Species-Specific Functioning of the *Pseudomonas* XcpQ Secretin: Role for the C-Terminal Homology Domain and Lipopolysaccharide[▽]

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Secretins are oligomeric proteins that mediate the export of macromolecules across the bacterial outer membrane. The members of the secretin superfamily possess a C-terminal homology domain that is important for oligomerization and channel formation, while their N-terminal halves are thought to be involved in system-specific interactions. The XcpQ secretin of *Pseudomonas* spp. is a component of the type II secretion pathway. XcpQ from *Pseudomonas alcaligenes* is not able to functionally replace the secretin of the closely related species *Pseudomonas aeruginosa*. By analysis of chimeric XcpQ proteins, a region important for species-specific functioning was mapped between amino acid residues 344 and 478 in the C-terminal homology domain. Two chromosomal suppressor mutations were obtained that resulted in the proper functioning in *P. aeruginosa* of *P. alcaligenes* XcpQ and inactive hybrids. These mutations caused a defect in the synthesis of the lipopolysaccharide (LPS) outer core region. Subsequent analysis of different LPS mutants showed that changes in the outer core and not the loss of O antigen caused the suppressor phenotype. High concentrations of divalent cations in the growth medium also allowed *P. alcaligenes* XcpQ and inactive hybrids to function properly in *P. aeruginosa*. Since divalent cations are known to affect the structure of LPS, this observation supports the hypothesis that LPS has a role in the functioning of secretins.

Pseudomonas aeruginosa is able to secrete proteins into the extracellular medium via at least five different pathways (16). Most of the exoproteins are secreted via the type II secretion system. This pathway, which is widely distributed among the *Proteobacteria*, involves two separate translocation steps (8, 26). First, the secreted proteins are directed to the Sec translocon in the cytoplasmic membrane by the presence of a signal sequence. Upon translocation across the inner membrane, the signal sequence is removed, and the mature protein is released in the periplasm. In this compartment, the exoproteins acquire a folded conformation with the help of specific chaperones and of general folding catalysts, before they are translocated across the outer membrane. Some type II substrates use the Tat system rather than the Sec system to cross the inner membrane (46), and in these cases folding may occur in the cytoplasm. The second step, transport of the folded exoproteins across the outer membrane, is mediated by the type II secretion machinery, which in *P. aeruginosa* consists of at least 12 different proteins, designated XcpA and XcpP to XcpZ (15, 16).

Since the Xcp secretion machinery transports folded exoproteins across the outer membrane, the secretion channel has to be large. Consistently, the only outer membrane component of the machinery, XcpQ, has been shown to form large channels (6). XcpQ belongs to a family of proteins generically designated the secretins. This family also includes members

that are involved in type III protein secretion, in the formation of type IV pili, in the production of filamentous phages, and in the uptake of DNA (20). Therefore, secretins constitute a very important group of macromolecule transporters in the outer membrane of gram-negative bacteria. Members of this family have been shown by electron microscopy to form large, ring-shaped, oligomeric complexes with a central cavity, which could represent the protein-conducting channel (2). The homology between members of the family is in their C-terminal halves (20). Therefore, this part of the secretins has been implicated in the formation of the ring-shaped structure and the transport channel, which was confirmed by limited proteolysis of XcpQ (6) and PulD of *Klebsiella oxytoca* (34). The N-terminal domains exhibit homology only in secretins that are involved in similar transport processes and could therefore play a role in the interaction with other components of the transport system or with the substrates. Studies performed with chimeric secretins have supported this notion, since they showed that the N termini of OutD of *Erwinia chrysanthemi* (3) and of the phage-encoded pIV secretin (10) are important for substrate specificity. Moreover, the EpsD secretin of *Vibrio cholerae* was shown to interact with the XcpP homolog EpsC via the N-terminal domain (27).

Pseudomonas alcaligenes is closely related to *P. aeruginosa* and also contains a complete *xcp* gene cluster, the products of which are involved in secretion of a lipase (21). Generally, type II secretion systems are highly specific, and secretion of heterologous exoproteins is rarely observed, even when they are expressed in closely related species. However, *P. aeruginosa* is able to secrete exoproteins of *P. alcaligenes* and vice versa (11), which indicates that the two secretion systems are closely related. Although most *xcp* genes of these two species can be

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TABLE 1. *Pseudomonas* strains and plasmids used

Strain or plasmid[a]	Relevant characteristics[b]	Reference or source
P. aeruginosa strains		
PAO25	*leu arg*	22
PAN1	PAO25 *xcpQ*::Gm	1
PAN15	PAN1, suppressor, LPS core deficient	This study
PAN16	PAN1, suppressor, LPS core deficient	This study
AK1012	PAO1, LPS core deficient	24
AK1401	PAO1, LPS B-band deficient	24
PAN38	AK1012 *xcpQ*::Gm	This study
PAN39	AK1401 *xcpQ*::Gm	This study
P. alcaligenes 93A		21
Plasmids		
pUC18	Amp^r, ColE1	NCCB
pMMB67EH	Amp^r, IncQ	18
pUR6500HE	pMMB67HE derivative, Km^r	17
pVDZ′2	Tet^r, IncP	12
pRK2013	Km^r Tra$^+$ Mob$^+$	14
pLAF600SB	*xcpPQ$_{alc}$* in PLAFR3	21
pB28	*xcpQ$_{aer}$* in pMMB67EH	1
pCK25	*xcpQ$_{aer}$* in pUR6500EH	This study
pCK27	*xcpP$_{alc}$* in pMMB67EH	This study
pCK28	*xcpQ$_{alc}$* in pMMB67EH	1
PCK29	*xcpP$_{alc}$* in pVDZ′2	This study
pCK31	*xcpPQ$_{alc}$* in pMMB67EH	This study
pCK32	*xcpQ$_{aer}$* and *xcpQ$_{alc}$* in pMMB67EH	This study
pUWL6	*xcpQ$_{aer}$* in pUC19	1
pUWL15	*xcpQ$_{F497}$* in pUC18	This study
pUWL20	*xcpQ$_{H13}$* in pUC18	This study
pKNWQ1	*xcpQ$_{aer}$*::Gm in suicide vector pKNG101, Sm^r	1

[a] *P. aeruginosa* strains PAN15, PAN16, PAN38, and PAN39 have been deposited in NCCB and are available as strains PC4313, PC4314, PC4319, and PC4320, respectively.

