Cleavage, Methylation, and Localization of the *Pseudomonas aeruginosa* Export Proteins XcpT, -U, -V, and -W

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Four components of the apparatus of extracellular protein secretion of *Pseudomonas aeruginosa*, XcpT, -U, -V, and -W (XcpT-W), are synthesized as precursors with short N-terminal leader peptides that share sequence similarity with the pilin subunit of this organism. A specialized leader peptidase/methylase, product of the *pilD* gene, has been shown to cleave the leader peptide from prepilin and to methylate the N-terminal phenylalanine of the mature pilin. Antibodies were prepared against XcpT-W and used to purify each of these proteins. Sequence analysis of XcpT-W has shown that these proteins, like mature pilin, contain N-methylphenylalanine as the N-terminal amino acid. Analysis of cellular fractions from wild-type and *pilD* mutant strains of *P. aeruginosa* showed that the precursor forms of XcpT-W are located predominantly in the bacterial inner membrane, and their localization is not altered after PilD-mediated removal of the leader sequence. These studies demonstrate that the biogenesis of the apparatus of extracellular protein secretion and that of type IV pili share a requirement for PilD. This bifunctional enzyme, acting in the inner membrane, cleaves the leader peptides from precursors of pilins and XcpT-W and subsequently methylates the amino group of the N-terminal phenylalanine of each of its substrates.

The extracellular secretion of proteins by gram-negative bacteria requires specialized machinery to allow for the selective passage of proteins across the normally impermeable outer membrane. *Pseudomonas aeruginosa*, a gram-negative opportunistic pathogen, is capable of exporting a variety of extracellular hydrolytic enzymes and toxins (i.e., exotoxin A, phospholipase C, elastase, alkaline phosphatase, and lipase) that can lead to tissue damage during the course of an infection. Precursors of these proteins are synthesized with a typical leader sequence and rely on components of the primary secretory pathway (*sec* genes and leader peptidase) for initial steps in their translocation to the periplasmic space. Further translocation to the extracellular supernatant requires the functions of a general export apparatus composed of at least eleven *xcp* gene products (13, 25). Similar export systems, composed of analogous gene products, have been found in at least four other gram-negative genera, including *Klebsiella* (*pul*) (20), *Erwinia* (*out*) (8), *Xanthomonas* (*xps*) (6), and *Aeromonas* (*exe*) (4).

We and others have cloned and sequenced four of the *P. aeruginosa* *xcp* genes, *xcpT*, *xcpU*, *xcpV*, and *xcpW* (referred to earlier as *pddA-D*, but the *xcp* nomenclature will be used in this report) and determined that all encode products having N-terminal amino acid residues that are highly homologous to sequences of the type IV pilin subunit of several gram-negative pathogens (3, 18). This region of the type IV pilin subunit displays several characteristic features, including the presence of a six- to eight-amino-acid N-terminal leader followed by the conserved sequence -Gly-Phe-Thr-Leu-(Leu or Ile)-Glu-. Following proteolytic cleavage of the leader sequence of pilin between the glycine and phenylalanine residues, N methylation of the phenylalanine residue occurs. This site-specific cleavage is carried out by an inner membrane peptidase, PilD (17). We have also recently shown that the N-methylation reaction is carried out by PilD

(24). In addition to its role in pilus biogenesis, PilD (XcpA) is required for extracellular protein export. Mutants in the *pilD* gene are unable to export exotoxin A, phospholipase C, elastase, or alkaline phosphatase (2, 23). Because of the similarities among XcpT, -U, -V, and -W (XcpT-W) and the type IV pilin subunit, we have proposed that the role of PilD in protein export is to cleave and methylate precursors of XcpT-W, leading to their maturation (18).

In this study, we prepared antibodies to XcpT-W and used them to demonstrate that PilD-dependent cleavage and methylation of XcpT-W occurs in vivo, accounting for the export-deficient phenotype of a *pilD* mutant. We also used the antibody preparations to determine the membrane localization of both precursor and mature forms of XcpT-W in PAK strains expressing single-copy chromosomal *xcpT-W* genes. Our results clearly show that cleavage and methylation of XcpT-W by PilD has little or no effect on membrane localization. These studies provide evidence that the maturation of these proteins bears a striking similarity to the early stages of biogenesis of type IV pili, suggesting that XcpT-W are themselves assembled into a physical structure resembling pili that is required for the formation and function of a protein export machinery.

