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Genetic and Functional Characterization of the Gene Cluster Specifying Expression of *Pseudomonas aeruginosa* Pili

TETSURO KOGA,† KARYN ISHIMOTO, AND STEPHEN LORY*

Department of Microbiology, School of Medicine, University of Washington, Seattle, Washington 98195

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The genetic organization of the gene cluster containing *pilA*, the structural gene for type IV pilin of *Pseudomonas aeruginosa*, as well as the accessory genes *pilB*, *pilC*, and *pilD*, has been studied. DNA sequences capable of initiating transcription when fused to a promoterless *lacZ* gene have been identified in the *pilA-pilB* and *pilB-pilC* intergenic regions. Unlike *pilA*, which requires *rpoN* (encoding the σ^{54} subunit of RNA polymerase) and products of two regulatory genes, *pilS* and *pilR*, expression of *pilB*, *pilC*, or *pilD* did not depend on any of these transcriptional regulators. Moreover, transcription of *pilA* from the *tac* promoter in an *rpoN* mutant background resulted in piliated bacteria, suggesting that the RpoN-based regulatory network is specific for *pilA* and does not control expression of any other genes necessary for formation of pili. Insertion of the Ω fragment containing strong transcriptional terminators into *pilB*, *pilC*, and *pilD* failed to have a polar effect on expression of downstream genes, as determined by the ability of each cloned gene to complement, in *trans*, the corresponding insertionally inactivated chromosomal copy. Insertions into *pilC*, however, resulted in decreased synthesis of PilD as determined by quantitation of PilD enzymatic activity in processing prepilin in vitro and by immunoassay. This finding suggests that PilD may require PilC for its optimal stability or correct membrane localization.

Pseudomonas aeruginosa is an opportunistic pathogen of humans responsible for severe and sometimes fatal infections in immunosuppressed individuals and in patients with cystic fibrosis. During the course of infection, a number of extracellular enzymes and surface components that contribute to pathogenicity are produced (6, 22). Among the various virulence factors, pili have been shown to play an important role during the initial stages of colonization of mucosal surfaces by mediating attachment of the bacterium to host epithelial cells (31). The pili of *P. aeruginosa* belong to the type IV class of pili, which are present on other gram-negative pathogens, such as *Moraxella bovis*, *Neisseria gonorrhoeae*, and *Dichelobacter nodosus* (10, 15–17). The subunits of type IV pili, pilins, are highly conserved at their amino termini and are synthesized as precursors with a 6- or 7-amino-acid N-terminal leader sequence. The export and assembly of this class of pili is preceded by cleavage of the leader sequence and N methylation of the newly formed N-terminal phenylalanine.

We have previously identified three genes, *pilB*, *pilC*, and *pilD*, that encode products responsible for processing and assembling pilin monomers into functional pili (19). PilD has been identified as the prepilin leader peptidase responsible for processing not only type IV pilins but also components of an extracellular protein secretion apparatus (20, 21). The function of the products of *pilB* and *pilC* is not known at this time; however, their role appears to be restricted to pilus biogenesis.

Expression of the pilin gene, *pilA*, in *P. aeruginosa* requires the alternative σ factor, RpoN, as well as the products of two regulatory genes, *pilS* and *pilR* (8, 9). The deduced amino acid sequences of PilS and PilR suggest that they are members of the family of two-component regulatory elements which utilize a phosphotransfer mechanism of signal transduction (25) whereby PilS is autophosphorylated and in turn transfers the phosphate to PilR.

In order to study the extent of regulation of the pilin structural genes and the genes encoding biogenesis functions, we have cloned the potential promoter fragments from each of the *pilB*, *pilC*, and *pilD* genes in front of a promoterless *lacZ* in broad-host-range plasmid vectors. These plasmids were transferred to wild type and various regulatory mutants of *P. aeruginosa*, and promoter activities were measured. Transcriptional termination mutants were also constructed by inserting the Ω fragment into *pilB*, *pilC*, and *pilD* genes, and complementation tests were then performed. These results showed that *pilB* and *pilC* have their own promoters; however, expression of *pilD* was influenced by *pilC*. Moreover, transcription of the accessory genes *pilB*, *pilC*, and *pilD* either is constitutive or is regulated independently from the pilin structural gene.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmid vectors used in this study are listed in Table 1. Figure 1 outlines the DNA fragments used to construct various complementing plasmids as well as those used to measure promoter activity. All cultures were grown on Luria broth (14) or minimal A salts (4) supplemented with 50 mM monosodium glutamate and 1% glycerol for *P. aeruginosa*. Antibiotics and concentrations were as follows: for *Escherichia coli*, tetracycline (20 μg/ml), ampicillin (100 μg/ml), and kanamycin (50 μg/ml); for *P. aeruginosa*, tetracycline (100 μg/ml) and carbenicillin (150 μg/ml).

