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Mutations in the Consensus ATP-Binding Sites of XcpR and PilB Eliminate Extracellular Protein Secretion and Pilus Biogenesis in *Pseudomonas aeruginosa*

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The process of extracellular secretion in *Pseudomonas aeruginosa* requires specialized machinery which is widely distributed among bacteria that actively secrete proteins to the extracellular medium. One of the components of this machinery is the product of the *xcpR* gene, which is homologous to *pilB*, a gene encoding a protein essential for the biogenesis of type IV pili. Both XcpR and PilB are characterized by the presence of a conserved ATP-binding motif (Walker sequence). The codons of highly conserved glycine residues within the Walker sequences of *xcpR* and *pilB* were altered to encode a serine, and the effects of these substitutions were examined. Bacteria expressing mutant XcpR or PilB were unable to secrete exotoxin A or assemble pili, respectively. In addition, high-level expression of mutant XcpR in wild-type *P. aeruginosa* led to a pleiotropic extracellular secretion defect, resulting in the periplasmic accumulation of enzymes that are normally secreted from the cell. These studies show that the putative ATP-binding sites of XcpR and PilB are essential for their functions in protein secretion and assembly of pili, respectively. Moreover, the observed dominant negative phenotype of mutant XcpR suggests that this protein functions as a multimer or, alternatively, interacts with another essential component of the extracellular protein secretion machinery.

Proteins secreted into the extracellular medium by gram-negative bacteria must cross a double membrane barrier, consisting of the cytoplasmic and outer membranes, separated by the periplasmic space. It has been well established, with *Escherichia coli* as a model system, that translocation of proteins bearing N-terminal signal peptides to the periplasm or to the outer membrane involves secretion machinery consisting of several of the *sec* gene products, one of two leader peptidases, and a number of cytoplasmic chaperones (reviewed in reference 32). This secretion pathway is conserved among all gram-negative bacteria. In contrast, extracellular protein secretion can occur by multiple mechanisms. One such mechanism is a two-step process whereby proteins bearing signal sequences are secreted across the cytoplasmic membrane via the *sec*-dependent pathway and then across the outer membrane by a distinct pathway known as the general secretion pathway (reviewed in reference 39). The general secretion pathway has been most extensively studied with *Klebsiella oxytoca*, in which the extracellular secretion of pullulanase requires machinery consisting of the products of at least 14 *pul* genes (*pulC* to *pulO* and *pulS*) (reviewed in reference 28). Alternatively, extracellular secretion of proteins lacking the N-terminal leader peptides, the prototype of which is the hemolysin of *E. coli*, are secreted by a *sec*-independent mechanism, utilizing as few as three proteins which are located in the cell envelope (reviewed in reference 39).

Pseudomonas aeruginosa is a gram-negative, opportunistic pathogen that secretes a number of virulence factors into the extracellular medium. Many of these virulence factors, such as alkaline phosphatase, exotoxin A, phospholipase C, lipase, and elastase, are secreted by the general secretion pathway, similar to that described for pullulanase secretion by *K. oxytoca* (36). The extracellular secretion functions of *P. aeruginosa* are encoded by the *xcp* genes (*xcpR* to *xcpZ*) and share homology with the *Klebsiella pul* genes (*pulE* to *pulM*) (1). Recently, a similar mechanism for extracellular secretion of enzymes has been described for other gram-negative bacteria, including *Erwinia* spp. and *Xanthomonas campestris* (reviewed in reference 29).

One of the interesting features of the components of the general secretion pathway is their similarity to determinants of type IV pilus biogenesis. The products of *xcpT* to *xcpW* (also called *pddA* to *pddD*), and their homologs in other bacteria, share extensive sequence similarity to the subunits of type IV pili. The sequence conservation is most pronounced in the short N-terminal leader peptide and the first 25 to 30 amino acids of the mature protein. These proteins are processed by a specialized leader peptidase (PilD/XcpA, PulO in *K. oxytoca*) (1, 25) which cleaves the short leader peptide (24) and N methylates the first amino acid of the mature protein (34). In addition, it has been shown that mutations in *pilD* lead to an accumulation of normally secreted proteins in the periplasm (33). The biogenesis of type IV pili also requires two additional proteins, PilB and PilC, and their respective homologs are also found among the products of the genes of the general secretion pathway, XcpR and XcpS in *P. aeruginosa* and PulE and PulF in *K. oxytoca* (1, 25).

The XcpR/PilB and PulE gene products are especially intriguing since they almost certainly function in extracellular protein secretion and assembly of pili from a cytoplasmic location. Their major distinguishing feature is the presence of a highly conserved region of an ATP-binding domain ("Walker box A") consisting of the sequence GXXGXGKT (38). A third PilB/XcpR homolog, PilT, in *P. aeruginosa* was recently described. PilT is involved in bacterial motility, called twitching motility, which is apparently mediated by the retraction of type IV pili (40). XcpR, PilB, and PilT therefore represent a family of proteins that play an as-yet-

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TABLE 1. Bacterial strains, phage, and plasmids

Strain, phage, or plasmid	Relevant traits[a]	Source or reference
Strain		
E. coli		
DH5α	*hsdR recA lacZYA* φ80 *lacZΔM15*	BRL[b]
XL-1 blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB*, *lacI*^{q}ADM15 Tn*10*]; Tet^r	Stratagene
RZ1032	Hfr; *dut ung thi-1 relA1, supE44, Zbd-279*::Tn*10*; Tet^r	A. Rattrey
P. aeruginosa		
PAK	Wild type	D. Bradley
PAKrif	Rif^r derivative of wt PAK	24
B24	Kan^r; Tn insertion upstream of secretion genes; wt control strain	24
B24ΔSca	Derivative of B24 with secretion (*xcp*) genes deleted	D. Nunn
Phage		
PO4	Pilus-specific lytic phage	D. Bradley
VCSM13	Interference-resistant helper phage	Stratagene
Plasmids		
pDN18/19	Tet^r broad-host-range cloning vectors	24
pDN19L	Secretion genes in pDN19; 9.2-kb *Eco*RI fragment (*xcpR* to *xcpZ*)	D. Nunn
pDN19B-EL	5′ deletion subclone of pDN19L; 7.2-kb *Bam*HI-*Eco*RI fragment of pDN19L (*xcpR* mutant)	D. Nunn
pRK2073	Str^r mobilizing plasmid	19
pGP1-2	Kan^r; thermoinducible T7 RNA polymerase gene	37
pMMD4	IPTG-inducible exotoxin A	35
pMMB67EH	Ap^r broad-host-range cloning vector; *lacI*^{q}/*tac* promoter	8
pBS[c] (KS/SK+)	Ap^r pBluescript phagemid cloning vectors	Stratagene
pTZ19A	Ap^r; pilus biogenesis accessory genes cloned into phagemid pTZ19R	D. Nunn

