

## Chapter 4

### **Interaction domains in the *Pseudomonas aeruginosa* type II secretory apparatus component XcpS**

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## ABSTRACT

*Pseudomonas aeruginosa* is an opportunistic pathogen, which secretes a wide variety of enzymes and toxins into the extracellular medium. Most exoproteins are exported by the type II secretion machinery, the Xcp system, which encompasses 12 different proteins. One of the core components of the Xcp system is the inner membrane protein XcpS, homologues of which can be identified in type II secretion machineries as well as in type IV piliation systems. In this study, XcpS was shown to be stabilized by co-expression of the XcpR and XcpY components of the machinery, demonstrating an interaction between these three proteins. By replacing segments of *P. aeruginosa* XcpS with the corresponding parts of its *Pseudomonas putida* counterpart, XcpS domains were identified that are important for species-specific functioning and thus represent putative interaction domains. The cytoplasmic loop of XcpS was found to be involved in the stabilization by XcpR and XcpY.

## INTRODUCTION

The ability to secrete proteins into the extracellular medium is important for the virulence of many plant, animal or human pathogens. In recent years, it has become clear that Gram-negative bacteria use a limited number of secretion mechanisms to secrete a large variety of extracellular proteins (45). One of these mechanisms is the type II secretion system (T2SS), which is widely distributed among Gram-negative bacteria, including pathogens such as *Vibrio cholerae*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Xanthomonas campestris*, *Erwinia chrysanthemi* and *Klebsiella oxytoca* (10). This system allows for the secretion of a range of degradative enzymes, including cellulases, pectinases, proteases, lipases, and toxins, such as aerolysin and cholera toxin.

Type II secretion is a two-step process. First, signal sequence-bearing exoproteins are translocated across the cytoplasmic membrane via the Sec or the Tat machinery (28, 48). After release in the periplasm, unfolded exoproteins adopt their tertiary conformation. Transport of the folded proteins across the outer membrane is the second step and takes place via the so-called secreton. The secreton is assembled from 12-16 different components, which are generically referred to as Gsp (general secretory pathway) proteins. In *P. aeruginosa*, type II secretion requires the products of 12 xcp genes, *xcpA* and *xcpP-Z* (22).

Homologues of several Xcp components are not only present in T2SSs, but also in type IV pilus biogenesis systems (29), in competence systems of Gram-positive bacteria (9), and in flagella and sugar-binding systems of various archaea (4, 36), suggesting a common evolutionary origin of these systems. The XcpTUVWX proteins show N-terminal sequence similarity to the type IV pilus subunit PilA and, therefore, they are designated pseudopilins (7, 44). Consistently, they have been demonstrated to be processed by the dedicated prepilin peptidase PilD/XcpA, which also processes the PilA precursor (35), and XcpT has been shown to assemble into a pilus-like structure upon over-production (16). The ATPase XcpR and the multispinning inner membrane component XcpS show considerable sequence similarity to PilB and PilC, respectively, which are both required for the formation of type IV pili (36). This similarity suggests that XcpR and XcpS may play key roles in the assembly of the pilus-like structure formed by the pseudopilins.

The cytoplasmic protein XcpR was shown to associate to the inner membrane via the N-terminal domain of the bitopic inner membrane component XcpY (2). XcpR contains a conserved Walker A-box motif that was shown to be indispensable for its function (47). Binding of ATP was recently shown to trigger oligomerization of the *X. campestris* XcpR homologue XpsE (43) probably into hexamers (11, 42). Knowledge of the role of XcpS in the secreton and its interactions with other Xcp components is rather limited. Recently, the components XcpRSY were shown to co-purify with his-tagged XcpZ after cross-linking (40), and yeast two-hybrid studies with *E. chrysanthemi* T2SS components revealed interactions of the N terminus of OutF, the XcpS homologue, with OutE, the XcpR homologue, and with the cytoplasmic segment of OutL, the XcpY homologue (39).

Here, we show that XcpS is highly unstable in the absence of other Xcp components, a characteristic that was used to establish interactions between this central component of the secreton and other Xcp proteins. In addition, hybrid proteins composed of *P. aeruginosa* XcpS and *Pseudomonas putida* XcpS were used to identify possible interaction domains.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Strains used in this study are listed in Table 1. *P. aeruginosa* and *Escherichia coli* strains were grown at 37°C in a modified Luria-Bertani (LB) broth (46). 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, carbenicillin 300 µg/ml. To induce the expression of genes cloned behind the *lac* or *tac* promoter, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM.

**Plasmids and DNA manipulations.** Plasmids used in this study are listed in Table 2. Recombinant DNA methods were performed essentially as described (41) using *E. coli* strain DH5α for routine cloning. Plasmids were introduced by the CaCl<sub>2</sub> procedure into *E. coli* (41) or by electroporation into *E. coli* and *P. aeruginosa* (18). PCRs were performed with the proof-reading enzyme *Pwo* DNA polymerase (Roche) and PCR products were cloned into pCRII-TOPO or pCR2.1-TOPO according to the manufacturer's

TABLE 1. Strains used in this study

Strain	Relevant characteristics	Source or reference
<b><i>E. coli</i></b>		
DH5 $\alpha$	<i>thi-1 hsdR17 gyrA96 recA1 endA1 glnV44 relA1 phoA8 phi80dlacZdelM15 <math>\lambda</math></i>	(27)
BL21(DE3)	<i>F' ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup>m<sub>B</sub><sup>+</sup>) dcm gal</i>	Novagen
<b><i>P. aeruginosa</i></b>		
PAO25	PAO1 <i>leu arg</i>	(26)
PAO1 $\Delta$ P	PAO1 $\Delta$ <i>xcpP</i>	(6)
PAN1	PAO25 $\Delta$ <i>xcpQ</i>	(5)
PAN11	PAO25 $\Delta$ <i>xcpR</i> $\Delta$ <i>lasB</i>	(8)
PAO1 $\Delta$ S	PAO1 $\Delta$ <i>xcpS</i>	This study
PAO1 $\Delta$ T	PAO1 $\Delta$ <i>xcpT</i>	(3)
PAO $\Delta$ U	PAO1 $\Delta$ <i>xcpU</i>	This study
PAO $\Delta$ V	PAO1 $\Delta$ <i>xcpV</i>	This study
PAO $\Delta$ W	PAO1 $\Delta$ <i>xcpW</i>	This study
PAO1 $\Delta$ X	PAO1 $\Delta$ <i>xcpX</i>	(7)
KS910	<i>xcpY51</i> mutant from PAO503	(49)
KS902	<i>xcpZ5</i> mutant from PAO503	(49)
DZQ40	PAO1 $\Delta$ <i>xcpP-Z</i>	(2)

