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The *Pseudomonas aeruginosa* Pathogenicity Island PAPI-1 Is Transferred via a Novel Type IV Pilus▽‡

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Pseudomonas aeruginosa **is a major cause of nosocomial infections, particularly in immunocompromised patients or in individuals with cystic fibrosis. The notable ability of *P. aeruginosa* to inhabit a broad range of environments, including humans, is in part due to its large and diverse genomic repertoire. The genomes of most strains contain a significant number of large and small genomic islands, including those carrying virulence determinants (pathogenicity islands). The pathogenicity island PAPI-1 of strain PA14 is a cluster of 115 genes, and some have been shown to be responsible for virulence phenotypes in a number of infection models. We have previously demonstrated that PAPI-1 can be transferred to other *P. aeruginosa* strains following excision from the chromosome of the donor. Here we show that PAPI-1 is transferred into recipient *P. aeruginosa* by a conjugative mechanism, via a type IV pilus, encoded in PAPI-1 by a 10-gene cluster which is closely related to the genes in the enterobacterial plasmid R64. We also demonstrate that the precursor of the major pilus subunit, PilS2, is processed by the chromosomally encoded prepillin peptidase PilD but not its paralog FppA. Our results suggest that the pathogenicity island PAPI-1 may have evolved by acquisition of a conjugation system but that because of its dependence on an essential chromosomal determinant, its transfer is restricted to *P. aeruginosa* or other species capable of providing a functional prepilin peptidase.**

The genomes of a number of microorganisms, primarily those that have a capability of changing and adapting to a wide range of environments, evolve by acquisition of novel genetic information in blocks of genes via a process referred to as horizontal gene transfer (HGT). Other bacterial species change their genetic repertoire minimally, principally those that have adapted to a particular environment and, in the case of pathogenic bacteria, to a specific host. For HGT-mediated acquisition of genes to occur, a recipient has to be in an environment where donor genetic material is available, such as different strains of the same species cohabitating a shared niche or growing in a large and diverse community of several hundred different microorganisms. Moreover, for bacteria to become successful recipients of foreign genetic material, they have to posses one of three mechanisms of HGT: natural competence for uptake of foreign DNA (transformation), the ability to be infected by transducing bacteriophages (transduction), or serving as recipients during conjugation of plasmids or mobilized chromosomal DNA (conjugation). Acquired genetic material can consist of individual genes, where they recombine into homologous sequences in the recipient genome and thus increase the genetic diversity. However, large blocks of hundreds of contiguous genes in elements called genomic islands can be also transferred between bacteria, allowing the recipient microorganisms to acquire a number of new traits by a single HGT event.

Previous studies comparing genomes of the opportunistic pathogen *Pseudomonas aeruginosa* pointed toward HGT as an important factor in its evolution (23). The genomes of all strains sequenced to date contain a significant fraction of horizontally acquired genes, in genomic islands and prophages, consisting of a few to several hundred. These islands can be recognized by the presence of certain signature features, such as an atypical nucleotide composition relative to the rest of the genome, location within predicted sites of chromosomal integration (*att* sites), and the presence of genes encoding bacteriophages and conjugation machineries. We have recently demonstrated that PAPI-1, a large *P. aeruginosa* genomic (pathogenicity) island, can be excised from its tRNA *att* site and that a copy can be transferred into a recipient, where it integrates into the same tRNA gene (27). Inspection of the genes in PAPI-1 and features of the transfer process, namely, an integrase-dependent excision and formation of a circular intermediate, suggested that PAPI-1 is an integrative and conjugative element and that it is likely transferred by a conjugative mechanism.

Here we extended our analysis of PAPI-1 by testing its transfer from a preselected group of *P. aeruginosa* PA14 mutants with insertions in each of the genes on the island. Among those mutants that were defective in PAPI-1 transfer, one group of genes encode homologs of type IV pilus proteins. While type IV pili have been found to be involved primarily in bacterial adhesion and twitching motility (24), the PAPI-1-encoded pilus is closely related to the conjugative apparatus of plasmid R64 (14). Moreover, we show that an essential posttranslational modification reaction, converting the precursor of the major pilin subunit encoded in PAPI-1 into a mature protein,

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is carried out by an enzyme encoded in the chromosome of the donor cells. The acquisition and adaptation of groups of genes and subsequent loss of an essential function may represent a novel evolutionary strategy, limiting horizontal transfer to a specific bacterial species.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. All the strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* strains and mutants were grown in Luria-Bertani (LB) broth supplemented with the appropriate antibiotic. For selection of *P. aeruginosa* mutants, the antibiotics used were as follows: irgasan, 25 μg/ml; gentamicin, 75 μg/ml; tetracycline, 75 μg/ml; and carbenicillin, 200 μg/ml for strain PA14 and 150 μg/ml for strain PAO1. For maintenance of plasmids or integrated elements, the antibiotic concentrations were lowered to 50 μg/ml for both gentamicin and tetracycline and 100 μg/ml for carbenicillin. When plasmids were maintained in *Escherichia coli*, the medium was supplemented with antibiotics at 30 μg/ml for gentamicin, 15 μg/ml for tetracycline, and 50 μg/ml for carbenicillin. If the expression of a cloned gene in the plasmid pMMB67 was required, isopropyl-β-D-thiopyranoside (IPTG) was added at a final concentration of 250 μM.

Construction of *P. aeruginosa* mutants. The in-frame deletion mutants were generated via homologous recombination using previously described gene replacement vectors and methods (12). All primers used for generating the deletion mutants are listed in Table SA1 in the supplemental material. DNA fragments of about 600 bp, from both up- and downstream of the target gene, were amplified and directly cloned in the vector pEX18Ap. The recombinant plasmids were conjugated from *E. coli* SM10 into *P. aeruginosa*. The carbenicillin-resistant (Cb^r) plasmid integrants were selected on LB plates containing the appropriate antibiotics. Merodiploids were resolved by plating on LB plates containing 6% sucrose. Deletion mutants were screened by PCR and confirmed by DNA sequencing. To insert the gentamicin resistance (Gm^r) gene in the middle of the target gene, an internal fragment of about 100 to 300 bp of each target gene was cloned in the suicide vector pEXG2. The Gm^r plasmid integrants were selected on LB agar plates containing gentamicin and irgasan. The insertion was confirmed by PCR. To insert the gene in the *att* site of the *P. aeruginosa* chromosome, the target gene was first cloned in delivery vector mini-CTX1 or mini-CTX-lacZ (13). The recombinant plasmids were then moved in *P. aeruginosa* from *E. coli* SM10. The backbone of the delivery vector was eliminated using the plasmid pFLP2 as previously described (12). The insertion of the target gene at the CTX phage *att* site was confirmed by PCR.

Transfer of PAPI-1. Transfer of PAPI-1 via liquid mating was performed as previously described with modifications (27). Briefly, the PAPI-1 donor and recipient strains were grown overnight in LB broth supplemented with appropriate antibiotics at 37°C on a shaker (300 rpm). The donor cells (optical density at 600 nm [OD_{600}] of 0.8) were then mixed with the recipient cells (OD_{600} of 0.4) in an Eppendorf tube, collected by spinning at 8,000 × *g* for 3 min, and resuspended in 1 ml of LB broth free of antibiotics. The cell suspension was transferred to a 15-ml culture tube and incubated at 37°C statically for 24 h prior to plating. Transconjugants were selected on LB agar plates containing gentamicin, carbenicillin, and 40 μg/ml of 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal). The transfer efficiency was calculated using the total number of transconjugants divided by the total recipients in the mating mixture.