MATERIALS AND METHODS

Bacterial strains and plasmids. The plasmids and bacterial strains used in this study are listed in Table 1. All cloning procedures were carried out with either *Escherichia coli* DH5 α or *E. coli* XL-1 as the host. Bacterial cultures were routinely grown in LB medium (14) or minimal A salts supplemented with 1% glycerol and 50 mM monosodium glutamate (5). Antibiotic concentrations were as follows: for *P. aeruginosa*, carbenicillin at 150 μ g/ml and neomycin at 100 μ g/ml; for *E. coli*, ampicillin at 100 μ g/ml and streptomycin at 50 μ g/ml.

Construction of XcpT-W fusion protein-encoding plasmids. The XcpT-W fusion protein-encoding plasmids were con-

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TABLE 1. Strains, vectors, and plasmids

Strain, vector, or plasmid	Relevant genotype or properties	Source or reference
Strains		
<i>E. coli</i>		
DH5 α	F ⁻ <i>endA1 thi-1 recA1 gyrA96 relA1 (lacZYA-argF)</i>	Bethesda Research Laboratories, Gaithersburg, Md.
XL-1	[F' <i>proAB, lacI^q ΔM15::Tn10</i>] <i>endA1 recA1 thi-1</i>	Stratagene, La Jolla, Calif.
RZ1032	<i>dut ung</i> strain for oligonucleotide-directed mutagenesis	11
<i>P. aeruginosa</i>		
PAK B24	PAK wild type with Tn5 insertion downstream of <i>pilD</i> gene; Km ^r	16
PAK B30	PAK <i>pilD</i> mutant with Tn5 insertion in <i>pilD</i> ; Km ^r	16
PAK XG9	PAK <i>xcpT-W</i> deletion mutant; Gm ^r	18
Cloning vectors		
M13mp19	Single-stranded filamentous phage vector	28
pMMB66EH	RSF1010-derived <i>tac</i> expression vector; Ap ^r	7
pMMB67HE	RSF1010-derived <i>tac</i> expression vector; Ap ^r	M. Bagdasarian
pATH3	TrpE fusion vector; Ap ^r	10
pMalCR1	MalE fusion vector; Ap ^r	New England Biolabs, Beverly, Mass.
Recombinant plasmids		
pPilA-XcpT	PilA-XcpT fusion plasmid; pMMB66EH based; Ap ^r	This study
pPilA-XcpW	PilA-XcpW fusion plasmid; pMMB66EH based; Ap ^r	This study
pMalE-XcpT	MalE-XcpT fusion plasmid; pMalCR1 based; Ap ^r	This study
pMalE-XcpU	MalE-XcpU fusion plasmid; pMalCR1 based; Ap ^r	This study
pMalE-XcpV	MalE-XcpV fusion plasmid; pMalCR1 based; Ap ^r	This study
pMalE-XcpW	MalE-XcpW fusion plasmid; pMalCR1 based; Ap ^r	This study
pTrpE-XcpU	TrpE-XcpU fusion plasmid; pATH3 based; Ap ^r	This study
pTrpE-XcpV	TrpE-XcpV fusion plasmid; pATH3 based; Ap ^r	This study

structured as follows and as shown in Fig. 1. An *EcoRI*-*HindIII* fragment containing the *pilA* gene was subcloned into similarly digested M13mp19 replicative-form DNA. A mutagenic oligonucleotide was used to generate an *XhoI* site at the site corresponding to the +4 and +5 positions of the mature PilA protein (changing Ile-Glu to Leu-Glu). Oligonucleotide-directed mutagenesis was carried out by the method of Kunkel et al. (11), as were all subsequent oligonucleotide-directed mutagenesis experiments. To generate a fusion of the PilA protein with XcpT, we subcloned a 1.9-kb *SphI*-*PstI* fragment containing the *xcpT* gene into *SphI*-*HindIII*-cut M13mp19 after filling in the *PstI* site of the *xcpT*-containing

fragment with T4 DNA polymerase and attaching a *HindIII* linker. Oligonucleotide-directed mutagenesis was used to remove an *XhoI* site within the coding sequence of *xcpT* and to subsequently introduce an *XhoI* site at the site corresponding to the +4 and +5 positions of the XcpT protein (changing Ile-Glu to Leu-Glu). The PilA-XcpT fusion was created by replacing the *XhoI*-*HindIII* fragment containing the 3' end of *pilA* with the *XhoI*-*HindIII* fragment containing the 3' end of the *xcpT* gene. The *EcoRI*-*HindIII* fragment containing the *pilA*-*xcpT* fusion was subcloned into the broad-host-range expression vector pMMB66EH to generate pPilA-XcpT. The MalE-XcpT fusion was generated by subcloning the *XhoI*-*HindIII* fragment containing the 3' sequences of XcpT into *SalI*-*HindIII*-digested pMalCR1 to give rise to pMalE-XcpT.

