Interstrain transfer of the large pathogenicity island (PAPI-1) of *Pseudomonas aeruginosa*

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The large *Pseudomonas aeruginosa* pathogenicity island PAPI-1 of strain PA14 is a cluster of 108 genes that encode a number of virulence features. We demonstrate that, in a subpopulation of cells, PAPI-1 can exist in an extrachromosomal circular form after precise excision from its integration site within the 3′ terminus of the tRNA^{Lys} gene. Circular PAPI-1 can reintegrate into either of the two tRNA^{Lys} genes, including the one that was used for integration of small pathogenicity island PAPI-2 in strain PA14. The excision requires PAPI-1-encoded integrase, a member of the tyrosine recombinase family. PAPI-1 Soj contains the conserved domains of proteins that are related to chromosome and plasmid partition. *soj* plays a role in maintaining PAPI-1 and mutations in *soj* result in the loss of PAPI-1 from *P. aeruginosa*. We further demonstrate that, during coculture, the PAPI-1-containing strains are able to transfer it into *P. aeruginosa* recipient strains that do not harbor this island naturally. After transfer, PAPI-1 integrates into either of the two tRNA^{Lys} genes. PAPI-1 encompasses many features of mobile elements, including mobilization and maintenance modules. Together with the virulence determinants, PAPI-1 plays an important role in the evolution of *P. aeruginosa*, by expanding its natural habitat from soil and water to animal and human infections.

evolution | horizontal gene transfer | integration

It is now well recognized that horizontal gene transfer (HGT) plays a key role in bacterial evolution. The acquisition and retention of blocks of DNA, encoding up to hundreds of genes, represents a rapid mechanism for evolution. HGT can have a more significant and immediate impact on the organism's phenotype when compared with a slower process such as accumulation of mutations within individual genes and subsequent selection for the advantageous phenotypes. DNA segments that did not coevolve with the core genome, often referred to as genomic islands (GIs), are acquired by HGT (1). In bacterial genomes, GIs are characterized by several signature features including atypical G+C content, proximity to transfer RNA (tRNA) genes, and presence of genetic determinants responsible for their mobilization and stable maintenance in the recipient. It is generally accepted that HGT-mediated gene acquisition involves one of the three mechanisms for DNA uptake: conjugation, transformation, and transduction.

One way to classify GIs is based on the functions they encode, such as additional metabolic activities, antibiotic resistance, or properties involved in a particular lifestyle including symbiosis or pathogenesis (2). Islands encoding virulence determinants (pathogenicity islands, PAIs) have been described in a wide variety of pathogens and, in many instances, have been implicated as the genetic determinants responsible for endowing a nonpathogenic species with virulence traits (3). Several GIs, termed PAGI-1, -2, and -3, were identified in different strains of the opportunistic pathogen *Pseudomonas aeruginosa* (4, 5). The majority of the proteins encoded within these islands have unknown function, which makes it difficult to assess the selection forces that facilitated their acquisition in the recipient strains. Recently, in *P. aeruginosa* PA14, a strain characterized by its ability to infect a broad range of plants, insects, and animals, two GIs, PAPI-1 and -2, have been shown to encode several virulence determinants (6). PAPI-1 and -2 are located adjacent to two tRNA^{Lys} genes, PA4541.1 and PA0976.1, respectively, according to the annotation of strain PAO1 (7). These tRNA genes presumably provided an *attB* site for integration of these islands after their acquisition. Mutations in a number of genes in the larger island PAPI-1 (108 kb) resulted in the attenuation of PA14 virulence in several infection models (6). Moreover, PAPI-1 carries several regulatory genes, including *pvrR*, which controls the biofilm formation of antibiotic resistant variants of *P. aeruginosa* that are associated with chronic infections in individuals with cystic fibrosis (CF) (8). The smaller island, PAPI-2 (11 kb), contains a gene encoding the potent cytotoxin ExoU and is likely a remnant of a larger island (9).

Integration of horizontally acquired islands into the recipient's chromosome is often followed by progressive decays in genes or DNA sites associated with their mobilization, such as mutations in mobility genes or deletions of *att* sites. For these reasons, experimental demonstration of a GI transfer has been difficult. Moreover, specific environmental conditions may be required for HGT to occur. Here we report the transfer of the *P. aeruginosa* pathogenicity island PAPI-1 into recipient *P. aeruginosa* strains after excision of the island and formation of a circular intermediate in the donor bacterium. The excision and/or transfer of PAPI-1 require a functional integrase gene (*int*) and an orthologue of the chromosome partitioning gene *soj*, which are located on the island. Mutations in *soj* lead to the deletion of PAPI-1 from strain PA14. This work implicates PAPI-1 as a key mobile genetic element, capable of dissemination among *P. aeruginosa* strains and contributing to their enhanced pathogenicity.