[a] Abbreviations: Tet^r, tetracycline resistant; Rif^r, rifampin resistant; wt, wild-type; Kan^r, kanamycin resistant; Str^r, streptomycin resistant; Ap^r, ampicillin resistant.
[b] BRL, Bethesda Research Laboratories.
[c] pBS, pBluescript.

unidentified role in extracellular secretion of proteins and assembly and disassembly of pili, respectively.

In order to investigate the structure-function relationship of XcpR and PilB, we engineered mutations in the *xcpR* and *pilB* genes to give products with amino acid substitutions in the conserved ATP-binding site. We show that these mutations lead to defects in extracellular protein secretion and pilus assembly, providing evidence that the ATP-binding site is required for the proper function of these proteins, very likely by hydrolyzing ATP to provide energy for the membrane translocation process.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains, plasmids, and phages used in this study are listed in Table 1. The wild-type control, strain B24, used for secretion studies involving XcpR, was a kanamycin-resistant derivative of *P. aeruginosa* wild-type strain PAK, carrying a transposon insertion upstream of the secretion genes. The secretion-deficient derivative of strain B24, B24ΔSca, was created by deleting a *Sca*I fragment containing all but the 5′ 100 bp of the secretion genes *xcpR* to *xcpZ*. The resulting fragment was subcloned into pBR322. The construct was introduced into *P. aeruginosa* B24 by conjugation and was subsequently integrated into the chromosome. A nonhemolytic (secretion-deficient) exconjugant was generated. The deletion of the *Sca*I fragment was confirmed by Southern analysis (22a).

Culture conditions. Cultures were routinely grown in Luria broth (21) or minimal A salts (6) supplemented with 50 mM monosodium glutamate and 1% glycerol for *P. aeruginosa*. For growth of *E. coli*, the minimal salts were supplemented with 0.2% glycerol and 0.001% thiamine; for labeling experiments, the minimal salts were supplemented with a mixture of 0.02% 18 amino acids lacking methionine and cysteine. Antibiotics (concentrations in micrograms per milliliter) were as follows: for *P. aeruginosa*, tetracycline (100), kanamycin (50), and carbenicillin (150); for *E. coli*, tetracycline (20), ampicillin (100), and kanamycin (50).

Enzymes and chemicals. All restriction enzymes and T4 DNA ligase were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Isotopes for labeling of polypeptides, [^{35}S]methionine (Express ^{35}S^{35}S protein labeling mix), and ^{125}I-protein A for immunoblots were purchased from Dupont, NEN Research Products, Boston, Mass. Sequenase version 2.0 was purchased from U.S. Biochemical Corp. (Cleveland, Ohio) and used as instructed by the manufacturer.

Cloning and site-directed mutagenesis. A 2.8-kb *Eco*RI-*Asu*II fragment from plasmid pDN19L, containing the coding region of *xcpR*, was subcloned into pBluescript KS+, resulting in plasmid pKSXcpR. The *pilB* coding region was obtained by subcloning a 2.1-kb *Kpn*I-*Nar*I fragment from pTZ19A into pBluescript SK+, resulting in plasmid pSK-PilB.

Uracil-containing templates for site-directed mutagenesis were prepared by using the helper phage VCSM13 to rescue single-stranded DNA from *E. coli* RZ1032 transformed with pKSXcpR or pSKPilB. Mutagenesis was carried out as described by Kunkel et al. (17). Oligonucleotide primers XcpRmut (5′CCACCGGCTCGΔGCAAGACCAC3′) and PilBmut (5′CCACCGGCTCGΔGCAAGACGGT3′) were used to make single base changes (underlined) in the putative ATP-binding sites of XcpR and PilB, respectively, resulting in plasmids pKSXcpRmut and pSKPilBmut.

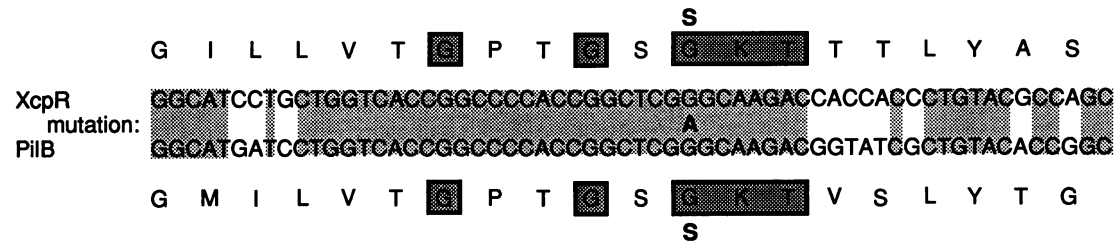

FIG. 1. Alignment of partial sequences of XcpR (amino acid residues 256 to 275) and PilB (amino acid residues 320 to 339). Identical bases in the DNA sequence are shaded to demonstrate the high degree of homology between the two genes. The consensus GXXGXGKT Walker box (38) consists of amino acid residues 262 to 269 in XcpR and residues 326 to 333 in PilB. The conserved, essential residues are indicated by shaded boxes. The mutation introduced into the DNA sequence by site-directed mutagenesis is indicated in boldface between the DNA sequences. The glycine to serine changes resulting from this mutation are indicated above and below the amino acid sequences.