The PilA-XcpW fusion-encoding plasmid was generated in a similar manner. A 1.8-kb *SalI* fragment containing the *xcpW* gene was subcloned into pUC19 in the orientation opposite that of the *lacZ'* gene. As the natural sequence of the *xcpW* gene contains a *XhoI* site at the positions corresponding to +4 and +5 of the XcpW protein, a *XhoI*-*HindIII* fragment was subcloned from this construction and ligated to the *XhoI*-*HindIII*-digested PilA construction described above. The *EcoRI*-*HindIII* fragment containing the *pilA*-*xcpW* fusion was subcloned into the broad-host-range expression vector pMMB66EH to generate pPilA-XcpW. The MalE-XcpW fusion was created by subcloning the *XhoI*-*HindIII* fragment containing the 3' sequences of XcpW into *SalI*-*HindIII*-digested pMalCR1 to give rise to pMalE-XcpW.

The MalE-XcpU and MalE-XcpV fusion-encoding plasmids were generated as follows. A 1.1-kb *XhoI* fragment containing the *xcpU* and *xcpV* genes was subcloned into the *SalI* site of M13mp19. A *XhoI* site was introduced at the positions corresponding to either +4 and +5 of the XcpU protein (changing Ile-Glu to Leu-Glu) or +4 and +5 of the

FIG. 1. Schematic diagram of the construction of XcpT-W fusion protein plasmids. Abbreviations for restriction endonuclease sites: E, *EcoRI*; H, *HindIII*; P, *PstI*; S, *SalI*; X, *XhoI*. Circles around restriction sites represent sites lost during cloning. ▼, the *PilD* cleavage site of PilA or proposed cleavage site of XcpT-W. Underlined amino acid residues represent +4 and +5 positions of XcpT-W and PilA. Ads, adsorbent; Ag, antigen.

XcpV protein by oligonucleotide-directed mutagenesis. The resultant plasmids were each digested with *Xho*I and *Hind*III. The *Xho*I-*Hind*III fragments containing the 3' end of the *xcpU* or *xcpV* gene were ligated to *Sal*I-*Hind*III-digested pMalCRI to give rise to pMalE-XcpU or pMalE-XcpV, respectively. Fusions of the C termini of XcpU and XcpV to TrpE N-terminal sequences were generated by ligating the same *Xho*I-*Hind*III fragments to *Sal*I-*Hind*III-digested pATH3 to give rise to pTrpE-XcpU and pTrpE-XcpV, respectively.

Plasmid pMMB-PCR was constructed by ligating an *Xba*I-*Eco*RI polymerase chain reaction product encoding *xcpT-W* (18) into *Xba*I-*Eco*RI-digested pMMB67HE.

Purification of fusion proteins. Plasmid pPilA-XcpT or pPilA-XcpW was introduced from *E. coli* DH5 α into PAK B24 strains by conjugal transfer, using pRK2073 (12) as a mobilizing plasmid. The procedure for purifying the fusion proteins was similar to that previously described for the purification of prepilin (17). Total membranes were prepared from isothiolgalactopyranoside (IPTG)-induced cultures by sonication and ultracentrifugation. Membrane proteins were separated by gel filtration in the presence of sodium dodecyl sulfate (SDS), and fractions containing the PilA-Xcp fusion proteins were pooled and precipitated with acetone.

Fusions of XcpT-W to the maltose-binding protein (MalE) were prepared from cultures of *E. coli* DH5 α containing either pMalE-XcpT or pMalE-XcpW and purified by affinity chromatography of cytoplasmic extracts as described by Kellermann and Ferenci (9).

Fusions of XcpU and XcpV to the TrpE protein were prepared from indole acrylate-induced cultures of *E. coli* DH5 α containing pTrpE-XcpU and pTrpE-XcpV by purification of inclusion bodies as described by Koerner et al. (10).

Antibody production. New Zealand White rabbits were immunized with the purified fusion proteins. A primary injection of 500 μ g of PilA-XcpT, PilA-XcpW, MalE-XcpU, or MalE-XcpV was given to each rabbit. After 1 month, a second injection of 100 μ g of each fusion protein was given to the respective rabbit. Serum was obtained from each rabbit 1 month following the second injection.

