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Multiple Roles of the Pilus Biogenesis Protein PilD: Involvement of PilD in Excretion of Enzymes from *Pseudomonas aeruginosa*

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In *Pseudomonas aeruginosa*, the genes *pilB*, *pilC*, and *pilD* encode proteins necessary for posttranslational modification and assembly of pilin monomers into pilus organelles (D. Nunn, S. Bergman, and S. Lory, J. Bacteriol. 172:2911–2919, 1990). We show that PilD, encoding a putative pilin-specific leader peptidase, also controls export of alkaline phosphatase, phospholipase C, elastase, and exotoxin A. *pilD* mutants accumulate these proteins in the periplasmic space, while secretion of periplasmic and outer membrane proteins appears to be normal. The periplasmic form of exotoxin A was fully mature in size, contained all cysteines in disulfide bonds, and was toxic in a tissue culture cytotoxicity assay, suggesting that in *pilD* mutants, exotoxin A was folded into its native conformation. The function of the other two accessory proteins, PilB and PilC, appears to be restricted to pilus biogenesis, and strains carrying mutations in their respective genes do not show an export defect. These studies show that in addition to cleaving the leader sequence from prepilin, PilD has an additional role in secretion of proteins that are released from *P. aeruginosa* into the surrounding media. PilD most likely functions as a protease that is involved in processing and assembly of one or more components of the membrane machinery necessary for the later stages of protein extracellular localization.

A sorting mechanism in gram-negative bacteria is responsible for localization of proteins into one of several compartments. Whereas most proteins synthesized in the cytoplasm are not secreted, a number of polypeptides are inserted into the inner or outer membrane, secreted into the periplasmic space, or released into the extracellular space. While extracellular localization of polypeptides is not common in members of the family *Enterobacteriaceae*, many other soil or pathogenic gram-negative bacteria actively excrete enzymes and toxins into their surrounding environment. Proteins that are released by gram-negative microorganisms often require a specialized export apparatus distinct from the normal secretion machinery used for membrane or periplasmic localization (8, 26).

The assembly of pili is also coupled to export of the monomeric pilin subunits across the bacterial membranes and subsequent incorporation of these subunits into an organelle that protrudes away from the bacterial cell. Formation of *Escherichia coli* pili appears to be a complex biogenesis process that involves the interaction of the monomers with various accessory polypeptides during the transfer across the membranes and periplasmic space (3).

We have recently identified three genes in *Pseudomonas aeruginosa* that encode the accessory pilus biogenesis proteins PilB, PilC, and PilD (25). Mutations in *pilB* and *pilC* allow synthesis of mature pilin which is equally partitioned between the inner and outer membrane. Mutations in *pilD* prevent processing of prepilin into mature pilin, and work in this laboratory has identified PilD as the prepilin leader peptidase (25a). The prepilin precursor contains a short, 6-amino-acid leader sequence which differs from typical secretion signal sequences of procaryotic cells (11). However, together with the N terminus of the mature protein, it may be part of an export signal necessary for membrane translocation during the initial stages of pilus biogenesis (27). This export pathway may be unique for type IV pilins and is likely to be shared by *Neisseria gonorrhoeae* (24), *Moraxella bovis* (22), and *Bacteroides nodosus* (23). These bacteria synthesize prepilins that are structurally related to *P. aeruginosa* pili.

We have examined the range of secreted proteins that may require expression of functional products of genes *pilB*, *pilC*, and *pilD*. The role of PilB and PilC appears to be restricted to the formation of pili. However, mutations in *pilD* prevented export of all extracellular proteins examined, while the same mutation had no effect on secretion of periplasmic or outer membrane proteins. These findings suggest that the putative prepilin-specific leader peptidase has a wide range of substrates that includes one or more of the components of the export machinery in *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains and vectors. *P. aeruginosa* PAK and various Tn*5* insertion mutants of this strain that are deficient in pilus biogenesis have been described previously (25). Strain E4 is a *pilB* mutant, strain E9 is a *pilC* mutant, and strain 2B18 is a *pilD* mutant. Strain B24 carries a Tn*5* insertion in a region 0.5 kb downstream of *pilD*, has no known phenotype, and was used as a wild-type control in some experiments. Plasmid pDN19XS was constructed by cloning a *Sal*I-*Xba*I fragment containing the *pilD* gene into the broad host range plasmid pDN19 (25). Plasmid pMMD4 was constructed by deleting ca. 300 bp from the 5′ region flanking exotoxin A in plasmid pMS150A (19) and cloning the exotoxin A gene into pMMB66EH (4), immediately adjacent to the *tac* promoter. Plasmid pMMD4 can express exotoxin A only when induced by isopropyl-β-D-thiogalactopyranoside (IPTG). Plasmids pDN19, pDN19XS, and pMMD4 were introduced into *P. aeruginosa* recipients by triparental mating, with pRK2073 (16) as a helper plasmid.

