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Assembly of XcpR in the Cytoplasmic Membrane Is Required for Extracellular Protein Secretion in *Pseudomonas aeruginosa*

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A broad range of extracellular proteins secreted by *Pseudomonas aeruginosa* use the type II or general secretory pathway (GSP) to reach the medium. This pathway requires the expression of at least 12 *xcp* gene products. XcpR, a putative nucleotide-binding protein, is essential for the secretion process across the outer membrane even though the protein contains no hydrophobic sequence that could target or anchor it to the bacterial envelope. For a better understanding of the relationship between XcpR and the other Xcp proteins which are located in the envelope, we have studied its subcellular localization. In a wild-type *P. aeruginosa* strain, XcpR was found associated with the cytoplasmic membrane. This association depends on the presence of the XcpY protein, which also appears to be necessary for XcpR stability. Functional complementation of an *xcpY* mutant required the XcpY protein to be expressed at a low level. Higher expression precluded the complementing activity of XcpY, although membrane association of XcpR was restored. This behavior suggested that an excess of free XcpY might interfere with the secretion by formation of inactive XcpR-XcpY complexes which cannot properly interact with their natural partners in the secretion machinery. These data show that a precise stoichiometric ratio between several components may be crucial for the functioning of the GSP.

Pseudomonas aeruginosa is an opportunistic pathogen causing chronic and acute infections in humans. Like many other bacterial pathogens of plants and animals, the virulence character of *P. aeruginosa* is multifactorial and associated with the elaboration of a large number of extracellular proteins with toxic or hydrolytic activities (25). During chronic infections in patients with cystic fibrosis, these enzymes have been implicated as important factors contributing directly or indirectly to the lung diseases (46). To be targeted to the surrounding medium, the secreted proteins must cross the two membranes enveloping this gram-negative bacterium. At least three distinct secretion pathways, each specialized for different substrate proteins, coexist in *P. aeruginosa* (14, 18, 48).

A majority of *P. aeruginosa* exoproteins are secreted by means of a two-step pathway (43), known as the type II or general secretory pathway (GSP) (34, 37). The first step of secretion, through the cytoplasmic membrane, is promoted by the presence of an N-terminal signal sequence and apparently occurs via a classical *sec*-dependent pathway (11). The second step, from the periplasm to the surrounding medium, requires the products of the *xcp* genes in *P. aeruginosa*. Genetic analysis of mutants defective for the secretion of proteases, lipase, and toxin A led to the identification of 12 genes (*xcpP* to *-Z* and *xcpA*/*pilD*) that are essential for protein translocation across the outer membrane (1, 5, 6, 14). Nucleotide sequence data revealed that these genes are homologous to the *pul* genes involved in pullulanase secretion by *Klebsiella oxytoca*, the first organism in which this pathway was identified (36). In recent years, similar sets of secretion genes have been found in a wide variety of gram-negative bacteria (20, 24, 31, 35).

The GSP mechanism involved in the outer membrane translocation step has not been determined, and there is little information about the structure or function of the majority of the secretion factors. XcpQ is an integral outer membrane protein that forms large ring-shaped homomultimers which may function as specialized pores (7). XcpT, -U, -V, and -W are structurally related to the type IV pilin subunits and are processed on the cytoplasmic face of the membrane by XcpA/PilD, a peptidase/methylase also required for pilus assembly (5, 30). An additional protein, XcpX, was recently shown to belong to the same family (the pseudopilins) and to also be processed by XcpA/PilD, although it contains an atypical N-terminal region (9). Four other proteins are located in the cytoplasmic membrane. XcpS is a polytopic protein with three transmembrane domains (3, 42); XcpP, -Y, and -Z span the membrane once, with their N termini in the cytoplasm (8). In XcpP and XcpZ, the transmembrane domain is close to the N terminus, so most of the protein is exposed to the periplasm. In contrast, the majority of XcpY is located in the cytoplasm, with a smaller domain extending into the periplasm. XcpR and its homologues have conserved sequences commonly found in nucleotide-binding proteins (Walker box A [45]). Mutations in these motifs result in defects in protein secretion (32, 33, 44), suggesting the involvement of these proteins in an energy-dependent step of the mechanism. Although one might expect such an activity to be performed by a membrane protein, a particularity of XcpR is an overall hydrophilic character suggesting a cytoplasmic location (5).