Results and Discussion

Identification of Extrachromosomal PAPI-1. The pathogenicity island PAPI-1 that harbors both plant and animal virulence genes was first described in *P. aeruginosa* strain PA14 at a location between PA4541 and PA4542 (6). Because strain PAO1 and a number of other strains lack this island, it appears that strain PA14 acquired the PAPI-1 horizontally in which it integrated into the chromosome at the *attB* site in the tRNA^{Lys} gene PA4541.1, generating two direct repeats (*attL* and *attR*) at both ends of the island (Fig. 1*A*). A set of PCR primers were designed to determine whether PAPI-1-like genomic islands are present in other *P. aeruginosa* strains at the same chromosomal location as in strain PA14, and whether they can excise from the chromosome (Fig. 1*A*). Primer pairs 4542F + intF and sojR + 4541F were used to amplify left- and right-junction sequences between the chromosome and the island, respectively;

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Abbreviations: CF, cystic fibrosis; GIs, genomic islands; HGT, horizontal gene transfer; PAIs, pathogenicity islands; PAPI-1, large *Pseudomonas aeruginosa* pathogenicity island; PAPI-2, small *Pseudomonas aeruginosa* pathogenicity island.

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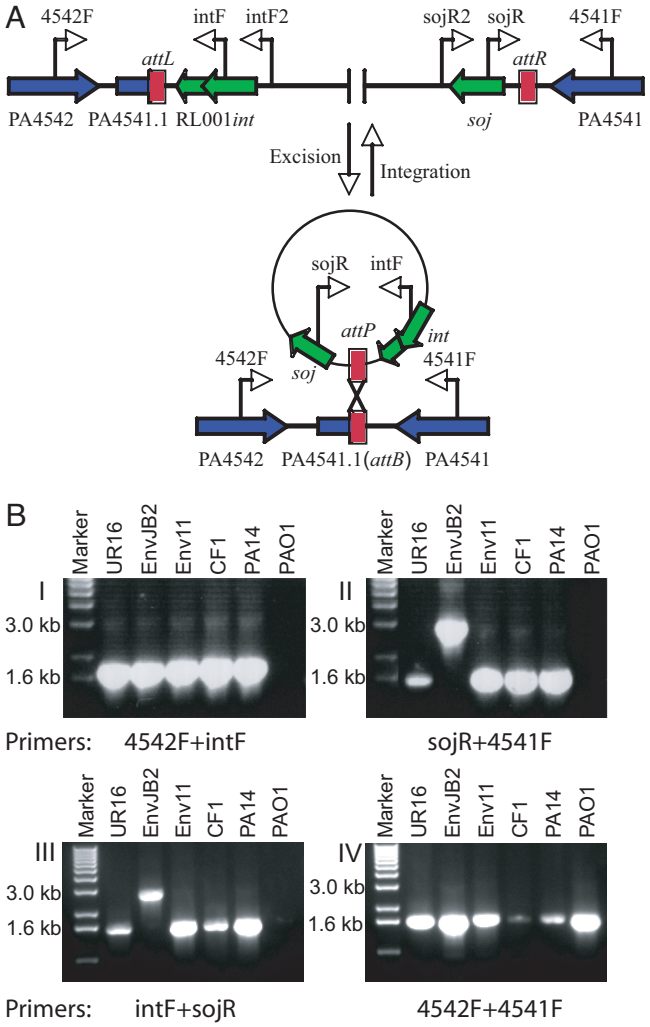

Fig. 1. Detection of integrated and circular PAPI-1 in *P. aeruginosa*. (*A*) Model for PAPI-1 excision from the chromosome. Open arrowheads indicate the primers used for detection of integrated and circular PAPI-1 in various *P. aeruginosa* strains. The genes are not drawn to scale. (*B*). I, II, III, and IV are PCR amplifications with primer pairs 4542F + intF, sojR + 4541F, intF+sojR, and 4542F + 4541F, respectively.

primer pair intF + sojR was used to detect the presence of a circular PAPI-1, and primer pair 4542F + 4541F was used to confirm the PAPI-1 excision (Fig. 1*A*). PCR products were detected in a number of clinical and environmental isolates with primer pairs 4542F + intF and sojR + 4541F, whereas no products were detected for strain PAO1 (Fig. 1*B*). This observation suggests that a PAPI-1-like island is present in a variety of *P. aeruginosa* strains at a conserved chromosomal location. The larger PCR product observed in strain EnvJB2 with primer pair sojF + 4541F indicates an additional insertion between the *soj* gene and the *attR* site (Fig. 1*B*). PCR products were also observed in all strains, except for PAO1, with the primer pair intF + sojR that is specific to the circular PAPI-1 (Fig. 1*B*). Moreover, PCR products that are identical in size to that of PAO1 were detected in all strains with primer pair 4542F + 4541F (Fig. 1*B*). These data demonstrate that PAPI-1 and related islands can excise from the chromosome and form a circular intermediate. Detection of both integrated and circular PAPI-1 suggests the populations of bacteria carrying PAPI-1-like islands are heterogeneous. Some cells may carry an integrated island, whereas other cells may carry a circular island.