Media and growth conditions. Bacteria were propagated at 37°C on low-phosphate TY medium (10) for analysis of alkaline phosphatase and phospholipase C. L broth (21) was used to grow bacteria for expression of exotoxin A. Medium

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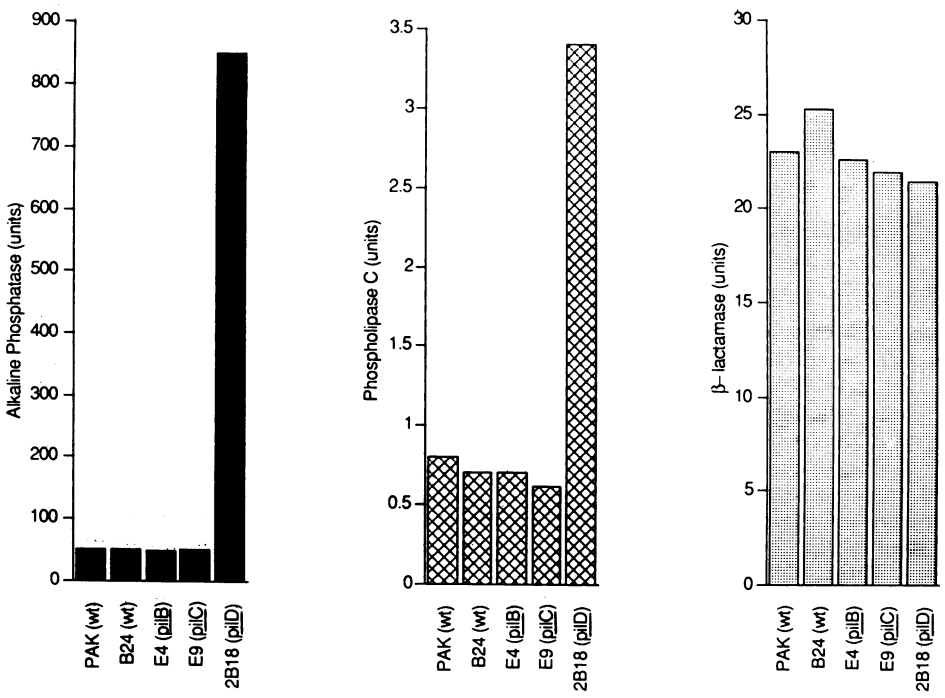

FIG. 1. Levels of synthesis of extracellular enzymes in the pilus biogenesis mutants. Bacteria were grown in low-phosphate medium to mid-logarithmic phase (A_{600}, 1.5). Prior to assay of alkaline phosphatase, phospholipase C, and β-lactamase activities as described in Materials and Methods, 5 ml of this culture was passed through a French pressure cell to lyse the bacteria. Strains PAK, B24 (wild type), E4 (*pilB*), E9 (*pilC*), and 2B18 (*pilD*) each carried plasmid pMMB66EH to provide constitutive synthesis of β-lactamase.

used for analysis of outer membrane protein G was dialyzed Trypticase soy broth (17) (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 1 mM $MgCl_2$ and 10 μM $FeSO_4$. To maintain plasmids, medium was supplemented with tetracycline (100 μg/ml) or carbenicillin (150 μg/ml) on L agar.

Enzyme assays. Alkaline phosphatase activity was measured by monitoring the rate of hydrolysis of *p*-nitrophenyl phosphate (Sigma Chemical Company, St. Louis, Mo.) (2). Phospholipase C was measured by using the chromogenic substrate *p*-nitrophenyl phosphorylcholine (Sigma) as previously described (1). β-Lactamase was assayed with nitrocefin (BBL) as a chromogenic substrate (12). Units of activity in subcellular fractions were measured after correcting the enzyme volumes in reaction mixtures for the bacterial mass in the culture.