In this work, we investigated the cellular localization of XcpR and found that the protein is associated with the cytoplasmic membrane through an interaction with XcpY. These

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Reference or source
E. coli strains		
TG1	*supE* Δ(*lac-proAB*) *thi hsdR*Δ*5* (F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *Z*ΔM15)	26
pop2136	*endA thi hsdR malT::cI857*	O. Raibaud, Institut Pasteur
P. aeruginosa strains		
PAO1	Prototroph, *chl-2*	Holloway collection
DZQ40	PAO1 with *xcpP* to *-Z* chromosomal deletion	This study
KS910-503	*xcpY51 met-9011*	47
Plasmids		
pUC19	ColE1 replicon, *lacI* ϕ80d*lacZ* Ap^r	49
pYZ4	ColE1 replicon, *oriF*1 p_{lacUV5} Km^r	50
pRK2013	ColE1 replicon, Tra$^+$ Mob$^+$ (RK2) Km^r	13
pLAFR3	pLAFR1 derivative, IncP1 *ori*T *cos* Tc^r	16
pMMB67HE	RSF1010 replicon, p_{tac} *lacI*^{q} Ap^r	17
pMMB190	RSF1010 replicon, p_{tac} p_{lacUV5} *lacI*^{q}, Ap^r	29
pAX24	pLAFR3 with *xcpP* to *-Z* on 20-kb DNA insert	15
pLFR4	1.8-kb *Pst*I-*Asu*II DNA insert carrying *xcpR* cloned in pLAFR3	This study
pMR1	Same insert as pLFR4 cloned in pMMB67HE	This study
pSB31	1.4-kb *Sal*I-*Sph*I insert carrying *xcpY* cloned in pMMB67HE	This study
pSB34	1.35-kb *Sal*I-*Pst*I insert carrying *xcpY* cloned in pYZ4	This study
pMYS	*xcpY′* (Met1→Pro234) cloned in pMMB190	This study
pSB72	*lasB*^{SP}-′*xcpY* (Arg259→stop) cloned in pMMB190	28
pMYR	1.35-kb *Sal*I-*Pst*I and 1.8-kb *Pst*I-*Asu*II DNA inserts carrying *xcpY* and *xcpR* cloned together in pMMB67HE	This study

findings corroborate and expand those previously obtained with EpsE and EpsL, the XcpR and XcpY homologues, respectively, in *Vibrio cholerae* (38). In addition, we present data indicating a stoichiometric relationship between XcpR and one or several additional components of the secretion system. These results are consistent with the idea of a multiprotein secretion complex organized within the envelope of gram-negative bacteria.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. The *Escherichia coli* K-12 strain TG1 was used as a host for cloning experiments and for expression of constructs carrying *xcpR* and *xcpY*. The *xcp*-deleted strain DZQ40 is a derivative of *P. aeruginosa* wild-type strain PAO1 constructed as follows. A 8.5-kb deletion between the *Sca*I-1 site in *xcpZ* and the *Sal*I-6 site in *xcpQ* was created by coligation of the 1.1-kb *Eco*RI-1–*Sca*I-1 and the 1.1-kb *Sal*I-6–*Sal*I-7 DNA fragments into pUC19 (Fig. 1). Cohesive ends between the two DNA fragments were generated by previous cloning in the pUC19 polylinker. This construction was introduced into *P. aeruginosa* PAO1 by electroporation (39). Since pUC19 cannot replicate autonomously in *P. aeruginosa*, its maintenance is dependent solely on its ability to integrate into the chromosome. Clones with plasmid integration were selected for on plates containing carbenicillin. The second homologous recombination event resulting in loss of the integrated plasmid and the *xcp* chromosomal region was generated during serial subcultures in the absence of antibiotic. Several carbenicillin-sensitive clones were obtained and tested for their secretion phenotype on tryptic soy agar plates containing either 1.5% skim milk (Difco Laboratories) or elastine (15). The 8.5-kb chromosomal deletion in clone DZQ40 was verified by Southern blot analysis (not shown).

The conjugative properties of pRK2013 (13) were used to transfer recombinant plasmids from *E. coli* to *P. aeruginosa* by triparental mating. Bacterial cells were grown at 37°C in Luria-Bertani broth for *E. coli* and in tryptic soy broth (TSB; Difco) for *P. aeruginosa*. When necessary, isopropyl-β-D-thiogalactopyr-

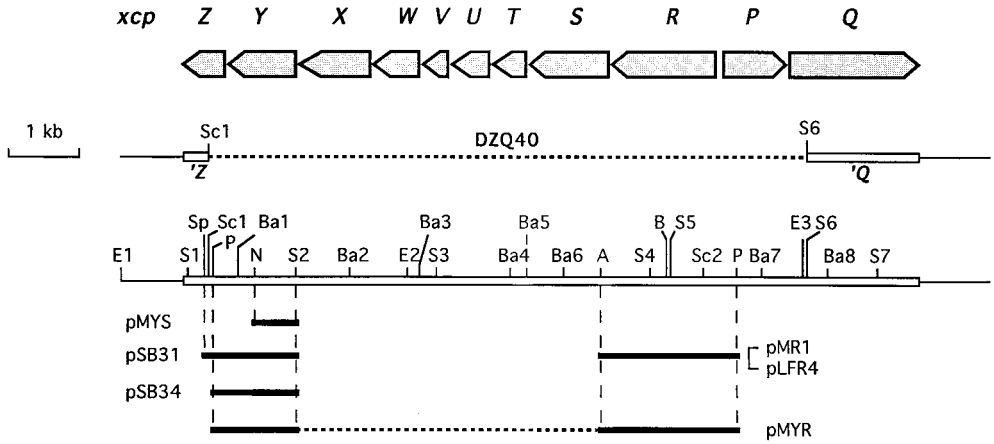


FIG. 1. Genetic organization and restriction map of the *P. aeruginosa xcp* region at 40 min. The 10.3-kb DNA fragment carrying the *xcpP* to *-Z* genes is shown by a double line; the 8.5-kb chromosomal sequence deleted in strain DZQ40 is shown by a dotted line; locations of plasmid subclones are indicated by bold lines. Restriction sites: A, *Asu*II; B, *Bam*HI; Ba, *Bal*I; E, *Eco*RI; N, *Not*I; S, *Sal*I; Sc, *Sca*I; Sp, *Sph*I; P, *Pst*I. Only the relevant positions are indicated for the *Asu*II, *Pst*I, and *Not*I restriction sites.