Expression of the PAPI-1 *pil* operon and *pilS2* gene. DNA fragments containing the putative *pil* operon promoter (upstream of the *pilL2* gene, pilL2P) and *pilS2* promoter (upstream of the *pilS2* gene, pilS2P) were PCR amplified from the strain PA14 using primer pairs described in Table SA1 in the supplemental material. DNA fragments were cloned into the vector mini-CTX-lacZ (13). The recombinant plasmids were then moved into strain PA14 by conjugation with *E. coli* SM10, and the backbone of mini-CTX-lacZ was eliminated using plasmid pFLP2 as previously described. β-Galactosidase activity was measured as described before (27).

Expression of PilS2 and tagged PilS2. The *pilS2* gene was amplified by PCR from strain PA14 using primers *pilS2*F and *pilS2*R (see Table A1 in the supplemental material) and cloned in plasmid pMMB67 (Tc^r). To fuse the tags with the PilS2 at its carboxyl terminus, the nucleotides encoding the various tags were incorporated in the primer *pilS2*R between the restriction site and the gene-specific sequence (5′GAACTGAGCCTGGATGATCG).

SDS-PAGE and Western blot analysis. *P. aeruginosa* strains PA14 Δ*pilS2*p*pilS2-His*, PA14 Δ*pilD*p*pilS2-His*, and PA14 Δ*pilD att*::*pilD* p*pilS2-His* were grown in LB with antibiotics and IPTG. The cells were collected and washed once with ice-cold Tris-HCl buffer (50 mM, pH 7.4), and the pellets were resuspended in the SDS-PAGE loading buffer at the concentration of 0.05 OD_{600} unit/μl. The samples were then boiled for 10 min, and supernatants were recovered by spinning the whole-cell lysates at 12,000 × *g* for 10 min. The proteins were separated on a 12% Bis-Tris gel (Novex; Invitrogen) and then blotted to a nitrocellulose membrane, and the PilS2 was detected using a mouse anti-oligo-His antibody (H-3; Sigma) at a dilution of 1:2,000, followed by horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG at a dilution of 1:5,000. Finally, the membrane was stained with Super SignalWest Pico chemiluminescent substrate (Pierce).

Transmission electron microscopy. *P. aeruginosa* strains PA14TnC2 Δ*pilA*Δ*pilS2*p*pilS2* and PA14TnC2 Δ*pilA*Δ*pilS2*p*pilS2-Flag* were grown in 1 ml of LB broth supplied with both antibiotics and IPTG statically at 37°C overnight. The grids were immersed in a drop of bacterial suspension for 5 min, fixed with 5% paraformaldehyde for 5 min, and then washed two times with phosphate-buffered saline (PBS), each for 5 min. After incubation with a 5% bovine serum albumin (BSA) solution in PBS for 15 min, the grids were incubated with mouse antibody against the Flag tag (Sigma) at a 1:50 dilution in 1% BSA for 30 min. After being briefly washed with PBS, the grids were further incubated with bridging antibody (rabbit anti-mouse) in 1% BSA for 30 min, followed by incubation with a protein A-gold (10 nm) solution for 20 min. After being extensively washed in PBS and water, the grids were stained with 1% uranyl formate and examined using a Tecnai G^2 Spirit transmission electron microscope.

RESULTS

The *pilS2* and *pilV2* genes carried on PAPI-1 are required for its interstrain transfer. To systematically identify genes that play a role in horizontal transfer of PAPI-1, mutations in individual genes within the island were retrieved from the collection of mapped *mariner* transposon insertions in *P. aeruginosa* strain PA14 (22). A total of 95 strains with transposon insertions were obtained from the PA14 mutant library. The location of the transposon in each PAPI-1 gene was confirmed by DNA sequencing. For those genes with no transposon mutants in the collection, mutations were generated by inserting a gentamicin acetyltransferase gene, *aacC1*, in the middle of each open reading frame (ORF). Strains with mutations in individual PAPI-1 genes were then used as donors in mating experiments to assess their ability to transfer PAPI-1 into the PAO1 recipient. Strain PA14TnC2, which harbors a *mariner* transposon at the 3′ end of the hypothetical gene *RL090* (PA14_59200) and is proficient in transfer, was used as a control. Insertions in a total of 55 genes showed reduced efficiency of PAPI-1 transfer compared to the control (see Table SA2 in the supplemental material). These 55 genes were grouped into six clusters. As expected, mutations in two genes that were characterized previously, *int* and *soj*, abolished PAPI-1 transfer (27). This screen also revealed that several PAPI-1 genes encoding homologs of proteins previously shown to be involved in the transmission of conjugative plasmids were also required for PAPI-1 transfer into the recipient PAO1 (see Table SA2 in the supplemental material). These include genes *RL003*, *RL022*, and *RL047*. *RL003* encodes a homolog of DNA relaxase, which mobilizes DNA for transfer by creating a nick at the origin of transfer (*oriT*) and covalently attaching to the 5′ phosphate of the nicked strand (34); *RL022* encodes a homolog of the VirB4 protein, which was shown to be required for conjugal transfer of the Ti plasmid in *Agrobacterium* (3); and *RL047* encodes a homolog of the TraG/TraD proteins, which function as a coupling protein in the type IV conjugative transfer system (33). Furthermore, PAPI-1 harbors a 10-gene cluster (*pilL2*, *-N2*, *-O2*, *-P2*, *-Q2*, *-R2*, *-S2*, *-T2*, *-V2*, and *-M2*) encoding determinants of type IV pili such as PilS2, the major subunit of the pilus filament; PilV2, the minor subunit of the