protocol. The oligonucleotides used are associated as an online supplement. With pAX24 as template, the oligonucleotide Osup, which primes upstream of the *xcpS* gene sequence, and either one of the oligonucleotides Osci, Ospi, Oscii, and Ospii, which prime at different positions within the *xcpS* gene sequence, were used to PCR amplify 3'-truncated *xcpS* genes. These oligonucleotides were designed in such a way that a SmaI site was introduced at the 3' end of the PCR product. Each DNA fragment was subsequently cloned into pCR2.1-TOPO yielding the pOSci, pOSpi, pOScii and pOSpii plasmids, respectively. These plasmids were linearized by SmaI-XbaI digestion. The 2.6-kb *phoA* gene cassette obtained by SmaI-XbaI digestion from pPHO7 was cloned into the linearized plasmids yielding the pOSciIPA, pOSpiIPA, pOSciiIPA and pOSpiiIPA plasmids, which encode 'PhoA fused at positions E114, G216, K310, and V404, respectively, of the XcpS protein. The *lacI* gene was PCR amplified with plasmid pET16b as a template using the primers PB7 and PB8, thereby introducing an NcoI restriction site downstream of the stop codon. The PCR product was cloned into the HincII site of pBC18R, which resulted in construct pCR-LacI and subsequently the SphI-NcoI fragment was introduced into the pBBR1-MCS5 vector, resulting in pYRC. With the oligonucleotides JAXcpS01for and JAXcpS02rev *xcpS* was amplified from

TABLE 2. Plasmids used in this study

Plasmid	Relevant characteristic*	Source or reference
pCR2.1-TOPO	Ap <sup>r</sup> ; Km <sup>r</sup> ; TOPO TA cloning vector	Invitrogen
pPHO7	Ap <sup>r</sup> ; <i>phoA</i> without ss-encoding part	(25)
pOScIPA	pCR2.1; <i>xcpSE114-‘phoA</i>	This study
pOSpIPA	pCR2.1; <i>xcpSG216-‘phoA</i>	This study
pOScIIPA	pCR2.1; <i>xcpSK310-‘phoA</i>	This study
pOSpIIPA	pCR2.1; <i>xcpSV404-‘phoA</i>	This study
pMMB67HE, EH	Ap <sup>r</sup> ; cloning vector; P <sub>tac</sub>	(23)
pMMB67HE-S	pMMB67HE; <i>xcpS</i>	This study
pCRII-TOPO	Ap <sup>r</sup> ; Km <sup>r</sup> ; TOPO TA cloning vector	Invitrogen
pCRII-S, -RS, -RZ	pCRII-TOPO; <i>xcpS</i> , <i>xcpRS</i> , or <i>xcpR-Z</i>	This study
pUC19	Ap <sup>r</sup> ; cloning vector	(50)
pUAWE6	pUC19; <i>xcpRSTUVW</i>	(12)
pAX24	<i>xcpP-Z</i> cluster in pLAFR3	(21)
pMPM-T4Ω	Tet <sup>r</sup> ; cloning vector; P <sub>araBAD</sub>	(32)
pMPM-T4S <sub>xx</sub>	pMPM-T4Ω; <i>xcpS</i>	This study
pMPM-K4Ω	Km <sup>r</sup> ; cloning vector; P <sub>araBAD</sub>	(32)
pMPM-K4S1	pMPM-K4Ω; <i>xcpS</i> ; <i>NcoI</i> site removed	This study
pBBR1-MCS5	Gm <sup>r</sup> ; cloning vector	(31)
pBC18R	Ap <sup>r</sup> ; <i>lacI</i>	Novagen
pCR-lacI	pBC18R; <i>lacI</i>	This study
pYRC	pBBR1-MCS5; <i>lacI</i>	This study
pYRC-R, -YZ, -RY, -RYZ	pYRC; <i>xcpR</i> , <i>xcpYZ</i> , <i>xcpRY</i> , or <i>xcpRYZ</i>	This study
pBluescript II SK(-)	Ap <sup>r</sup> ; cloning vector	Stratagene
pMEK45	pBluescript SK(-); truncated <i>xcpSI165</i>	This study
pET16b	Ap <sup>r</sup> ; expression vector; T7 promoter	Novagen
pMEK49	pET16b; encoding His <sub>6</sub> -XcpSI165	This study
pEMBL18, 19	Ap <sup>r</sup> ; cloning vector	(15)
pAG102	pMMB67HE; <i>P. putida xcpRS</i>	(14)
pAG55	pEMBL19; <i>P. putida xcpS</i>	This study
pAG403	pEMBL18; <i>P. aeruginosa xcpS</i>	This study
pESH5, -6	pEMBL19; <i>xcpS</i> hybrid gene 5, or 6	This study
pESH7, -104, -106, -108, -109, -110	pEMBL18; <i>xcpS</i> hybrid gene 7, 104, 106, 108, 109, or 110	This study
pMSP31	pMMB67HE; <i>P. putida xcpS</i>	This study
pMSA21	pMMB67HE; <i>P. aeruginosa xcpS</i>	This study

pMSH-6, -104, -106, -108, -109, -110	pMMB67EH; <i>xcpS</i> hybrid gene 6, 104, 106, 108, 109, or 110	This study
pMPM-6, -104, -106, -108, -109, -110	pMPM-K4Ω; <i>xcpS</i> hybrid gene 6, 104, 106, 108, 109, or 110	This study
pKNG101	Sm <sup>r</sup> ; <i>oriR6K sacBR mobRK2</i>	(30)
pKN-ΔU	pKNG101; <i>xcpT'V'</i>	This study
pKN-ΔV	pKNG101; <i>xcpU'W'</i>	This study
pKN-ΔW	pKNG101; <i>xcpV'X'</i>	This study
pRK2013	Km <sup>r</sup> ; <i>ori ColE1, Tra<sup>+</sup> Mob<sup>+</sup></i>	(19)

