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Membrane Topology of Three Xcp Proteins Involved in Exoprotein Transport by *Pseudomonas aeruginosa*

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Xcp proteins constitute the secretory apparatus of *Pseudomonas aeruginosa*. Deduced amino acid sequence of *xcp* genes, expression, and subcellular localization revealed unexpected features. Indeed, most Xcp proteins are found in the cytoplasmic membrane although *xcp* mutations lead to periplasmic accumulation of exoproteins, indicating that the limiting step is translocation across the outer membrane. To understand the mechanism by which the machinery functions and the interactions between its components, it is valuable to know their membrane organization. We report data demonstrating the N_{in}-C_{out} topologies of three general secretion pathway components, the XcpP, -Y, and -Z proteins.

Pseudomonas aeruginosa is a gram-negative bacterium which secretes several virulence factors (9) via a two-step mechanism. The first step is the signal peptide-dependent inner membrane translocation (15), and the second step requires additional components for translocation of the secreted proteins across the outer membrane. This pathway is well conserved among gram-negative bacteria (4) and was named general secretion pathway (11). In *P. aeruginosa*, the secretion apparatus includes 12 proteins, encoded by the *xcp* genes (18). Only one, XcpQ, is an outer membrane protein (1). XcpT, -U, -V, and -W present strong similarities with the N terminus of the major subunit of type IV pili (PilA) from *P. aeruginosa* (2). They are synthesized as inner membrane precursors from which the leader peptide is cleaved by the inner membrane peptidase XcpA (PilD) (2), a polytopic inner membrane protein. The mature forms of these pilin-like Xcp components may then be assembled into a complex structure forming the secretion apparatus. XcpR and XcpS are homologous to PilB and PilC, respectively, two proteins which are essential for type IV pilus biogenesis in *P. aeruginosa* (10) and thus by analogy are presumably involved in the biogenesis of the pilin-like Xcp components. XcpS is a polytopic inner membrane protein, and XcpR carries a Walker motif (20). The remaining proteins, XcpP, -X, -Y, and -Z, are inner membrane components, the functions of which in *P. aeruginosa* have not been identified. However, it was recently suggested that, in *Vibrio cholerae*, the homolog of XcpY (EpsL) could be involved in membrane association of EpsE (XcpR homolog) (14). An intriguing aspect of this model is that even though the majority of Xcp proteins are localized in the inner membrane, *xcp* mutations lead to periplasmic accumulation of mature exoproteins and thus block outer membrane translocation. To elucidate the functions and possible interactions of XcpP, -Y, and -Z, we investigated their membrane topology with alkaline phosphatase or β-lactamase as a topology probe.

Isolation of *xcpY-blaM* and *xcpY-phoA* fusions. XcpY is a protein of 382 residues. It is located in the cytoplasmic membrane (6), and computer analysis, using the TOPPRED algorithm of G. von Heijne (19), predicted three potential transmembrane segments. Segment II (between residues 234 and 254) was proposed as certain, and segments I (1 to 21) and III (303 to 323) were proposed as tentative. The four possibilities, in which segment II is always a transmembrane segment, are presented in Fig. 1A. We constructed three in vitro gene fusions with *blaM* and *phoA* as reporter genes (Fig. 1B). The 1.2-kb *Sal*I-*Pvu*II DNA fragment from pUEX385 (*xcpX*, *-Y*, and *-Z* cloned in pUC19) (4), which contains the almost complete *xcpY* gene, was inserted between the *Sal*I and *Pvu*II sites of pJBS633 (3). The construct resulted in the fusion of mature β-lactamase to residue Q-363 of XcpY, downstream from the third tentative transmembrane segment (pSB3). The hybrid gene sequence was checked by using the primer AFO1 (5′-CTCGTGCACCCAACTGA-3′), which hybridizes between codons Q-16 and R-20 of mature β-lactamase and extends in the direction of the fusion junction. The recombinant protein was identified by immunoblot analysis (data not shown) and was able to protect *Escherichia coli* against ampicillin (up to 500 μg/ml on plates). We concluded that XcpY exposes its C terminus to the periplasmic side of the membrane, which rules out prediction γ (Fig. 1A). An *xcpY-phoA* gene fusion was generated by inserting the 2.6-kb *Xma*I DNA fragment of pPHO7 (7), carrying the coding region for mature alkaline phosphatase, into the *Not*I site of pUEX385 (pSB4). This resulted in a fusion at codon W-232, between segments I and II of XcpY. The sequence was checked by using AFO2 (5′-GC CCTGAGCAGCCCGGTT-3′), which hybridizes between codons G-9 and N-14 of mature alkaline phosphatase. The corresponding *E. coli* transformants were white on XP medium (5-bromo-4-chloro-3-indolylphosphate), indicating that PhoA was exposed on the cytoplasmic side of the membrane (8). The hybrid protein was readily detected by immunoblot analysis with a polyclonal antiserum directed against PhoA (data not shown). This eliminates hypothesis δ (Fig. 1A). The only known bacterial inner membrane protein having a large N-terminal periplasmic domain, not preceded by a translocation signal, is the proW protein of *E. coli* (21). In this case, there are a large number of negatively charged residues in the N tail. This is not the case for XcpY in hypothesis β (Fig. 1A). To definitely eliminate this possibility, we made a fusion by using PCR methodology. The primer AFO15 (5′-TCGCGGATC CTCGGGAAACAA-3′), hybridizing between codons L-275