TABLE 1. *P. aeruginosa* strains and plasmids used in this study

Strain or plasmid	Antibiotic resistance[a]	Description	Source, reference, or accession no.
E. coli SM10	None	Host strain for plasmids pEXAp18, pMMB67, mini-CTX, and mini-CTX-lacZ and their derivatives	Lab collection
P. aeruginosa strains			
PAO1	None	Laboratory strain	Lab collection
PAO1pilL2P	None	Strain PAO1 with the DNA fragment containing putative promoter of the PAPI-1 *pil* operon and a promoterless *lacZ* gene inserted at the CTX phage *att* site on the chromosome; the backbone of the plasmid mini-CTX-lacZ was eliminated	This study
PAO1pilS2P	None	Strain PAO1 with a DNA fragment containing the putative promoter of the *pilS2* gene and a promoterless *lacZ* gene inserted at the CTX phage *att* site on the chromosome; the backbone of the plasmid mini-CTX-lacZ was eliminated	This study
PAO1Bla6	Cb^r	Strain PAO1 with genes *bla* and *lacZ* inserted at the CTX phage *att* site on the chromosome	27
PA14	None	Burn isolate	27
PA14pilL2P	None	Strain PA14 with a DNA fragment containing the putative promoter of the PAPI-1 *pil* operon (upstream of the *pilL2* gene) and a promoterless *lacZ* gene inserted at the CTX phage *att* site on the chromosome; the backbone of the plasmid mini-CTX-lacZ was eliminated	This study
PA14pilS2P	None	Strain PA14 with a DNA fragment containing the putative promoter of the *pilS2* gene and a promoterless *lacZ* gene inserted at the CTX phage *att* site on the chromosome; the backbone of the plasmid mini-CTX-lacZ was eliminated	This study
PA14 Δ*pilS2*p*pilS2-His*	Tc^r	Deletion mutant PA14 Δ*pilS2* carrying the cloned *piS2-His* in plasmid pMMB67Tc (p*pilS2-His*)	This study
PA14 Δ*pilD*Δ*pilS2*p*pilS2-His*	Tc^r	Deletion mutant PA14 Δ*pilD* Δ*pilS2* carrying the cloned *piS2-His* in plasmid pMMB67Tc (p*pilS2-His*)	This study
PA14 Δ*pilD*att::*pilD*Δ*pilS2*p*pilS2-His*	Tc^r	Strain PA14 Δ*pilD*Δ*pilS2*p*pilS2-His* with the *pilD* gene driven by Ptac inserted at the *att* site on the chromosome; the backbone of the mini-CTX was eliminated	This study
PA14TnC2	Gm^r	Strain PA14 with a transposon MAR2×T7 inserted at nucleotide 1634 of PAPI-1 gene RL090 (PA14_59200)	22
PA14TnC2 Δ*pilS2*	Gm^r	In-frame deletion of the PAPI-1 *pilS2* gene in strain PA14TnC2	This study
PA14TnC2 Δ*pilV2*	Gm^r	In-frame deletion of the PAPI-1 *pilV2* gene in strain PA14TnC2	This study
PA14TnC2 Δ*pilA*	Gm^r	In-frame deletion of the *pilA* gene in strain PA14TnC2	This study
PA14TnC2 Δ*flp*	Gm^r	In-frame deletion of the *flp* gene in strain PA14TnC2	This study
PA14TnC2 Δ*fppA*	Gm^r	In-frame deletion of the *fppA* gene in strain PA14TnC2	This study
PA14TnC2 Δ*pilD*	Gm^r	In-frame deletion of the *pilD* gene in strain PA14TnC2	This study
PA14TnC2 Δ*pilA*Δ*pilS2*	Gm^r	In-frame deletion of both the *pilA* and *pilS2* genes in strain PA14TnC2	This study
PA14TnC2 Δ*pilS2*p*pilS2*	Gm^r Tc^r	Strain PA14TnC2 Δ*pilS2* carrying cloned *pilS2* on plasmid pMMB67Tc (p*pilS2*)	This study
PA14TnC2 Δ*pilV2*p*pilV2*	Gm^r Tc^r	Strain PA14TnC2 Δ*pilV2* carrying cloned *pilV2* on plasmid pMMB67Tc (p*pilV2*)	This study
PA14TnC2 Δ*pilS2*vector	Gm^r Tc^r	Strain PA14TnC2 Δ*pilS2* carrying plasmid pMMB67Tc	This study
PA14TnC2 Δ*pilV2*vector	Gm^r Tc^r	Strain PA14TnC2 Δ*pilV2* carrying plasmid pMMB67Tc	This study
PA14TnC2 Δ*pilD*p*pilD*	Gm^r Tc^r	Strain PA14TnC2 Δ*pilD* carrying the cloned *pilD* gene on plasmid pMMB67Tc (p*pilD*)	This study
PA14TnC2 Δ*pilD*p*fppA*	Gm^r Tc^r	Strain PA14TnC2 Δ*pilD* carrying the cloned *fppA* gene on plasmid pMMB67Tc (p*fppA*)	This study
PA14TnC2 Δ*pilD*vector	Gm^r Tc^r	Strain PA14TnC2 Δ*pilD* carrying plasmid pMMB67Tc	This study
PA14TnC2 Δ*pilA*Δ*pilS2*vector	Gm^r Tc^r	Strain PA14TnC2 Δ*pilA*Δ*pilS2* carrying plasmid pMMB67Tc	This study
PA14TnC2 Δ*pilA*Δ*pilS2*p*pilS2*	Gm^r Tc^r	Strain PA14TnC2 Δ*pilA*Δ*pilS2* carrying plasmid p*pilS2*	This study
PA14TnC2 Δ*pilA*Δ*pilS2*p*pilS2-Flag*	Gm^r Tc^r	Strain PA14TnC2 Δ*pilA*Δ*pilS2* carrying plasmid p*pilS2-Flag*, in which the Flag tag is fused with PilS2 at its C terminus	This study
Plasmids			
pEXG2	Gm^r	Gene replacement vector for constructing deletion or insertion mutants of *P. aeruginosa*	28
pEX18Ap	Ap^r	Gene replacement vector for constructing deletion or insertion mutants of *P. aeruginosa*	12
pFLP2	Ap^r	Plasmid used for resolving the backbone of mini-CTX-lacZ	12
Mini-CTX	Tc^r	Gene delivery vector for inserting genes at the CTX phage att site on the *P. aeruginosa* chromosome	13
CTX-Ptac*pilD*	Tc^r	The *pilD* gene and *Ptac* were cloned in the vector mini-CTX	This study

Continued on following page

TABLE 1—*Continued*

Strain or plasmid	Antibiotic resistance[a]	Description	Source, reference, or accession no.
Mini-CTX-lacZ	Tc^r	Gene delivery vector for inserting genes at the CTX phage *att* site on the *P. aeruginosa* chromosome	AF140579
CTXpilL2P	Tc^r	A DNA fragment containing the putative promoter of the PAPI-1 *pil* operon was cloned in the vector mini-CTX-lacZ	This study
CTXpilS2P	Tc^r	A DNA fragment containing the putative promoter of the PAPI-1 *pilS2* gene was cloned in the vector mini-CTX-lacZ	This study
pMMB67Tc	Tc^r	Replicative plasmid in *P. aeruginosa*, used for complementary analysis in *P. aeruginosa* deletion mutants	40
p*pilS2*	Tc^r	The *pilS2* gene was cloned in plasmid pMMB67Tc	This study
p*pilV2*	Tc^r	The *pilV2* gene was cloned in plasmid pMMB67Tc	This study
p*pilD*	Tc^r	The *pilD* gene was cloned in plasmid pMMB67Tc	This study
p*pilS2-His*	Tc^r	The *pilS2* gene and His tag at the C terminus were cloned in plasmid pMMB67Tc	This study
p*pilS2-Flag*	Tc^r	The *pilS2* gene and Flag tag at the C terminus were cloned in plasmid pMMB67Tc	This study

[a] Ap^r, ampicillin resistance; Gm^r, gentamicin resistance; Cb^r, carbenicillin resistance; Tc^r, tetracycline resistance.

pilus filament and the various pilus assembly accessory proteins (Table 2). Insertion of the transposon into any of 10 *pil* genes leads to a significant loss of PAPI-1 transfer (Table 2), suggesting that they may be part of the PAPI-1 conjugation apparatus. Examination of the gene organization of the 10 *pil* genes suggests that they are likely transcribed as an operon (Fig. 1, *Pa* PAPI-1). Because of the potential polar effects of the transposon insertion, in-frame deletions of two genes encoding the major and minor pilus subunit proteins, *pilS2* and *pilV2*, were generated. Similar results were observed when using PA14 Δ*pilS2* or PA14 Δ*pilV2* as with the PAPI-1 donor strain; however, the PAPI-1 transfer efficiency was restored for both mutants once the corresponding gene was provided in *trans* (Table 2).