Enzymes and chemicals. All restriction enzymes and T4 DNA ligase were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Isotopes for labeling probes ([α-^{32}P]dCTP) and ^{125}I-labeled protein A were purchased from Dupont, NEN Research Products, Boston, Mass.

* Corresponding author.
† Present address: Department of Food Microbiology, Tokushima University School of Medicine, Tokushima, Japan.

TABLE 1. Bacterial strains, phage, and plasmids

Strain, phage, or plasmid	Relevant traits	Source or reference
E. coli DH5α	*hsdR recA lacZYA80 lacZ*M15	Bethesda Research Laboratories
P. aeruginosa		
PAK	Wild type	D. Bradley
PAK-N1G	*rpoN* mutant	29
PAK-R1	PAK pilin regulatory mutant with Tn*5*G insertion in *pilR*	9
PAK-RA	PAK pilin regulatory mutant with Tn*5*G insertion in *pilS*	9
B24	Tn*5* insertion in region 0.5 kb downstream of *pilD*; no known phenotype; used as wild-type control in some experiments; PO4 phage sensitive	19
E4	Tn*5* insertion mutant into *pilB*; PO4 phage resistant	19
E3	Tn*5* insertion mutant into *pilC*; PO4 phage resistant	19
E20	Tn*5* insertion mutant into *pilC*; PO4 phage resistant	19
2B18	Tn*5* insertion mutant into *pilD*; PO4 phage resistant	19
PAK-B Ω	Ω insertion mutant into *pilB*	This study
PAK-C Ω	Ω insertion mutant into *pilC*	This study
PAK-D Ω	Ω insertion mutant into *pilD*	This study
Phage PO4	Pilus-specific lytic phage	D. Bradley
Plasmids		
pUC18	Ap^r cloning vector	1
pUC18cm	Cm^r cloning vector	29
pUC19*mob*	pUC19 containing *mob* region of broad-host-range plasmid pRP4	19
pUC19 Ω	2.0-kb *Sma*I fragment (Ω) from pHP45 Ω (23) cloned into pUC19	Stephen Lory
pEX1	4.0-kb *Xba*I fragment containing *pilB*, *pilC*, and *pilD* genes cloned into pUC19 (U.S. Biochemicals)	19
pDN19*lac* Ω	Broad-host-range plasmid containing promoterless *lacZ* gene	29
pMS27A	1.2-kb *Hin*dIII fragment containing pilin structural gene cloned into pUC18	27
pMS*tac*27PD	*pilA* transcribed from *tac* promoter	26
pUC4K	Kanamycin cassette	Pharmacia
pRK2073	Mobilizing plasmid	13

DNA manipulations and analyses. *P. aeruginosa* chromosomal DNA was extracted as described by Strom and Lory (27). Plasmid DNA was prepared by the method of Birnboim and Doly (2) for routine small-scale isolation and large-scale isolations followed by cesium chloride-ethidium bromide density gradients (14). DNA was digested with restriction enzymes and analyzed by agarose gel electrophoresis. DNA purification from agarose gels by electroelution was done essentially as described by Maniatis et al. (14). DNA fragments were blotted onto Nytran sheets (Schleicher & Schuell, Keene, N.H.) by the method of Southern (24). The probes were radiolabeled by the random priming method with a kit purchased from Bethesda Research Laboratories.

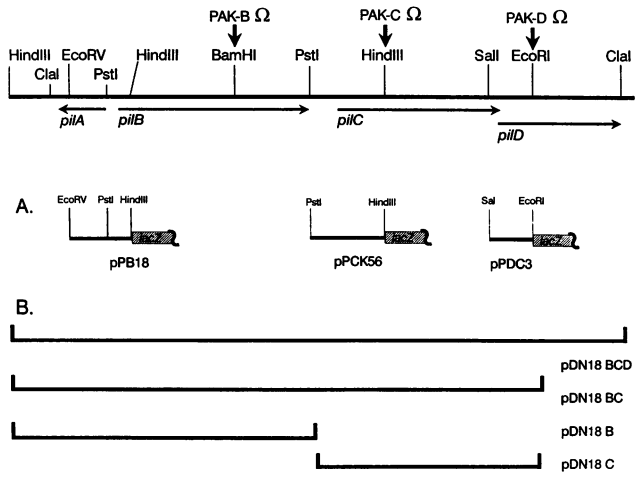

FIG. 1. Restriction map of the chromosomal region containing *pilA*, *pilB*, *pilC*, and *pilD* and various plasmid constructs derived from this region. Indicated are the locations of the Ω inserts in the *pilB*, *pilC*, and *pilD* mutants. (A) Fragments used to construct *lacZ* fusions. *Bam*HI is derived from pMS27 and used to isolate the promoter-containing plasmid as described in Materials and Methods. (B) The full-length 4.0-kb *Xba*I fragment derivative pDN18BCD and the three deletion derivatives pDN18BC, pDN18B, and pDN18C are shown in relation to the full-length 4.0-kb *Xba*I fragment.

SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting. Membranes or cell extracts were separated on 15% polyacrylamide gels by using the system of Laemmli (12). For assay of prepilin leader peptidase activity, samples were electrophoresed in sodium dodecyl sulfate (SDS)–tricine–15% polyacrylamide gels as described previously (20). Following electrophoresis, proteins were transferred onto nitrocellulose sheets for immunoblot analysis. The blots were reacted with rabbit anti-PilD-derived peptide antibody (20) or mouse anti-pilin antibody, and bound immunoglobulins were identified by reaction with ^{125}I-protein A.

Phage sensitivity tests. The suspensions of *P. aeruginosa* strains to be tested (10^8 cells per ml) were streaked onto an L-agar plate containing tetracycline (100 μg/ml), after which a 5-μl sample of a suspension of the pilus-specific phage PO4 (10^{10} PFU/ml) was spotted onto the bacteria (3). Resistance was detected as unrestricted growth occurring after phage spotting.

Cell fractionations. The total membranes of *P. aeruginosa* were isolated as described by Hancock and Nikaido (7).

Prepilin cleavage assays. Prepilin cleavage activities were determined as described by Nunn and Lory (20). The cleavage activity was considered the percentage of prepilin con-

verted to the mature form as estimated by staining with Coomassie blue R.

β-Galactosidase assays. Expression of the *lacZ* gene under control of the *pilB*, *pilC*, or *pilD* promoter was measured by using β-galactosidase assays as described by Miller (18).

Construction of fusions between the *pilB*, *pilC*, and *pilD* promoter regions and *lacZ*. To determine the promoter activities of the three genes, the vector pDN19*lac* Ω (29) was used. This broad-host-range plasmid contain a promoterless *lacZ* gene just downstream of *Eco*RI and *Bam*HI sites into which potential promoter fragments can be cloned. To prevent transcription from the resident *lac* promoter from pDN19 (19), the 2.0-kb Ω fragment from pHP45 Ω (23) was inserted into the single *Hin*dIII site of pDN19.

To determine the *pilB*, *pilC*, and *pilD* promoter activities, transcriptional fusions of the potential promoter fragments from three genes to the *lacZ* gene in pDN19*lac* Ω were created. For the *pilB* promoter-*lacZ* fusion, pMS27A (27), which contains both a pilin structural gene and a part of the *pilB* gene, was digested with *Eco*RV and *Bam*HI. The 0.7-kb fragment was cloned into *Sma*I-*Bam*HI-cut pUC18cm. The cloned fragment was excised as an *Eco*RI-*Bam*HI fragment and ligated into *Eco*RI-*Bam*HI-digested pDN19*lac* Ω, resulting in plasmid pPB18. To construct the *pilC* promoter-*lacZ* fusion, the 0.5-kb *Pst*I-*Hin*dIII fragment from pEX1 was cloned into *Pst*I-*Hin*dIII-digested pUC18. This plasmid was digested with *Hin*dIII, blunt ended with the Klenow polymerase, and ligated with the kanamycin resistance determinant from pUC4K, which was digested with *Eco*RI and also blunt ended, resulting in plasmid pKM1. The 0.5-kb *Bam*HI fragment of pKM1 was cloned into the *Bam*HI site of pDN19*lac* Ω, resulting in pPCK56, in which the direction of *pilC* transcription is the same as that of *lacZ*. A *pilD* promoter-*lacZ* fusion plasmid was constructed as follows. The ends of a 0.4-kb *Sal*I-*Eco*RI fragment from pEX1 were filled in, and the fragment was then cloned into *Sma*I-digested pUC18. The clone, which is oriented such that the blunt-ended *Sal*I is immediately to the right of the *Eco*RI site of pUC18, was identified, and the 0.4-kb *Eco*RI-*Bam*HI fragment was isolated from this construction. The 0.4-kb fragment was cloned into *Eco*RI-*Bam*HI-digested pDN19*lac* Ω, creating pPDC3. For analysis of promoter expression, plasmids were transferred to *P. aeruginosa* with triparental mating as described previously (5). pRK2073 was a mobilizing plasmid, and PAK, PAK-N1G, PAK-R1, and PAK-RA were recipients. Selection was on a minimal A plate containing 0.05 M monosodium glutamate, 1% glycerol, and tetracycline (100 μg/ml) or on an L-agar plate containing tetracycline (100 μg/ml) and gentamicin (25 μg/ml).