These mutations, as well as the wild type, were then subcloned into broad-host-range vectors for introduction into *P. aeruginosa*. A 3-kb *Bam*HI fragment from pKSXcpR and the corresponding fragment from pKSXcpRmut were subcloned into pDN19B-EL to generate pLT1 and pLT2, respectively, and 2.1-kb *Kpn*I-*Nar*I fragments from pSKPilB and pSKPilBmut were subcloned into pDN18 to generate pLTB1 and pLTB2. In addition, an approximately 3-kb *Eco*RI-*Stu*I fragment from pKSXcpR and a 3-kb *Eco*RI-*Kpn*I fragment from pKSXcpRmut were subcloned into the broad-host-range vector pMMB67EH, which carries an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *tac* promoter (8), generating pMMR and pMMRmut, respectively.

DNA manipulations and analyses. All plasmid DNA from *E. coli* was prepared by the method of Birnboim and Doly (3) for both large- and small-scale isolations. Plasmid DNA was further purified with CsCl-ethidium bromide density gradients.

The presence of the engineered mutations was confirmed by sequence analysis of plasmids pKSXcpR, pKSXcpRmut, pSKPilB, and pSKPilBmut. Sequencing was carried out with single-stranded or double-stranded templates by the dideoxy method of Sanger et al. (30). Oligonucleotide primers XcpR (5'GGTATTCGATCGGATCC3') and PilB (5'GCACTCAA GCAGCCGCA3') were employed.

Genetic techniques. Plasmids were introduced into *P. aeruginosa* by triparental matings, using pRK2073 as a mobilizing plasmid (19). Transconjugants were isolated after selection on L or minimal agar plates containing the appropriate antibiotics.

Cell fractions. For determining the localization of exotoxin A, *P. aeruginosa* was grown in L broth with the appropriate antibiotics to an optical density at 600 nm of 0.4 to 0.5. Fresh medium was added and supplemented with 4 mM IPTG to induce expression of exotoxin A. Cells were grown for an additional 1.5 h and were harvested by centrifugation. The culture supernatants were saved as the extracellular fraction. The cell pellets were washed with cold 30 mM Tris (pH 8.0) and resuspended in 50 mM Tris (pH 7.2)–0.2 M $MgCl_2$. Periplasmic fractions were prepared in the manner of Hoshino and Kageyama (12). Briefly, the suspensions were treated twice as follows: incubation at 30°C for 30 min, chilling on ice, incubation at room temperature for 15 min. The cell pellets, representing the cell-associated fraction, were resuspended in 30 mM Tris (pH 8.0) and lysed by sonication. The total protein of each fraction was precipitated with 10% trichloroacetic acid, using 0.01% skim milk as a carrier. The pellets were washed with 100% acetone and resuspended in distilled water.

SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting. Samples were denatured by boiling in 2% sodium dodecyl sulfate (SDS)–5% 2-mercaptoethanol–62.5 mM Tris (pH 6.8). Preparations were separated on tricine-SDS–10% polyacrylamide gels (31) or SDS–10% polyacrylamide gels by the system of Laemmli (18). Gels were either stained with Coomassie blue R or immunoblotted as follows. The proteins in the gels were electrophoretically transferred to nitrocellulose filters (37). The filters were then treated with 5% nonfat dry milk in phosphate-buffered saline, incubated with mouse polyclonal anti-exotoxin A sera, washed, probed with ^{125}I-protein A, and autoradiographed.

T7 expression experiments. A T7 RNA polymerase-promoter gene expression system was used to express proteins encoded by plasmids pKSXcpR, pKSXcpRmut, pSKPilB, and pSKPilBmut in *E. coli*. pGP1-2 served as a source of thermoinducible T7 polymerase (35). Polypeptides, labeled with [^{35}S]methionine, were analyzed by SDS-PAGE and autoradiography.

Phage sensitivity tests. Suspensions of *P. aeruginosa* strains to be tested were streaked onto L agar plates containing antibiotics. A 5-μl sample of a suspension of the pilus-specific phage PO4 was applied to the center of the steak. Sensitivity was defined as no growth, resulting from cell lysis, in the area of phage inoculation.

Enzyme assays. *P. aeruginosa* PAK cultures carrying the appropriate plasmids were grown in low-phosphate medium (13) with 2 mM IPTG until they reached an optical density at 600 nm of 2.0 to 3.0. Extracellular, periplasmic, and cell-associated fractions were prepared. Phospholipase C activity was measured with the chromogenic substrate *p*-nitrophenylphosphorylcholine (Sigma Chemical Company, St. Louis, Mo.) (2). β-Lactamase and alkaline phosphatase activities were assayed by monitoring the rates of hydrolysis of nitrocefin (Bethesda Research Laboratories) and *p*-nitrophenyl phosphate (Sigma), respectively (4, 14). Activities of the enzymes in each fraction were corrected for the bacterial mass of the culture.

RESULTS

Mutagenesis of *xcpR* and *pilB*. Oligonucleotide site-directed mutagenesis was carried out to change a codon for one of the essential glycines of the XcpR and PilB Walker boxes to one encoding a serine (Fig. 1). Changes of the invariant amino acids of the ATP-binding site have been shown to abolish ATP binding in many proteins, including the RecD subunit of the RecBCD enzyme and the histidine permease (HisP) of *E. coli* (16, 22). The wild-type and mutant forms of *xcpR* and *pilB* were subsequently subcloned

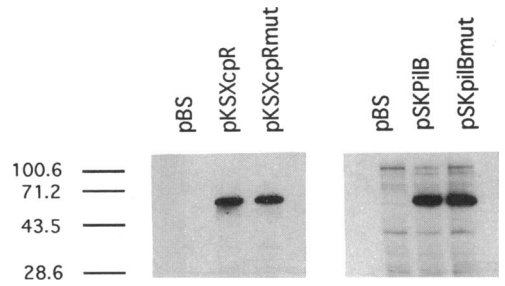

FIG. 2. Autoradiogram of T7 expression of XcpR and PilB. The proteins expressed by plasmids pBluescript (pBS), pKSXcpR, pKSXcpRmut, pSKPilB, and pSKPilBmut were labeled with [^{35}S]methionine and run on SDS-polyacrylamide gels. Numbers represent the positions of molecular mass markers (in kilodaltons). All clones expressed high levels of a protein in the expected size range of XcpR (55.5 kDa) or PilB (62.4 kDa).

into broad-host-range vectors for introduction into *P. aeruginosa*.