\*Ap, ampicillin; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Tet, tetracycline; ss, signal sequence. The *xcpS* hybrid 5 contains the first 626 bp of *P. aeruginosa xcpS*, and the last 585 bp of *P. putida xcpS*; hybrid 7 contains the first 626 bp and the last 132 bp of *P. aeruginosa xcpS* and 485 bp of *P. putida xcpS*. The proteins encoded by the other *xcpS* hybrids are schematically depicted in Fig. 3.

pAX24 as template and cloned into pCRII-TOPO resulting in construct pCRII-S. The XbaI-EcoRI insert was subsequently ligated into XbaI-EcoRI-digested pMMB67HE resulting in pMMB67HE-S. With the oligonucleotides JAXcpR01for and JAXcpR02rev *xcpR* was amplified from pAX24 as template and cloned into pCRII-TOPO resulting in construct pCRII-R. The HindIII-XbaI, and the XbaI-EcoRI inserts from pCRII-S and pCRII-R were ligated together in HindIII-EcoRI-digested pRCII-TOPO yielding pCRII-RS. With oligonucleotides JAXcpY02for and JAXcpZ02rev a DNA fragment containing the genes *xcpYZ* was amplified from pAX24 as template and the product was cloned into pCRII-TOPO resulting in pCRII-YZ. The EcoRI-XbaI product of pCRII-YZ was ligated into EcoRI-XbaI-digested pUC19, resulting in pUC-YZ. The HindIII-EcoRI product of pCRII-RS and the EcoRI-XbaI product of pUC-YZ were ligated into HindIII-XbaI-digested pUC19, which resulted in pUC-RSYZ. The HindIII-EcoRI product of pCRII-RS was inserted in HindIII-EcoRI-digested pUC19, resulting in pUC-RS. Ligation of the 3.5-kb BamHI-EcoRI and the 2.1-kb EcoRI-NotI product of pAX24 into BamHI-EcoRI-digested pUC-RSYZ resulted in pUC-RZ. The HindIII-XbaI insert of pUC-RZ was introduced in HindIII-XbaI-digested pCRII-TOPO, resulting in pCRII-RZ. The *xcpS* gene was transferred as an XbaI-XhoI fragment from pCRII-S to XbaI-XhoI-digested pMPM-T4Ω, which resulted in pMPM-T4S<sub>xx</sub>. To remove the additional start codon generated by the NcoI site, this construct was digested with NcoI and incubated with T4 polymerase. Removal of the NcoI-site was confirmed by restriction analysis. The BamHI-PstI fragment of this construct was introduced into BamHI-PstI-digested pMPM-K4Ω resulting in



pMPM-K4S1. Construct pYRC-R contains *xcpR* from pCRII-R inserted as a HindIII-XbaI fragment into pYRC. Construct pYRC-YZ contains *xcpYZ* from pCRII-YZ inserted as an EcoRI-XbaI fragment into pYRC. Introduction of *xcpR* as a HindIII-EcoRI fragment from pCRII-R into HindIII-EcoRI-digested pYRC-YZ resulted in pYRC-RYZ. The genes *xcpRY* were subsequently introduced into pYRC as a HindIII-PstI fragment from pYRC-RYZ, resulting in pYRC-RY. Constructs pMSA21 and pAG403 contain *P. aeruginosa xcpS* as a 2.0-kb Sall-XhoI fragment from pAX24 inserted in pMMB67HE and pEMBL18, respectively. With the oligonucleotides MK01 and MK02, the first 495 bp of *xcpS* was amplified from pAG403 and a stop codon was generated. The resulting PCR fragment was introduced into SmaI-digested pBluescript SK(-) yielding pMEK45. Construct pMEK45 was digested with BamHI and the fragment with truncated *xcpS* was introduced into BamHI-digested pET16b, resulting in pMEK49. Constructs pMSP31 and pAG55 contain *P. putida xcpS* as a 2.3-kb SphI fragment from pAG102 inserted in pMMB67EH or pEMBL19, respectively. The hybrid on pESH5 was constructed in two steps. First, a PCR fragment was obtained containing the last 585 bp of *P. putida xcpS* with the use of oligonucleotides PPCS and the pUC reverse primer with pAG55 as template, and the HincII-HindIII-digested PCR product was cloned in HincII-HindIII-digested pEMBL19. The resulting construct was linearized with HincII, and a 1.2-kb HincII fragment from pAG403, containing the first 626 bp of *P. aeruginosa xcpS* was inserted in the correct orientation. The hybrid on pESH6 was obtained by replacing the 1.1-kb AsuII fragment of pESH5 by the corresponding fragment of pAG403. Similarly, pESH7 was obtained by replacing the 1.1-kb AsuII fragment of pAG403 by the corresponding fragment of pESH5. A HindIII-BamHI-digested PCR fragment obtained with template pAG403 and oligonucleotides AGA1 and AGA3, and a BamHI-EcoRI-digested PCR fragment, obtained using oligonucleotides AGP1 and AGA2 with pESH7 as template, were cloned together in pEMBL18 resulting in pESH110. To construct pESH106, PCR was performed using oligonucleotides PPNS2 and AGP8 with pAG55 as template to amplify the first 393 bp of *P. putida xcpS*. This product was used in a second PCR containing further *P. aeruginosa xcpS* on pAG403 as a template and oligonucleotides AGA9 and PPNS2. Finally, the product of the second PCR was digested with HindIII and SphI and cloned in HindIII-SphI-digested pAG403. The hybrid genes on pESH108, pESH104, and pESH109 were constructed using a 3-step PCR