Construction of strains carrying the Ω cassette in the pilus biogenesis genes. To determine whether *pilB*, *pilC*, and *pilD* genes were cotranscribed, the omega fragment (Ω) containing transcriptional terminators was inserted into each gene. The location of each insert is shown in Fig. 1. First, pUC19*mob* was digested with *Bam*HI, *Hin*dIII, and *Eco*RI; protruding ends were filled in with Klenow polymerase and then digested with *Xba*I. The 4.0-kb *Xba*I fragment containing *pilB*, *pilC*, and *pilD* genes from pEX1 was cloned into each vector, creating in turn pUC19*mob*XB, pUC19*mob*XC, and pUC19*mob*XD, respectively. To clone the Ω fragment into the *pilB* site, the *Bam*HI-cut fragment was inserted into the single *Bam*HI site of pUC19*mob*XB, resulting in pUC19*mob*XBΩ. In order to insert the Ω fragment into the *Hin*dIII site of *pilC*, pUC19*mob*XC was digested with *Bam*HI and the 1.1-kb *Bam*HI fragment containing the *Hin*dIII site was removed; the *Hin*dIII-digested Ω fragment was then inserted into this plasmid, resulting in pUC19*mob*XCΩ. To clone the Ω fragment into the *pilD* site, pUC19*mob*XD was digested with *Eco*RI and filled in, and then the *Sma*I-cut Ω fragment was cloned into this site, creating pUC19*mob*XDΩ. To introduce the Ω-digested mutants into the *P. aeruginosa* chromosome by gene replacement, these recombinant plasmids were transformed into *E. coli* DH5α. These plasmids were then mobilized into a *P. aeruginosa* PAK by triparental mating, using pRK2073 as a helper plasmid. The exconjugants were selected after plating of the mating mixture onto a minimal A plate containing 0.05 M monosodium glutamate, 1% glycerol, and streptomycin (600 μg/ml). Colonies that arose were tested for sensitivity to carbenicillin. Such clones were presumptive candidates for mutant strains that contained in their chromosome the Ω fragment, having replaced the homologous sequence by recombination along sequences flanking both sides of the Ω fragment. The correct gene replacement was confirmed by Southern analyses of restriction digests of DNA isolated from each of the mutants with radiolabeled 4.0-kb *Xba*I fragment containing *pilB*, *pilC*, and *pilD* genes as probe.

RNA isolation and primer extension analysis. Total RNA was isolated as described by von Gabain et al. (30) with some modifications. The cultures were grown in L broth at 37°C until they reached an optical density at 600 nm of 1.2. The cells were pelleted by centrifugation and washed in ice-cold 10 mM sodium acetate–0.15 M sucrose–0.1 mg of heparin per ml–0.01% (vol/vol) diethylpyrocarbonate. The cells were lysed by adding 0.5% (wt/vol) SDS and heated to 65°C. Hot phenol saturated with 20 mM sodium acetate (pH 4.8) was then added, and the lysate was shaken for 5 min at 65°C, after which it was submerged in an ice bath for 10 min. The aqueous (top) phase was separated from the phenol by centrifugation at 12,000 × *g*, and phenol extraction was repeated three more times and followed by two chloroform extractions. The RNA was precipitated with 0.3 M sodium acetate–2.2 volumes of ethanol. The precipitate was suspended in 10 mM Tris HCl–10 mM Mg acetate (pH 7.6).

Primer extension was done as described previously (29). The oligonucleotide used to prime the synthesis was 5′-CGAGCTGTCGGGACAGACCG-3′, starting at the 25th bp of the *pilB* coding sequence. The oligonucleotide was labeled with [γ-^{32}P]phosphate and polynucleotide kinase as described elsewhere (1). The labeled oligonucleotide (2 ng) was added to 50 μg of RNA in a total volume of 40 μl containing 50 mM Tris HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, and 3 mM $MgCl_2$. Hybridization of the primer to RNA was accomplished by heating the sample to 65°C for 5 min and then cooling it to room temperature. A cocktail of all four nucleotides was added to a concentration of 0.5 mM, and then 200 U of murine leukemia virus reverse transcriptase (BRL) was added. The mixture was incubated for 30 min at 40°C. An aliquot was then analyzed on a 6% urea-polyacrylamide sequencing gel along with a dideoxy sequencing ladder. To obtain the sequencing ladder, 2.0 μg of CsCl-purified plasmid pMS6 (27) was denatured in 0.5 M NaOH and then, after addition of 0.1 volume of Na acetate, precipitated in 4 volumes of ethanol. This denatured plasmid was used as a template for a sequencing reaction, primed with the same oligonucleotide as that employed in the primer extension reaction, that used [α-^{32}P]ATP and the Sequenase kit (U.S. Biochemical).

