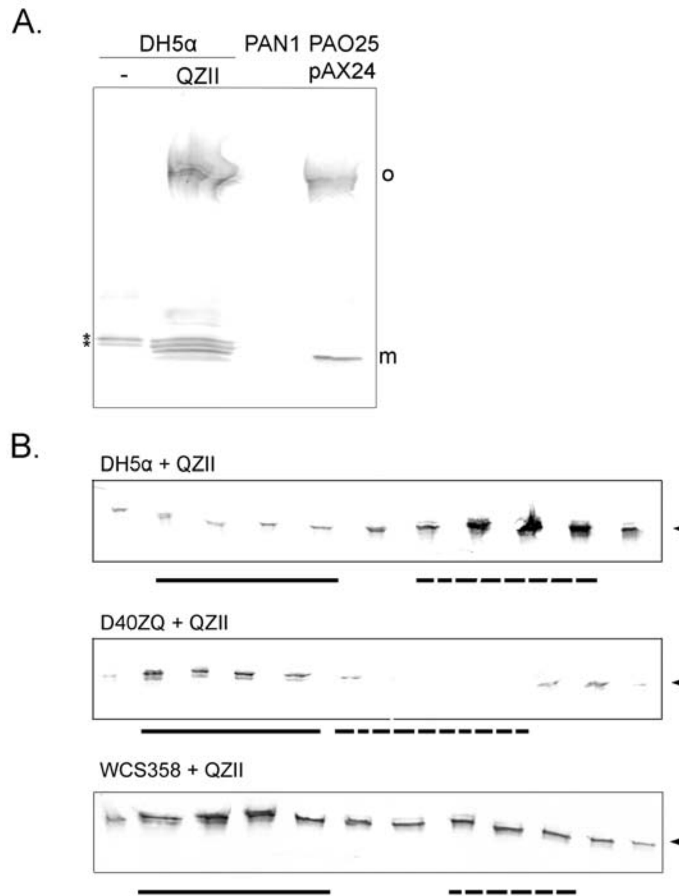

FIG. 2. Oligomerization and localization of XcpQ. (A) Non-boiled whole cell lysates of DH5α containing the empty vector pYRC (-), or producing XcpQ from pYRC-QZII (QZII), the *P. aeruginosa xcpQ* mutant (PAN1), and the wild-type *P. aeruginosa* strain PAO25 overproducing the Xcp system from pAX24 (pAX24) were separated by SDS-PAGE on a 3-9% acrylamide gradient gel and analyzed by Western blotting using XcpQ-specific antiserum. The position of oligomeric XcpQ is indicated with 'o', of the monomeric form with 'm', and of cross-reacting bands with asterisks. (B) Cell envelope fractions of French press lysates of DH5α, *P. aeruginosa* lacking the *xcpP-Z* gene cluster (D40ZQ), and *P. putida* WCS358, all producing XcpQ from pYRC-QZII were applied onto 30-55% sucrose gradients, which were centrifuged and fractionated as described in experimental procedures. Samples from all fractions were analyzed on 3-9% acrylamide gradient gels and Western blotting with anti-XcpQ antiserum. The samples representing the inner (dashed lines) and outer membranes (solid lines) were identified based on NADH-oxidase activity and the presence of porins, respectively. Arrowheads indicate the position of oligomeric XcpQ.

Subsequently, another strategy was employed to target XcpQ to the *E. coli* outer membrane, employing a well-known pilotin. PulD is the XcpQ homologue of the T2SS of *K. oxytoca* and PulS is the cognate pilotin, which stabilizes PulD and promotes its outer membrane localization (33). Daefler *et al.* (15) showed that fusion of the C-terminal 65 amino acid residues of PulD to the secretin pIV of the filamentous phage f1 rendered the fusion protein $pIVD_{65}$ dependent on PulS for stability and for proper localization. Analogously, a gene fusion was constructed encoding a chimeric protein in which the C-terminal 55 amino acid residues of XcpQ are replaced by the corresponding C-terminal 65 amino acid residues of PulD. Upon production of the hybrid protein $XcpQD_{65}$ from pUC-QD_{65}Z in *E. coli*, a product was detected by immunoblotting that migrated faster in the gel than wild-type XcpQ (Fig. 3A), suggesting that the C-terminal PulD domain was proteolytically removed. Co-production of PulS from pCHAP580 resulted in the detection of $XcpQD_{65}$ migrating at a position similar to that of wild-type XcpQ (Fig. 3A). Thus, PulS appears to protect the C-terminal PulD domain of $XcpQD_{65}$ against proteolytic degradation. Interestingly, co-production of $XcpQD_{65}$ and PulS also resulted in pronouncedly increased levels of monomeric $XcpQD_{65}$ in non-boiled samples, without concomitant increase in the amount of oligomers (Fig. 3B). Localization studies showed that the $XcpQD_{65}$ oligomers were predominantly present in the inner membrane fractions in the absence of PulS (Fig. 3C). When $XcpQD_{65}$ and PulS were produced simultaneously, the $XcpQD_{65}$ oligomers (Fig. 3C) and PulS (results not shown) co-localized predominantly with the outer membrane markers. Thus, $XcpQD_{65}$ can be targeted to the outer membrane of *E. coli* in a PulS-dependent manner.