protocol as described before (24). Plasmids pAG55 and pAG403 were used as templates for *P. putida* and *P. aeruginosa* DNA, respectively. Final products were obtained using oligonucleotides AGA7 and AGA2, digested with BamHI and EcoRI and cloned into pEMBL18. Specific oligonucleotides were as follows: AGP2, AGP7, AGP2B and AGP7B for pESH108; AGP6, AGP3, AGP6B and AGP3B for pESH104; AGP4, AGP5, AGP4B and AGP5B for pESH109. All fusions were verified by nucleotide sequencing. In the pESH-series of plasmids, the *xcpS* gene fusions are cloned on pEMBL18 or -19 in the orientation opposite to the *lac* promoter. The fusions were recloned in the proper orientation behind the *tac* promoter into pMMB67EH resulting in pMSH-6, or into pMMB67HE resulting in pMSH-104, -106, -108, -109, and -110. The hybrid gene 110 was PCR amplified with the oligonucleotides JAXcpS01for and JAXcpS02rev, the genes 104, 108 and 109 with the oligonucleotides JAXcpS03for and JAXcpS02rev with the pMSH plasmids as template DNA. The resulting products were ligated into pCRII-TOPO. The inserts were subsequently recloned as HindIII-EcoRI fragments into HindIII-EcoRI-digested pMPM-K4S1. The hybrid gene 6 was cloned as a HindIII-EcoRI fragment from pMSH-6 into pUC19. Hereafter, hybrid 6 was introduced as PvuII-HindIII fragment in PvuII-HindIII-digested pMPM-K4S1. The resulting constructs were named pMPM-6, -104, -106, -108, -109, and -110.

**Construction of chromosomal *P. aeruginosa xcp* mutants.** The mutants PAO $\Delta$ U ( $\Delta xcpU$ ), PAO $\Delta$ V ( $\Delta xcpV$ ) and PAO $\Delta$ W ( $\Delta xcpW$ ) were constructed using the approach previously described (16, 17). Briefly, 500-bp DNA fragments upstream and downstream the target genes were PCR amplified. The oligonucleotides were designed for amplifying fragments with overlapping 3' and 5' ends. For the *xcpU* deletion, the upstream fragment was obtained with the oligonucleotides XcpT'5 and XcpT'6, and the downstream region with XcpV'3 and XcpV'4. For the *xcpV* deletion, the upstream fragment was obtained with the couple XcpU'6 and XcpU'7, and the downstream region with XcpW'3 and XcpW'4. For the *xcpW* deletion, the upstream fragment was obtained with the couple XcpV'5 and XcpV'6, and the downstream region with XcpX'3 and XcpX'4. In each case, the two fragments obtained were fused by performing an overlap PCR: the two fragments were mixed, melted and annealed with the most upstream and downstream primers to perform a second PCR. The resulting PCR product was cloned into pCR2.1-TOPO. A 1.000-bp BamHI-ApaI DNA fragment was then subcloned into the suicide vector pKNG101. The resulting

constructs were transferred to *P. aeruginosa* by mobilization with pRK2013. The strains in which the chromosomal integration event occurred were selected on *Pseudomonas* isolation agar plates containing 2 mg/ml streptomycin. Excision of the plasmid, resulting in the deletion of the chromosomal target gene, was performed after selection on LB plates containing 5 % (wt/vol) sucrose. Clones that became sucrose resistant and streptomycin sensitive were confirmed to contain the gene deletion by PCR analysis. For the construction of PAO1 $\Delta$ S, an internal 1.1-kb *Asu*II fragment in *xcpS* on plasmid pUAWE6 was deleted. The gene with the deletion was cloned into the suicide vector pKNG101. The pKNG101 derivative was introduced in PAO1, and an *xcpS* deletion mutant resulting from double crossover was obtained as previously described (14).

**Enzyme assays.** Alkaline phosphatase activity was assayed by growth of strains on LB agar plates containing 0.4 mg/ml 5-bromo-4-chloro-3-indolylphosphate (XP). Secretion of elastase was analyzed qualitatively on LB agar plates with a top layer containing 1% elastin (Sigma). After overnight growth, the plates were screened for the presence of haloes around the colonies. For quantitative measurements, the colorimetric elastin-Congo red assay (34) was used. Briefly, 250  $\mu$ l of culture supernatant of cells grown overnight in the presence of IPTG were incubated for 2 h at 37°C with 10 mg/ml elastin-Congo red (Sigma) dissolved in assay buffer (0.045 M Tris/HCl, 1.5 mM CaCl<sub>2</sub>, pH 7.2), the reaction was stopped by the addition of 500  $\mu$ l of 0.7 M NaH<sub>2</sub>PO<sub>4</sub>, pH 6.0. After centrifugation, absorbance in the supernatant was measured at 495 nm.

**SDS-PAGE and immunoblot analysis.** Bacterial cells were suspended in SDS-PAGE sample buffer (2% SDS, 5%  $\beta$ -mercaptoethanol, 10% glycerol, 0.02% bromophenol blue, 0.1 M Tris/HCl, pH 6.8). Whole cell lysates were heated for 10 min at 95°C and proteins were separated on gels containing 10 % acrylamide. Proteins were transferred to nitrocellulose membranes by semi-dry electroblotting for immunodetection. The primary antiserum directed against XcpS was used at a 1:1000 dilution. Alkaline phosphatase-conjugated goat anti-rabbit IgG antiserum (Biosource international) was used as secondary antibody, unless otherwise indicated. Alkaline phosphatase-conjugated antibodies were detected by staining with XP and nitroblue tetrazolium. When peroxidase-conjugated goat anti-rabbit IgG antiserum (Biosource international) was used, detection was carried out with chemiluminescence (Pierce). The XcpS antiserum was raised in a rabbit against His-tagged XcpS produced in BL21(DE3) from pMEK49.

Briefly, BL21(DE3) carrying pMEK49 was grown overnight, cells were harvested by centrifugation, resuspended in TEN buffer (100 mM NaCl, 1 mM EDTA, and 50 mM Tris/HCl, pH 8.0), and sonicated. The lysate was centrifuged for 30 min at 10,000 g at 4 °C and the pelleted inclusion bodies were resuspended in 8 M urea in TEN buffer. After centrifugation for 15 min at 3,000 g, the solubilized His-tagged XcpS was purified with Ni-NTA beads (Qiagen) according to the manufacturer's protocol.

**Bioinformatic predictions.** For bioinformatic predictions, the TopPred program ([bioweb.pasteur.fr/seqanal/interfaces/toppred.html](http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html)) was used.