To verify that the mutations introduced into *xcpR* and *pilB* had not altered the size and stability of the mutated gene products, the levels of expression of *xcpR*, *pilB*, and their mutant counterparts were compared in a coupled T7 promoter-polymerase system (35). Labeled extracts, when analyzed by PAGE (Fig. 2), showed that the mutant forms of XcpR and PilB were equally well synthesized and were of the same size (ca. 60,000 Da) as the wild-type forms.

Expression of mutant *xcpR* in *P. aeruginosa*. In order to determine the effect of the engineered *xcpR* mutation on extracellular protein secretion, pLT1 (carrying wild-type *xcpR* to *xcpZ*) and pLT2 (carrying mutant *xcpR* and wild-type *xcpS* to *xcpZ*) were introduced into a wild-type strain (B24) and a secretion-defective strain (B24ΔSca) of *P. aeruginosa*. These bacteria also carried plasmid pMMD4, which expresses exotoxin A from the *tac* promoter. After induction of exotoxin A synthesis with IPTG, the locations of exotoxin A antigen in various cellular compartments were compared.

An immunoblot of extracellular, periplasmic, and cell-associated fractions, using anti-exotoxin A antibody, is shown in Fig. 3. As expected, mutant B24ΔSca carrying vector pDN19 accumulated exotoxin A in the periplasm, while exotoxin A synthesized in wild-type strain B24 was found entirely in the culture media, without any periplasmic accumulation. When the wild-type copy of *xcpR* (plasmid pLT1) was introduced into strain B24ΔSca, extracellular secretion of exotoxin A was restored, with approximately 90% of the total toxin being released into the medium and some still remaining in the periplasm. The presence of additional copies of wild-type *xcpR* in wild-type strain B24 had no effect on the export of exotoxin A from this strain.

Expression of the mutant form of *xcpR* from plasmid pLT2 in strain B24ΔSca failed to restore extracellular secretion of exotoxin A. Interestingly, expression of mutant XcpR in wild-type *P. aeruginosa* led to a modest defect in exotoxin A secretion into the medium. In this strain, approximately 30% of the exotoxin A synthesized was found in the periplasm, suggesting that the mutant form of *xcpR* interfered with normal extracellular protein secretion.

To further investigate whether the mutant form of XcpR did indeed cause a dominant negative phenotype in the wild-type background, the wild-type and mutant *xcpR* genes were subcloned into pMMB67EH. In these constructs, the transcription of *xcpR* is directed by an IPTG-inducible *tac*

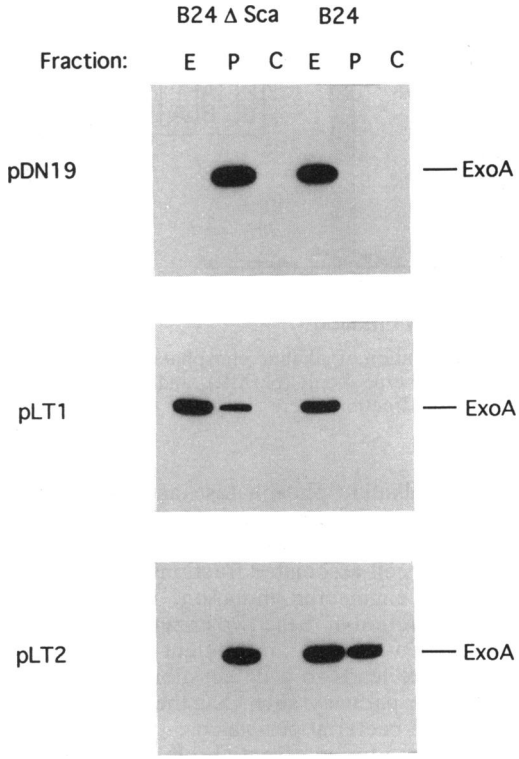

FIG. 3. Autoradiogram of Western blot analysis of exotoxin A localization. Extracellular (E), periplasmic (P), and cell-associated (C) fractions from protein secretion-deficient strain B24ΔSca and from wild-type strain B24, each carrying the vector alone (pDN19) or plasmid pLT1 or pLT2, were run on SDS-polyacrylamide gels and immunoblotted with mouse polyclonal anti-exotoxin A sera and labeled with ^{125}I-protein A.

promoter of the plasmid vector. High-level expression of mutant *xcpR* in wild-type *P. aeruginosa* resulted in a complete block of the extracellular secretion of exotoxin A, which accumulated in the bacterial periplasm and cofractionated with β-lactamase (Fig. 4). In contrast, the bacteria carrying vector pMMB67EH secreted exotoxin A from the cell. Expression of the wild-type *xcpR* gene, under the same conditions, had no effect on exotoxin A export.

In order to assess whether mutant XcpR interferes with extracellular secretion of other *P. aeruginosa* proteins, the

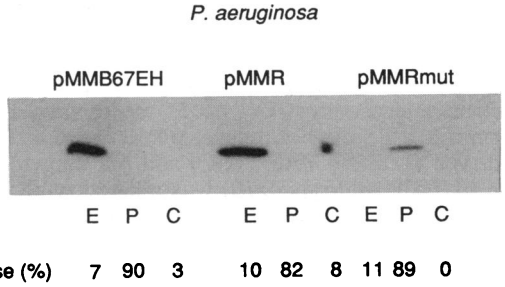

FIG. 4. Western blot analysis of exotoxin A localization in wild-type cells expressing vector pMMB67EH, wild-type XcpR (pMMR), and mutant XcpR (pMMRmut). The percentage of β-lactamase cofractionating with each cellular fraction (extracellular [E], periplasmic [P], and cell associated [C]) is indicated below each lane.