anoside (IPTG) was added in early exponential-phase cultures. Antibiotics used were tetracycline (100 μg/ml) and carbenicillin (300 μg/ml) for *P. aeruginosa* and tetracycline (20 μg/ml) and ampicillin (50 μg/ml) for *E. coli*.

Plasmid and DNA procedures. Plasmid DNA isolation and manipulations were as described by Maniatis et al. (26). The transmembrane and C-terminal domains of XcpY were deleted by blunt-end ligation between the filled-in *Not*I site in *xcpY* cloned in pSB34 and the filled-in *Xma*I site in the polylinker of pYZ4 vector. Insertion of an in-frame stop codon following the *xcpY′* sequences was generated by the procedure of Fellay et al. (12), using the Ω element carried on pH45Ω. The truncated *xcpY* was subsequently transferred into pMMB190 as a 0.7-kb *Sal*I-*Hin*dIII fragment by using restriction sites provided by pYZ4 and the Ω element.

Antisera production. XcpR antiserum against a fusion protein expressed under control of the bacteriophage lambda p_R promoter in the plasmid vector pEX3 was raised in a rabbit (41). The 0.6-kb *Sal*I-*Asu*II DNA fragment containing the 3′ region of *xcpR* was cloned in frame into the pEX3 polylinker sequence, resulting in a *cro′-lacI′-lacZ′-′xcpR* gene fusion. *E. coli* pop2136, which carries the gene coding for the cIts857 repressor, was transformed with the recombinant plasmid. To isolate the fusion protein, cells were grown to log phase at 30°C and transferred at 42°C for 2 h. Subsequently, cells were pelleted and lysed as described by Kusters et al. (23). The hybrid protein was recovered under an aggregated form after lysate centrifugation and loaded onto a preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel. After polyacrylamide gel electrophoresis (PAGE), the protein band was stained with 1 M potassium acetate, cut out, and electroeluted in an ISCO sample concentrator cup. Purified hybrid protein (approximately 0.2 mg) was emulsified with Freund's adjuvant (Sigma) and injected subcutaneously into an adult rabbit. Booster injections were given at 3-week intervals.

Antibodies directed against XcpY were obtained by using a glutathione *S*-transferase (GST) fusion protein. A construction coding for a GST-XcpY protein fusion was generated by cloning a DNA fragment containing entire *xcpY* into pGEX-2T (40). PCR was used to isolate the coding sequence of *xcpY* and create in-frame gene fusion. The hybrid protein was purified by affinity chromatography on glutathione-agarose beads as described elsewhere (9).

Subcellular fractionation and membrane analysis. *P. aeruginosa* cells were grown until the transition between the late exponential phase and the beginning of stationary phase. Expression of the *xcp* genes increases during this period of growth, thereby facilitating immunodetection of XcpR (10). *E. coli* was grown to mid-exponential phase. Approximately 2×10^9 cells were harvested by low-speed centrifugation, and pellets were washed and resuspended in 10 mM Tris-HCl (pH 8). The cells were then disrupted by sonication (three pulses of 10 s each) in the presence of 1 mM phenylmethylsulfonyl fluoride and 1 mM EDTA to inhibit proteolytic activities. Unbroken cells and cellular debris were removed by low-speed centrifugation, and soluble and membrane fractions were separated by ultracentrifugation for 60 min at 100,000 × *g* (Beckman TLA-45 rotor). Cytoplasmic and periplasmic proteins in the supernatant were precipitated with 10% (wt/vol) trichloroacetic acid. Pelleted proteins were washed with ice-cold acetone, resuspended in sample buffer, and examined by SDS-PAGE and immunoblotting.

Protein extractions were carried out by using membranes resuspended in 10 mM Tris-HCl (pH 8)–0.4 mM phenylmethylsulfonyl fluoride. Incubation in the presence of 1.5 or 5 M urea–1 M NaCl–0.1 M NaOH–0.1 M Na_2CO_3 (pH 11)–2% Triton X-100 containing 1 mM $MgCl_2$ or 2% sarcosyl (*N*-lauroylsarcosine) was performed on ice for 30 min. Samples were centrifuged at 100,000 × *g* for 1 h. Proteins in the pellet were solubilized in SDS-PAGE sample buffer. The supernatants were fivefold diluted; proteins were precipitated with trichloroacetic acid and resuspended in sample buffer.