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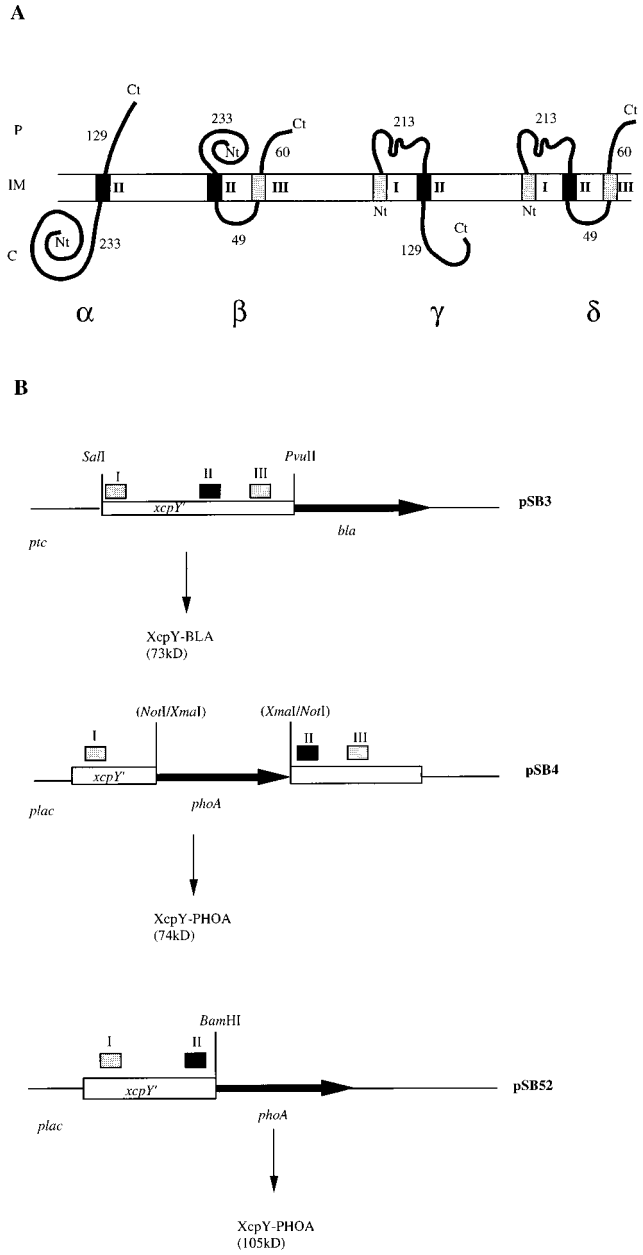

FIG. 1. (A) Four possible topologies for XcpY. P, periplasm; IM, inner membrane; C, cytoplasm. The number of amino acids contained in each extramembrane domain is indicated. The N and C termini are labelled Nt and Ct, respectively. (B) Construction of *xcpY-bla* and *xcpY-phoA* gene fusions. The size of the fusion protein is indicated in parentheses. The reporter gene is represented by the thick black arrow. *ptc* and *plac* are the promoters of the tetracycline resistance gene and the *lac* operon, respectively. ▨, tentative transmembrane segment; ■, certain transmembrane segment.