[b] Gm, gentamicin; Amp, ampicillin; Km, kanamycin; Tet, tetracycline; Sm, streptomycin.

functionally exchanged, expression of the *xcpQ* gene from *P. alcaligenes* does not efficiently complement the secretion defect of an *xcpQ* mutant of *P. aeruginosa* (11). In this study, we constructed chimeric XcpQ proteins to identify the domain that is involved in species-specific functioning of the XcpQ protein. Furthermore, to identify host factors important for the lack of exchangeability, we isolated and characterized suppressor mutations that allowed proper functioning of *P. alcaligenes* XcpQ in *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. *Escherichia coli* strains DH5α and MC1061 were obtained from The Netherlands Culture Collection of Bacteria (NCCB). DH5α was used for general cloning purposes, and MC1061 was used for isolation of chimeric *xcpQ* genes. The *Pseudomonas* strains used in this study are listed in Table 1. The *P. aeruginosa* and *E. coli* strains were grown at 37°C in a modified Luria-Bertani (LB) broth (43). *P. alcaligenes* was grown as previously described (21). Plasmids used in this study are listed in Table 1 and Fig. 1. Genes derived from *P. aeruginosa* and *P. alcaligenes* are indicated by the subscripts *aer* and *alc*, respectively, in the gene designations. The *P. aeruginosa*-infecting bacteriophages F116L and E79 were obtained from the NCCB and Bengt Wretlind, respectively. Bacteriophage PAF3 was isolated from sewage water obtained at the wastewater purification system in Houten, The Netherlands. Phage-resistant mutants were isolated by plating 0.1 ml of an overnight

culture together with 0.1 ml of a phage stock solution (10^{10} to 10^{11} PFU/ml) in a 5-ml agar top layer. Subsequently, the plates were grown for 30 h at 37°C, potential phage-resistant mutants were streaked twice on fresh LB medium plates, and the lipopolysaccharide (LPS) patterns were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Transduction experiments were performed with phage F116L. Chromosomal *xcpQ* mutations were obtained by using the suicide plasmid pKNWQ1, which contains part of the *xcpQ* gene and a gentamicin resistance cassette, as described previously (1). Plasmids were introduced by the CaCl$_2$ procedure into *E. coli* (38) and by triparental mating into *Pseudomonas* strains using the conjugative helper plasmid pRK2013. For plasmid maintenance or selection, 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); and for *P. aeruginosa*, piperacillin (40 μg/ml), kanamycin (50 μg/ml), tetracycline (80 μg/ml), and gentamicin (40 μg/ml). Sensitivity to antibiotics and EDTA was determined as described previously (45).

Isolation and characterization of chimeric *xcpQ* genes. Plasmid isolation and DNA manipulations were performed using standard procedures (38). Plasmid pCK27 was constructed by cloning the EcoRI/SalI fragment of pLAF600SB containing the *xcpP$_{alc}$* gene in the EcoRI/SalI-digested cloning vector pMMB67EH. The *xcpP$_{alc}$* gene was subsequently recloned into the vector pVDZ′2 using the EcoRI/HindIII sites, resulting in plasmid pCK29. Plasmid pCK31, carrying the *xcpPQ$_{alc}$* genes, was obtained by cloning the EcoRI/SphI fragment of pCK27 and the EcoRI/HindIII fragment of pCK28 in SphI/HindIII-digested pMMB67EH by three-point ligation. The *xcpQ$_{aer}$* gene was obtained by digesting pUWL6 with HindIII/SacI and cloning of the fragment with *xcpQ$_{aer}$* in the same sites of the pUR6500HE vector, resulting in plasmid pCK25.

An in vivo recombination strategy (42) was used to construct hybrid genes. The *xcpQ$_{aer}$* and *xcpQ$_{alc}$* genes were cloned in tandem in the pMMB67EH vector with a unique restriction site between the two genes. First, the vector was digested with EcoRI/BamHI and religated after the overhanging ends were filled in, which resulted in a cloning vector lacking most of the multiple cloning site. The *xcpQ$_{alc}$* gene was obtained from plasmid pCK28 with KpnI/HindIII, and the *xcpQ$_{aer}$* gene was obtained from plasmid pCK25 using PstI and KpnI. The two genes were introduced by three-point ligation into the altered pMMB67EH vector digested
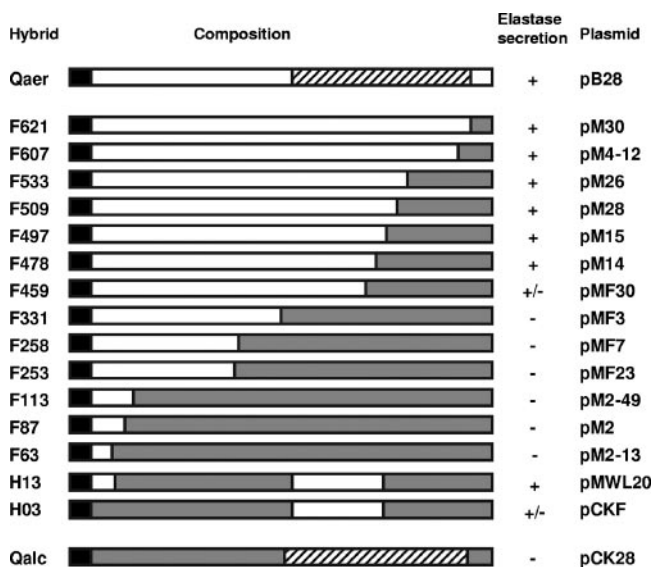

FIG. 1. Schematic diagrams of the different (chimeric) XcpQ proteins. Open bars, XcpQ$_{aer}$; shaded bars, XcpQ$_{alc}$; solid bars, signal sequences; cross-hatched bars, C-terminal domain that is conserved in the various members of the secretin family. The designations of the proteins are indicated on the left; in each case the number refers to the position of the amino acid residue at the fusion site (the numbering includes the signal sequence). The corresponding plasmids are indicated on the right. The functionality of XcpQ was based on the ability of the *xcpQ* mutant PAN1 supplemented with the different *xcpQ* genes to form haloes on elastin-containing plates after 24 h of growth. +, formation of haloes whose sizes were similar to the sizes of haloes formed by the wild-type strain; +/−, formation of smaller haloes; −, no halo formation.

with PstI and HindIII. The resulting plasmid, pCK32, contained the two genes in tandem with a unique KpnI site between them. To obtain $xcpQ$ chimeras in vivo, plasmid pCK32 was linearized with KpnI. After inactivation of the restriction enzyme, the overhanging ends were made blunt with T4 DNA polymerase. The linear plasmid was used for electroporation of *E. coli* strain MC1061. Alternatively, pCK32 was linearized with SmaI and used for electroporation. Linear plasmids cannot be replicated in vivo, but the linear plasmids can become circular by recombination at homologous sites in the plasmids, resulting in the desired chimeric genes (42). The plasmids were isolated from transformants and subjected to restriction enzyme analysis to identify clones in which a recombination event had occurred. The nucleotide sequences of the relevant fragments subsequently showed the exact fusion sites. The chimeras obtained are shown schematically in Fig. 1.