P. aeruginosa inner and outer membranes were separated as described previously (4). Briefly, cells were grown in TSB, harvested, and disrupted in a French pressure cell (40,000 lb/in^2). Unbroken cells were removed, and the lysate was applied to a discontinuous sucrose gradient. After centrifugation for 16 h in a Beckman SW41Ti rotor at 183,000 × *g*, fractions were collected from the top.

SDS-PAGE and immunoblotting. Samples were solubilized by heating for 5 min at 95°C in sample buffer (2% SDS, 0.75 M β-mercaptoethanol, 10% glycerol, 60 mM Tris-HCl [pH 6.8], 0.02% bromophenol blue) prior to electrophoresis on 11% acrylamide gels. Proteins were electrophoretically transferred onto nitrocellulose membranes (Transblot SD apparatus; Bio-Rad). Filters were blocked with 5% nonfat milk in Tris-buffered saline and then incubated with appropriate antisera. Immunodetection of XcpR and XcpZ in *P. aeruginosa* was performed in the presence of concentrated cell extracts of the *xcpP* to *-Z* deletion strain DZQ40 in order to reduce the immunoreaction background. Antiserum against elastase was obtained as described elsewhere (21). Reactions with antisera were developed with secondary antibodies conjugated to horseradish peroxidase and visualized by chemiluminescence (Pierce).

RESULTS

Subcellular localization of XcpR in *P. aeruginosa*. Among the 12 identified Xcp components (XcpP to -Z encoded by the 40-min locus [Fig. 1] and XcpA/PilD [4]), XcpR is the only protein that lacks a hydrophobic domain and exhibits the general characteristics of a cytoplasmic protein (5). To determine its cellular localization, soluble and membrane fractions of *P. aeruginosa* wild-type strain PAO1 were analyzed by SDS-PAGE followed by immunodetection with anti-XcpR antibody. As shown in Fig. 2A, XcpR was confined to the membrane fraction (lane 4) and was not detected in the fraction containing cytoplasmic and periplasmic proteins (lane 3). Separation of membrane fractions by centrifugation through a sucrose gradient showed that XcpR is associated with the low-density fractions corresponding to the cytoplasmic membrane. The nature of the association was further examined by incubating corresponding fractions in the presence of various reagents and studying the susceptibility of XcpR to extraction. Alkali treatments (0.1 N NaOH, 0.1 M Na_2CO_3 [pH 11]), high salt (1 M NaCl), or protein denaturant (5 M urea) released almost all XcpR, suggesting that it is peripherally bound to the cytoplasmic membrane. Treatment of membranes with the nonionic detergent Triton X-100 resulted in complete solubilization (data not shown).

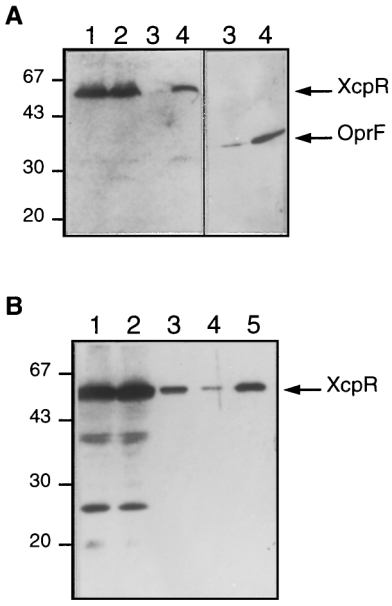


FIG. 2. Subcellular localization of XcpR in *P. aeruginosa*. Soluble and membrane fractions were prepared as described in Materials and Methods. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunodetected with anti-XcpR serum. (A) Fractionation of wild-type PAO1 cells. Lanes: 1, total cells; 2, cell lysate; 3, soluble fraction; 4, membrane fraction. Immunodetection of the outer membrane protein OprF is shown as a fractionation control (right panel). Positions of protein molecular mass markers are indicated on the left in kilodaltons. (B) Fractionation of DZQ40/pMR1(*xcpR*) cells grown in the presence of 2 mM IPTG. The membrane pellet was extracted with 2% Triton X-100 in the presence of 1 mM $MgCl_2$ and centrifuged again. Lanes: 1, total cell lysate; 2, soluble fraction; 3, membrane fraction; 4, soluble material after Triton X-100 extraction of the membrane fraction; 5, insoluble material after Triton X-100 extraction. Positions of molecular mass markers are indicated on the left in kilodaltons.