Two approaches were used to affinity purify antibodies specific for the Xcp proteins. In the first approach, purified MalE-XcpT and MalE-XcpW fusion proteins were each coupled to a solid support (AminoLink; Pierce Chemical Co., Rockford, Ill.) according to the manufacturer's directions. After an initial ammonium sulfate fractionation, the appropriate crude antiserum was applied to the XcpT- or XcpW-coupled solid supports. Adsorbed antibodies were eluted with 0.3 M glycine (pH 3.5), and the eluate was neutralized with 1 M Tris-HCl (pH 8.0). After an overnight dialysis with phosphate-buffered saline (PBS; 50 mM sodium phosphate, 150 mM sodium chloride), the antibodies were concentrated to 10 mg/ml by ultrafiltration. A second approach was used to purify antibodies against XcpU and XcpV. One milligram of each of the purified TrpE-XcpU and TrpE-XcpV fusion proteins was run on preparative SDS-polyacrylamide gels, and the fusion proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) paper as described by Matsudaira (15). The protein-paper conjugates were incubated with crude antiserum to the respective Xcp protein for 16 h at 4°C with gentle agitation. The paper strips were then washed extensively with PBS, and the adsorbed antibodies were eluted with 0.3 M glycine (pH 3.5). After neutralization and dialysis, the antibody

preparations were concentrated to a final protein concentration of 10 mg/ml by ultrafiltration.

Antibody to PilD was prepared as previously described (17). Monoclonal antibody to the *P. aeruginosa* outer membrane protein OprF was kindly provided by Richard Darveau (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, Wash.).

Immunoblotting procedures. Membrane fractions and samples were solubilized by boiling for 5 min in sample buffer (2% SDS, 1% 2-mercaptoethanol, 10% glycerol, 100 mM Tris-HCl [pH 8.0]) and separated on 10 or 15% polyacrylamide-SDS-tricine gels (21). The separated proteins were electrophoretically transferred to nitrocellulose filter paper by the method of Towbin et al. (26). The filters were blocked with 2% nonfat dried milk, incubated with the appropriate antibody preparation in PBS, and detected with [125 I]protein A and autoradiography.

Immunoprecipitation. To obtain sufficient XcpT-W proteins for N-terminal protein sequencing, total membranes were prepared from an IPTG-induced strain of PAK B24 containing plasmid pMMB-PCR. For each immunoprecipitation, an aliquot of membranes containing approximately 100 mg of total protein was solubilized in 5 ml of 1% Triton X-100–50 mM Tris-HCl (pH 7.5)–150 mM NaCl. After ultracentrifugation of the solubilized material, the clarified supernatants were incubated with 100 μ g of each of the affinity-purified antibodies to XcpT-W at 4°C overnight with gentle agitation. A 100- μ l volume of a protein A-Sepharose suspension (Sigma Chemical, St. Louis, Mo.) was added to each preparation, which was allowed to further incubate for 4 h at 4°C. The protein A-Sepharose beads were then washed extensively with PBS by repeated low-speed centrifugation. After a final centrifugation, 100 μ l of SDS-containing sample buffer (minus 2-mercaptoethanol) was added to the beads, which were heated to 65°C for 10 min.

Protein sequencing. The immunoprecipitated XcpT-W proteins were separated on a 10% polyacrylamide-SDS-tricine gel, and the separated proteins were electrophoretically transferred to PVDF paper. The membranes were stained with Coomassie blue R to detect the transferred proteins. Bands corresponding to the predicted molecular weight of each of the XcpT-W proteins were cut out and used for automated amino-terminal amino acid sequencing by sequential phenylisothiocyanate (PTH) degradation on an Applied Biosystems model 477A gas-phase analyzer. Samples were analyzed by high-pressure liquid chromatography (HPLC) through a 5-mm C_{18} column in a gradient of 5% tetrahydrofuran and acetonitrile as follows: 11% acetonitrile, 0.4 min; 11% acetonitrile to 35% acetonitrile, 18 min; 35% acetonitrile to 35% acetonitrile, 27.1 min; and 36% acetonitrile to 65% acetonitrile, 35 min. The flow rate was 0.21 ml/min at 35°C; detection was at 267 nm. PTH derivatives of phenylalanine and *N*-methylphenylalanine were used as standards. The identities of the first five residues were determined for each sample.

Membrane localization. Two methods were used to determine the membrane localization of XcpT-W as well as of the cytoplasmic membrane marker PilD and the outer membrane marker OprF. The first, sucrose density centrifugation, was used to separate total membranes as follows. For each fractionation, cells of either PAK B24 or B30 were grown in 400 ml of medium A with 1% glycerol, 50 mM monosodium glutamate, and 1% Casamino Acids until an optical density at 600 nm of 1.2 was reached (late log phase). Cells were immediately cooled on ice and harvested by centrifugation at 4°C. The cell pellet was resuspended in 6 ml of cold 50 mM