The chimeric $xcpQ_{H13}$ gene was created by a modified version of the QuikChange mutagenesis procedure (Stratagene). First, $xcpQ_{F497}$ was subcloned in pUC18, resulting in pUWL15. Subsequently, an internal fragment of $xcpQ_{alc}$ was amplified by PCR, using primers WILQF1 (5′-TCCGCGAATTCATCGAC CAG-3′) and WILQF3 (5′-CAGCAGGACCAGGGCATTG-3′), both of which were designed to hybridize with $xcpQ_{aer}$ as well as with $xcpQ_{alc}$. The resulting PCR product was extracted with phenol and purified on an agarose gel by using Jetsorb (Genomed). Subsequently, this material was used as a megaprimer in a QuikChange mutagenesis protocol with pUWL15 as the template. This protocol was performed with *Pwo* polymerase (Boehringer) using 18 10-min amplification steps. The final PCR product was treated with DpnI and used to transform DH5α cells. The clone that was isolated, which contained $xcpQ_{H13}$, was subjected to restriction enzyme analysis and DNA sequencing and was designated pUWL20. Subsequently, the $xcpQ_{H13}$ gene was introduced into pMMB67EH by using restriction enzymes XbaI and HindIII, resulting in plasmid pMWL20. This plasmid was used to construct pCKF (carrying $xcpQ_{H03}$) by replacing the SmaI/HindIII fragment, encoding the N-terminal domain of $xcpQ_{H13}$, with the corresponding fragment of pCK28 ($xcpQ_{alc}$).

Isolation of suppressor mutations. Suppressor mutants were selected on the basis of their ability to secrete the staphylolytic enzyme LasA. Cells of strain PAN1 carrying plasmid pM2 (Fig. 1) were treated for 90 min with ethyl methanesulfonate. Subsequently, the bacteria were washed with 5% NaS_2O_3 and 0.9% NaCl, incubated in LB medium for 2 h at 37°C, and plated on selection plates. These plates contained 25 ml of 10-fold-diluted LB medium in M9 medium (without an additional carbon source), piperacillin, 1.5% agar, and heat-killed *Staphylococcus aureus* cells from a 10-ml overnight culture, which were pelleted and washed. After overnight growth, colonies of LasA-secreting bacteria could be identified by their larger sizes and by the small clear halo surrounding each colony. Putative suppressor mutants were subsequently analyzed for the secretion of elastase on elastin plates as described previously (5).

SDS-PAGE and immunoblotting. Proteins were separated by SDS-PAGE (29), with 0.2% SDS in the running gel. The proteins were stained in the gel with Coomassie brilliant blue, or they were transferred onto nitrocellulose membranes by semidry electroblotting. To improve immunodetection, the oligomeric XcpQ complexes were denatured in situ in the gel prior to the blotting procedure. To do this, the gels were incubated for 30 min in 10% trichloroacetic acid (TCA) and washed four times in distilled water and then in SDS-PAGE electrode buffer until the pH of the electrode buffer was reached. After blotting, the different XcpQ variants were visualized by immunodetection with antibodies directed against an N-terminal fragment of XcpQ (amino acid residues 12 to 100 of the mature protein) (1). Peroxidase activity on blots was detected by staining with 4-chloro-1-naphthol and H_2O_2, unless indicated otherwise.

Cell fractionation. Secreted proteins were isolated by precipitation from the supernatant fractions of cell cultures with 5% TCA. Cell envelopes were harvested by centrifugation (20 min, 4°C, 20,000 × *g*) after ultrasonic disruption of *P. aeruginosa* cells in the presence of 1% NP-40 (Igepal CA-630) (32) and the Complete protease inhibitor cocktail (Boehringer Mannheim) to keep endogenous elastase inactive. For separation of inner and outer membranes, cells grown overnight in LB medium were pelleted by centrifugation and resuspended in a solution containing 0.25 M sucrose, 0.5 mM EDTA, 100 mM Tris-HCl (pH 8), and 60 μg/ml lysozyme (Merck). The mixture was incubated for 1 h at room temperature. After addition of dithiothreitol (DTT) and phenylmethylsulfonyl fluoride, both to a final concentration of 2 mM, the spheroplasts were collected by centrifugation for 75 min at 150,000 × *g* at 4°C, resuspended in H_2O with 2 mM DTT and 1 mM phenylmethylsulfonyl fluoride, and disrupted by 20 s of ultrasonication. The membranes were pelleted by centrifugation for 75 min at 150,000 × *g* at 4°C, resuspended in 5 mM EDTA (pH 7.5)–2 mM DTT, and layered on a discontinuous sucrose gradient containing 30 to 55% (wt/wt) sucrose in 5 mM EDTA (pH 7.5)–2 mM DTT. The gradients were centrifuged at 35,000 rpm in an SW41 rotor (Beckman) for 20 h at 4°C and fractionated. NADH

oxidase activity was determined as described previously (35). The presence of OprF in the different fractions was evaluated by SDS-PAGE.

Purification and analysis of LPS. Routinely, LPS patterns in proteinase K-treated cell envelopes were analyzed by SDS-PAGE on 16% acrylamide gels. After separation, LPS was visualized in the gels by silver staining (44). For detailed analysis of the LPS composition, bacteria were grown in 10-liter cultures. Cell pellets were collected and washed with 0.9% NaCl and then with distilled water prior to lyophilization. The lyophilized cells were washed overnight with ethanol and then twice with acetone and finally with diethyl ether. Subsequently, the extracted cell debris was dried overnight in air. The dried material (10 to 15 g) was resuspended in 250 ml distilled water supplemented with DNase (10 mg) and RNase (10 mg) and stirred overnight at room temperature. Subsequently, proteinase K (10 mg) was added, and this was followed by incubation for 16 h at room temperature. The suspension was dialyzed against distilled water for 5 days at 4°C, and the insoluble material was collected by centrifugation, washed twice with acetone (250 ml), and dried in air. *P. alcaligenes* LPS was extracted with 45% hot phenol (47), and *P. aeruginosa* LPS was isolated by a modified phenol-chloroform-petroleum ether extraction method (19).