Localization of XcpR in the absence of other Xcp proteins. Anticipating that XcpR might be membrane associated in *P. aeruginosa* through interaction with one or several Xcp proteins, we sought to determine its localization in the absence of the components encoded by the *xcpP* to *-Z* gene cluster. The 1.8-kb *Pst*I-*Asu*II DNA fragment that carries the *xcpR* gene was cloned into pMMB67HE to give pMR1 (Fig. 1). In this construction, *xcpR* is expressed under the control of the p_{tac} promoter. Plasmid pMR1 was introduced by triparental mating into strain DZQ40, a PAO1 derivative which is chromosomally

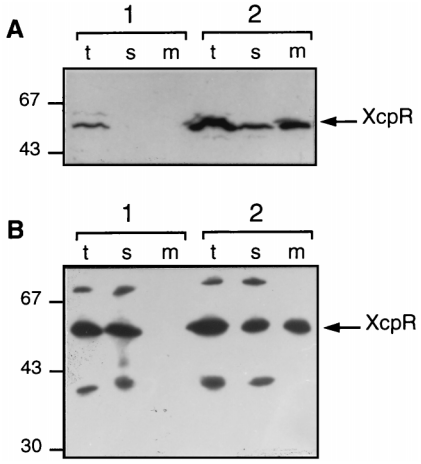


FIG. 3. Membrane association of XcpR in the presence of XcpY. (A) Expression of XcpR or of XcpR and XcpY in *P. aeruginosa*. Lane sets: 1, strain DZQ40/pLFR4(*xcpR*)/pMMB67HE; 2, strain DZQ40/pLFR4(*xcpR*)/pSB31-(*xcpY*). (B) Expression of XcpR in *E. coli* in the absence or presence of XcpY. Lane sets: 1, strains TG1/pMR1(*xcpR*)/pYZ4; 2, TG1/pMR1(*xcpR*)/pSB34(*xcpY*). Cells were grown in the presence of IPTG (2 mM for *P. aeruginosa*; 0.1 mM for *E. coli*) for specific expression of plasmid-encoded genes, and total cells (t) were fractionated into soluble (s) and membrane (m) fractions. Samples were analyzed by SDS-PAGE and immunoblotting with anti-XcpR serum. The band of lower molecular size corresponds to the pMR1-encoded LacI repressor (38.6 kDa) which is recognized by the serum. Positions of molecular mass markers are indicated on the left in kilodaltons.

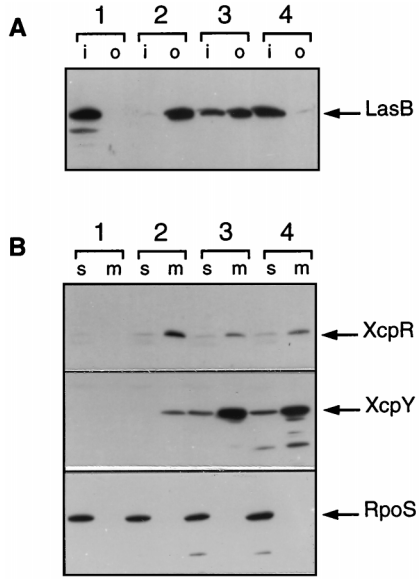


FIG. 4. Dosage-dependent complementation and interference in a *P. aeruginosa xcpY51* mutant. Strain KS910-503 (*xcpY51*) containing plasmid pMMB67HE (control; lane set 1) or pSB31(*xcpY*) (lane sets 2 to 4) was grown in TSB in the absence (lane sets 1 and 2) or presence of 0.1 (lane set 3) or 2 (lane set 4) mM IPTG. Cells and extracellular medium were separated by centrifugation. (A) Whole cells (i) and proteins in culture supernatants (o) were analyzed by SDS-PAGE and immunoblotting with anti-LasB serum. (B) Cells were disrupted by sonication and fractionated by centrifugation as described in Materials and Methods. Samples of soluble (s) and membrane (m) fractions corresponding to equivalent amounts of cells were analyzed by SDS-PAGE followed by immunoblotting with anti-XcpR and anti-XcpY sera. Immunodetection of the cytosolic RpoS sigma factor was performed as a fractionation control.

deleted for the *xcpP* to *-Z* genes (see Fig. 1 and Materials and Methods for details on strain construction). Following DZQ40/pMR1 cell fractionation and immunodetection, the XcpR protein was detected mainly in the soluble fraction (Fig. 2B, lanes 2 and 3). The low amount of protein found in the particulate fraction was not membrane associated because it was only weakly soluble in 2% Triton X-100 (Fig. 2B, lanes 4 and 5). Such behavior suggested that upon overproduction, a minor fraction of XcpR could form aggregates sedimenting with membrane vesicles, as suggested earlier for the homologous PulE protein of *K. oxytoca* (32). These results indicate that the association of XcpR to the cytoplasmic membrane of *P. aeruginosa* requires the expression of other Xcp proteins encoded by the 40-min locus.

Role of other Xcp proteins in the association of XcpR to inner membrane. The previous identification of an interaction between EpsL and EpsE, the XcpY and XcpR homologues in *V. cholerae* (38), was suggestive of a possible role of the XcpY protein in the membrane localization of XcpR. To test this hypothesis, the cloned *xcpR* and *xcpY* genes were expressed in the genetic background of the deletion strain DZQ40. Plasmid pLFR4, which carries *xcpR* under control of the native promoter, was mobilized into DZQ40. Figure 3A shows that XcpR was detected in cells of DZQ40/pLFR4 but was not recovered after the cell fractionation procedure (lane set 1). In contrast, the results in Fig. 2B show that XcpR overexpressed under p_{tac} promoter control in DZQ40 was stable during cell fractionation and recovered mainly in the soluble fraction. Possibly, a high expression level causes the accumulation of XcpR under a state, different from the native one, that increases the stability of the protein. Introduction of a second plasmid (pSB31, carrying *xcpY*) into DZQ40/pLFR4 led to increase in the level of XcpR. Under these conditions, XcpR was found to cofractionate mainly with the pellet (Fig. 3A, lane set 2), showing that XcpY alone can promote its association with the membrane of *P. aeruginosa*.