Cell fractionation. After the release of periplasmic space contents from concentrated cells following the $MgCl_2$ treatment method of Hoshino and Kageyama (9), cells were further separated into cytoplasmic and membrane fractions following the method of Hancock and Nikaido (6). Outer membranes were separated from total cell lysates as previously described by Hancock and Carey (5).

SDS-polyacrylamide gel electrophoresis and Western immunoblots. Proteins were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels (13) and transferred to nitrocellulose filters by the method of Towbin et al. (29). Rabbit polyclonal antisera against elastase and mouse monoclonal antibodies against the outer membrane protein G (OprG) of *P. aeruginosa* were a gift from Donald Woods, University of Calgary. Mouse antisera against purified exotoxin A were prepared in this laboratory.

Detection of elastase. Cultures were grown in 5% peptone–0.5% Trypticase soy broth and subjected to fractionation into subcellular compartments as described above or were spotted on the same medium containing 2% agar and 0.1% elastin (Sigma); cultures were then incubated for 2 days at 37°C and 7 additional days at room temperature until clearing of the elastin was visible.

RESULTS

Synthesis and localization of extracellular proteins by *P. aeruginosa* mutants in pilus biogenesis determinants. We have examined representative strains of *P. aeruginosa* PAK that carry transposon insertions in the chromosomal loci of genes *pilB*, *pilC*, and *pilD* (25). Levels of two extracellular enzymes, alkaline phosphatase and phospholipase C, were found to be unchanged in total cell extracts of strains E4 (*pilB*) and E9 (*pilC*) (Fig. 1). These levels were comparable with those of strain B24, which served as a wild-type control. In contrast, the levels of these same two enzymes in strain 2B18 (*pilD*) were found to be significantly higher. Activity of alkaline phosphatase in strain 2B18 was 15- to 17-fold higher than the activity in the other *pil* mutants or in the wild type. Similarly, analysis of phospholipase C activity in the *pilD* mutant revealed a seven- to ninefold increase over activities detected in strain E4, E9, or B24. The observed derepression of extracellular enzymes in a *pilD* background was not observed for β-lactamase, a periplasmic enzyme; all four strains showed comparable levels of β-lactamase activity.

The localization of these normally secreted polypeptides was also examined in the different *pil* mutant backgrounds. Cultures of individual strains were fractionated into extracellular, periplasmic, cytoplasmic, and membrane fractions.