Integration of PAPI-1 at the *attB* Site in PA0976.1. There are two $tRNA^{Lys}$ genes in the *P. aeruginosa* genome, PA0976.1 and PA4541.1 (7). In strain PA14, downstream of PA0976.1 is the small *P. aeruginosa* pathogenicity island PAPI-2 that encodes the cytotoxic protein ExoU (6). In other *P. aeruginosa* strains, various sizes of *exoU* containing islands (ExoU islands) were identified at the same chromosomal location (9). Because in strain PA14, the *attB* sequence in PA0976.1 is identical to the *attL* of PAPI-1, it is conceivable that after excision, the circular PAPI-1 can integrate into the chromosome at the *attB* site in PA0976.1. Therefore, a set of primers was designed to test this hypothesis. The PAPI-1-specific primer PAPI-1R and the primer 976F were combined to amplify the junction between PA0976 and PAPI-1; the PAPI-2-specific primer PAPI-2R and the primer sojR167 were combined to amplify the junction between the two islands, PAPI-1 and -2 (Fig. 2*A*). PCR products were observed with both primer pairs for strain PA14 (Fig. 2*B*). Sequencing of PCR products confirmed the presence of PAPI-1 between PA0976.1 and PAPI-2. These data suggest that, in strain PA14, PAPI-1 excision is a reversible process, and the circular PAPI-1 can integrate into chromosome at either of the two $tRNA^{Lys}$ genes.

The two $tRNA^{Lys}$ genes used by PAPI-1 and -2 for their integration were identified as "hot spots" for insertion and excision of large genetic elements in several *P. aeruginosa* strains. For example, in clone K strains, the large plasmid pKLK106 was capable of sequentially recombining with either of the two $tRNA^{Lys}$ genes, resulting in genomic rearrangements in sequential K isolates obtained from the airway of a CF patient (10). In clone C strains, the plasmid pKLC102 reversibly incorporated only into PA4541.1 but not PA0976.1, which was occupied by a 23-kb genomic island named PAGI-4 (11). The integration of PAPI-1 at the site that was used for integration of PAPI-2 suggests that this site is conserved and could be used for acquiring multiple genetic elements. Formation of tandem arrays of *Vibrio cholerae* integrating conjugative elements SXT and R391 after their transfer was previously reported, and this arrangement appeared to be stably maintained for many generations (12). Therefore, in a recipient cell, the *attB* site can serve as a platform to build composite GIs, which are assembled by sequentially acquired independent units. The minimal requirement, in the absence of active exclusion mechanisms, is compatibility of *attP* and *attB* sites as substrates for integrase-mediated recombination. The formation of tandem arrays by sequential capture of gene cassettes peripherally resembles the model proposed for the for-

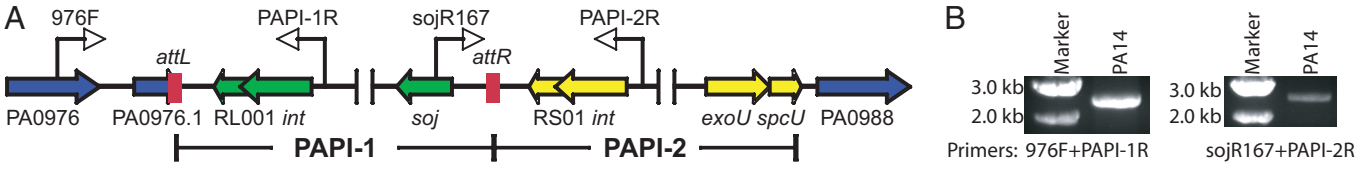

Fig. 2. Detection of the composite island adjacent to PA0976.1 in strain PA14. (*A*) Schematic diagram shows the gene organization between PA0976 and PA0988 after integration of PAPI-1 at PA0976.1. Open arrows indicate the primers used to amplify the junctions between chromosome and PAPI-1 after PAPI-1 integrates into the PA0976.1. (*B*) PCR amplifications with primer pairs 976F + PAPI-1R and sojR167 + PAPI-2R.