Although it seems clear that XcpY is required for the subcellular location of XcpR, the effect of XcpY could still be indirect, with some other, unidentified component providing a link between XcpY and XcpR. To further address this question, we examined the localization of XcpR in the heterologous genetic background of *E. coli*. When expressed alone in strain TG1/pMR1, XcpR was detected exclusively in the soluble fraction (Fig. 3B, lane set 1). The *xcpY* gene cloned on the compatible plasmid pSB34 was introduced into TG1/pMR1. Immunodetection after cell fractionation showed that approximately half of XcpR was membrane associated (Fig. 3B, lane set 2). The dual localization of XcpR in membrane and soluble fractions observed here might be related to some limitation in the number of XcpY molecules inserted into the cytoplasmic membrane.

Dosage-dependent complementation and interference in the *xcpY51* mutant. Previous studies have shown that introduction of a wild-type *xcpY* gene in the *xcpY51* mutant strain restores the secretion phenotype (14). To analyze the effect of the *xcpY51* complementation on the localization of XcpR, we mobilized plasmid pSB31, carrying *xcpY* under p_{tac} control, into strain KS910-503 (*xcpY51*). As shown in Fig. 4B, XcpY was not detected in extracts of the control strain KS910-503/pMMB67HE (lane set 1). Sequencing of chromosomal DNA from *xcpY51* identified a frameshift mutation that is responsible for the synthesis of a modified protein with a basic C-terminal domain that probably impairs membrane insertion and causes a rapid degradation (28). The presence of the wild-type *xcpY* gene in *trans* allowed production of a detectable amount of XcpY in KS910-503/pSB31 without IPTG induction (Fig. 4B, lanes 2). Protease plate assay (not shown) and im-

munodetection of LasB elastase, the major protease secreted by the Xcp pathway (43), showed that the secretion defect of *xcpY51* was complemented (Fig. 4A, lane set 2). It is known that LacI repression of the p_{tac} promoter is incomplete in *P. aeruginosa* (2), and the basal expression level of *xcpY* from uninduced p_{tac} on pSB31 thus appears sufficient for phenotypic complementation of the *xcpY51* mutation. This complementation was correlated with the presence of XcpR in the membrane fraction, whereas trace amounts of XcpR were detected only in the soluble compartment of the control strain (Fig. 4B, lane sets 1 and 2).

To examine the effects of an increased expression of *xcpY*, we repeated the experiment in the presence of inducer. Addition of 0.1 mM IPTG during the growth of KS910-503/pSB31 resulted in a higher level of XcpY (Fig. 4B, lane set 3) and in a partial secretion defect, as shown by the accumulation of mature-sized LasB in total cell fraction (Fig. 4A, lane set 3). This result suggested that the overproduced XcpY might interfere with normal extracellular protein secretion. A small amount of XcpY, possibly representing molecules that failed to enter the export pathway to be inserted into the cytoplasmic membrane, was detected in the soluble fraction of induced cells. Immunodetection of XcpR showed that induced cells contained reduced amounts of the protein but did not reveal a marked difference in its subcellular distribution, indicating that the overexpressed XcpY efficiently binds XcpR to the membrane. The secretion defect was not the result merely of a decreased level of XcpR, because a stronger induction by 2 mM IPTG resulted in a complete block of elastase secretion (Fig. 4A, lane set 4) whereas the amount of XcpR was unchanged (Fig. 4B, lane set 4).

Since the XcpR-XcpY complexes were formed, we assumed that one or several other components of the secretion machinery were becoming limiting for interaction or coassembly with XcpY, XcpR, or both, so that the chromosomally encoded XcpR was displaced from the functional machinery by the excess of membrane-bound XcpY located outside the active secretion complexes. To test this hypothesis, we reasoned that if the negative effect is brought about by sequestration of XcpR from functional complexes, it should be overcome by the concomitant overexpression of XcpR. To this end, the *xcpR* and *xcpY* genes were cloned together under p_{tac} control into pMMB67, to give pMYR. This construct was introduced into PAO1, and the effects of IPTG induction were analyzed in comparison to PAO1 carrying *xcpY* alone on pSB31. In the absence of induction, both strains secreted LasB into the extracellular medium (Fig. 5A, lane sets 1 and 3). In the presence of 2 mM IPTG, the LasB elastase produced by PAO1/pSB31 was accumulated inside the cells (lane set 2), while the protein was secreted by PAO1/pMYR and not detected in the cellular compartment (lane set 4). Immunodetection of XcpY and XcpR showed that the two proteins were efficiently overproduced from pMYR (Fig. 5B). We thus concluded that the increased amount of XcpR allows the restoration of a number of active secretion complexes because it can saturate the excess of XcpY responsible for the secretion interference.