Next, we tested whether the Xcp system produced from pYRC-QD_{65}Z was functional when PulS was co-produced. Therefore, pMPM-K4-201A, which encodes LipA, Lif, and XcpA, was introduced as a third construct into DH5α already containing pYRC-QD_{65}Z and pCHAP580, and extracellular lipase activity was determined under several induction conditions. However, no lipase activity was detected in the culture supernatants (data not shown). Apparently, mistargeting of XcpQ is not the only obstacle in the functioning of the Xcp apparatus in *E. coli*.

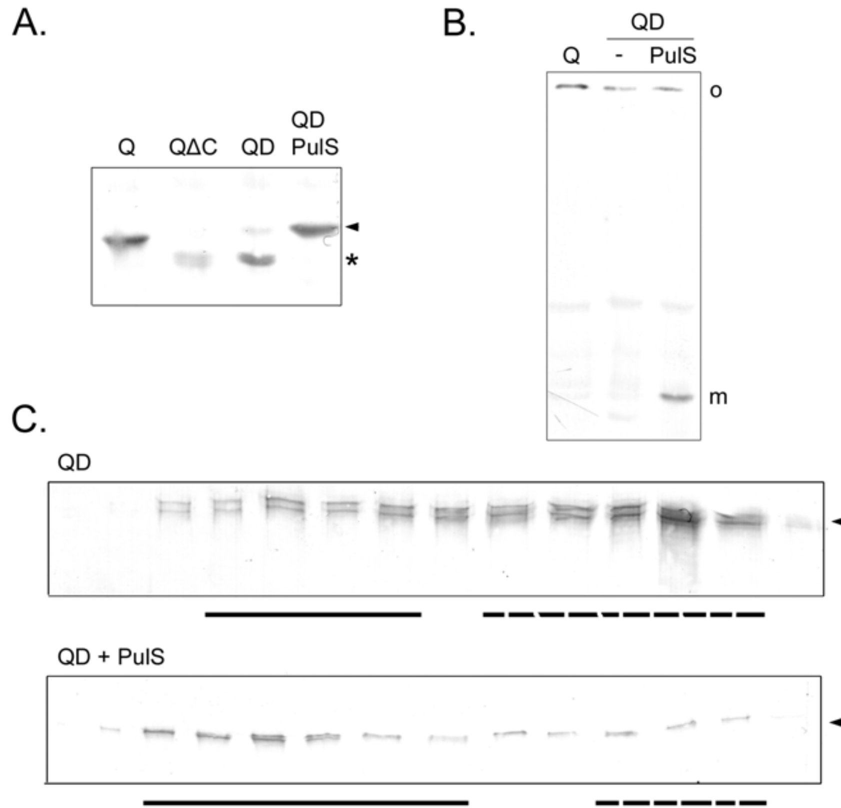

FIG. 3. Stability and localization of different XcpQ variants in *E. coli*. (A) Cell envelopes of DH5α containing pUC-QZII carrying wild-type *xcpQ* (Q), pUC-$Q_{\Delta C35}$Z carrying *xcpQ*$_{\Delta C35}$ (QΔC), pUC-QD_{65}Z carrying *xcpQD*$_{65}$ (QD), or pUC-QD_{65}Z together with pCHAP580 carrying *pulS* (QD PulS) were boiled for 10 min in sample buffer and analyzed on an 8% acrylamide gel, followed by Western blotting and immunodetection with XcpQ-specific antiserum. The position of monomeric XcpQ is indicated with an arrowhead, and of a degradation product with an asterisk. (B) Non-boiled cell envelopes of DH5α containing pUC-QZII (Q), pUC-QD_{65}Z (QD) with or without pCHAP580 (PulS) were separated on an 8% acrylamide gel and analyzed by Western blotting and immunodetection with XcpQ-specific antiserum The position of oligomeric XcpQ is indicated with 'o', and of the monomeric form with 'm'. (C) Cell envelope fractions of French press lysates of DH5α containing pUC-QD_{65}Z (QD) with or without pCHAP580 (PulS) were applied onto 30-55% sucrose gradients, which were centrifuged and fractionated. Samples from all fractions were analyzed on 3-9% acrylamide gradient gels, followed by Western blotting using XcpQ-specific antibodies. The samples representing the inner (dashed lines) and outer membranes (solid lines) were identified based on NADH-oxidase activity and the presence of porins, respectively. An arrowhead indicates the position of oligomeric XcpQ.