Table 1. Functional analysis of *int* and *soj* on PAPI-1 excision by PCR amplifications

Strains or mutants*	Primer pairs					
	4542F + intF2† (junction between PA4542 and PAPI-1)	sojR + 4541F (junction between PA4541 and PAPI-1)	intF2†+sojR (circular PAPI-1)	4542F + 4541F (empty *attB* site)	976F + PAPI-1R (junction between PA0976 and PAPI-1)	sojR167 + PAPI-2R (junction between PAPI-1 and PAPI-2)
PA14	+	+	+	+	+	+
PA14Δ*int*	+	+	−	−	−	−
PA14Δ*int* p*int*	+	+	+	+	+	+
PA14Δ*int* vector	+	+	−	−	−	−
	4542F + intF	sojR2†+4541F	intF + sojR2†	4542F + 4541F	976F + PAPI-1R	sojR167 + PAPI-2R
PA14	+	+	+	+	+	+
PA14Δ*soj*	−	−	−	+	−	−
PA14p*soj*	+	+	+	+	+	+
PA14p*soj*Δ*soj*	+	+	+	+	+	−‡
PA14 vector	+	+	+	+	+	+

"+" indicates a positive PCR amplification, whereas "−" indicates no PCR products. The gel images of PCR amplification are presented in SI Fig. 4. The chromosomal locations of primers are described in Figs. 1*A* and 2*A*.

*A detailed description of each mutant is listed in SI Table 3. p*int* and p*soj* are cloned *int* and *soj* on plasmid pPSV35, respectively. Vector is plasmid pPSV35.

†Because primer sites intF and sojR are not present in mutant strains PA14Δ*int* and PA14Δ *soj*, respectively, primer intF2, located upstream of *int*, and primer sojR2, located downstream of *soj*, are used.

‡Primer sojR167 is not present in mutant strain PA14p*soj*Δ*soj*.

mation of superintegrons (13), which could provide an important mechanism for rapid genome evolution.

The *int* Gene Is Required for PAPI-1 Excision. One common feature of PAIs is the presence of the mobility genes that were presumably involved in mobilizing the PAIs into the chromosome (14). The PAPI-1 *int*, separated from PA4541.1 by a hypothetical gene RL001, encodes a 427-aa integrase (Fig. 1*A*). This integrase belongs to the tyrosine recombinase family that catalyzes a site-specific recombination between a chromosomal site (*attB*) and a similar or identical sequence (*attP*) found on mobile genetic elements including bacteriophages, insertion sequences, and transposons (15, 16). To explore the biological functions of PAPI-1 *int*, we generated an *int* deletion mutant of strain PA14 (PA14Δ*int*) and examined the excision of PAPI-1 in this mutant strain.

PCR products were observed by using primer pairs 4542F + intF2 and sojR + 4541F in mutant PA14Δ*int*; however, none were detected with primer pairs intF2 + sojR or 4542F + 4541F (Table 1). Excision of PAPI-1 was restored in mutant PA14Δ*int* by expressing the *int* gene from a plasmid (Table 1, PA14Δ*int* p*int*). These observations suggested that deletion of *int* in strain PA14 locked PAPI-1 at its chromosomal location. Therefore, Int is required for PAPI-1 excision, presumably by catalyzing the site-specific recombination between the *attR* and *attL* sites. Furthermore, no integrated PAPI-1 was detected adjacent to the other $tRNA^{Lys}$ gene (PA0976.1) in mutant PA14Δ*int*, which suggests that circular PAPI-1 is required for mobility of PAPI-1 between the *attB* sites in the two $tRNA^{Lys}$ genes (Table 1). Strain PA14 carries another *int* gene, located on PAPI-2 (6). Although the two proteins share 96% identity, the PAPI-2 *int* did not complement the deletion of PAPI-1 *int* in the mutant PA14Δ*int*. It is conceivable that the PAPI-2 encoded integrase either is not expressed or is defective.

The *soj* Gene Is Required for Maintenance of PAPI-1. The *soj* gene on PAPI-1 (Fig. 1*A*) encodes a protein implicated in chromosomal partition and is also related to the ParA family of proteins, which in conjugation with ParB are responsible for correct segregation of low-copy plasmids during cell division (17–19). A homologue of PAPI-1 *soj* is also present in plasmid pKLC102, which has been suggested to play a role in its maintenance (11). To explore the biological functions of PAPI-1 *soj*, the excision of PAPI-1 was examined in a *soj* deletion mutant of strain PA14 (PA14Δ*soj*). Deletion of *soj* resulted in complete loss of PAPI-1 in strain PA14, leaving the junction between PA4542 and PA4541 empty (Table 1). When a plasmid carrying *soj* was introduced into strain PA14 before deleting the PAPI-1 *soj*, the integrated PAPI-1 at both sites (PA4541.1 and PA0976.1) as well as the circular PAPI-1 were all detected (Table 1, PA14p*soj*Δ*soj*). Therefore, Soj is responsible for the maintenance of PAPI-1, presumably by stabilizing the circular PAPI-1. In the absence of Soj, PAPI-1 continually excises from the chromosome and eventually is lost from the entire population.