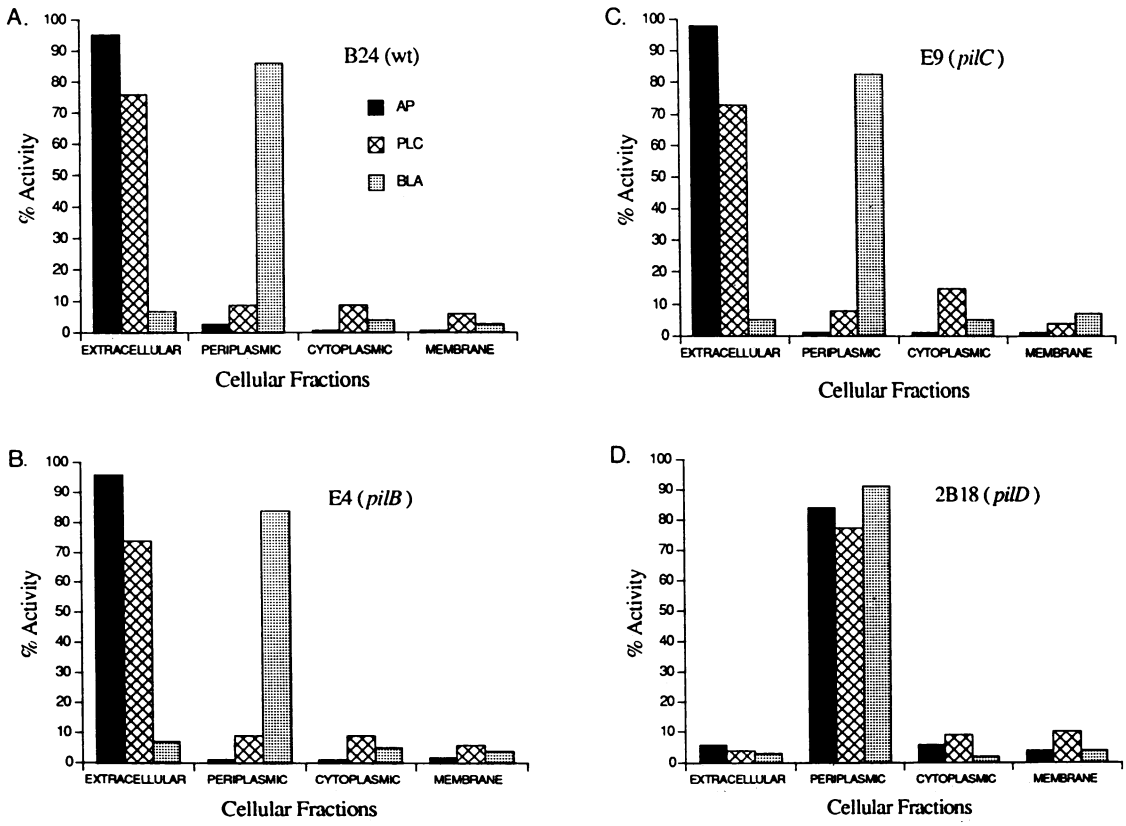

FIG. 2. Distribution of exported enzymes in the wild type and in *pilB*, *pilC*, and *pilD* mutants. Strains B24, E4, E9, and 2B18 carrying pMMB66EH were grown in the low-phosphate medium and were harvested at mid-logarithmic stage of growth. The cells were fractionated into periplasmic, membrane, and cytoplasmic compartments. Levels of each of the enzymes were determined as described in Materials and Methods. Abbreviations: AP, alkaline phosphatase; PLC, phospholipase C; BLA, β-lactamase.

The distribution of selected proteins in each compartment was determined. Activities of alkaline phosphatase and phospholipase C were found exclusively in the extracellular fractions of *pilB*, *pilC*, and the wild-type control strains (Fig. 2A, B, and C). The same enzymes, however, were localized in the periplasmic fraction of the *pilD* mutant 2B18, where they were found to cofractionate with β-lactamase (Fig. 2D).

The alterations of export of alkaline phosphatase and phospholipase C were due to the inability of strain 2B18 to express functional PilD. Full restoration of the export defect was accomplished by the introduction of plasmid pDN19XS, containing the cloned *pilD* gene, into *P. aeruginosa* 2B18 (Fig. 3). The total levels of these enzymes in the culture fluids of strain 2B18(pDN19XS) were also comparable to those of the wild type (data not shown).

A third secreted enzyme, elastase, was also examined for its localization in the different *pil* mutants. We were unable to examine the distribution of elastase in subcellular fractions, because *P. aeruginosa* PAK is a low elastase producer compared with other proteolytic strains such as PAO1, and

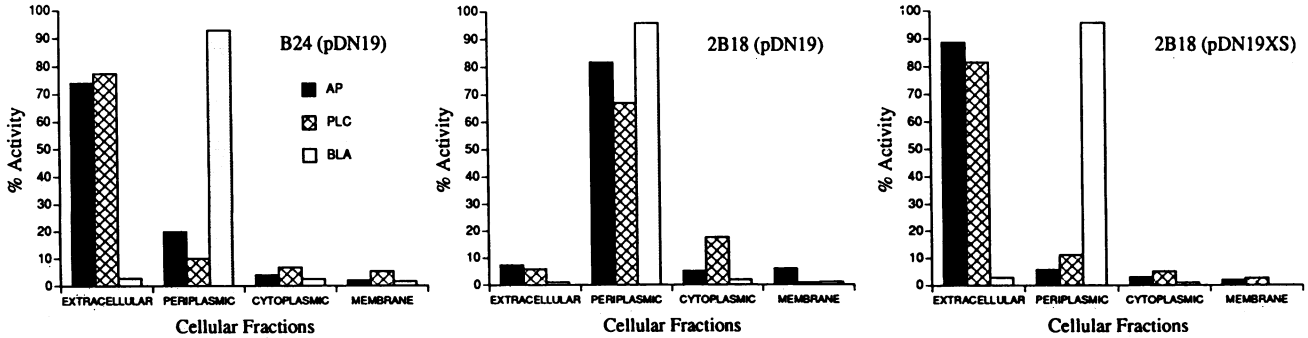

FIG. 3. Complementation of the export defect in strain 2B18 by the cloned *pilD* gene. Strains B24(pDN19), 2B18(pDN19), and 2B18(pDN19XS) were grown in low-phosphate medium supplemented with 1 μg of imipenem per ml and 100 μg of tetracycline per ml. Cells were harvested and fractionated, and enzymes were assayed as described in Materials and Methods. Abbreviations: AP, alkaline phosphatase; PLC, phospholipase C; BLA, β-lactamase.