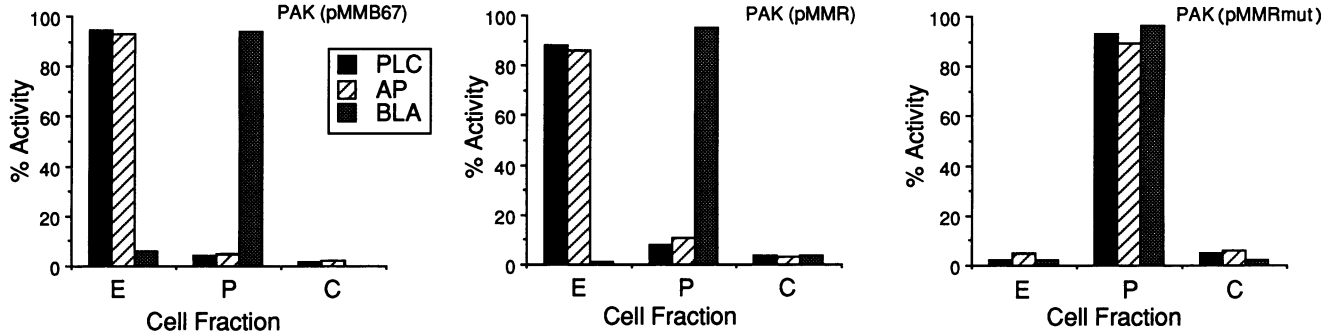

FIG. 5. Localization of alkaline phosphatase (AP), phospholipase C (PLC), and β-lactamase (BLA) in wild-type cells expressing vector pMMB67EH, wild-type XcpR (pMMR), and mutant XcpR (pMMRmut). Enzyme activities in the extracellular (E), periplasmic (P), and cell-associated (C) fractions were assayed.

localization of alkaline phosphatase and phospholipase C was determined in bacteria expressing the mutant *xcpR* gene. Quantitative enzyme assays from extracellular, periplasmic, and cell-associated fractions demonstrated that XcpR, with its engineered mutation, interferes with the extracellular secretion of these two enzymes as well (Fig. 5). The presence of wild-type and mutant XcpR in the same bacteria therefore leads to a defect along the extracellular protein secretion pathway such that the export of proteins terminates in the bacterial periplasm.

Further evidence for an essential role for XcpR in protein secretion was obtained from studies involving a strain of *P. aeruginosa* carrying the engineered mutation in the chromosomal copy of *xcpR*. In this strain, exotoxin A localized to the periplasm (data not shown). This result indicates that the glycine residue of the conserved ATP-binding site is required for XcpR function and thus for extracellular protein secretion.

Expression of mutant *pilB* in *P. aeruginosa*. In order to test the effect of the glycine to serine substitution on PilB function, we introduced plasmids pLTB1 (wild-type *pilB*) and pLTB2 (mutant *pilB*) into *P. aeruginosa* E4, which carries a transposon insertion in the *pilB* gene. Strain E4 does not assemble pili and is therefore resistant to the killing action of the pilus-specific phage PO4 (23). *P. aeruginosa* E4 carrying the wild-type *pilB* gene encoded on plasmid pLTB1 became susceptible to infection and lysis by the PO4 phage, while the same strain carrying the mutated *pilB* on plasmid pLTB2 remained resistant to phage killing (Fig. 6). The continued resistance to phage killing in E4 carrying pLTB2 indicates that the mutant form of PilB is unable to complement the piliation defect in this *pilB* mutant strain.

To verify that the differential killing effect of the PO4 phage in E4 expressing wild-type and mutant *pilB* was due to the piliation state of the cells, we examined the bacteria by electron microscopy (Fig. 7). *P. aeruginosa* E4 carrying plasmid pDN18 did not display pili, as seen at the poles of wild-type *P. aeruginosa* PAK (data not shown). Strain E4 with pLTB1 (wild-type *pilB*) was piliated, with pili comparable in number and length to those on wild-type *P. aeruginosa*. In contrast, E4 expressing pLTB2 (mutant *pilB*) remained nonpiliated. These results confirm that the conserved glycine in the ATP-binding site of PilB is essential for its function in pilus biogenesis. The expression of mutant PilB in wild-type *P. aeruginosa* was also examined. Electron microscopic observation of negatively stained bacteria showed pili that were normal in appearance, number, and overall distribution. We cannot, however, exclude the possibility that a dominant negative effect of mutant PilB was exerted at a level other than the morphological appearance of pili on the bacterial surface.

DISCUSSION

Protein secretion in gram-negative bacteria requires proteins to cross two membranes separated by the periplasmic space. One pathway of secretion, the general secretion pathway, has been characterized in *K. oxytoca* and has been shown to involve at least 14 of the *pul* genes of *K. oxytoca* (28). Homologs of the *pul* genes have been found in many bacteria, including *Erwinia chrysanthemi*, *Erwinia carotovora*, *P. aeruginosa*, and others (reviewed in reference 29). *P. aeruginosa* contains three homologs of one of the *pul* genes (*pulE*): *xcpR*, *pilB*, and *pilT*. All of these genes encode

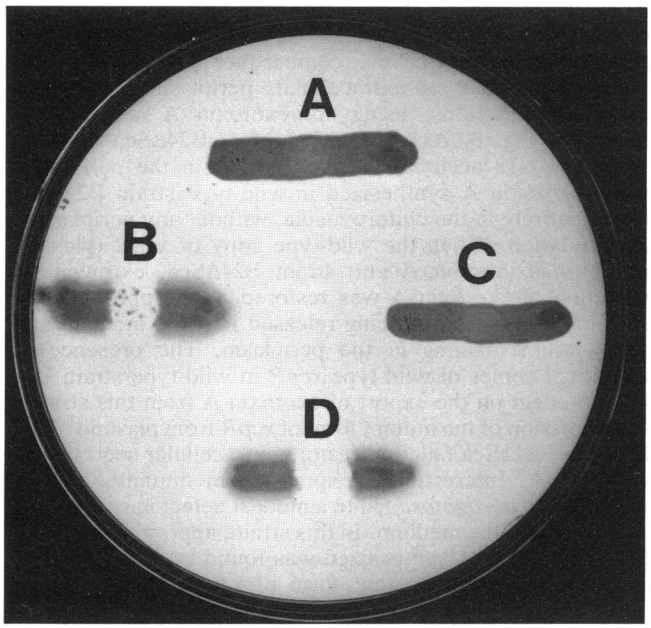

FIG. 6. Phage sensitivity assay. Nonpiliated (PilB$^-$) strain E4 (A), E4 carrying plasmids pLTB1 (B) and pLTB2 (C), and wild-type *P. aeruginosa* PAKrif (D) were tested for sensitivity to the pilus-specific phage PO4. Phage sensitivity was defined as an area of no growth, resulting from cell lysis, at the site of phage inoculation.