TABLE 2. Transcription of various promoter-containing fragments in wild-type, *rpoN*, *pilR*, and *pilS* backgrounds

Plasmid	β-Galactosidase activity (U)[a]			
	PAK (wild type)	PAK-N1G (*rpoN* mutant)	PAK-R1 (*pilR* mutant)	PAK-RA (*pilS* mutant)
pPB18 (*pilB-lacZ*)	288	501	117	134
pPCK56 (*pilC-lacZ*)	122	259	145	113
pPDC3 (*pilD-lacZ*)	46	101	64	49
pDN19*lac* Ω (vector control)	35	29	28	26

[a] As defined by Miller (18).

RESULTS

Promoter activities of *pilB*, *pilC*, and *pilD* genes. To determine whether *pilB*, *pilC*, and *pilD* genes are transcribed independently or as an operon, we cloned DNA fragments from intergenic regions of *pilB*, *pilC*, and *pilD* (Fig. 1A) into the promoter probe vector pDN19*lac* Ω. These constructs were then introduced into *P. aeruginosa* PAK (wild type), PAK-N1G (*rpoN* mutant), PAK-R1 (*pilR* mutant), and PAK-RA (*pilS* mutant) and analyzed for β-galactosidase activity. Results of these analyses are shown in Table 2. In each case, the transcription of the *lacZ* gene directed by the insert was above background (the vector without insert). Plasmids pPB18 and pPCK56, which contained potential *pilB* or *pilC* promoter sequence, respectively, could direct synthesis of the *lacZ* gene, giving β-galactosidase with relatively modest activity (113 to 501 U) in PAK (wild type) and other mutants. In contrast to these plasmids, plasmid pPDC3, which contained a potential *pilD* promoter sequence, produced low levels (46 to 101 U) of β-galactosidase in these strains, but these activities are slightly higher than those of *lacZ* fusion vector pDN19*lac*. These results indicate that *pilB* and *pilC* very likely contain functional promoters, while *pilD* transcription may be linked to that of *pilC*. Alternatively, *pilD* may be transcribed by a relatively weak promoter.

Comparison of promoter activities of the various constructs in *rpoN*, *pilS*, and *pilR* mutant backgrounds, also shown in Table 2, revealed that while some variation in the levels of β-galactosidase occurred in each mutant, there was no detectable decrease in expression of the various fusions, as would be expected if σ^{54} with its cognate transcriptional activators was involved in expression of *pilB*, *pilC*, and *pilD*. Thus, the genes encoding the accessory genes are not under the same transcriptional control as *pilA*, the structural gene for the major structural subunit. Interestingly, the expression of all three fusions in the *rpoN* mutant background resulted in an approximately twofold increase in β-galactosidase activity, which may be due to increased availability of the core RNA polymerase for overall transcription.

We have attempted to determine the sites of initiation of pilin biogenesis genes by using primer extension analysis. Primers originating in *pilB* gave the only detectable extended product. The site of initiation of transcription of *pilB* was 26 bp from the initiating methionine (Fig. 2). The same extended product was detected in RNAs isolated from wild type and the *rpoN* mutant, confirming the *rpoN*-independent expression of the *pilB-lacZ* fusion. The site of initiation of transcription is preceded by a typical −10 sequence and a sequence at position −35 which showed less similarity with canonical sequences found at this position (7a). Examination of the sequence upstream from the transcriptional start site

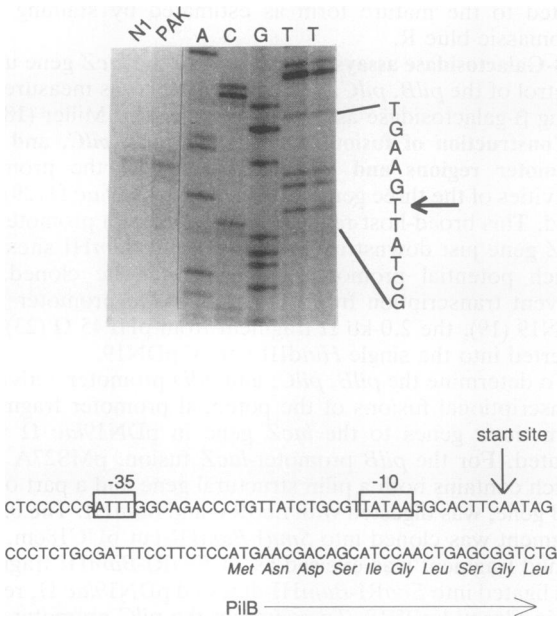

FIG. 2. Site of initiation of transcription of *pilB*. The arrow indicates the extended product from RNA isolated from wild-type PAK and the *rpoN* mutant PAK-N1. The −10 and −35 regions of the putative promoter sequence are boxed.

failed to identify a conserved sequence (GG-N_{10}-GC) at positions −12 to −24 that is found in all promoters of RpoN-transcribed genes (11), which suggests that the *pilB* promoter is not recognized by RpoN but is most likely transcribed by RNA polymerase with the σ^{70} subunit.