## RESULTS

**Topology of the XcpS protein.** Bioinformatic predictions suggested that XcpS contains three transmembrane segments, which separate a large cytoplasmic N-terminal domain (residues 1-173), a short periplasmic loop (residues 193-220), a large cytoplasmic loop (residues 240-376), and a short periplasmic C-terminal domain (residues 396-405) (Fig. 1). We constructed *xcpS-phoA* hybrid genes to experimentally test this topology. The leaderless *phoA* gene was fused to the 3'-end of *xcpS* fragments truncated at positions corresponding to residues E114, G216, K310, and V404 in the XcpS protein, yielding the *xcpSE114-phoA*, *xcpSG216-phoA*, *xcpSK310-phoA*, and *xcpSV404-phoA* gene fusions (Fig. 1). The recombinant plasmids were introduced into *E. coli* DH5 $\alpha$  and the strains were plated on LB agar containing XP to probe alkaline phosphatase activity. Colonies of cells carrying the plasmids encoding XcpSG216-PhoA and XcpSV404-PhoA were blue on these plates, whereas those of cells producing XcpSE114-PhoA and XcpSK310-PhoA remained white. This observation located the G216 and V404 residues on the periplasmic side of the cytoplasmic membrane, and residues E114 and K310 in the cytoplasm, in agreement with the predicted topology (Fig. 1).

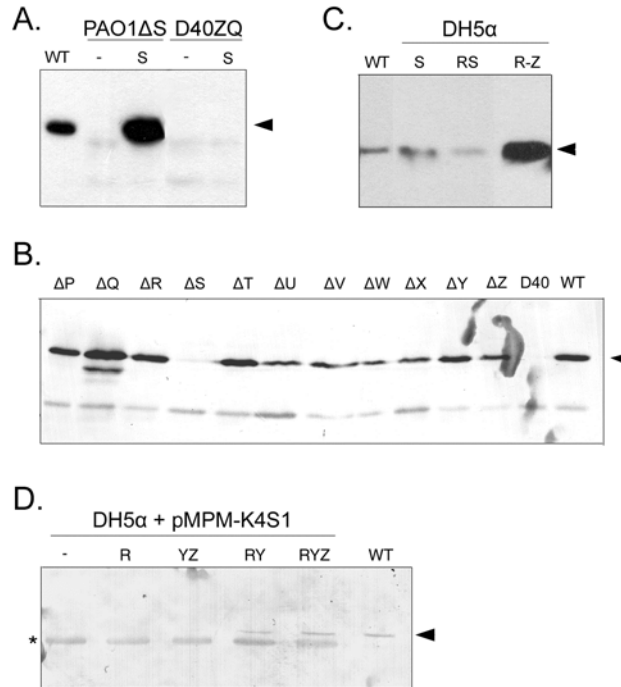
**XcpS is stabilized by other Xcp components.** When pMMB67HE-S carrying the *xcpS* gene under control of the *tac* promoter was introduced in a *P. aeruginosa xcpS* mutant (PAO1 $\Delta$ S), the secretion defect was complemented (results not shown) and production of XcpS was readily detectable by immunoblot analysis (Fig. 2A). However, when the plasmid



**FIG. 1. Topology of the XcpS protein.** A topology model is shown at the top. Transmembrane segments (TM) are indicated in grey, and the cytoplasmic (cyto) and periplasmic (peri) domains in white. To verify the topology, plasmids containing various *xcpS-phoA* fusions (fusion sites are indicated) were introduced into *E. coli* DH5a and activity of the periplasmic marker alkaline phosphatase (PhoA) was assayed by growth on LB agar containing XP. +, blue colouring of colonies; -, absence of blue colouring.

was introduced in strain DZQ40, which lacks the entire *xcp* gene cluster, XcpS was not detectable (Fig. 2A), which indicates that the protein is unstable in the absence of other Xcp components. To identify the Xcp protein(s) involved in XcpS stabilization, the levels of XcpS were determined in various non-polar *xcp* mutants. Remarkably, similar amounts of XcpS as in the wild-type strain were detected in all mutant strains, except that the *xcpQ* mutant produced reproducibly more XcpS than the other strains (Fig. 2B). The latter phenomenon was not further investigated. The observation that XcpS was not detected in the DZQ40 strain when expressed from a plasmid, but was present in all single mutants suggested that more than one component can interact with and stabilize XcpS.

**XcpS is stabilized by XcpRY.** XcpS production was also studied in the heterologous host *E. coli*. XcpS was detectable in cells with the *xcpS* gene on the high copy-number construct pCRII-S (Fig. 2C). However, the levels largely increased when the protein was produced from plasmid pCRII-RZ, which contains the entire *xcpR-Z* operon (Fig. 2C). Although other interpretations are possible, this result is consistent with the idea that production of other Xcp proteins can stabilize XcpS. An interaction between