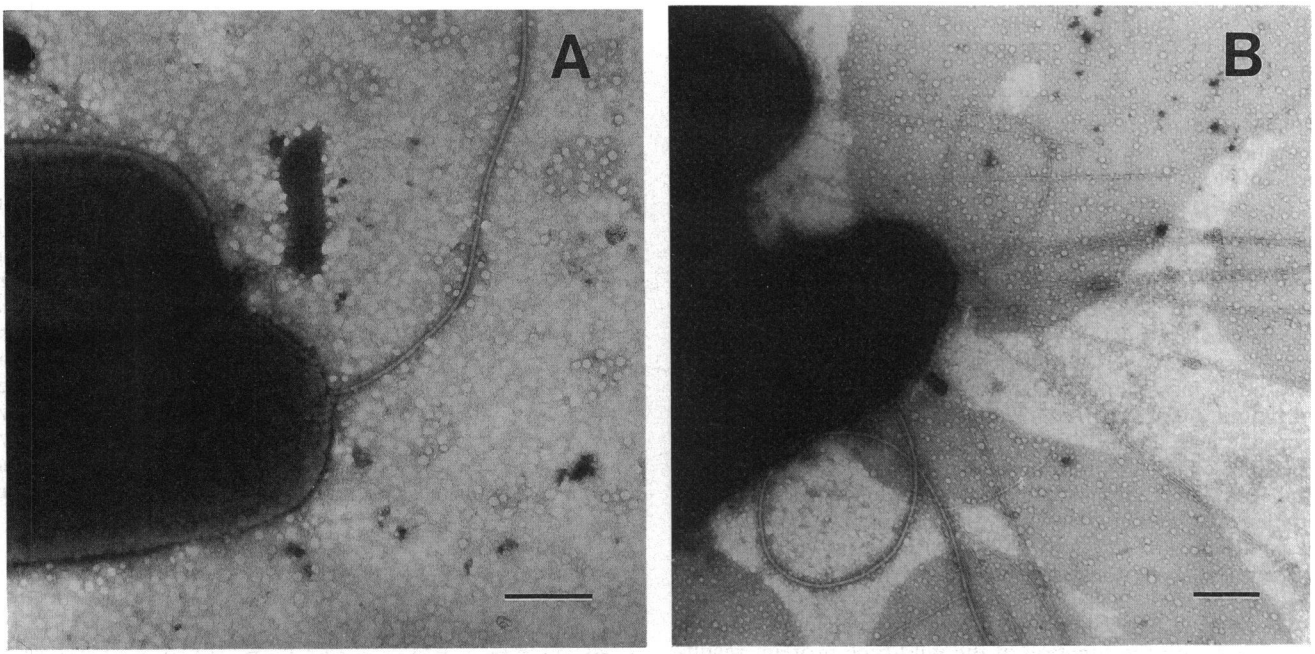

FIG. 7. Transmission electron micrographs of *P. aeruginosa* E4 expressing mutant PilB (A) and wild-type PilB (B). Overnight cultures were resuspended in 10 mM $MgCl_2$ and negatively stained with phosphotungstic acid (pH 7.2). Cells were viewed through a JEOL 1200 transmission electron microscope operated at 60 kV. Bars = 200 nm.

cytoplasmic proteins that contain a consensus sequence found in many proteins that bind ATP (Walker box). In this study, site-directed mutagenesis was used to alter one of the residues in the Walker boxes of XcpR and PilB, and the effects of these mutations were analyzed.

Here we have demonstrated that the alteration of a single amino acid in the putative ATP-binding sites of these proteins eliminated their normal functions. In the case of PilB, a serine for glycine substitution in the Walker box eliminated pilus assembly, while a corresponding change in XcpR eliminated the extracellular secretion of a number of *P. aeruginosa* enzymes. These results suggest that ATP is involved in the processes of pilus assembly and extracellular protein secretion. XcpR and PilB may be ATPases involved in providing energy for some aspect of protein secretion and pilus assembly, respectively. Alternatively, these proteins could be kinases involved in carrying out an essential phosphorylation event. In either case, alteration of the ATP-binding sites of XcpR and PilB would render them incapable of carrying out their normal functions.

Attempts to demonstrate ATP binding by XcpR and PilB overproduced in *E. coli*, using UV light-induced cross-linking of [^{32}P]ATP, were not successful (37b). It is conceivable that XcpR and PilB do not possess an inherent ability to bind ATP but that the binding or hydrolysis of ATP is dependent on the interaction of these proteins with components of the machinery of extracellular secretion or pilus biogenesis, respectively. Alternatively, interaction with ATP may occur as part of the process of protein translocation across the membrane: extracellular secretion of enzymes for XcpR and assembly of pilin subunits into pili for PilB. Experiments with in vitro-reconstituted *E. coli* histidine and maltose transport systems have demonstrated that ATP hydrolysis by the cytoplasmic HisP and MalK proteins, respectively, requires the presence of the membrane-associated components as well as the ligated periplasmic transport proteins (7, 26). Similarly, during protein secretion in *E. coli*, ATP hydrolysis by the SecA protein depends on its interaction with SecY-containing lipid vesicles as well as export-competent secretory proteins (20). Demonstration of protein-ATP interaction in vitro with XcpR and PilB may, therefore, require the development of a more complete protein translocation system.

ATP-binding proteins are coupled to many distinct biological processes. It is becoming increasingly evident that the ATP-binding proteins involved in many diverse transport systems belong to a superfamily of related ATP-binding cassette transporters. The superfamily currently consists of more than 30 proteins. It is clear that ATP hydrolysis by such ATP-binding cassettes is coupled to many transport processes (reviewed in references 10 and 27). While ATP hydrolysis has been demonstrated for several of these proteins, one cannot exclude the possibility that putative interaction with ATP by XcpR and PilB may be part of a phosphorylation reaction or a nucleotide-dependent regulatory event. Alternatively, XcpR and PilB may interact with a nucleotide other than ATP.