The PAPI-1 *pil* gene cluster is related to the *pil* operon of the conjugative plasmid R64. The PAPI-1 *pil* gene cluster is 10,645 bp with an average GC content of 64.3%, which is slightly higher than that of the PAPI-1 island (59.7%) (see Table SA3

TABLE 2. Putative functions of PAPI-1 Pil proteins

Gene	Gene product	Mutant strain(s)[a]	Transfer efficiency	Conserved domain[b]	Homologous component(s) of TFp/T4SS	Description (reference)
PA14_59240	PilL2	PA14TnΩ241*pilL2*	$<10^{-8}$	None	Type IV lipoprotein PilL	Lipoprotein (29)
PA14_59250	PilN2	PA14TnΩ47*pilN2*	$<10^{-8}$	Secretin, bacterial type II and III secretion system protein (pfam00263)	Type IV pilus secretin protein PilN	Secretin (29)
PA14_59270	PilO2	PA14TnΩ111*pilO2*	$<10^{-7}$	PAP_PilO, pilin accessory protein (pfam06864)	Pilus accessory protein PilO	Outer membrane protein (30)
PA14_59280	PilP2	PA14TnΩ344*pilP2*	$<10^{-7}$	Pilus_PilP superfamily (pfam11356)	Pilus assembly protein PilP	Periplasmic or outer membrane protein (30)
PA14_59290	PilQ2	PA14TnΩ241*pilQ2*	$<10^{-7}$	P-loop nucleoside triphosphatase superfamily	TFp pilus assembly protein PilT, pilus retraction ATPase (COG2805)/VirB11-like ATPase (cd01130)	ATPase
PA14_59310	PilR2	PA14TnΩ37*pilR2*	$<10^{-7}$	GSPII_F, bacterial type II secretion system protein F domain (pfam00482)	Plasmid R64 integral membrane protein PilR	Membrane protein (30)
PA14_59320	PilS2	PA14TnΩ103*pilS2*	$<10^{-7}$	PilS superfamily (pfam08805)	Type IV prepilin PilS	Pilin protein
		PA14 Δ*pilS2*	$<10^{-8}$			
		PA14 Δ*pilS2*p*pilS2*	1.7×10^{-5}			
PA14_59340	PilT2	PA14TnΩ44*pilT2*	$<10^{-7}$	P-loop nucleoside triphosphatase superfamily	TFp pilus assembly protein PilT, pilus retraction ATPase (COG2805)/VirB11-like ATPase (cd01130)	ATPase
PA14_59350	PilV2	PA14TnΩ103*pilV2*	$<10^{-7}$	Shufflon_N superfamily (pfam04917)	Plasmid R64 minor pilin subunit PilV	Shufflon protein (17)
		PA14 Δ*pilV2*	$<10^{-8}$			
		PA14 Δ*pilV2*p*pilV2*	8.5×10^{-5}			
PA14_59360	PilM2	PA14TnΩ59*pilM2*	$<10^{-8}$	PilM (pfam07419)	Plasmid R64 inner membrane protein PilM	Membrane protein (30)

[a] The number indicates the insertion site of the transposon in the gene.
[b] Revealed by PSI-BLAST analysis.

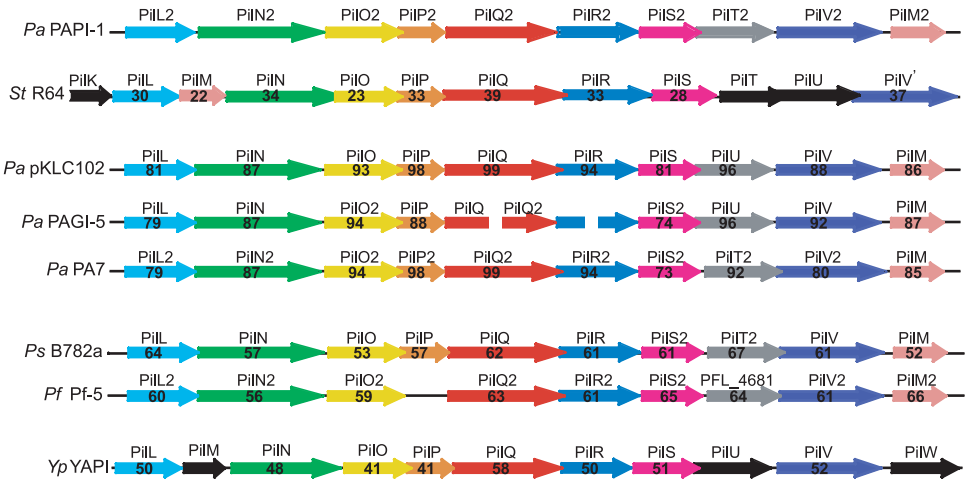

FIG. 1. Conservation of the PAPI-1 *pil* gene clusters in different microorganisms. The putative function of each PAPI-1 pilus protein is presented in Table 2. Homologous proteins in various bacteria are indicated by the same color. The numbers indicate the sequence similarity of each protein with its PAPI-1 homolog. The black arrows represent those genes lacking homologs in the PAPI-1 island. The gaps in PilQ2 and PilR2 of the *P. aeruginosa* (*Pa*) PAGI-5 represent the disruptions of ORFs by insertions. The *pil* cluster sequences were retrieved from GenBank: PAPI-1, accession no. AY273869; pKLC102, AY257538; PAGI-5, EF611301; R64 from *Salmonella enterica* serovar Typhimurium (*St*), NC_005014; and YAPI from *Yersinia pseudotuberculosis* (*Yp*), AJ627388. The *pil* cluster sequence of *P. aeruginosa* strain PA7, *P. syringae* (*Ps*) strain B782a, and *P. fluorescens* (*Pf*) strain Pf-5 were obtained from the *Pseudomonas* Genome Database (38).

in the supplemental material). To infer the putative function and evolutionary origin of the PAPI-1 pili, BLAST analysis was performed for each PAPI-1 pilus protein. Strikingly, PAPI-1 Pil protein showed high similarities to the components of the type IV pilus (TFp) (PilL2, -N2, -O2, -P2, -Q2, -R2, -S2, -T2, -V2, and -M2) and of the type IV secretion system (T4SS) (PilQ2 and PilT2) (Table 2). Further exploration of the PAPI-1 *pil* operon revealed that it is closely related to the *pil* operon of conjugative plasmid R64 (Fig. 1). In the plasmid R64, 12 genes of the *pil* operon (from *pilK* to *pilV'*), which encodes both structural and assembly components of a thin pilus, are required for its liquid mating (Fig. 1, *St* R64) (14, 42). Homologs of nine PAPI-1 Pil proteins were found in plasmid R64. Besides PilS2 and PilV2, which are homologs of the major and minor subunits of R64 thin pilus, PilS and PilV, they are PilL2, a type IV lipoprotein; PilN2, a type IV pilus secretin protein; PilO2, a pilus accessory protein; PilP2, a pilus assembly protein; PilQ2, a pilus retraction ATPase; PilR2, an integral membrane protein and PilM2, an inner membrane protein (Table 2). The R64 PilU is a prepilin peptidase, which catalyzes the cleavage of the R64 PilS prepilin protein prior to it assembling into the mature pilus (1). No homolog of R64 PilU was identified on the PAPI-1 island; however, R64 PilU is distantly related to PA14 PilD (PA14_58770, 27% identity), which is involved in processing of prepilin protein PilA, the major subunit of *P. aeruginosa* polar pili (36).