Chemical analysis of LPS. Gas-liquid chromatography (GLC) was performed with a Hewlett-Packard model 5890 Series II instrument equipped with a 30-m SPB-5 capillary column (Supelco, Germany) using a temperature gradient which increased from 150 to 320°C at a rate of 5°C min^{-1}. Monosaccharides were analyzed by GLC as the alditol acetates after hydrolysis of LPS with 0.1 M HCl for 48 h at 100°C for neutral sugars (39) or with 10 M HCl for 30 min at 80°C for amino sugars (13). Following hydrolysis, sugars were dried by evaporation in a vacuum, reduced with $NaBH_4$, and acetylated with acetic anhydride in pyridine (30 min, 85°C). Fatty acids were analyzed by GLC as their methyl esters, liberated after strong methanolysis (2 M HCl–methanol, 120°C, 24 h) and extraction with chloroform. Total hexosamine contents were determined after acid hydrolysis (4 M HCl, 16 h, 100°C) using the Morgan-Elson reaction as described previously (41). Amino acids and amino sugars were identified as their phenylisothiocyanate derivatives by reversed-phase high-performance liquid chromatography using a Waters PICO-TAG system (Waters, Eschborn, Germany) (7, 9). 3-Deoxy-D-*manno*-octulosonic acid contents were determined by the thiobarbituric acid assay using the modified method (4). Total phosphate was quantified by the ascorbic acid method (31).

RESULTS

Construction and functionality of chimeric XcpQ secretins. Although *P. alcaligenes* XcpQ (XcpQ$_{alc}$) is highly homologous to its counterpart in *P. aeruginosa*, exhibiting 69% identity (21), expression of $xcpQ_{alc}$ in an $xcpQ$ mutant of *P. aeruginosa* does not restore protein secretion via the type II secretion pathway (11). In order to identify the amino acid residues responsible for the species-specific functioning, hybrids between $xcpQ_{alc}$ and $xcpQ_{aer}$ were constructed. Since we expected that the more variable N-terminal domain would be involved in species specificity, we constructed chimeric genes that encoded different N-terminal fragments of XcpQ$_{aer}$ and the complementing C-terminal domain of XcpQ$_{alc}$. The hybrid genes were obtained by in vivo recombination (see Materials and Methods), and the fusion sites were localized by restriction enzyme mapping and nucleotide sequencing (Fig. 1). The numbers in the designations of the hybrid proteins indicate the positions in the amino acid sequence of the XcpQ precursor where the fusions occurred. All the $xcpQ$ genes were under control of the *tac* promoter.

Subsequently, the constructs containing the hybrid genes were mobilized into *P. aeruginosa* strain PAN1, in which the chromosomal $xcpQ$ gene is inactivated by insertion of a gentamicin resistance gene, and they were tested to determine the abilities to complement the secretion defect of this strain. Secretion of the type II substrate elastase was examined on elastin-containing plates, on which secretion-proficient strains
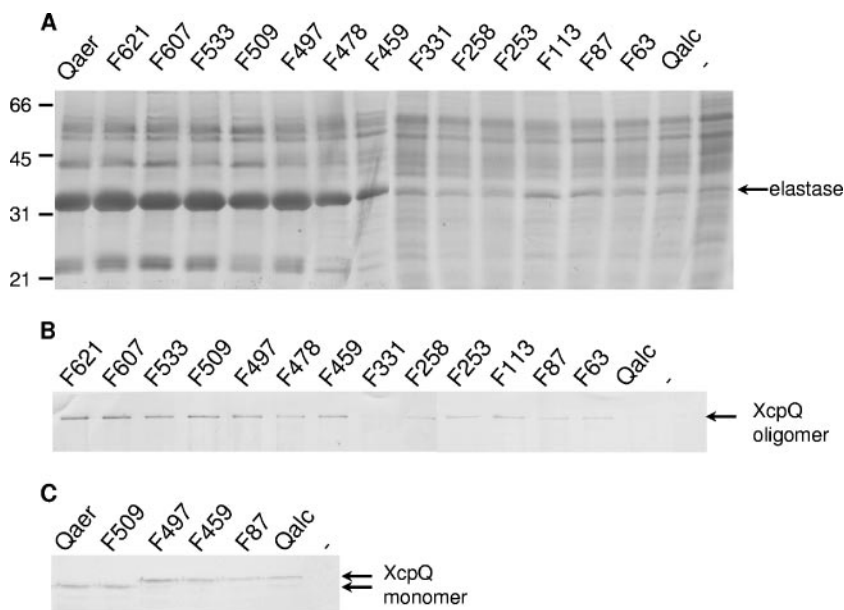
FIG. 2. Functionality and complex formation for the different XcpQ secretins. (A) Extracellular protein profiles of *P. aeruginosa* strain PAN1 with constructs encoding the different XcpQ variants or with the empty vector (lane −). Secreted proteins were precipitated with 5% TCA and separated by SDS-PAGE. The position of the major protein secreted by the type II pathway, elastase, is indicated on the right, and the positions of the molecular mass standard proteins (in kDa) are indicated on the left. (B and C) Cell envelopes (B) or whole-cell preparations (C) of strain PAN1 with constructs encoding different XcpQ variants or with the empty vector (lane −) were subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes and visualized by immunodetection with polyclonal antibodies directed against XcpQ. The positions of the XcpQ oligomers and monomers are indicated on the right.

formed haloes around the colonies (Fig. 1), and by SDS-PAGE analysis of the extracellular protein profiles, in which elastase was the most prominent band (Fig. 2A). The hybrids containing an N-terminal pre-XcpQ$_{aer}$ fragment consisting of 478 or more amino acid residues were able to restore the secretion of elastase very efficiently. The XcpQ$_{F459}$ hybrid exhibited partial complementation, whereas all the fusions with an N-terminal pre-XcpQ$_{aer}$ fragment consisting of less than 331 amino acid residues were not functional. Thus, the protein segment between residues 331 and 478 of XcpQ$_{aer}$ appears to contain an important determinant for species-specific functioning.

To investigate whether additional specificity determinants are present in the N-terminal domain of XcpQ, we constructed two additional chimeric genes, encoding the H13 and H03 hybrids (Fig. 1), in vitro. The resulting hybrids contained the same C-terminal segment of XcpQ$_{alc}$ that was present in hybrid F497 and another XcpQ$_{alc}$ segment extending from either amino acid residue 1 (in H03) or residue 68 (in H13) up to residue 344. Both genes were able to complement the secretion defect of strain PAN1 (Fig. 1), although the gene encoding H03 did not restore secretion completely. We concluded that the region between amino acid residues 344 and 478 plays an important role in preventing XcpQ$_{alc}$ from functioning in *P. aeruginosa*. All of this region is located in the C-terminal secretin homology domain, which encompasses amino acid residues 326 to 606 of the XcpQ$_{aer}$ precursor.