RpoN-independent expression of pili. In order to determine whether any genes involved in biogenesis of pili are controlled by RpoN, we introduced plasmid pMS*tac*27PD, carrying *pilA* under the control of the *tac* promoter (26), into wild type and the *rpoN* mutant of *P. aeruginosa*. If one or several essential biogenesis functions are controlled by RpoN, high-level synthesis of the pilin subunits should not allow assembly of functional pili in an *rpoN* mutant. When the *tac* promoter was induced by isopropyl-β-D-thiogalactopyranoside (IPTG), synthesis of pilin in the *rpoN* mutant PAK-N1 was comparable to that in the wild-type PAK (Fig. 3). When piliation of these strains was examined by using the PO4 phage sensitivity assay, the previously phage-resistant mutant PAK-N1 was now sensitive to the killing activity of the PO4 phage. Examination of PAK-N1(pMS*tac*27PD) by electron microscopy revealed that it now contained polar pili, while the same mutant carrying the vector was not piliated (data not shown). It therefore appears that RpoN is required for transcription of the pilin structural subunit but for none of the genes encoding biogenesis functions.

Isolation and characterization of mutant strains carrying Ω insertion. In order to assess the organization of the *pilB-pilC* gene cluster, we created mutations in this region with the Ω fragment carrying transcriptional terminators. The Ω fragment was inserted into specific restriction sites within *pilB*, *pilC*, and *pilD* (Fig. 1) and was introduced into the *P. aeruginosa* chromosome by a gene replacement. The insertion of the Ω fragment into each of the accessory genes yielded mutations that were unable to assemble pili (data not shown), confirming the phenotypes observed for the Tn*5*-generated mutants in the same genes (19). Moreover, all of

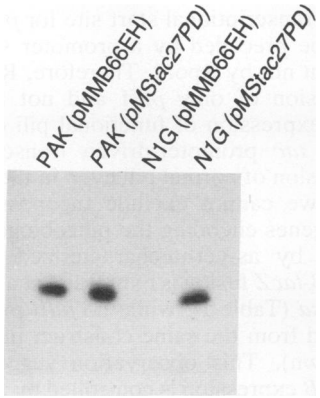

FIG. 3. Synthesis of pilin in wild type and *rpoN* mutants. Extracts of *P. aeruginosa* PAK (wild type) and N1G (*rpoN* mutant) carrying the clone of pilin gene under control of the *tac* promoter (pMStac27PD) or vector (pMMB66EH) were analyzed by Western immunoblotting as described in Materials and Methods.

the insertion mutants were resistant to the lytic action of the pilus-specific phage PO4.

The effect of Ω insertions on expression of downstream genes was examined by complementation analysis with plasmids carrying segments of the *pilBCD* gene cluster. The 4.0-kb *Xba*I fragment was subcloned into pDN18 in an orientation opposite to that of the vector promoter, resulting in plasmid pDN18BCD. When plasmid pDN18BCD was introduced into any of the Ω-fragment-carrying mutants, assembly of functional pili was restored and the transconjugants became sensitive to the PO4 phage. Pilus formation (PO4 sensitivity) was not restored in any of the mutants that carried the vector pDN18. Three deletion derivatives of the *Xba*I fragment, pDN18BC, pDN18B, and pDN18C, were generated (Fig. 1B). These constructions were introduced into the Ω-inserted mutants and were then tested for the ability to complement phage-resistant (nonpiliated) mutants to phage sensitivity (Fig. 4); this is summarized in Table 3.

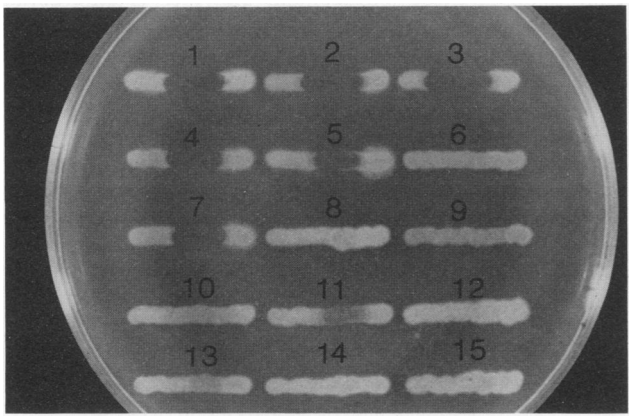

FIG. 4. Sensitivity to PO4 phage killing of Ω insertion mutants complemented by various deletion derivatives. Strains: 1, PAK-B Ω(pDN18 BCD); 2, PAK-C Ω(pDN18 BCD); 3, PAK-D Ω(pDN18 BCD); 4, PAK-B Ω(pDN18 BC); 5, PAK-C Ω(pDN18 BC); 6, PAK-D Ω(pDN18 BC); 7, PAK-B Ω(pDN18 B); 8, PAK-C Ω(pDN18 B); 9, PAK-D Ω(pDN18 B); 10, PAK-B Ω(pDN18 C); 11, PAK-C Ω(pDN18 C); 12, PAK-D Ω(pDN18 C); 13, PAK-B Ω(pDN18); 14, PAK-C Ω(pDN18); 15, PAK-D Ω(pDN18).