Competitive inhibition by the soluble domains of XcpY. XcpY is an inner membrane protein with a single transmembrane segment which connects two hydrophilic domains (8). This topology is consistent with an interaction between XcpR and the N-terminal cytoplasmic domain of XcpY. To determine if this was the case, we constructed a 3′-truncated *xcpY* gene that lacks the sequences for the transmembrane and the C-terminal periplasmic regions. We introduced the truncated allele carried on pMYS in PAO1 and examined whether production of the cytoplasmic portion of XcpY ($XcpY^N$) could

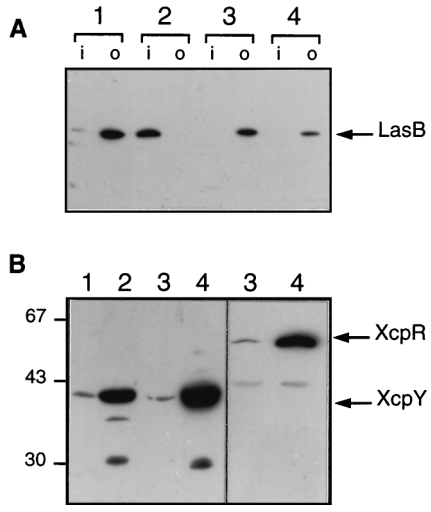


FIG. 5. Phenotypic suppression of interference by XcpR overexpression. Wild-type strain PAO1 containing plasmid pSB31 (*xcpY*; lane sets 1 and 2) or pMYR (*xcpR xcpY*; lane sets 3 and 4) was grown in the absence (lane sets 1 and 3) or presence (lane sets 2 and 4) of 2 mM IPTG. (A) Proteins in cell lysates (i) or culture supernatants (o) were separated by SDS-PAGE, blotted, and probed with anti-LasB serum. (B) Equivalent amounts of cellular proteins were analyzed by SDS-PAGE and detected with anti-XcpY (left) or anti-XcpR (right) serum. Positions of molecular mass markers are indicated on the left in kilodaltons.

compete for the XcpR-XcpY interaction. Data in Fig. 6B show that upon increasing synthesis of the $XcpY^N$ product by induction of p_{tac} on pMYS, the majority (lane set 2) or the totality (lane set 3) of XcpR partitioned with the soluble fraction. Correlated with the displacement of XcpR from the membrane, secretion of extracellular LasB was impaired or abolished (Fig. 6A, lane sets 2 and 3). These results strongly indi-

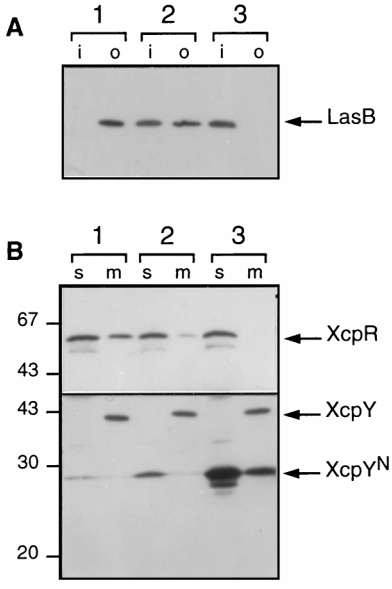


FIG. 6. Dominant negative effect of truncated XcpY protein. Strain PAO1 carrying pMYS (*xcpY′*) was grown in TSB in the absence (lanes 1) or presence of 10 (lane set 2) or 100 μM (lane set 3) IPTG. (A) Same as for Fig. 5A. (B) Cellular proteins were separated by SDS-PAGE and immunodetected with anti-XcpR (top) or anti-XcpY (bottom) serum. The truncated form of XcpY ($XcpY^N$) is indicated. Positions of molecular mass markers are indicated on the left in kilodaltons.

cate that the truncated XcpY exerts a dominant negative effect on secretion by competing with chromosomally encoded XcpY for the formation of XcpR-XcpY active complexes. Remarkably, the low level of $XcpY^N$ produced in the absence of inducer was nevertheless sufficient to displace approximately half of XcpR from the membrane, while secretion was apparently not impaired (Fig. 6A, lane set 1). Thus, the secretory pathway can tolerate a large decrease in the number of functional XcpR molecules without effect on the efficiency of extracellular release, suggesting that the step involving XcpR is not limiting under the conditions used.

The accumulation of soluble forms of XcpR in the presence of $XcpY^N$ indicates that XcpR has no permanent interaction with another membrane protein than XcpY. However, as the effects of XcpY overproduction (Fig. 4) support the existence of at least one additional partner interacting with the XcpR-XcpY complex, we considered the possibility that the C-terminal domain of XcpY is involved in interactions with some other component(s) in the periplasm. Plasmid pSB72 carries an in-frame gene fusion between the sequences encoding the LasB signal peptide ($LasB^{SP}$) and those corresponding to the C-terminal periplasmic domain of XcpY ($XcpY^C$; residues 259 to 382) (28). When the $LasB^{SP}$-$XcpY^C$ hybrid was expressed in PAO1, the LasB signal peptide was cleaved off and the $XcpY^C$ domain was immunodetected in the periplasm. However, LasB secretion was unaffected by the accumulation of this periplasmic form of XcpY (not shown).