Characteristics of the oligomers formed by the chimeric XcpQ secretins. To study the production of the chimeric proteins and their abilities to form oligomers, cell envelope preparations were analyzed by SDS-PAGE, followed by immunoblotting. Most of the chimeric proteins were detected as

high-molecular-weight oligomeric complexes; the only exception was the hybrid protein XcpQ$_{F331}$, which did not seem to form any oligomers (Fig. 2B). However, the amounts of oligomeric complexes formed by the nonfunctional fusions were smaller than the amounts formed by the functional fusions; in particular, the complexes formed by XcpQ$_{alc}$ were hardly visible after immunodetection (Fig. 2B). Analysis of whole-cell preparations revealed that a small proportion of XcpQ was present in the monomeric form. Interestingly, the amounts of monomers were the same for all the XcpQ variants, as shown in Fig. 2C for a number of representative proteins, indicating that a constant pool of stable XcpQ monomers was present in the cells, which may have represented assembly intermediates.

To determine whether there is a direct correlation between functionality and the amount of oligomer, PAN1 producing the inactive fusion protein XcpQ$_{F87}$ from pM2 was grown in the presence of various concentrations of isopropyl-β-D-thiogalac-topyranoside (IPTG). Addition of IPTG to the growth medium resulted in an increase in the amount of the XcpQ complex (Fig. 3A); however, this did not lead to restoration of elastase secretion (Fig. 3B). In contrast, addition of IPTG was not required to obtain type II-mediated secretion in the case of the active fusions (shown for fusion protein XcpQ$_{F509}$ in Fig. 3A and B). These results show that the failure of the hybrids to function was not correlated with a quantifiable defect in oligomer formation.

The inability of the inactive fusions to function in *xcpQ* mutant strain PAN1 could have been due to incorrect targeting to the outer membrane. To examine this possibility, the subcellular localizations of the oligomers of the functional hybrid protein XcpQ$_{F509}$ and the nonfunctional protein XcpQ$_{F87}$
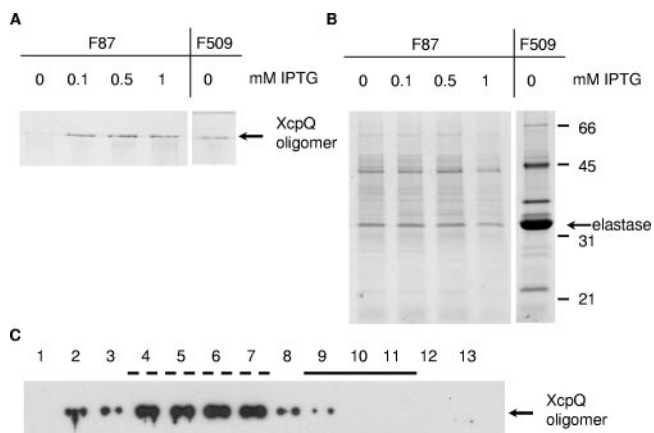
FIG. 3. Characterization of the nonfunctional hybrid protein XcpQ$_{F87}$. The *xcpQ* mutant PAN1 with pM2 encoding the nonfunctional hybrid protein XcpQ$_{F87}$ was grown to an optical density at 660 nm of 2.3, after which IPTG was added at the concentrations indicated above the lanes, and growth was continued for 2 h. PAN1(pM28) producing the active fusion protein XcpQ$_{F509}$ was grown without IPTG. To determine the production of XcpQ oligomers, cell envelopes were isolated and subjected to SDS-PAGE and Western blotting with polyclonal antibodies directed against XcpQ (A). To determine the functionality of XcpQ hybrids, secreted proteins were precipitated with 5% TCA and separated by SDS-PAGE (B). The position of the major protein secreted via the type II pathway, elastase, and the positions of the molecular mass standard proteins (in kDa) are indicated on the right. (C) Cellular localization of XcpQ$_{F87}$. A cell envelope preparation of *P. aeruginosa* PAN1(pM2) expressing XcpQ$_{F87}$ was applied to a 30 to 55% sucrose gradient, which was centrifuged and fractionated. Samples from all fractions were analyzed by SDS-PAGE, and proteins were transferred to a nitrocellulose membrane and visualized by immunodetection using antibodies against XcpQ and enhanced chemiluminescence for detection. The fractions that represented the inner membrane (solid line) and outer membrane (dashed line) were identified based on NADH oxidase activity and the presence of OprF, respectively. The position of the XcpQ oligomers is indicated on the right.

were determined. Neither XcpQ protein could be detected in the soluble fraction (data not shown). The membrane fractions were analyzed further by sucrose gradient centrifugation to separate the inner and outer membranes. As expected, the functional XcpQ$_{F509}$ oligomeric complexes were found in the fractions containing the outer membrane proteins (data not shown). However, the oligomers of the inactive hybrid XcpQ$_{F87}$ protein also fractionated with the outer membranes (Fig. 3C). These results show that the oligomers formed by this chimeric protein were targeted to the outer membrane.

Isolation of suppressor mutations. The malfunctioning of the XcpQ$_{alc}$ protein in *P. aeruginosa* is probably due to aberrant interactions with components of the heterologous host. We first examined whether XcpP might be such a component, since several reports have indicated that XcpP and its homologs in other bacterial species interact with their cognate secretins (26, 27). Therefore, we introduced a plasmid, pCK29, containing *P. alcaligenes xcpP* into strain PAN1 producing in *trans* one of the various inactive XcpQ hybrids and examined the protein secretion profiles by SDS-PAGE. However, no restoration of protein secretion was observed (results not shown).

As an alternative approach, we isolated suppressor muta-

tions in strain PAN1 that allowed functioning of an inactive XcpQ hybrid protein. The selection procedure was based on the ability of the type II-secreted LasA protein, also known as staphylolytic enzyme, to lyse *S. aureus* cells. LasA-secreting cells exhibit increased growth on plates containing *S. aureus* cells and clearing zones around the colonies. Cells of strain PAN1 containing plasmid pM2, which encodes the inactive hybrid protein XcpQ$_{F87}$, were mutagenized with ethyl methanesulfonate and plated on plates containing *S. aureus* cells. Twelve fast-growing colonies were analyzed on elastin-containing plates. Two of these colonies exhibited extracellular elastase activity, and they were subsequently cured of pM2. The cured strains, designated PAN15 and PAN16, were secretion defective, whereas reintroduction of pM2 into both mutants, but not reintroduction of pM2 into PAN1, resulted in elastase secretion, as revealed by halo formation on elastin plates (results not shown) and by SDS-PAGE analysis of the extracellular protein profiles (shown for PAN16 in Fig. 4A). These results show that the suppressor mutations were located on the chromosome.