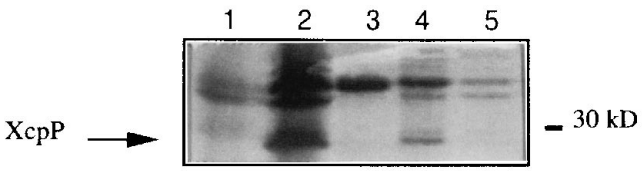

FIG. 2. Subcellular fractionation of the XcpP protein. Lanes 1 and 2, total cells; lane 3, outer membrane fraction; lane 4, inner membrane fraction; lane 5, soluble fraction. XcpP is indicated by an arrow. The band observed in lane 3 corresponds to the major outer membrane porins OmpF and OmpC. The molecular mass standard is indicated on the right.

and E-278, between segments II and III, was used to amplify the region encoding the N-terminal part of XcpY, which was then fused to the *phoA* gene (pSB52). All recombinant clones were blue on XP plates. In conclusion, XcpY is a type II bitopic inner membrane protein with a single transmembrane segment between residues 234 and 254. The N terminus is in the cytoplasm (233 residues) and the C terminus (129 residues) exposed to the periplasm (hypothesis α [Fig. 1A]).

Isolation of *xcpP*- and *xcpZ-phoA* fusions. XcpP and -Z are proteins of 235 and 174 residues, respectively. Computer analysis (TOPPRED) predicts that each protein has one transmembrane segment: positions 31 to 51 in XcpP and positions 33 to 53 in XcpZ. Only XcpZ has been shown experimentally to be an inner membrane component (6). The *xcpP* gene on a 1.5-kb *Sal*I-*Eco*RI DNA fragment from pUAWB5 was placed under the T7 promoter of pT7.6 (17), yielding pSB9. *E. coli* BL21(DE3) cells (16) containing the plasmid control pT7.6 (Fig. 2, lane 1) or plasmid pSB9 (Fig. 2, lanes 2 to 5) were induced for expression and pulse-labelled for 30 s with [^{35}S]methionine, and subcellular fractions were obtained as previously described (2, 6). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in gels containing 11% acrylamide and exposed to Kodak X-ray film after drying. The experiment clearly showed that XcpP is a cytoplasmic membrane protein (Fig. 2). We constructed three appropriate in vitro fusions to determine the orientation of the two proteins in the membrane (Fig. 3). The *phoA* cassette of pPHO7 was obtained as a 2.6-kb *Sma*I fragment and ligated into the *Msc*I site of the *xcpP* gene. The fusion junction was thus at codon L-173 of XcpP downstream from the region encoding the transmembrane segment (Fig. 3A). The *E. coli* transformants were strongly blue on XP plates, indicating that the C terminus, which represents most of the protein (189 residues), spans the periplasm. The *phoA* cassette as an *Xma*I fragment was inserted into the *Apa*I site of *xcpZ* at codon L-11, upstream from the region encoding the transmembrane segment (Fig. 3B). *E. coli* transformant colonies were white on XP plates. The hybrid protein was clearly detected by immunoblot analysis (Fig. 4), indicating that the lack of activity is linked to the localization of the PhoA part of the fusion and not to a low expression level. Subcellular fractionation showed that the product was mainly recovered in the cytoplasmic fraction, while the alkaline phosphatase, constitutively expressed from *E. coli* BW3627 (B. Wanner collection), was correctly localized to the periplasmic fraction (Fig. 4). The DNA sequence of both constructions was checked by using the AFO2 primer. An additional *xcpZ-bla* gene fusion was constructed. The 1-kb *Hin*cII DNA fragment from pUEX385, which contains the almost complete *xcpZ* gene, was inserted between the *Eco*RV and *Pvu*II sites of pJBS633 (Fig. 3C). The hybrid resulted in the fusion of mature β-lactamase to residue V-120 of XcpZ, thus downstream from the transmembrane segment. As expected, the recombinant protein was able to protect *E. coli* cells against ampicillin (up to 300 μg/ml on plates). Thus, like XcpP, the C terminus of XcpZ (126 residues) is in the periplasmic space (Fig. 3D).

Many gram-negative bacteria possess a general secretory pathway by which proteins with peptide signal are secreted. Data obtained for many gram-negative bacteria including *Klebsiella oxytoca*, *Erwinia chrysanthemi* or *Erwinia carotovora*, *Xanthomonas campestris*, *Aeromonas hydrophila*, *V. cholerae*, and *Pseudomonas putida* and *P. aeruginosa* point out the complexity