TABLE 3. Complementation of mutants to phage sensitivity by deletion derivatives

Deletion delivative	Complementation by mutant:		
	PAK-B Ω	PAK-C Ω	PAK-D Ω
pDN18BCD	+	+	+
pDN18BC	+	+	−
pDN18B	+	−	−
pDN18C	−	+	−
pDN18	−	−	−

Plasmid pDN18BC (containing intact *pilB* and *pilC*) was able to restore pilus formation and phage sensitivity in mutants PAK-B Ω and PAK-C Ω but not in PAK-D Ω. Plasmids pDN18B and pDN18C complemented the piliation defect in mutants PAK-B Ω and PAK-C Ω, respectively. This complementation pattern suggests that insertions in *pilB* and *pilC* genes do not exert polar effects on downstream genes and that transcription of *pilB*, *pilC*, and *pilD* is very likely initiated from their own promoters.

Synthesis of *pilD* gene product in Ω insertion mutants. The effect of Ω insertion mutation on the synthesis of the *pilD* gene product was further examined. PilD is a prepilin leader peptidase, and expression of *pilD* can be assessed by examination of prepilin processing in bacteria, assays of prepilin cleavage in vitro, and immunoassay for PilD antigen.

The total membranes of the Ω insertion mutants as well as those of similar Tn*5* insertion mutants were fractionated by SDS-PAGE and immunoblotted with anti-PilD-derived peptide antibody. The amount of PilD synthesized in a strain with a transposon insertion in *pilB* (E4) or the Ω insertion mutant PAK-B Ω was comparable to that of wild-type *P. aeruginosa* (Fig. 5A). In contrast, the Tn*5* insertions into *pilC* (mutants E3 and E20) and the Ω-generated mutant PAK-CΩ resulted in decreased production of PilD. Transposon or Ω fragment disruptions of the *pilD* gene in 2B18 or PAK-D resulted in complete absence of *pilD* product.

Given the variability of the amounts of PilD present in *pilC* mutants, the extent of prepilin processing was examined in the various mutant strains. Extracts of the same mutants used for detection of PilD antigen were fractionated by SDS-PAGE and after immunoblotting were developed with mouse antipilin sera (Fig. 5B). With the exception of the two *pilD* insertions, all of the mutant strains synthesized and

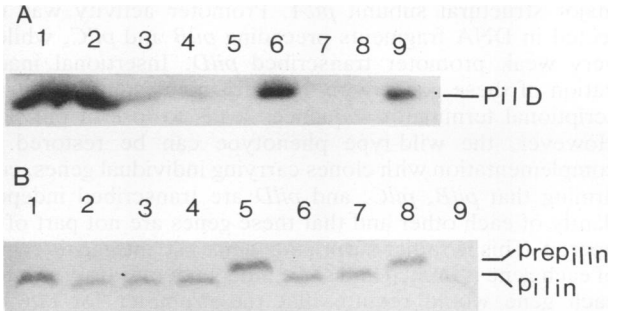

FIG. 5. Immunoblot analysis of total membranes from *P. aeruginosa* wild type (strain B24), Tn*5* insertion mutants (E4, E3, E20, and 2B18), and Ω insertion mutants (PAK-B Ω, PAK-C Ω, and PAK-D Ω). Blots were incubated with anti-PilD antiserum (A) or (B). Lanes: 1, B24; 2, E4; 3, E3; 4, E20; 5, 2B18; 6, PAK-B Ω; 7, PAK-C Ω; 8, PAK-D Ω; 9, purified PilD (A) or purified pilin (B).

TABLE 4. Prepilin cleavage activities of various mutants defective in pilus biogenesis

Strain	Genotype	Prepilin cleavage activity (%)[a]
B24	wt	76.2
E4	*pilB* mutant	90.8
E3	*pilC* mutant	13.4
E20	*pilC* mutant	25.8
2B18	*pilD* mutant	0
PAK-B Ω	*pilB* mutant	84.5
PAK-C Ω	*pilC* mutant	7.3
PAK-D Ω	*pilD* mutant	0

[a] Cleavage activities were determined as described by Nunn and Lory (20). Prepilin cleavage activity was the percentage of prepilin converted to pilin as estimated by staining with Coomassie blue R.

processed pilin to the same extent as the wild type. PAK-D Ω and 2B18 synthesized a higher-molecular-weight form of the pilin subunit (unprocessed prepilin). Since the product of *pilD* has been identified as a prepilin leader peptidase (20), it is obvious that all the mutants except PAK-D Ω and 2B18 synthesized the *pilD* gene product, which was capable of processing all of the prepilin in spite of low levels of PilD in the *pilC* insertion mutants.