All the constructs containing different *xcpQ* genes were introduced into PAN16, and the extracellular protein patterns of the transconjugants were analyzed. Most of the fusions that were inactive in PAN1 were able to restore, at least to some extent, protein secretion in the suppressor mutant PAN16 (shown for XcpQ$_{F87}$ in Fig. 4A); the only exception was XcpQ$_{F331}$, which, like the results obtained for PAN1 (Fig. 2B), did not form any detectable oligomers in this strain (data not shown). Even the wild-type XcpQ$_{alc}$ protein expressed from plasmid pCK28 was functional in PAN16 (Fig. 4A), although the efficiency of secretion was relatively low and the XcpQ$_{alc}$ oligomers formed were hardly visible (Fig. 4B). The plasmids encoding the intact XcpQ$_{aer}$ protein, the functional XcpQ$_{F497}$ fusion protein, or the partially functional XcpQ$_{F459}$ fusion protein restored secretion in the suppressor mutant to levels similar to those in the PAN1 strain (Fig. 4A). In contrast, introduction of the empty vector pMMB67HE into strain PAN1 or into the suppressor strain PAN16 did not result in secretion of elastase and other type II-dependent exoproteins (Fig. 4A).

Analysis of the cell envelopes showed that the yield of oligomers formed by XcpQ$_{alc}$ or XcpQ$_{F87}$ in the suppressor strain was slightly greater than the yield in PAN1, whereas the amounts of XcpQ$_{aer}$, XcpQF$_{F497}$, and XcpQ$_{F459}$ oligomers were the same in both strains (Fig. 4B).

LPS biosynthesis is affected in suppressor strains PAN15 and PAN16. To identify the chromosomal mutations responsible for the suppressor phenotype, the obvious candidates (i.e., the genes encoding components of the type II secretion machinery) were evaluated first. Since the *xcpP* and *xcpRSTU VWXYZ* genes are adjacent to *xcpQ* on the chromosome, transduction experiments were performed. However, the suppressor phenotype could not be cotransduced with the *xcpQ*::Gm allele, which indicated that the suppressor mutation is not located in the *xcp* gene cluster.

Analysis of the cell envelope protein profiles of PAN15 and PAN16 by SDS-PAGE revealed no significant differences compared with the profile of PAN1 (data not shown). Also, the growth characteristics of the mutants were not altered. In addition, the sensitivities of the two suppressor strains to various
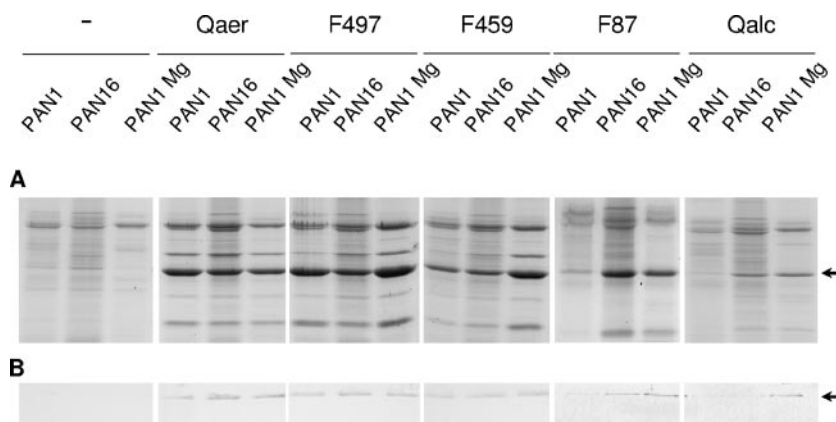
FIG. 4. Effects of the suppressor mutation and divalent cations on the functionality and production of various XcpQ proteins. *P. aeruginosa* strains PAN1 and PAN16 containing the empty vector (−) or *xcpQ_aer*, *xcpQ_F497*, xcpQ_F459, *xcpQ_F87*, or *xcpQ_alc* on plasmids were grown in normal LB medium or LB medium supplemented with 5 mM $MgCl_2$ (Mg). Extracellular proteins were precipitated with 5% TCA and separated by SDS-PAGE (A). The position of the major protein secreted via the type II pathway, elastase, is indicated by an arrow. (B) Amounts of XcpQ oligomers produced by the strains. Cell envelopes were subjected to SDS-PAGE followed by Western blotting with polyclonal antibodies directed against XcpQ. The position of the XcpQ oligomers is indicated by an arrow.

antibiotics (ampicillin, polymyxin B, and kanamycin) and EDTA did not differ from the sensitivities of PAN1, which indicates that the integrity of the outer membrane was not grossly affected. Subsequently, the LPS patterns of the different strains were analyzed by SDS-PAGE, using the well-characterized LPS mutants AK1012, which produces LPS molecules consisting of the lipid A part and the inner core domain, and AK1401, which is deficient only in the synthesis of the B-band O antigen, as references (Fig. 5, lanes 2 and 3, respectively). Both wild-type strain PAO1 and the original *xcpQ* mutant PAN1 produced intact lipid A-core molecules (Fig. 5, lanes 1 and 4, respectively) and O-antigen subunits (results not shown). In contrast, LPS isolated from strains PAN15 and PAN16 clearly exhibited increased mobility on SDS-PAGE gels (Fig. 5, lanes 5 and 6), similar to the mobility observed for the LPS of strain AK1012, which indicated that there was a defect in the synthesis of the LPS core region. Consistently, PAN15 and PAN16 were resistant to the *P. aeruginosa*-infecting phage E79, which uses LPS as its receptor (25).

To obtain more insight into the LPS structure required for

XcpQ_alc to function, the LPS of *P. aeruginosa* strains PAN1 and PAN16 were isolated, and their chemical compositions were determined (Table 2). Differences between PAN1 and the suppressor mutant were found in the content of glucose and rhamnose, which are components of the outer core. The composition of PAN16 LPS is consistent with the proposed LPS structure of the outer core mutant AK1012 (37) and is shown in Fig. 6. The chemical composition of *P. alcaligenes* LPS was also determined (Table 2). Interestingly, the LPS of this bacterium is characterized by a lower glucose content. Since glucose residues are found in the outer core and are not present in the suppressor mutant, their presence in wild-type *P. aeruginosa* LPS could be responsible for the malfunctioning of XcpQ_alc.