Functionality of XcpQD$_{65}$ in *P. aeruginosa*. The functionality and PulS dependency of XcpQD$_{65}$ were also analysed in the *P. aeruginosa xcpQ* mutant strain PAN1, into which pMMB67-QD$_{65}$ was introduced for this purpose. The C terminus of PulD appeared to render the fusion protein prone to degradation. In contrast to *E. coli*, where the XcpQ moiety of the fusion protein was protected (Fig. 3A), the complete protein appeared to be degraded in *P. aeruginosa*, since no degradation products were detected (Fig. 4A). When PulS was co-produced from pBBR1-pulS, the amount of XcpQD$_{65}$ detected was similar to wild-type XcpQ levels (Fig. 4A). To study functionality, PAN1 producing XcpQD$_{65}$ in the presence or absence of PulS was grown on LB agar plates containing elastin and halo formation was evaluated (Fig. 4B). XcpQD$_{65}$ complemented the secretion defect of the *xcpQ* mutant when PulS was co-produced, but was not functional in the absence of PulS. In conclusion, replacement of the C-terminal part of XcpQ by that of PulD resulted in a protein that requires PulS for stability and functionality. Whether the chimeric secretin also depended on PulS for outer membrane targeting in *P. aeruginosa* could not be determined, because of the instability of XcpQD$_{65}$ in the absence of PulS.

DISCUSSION

By placing the *P. aeruginosa xcp* genes under control of the *lac* promoter, the components of the Xcp system could be produced in *E. coli*. However, the proteins did not assemble into a functional secretion system. In contrast, the *P. aeruginosa* Xcp system could be reconstituted in *P. putida*. Thus, the assembly of the Xcp secreton from pYRC-QZII appeared host-specific. The two heterologous hosts differed in their ability to target the secretin XcpQ to the outer membrane. While XcpQ was correctly localized in the outer membrane in *P. putida*, it was associated with the inner membrane in *E. coli*. In *E. coli*, the XcpQ oligomers appeared to be present in the membranes and not in aggregates, since they remained associated with the inner membrane fractions in flotation experiments and were not solubilized by 8 M urea (data not shown). Remarkable in this respect is the observation that, in our hands, incorrectly localized XcpQ oligomers did not induce PspA production.

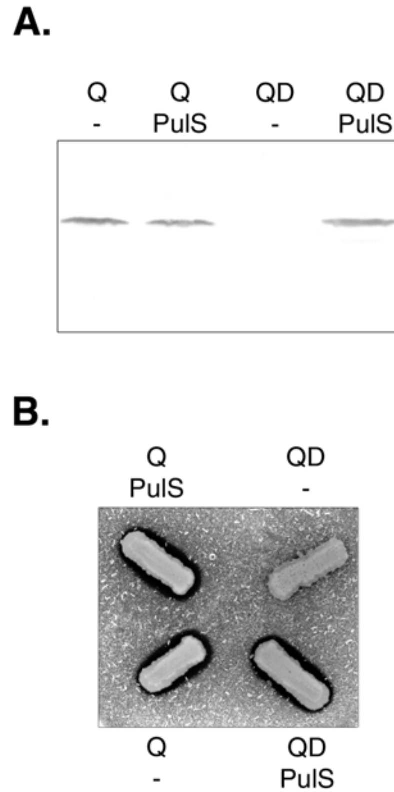

FIG. 4. Production and functionality of XcpQD$_{65}$ in *P. aeruginosa.* (A) Cell envelopes of the *P. aeruginosa xcpQ* mutant PAN1 producing wild-type XcpQ from pB28 (Q) or XcpQD$_{65}$ from pMMB67-QD$_{65}$ (QD), and co-producing PulS from pBBR1-pulS (PulS) or containing the empty vector pBBR1-MCS (-) were analyzed by SDS-PAGE, followed by Western blotting using XcpQ-specific antibodies. (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.