Tris-HCl (pH 8.0)–10 mM MgCl₂–50 µg of DNase I per ml–50 µg of RNase I per ml. The resuspended cells were broken by two passages through a French pressure cell at 20,000 lb/in², and the preparation was separated from unbroken cells by low-speed centrifugation. The clarified supernatant was placed on a sucrose gradient consisting of 1 ml of 70% (wt/vol) sucrose and 5.5 ml of 15% sucrose. The gradients were centrifuged for 2 h at 35,000 rpm in an SW40.1 rotor. After centrifugation, the bottom 2.5 ml of each was placed on a sucrose gradient consisting of 1 ml of 70% (wt/vol), 1 ml of 67%, 1 ml of 64%, 1 ml of 61%, 1 ml of 58%, 1 ml of 56%, 1 ml of 49%, and 1 ml of 46% sucrose. The gradients were centrifuged for 24 h at 35,000 rpm. Ten 1-ml fractions were taken from each through the bottom of the tube. An equal volume of each fraction was precipitated with trichloroacetic acid, resuspended in SDS sample buffer, and separated by SDS-polyacrylamide gel electrophoresis (PAGE) for immunodetection.

The second method to separate cytoplasmic membrane proteins from outer membrane proteins is based on the selective solubilization of cytoplasmic membrane proteins by the detergent Sarkosyl. Total membranes were prepared from PAK B24 and PAK B30 by sonication and centrifugation of cells resuspended in 200 mM Tris-HCl (pH 7.5)–20 mM EDTA–0.4 mM phenylmethylsulfonyl fluoride. One-milliliter aliquots of membranes (~30 mg of total protein) were resuspended in 20 mM Tris-HCl (pH 7.6) and mixed with 1 ml of either 0.2, 1.0, or 4.0% Sarkosyl in 20 mM Tris-HCl (pH 7.6) to give a final Sarkosyl concentration of 0.1, 0.5, or 2.0%, respectively. The solubilized membranes were centrifuged to separate insoluble from soluble proteins. The insoluble pellets were resuspended in 2.0 ml of 20 mM Tris-HCl (pH 7.6). The Sarkosyl-soluble and -insoluble fractions were mixed with SDS-containing sample buffer and separated by SDS-PAGE for immunodetection.

RESULTS

Production of antibodies to XcpT-W. To obtain antibodies to each of the four export proteins, XcpT-W, various strategies were used to overexpress these proteins for use as immunogens. Initial attempts to use strong promoters, such as the regulated *tac* promoter of plasmid pMMB66EH, did not significantly enhance expression of these proteins, while a similar construction with *pilA*, the structural gene for pilin, led to overexpression of pilin protein such that it accounted for at least 50% of the total membrane protein (17). Gene fusions of the 400 bp upstream of the *pilA* gene up to and including the initiating codon with the coding sequences of the four *xcp* genes were constructed, but they also did not give detectable levels of expression.

When we examined the nucleotide sequence of several type IV pilins, which can be readily overproduced in a number of bacterial hosts, we noted a conserved sequence of five adenines in the region encoding the –3 and –2 amino acids of the leader sequence. Assuming that these sequences may be involved in determining the expression levels of this gene, we devised a strategy for generating PilA-Xcp gene fusions that would allow for the expression of native XcpT-W proteins after cleavage of the PilA-derived leader sequence by PilD, with the exception of a conservative substitution of leucine for isoleucine at the +4 position of XcpT and XcpU (plasmid constructions detailed in Materials and Methods and Fig. 1).

The four PilA-Xcp fusion constructs were introduced into PAK B24 (wild type) and B30 (*pilD* mutant). Total mem-

FIG. 2. PilD-dependent cleavage of XcpT-W. Shown are immunoblots of membrane proteins prepared from PAK B24 (wild type [wt]), PAK B30 (*pilD*), and PAK XG9 (Δ xcpT-W) and separated by SDS-PAGE. XcpT-W proteins were determined with specific antisera prepared as described in Materials and Methods and detected with [¹²⁵I]protein A and autoradiography.

branes were prepared from these strains and analyzed on Coomassie-stained SDS-polyacrylamide gels. High levels (>50%) of proteins corresponding to the predicted molecular sizes of XcpT (15 kDa) and XcpW (26 kDa) were detected in membranes prepared from PAK B24. Slightly larger proteins were seen in the membranes of the *pilD* mutant strain expressing the same construct (data not shown), indicating that the hybrid proteins were substrates for PilD-mediated cleavage. XcpT and XcpW proteins were purified from membranes prepared from PAK B24 expressing the appropriate constructs as described in Materials and Methods. Purified XcpT and XcpW were used as immunogens.

In contrast, no detectable levels of the PilA-XcpU or PilA-XcpV fusion protein could be seen by SDS-PAGE. Furthermore, the PilA-XcpV fusion also appeared to be lethal to the wild-type but not the *pilD* mutant strain, as cultures stopped growing upon addition of IPTG to induce the expression of the PilA-XcpV fusion. As an alternative for the expression of XcpU and XcpV, the same C-terminus-encoding fragments used to generate the unsuccessful PilA fusions were cloned into the MalE (maltose-binding protein) fusion vector, pMalCRI (Fig. 1). These constructs expressed high levels of a protein in *E. coli* corresponding to the predicted molecular weight of the fusion. The MBP-XcpU and MBP-XcpV fusion proteins were purified and used as immunogens.