In order to assess the amount of the *pilD* gene product of these strains quantitatively, prepilin cleavage activities were determined as described by Nunn and Lory (20). The prepilin cleavage activity correlated well with the amount of PilD detected by immunoblotting. Wild-type strain E4 and *pilB* mutant PAK-B Ω strains expressed a high level of prepilin cleavage activities (Table 4). Strains E3 and E20, with Tn*5* in *pilC*, showed significantly reduced cleavage activities. Another *pilC* mutant, PAK-C Ω, expressed the lowest detectable levels of prepilin cleavage activity. These results show that while *pilD* does not appear to be cotranscribed with *pilC*, insertional inactivation of *pilC* influences the levels of PilD polypeptide synthesized.

DISCUSSION

In this report, we describe localization of promoters in the gene cluster encoding *pilB*, *pilC*, and *pilD*, the genes required for biogenesis of type IV pili of *P. aeruginosa*. The DNA fragments containing a potential promoter of these genes were subcloned into a promoter-probe vector and then introduced into wild-type *P. aeruginosa* as well as into strains with mutations in genes required for expression of the major structural subunit *pilA*. Promoter activity was detected in DNA fragments preceding *pilB* and *pilC*, while a very weak promoter transcribed *pilD*. Insertional inactivation of these genes with an Ω fragment containing transcriptional terminator sequences leads to loss of piliation. However, the wild-type phenotype can be restored by complementation with clones carrying individual genes, confirming that *pilB*, *pilC*, and *pilD* are transcribed independently of each other and that these genes are not part of an operon. This is rather surprising, since the intragenic region of each gene is limited and since independent transcription of each gene would require that the promoter for *pilC* be located within the *pilB* coding sequence and that the promoter for *pilD* be located within the *pilC* coding sequence. Moreover, transcription from *pilB*, *pilC*, or *pilD* promoters was not affected by chromosomal mutations in the genes required for *pilA* expression, namely, *rpoN*, which encodes σ^{54}, or in the genes for two transcriptional activators, *pilS* and *pilR*. The transcriptional start site for *pilB* was mapped and found to be preceded by a promoter sequence recognized by σ^{70} but not by RpoN. Therefore, RpoN appears to control expression of only *pilA* and not any other gene necessary for expression of functional pili on the bacterial surface, since *tac* promoter-driven transcription of *pilA* leads to expression of normal pili even in the *rpoN* mutants. At this time, we cannot exclude the possibility that the expression of genes encoding the pilus biogenesis functions are controlled by as-yet-uncharacterized regulatory elements. The *pilB-lacZ* fusion is expressed at a significant level in *P. aeruginosa* (Table 1), while no *pilB* promoter activity can be detected from the same construct in an *E. coli* host (data not shown). This observation suggests that in *P. aeruginosa*, *pilB* expression is controlled by a transcriptional activator which is absent in *E. coli*.

We have observed a significant inhibitory effect on levels of *pilD* by insertions in the adjacent gene, *pilC*. This opens a formal possibility that *pilC* and *pilD* are cotranscribed from a *pilC* promoter. Only low levels of *pilD* promoter activity were detected from an intergenic *pilC-pilD* region. Complementation of *pilC* insertions by cloned *pilC* could be due to weak transcription from a promoter sequence near the 3′ end of *pilC*. Interestingly, low levels of PilD had no effect on the efficiency of prepilin cleavage in bacteria; complete processing of prepilin to its mature form was observed when the level of PilD was as low as 7% of that of wild type (Table 4). PilD is also responsible for the processing of four proteins that are components of the extracellular secretion machinery (21). None of the insertions in *pilC* which resulted in decreased levels of PilD affected secretion of extracellular enzymes (28), demonstrating that all of the recognized substrates of PilD are efficiently processed. These studies therefore suggest that PilD is synthesized in *P. aeruginosa* in at least 10-fold excess over what is needed for processing prepilin and precursors of components of the extracellular secretion machinery.

It is not surprising that *pilD* is not part of a regulon controlling expression of genes necessary for pilus biogenesis. PilD is required for maturation of four polypeptides that are components of a membrane machinery responsible for extracellular secretion of a variety of enzymes, and the expression of these extracellular enzymes is differentially regulated. It is conceivable that PilD is synthesized at constant levels without any influence from the external growth environment. The influence of insertions in *pilC* on levels of PilD is still not clear. It is conceivable that PilC is needed for optimal localization or stabilization of PilD, and the absence of PilC may result in only a small fraction of newly synthesized PilD stably inserted into the bacterial membrane. Studies of the precise role of PilB and PilC in biogenesis of pili are currently under way.

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