Induction of PspA synthesis is a common response to conditions that affect the integrity of the inner membrane and has been reported to occur in response to mistargeting of various secretins (1, 15, 33, 38). Akrim *et al.* (1) have shown that production of *P. aeruginosa* XcpPQ from the T7 promoter did induce PspA synthesis. Whether this discrepancy relates to differences in the production levels or to the co-production of the other Xcp components is currently under investigation.

In an attempt to achieve proper targeting of XcpQ in *E. coli*, we deleted the C-terminal 35 amino acid residues of XcpQ, or, in another approach, we replaced a C-terminal segment by the corresponding part of the secretin PulD. $XcpQ_{\Delta C35}$ still accumulated in the inner membrane, but $XcpQD_{65}$ was targeted to the outer membrane in a PulS-dependent manner. Remarkably, co-production of PulS with $XcpQD_{65}$ also resulted in a significantly increased monomer:oligomer ratio compared to that found for wild-type XcpQ. This result indicates that either PulS or the C-terminal segment of PulD functions by delaying oligomerization, thereby possibly facilitating the transport of $XcpQD_{65}$ in a monomeric state across the peptidoglycan layer.

Although $XcpQD_{65}$ localized to the outer membrane when PulS was co-produced, the Xcp proteins still failed to assemble into a functional T2SS in *E. coli*. The non-functionality might result from abnormal stoichiometry of the components. Normally, *xcpR-Z* and *xcpPQ* are expressed from different promoters (12), which regulate both onset and production levels. However, the Xcp system produced from pYRC-QZII was functional in *P. aeruginosa* and in *P. putida*. Possibly, the problem relates to the production level of the prepilin peptidase XcpA relative to the other components. Although the vast majority of XcpT was found in the mature form in overnight grown *E. coli* cells (data not shown), we cannot exclude the possibility that processing was not sufficiently efficient to generate a functional Xcp system. Of note, we observed that small amounts of processing-defective pseudopilin subunits can strongly interfere with secretion in an otherwise wild-type *P. aeruginosa* strain (Chapter 3). Alternatively, a host-specific factor may be required for proper functioning of the machinery. For example, the structure of the LPS in the outer membrane has been shown to influence the functionality of the Xcp machinery (42).

The *xcpQD₆₅* hybrid gene was able to complement an *xcpQ* mutation in *P. aeruginosa*, showing that the chimeric protein is functional. The functionality and stability of this protein were, however, strictly dependent on PulS. In the absence of the pilotin, the $XcpQD_{65}$ secretin was completely degraded. In stark contrast, the major portion of the protein was stable in *E. coli*, where only the PulD moiety appeared to be removed. Similarly, the C-terminally truncated XcpQ was detectable in *E. coli* but not in *Pseudomonas*. Possibly, oligomerization occurs fast in *E. coli*, thereby protecting XcpQ against proteolytic degradation and, at the same time,

resulting in aberrant targeting of the XcpQ oligomers, e.g. by preventing passage of the peptidoglycan layer. In *P. aeruginosa*, oligomerization may be delayed until after the passage of the peptidoglycan, thereby rendering the XcpQD_{65} protein more susceptible to degradation. In agreement with this assumption substantial amounts of XcpQ are present in the monomeric form in *Pseudomonas* (6, 10). Maintaining the monomeric state may be accomplished by the activity of a chaperone, which, in that case, would still bind to the mutant XcpQ lacking the C-terminal segment, as has also been reported for the binding of pilotin YscW to secretin YscC (11).

Does the secretin XcpQ require a so-far unidentified pilotin for targeting to the outer membrane? The fact that the protein is not correctly localized to the outer membrane in *E. coli* points in that direction, especially since targeting could be achieved artificially by co-expression of PulS with the XcpQD_{65} hybrid. Interestingly, deletion of the C terminus renders XcpQ highly unstable in *Pseudomonas*, but not in *E. coli*. The latter finding may indicate that XcpQ contains multiple interaction sites, e.g. an interaction site to maintain a monomeric state, which does not require the C terminus, and a C-terminal interaction site that stabilizes the protein in *Pseudomonas*. Interestingly, Hamood *et al.* (30) reported the isolation of a *P. aeruginosa* TT2S-defective mutant, which could neither be complemented by *xcpP-Z* nor by *xcpA*, hinting at the existence of additional gene(s) required for secretion. The putative pilotin should also be present in *P. putida*, since XcpQ was localized properly in this host. *P. putida* does possess a T2SS, but this system is not very related to that of *P. aeruginosa*. The homology between *P. putida* XcpQ and *P. aeruginosa* XcpQ is not high (37% identity) and their C termini are very different (18). Thus, in contrast to the known pilotins, *Pseudomonas* may use a more general system for targeting secretins to the outer membrane.

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