Functionality of XcpQ_alc in *P. aeruginosa* is dependent on LPS core mutations or the presence of divalent cations. To verify that the suppressor phenotype of strains PAN15 and
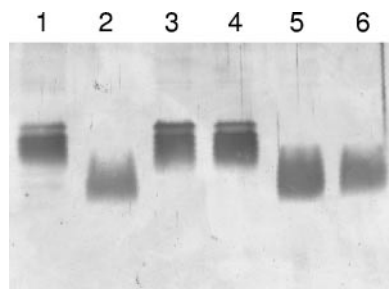

FIG. 5. LPS patterns of *P. aeruginosa* strains. Partially purified LPS of *P. aeruginosa* PAO1 (wild type) (lane 1), AK1012 (core mutant) (lane 2), AK1401 (B-antigen mutant) (lane 3), PAN1 (lane 4), and suppressor strains PAN15 (lane 5) and PAN16 (lane 6) were separated by SDS-PAGE and visualized by silver staining. LPS molecules containing multiple repeating units of the O antigens were not detected due to the small amounts of LPS loaded.

TABLE 2. Composition of LPS isolated from *P. aeruginosa* PAN1 and PAN16 and *P. alcaligenes* 93A

Component[a]	Concn (nmol/mg) in:		
	Strain PAN1	Strain PAN16	Strain 93A
Total HexN	631	663	445
Total GlcN	427	553	321
GalN	225	290	161
Ala	199	278	214
Kdo	231	303	237
P	1,612	2,176	1,261
Glc	703	117	345
Hep	118	195	109
Rha	527	90	1438
10:0(3-OH)	161	239	170
12:0	115	159	247
12:0(2-OH)	200	292	
12:0(3-OH)	265	380	241
16:0	31	39	

[a] HexN, hexosamine; GlcN, glucosamine; GalN, galactosamine; Kdo, 3-deoxy-D-*manno*-octulosonic acid; Glc, glucose; Hep, L-*glycero*-D-*manno*-heptose; Rha, rhamnose.
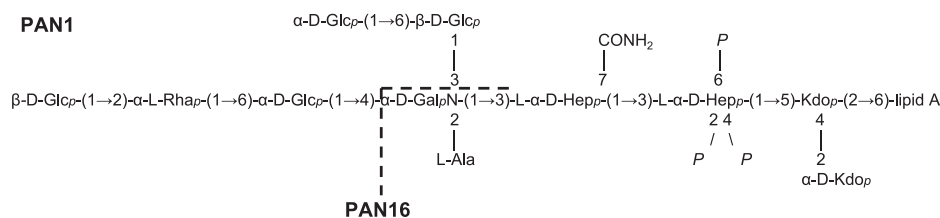
FIG. 6. Structural model of the LPS alteration in suppressor mutant PAN16. The structures of PAN1 and PAN16 LPS are based on data obtained previously (37) and data reported here. Kdo, 3-deoxy-D-*manno*-octulosonic acid.

PAN16 correlates with the defect in the synthesis of the LPS core region, *xcpQ* deletion mutant derivatives of the LPS mutants AK1012 and AK1401 were constructed. As expected, the resulting strains, designated PAN38 and PAN39, respectively, were secretion deficient (Table 3). Subsequently, plasmids carrying different *xcpQ* genes were introduced into PAN38 and PAN39, and the secretion profiles were evaluated. Introduction of pB28 encoding XcpQ$_{aer}$ restored elastase secretion in both strains, although the levels of secretion were slightly lower than those of strain PAO25 (Table 3). Introduction of plasmids encoding either XcpQ$_{alc}$ or the inactive XcpQ$_{F87}$ hybrid protein into PAN39 did not result in complementation of the *xcpQ* mutant phenotype. However, both plasmids restored the secretion defect in strain PAN38, which confirmed that mutations affecting LPS outer core biosynthesis facilitate functioning of the inactive XcpQ hybrids. To substantiate this conclusion, we selected mutants of strain PAN1 containing plasmid pM2 (*xcpQ*$_{F87}$) that were resistant to the LPS-specific phage PAF3. Nine of 12 mutants isolated had an altered LPS core, whereas the other mutants only had a defect in O-antigen biosynthesis, as revealed by SDS-PAGE analysis of proteinase K-treated cell envelope preparations (results not shown). In contrast to the three O-antigen mutants, all nine mutants with a defect in outer core biosynthesis had a suppression phenotype, as determined by halo formation on elastin plates. Together, these results show that defects in the LPS outer core, rather than the loss of O antigen, allow the nonactive hybrid XcpQ proteins to become functional.

Since the structure of LPS can also be influenced by the growth conditions, the activity of the nonfunctional hybrid protein XcpQ$_{F87}$ in the *xcpQ* mutant PAN1 was determined at different temperatures and in medium with various concentra-

tions of salt or divalent cations. The temperature or different salt concentrations did not influence the functionality of XcpQ$_{F87}$. However, addition of Mg^{2+} or Ca^{2+} to the growth medium increased the ability of XcpQ$_{F87}$ to restore elastase secretion in PAN1 (Fig. 4A) and to form oligomers (Fig. 4B). The effect of the divalent cations on the functionality of this hybrid was similar to the effect of the LPS mutations. Divalent cations also stimulated protein secretion in strain PAN1 producing the XcpQ$_{alc}$ protein (Fig. 4A) or the other inactive chimeric hybrids (data not shown). The presence of divalent cations did not alter the extracellular protein profiles of PAN1 producing XcpQ$_{aer}$ or the active fusion protein XcpQ$_{F497}$ (Fig. 4A). In contrast, the functionality of the partially functional fusion protein XcpQ$_{F459}$ was improved by the presence of Mg^{2+} (Fig. 4A).

DISCUSSION

P. aeruginosa and *P. alcaligenes* are closely related organisms that both secrete proteins via the type II secretion pathway. Previously, it has been shown that most of the components of the Xcp machinery of *P. alcaligenes* can replace their *P. aeruginosa* counterparts; the exceptions are XcpP and XcpQ. Only when the genes encoding XcpP and XcpQ were expressed simultaneously was some complementation observed (11). However, this complementation was observed only when the cells were grown on plates and not when the cells were grown in liquid cultures. In this study, we determined the region of the XcpQ$_{alc}$ protein that is responsible for species-specific functioning of the secretin and isolated suppressor mutations on the chromosome of *P. aeruginosa* that allowed XcpQ$_{alc}$ to be functional.