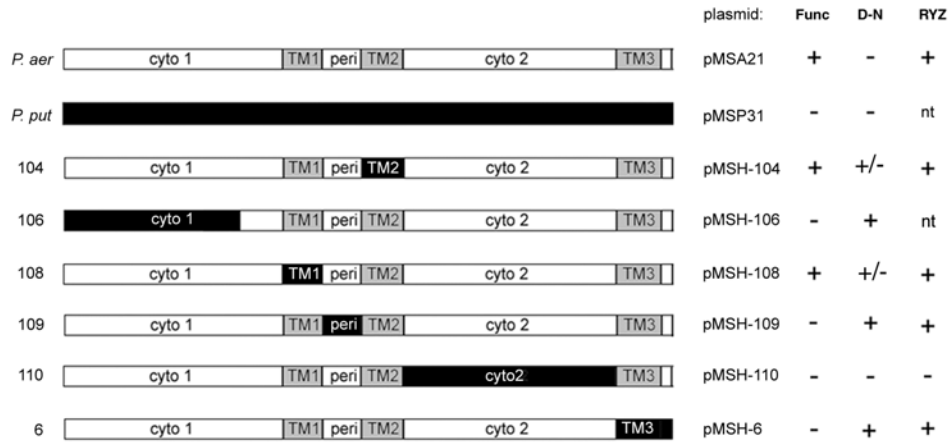


**FIG. 2.** XcpS production in the presence or absence of other Xcp components. Whole cell lysates were analysed by SDS-PAGE, followed by Western blotting using anti-XcpS antiserum. Immunoanalysis was performed as described in Methods (B and D) or with peroxidase-conjugated goat anti-rabbit IgG antiserum and chemiluminescence (A and C). PAO25 (WT) was included in all panels as reference and the position of XcpS is indicated with an arrow. (A) *P. aeruginosa* *xcpS* mutant PAO1ΔS and strain DZQ40, which lacks the entire *xcpP-Z* operon, either expressing XcpS from pMMB67HE-S (S) or containing the empty vector (-). Cells were grown overnight with IPTG to induce the *tac* promoter. (B) Chromosomal expression of XcpS in the wild-type strain PAO25 (WT), the DZQ40 (D40) mutant lacking *xcpP-Z* and different non-polar *xcp* mutants (indicated with the last letter of the gene designation) of *P. aeruginosa*. (C) *E. coli* DH5α with the constructs pCRII-S (S), pCRII-RS (RS), or pCRII-RZ (RZ). Overnight cultures were diluted to an optical density of 0.1 at 600 nm, grown for 1 h and subsequently induced for 2.5 h by the addition IPTG. (D) *E. coli* DH5α carrying plasmid pMPM-K4S1 combined with the empty vector pYRC (-), pYRC-R (R), pYRC-YZ (YZ), pYRC-RY (RY), or pYRC-RYZ (RYZ). A prominent cross-reacting protein in *E. coli* is indicated with an asterisk (\*).

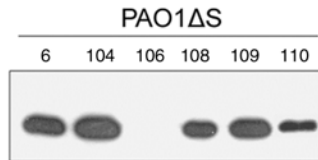
OutF and OutE, the *E. chrysanthemi* homologues of XcpS and XcpR, respectively, has been established by the yeast two-hybrid system (39). Production of XcpR together with XcpS from construct pCR-RS, however, did not increase XcpS levels compared to its production from pCRII-S (Fig. 2C). Other good candidates for an interaction with XcpS are the inner membrane components XcpY and XcpZ. To study if these proteins are important for XcpS stabilization, an experiment was performed in *E. coli* with *xcpS* under the control of an arabinose-inducible promoter on the high copy-number plasmid pMPM-K4S1 in combination with a second plasmid encoding XcpR, XcpYZ, XcpRY, or XcpRYZ. When cells were grown with arabinose, considerable amounts of XcpS were already detected in the strain carrying only pMPM-K4S1 (data not shown). However, at low expression levels, *i.e.* in the absence of inducer, XcpS was barely detectable in this strain (Fig. 2D). Co-production of XcpR or XcpYZ did not alter XcpS levels, but the presence of constructs pYRC-RY or pYRC-RYZ containing the *xcpRY* and *xcpRYZ* genes, respectively, considerably increased the quantity of XcpS. The construct with the *xcpRYZ* genes was slightly more effective in increasing the XcpS levels than the one carrying only the *xcpRY* genes. Production of XcpY and/or XcpZ from pYRC-RY, pYRC-YZ and pYRC-RYZ was confirmed by immunoblotting (results not shown). The amount of XcpR could not be determined because no antiserum was available. It can be concluded from these experiments that XcpR and XcpY together can, at least partially, stabilize XcpS.

**Analysis of *P. aeruginosa* - *P. putida* XcpS chimeras.** The XcpS proteins of *P. aeruginosa* and *P. putida* are similar in size and share 44% amino acid sequence identity. In contrast to a plasmid carrying *P.aeruginosa xcpS* (pMSA21), introduction of a construct carrying the *xcpS* gene of *P. putida* (pMSP31) into the *P. aeruginosa xcpS* mutant did not restore elastase secretion (see below), presumably because the heterologous XcpS does not properly interact with other components of the Xcp machinery. To identify putative interaction domains in XcpS, a series of chimeric genes was constructed in which various parts of *P. aeruginosa xcpS* were replaced by the corresponding parts of *P. putida xcpS*. A schematic representation of the proteins encoded by the hybrid genes is depicted in Fig. 3A. Production of all hybrid proteins, except for hybrid 106, could be confirmed by immunoblotting (Fig. 3B). The XcpS antiserum used was raised against the N-terminal cytoplasmic region of *P. aeruginosa* XcpS

A.



B.



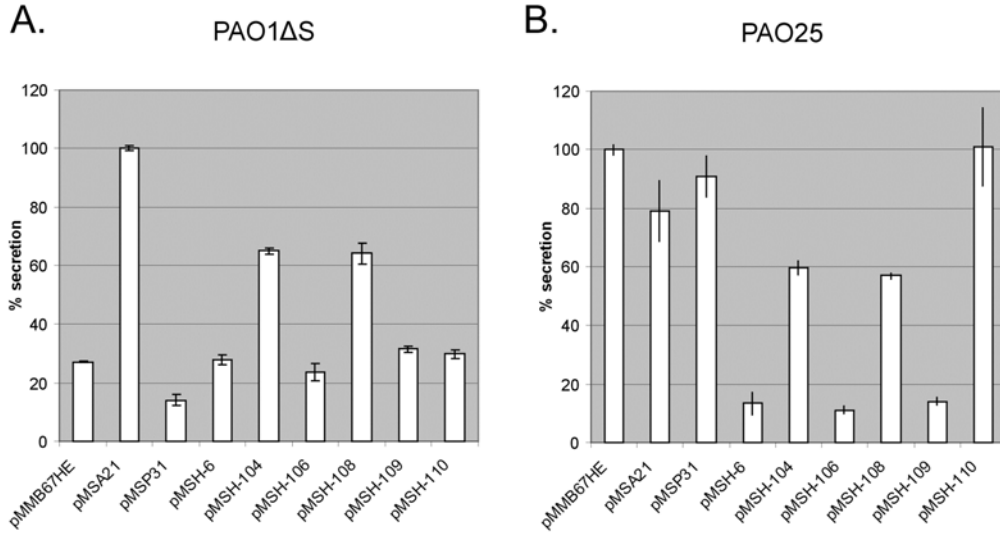
**FIG. 3. *P. aeruginosa* / *P. putida* XcpS hybrids. (A) Schematic representation of XcpS hybrids. *P. aeruginosa* sequences are depicted in white or in gray for transmembrane segments, *P. putida* sequences in black. Transmembrane segments (TM), and the cytoplasmic (cyto) or periplasmic (peri) domains are indicated. The nomenclature of the hybrid proteins is shown on the left, and of plasmids encoding the proteins on the right. Functionality of the hybrids (func) is based on their ability to complement the defect in elastase secretion of PAO1ΔS. +, complementation; -, no complementation. The dominant-negative effect (D-N) of the hybrids is based on their effect on elastase secretion in the wild-type strain. -, no effect on secretion; +, strong inhibition of secretion; +/-, partial inhibition of secretion. The column RYZ indicates the stabilization of the hybrids by XcpRYZ in *E. coli*. +, stabilization; -, no stabilization; nt, not tested. (B) Production of XcpS hybrids in the *P. aeruginosa xcpS* mutant. Whole cell lysates of *P. aeruginosa xcpS* mutant PAO1ΔS expressing *xcpS* hybrids from pMSH-6 (6), pMSH-104 (104), pMSH-106 (106), pMSH-108 (108), pMSH-109 (109), or pMSH-110 (110) were analysed by SDS-PAGE, followed by Western blotting. Immunodetection was carried out using XcpS-specific antiserum, peroxidase-conjugated goat anti-rabbit IgG antiserum and chemiluminescence. Cells were grown overnight in the presence of IPTG.**