PAPI-1 Soj may function like other ParA proteins, which, along with ParB, coordinate segregation of low-copy plasmids (17). Reexamination of PAPI-1 genes revealed that gene RL102 encodes a protein containing a ParB-like nuclease domain (pfam02195). However, we were unable to identify a putative replication origin, although several genes related to DNA replication are present on PAPI-1, such as *dnaB* (RL109), *ssb* (RL095), and *topA* (RL092). Because there is no evidence that circular PAPI-1 replicates, PAPI-1 Soj may perform a novel function, such as protecting circular PAPI-1 directly from degradation, or indirectly, by promoting the integration of circular PAPI-1 into the chromosome, thus preventing its loss from the cell. Interestingly, homologues of both *int* and *soj* are found in a number of GIs from β- or γ-*Proteobacteria*. These two genes are often located at the opposite ends of the island (20). The conservation of *soj* in various GIs suggests a similar function of this gene in other islands as it is in PAPI-1.

That the PCR product amplified with the primer pair 4542F + 4541F from strain PA14 was identical in size to that from strain PAO1 (Fig. 1*B*) suggests the excision of PAPI-1 likely occurs by recombination between the *attL* and *attR* sites. Excision of PAPI-1 in mutant PA14Δ*soj* should restore the intergenic chromosomal region to a sequence as it was before the acquisition of PAPI-1. This region was sequenced by using the PCR product amplified from mutant PA14Δ*soj*. The *attB* site in PA14Δ*soj* was identical to the *attL* of PAPI-1 and the *attB* in PA4541.1 of strain PAO1 [supporting information (SI) Fig. 5]. These data confirmed that excision of PAPI-1 occurred by a precise recombination between the *attL* and the *attR* sites in PAPI-1. The restored intergenic region between PA4541 and PA4542 in mutant PA14Δ*soj* is similar to that in strain PAO1, where the conserved *attB* site in PA4541.1 can be used to insert genetic elements including PAPI-1. Although acquisition of PAPI-1 may provide a selective advantage in enhancing the virulence of certain strains, it is equally conceivable that in certain environments, survival of strains lacking this island is favored.

Elimination of PAPI-1 could occur by a block in *soj* gene expression, or by a mutation in the *soj* gene.

Transcriptional Analysis of *soj*. Because *soj* is required for maintenance of PAPI-1, we hypothesize that the expression of *soj* may be different after PAPI-1 is excised. Therefore, DNA fragments upstream of *soj* that might contain putative promoters were cloned, and their activity in directing transcription of a promoter-less *lacZ* gene was tested (Fig. 3*A*). Fragments sojP1 and sojP2 were amplified from the integrated PAPI-1, and fragments sojP3 and sojP4 were amplified from the circular PAPI-1 (Fig. 3*A*). Along with *lacZ*, each fragment was inserted into the CTX phage *att* site in the PA14 chromosome by using an integrative vector miniCTX1-lacZ (GenBank accession no. AF140579). The promoter activity was determined by measuring the β-galactosidase levels during both the exponential and early stationary growth phases. The β-galactosidase activity was detected only for sojP3 and sojP4 but not for either sojP1 or sojP2 (Fig. 3*B*). These data suggest that *soj* is expressed only when PAPI-1 is excised and assume a circular form, which orients the promoter, located at the opposite end of the PAPI-1, in the same direction as *soj*. Higher β-galactosidase activity was detected in the early stationary phase, which indicated that these promoters might be regulated by environmental conditions and/or cells density. Moreover, because the activity of the promoter in sojP4 fragment was higher than that in sojP3 fragment at both the exponential and stationary growth phases, it is conceivable that RL001 encodes a transcriptional activator, or it contains sequences serving as a binding site for an as-yet-unidentified transcriptional activator for *soj*.

To assess the frequency of PAPAI-1 excision in strain PA14, we used the activity of the *soj* promoter as a reporter for the cells carrying the circular PAPI-1. The GFP gene (*gfp*) was inserted into a site immediately after the translational terminator of *soj* in mutant PA14Δ*int* in which PAPI-1 is locked at its chromosomal site (Table 1), generating mutant PA14Δ*int soj*::*gfp*. The empty vector (used as control) and cloned *int* (p*int*) were then introduced in mutant PA14Δ*int soj*::*gfp*, respectively. The fraction of PA14 expressing GFP was quantified by using flow cytometry in control cells (Vector) and in cells expressing the gene *int* (p*int*) (Fig. 3*C*). In the absence of *int*, no GFP-positive cells were detected (Fig. 3*C* Vector). However, when *int* was supplied, ≈0.16% cells were producing GFP (Fig. 3*C*, p*int*), which indicated that only a small fraction of the PA14 population underwent PAPI-1 excision under the condition examined.