The 10 PAPI-1 pilus proteins are well conserved in several *P. aeruginosa* strains that carry this island, including PA2192, C3710, PACS2, PA7, and PSE9 (PAGI-5), and in *P. aeruginosa* clone C strains that carry a pKLC102-like element (4, 15, 23). The sequence similarities at the amino acid level range from 73% (Fig. 1, PilS2 of PA7) to 99% (Fig. 1, PilQ2 of PA7 and pKLC102). It is noteworthy that the PilU annotated in pKLC102, PAGI-5, and strain PA7 is a homolog of PAPI-1 PilT2 (ATPase) but is not a homolog of PilU from the pathogenicity island YAPI or plasmid R64, in which PilU is a prepilin peptidase (Fig. 1). Although the nucleotide sequences of PAGI-5 *pilQ2* and *pilR2* are very similar to those in PAPI-1, there are several insertions in both genes resulting in frameshift mutations (Fig. 1, indicated by interrupted ORFs). Homologs of 10 PAPI-1 pilus proteins were also found in *Pseudomonas syringae* strain B728a (from Psyr_1509 to Psyr_1518), whereas in *Pseudomonas fluorescens* Pf-5, homologs of nine PAPI-1 pilus proteins are present but there is no homolog PAPI-1 PilP2, which is a putative periplasmic protein (Table 2). Furthermore, homologs of eight PAPI-1 pilus proteins were found in the YAPI pathogenicity island of *Yesinia pseudotuberculosis* (7) (Fig. 1, *Yp* YAPI) (PilL, PilN, PilO, PilP, PilQ, PilR, PilS, and PilV). This observation suggests that the PAPI-1 *pil* gene cluster and the determinants of the plasmid R64 conjugation apparatus may have been acquired from a common ancestor but that over time they evolved to adapt to their cognate mobile elements by selective loss or acquisition of additional genes. The absence of *pilK*, *pilT*, and *pilU* in all *Pseudomonas* strains suggests that the *pil* cluster passed through an ancestor where these three genes were lost before entering the *Pseudomonas* lineage.

The PAPI-1 *pilS2* gene encodes a major pilin subunit of the type IVb pilus. PAPI-1 *pilS2* encodes a 176-amino-acid protein containing a conserved PilS superfamily domain (Table 2), suggesting that it is a precursor of the major pilus subunit protein. In addition to the PAPI-1 *pil* gene cluster, all *P. aeruginosa* strains analyzed to date, including PA14, harbor two prepilin genes, *pilA* and *flp* (Fig. 2A). The PilA protein is the member of the type IVa pilus family, and it is assembled into a filament at the pole of the rod-shaped cell (9). These polar pili are widely distributed in *Proteobacteria*, and in *P. aeruginosa* they have been shown to play a role in twitching motility, adhesion to epithelial cells, and promotion of biofilm formation (6, 26, 31, 41). The Flp protein belongs to the Flp/Fap

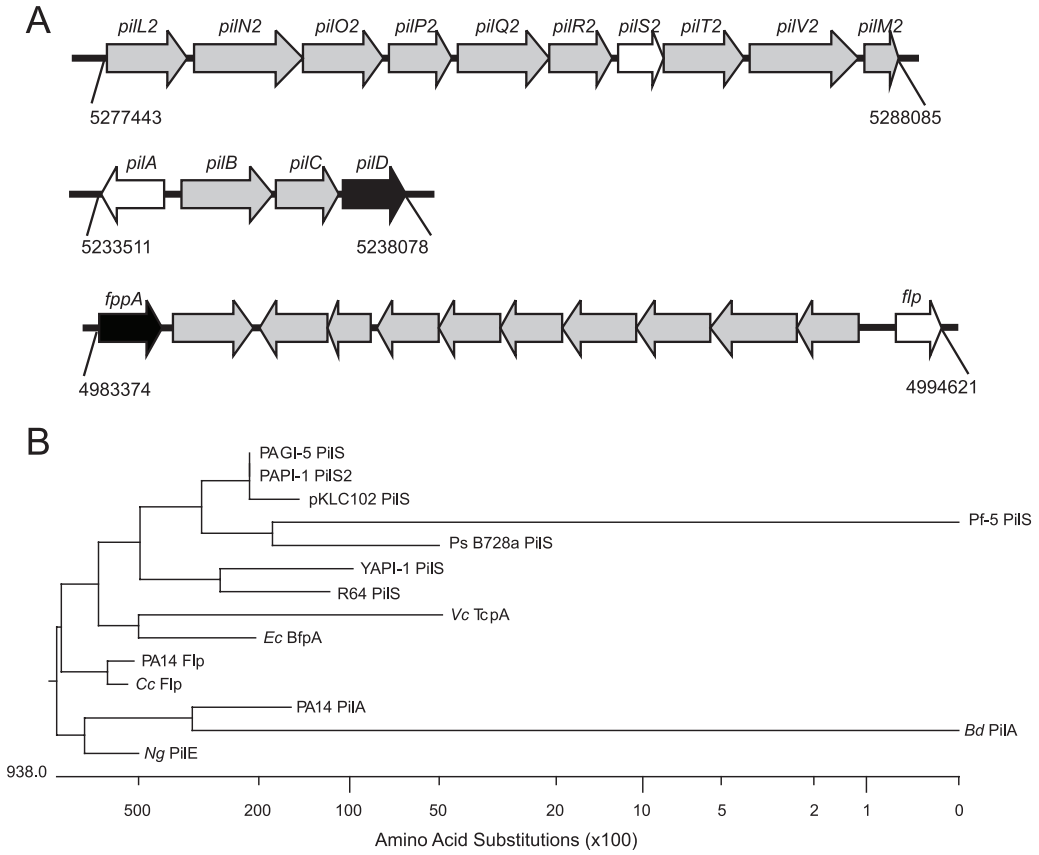

FIG. 2. (A) Chromosomal locations of three *P. aeruginosa* pilin genes, *pilA*, *flp*, and *pilS2*, in strain PA14. The numbers indicate the chromosome positions. (B) Phylogenetic analyses of the three *P. aeruginosa* pilin proteins and some pilin proteins from other organisms. The alignment was performed by the Jotun Hein method using DNASTAR Lasergene software. The pilin protein sequences were retrieved from GenBank. The type IVb pilin proteins are represented by PAPI-1 PilS2 (accession o. YP_792926), pKLC102 PilS (AAP22599), PAGI-5 PilS (translated protein from EF611301, protein ID ABR13424.1), PilS of *P. fluorescens* Pf-5 (*Pf*) (YP_261763), PilS of *P. syringae* B728a (*Ps*) (AAY36562), R64 PilS (NP_863479), YAPI-1 PilS (YP_001402683), BfpA of *Escherichia coli* (*Ec*) (YP_001965379), TcpA of *Vibrio cholerae* (*Vc*) (AAA88688), *P. aeruginosa* Flp (YP_792651), and Flp of *Caulobacter crescentus* (*Cc*) (AAF40189). Type IVa pilin proteins used in the analysis are PA14 PilA (YP_792871), PilA of *Burkholderia dolosa* (*Bd*) (ZP_04944829), and PilE from *Neisseria gonorrhoeae* (*Ng*) (CAA47349).

family, and in *P. aeruginosa*, the Flp pili have also been implicated in adhesion and biofilm formation (8). ClustalW analyses of the various major prepilins revealed that the evolutionary relationship is based on the distinction of the type IVa and type IVb families. Within the type IVb group, the PAPI-1 PilS2, along with its homologs found in other mobile elements of *P. aeruginosa* and in *P. fluorescens* and *P. syringae*, form a very closely related group (Fig. 2B). This group of pilus proteins is also related to the pilin protein of enterobacterial origin (R64 PilS) and somewhat less so to the various Flp pilins (Fig. 2B). This suggests that the PAPI-1-encoded pilus is a member of an evolutionarily related pilus family that is widespread among bacterial species, where these proteins participate in horizontal gene transfer.