Considering the homology between XcpR and PilB, it seems possible that XcpR may be involved in assembly of the secretion apparatus in a manner similar to the involvement of PilB in pilus assembly. In fact, there are many similarities between the two systems. In addition to the observed sequence similarity between PilB/PilC and XcpR/XcpS and the processes of protein export and pilus biogenesis, the amino termini of four components of the protein secretion machinery, XcpT to XcpW, are homologous to the same region of the pilin subunit. Leader peptide cleavage from precursors and N methylation of the mature forms of all five of these proteins are carried out by a single bifunctional enzyme, PilD (34). It is an attractive hypothesis that the *xcpT* to *xcpW* gene products are assembled into a pilus-like structure by the accessory proteins XcpR and XcpS, al-

though there is no experimental evidence of physical interaction between any of these proteins, nor is there evidence for the existence of a pilus-like structure. It should not be surprising that protein secretion and pilus assembly are so closely related, in that pilus assembly is, in essence, a secretion process, whereby the pilin subunits must cross the cell envelope in order to be assembled into functional pili.

The striking similarities between these processes lead to the question, why are there two functional homologs, XcpR and PilB, in *P. aeruginosa*? We have previously shown that mutations in *pilB* have no effect on extracellular protein secretion (33) and, conversely, that *xcpR* mutants of *P. aeruginosa* are piliated (37a). It is clear that one does not compensate for the loss of the other, since it is possible to determine loss-of-function mutations in either gene. It is intriguing to consider the specificity of each system considering the high degree of homology between XcpR and PilB. The differences which do exist in the primary sequences of each of these proteins may reflect the specificity of the processes that they control, and these distinct domains may be involved in interactions with the other components of the extracellular secretion machinery or the pilus biogenesis determinants.

We observed that expression of the mutant form of XcpR interfered with the function of the wild-type protein, resulting in an increased level of periplasmic localization of exotoxin A. Additionally, when the mutant XcpR was expressed at an extremely high level in a wild-type background, it led to the complete elimination of extracellular protein secretion for three different enzymes, exotoxin A, phospholipase C, and alkaline phosphatase. There are two possible explanations for this dominant negative effect. One possibility is that XcpR may be interacting with another component of the secretion process, encoded by the *xcp* gene cluster. Overexpression of the mutant form would cause a dominant negative effect since the wild-type protein would be competing with the mutant protein, of which there are more copies, for this essential interaction. The manner in which the first set of experiments (Fig. 3) were carried out suggests that this may not be the case. When the mutated form of XcpR was introduced into the wild-type cells, it was done so in the context of introducing wild-type copies of the other secretion genes (*xcpS* to *xcpZ*) on the same plasmid (clone pLT2). All the genes were expressed constitutively. If the observed dominant negative effect was the result of an interaction of the mutant XcpR with one or more of the proteins encoded by *xcpS* to *xcpZ*, it seems likely that the overexpression of these wild-type components would compensate for the interference by the mutant XcpR in the wild-type *xcpR* background. This does not rule out the possibility that XcpR is interacting with some component not included in the *xcpR* to *xcpZ* gene cluster, however.

Alternatively, XcpR proteins could form a multimer in its active form. Other members of the ATP-binding cassette superfamily are known to act as dimers in three possible ways: (i) as a homodimer of a single conserved component, as demonstrated by the HisP component of the histidine permease of *E. coli* (15); (ii) as a heterodimer of two separate and homologous conserved components, as in the case of the oligopeptide permease of *Salmonella typhimurium* (11); (iii) as an intramolecular heterodimer of a single protein encoding two homologous halves, which is typical of the eukaryotic transporters belonging to this family, such as the multidrug resistance protein of mammalian cells (5, 9). If XcpR normally functions as a homodimer, the dominant negative effect would result from the formation of heterodimers, consisting of wild-type and mutant subunits, which are incapable of carrying out their normal functions. In the case of expression of mutant XcpR from the *tac* promoter in a wild-type background, the severe overexpression of the mutant form would saturate the interactions of wild-type subunits, thereby causing a block in extracellular protein secretion.

The precise roles of XcpR and PilB in extracellular protein secretion and pilus assembly are unclear. The presence of a putative ATP-binding site which is required for the function of these proteins suggests that they are involved in energizing the translocation of proteins across the outer membrane by the hydrolysis of ATP. Because of their likely cytoplasmic location, XcpR and PilB must interact with additional proteins in the bacterial cell envelope in order to channel energy to a process in the outer membrane. Alternatively, XcpR and PilB may participate in protein export indirectly, mediating an energy-dependent step in the assembly of the machineries of extracellular protein secretion and pilus biogenesis.

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REFERENCES

1. **Bally, M., A. Filloux, M. Akrim, G. Ball, A. Lazdunski, and J. Tommassen.** 1992. Protein secretion in *Pseudomonas aeruginosa*: characterization of seven *xcp* genes and processing of secretory apparatus components by prepilin peptidase. Mol. Microbiol. **6**:1121–1132.
2. **Berka, R. M., G. L. Gray, and M. L. Vasil.** 1981. Studies of phospholipase C (heat-labile hemolysin) in *Pseudomonas aeruginosa*. Infect. Immun. **34**:1071–1074.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
4. **Brickman, E., and J. Beckwith.** 1975. Analysis of the regulation of *E. coli* alkaline phosphatase synthesis using deletions and ϕ80 transducing phages. J. Mol. Biol. **96**:307–316.
5. **Chen, C. J., J. E. Chin, K. Ueda, D. P. Clark, I. Pastan, M. M. Gottesman, and I. B. Roninson.** 1986. Internal duplication and homology with bacterial transport proteins in the *mdr1* (P-glycoprotein) gene from multidrug-resistant human cells. Cell **47**:381–389.
6. **Davis, B. D., and E. S. Mingioli.** 1950. Mutants of *Escherichia coli* requiring methionine or vitamin B_{12}. J. Bacteriol. **60**:17–28.
7. **Dean, D. A., A. L. Davidson, and H. Nikaido.** 1989. Maltose transport in membrane vesicles of *Escherichia coli* is linked to ATP hydrolysis. Proc. Natl. Acad. Sci. USA **86**:9134–9138.
8. **Fürste, J. P., W. Pansegrau, R. Frank, H. Bloecker, P. Scholz, M. Bagdasarian, and E. Lanka.** 1986. Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. Gene **48**:119–131.
9. **Gros, P., J. Croop, and D. Housman.** 1986. Mammalian multidrug resistance gene: complete cDNA sequence indicates strong homology to bacterial transport proteins. Cell **47**:371–380.
10. **Higgins, C. F., S. C. Hyde, M. M. Mimmack, U. Gileadi, D. R. Gill, and M. P. Gallagher.** 1990. Binding protein-dependent transport systems. J. Bioenerg. Biomembr. **22**:571–592.
11. **Hiles, I. D., M. P. Gallagher, D. J. Jamieson, and C. F. Higgins.** 1987. Molecular characterization of the oligopeptide permease of *Salmonella typhimurium*. J. Mol. Biol. **195**:125–142.
12. **Hoshino, T., and M. Kageyama.** 1980. Purification and properties of a binding protein for branched-chain amino acids in *Pseudomonas aeruginosa*. J. Bacteriol. **141**:1055–1063.
13. **Inouye, H., S. Michaelis, A. Wright, and J. Beckwith.** 1981. Cloning and restriction mapping of the alkaline phosphatase