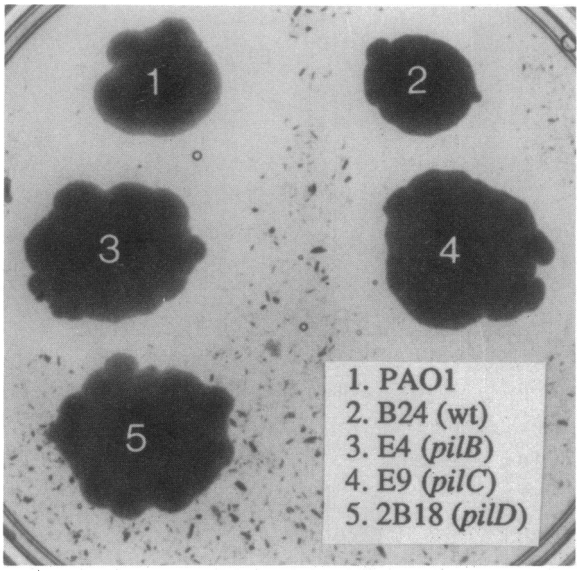

FIG. 4. Secretion of elastase in the pilus biogenesis gene mutants. The different strains shown were spotted on 5% peptone–0.5% Trypticase soy agar containing elastin and incubated as described in Materials and Methods.

detection of elastase in the culture supernatants was not possible on immunoblots or by using standard elastase chromogenic substrates. However, after 2 days of incubation at 37°C and 7 days at room temperature, clearing of elastin on a solid medium was visible in all strains, with the exception of the *pilD* mutant (Fig. 4). Therefore, the *pilD* mutation also prevents the export of elastase across the outer membrane into the extracellular environment.

We have also examined the localization of exotoxin A in the same mutant strains, carrying a cloned exotoxin A gene under control of the *tac* promoter (pMMD4). Following induction with IPTG, the cells were subjected to the same fractionation procedure as described above. The distribution of exotoxin A, as measured by immunoblot analysis of aliquots of each subcellular fraction, was identical to those observed for the extracellular enzymes (Fig. 5). The *pilB* and *pilC* mutants released exotoxin A as efficiently as the wild-type control strain, whereas the *pilD* mutant localized the toxin into its periplasmic space. As judged by the intensity of bands on immunoblots, the amount of exotoxin A antigen in the periplasmic space of strain 2B18 was comparable to the amount released by the wild type or the other *pil* mutants. The electrophoretic mobility of exotoxin A isolated from the periplasmic space of *pilD* was identical to that of exotoxin A released into the culture medium by *pilB*, *pilC*, or wild-type strains (Fig. 5), suggesting that the *pilD* mutation does not block removal of the 25-amino-acid signal peptide of the exotoxin A precursor (19).

The localization defect of the *pilD* mutant was restricted only to extracellular proteins. Chromosome- or plasmid-encoded β-lactamase secretion was not affected. Examination of bacteria by electron microscopy revealed that while *pilD* mutants lacked pili, they all displayed prominent polar flagella. In addition, examination of outer membrane fractions of all *pil* mutants for the presence of the outer membrane protein OprG by Western immunoblotting revealed no alteration in the amounts of this protein, as compared with those in the wild-type control strains (data not shown).

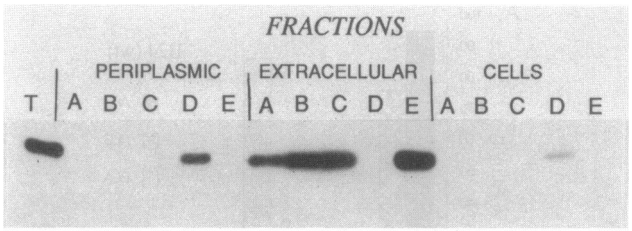

FIG. 5. Cellular localization of exotoxin A in *P. aeruginosa* PAK wild type and pilus biogenesis mutants containing pMMD4 after IPTG induction. Wild-type *P. aeruginosa* PAK and strains B24, E4 (*pilB*), E9 (*pilC*), and 2B18 (*pilD*) (all containing pMMD4) were grown in L broth at 37°C to an A_{600} of 1.5, after which they were induced with 1 mM IPTG for 2 h. Cells were harvested and fractionated into the different cellular compartments and electrophoresed on SDS–7.5% polyacrylamide gels, which was followed by Western transfer and immunoblotting with antitoxin and ^{125}I-protein A (New England Nuclear, Boston, Mass.). Lanes show purified exotoxin A (T) and the periplasmic, extracellular, and cell (after extraction of periplasmic space proteins) fractions derived from strains B24 (lane A), E4 (lane B), E9 (lane C), 2B18 (lane D), and the wild type (lane E).