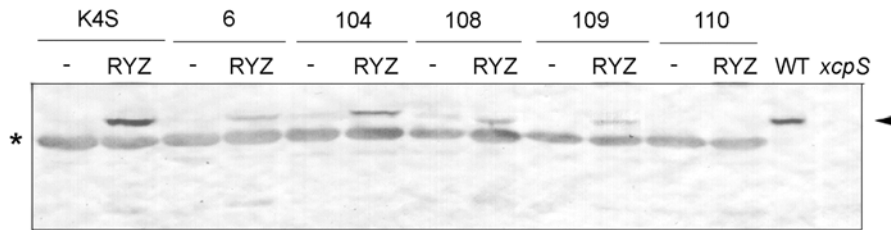
and did not cross-react with *P. putida* XcpS. Hybrid 106 contains the N-terminal domain of *P. putida xcpS* (Fig. 3A), which explains the lack of detection of the corresponding protein on the immunoblot (Fig. 3B). However, the presence of plasmid pMSH-106 in the wild-type strain affected secretion (see below), showing that also this chimeric protein was produced.

The functionality of the XcpS hybrids could not be tested in *P. putida*, since the substrates of its Xcp machinery have not been characterized (13, 14). In *P. aeruginosa*, the hybrids were tested for their ability to complement the defect of elastase secretion in an *xcpS* mutant, using elastin-Congo red as a substrate for the enzyme. Hybrids 104 and 108, in which the second and the first transmembrane segment, respectively, of XcpS is substituted, were functional in secretion, although they appeared less efficient than wild-type *P. aeruginosa* XcpS (Fig. 4A). All other hybrids were found to be non-functional. The fusions were also produced in wild-type *P. aeruginosa* PAO25 to detect any dominant-negative effect on secretion. Fusions 6, 106 and 109, in which the third transmembrane domain, the N-terminal cytoplasmic domain and the small periplasmic loop, respectively, are exchanged, all strongly inhibited secretion. Hence, these non-functional fusions were still capable of interacting with other Xcp components, although they do not assemble into a functional apparatus. The non-functional chimeric protein 110, which contains the large cytoplasmic loop of *P. putida* XcpS, did not have such a negative effect on secretion, indicating that this protein is not able to engage stably into an interaction with other Xcp components. Finally, production of the functional hybrids 104 and 108 in wild-type *P. aeruginosa* reduced secretion to some extent, suggesting that they were somewhat less functional compared with wild-type XcpS (Fig. 4B).

**The cytoplasmic loop of XcpS is involved in the stabilization by XcpRY.** The data presented above suggested that the cytoplasmic loop of XcpS is important for interaction with other Xcp components. To determine whether this part of the protein is required for the stabilization of XcpS by the XcpRYZ proteins, the hybrid gene 110 was cloned into pMPM-K4 $\Omega$ , resulting in pMPM-110. This construct was introduced into *E. coli* and stabilization was studied by production of XcpRYZ *in trans*. The protein was detectable on immunoblots when the cells were grown with L-arabinose (data not shown). However, at low expression levels in the absence of L-



**FIG. 4.** Extracellular elastase activity of *P. aeruginosa* strains expressing the XcpS hybrids or containing the empty vector. Elastase activity was determined with the elastin-Congo red assay. Bars represent the average of three independent experiments and standard deviations are indicated. Expression of the hybrids was induced by the addition of IPTG. Elastase activity is presented as percentage of the activity measured in PAO1ΔS complemented with wild-type XcpS (A), or in PAO25 containing the empty vector (B).



**FIG. 5.** Effect of production of XcpR, XcpY and XcpZ *in trans* on the stability of XcpS hybrids in *E. coli*. Whole cell lysates of *E. coli* containing plasmid pYRC-RYZ (RYZ), or the empty vector pYRC (-) combined with pMPM-K4S1 (K4S), pMPM-6 (6), pMPM-104 (104), pMPM-108 (108), pMPM-109 (109), or pMPM-110 (110) were analysed by SDS-PAGE, followed by Western blotting. Immunodetection was carried out using XcpS-specific antiserum. PAO25 (WT) and PAO1ΔS (*xcpS*) were included as references. The position of XcpS is indicated with an arrow, and a prominent cross-reacting protein with an asterisk (\*).

arabinose, this chimeric XcpS was not detectable and the amounts of the protein did not increase to detectable levels upon co-production of XcpRYZ (Fig. 5). In contrast, all other chimeric proteins were stabilized by co-production of XcpRYZ (Fig. 5). Hence, the cytoplasmic domain of XcpS, between residues 240 and 376 appears to be important for interaction with XcpRYZ.