The PAPI-1-encoded pilus is most likely the component of a conjugation machinery; however, we could not exclude the possibility that either the chromosomally encoded PilA pilus or Flp pilus could substitute for the PilS2 pilus during PAPI-1 transfer. To determine which one of the three type IV pili is used by PAPI-1 during transfer into recipient cells, in-frame deletions of three pilin genes, *pilS2*, *pilA*, and *flp*, were generated in strain PA14TnC2. Each mutant was used as a donor strain, and the efficiency of PAPI-1 transfer into the PAO1 recipient was compared to that from the wild-type strain PA14TnC2. Absence of either PilA or Flp had no effect on PAPI-1 transfer, whereas deletion of the *pilS2* gene abolished PAPI-1 transfer completely (Table 3). Therefore, in spite of the presence of other type IV pili genes in the chromosome of *P. aeruginosa*, the transfer of PAPI-1 is mediated exclusively by the PilS2 pilus encoded in the island.

Processing of prepilin PilS2 by the PilD peptidase. Regardless of the diversity in their functions and distribution of type

TABLE 3. Transfer of PAPI-1 from PA14 with mutations in various pilin genes into PAO1

Donor strain	Transfer efficiency
PA14TnC2 (Gm^r)	7.0×10^{-5}
PA14TnC2 Δ*pilA* (Gm^r)	5.4×10^{-5}
PA14TnC2 Δ*flp* (Gm^r)	7.9×10^{-5}
PA14TnC2 Δ*pilS2* (Gm^r)	$<10^{-8}$
PA14TnC2 Δ*pilS2*p*pilS2* (Gm^r Tc^r)	1.7×10^{-5}
PA14TnC2 Δ*pilS2*vector (Gm^r Tc^r)	2.7×10^{-8}

FIG. 3. (A) Leader peptides of representative type IV prepilin proteins. The arrow indicates the site of processing by the prepilin peptidase. (B) Processing of the PilS2 leader peptide by chromosomally encoded PilD. Western immunoblot analysis of cleavage of His_6-tagged PilS2 in strain PA14 Δ*pilS2*p*pilS2-His* expressing wild-type levels of PilD (lane 1), in a PilD-deficient mutant (PA14 Δ*pilD*Δ*pilS2*p*pilS2-His* (lane 2), and in the complemented strain PA14 Δ*pilD*Δ*pilS2att*::*pilD* p*pilS2-His* (lane 3) is shown.

IV pili, one of the common features shared by all type IV pili during their biogenesis is the requirement for posttranslational processing of the precursor of the major subunit. The amino-terminal leader peptide from prepilin protein is cleaved by a specific prepilin peptidase prior to assembly of the monomers into a pilus (35). The cleavage sites of all prepilin proteins are conserved (Fig. 3A), with an invariant glycine residue at position −1 and position +1 most frequently containing a phenylalanine, although different amino acids can be tolerated at this position without affecting processing (36). Since we could not identify a gene encoding a prepilin peptidase homolog in the PAPI-1 island, we hypothesized that the processing of pre-PilS2 into its mature form is carried out by one of the two chromosomally encoded prepilin peptidases, PilD or FppA, which are responsible for the processing of the precursors of PilA or Flp, respectively. We engineered *pilD* and *fppA* deletions in *P. aeruginosa* PA14TnC2 and tested the mutant strains for PAPI-1 transfer into PAO1. Transfer of PAPI-1 was blocked when PA14TnC2 Δ*pilD* was used as a donor, while PA14TnC2 Δ*fppA* served as an efficient donor of PAPI-1 (Table 4), indicating that the PilS2 precursor is the substrate of PilD.

To confirm that PilD is responsible for the cleavage of the PilS2 leader peptide, a sequence specifying a His_6 tag was fused to the C-terminal coding sequence of the *pilS2* gene and processing of this hybrid protein in both the wild-type strain (PA14 Δ*pilS2*p*pilS2-His*) and a PilD-deficient strain (PA14 Δ*pilD*Δ*pilS2*p*pilS2-His*) was examined in a Western immunoblot of cell extracts. In the strain lacking PilD, a larger protein was observed than in the strain expressing the wild-type *pilD* gene (Fig. 3B, lane 2). The size of this larger protein was approximately equivalent to the predicted size of the His_6-tagged prepilin PilS2 (17.9 kDa), suggesting that the cleavage of the 14-amino-acid leader peptide was blocked in this strain. Supplying the *pilD* gene in *trans* restored the cleavage, leading to the production of the 16.4-kDa mature pilin protein PilS2 (Fig. 3B, lane 3), which is identical in size to the protein produced by the wild-type strain (Fig. 3B, lane 1). This result confirmed the role of the chromosomally encoded PilD in PAPI-1 transfer. Overproduction of FppA did not complement the *pilD* mutation, suggesting that PilD-mediated cleavage is substrate specific (Table 4). This cooperative action between the products of genes located on a mobile element (PAPI-1 *pil* genes) and on the host chromosome (*pilD*) in assembling the *P. aeruginosa* conjugation apparatus is an example of adaptation of mobile elements to their host strains.

Expression of PAPI-1 *pil* genes. Genes whose products are involved in the biogenesis of type IVb pili are often organized into operons and expressed coordinately. Inspection of the DNA sequence of the *pil* locus revealed two potential promoters located upstream of the first *pil* gene, *pilL2*, and one putative promoter located upstream of the *pilS2* gene, each containing putative −10 and −35 sequences (see Fig. S1A in the supplemental material). The expression of the putative promoter of the PAPI-1 *pil* operon (promoter located upstream of the *pilL2* gene, pilL2P) and the putative promoter of the *pilS2* gene (pilS2P) was examined during their aerobic growth in rich medium (LB). Similar growth rates were observed for strains carrying cloned pilL2P and pilS2P (data not shown). The expression of both promoters increased with cell density, and the highest expression levels for both promoters were observed at the late stationary phase in strain PA14 (Fig. 4, filled bars), suggesting that the *pil* operon might be the target of stationary-phase regulators. The expression of the *pilS2* promoter was consistently higher than that of the putative *pil* operon, which could reflect the stoichiometric relationship between PilS2, the major component of the pilus structure, and the relatively fewer components needed to assemble the filament. Similar expression trends for both promoters were observed in strain PAO1 (Fig. 4, open bars), suggesting that the expression of the *pil* genes does not require any of the PAPI-1-encoded regulators.