Conformation of the periplasmic form of exotoxin A. Exotoxin A, extracted from the periplasmic space of the *pilD* mutant 2B18, was further studied to determine whether folding and formation of correct intrapolypeptide disulfide bonds occurred without export to the extracellular space. Disulfide bond formation was assessed by comparing the electrophoretic mobilities of the reduced and alkylated form of exotoxin A with that exported by wild-type *P. aeruginosa*. The mobility on SDS-polyacrylamide gels of periplasmic exotoxin A from the *pilD* mutant, analyzed in the presence and absence of the reducing agent dithiothreitol, was identical to the mobility of the exported protein from wild-type *P. aeruginosa* (Fig. 6).

Correct folding of exotoxin A can also be assessed by cytotoxicity to cultured cells, since only exotoxin A in its native conformation can enter and kill eucaryotic cells (18). A dose-response toxicity curve of exotoxin A is shown in Fig. 7. Toxins derived from both the culture supernatant of

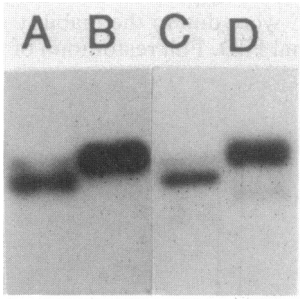

FIG. 6. Effects of disulfide bond reduction on mobility of exotoxin A in SDS-polyacrylamide gels. Exotoxin A present in the culture supernatant of IPTG-induced *P. aeruginosa* B24(pMMD4) (lanes A and B) and in the periplasmic space fraction of *P. aeruginosa* 2B18(pMMD4) (lanes C and D) was denatured at room temperature in 1% SDS, with (lanes B and D) and without (lanes A and C) 10 mM dithiothreitol. Samples were treated with 50 mM iodoacetamide to block reformation of disulfide bonds and then electrophoresed on SDS–7.5% polyacrylamide gels, which was followed by Western transfer and immunoblotting with antitoxin and ^{125}I-protein A.

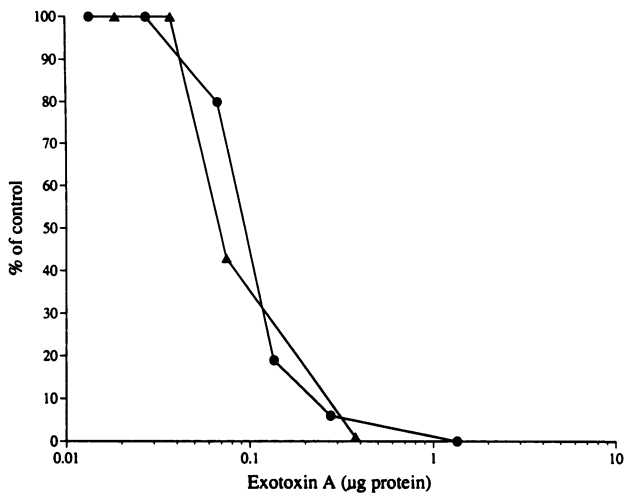

FIG. 7. Comparison of inhibition of protein synthesis of HeLa cells by exotoxin A derived from the periplasmic space in strain 2B18(pMMD4) versus supernatant culture medium from B24(pMMD4). Monolayers of HeLa cells in vials with a surface area of 1 cm^2 and containing 0.5 ml of medium were exposed to various amounts of exotoxin A for 20 h. Inhibition of protein synthesis was determined after a 30-min pulse with ^{35}S-Trans label (ICN Biomedicals, Costa Mesa, Calif.). Cells were washed of the label once with phosphate-buffered saline followed by direct precipitation of the monolayers with 10% trichloroacetic acid and addition of the liquid scintillation cocktail Aquasol II (NEN). The radioactivity incorporated into acid-insoluble protein was determined by liquid scintillation. ●, Exotoxin A from the supernatant of *P. aeruginosa* PAK B24(pMMD4); ▲, exotoxin A from the periplasmic space fraction of *P. aeruginosa* 2B18(pMMD4). Culture conditions and isolation of these fractions are described in the legend to Fig. 5.

the wild type and the periplasm of the *pilD* mutant of *P. aeruginosa* PAK equally inhibited protein synthesis in HeLa cells with a 50% inhibitory concentration of approximately 50 ng/ml.