To affinity purify antisera directed against only the Xcp portions of the fusion proteins, a second set of Xcp fusion proteins was prepared for each. MalE-XcpT and MalE-XcpW fusion-expressing plasmids were constructed as described for Fig. 1. To generate alternate fusions for XcpU and XcpV, the C-terminus-encoding fragments of each were cloned into the TrpE fusion vector, pATH3 (Fig. 1). Fusion proteins were prepared from each of the four constructs and used to affinity purify antibodies directed against the Xcp protein portion of the original antigen preparation.

PilD-dependent cleavage of XcpT-W. To examine the cleavage of the XcpT-W proteins in *P. aeruginosa* strains expressing them under nonoverproducing, physiological conditions, we isolated membranes from a wild-type and *pilD* mutant strain of PAK and compared the relative sizes of these proteins by immunoblotting with their respective antibodies. Immunoblots of membranes from wild-type PAK (B24), probed with the individual antibodies, show a single immunoreactive protein of the molecular size expected for each of the XcpT-W proteins (Fig. 2). Membranes from the *pilD* mutant (B30) show proteins of slightly larger size,

FIG. 3. Immunoprecipitation of XcpT-W. Shown is a Coomassie-stained SDS-polyacrylamide gel of membrane proteins prepared from PAK B24(pMMB-PCR) immunoprecipitated with the indicated Xcp-specific antisera. The position of each is noted. The predicted sizes of XcpT-W are 14.5, 18.5, 13.7, and 26.3 kDa, respectively. Lane MW, molecular weight standards.

corresponding to unprocessed XcpT-W. Membranes from strain XG9, carrying a chromosomal deletion of *xcpT-W*, did not react with any of the antibodies, demonstrating the specificity of the affinity-purified antibodies.

Methylation of XcpT-W in vivo. We have previously determined that PilD, in addition to its role as the prepilin peptidase, carries out the N methylation of the type IV pilin. It was also shown that PilD can methylate that N-terminal phenylalanine of XcpW in vitro (24). To determine whether methylation of XcpT-W occurs in vivo, each of the antibody preparations was used to immunoprecipitate the respective Xcp protein from Triton-solubilized total membranes of a wild-type strain of PAK containing plasmid pMMB-PCR, which expresses XcpT-W under the control of the *tac* promoter. Immunoprecipitated proteins were electrophoretically separated and transferred to PVDF paper for sequential Edman degradation. A Coomassie-stained SDS-polyacrylamide gel showing samples of the immunoprecipitated proteins is presented in Fig. 3. An HPLC elution profile of the first residue and the relative positions of phenylalanine and N-methylphenylalanine standards are shown for each of the Xcp proteins in Fig. 4. In each case, the first amino acid of the mature Xcp protein is an N-methylphenylalanine, as would be predicted if PilD-dependent cleavage and methylation of these proteins had occurred. The amino acid sequences of the first five turns were as follows: XcpT, N-methyl-Phe-Thr-Leu-Ile-Glu-; XcpU, N-methyl-Phe-Thr-Leu-Ile-Glu-; XcpV, N-methyl-Phe-Thr-Leu-Leu-Glu-; and XcpW, N-methyl-Phe-Thr-Leu-Leu-Glu-. These sequences are in exact agreement with the deduced amino acid sequence of the *xcpT-W* gene products.

Localization of XcpT-W. To determine the location of XcpT-W in PAK strains expressing these proteins under physiological conditions and to determine whether removal of their leader sequence leads to an alteration in localization, inner and outer membrane fractions were obtained from the wild-type (B24) and *pilD* mutant (B30) strains of PAK. Two independent procedures, Sarkosyl solubilization and sucrose density gradient centrifugation, were used to obtain inner and outer membrane fractions. Proteins from these fractions were separated by SDS-PAGE, electrophoretically transferred to nitrocellulose, and probed with anti-XcpT-W antibodies. Immunoblots of the fractions were also probed with antibodies to the outer membrane protein OprF and, where appropriate, to PilD, which we have previously determined to be located exclusively in the inner membrane.

FIG. 4. Analyses of the amino-terminal residues of XcpT-W. Shown are chromatograms of the first cycle of PTH-labeled Edman degradation products separated by HPLC. The arrows labeled Phe and N-MethylPhe represent the elution times of PTH-phenylalanine and PTH-monomethylphenylalanine standards determined before each run.