PAPI-1 encoded type IVb pilus. In most microorganisms, type IV pili are thin, long, flexible filaments emanating from the bacterial surface. In *P. aeruginosa*, the PilA pili (type IVa) are polar and are located at the same cell pole as the flagellum (9). When expressed, the Flp pili (type IVb) appear to be bundled filaments with a twisted appearance (8). To reveal the morphological characteristics of PAPI-1-encoded pilus, various epitope tags (Flag, cMyc, HA, Strep, and Flash) were fused to the C terminus of PilS2 to generate various C-tagged PilS2 protein. We then expressed various C-tagged pilin proteins in a strain that is deficient in both PilS2 and PilA (PA14TnC2 Δ*pilS2*Δ*pilA*) and examined whether the PAPI-1 transfer efficiency was restored. Full complementation of the transfer was observed only when these bacteria were expressing the Flag-tagged PilS2 (data not shown). Therefore, the strain express-

TABLE 4. PilD, but not FppA, is required for PAPI-1 transfer

Donor strain	Transfer efficiency
PA14TnC2 (Gm^r)	7.0×10^{-5}
PA14TnC2 Δ*fppA* (Gm^r)	3.0×10^{-5}
PA14TnC2 Δ*pilD* (Gm^r)	$<10^{-8}$
PA14TnC2 Δ*pilD*p*pilD* (Gm^r Tc^r)	1.7×10^{-5}
PA14TnC2 Δ*pilD*p*fppA* (Gm^r Tc^r)	2.5×10^{-8}
PA14TnC2 Δ*pilD*vector (Gm^r Tc^r)	2.1×10^{-8}

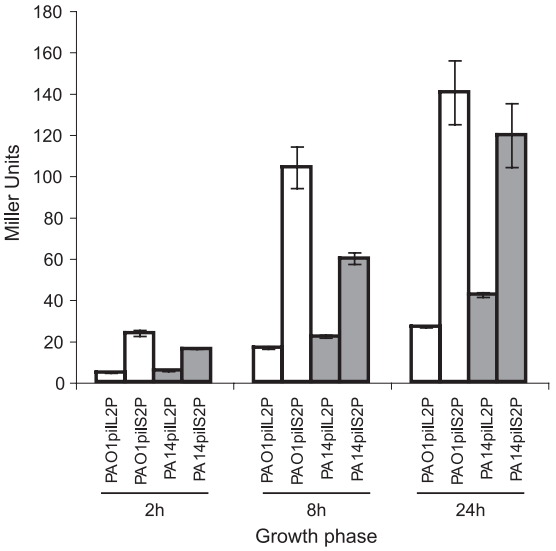

FIG. 4. Expression of the PAPI-1 *pil* genes. β-Galactosidase levels in *P. aeruginosa* strains PAO1 (open bars) and PA14 (filled bars) carrying promoter-*lacZ* fusions are shown. The putative promoters of the PAPI-1 *pil* operon (pilL2P) and the *pilS2* gene (pilS2P) were fused to the reporter *lacZ* gene, and the expression of β-galactosidase was monitored in both PAO1, a strain lacking of the PAPI-1 island, and strain PA14. Error bars indicate standard deviations.

ing Flag-tagged PilS2 pilin protein (PA14TnC2 Δ*pilS2*Δ*pilA* p*pilS2-Flag*) was used to examine the morphology of the PAPI-1 pili by transmission electron microscopy following immunogold labeling of bacteria using Flag-specific antibody (Fig. 5). PAPI-1-encoded pili exhibit typical characteristics of type IVb pili, i.e., thin, long, and bundling filaments, but they also appeared quite curly (Fig. 5B). The Flag tag-mediated gold labeling appears to be specific, and no immunogold particles were specifically associated with any structures protruding from PA14TnC2 Δ*pilS2*Δ*pilA* p*pilS2* cells expressing the same construct lacking the Flag tag on PilS2 (Fig. 5A). In individual cells, the PAPI-1 pili appeared in a limited number of bundles, but unlike the type IVa pili of *P. aeruginosa*, their surface location was not exclusively polar. While in most instances the PAPI-1 pili were observed attached to the sides of the rod-shaped cells, they were occasionally present at the poles as well.

DISCUSSION

Horizontal gene transfer has been recognized as one of the major mechanisms driving the evolution of microorganisms and plays a key role in their ability to adapt to various environments through rapid, one-step acquisition of several new functions. Sequence analyses of several *P. aeruginosa* genomes revealed that they have a mosaic structure consisting of a core genome of ca. 5,000 genes and a variable accessory component that can contain anywhere from several hundred up to a thousand additional genes (23). The majority of the accessory genes are not randomly scattered through the chromosome but are instead found in clusters (genomic islands) located at conserved loci. These observations suggest that the majority of the accessory genome was horizontally acquired. This hypothesis is supported by the observations that these islands are frequently located next to tRNA genes and that in some instances, their excision and transfer can be demonstrated (10, 21, 27). In the genomes of certain strains, phage-related elements can be identified in such islands, clearly pointing toward their origin via HGT (39).

We have previously demonstrated that the *P. aeruginosa* pathogenicity island PAPI-1, which is associated with virulence

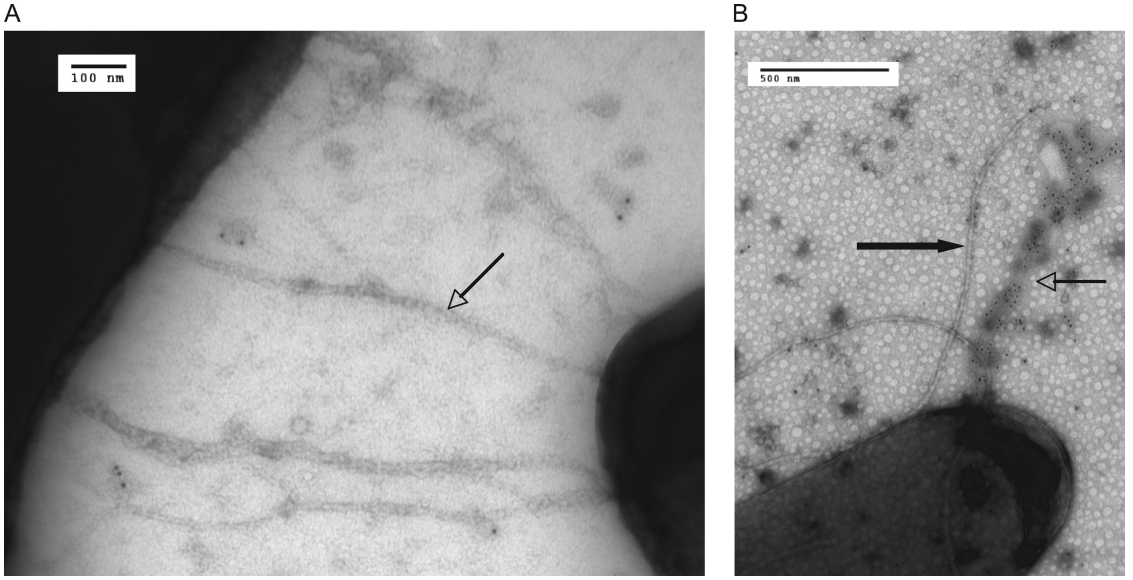

FIG. 5. Examination of PAPI-1-encoded pili by transmission electron microscopy (TEM). Long, thin, bundled pili of the control cells (PA14TnC2 Δ*pilS2*Δ*pilA* p*pilS2*) (A) and gold-labeled PAPI-1 pili (arrow with open head) of Flag-tagged cells (PA14TnC2 Δ*pilS2*Δ*pilA* p*pilS2*-flag) beside the flagella (filled arrow) (B) are seen. The immunogold labeling appears to be specific to the Flag tag, since there is little labeling of the native pili (no Flag-tagged PilS2) (A).