DISCUSSION

We have examined the effects of mutations in each of the genes encoding the pilus biogenesis functions on the localization of a number of secreted *P. aeruginosa* proteins. From the results of this survey, we conclude that in *P. aeruginosa*, accessory functions encoded by the *pilB* and *pilC* genes appear to be pilin specific. In contrast, *pilD* encodes a protein that is required for correct localization of a wide range of extracellular polypeptides, including exotoxin A, alkaline phosphatase, phospholipase C, and elastase. This pleiotropic effect on the secretion of *P. aeruginosa* extracellular proteins is similar to that described for mutants in several *xcp* genes (14). Export of periplasmic and outer membrane proteins appears to utilize a pathway which is PilD independent. In addition to the protein localization defect of the *pilD* mutant, the overall activities of alkaline phosphatase and phospholipase C in this mutant greatly exceeded the levels detected in wild-type and *pilB* or *pilC* strains (Fig. 1). These findings suggest that PilD may be involved in the control of synthesis of exported proteins. Alternatively, failure to export proteins results in their accumulation in an enclosed compartment that differs from the extracellular medium, in which a substantial fraction of alkaline phosphatase or phospholipase C may be inactivated or degraded.

PilD has been previously shown to be required for processing prepilin to pilin (25). More recently, we have shown that the product of PilD is an endopeptidase with a substrate specificity for proteins with leader sequences found on prepilin or related proteins, which includes precursors of other type IV methylphenylalanine pilins (25a). The export block in *pilD* mutants, limited to extracellular proteins, is somewhat surprising, because precursors of these proteins contain typical secretion signal sequences that likely are substrates for the leader peptidase which is different from the *pilD* product. Furthermore, the exotoxin A found in the periplasmic space of *pilD* mutants appears to be identical in size to the mature protein, suggesting that *pilD* influences export at stages subsequent to removal of the secretion signal sequences. The role of PilD in export of exotoxin A, phospholipase C, alkaline phosphatase, and elastase is very likely indirect. PilD proteolytic activity is probably responsible for cleavage of leader peptides from newly synthesized components of the export machinery during assembly of the export apparatus in the bacterial cell envelope. The periplasmic localization of exported proteins in *pilD* mutants may be the consequence of an aberrant export pathway, or alternatively, exported proteins naturally transit this compartment during export, and the mutation in PilD prevents their further translocation across the outer membrane.

Secreted proteins are capable of membrane translocation only while they are in an unfolded, export-competent conformation, which is maintained by a class of cytoplasmic proteins called molecular chaperones (15). Our finding that exotoxin A in the *pilD* mutant is apparently in its native conformation, with the four pairs of disulfide bonds correctly formed, raises the possibility that the export block in the *pilD* mutant is due to folding of the toxin, which prevents its release from the periplasmic space. One component of the export machinery, therefore, could be a chaperonelike protein that prevents folding of mature exotoxin A and facilitates its transfer from cells via the adhesion zones between the inner and outer membrane (20) or by passage through the periplasmic space and the outer membrane. Recently, Tennent et al. (28) demonstrated the existence of a periplasmic chaperone protein (PapJ) which is required for correct assembly of pili by uropathogenic *E. coli*. PapJ shares a homology with a number of proteins involved in macromolecular or sugar transport, cell division, and ATP generation (7). It is conceivable that the excretion apparatus of *P. aeruginosa* contains such chaperones and that assembly of this apparatus into a functional membrane or periplasmic complex is controlled by PilD.

Accumulated evidence from studies of export of proteins from gram-negative bacteria suggests that the presence of the second membrane (the outer membrane) necessitates a need for a specialized export apparatus. Components of such machinery may function completely independently of the normal secretion machinery. It is likely, however, that extracellular localization is an extension of the normal export pathway, involving proteins that are substrates of the PilD peptidase.

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