PAPI-1 Is Transmissible Between *P. aeruginosa* Strains. The detection of a circular PAPI-1 in PA14 suggested it might represent the intermediate associated with cell-to-cell transmission. We therefore examined the transmissibility of PAPI-1 from the strain PA14 to PAO1, a strain that does not harbor PAPI-1 naturally. The donor strain PA14 140495 (Gm^R) contains a MAR2 × T7 transposon insertion in the hypothetical gene RL048 in PAPI-1 (21). The recipient PAO1 was engineered to be carbenicillin-resistant by inserting a Cb^R cassette into its CTX phage *att* site by using the vector miniCTX-lacZ, thus providing a second screening marker, *lacZ*, for the recipient strain (PAO1Cb^R*lacZ*). Several mating conditions were examined, including surface mating on agar plates, mating in static liquid cultures, and heat treatment of the recipient, presumably resulting in inactivation of restriction systems (22). Transfer of PAPI-1 was detected at frequencies ranging from 3.1×10^{-7} to 5.4×10^{-4} (Table 2). PAPI-1 transfer in static liquid cultures appeared to be more efficient than on a solid surface. Transfer efficiency from strain PA14 to strain PAO1 significantly improved when the recipient cells were subjected to heat treatment (42° for 2 h). Using primers described previously (Fig. 1*A*), both circular and integrated PAPI-1 were detected in recipient cells. In addition, integrated PAPI-1 was detected at the *attB* site in either of the two $tRNA^{Lys}$ genes (data not shown). Once strain PAO1 acquired PAPI-1, it could serve as a donor for other PAO1 strains, but the transfer efficiency was not improved by heat treatment of the recipient cells before mating (Table 2). Transfer of PAPI-1 from strain PA14 140495 was also observed by using other *P. aeruginosa* strains as recipients, including CF6–1, a CF isolate from Hannover, Germany, and M5C1, a non-CF clinical isolate from Colombia (SI Table 3).