Isolation of the chimeric proteins XcpQ$_{aer}$ and XcpQ$_{alc}$ enabled us to pinpoint a domain determining the nonfunctionality of XcpQ$_{alc}$ in *P. aeruginosa*. Interestingly, this region was found to be in the C-terminal homology domain. This result was not expected, since it is the N-terminal half of secretins that is thought to be involved in system-specific interactions. The region identified is 74% identical in XcpQ$_{alc}$ and XcpQ$_{aer}$. Moreover, the experiments also revealed that the chimeric secretin XcpQ$_{F478}$ was completely functional, whereas with XcpQ$_{F459}$ there was only partial restoration of secretion in the *P. aeruginosa xcpQ* mutant. These two XcpQ variants differ at only a single amino acid residue. Whereas XcpQ$_{F478}$ contains a lysine residue at position 460 (the numbering includes the signal sequence), XcpQ$_{F459}$ contains a glutamate residue at the equivalent position. Therefore, this lysine appears to be important for proper functioning of the secretin in *P. aeruginosa*.

With the exception of XcpQ$_{F331}$, all the fusions were capable

TABLE 3. Complementation of LPS mutant strains with *xcpQ* wild-type genes and chimeras[a]

Strain	Mutation[b]	Secretion with different *xcpQ* genes				
		None	*xcpQ*$_{aer}$	*xcpQ*$_{alc}$	*xcpQ*$_{F87}$	*xcpQ*$_{F497}$
PAO25	None	++	++	++	++	++
PAN1	*xcpQ*	−	++	−	−	++
PAN15	*xcpQ*, suppressor	−	++	+	++	++
PAN16	*xcpQ*, suppressor	−	++	+	++	++
PAN38	*xcpQ*, core	−	+	+	+	+
PAN39	*xcpQ*, O antigen	−	+	−	−	+

[a] Secretion was analyzed on elastin plates by evaluating halo formation after 24 h of incubation and by analyzing extracellular protein profiles by SDS-PAGE. ++, secretion comparable to that of wild-type PAO25 cells; +, decreased but significant secretion; −, no significant secretion.
[b] core, mutation causing a defective LPS outer core; O antigen, mutation causing a lack of O antigen.

of oligomerization. Although the amounts of the inactive XcpQ proteins were generically smaller than the amounts of the active proteins, we demonstrated that the reduced levels of expression were not responsible for the secretion defect. Furthermore, localization studies showed that the oligomers formed by the nonfunctional hybrid protein $XcpQ_{F87}$ colocalized with the outer membrane markers. The cells also contained a monomeric XcpQ pool, which was the same size for all the variants. This result suggests that the monomeric form of the inactive fusions is stable but that reduced efficiency during the subsequent stages in the biogenesis process makes the assembly intermediates susceptible to proteolytic degradation.

The nonfunctionality of $XcpQ_{alc}$ and the inactive fusions in *P. aeruginosa* might be explained by inappropriate interactions with host factors. Interestingly, mutations affecting the biosynthesis of the outer core region of LPS enabled $XcpQ_{alc}$ and inactive XcpQ hybrids to function in *P. aeruginosa*. It should be noted, however, that the functionality of $XcpQ_{alc}$ in the suppressor strain was lower than that of the hybrid proteins, which indicates that the N-terminal 63 amino acid residues may also contribute to the species specificity. Consistently, fusion protein $XcpQ_{H03,}$ which has the N terminus of $XcpQ_{alc}$, was not as functional as $XcpQ_{H13}$, which is similar except that the N-terminal 63 amino acid residues correspond to the residues in $XcpQ_{aer}$ (Fig. 1). The XcpP protein is a good candidate to interact with the N terminus. However, we did not observe any restoration of secretion in the *xcpQ* mutant during coexpression of $XcpP_{alc}$ with its cognate secretin, which may have been due to competition between $XcpP_{alc}$ and the endogenous XcpP for binding to other Xcp components. Presumably, both the correct LPS and the cognate XcpP are required for optimal functioning.

The stimulatory effect of the LPS mutation on the functionality of inactive hybrids could be either direct or indirect. In the case of the latter possibility, the alterations in the outer core might have pleiotropic effects, resulting in stimulation of protein folding or reduced activity of proteases in the periplasm. Alternatively, improved secretion in the LPS mutants could be due to an altered interaction between LPS and XcpQ. The following findings support this hypothesis. (i) The inactive secretins form outer membrane-associated oligomers, indicating that they are correctly folded. (ii) The region involved in species specificity is present in the C-terminal homology domain of the secretin that is thought to be located in the outer membrane. Therefore, this part of the protein is probably in close contact with LPS. (iii) It has been shown previously that a different LPS mutation affects the functioning of $XcpQ_{aer}$ negatively (33). Hence, mutations affecting the LPS structure can have opposite effects on the functionality of XcpQ in *P. aeruginosa*, namely, a negative effect on the endogenous secretin and a positive effect on the $XcpQ_{alc}$ secretin, which indicates that there is a direct interaction between LPS and XcpQ. (iv) The addition of divalent cations also allows the $XcpQ_{alc}$ secretin to function in *P. aeruginosa*. Divalent cations are known to affect LPS structure, either directly by influencing the charge and/or packaging of the LPS (40) or indirectly by downregulating LPS-modifying enzymes (36).

The ability of LPS to affect the functioning of outer membrane proteins has been reported previously. For instance, the outer membrane protease OmpT requires LPS for activity (28). The secretin-LPS interaction could also affect the interpretation of data obtained in several previous studies, in which secretion was studied in heterologous hosts. For example, the OutD secretin of *Erwinia carotovora* was shown to be unable to mediate the secretion of *E. chrysanthemi* exoproteins in a reconstituted pathway in *E. coli*, although the gene was expressed efficiently (30). However, the same protein did function correctly in an *E. chrysanthemi* background, indicating that an inappropriate interaction with LPS might play a role.

In conclusion, a part of the $XcpQ_{alc}$ protein that is responsible for the inability of this secretin to function properly in the closely related host *P. aeruginosa* is located in the C-terminal secretin homology domain. This inability to function properly can be suppressed by alterations in the outer core of the LPS of *P. aeruginosa* or by the presence of divalent cations, indicating that changes in LPS structure influence the functioning of these protein transport channels. In future experiments we will try to identify the chromosomal mutations in the suppressor mutants and determine the precise role of LPS in the functioning of the XcpQ secretin.

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