## DISCUSSION

The T2SS is a complex apparatus that spans the cell envelope and is composed of up to 16 different proteins (20). The mode of action of these machines is still largely enigmatic. Insight in the interactions between the constituents of the secretin might provide important clues on the assembly of the apparatus. In this study, we used the instability of XcpS to identify interactions with other Xcp components. This approach has been successfully applied before to show association between XcpY and XcpZ (33), XcpY and XcpR (2), and XcpP and XcpQ (6).

XcpS expressed in *E. coli* was stabilized by co-expression of both XcpR and XcpY simultaneously and not when one of these proteins was co-expressed alone. These results indicate that XcpSRY form a ternary complex in which XcpS interacts directly with either one of the partner proteins or with both of them. However, since interactions between OutE and OutF, the *E. chrysanthemi* XcpR and XcpS homologues, and between OutF and OutL, the XcpS and XcpY homologues, have been reported (39), it is conceivable that both XcpR and XcpY interact directly with XcpS. In that case, the role of XcpY in the stabilization of XcpS may be dual: (i) to interact directly with XcpS and (ii) to dock XcpR to the inner membrane (2, 37, 38), thereby facilitating an interaction between XcpR and XcpS. Noteworthy in this respect is that modelling of the X-ray crystal structure of the cytoplasmic fragment of the XcpY homologue EpsL together with that of a fragment of the XcpR homologue EpsE of the *V. cholerae* T2SS resulted in only partial filling of the groove between EpsL domains II and III (1). This observation hints at a missing protein in the modelled complex, which may be the cognate XcpS homologue. The XcpR homologue OutE of the *E. chrysanthemi* system has been shown to undergo a change in conformation that requires the XcpY homologue OutL and *vice versa* (38). This conformational change may be required to enable the interaction with

the cognate XcpS homologue. We observed that production of the XcpZ protein together with XcpRY somewhat further elevated XcpS levels, which may be related to the stabilizing effect of XcpZ on XcpY (33). However, we cannot exclude a direct interaction between XcpS and XcpZ. The existence of an XcpRSYZ sub-complex is in agreement with a recent publication showing the co-purification of XcpRSY with his-tagged XcpZ after cross-linking (40). It should be stressed that XcpS production was substantially higher from a construct carrying *xcpR-Z* than upon coexpression of only *xcpRYZ*. Since this increase did not correlate with an increase in XcpY production (data not shown), other Xcp components beside XcpY and XcpR appear to play role in XcpS stabilization.

The *P. putida xcpS* gene could not complement an *xcpS* mutation in *P. aeruginosa*, probably because it fails to interact properly with other Xcp components in the heterologous host. We used this observation to identify regions in the protein that are important for the species-specific functioning and that thus likely represent interaction domains. For that purpose, a series of chimeric *xcpS* genes was constructed and the results of these studies are summarized in Fig. 3A. The first two transmembrane segments of XcpS could be replaced by those of *P. putida* XcpS without loss of function. The similarities between the amino acid sequences of the *P. aeruginosa* and *P. putida* transmembrane segments one and two are 11 and 20%, respectively. This low level of similarity and the fact that they can be functionally exchanged shows that these segments are not involved in species-specific interactions. The third transmembrane segment including the last few periplasmic residues (hybrid 6) could not be replaced, although on an elastin-containing plate this hybrid appeared still partially functional as evidenced by the formation of a small halo around the colonies (data not shown). The production level of this hybrid was similar to those of the other hybrids; therefore, augmented instability does not seem to be the reason of its non-functionality. Hence, the last membrane-spanning segment and/or the C-terminal periplasmic residues appear to be involved in the species-specific functioning of XcpS, and thus likely in the interaction with other Xcp components. Similarly, replacement of the large N-terminal cytoplasmic domain (hybrid 106) and of the short periplasmic loop (hybrid 109) resulted in loss of functionality. Production of fusions 6, 106 and 109 in the wild-type strain interfered with secretion, which shows that these proteins still have the right conformation to interact with at least one other component of the secretion machinery but interfere with the formation of a

functional complex. Consistently, these proteins were still stabilized by XcpRYZ, and the non-functionality of these hybrids must be explained by inappropriate subsequent interactions with Xcp components.

Expression of fusion 110, in which the large cytoplasmic loop is replaced, did not complement the secretion defect of the *xcpS* mutant and did not display a dominant-negative effect on secretion in the wild-type strain. Apparently, this fusion is no longer stably incorporated in the secreton. When expressed in *E. coli*, fusion 110 was found to be the only hybrid that was no longer stabilized by co-expression of XcpRYZ. This result suggests that the cytoplasmic loop of XcpS is an essential segment for interaction with these Xcp components. In contrast, the N-terminal part of the XcpS homologue OutF was found to interact with the XcpR homologue OutE of *E. chrysanthemi* in yeast two-hybrid experiments (39). Possibly, both cytoplasmic segments of XcpS participate in the interaction with XcpR, but the cytoplasmic loop between residues 239 and 379 suffices for the stabilization effect. On the other hand, these results can also reflect the dynamic nature of the secreton, in which interactions change during assembly.

In summary, multiple domains of XcpS play a role in the species-specific functioning of this protein, suggesting that XcpS interacts with several other components at both sides of the cytoplasmic membrane. Multiple interactions may also provide an explanation for our observation that XcpS is unstable when the entire *xcp* gene cluster is absent, but not when individual *xcp* genes were missing. Likely, more than one component can stabilize the XcpS protein. The sensitivity of XcpS and other Xcp proteins to proteolytic degradation may be important to ensure the correct order of interactions during assembly of the secreton. Based on the current knowledge, we propose this assembly to occur in the following steps. XcpZ recruits XcpY resulting in a more or less stable complex in the cytoplasmic membrane (33). XcpY on its turn forms a docking site for the cytoplasmic ATPase XcpR, which then associates with the inner membrane (2). Docking of XcpR to the inner membrane results in conformational changes in both XcpY and XcpR (38). The XcpRYZ subcomplex subsequently engages with XcpS, rendering the latter less prone to degradation. This results in an inner membrane complex that might act as a platform for the assembly of a pilus-like structure. Our next goal will be to identify the other interaction partner(s) of XcpS.

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