Quantitative immunoblot analysis of the distribution of precursor and mature forms of XcpT-W in fractions obtained by sucrose density centrifugation of membranes from the wild-type and *pilD* mutant strain of PAK is shown in Fig. 5A, and representative immunoblots are shown in Fig. 5B. XcpT-W were consistently found in the fractions containing the majority of the membranes of lighter density prepared from the wild-type strain and cofractionated with the inner membrane marker PilD. Only a small proportion of these proteins fractionated with the denser membrane fractions where the outer membrane protein OprF can be seen. Moreover, processing of the precursors of XcpT-W to the mature form by PilD did not alter membrane localization, as both the precursor and mature forms of XcpT-W were consistently found in the same region of the density gradient.

To further confirm the localization of XcpT-W, we used selective detergent solubilization of total membranes isolated from the wild-type and *pilD* mutant strains. In this procedure, cytoplasmic membrane proteins are solubilized with the detergent Sarkosyl, while outer membrane proteins remain insoluble. Three increasing concentrations of Sarkosyl were used to ensure that minor differences in solubility were detected. Figure 6 shows immunoblots of wild-type and *pilD* membrane proteins solubilized with either 0.1, 0.5, or 2.0% Sarkosyl and detected with antibody to OprF, PilD (wild type only), each of the four Xcp proteins, and pilin (PilA). As can be seen, while the outer membrane marker protein OprF remains insoluble throughout the increasing concentrations of Sarkosyl, XcpT-W and pilin are completely soluble in 0.5 and 2% Sarkosyl, whether expressed from a wild-type or *pilD* mutant background. Taken together, these observations strongly suggest that PilD cleav-

FIG. 5. Membrane localization of XcpT-W as determined by sucrose density centrifugation. Membranes were prepared from wild-type (B24) and *pilD* mutant (B30) PAK strains and separated by sucrose density centrifugation. Proteins from each of 10 fractions were separated by SDS-PAGE, electrophoretically transferred to nitrocellulose, and detected with the appropriate antisera. (A) After detection of bound antibodies with [125 I]protein A and autoradiography, the density of the exposed region of film was determined for each fraction (Fotoanalyzer II and Collage; Fotodyne, New Berlin, Wis.). Each line of the graphs represents the distribution of Xcp protein in the strain indicated per fraction relative to the total protein detected across all fractions (% of total detected). Localization of the outer membrane marker OprF in the wild-type (wt) or *pilD* mutant strain, or the inner membrane marker PilD in the wild-type strain, is shown as a dashed line in each panel. Values for OprF and PilD are also duplicated for comparison in each graph. (B) Autoradiographs of representative immunoblots. i, PAK B24 (wild-type) membranes probed with α OprF; ii, PAK B30 (*pilD*) membranes probed with α OprF; iii, PAK B24 membranes probed with α PilD; iv, PAK B24 membranes probed with α XcpT; v, PAK B30 membranes probed with α XcpT.

age and methylation of XcpT-W or pilin have little or no effect on their apparent membrane localization.

DISCUSSION

We and others have recently identified four proteins in *P. aeruginosa*, XcpT-W (formerly referred to as PddA-D), that comprise a machinery for extracellular protein secretion (3, 18). One of the distinguishing features of these four proteins is their similarity to type IV prepilins. This similarity resides at the amino termini of the precursor forms of these proteins and includes a six- to eight-amino-acid leader sequence followed by a consensus sequence of Gly-Phe-Thr-Leu-(Leu or Ile)-Glu. It is this site that we have determined to be the recognition site for PilD, the previously described prepilin leader peptidase (22). Precursors of XcpU and XcpV, when

overexpressed from the T7 promoter in rifampin-treated *P. aeruginosa* strains, have been shown to be processed by PilD (XcpA) (3, 18). In this study, we have developed specific antibodies to XcpT-W and have shown that all four proteins, expressed under physiological conditions, require PilD for cleavage of their respective leader sequences *in vivo*.

The availability of the specific antibodies to XcpT-W has allowed us to use immunoaffinity techniques to purify quantities of these proteins for sequence analysis. The N-terminal amino acid of XcpT-W was identified as *N*-methylphenylalanine, another distinguishing characteristic of the type IV pilins, further demonstrating the similarity between these proteins. Since we have previously shown that *in vitro*, PilD is capable of transferring the methyl group of *S*-adenosylme-

FIG. 6. Membrane localization of XcpT-W and PilA as determined by Sarkosyl solubilization. Total membranes prepared from PAK B24 (wild type) and PAK B30 (*pilD*) strains were solubilized with increasing concentrations of Sarkosyl and separated into soluble and insoluble fractions. These fractions were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the indicated antisera. Odd-numbered lanes were prepared from B24; even-numbered lanes were prepared from B30. Lanes: 1 and 2, 0.1% Sarkosyl-soluble fractions; 3 and 4, 0.1% Sarkosyl-insoluble fractions; 5 and 6, 0.5% Sarkosyl-soluble fractions; 7 and 8, 0.5% Sarkosyl-insoluble fractions; 9 and 10, 2.0% Sarkosyl-soluble fractions; 11 and 12, 2.0% Sarkosyl-insoluble fractions.