structural gene (*phoA*) of *Escherichia coli* and generation of deletion mutants in vitro. J. Bacteriol. **146**:668–675.
14. **Jones, R. N., H. W. Wilson, and W. J. Novick, Jr.** 1982. In vitro evaluation of pyridine-2-azo-*p*-dimethylaniline cephalosporin, a new diagnostic chromogenic reagent, and comparison with nitrocefin, cephacetrile, and other beta-lactam compounds. J. Clin. Microbiol. **15**:677–683.
15. **Kerppola, R. E., V. K. Shyamala, P. Klebba, and G. F. Ames.** 1991. The membrane-bound proteins of periplasmic permeases form a complex. Identification of the histidine permease HisQMP complex. J. Biol. Chem. **266**:9857–9865.
16. **Korangy, F., and D. A. Julin.** 1992. Alteration by site-directed mutagenesis of the conserved lysine residue in the ATP-binding consensus sequence of the RecD subunit of the *Escherichia coli* RecBCD enzyme. J. Biol. Chem. **267**:1727–1732.
17. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154**:367–382.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
19. **Leong, S. A., G. S. Ditta, and D. R. Helinski.** 1982. Heme biosynthesis in *Rhizobium*. Identification of a cloned gene coding for delta-aminolevulinic acid synthetase from *Rhizobium meliloti*. J. Biol. Chem. **257**:8724–8730.
20. **Lill, R., K. Cunningham, L. A. Brundage, K. Ito, D. Oliver, and W. Wickner.** 1989. SecA protein hydrolyzes ATP and is an essential component of the protein translocation ATPase of *Escherichia coli*. EMBO J. **8**:961–966.
21. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Mimura, C. S., A. Admon, K. A. Hurt, and G. F. Ames.** 1990. The nucleotide-binding site of HisP, a membrane protein of the histidine permease. Identification of amino acid residues photoaffinity labeled by 8-azido-ATP. J. Biol. Chem. **265**:19535–19542.
22a. **Nunn, D.** Unpublished data.
23. **Nunn, D., S. Bergman, and S. Lory.** 1990. Products of three accessory genes, *pilB*, *pilC*, and *pilD*, are required for biogenesis of *Pseudomonas aeruginosa* pili. J. Bacteriol. **172**:2911–2919.
24. **Nunn, D. N., and S. Lory.** 1991. Product of the *Pseudomonas aeruginosa* gene *pilD* is a prepilin leader peptidase. Proc. Natl. Acad. Sci. USA **88**:3281–3285.
25. **Nunn, D. N., and S. Lory.** 1992. Components of the protein-excretion apparatus of *Pseudomonas aeruginosa* are processed by the type IV prepilin peptidase. Proc. Natl. Acad. Sci. USA **89**:47–51.
26. **Petronilli, V., and G. F. Ames.** 1991. Binding protein-independent histidine permease mutants. Uncoupling of ATP hydrolysis from transmembrane signaling. J. Biol. Chem. **266**:16293–16296.
27. **Pugsley, A. P.** 1992. Superfamilies of bacterial transport systems with nucleotide binding components in prokaryotic structure and function: a new perspective, p. 223–248. *In* S. B. Monohan, C. Dow, and J. A. Cole (ed.), Society for General Microbiology Symposium, vol. 47. Cambridge University Press, Cambridge.
28. **Pugsley, A. P., C. d'Enfert, I. Reyss, and M. G. Kornacker.** 1990. Genetics of extracellular protein secretion by gram-negative bacteria. Annu. Rev. Genet. **24**:67–90.
29. **Salmond, G. P. C., and P. J. Reeves.** 1993. Membrane traffic wardens and protein secretion in Gram-negative bacteria. Trends Biochem. Sci. **18**:7–12.
30. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
31. **Schaegger, H., and G. von Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. **166**:368–379.
32. **Schatz, P. J., and J. Beckwith.** 1990. Genetic analysis of protein export in *Escherichia coli*. Annu. Rev. Genet. **24**:215–248.
33. **Strom, M. S., D. Nunn, and S. Lory.** 1991. Multiple roles of the pilus biogenesis protein PilD: involvement of PilD in excretion of enzymes from *Pseudomonas aeruginosa*. J. Bacteriol. **173**:1175–1180.
34. **Strom, M. S., D. N. Nunn, and S. Lory.** 1993. A single bifunctional enzyme, PilD, catalyzes cleavage and N-methylation of proteins belonging to the type IV pilin family. Proc. Natl. Acad. Sci. USA **90**:2404–2408.
35. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82**:1074–1078.
36. **Tommassen, J., A. Filloux, M. Bally, M. Murgier, and A. Lazdunski.** 1992. Protein secretion in *Pseudomonas aeruginosa*. FEMS Microbiol. Rev. **9**:73–90.
37. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
37a. **Turner, L. R.** Unpublished data.
37b. **Turner, L. R., and S. Lory.** Unpublished data.
38. **Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay.** 1982. Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. **1**:945–951.
39. **Wandersman, C.** 1992. Secretion across the bacterial outer membrane. Trends Genet. **8**:317–322.
40. **Whitchurch, C. B., M. Hobbs, S. P. Livingston, V. Krishnapillai, and J. S. Mattick.** 1991. Characterisation of a *Pseudomonas aeruginosa* twitching motility gene and evidence for a specialised protein export system widespread in eubacteria. Gene **101**:33–44.