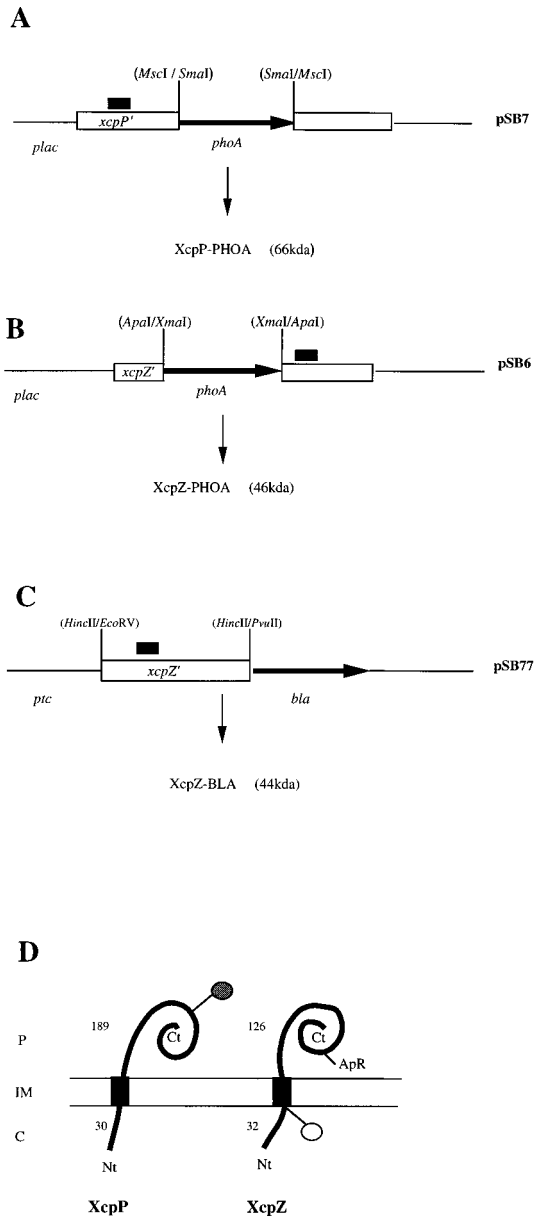

FIG. 3. Construction of *xcpP-phoA* (A), *xcpZ-phoA* (B), and *xcpZ-bla* (C) gene fusions, leading to the determination of the membrane organization of XcpP and XcpZ proteins as shown in panel D. Symbols and abbreviations are as for Fig. 1B. ● and ○ or ApR in panel D indicate the position of alkaline phosphatase or β-lactamase, respectively, in the hybrid protein. The dotted or white circle indicates that the strain containing the corresponding gene fusion is blue or white, respectively, on XP medium.

of the secretion machinery and its conservation among these various organisms. The relatedness among proteins of the various systems, as assessed from sequence similarities, implies that these proteins present similar topologies and functions. Detailed characterization of all the components will be necessary to understand how they assemble and interact in the cell envelope. Topological studies are under way for the Out proteins of *E. chrysanthemi* (13). We report the topologies of three general secretion pathway proteins, briefly described in the Pul system (5, 12) but not yet determined in the Out system, and thus we contribute to confirming the general picture of this secretion pathway. The three proteins XcpP, -Y, and -Z each

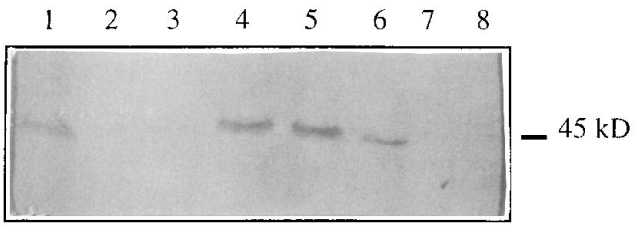

FIG. 4. Subcellular fractionation of the XcpZ-PhoA hybrid (lanes 1 to 4) and alkaline phosphatase (lanes 5 to 8). Lanes 1 and 5, total cells; lanes 2 and 6, periplasmic fraction; lanes 3 and 7, cell envelope; lanes 4 and 8, cytoplasmic fraction. The molecular mass standard is indicated on the right.

have a single transmembrane segment and adopt an N_{in}-C_{out} topology. In the case of XcpY, our experiments clearly demonstrate a large N-terminal domain in the cytoplasm. This topology is in good agreement with the presumed function of this protein as determined from recent work with *V. cholerae* in which it was shown that EpsL (XcpY homolog) interacted with the EpsE autokinase (XcpR homolog), located in the cytoplasm (14). Interestingly, in both XcpP and -Z proteins the larger moiety is in the periplasm. Although the function of these two proteins has yet to be determined, their C termini may interact with other components of the Xcp machinery on the periplasmic side of the membrane and possibly with the outer membrane protein XcpQ. An investigation of their putative interactions may be fruitful.

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