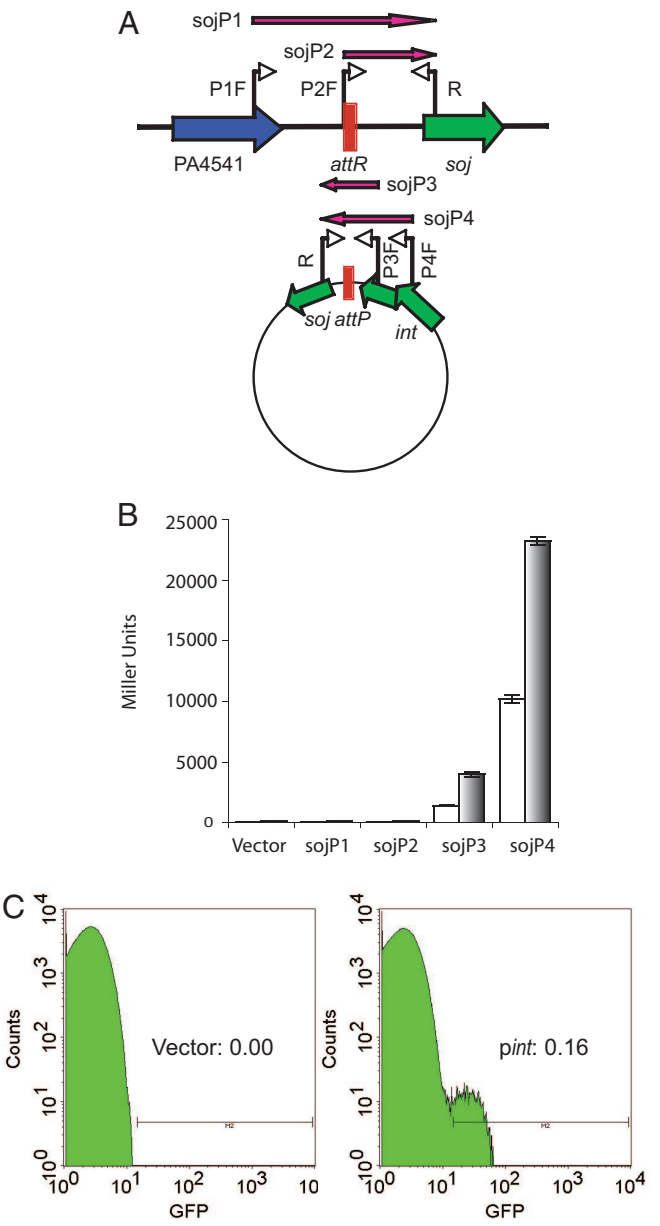

Fig. 3. Transcriptional analysis of *soj* and quantification of PA14 cells carrying circular PAPI-1. (*A*) Chromosomal locations of various *soj* promoter-containing fragments. The red arrows indicate the orientation of the fragments relative to the *lacZ* reporter gene. Open arrows indicate the primers used to amplify various *soj* promoter-containing fragments. Primer pairs P1F + R, P2F + R, P3F + R, and P4F + R were used to amplify DNA fragments sojP1, sojP2, sojP3, and sojP4, respectively. (*B*) Determination of β-galactosidase at midexponential (open bars) and early stationary phases (filled bars) in strains PA14/*lacZ* (vector), PA14sojP1/*lacZ* (sojP1), PA14sojP2/*lacZ* (sojP2), PA14sojP3/*lacZ* (sojP3), and PA14sojP4/*lacZ* (sojP4). (*C*) Quantification of the proportion of cells that carry circular PAPI-1 by flow cytometry. Vector is the strain PA14Δ*int soj*::*gfp* transformed with empty vector pPSV35. p*int* is the strain PA14Δ*int soj*::*gfp* transformed with cloned *int* on pPSV35. The percentage of GFP-positive cells (0.16%) is the average of three independent measurements.

Table 2. Transfer of PAPI-1 between *P. aeruginosa* strains

Donor strain (marker)*	Recipient strain (marker)*	Transfer conditions	Transfer frequency
PA14 140495 (Gm^R)	PAO1Cb^R/*lacZ* (Cb^R)	Plate mating	ND†
		Plate mating with heat shock	6.6×10^{-7}
		Liquid mating	6.6×10^{-5}
		Liquid mating with heat shock	5.4×10^{-4}
PAO1 PAPI-1 (Gm^R)	PAO1Tn1150 (Tc^R)	Plate mating	3.1×10^{-7}
		Plate mating with heat shock	3.5×10^{-7}
		Liquid mating	4.5×10^{-6}

*Antibiotic resistance: Gm^R, gentamicin resistance; Cb^R, carbenicillin resistance; Tc^R, tetracycline resistance.
†ND, not detected.

Although the molecular mechanism of PAPI-1 transfer is not yet fully understood, it is unlikely that PAPI-1 is transferred by transformation. Including DNase I in the static mating solution at a concentration that completely digested the spiked plasmid DNA (100 ng/ul) did not alter PAPI-1 transfer from strain PA14 to strain PAO1. Furthermore, no PAPI-1 transfer was observed when the PA14 cell-free culture medium was incubated with strain PAO1, which excluded phage-mediated transduction. Therefore, conjugation is the most likely mechanism for interstrain transfer of PAPI-1. PAPI-1 carries several genes that may play a role in its conjugative transfer. For example, genes (RL077–RL086), encoding proteins involved in the production of type IV B pili, may be responsible for the formation of mating pairs, because these pili of plasmid R64 were shown to be required for its conjugative transfer (23). PAPI-1 gene RL003 encodes an orthologue of conjugative relaxase, which may function as a pilot protein during conjugation (24). The gene product of PAPI-1 RL022 contains the conserved domain of FtsK/SpoIIIE protein family (pfam01580) and the VirB4 domain (COG3451), which may serve as a coupling protein for transport of DNA through the conjugation channel (24).

We therefore hypothesize that PAPI-1 transfer resembles the movement of integrative and conjugative elements (ICEs), a group of self-transmissible mobile genetic elements that distribute widely in bacteria and contribute greatly to lateral gene flow in prokaryotes (25). In the genus of *Pseudomonas*, the *clc* element of *Pseudomonas* sp. strain B13 is the other known self-transferable ICE. Like PAPI-1, the *clc* element is normally integrated into the bacterial chromosome, and it can excise at low frequency and self-transfer to a new strain in which it reintegrates (26). The *clc* element also carries an integrase gene, and the gene encoding a chromosome partitioning-related protein, located at the opposite ends of the island (27). The P4 integrase of the *clc* element is known to be responsible for mobilization of the island (28). Sequence comparison revealed a common evolutionary origin between PAPI-1 and the *clc* element but with a substantial evolutionary divergence (20). PAPI-1, along with pKLC102, represents the cluster of GIs that encode XerCD type integrases and use $tRNA^{Lys}$ for integration. The *clc* element is more closely related to PAGI-2 and -3, the two $tRNA^{Gly}$-associated GIs in *P. aeruginosa* (27). To date, only PAPI-1 and the *clc* element are known to be transferable in *Pseudomonas* bacteria, thus providing two good models to elucidate the molecular mechanism of horizontal gene transfer in a natural environment and in infected hosts.