DISCUSSION

In this report we show that XcpR, despite its hydrophilic character, is associated with the cytoplasmic membrane in *P. aeruginosa*. Membrane association is not an intrinsic property of XcpR but requires the *xcpY* gene product. XcpY spans the membrane once (8) and has a large N-terminal domain that faces the cytoplasm and likely interacts with XcpR. Consistent with this inference, the expression in *trans* of the soluble N-terminal region of XcpY resulted in a cytoplasmic location of XcpR and was inhibitory to protein secretion.

Previous studies by Sandkvist et al. (38) established that EpsE, the *V. cholerae* XcpR homologue, is associated to the cytoplasmic membrane through interaction with EpsL, the XcpY homologue in this bacteria. Our data obtained with the *P. aeruginosa* system support the prediction that the pattern of interactions taking place during the secretion process must be conserved in the GSP from different bacteria. However, it is notable that the *K. oxytoca* XcpR homologue, PulE, has been found associated with the particulate fraction in a reconstituted system in *E. coli* regardless of whether the other Pul functions were expressed. As suggested by Possot and Pugsley (32), this discrepancy could be related to a peculiar propensity of PulE to form small aggregates that associate with membrane vesicles when the protein is produced in the absence of the complete set of Pul secretion proteins.

Beside anchoring the XcpR protein to the cytoplasmic membrane, XcpY also appears to be required for its accumulation in *P. aeruginosa*. The cellular level of plasmid-expressed XcpR in the *xcpP* to *-Z* chromosomal deletion strain is considerably increased by the expression of a wild-type *xcpY* allele in *trans*. Similarly, the level of XcpR is very low in the *xcpY*-defective KS910-503 strain but restored to a wild-type level in the presence of XcpY. The reason for the apparent instability is unclear but may be due to an altered conformation in the absence of XcpY resulting in susceptibility to cellular proteases. In the *V. cholerae* system, the expression of EpsL also resulted in stabilization of the membrane-associated EpsE (38).

The relative stoichiometry between XcpR and XcpY seems to be crucial for the functioning of the secretion machinery. Overexpression of XcpY causes a negative secretion phenotype, although the membrane localization of XcpR is apparently not altered. Our interpretation of these observations is that the amount of chromosomally encoded XcpR is sequestered by the large majority of XcpY molecules that have failed to appropriately interact with the other component(s) of the secretory apparatus. The excess of XcpY might thus interfere with the assembly of the system because limiting amounts of other interacting Xcp proteins may not be able to provide final active secretion complexes. Concomitant overexpression of XcpR can suppress the inhibitory effect very likely by reestablishing a stoichiometric equilibrium with XcpY, so that the population of XcpY molecules correctly located can be saturated by XcpR. Consistent with the notion of at least a second protein-protein interaction involving XcpY, recent work of this laboratory indicates that XcpY has additional interactions with XcpZ (28). Furthermore, interference by XcpY overproduction appears to be partially relieved by an increased level of XcpZ, suggesting that this protein could be required for activity of the XcpR-XcpY binary complexes or their targeting to secretion sites. Of course, the observed relief of interference could be related to more indirect events; further work is needed to determine the primary effect of XcpZ overexpression that might lead to the phenotypic suppression effect.

Current data are insufficient to propose a definitive model for the function of XcpR family members in GSP. These proteins have a typical Walker box A which is essential for their activity (32, 38, 44). Although attempts to demonstrate ATPase activity were not successful (27, 38), these proteins could carry out functions related to an energetic step of the secretory pathway. By analogy with the role of the homologous protein PilB in type IV pilus assembly, it has been proposed that XcpR participates in the membrane organization of the five so-called pseudopilins XcpT, -U, -V, -W, and -X into a polymeric structure spanning the periplasm (5, 19, 34). The recent isolation of a mutation of *xcpR* that is capable of suppressing a temperature-sensitive allele of *xcpT* supports the possibility of an interaction between XcpR and pseudopilins (22). It might be also that XcpR participates in a gatekeeping function and somehow regulates the opening of the outer membrane pore constituted by XcpQ (7), possibly by modulating the polymeric state of the pseudopilins that could act as a plug in the absence of exoprotein movement through the channel. Whether it is required for steps in the assembly of a macromolecular Xcp complex or for the regulated activity of the secretory apparatus, XcpR probably does not act directly in the cytoplasm. On the basis of the association between XcpR and XcpY reported here, one appealing possibility is that XcpY contributes to coupling an energy-dependent activity of XcpR to the process of outer membrane translocation, maybe by transmitting a conformational change to upper components of the system. Further understanding of XcpR and XcpY interactions is required before such functional aspects can be addressed.

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