traits (11), can be mobilized from a donor chromosome and transferred into a recipient lacking this island (27). We demonstrated that transfer of the island occurred only when donor and recipient cells were cocultured and not by transfer from a donor bacterium-free culture medium, suggesting that the mechanism of PAPI-1 transfer is conjugation. Here we further characterized the conjugation machinery of PAPI-1, a thin pilus that is highly related to the thin pilus of conjugative plasmid R64 and required for its liquid mating (14). A number of plasmid-based conjugative transfer systems, exemplified by the F pilus encoded by the F sex factor of *E. coli*, are required for the initiation of cell-to-cell contact during conjugation and formation of mating pairs. Moreover, many of the core proteins involved in the biogenesis of F pili are related to the components of the Icm/Dot system of *Legionella* (collectively referred to as the type IV secretion system [T4SS]) and to the conjugation machinery involved in the transfer of T-DNA into plants by *Agrobacterium tumefaciens* (2, 20). Interestingly, the R64 conjugation system includes a number of orthologs of the T4SS (18); however, these are largely absent from the PAPI-1 island. In addition to the VirB11-like proteins PilQ2 and PilT2, there are only two PAPI-1-encoded proteins related by sequence to the components of the T4SS: *RL022* encodes a VirB4-like protein, which may function as a pilot protein, and RL047 encodes a VirD4-like coupling protein. These two proteins are both presumably involved in the conjugative transfer of PAPI-1 (32). Our data suggest that PAPI-1, during its evolutionary history, assembled an unique conjugative machinery using a "mix-and-match" approach, by capturing genes for components of the type IV pilus biogenesis proteins and the T4SS components from various distantly related bacterial species. Another difference between PAPI-1 and the F plasmid is that, based on electron microscopic observations, PAPI-1 pili appear to be expressed by only a subpopulation of cells. This is consistent with our previous observation that only a small portion of cells carry a circular PAPI-1 and therefore appear to be conjugation proficient (27). We observed that the expression of both the PAPI-1 *pil* operon and the *soj* gene is greatly enhanced at the stationary phase, but it remains unclear which environmental factors control the expression of PAPI-1 pili and the transfer of the PAPI-1 island. A putative Crp binding site (AAATAACA) was identified in the first putative promoter of the gene *pilL2* (see Fig. S1A and Table SA4 in the supplemental material), which may suggest that the expression of this PAPI-1 gene and therefore the conjugation may be subject to the regulation of cyclic AMP (cAMP) and the chromosomally encoded Crp transcriptional regulator (5, 37).

Examination of the DNA sequences of PAPI-1 *pil* regions revealed the presence of a short, near-perfect direct repeat (10/11 bp) flanking the *pil* cluster (see Fig. S2A in the supplemental material), suggesting that the *pil* gene cluster could be a result of HGT. The PAPI-1 *pil* gene cluster is well conserved in other *P. aeruginosa* strains (see Table SA3 in the supplemental material), suggesting that PAPI-1 and the related composite island PAGI-5 have very likely evolved from a plasmid related to pKLC102, which may have served as the ancestor for several additional *P. aeruginosa* genomic islands (19). Based on our comparison of related *pil* clusters in different organisms (Fig. 1), a complete set of genes required for conjugation is very likely expressed only by plasmid R64, making it a truly self-transmissible element, while others carry incomplete sets of *pil* genes. In all strains of *P. aeruginosa* examined here, no homolog of R64 PilU, a prepilin peptidase, could be identified within the *pil* gene cluster. Additionally, in the *P. aeruginosa* PAGI-5 there are frameshift mutations in the *pilQ* and *pilR* genes due to insertions. A survey of the distribution of pKLC102 sequences in a larger collection of *P. aeruginosa* strains by microarray hybridization identified several strains that contained PAPI-1 *pil*-related sequences, but individual genes were missing (16). Similarly, the *pil* locus of *P. syringae* strain B782a lacks the *pilU* gene, while in the *pil* gene cluster of *P. fluorescens* Pf-5, in addition to *pilU*, the *pilP*2 gene is missing. Moreover, the *Yersinia* YAPI cluster differs from R64 by its lack of *pilT*. Unless a functionally redundant protein encoded in the rest of the genome can provide the missing conjugative function, the deletion of an individual *pil* gene may contribute to immobilization of these HGT-acquired elements in the host strain.

The intriguing feature of the evolution of the type IV pilus apparatus in PAPI-1 is the apparent loss of essential functions necessary for the assembly of the organelle, which is compensated for by the product of a functionally redundant chromosomal gene. Since the PilS2 protein, the major subunit of the PAPI-1 pilus, is synthesized as a precursor with a 14-amino-acid leader peptide, its removal is essential for the biogenesis of the organelle. We demonstrated that the removal of this leader sequence from premature PilS2 was catalyzed by PilD, the prepilin peptidase encoded by a different (type IVa) *pil* gene cluster in the core genome. The consequence of utilization of a product of the chromosomal gene for the formation of the conjugative apparatus makes PAPI-1 not a truly self-transmissible element like the plasmid R64, since it restricts its transmission only from those donors that express a compatible prepilin peptidase. The observation that the transfer of PAPI-1 is dependent on PilD but not on FppA, a prepilin peptidase for *P. aeruginosa* prepilin Flp, suggests that unlike PilD, FppA has fairly narrow substrate specificity. In fact, PilD can process not only a variety of substrates found in *P. aeruginosa*, including precursors of PilA and XcpT-W, the components of the type II secretion apparatus (25), and the PAPI-1 PilS2, but also the major subunit of bundle-forming pilus (BfpA) found in enteropathogenic *Escherichia coli* (43). Therefore, it is conceivable that other organisms that express a compatible prepilin peptidase could serve as hosts and donors for transmission of the PAPI-1 island.

From the evolutionary prospective, we envision a scenario where an early form of the PAPI-1 element, perhaps in an organism other than *P. aeruginosa*, lacked machinery for interstrain transfer although it could have utilized a conjugation machinery encoded on another mobile element present in the same cell. At some point, PAPI-1 acquired a complete cluster of genes specifying type IVb pili. This eventually allowed it to be acquired by an ancestral *Pseudomonas* strain which already possessed determinants for type IVa pili. The ortholog of the prepilin peptidase gene in PAPI-1 was lost, resulting in dependence, in donor cells, on the chromosomal *pilD* gene product. This event restricted its mobility to *P. aeruginosa* or other *Pseudomonas* species that were capable of providing an enzyme for processing of the PilS2 precursor. The evolutionary history of PAPI-1 provides another example of the conse-

quences of gene loss during evolutionary adaptation of genomic elements to a limited number of hosts. However, we cannot exclude another scenario, i.e., that the original type IVb pilus clusters in PAPI-1 and R64 lacked the ancestral *pilD* gene and R64 acquired it during passage through a host possessing this gene. Regardless of the sequence of events leading to a loss or acquisition of an essential chromosomal function for HGT, this scenario may represent a previously unrecognized mechanism for limiting or expanding the range of bacteria that can participate in the exchange of genetic elements.

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