thionine onto the N-terminal phenylalanine of cleaved pilin and XcpW (24), it is certain that PilD also carries out the N methylation of XcpT-W in vivo. The significance of the N methylation of pilin and XcpT-W is not clear, but it is conceivable that this modification is required for the biogenesis of pili or an export apparatus or instead acts to protect these proteins from degradation by cellular proteases. Alternatively, methylation of XcpT-W may be essential for the function of the assembled export apparatus by enabling an interaction with other Xcp proteins, cellular components such as phospholipids, or the exported proteins themselves.

By two independent fractionation procedures, we have determined the cellular location of XcpT-W, expressed under physiological conditions, in a wild-type and a *pilD* mutant strain of *P. aeruginosa*. In both cases, these proteins were found to be located predominantly in cytoplasmic membrane fractions. No apparent differences in localization were found after cleavage of the leader sequence and methylation by PilD. These results are in contrast to the findings of Bally et al. (3), who concluded that cleavage of the leader sequence of XcpV by XcpA (PilD) leads to a change in the localization of XcpV from the inner to outer membrane, as determined by Sarkosyl solubilization. The reason for this discrepancy is unclear but may have resulted from the overexpression of XcpU under nonphysiological conditions (treatment with rifampin at 42°C), leading to aberrant localization.

Our results lead us to conclude that the role of the XcpT-W leader sequence and cleavage and methylation of the proteins is not to direct their membrane localization but instead to coordinate their proper polymerization into a

multicomponent structure. Although the subunit composition of such a structure is unknown and may consist of additional Xcp proteins, preliminary results of immunoblotting experiments show that the relative amounts of XcpT, XcpU, XcpV, and XcpW are approximately 16:1:1:4, respectively (data not shown). This, however, represents only the relative abundance of each protein in the membrane fractions and does not necessarily reflect the stoichiometry of these proteins in a polymerized structure. In respect to the relatively low level of the XcpU and XcpV proteins, it is interesting to note the deleterious effect of leader peptide cleavage from the PilA-XcpV hybrid and to speculate that this apparent lethality is in some way related to the role that XcpV, at normal levels, plays in the protein export apparatus. This lethality may reflect the interaction of the mature form of this protein with an essential cellular component or may be due to an unusual sensitivity of the membrane to perturbation by the mature form of XcpV.

Recent reports from a number of laboratories have described complex gene systems required for DNA uptake and extracellular protein export in other microorganisms. In each case, three or four of the proteins required for these biological processes also have the distinguishing characteristics of the type IV pilin subunit. These include the ComG3-5 proteins of *Bacillus subtilis*, required for DNA uptake during competence (1); PulG-J, required for pullulanase export by *Klebsiella oxytoca* (19); and OutG-J, required for export of cellulases and pectate lyases by *Erwinia* species (8). As would be expected, these systems also encode gene products with a high degree of homology to PilD (ComC, PulO, and OutO, respectively), which would be expected to carry out the cleavage and methylation of their respective pilin-like proteins. Pugsley and Dupuy (20) have, in fact, confirmed that the *K. oxytoca* PulG protein is processed by the PilD homolog PulO.

We now know of five proteins in *P. aeruginosa* that are substrates for PilD. It is conceivable that other type IV pilin-like proteins are present in this organism and may participate in the biological processes that have been ascribed to this family of proteins. An understanding of the pilus biogenesis system will be required to gain an insight into the form and function of what appears to be a complex but widespread apparatus for the export and import of macromolecules. Very clear homologies exist not only between the pilin-like proteins and the prepilin leader peptidases in each of these various transport systems but also between the pilus biogenesis genes PilB and PilC and other gene products of the Xcp and other systems (XcpR and XcpS; ComG1 and ComG2; PulE and PulF; OutE and OutF). It is very likely that these proteins have roles similar to those of PilB and PilC and are required for the assembly of XcpT-W or their counterparts into a pilus-like structure. It would be expected that the assembly of such a structure would lead to a detectable alteration in the membrane or cellular localization of its components. Alternatively, an assembled structure may be transient and exist only during the transfer of exported proteins across the cell wall. The recently described PilT protein, required for the retraction of the *P. aeruginosa* pilus, is also highly homologous to PilB (27). This similarity suggests that assembly and disassembly of the macromolecular transport apparatus may play an essential role in its function.

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