Conclusions

The study described here sheds light on an evolutionary mechanism that shapes the genome of the opportunistic pathogen *P. aeruginosa*. We have demonstrated that PAPI-1, a large pathogenicity island present in a significant fraction of *P. aeruginosa* isolates, retains its mobility and can be transferred to a number of recipients. We have also shown that PAPI-1 can excise and form an extrachromosomal circular element that can integrate into the chromosome at either of the two $tRNA^{Lys}$ genes (PA0976.1 and PA4541.1). Our finding that PAPI-1 can integrate into the chromosome at the *attB* site in PA0976.1 resulting in a composite island containing both PAPI-1 and -2 in strain PA14 provides the mechanistic basis for the assembly of mosaic genomic islands. Such islands would retain their mobility by maintaining intact *att* sites at the borders of the composite element and a transfer origin that would be recognized by the appropriate transfer apparatus. The composite island could be self-transmissible, or it could use various accessory elements encoded in other mobile genetic elements already present in the donor cells. We have also demonstrated that two genes, *int* and *soj*, located at the opposite ends of PAPI-1, play an important role in mobilizing PAPI-1. Functional integrase is required for the PAPI-1 excision, whereas *soj*, a homologue of plasmid and chromosomal partitioning genes, is responsible for the maintenance of PAPI-1. Moreover, *soj* is expressed only when PAPI-1 assumes a circular intermediate, implying a role for *soj* in stabilizing the circular PAPI-1 when it is in a form that is very likely required for transfer.

Depending on the selective advantages introduced in the recipient by the acquired island, the element can undergo decay, where the portions of the island carrying unnecessary or deleterious genes accumulate mutations or undergo deletions. PAPI-1 is no exception. Several *P. aeruginosa* isolates were shown to carry only a portion of this island (6). Mutations in mobility genes may lead to permanently fixing the island in the recipient's chromosome. For example, mutations in PAPI-1 *int* will block PAPI-1 excision and formation of a circular intermediate, thus abolishing its ability to transfer. Furthermore, mutations in or deletions of any *att* sites, located on the borders of the integrated island, would also inhibit any further mobility of this island. We have previously shown that some ExoU islands undergo substantial decay, generating derivates of various size ranging from 80 to 8.9 kb (9). Finally, our work, which demonstrated the transfer of a large pathogenicity island, provides a basis to elucidate the molecular mechanism of HGT and to determine the consequences of HGT during the infection, where suitable animal models can be used to study the evolution of pathogens. Because HGT, including the acquisition of new virulence traits, requires the coexistence of donor and recipient cells, it can take place only in genetically heterogeneous microbial communities such as biofilms or in certain diseases where mixed bacterial infections occur. Understanding how to prevent the acquisition of virulence determinants may also provide new insights into therapeutic strategies.

Materials and Methods

Strains, Plasmids, and Culture Conditions. *P. aeruginosa* isolates used in this study include a laboratory strain (PAO1), environmental isolates, clinical non-CF isolates, and CF isolates (SI Table 3). All plasmids used in this study and their sources are listed in SI Table 3. All strains were grown in LB medium supplemented with the appropriate antibiotic. All primers used in this study are described in SI Table 4.

***P. aeruginosa* Mutants.** Both deletion and insertion mutants were generated by homologous recombination using gene replacement vectors pEX18Ap (29) or pEXG2 (30). For deletions, ≈800-bp DNA fragments, from both up- and downstream of the target gene, were amplified and directly cloned into pEX18Ap or pEXG2. To insert *gfp* into a site immediately after the translational terminator of *soj* in mutant PA14Δ*int*, ≈800 bp upstream of the insertion site and the gene *gfp* from pTGL3 (31) were first cloned into plasmid pUC19 and then subsequently cloned into pEXG2 along with the 800-bp downstream fragment of the *gfp* insertion site. The recombinant plasmids were conjugated from *Escherichia coli* SM10 into *P. aeruginosa*. The Cb^R (pEX18Ap) or Gm^R (pEXG2) plasmid-integrants were selected on LB plates containing the appropriate antibiotic. Merodiploids were resolved by plating on LB plates containing 6% sucrose (29). Deletion mutants were screened by PCR and confirmed by DNA sequencing. Plasmid pPSV35 (30) was used for complementation analyses in *P. aeruginosa* deletion mutants.

Analysis of *soj* Promoter Activity. Four DNA fragments containing the putative *soj* promoter (sojP1: 699 bp; sojP2: 421 bp; sojP3: 452 bp and sojP4: 1,088 bp) were PCR amplified from strain PA14 using primer pairs described in Fig. 3*A* and cloned into vector miniCTX-lacZ [AF140579 (32)]. After they were moved into strain PA14, the backbone of miniCTX-lacZ was eliminated by using plasmid pFLP2 as described (29). β-Galactosidase activity was measured at both the exponential and stationary growth phases as described before (33).

Flow Cytometry Analysis. Both strains PA14Δ*int soj*::*gfp* vector and PA14Δ*int soj*::*gfp* p*int* were grown overnight at 37°C. Cells were washed with PBS buffer, suspended to a concentration of 0.5 OD_{600}, and analyzed on a FACScalibur microflow cytometer (BD Biosciences, Franklin Lakes, NJ). Both forward- and side-scatter parameters were adjusted to eliminate the cell debris. GFP was detected in the FL1 channel (530/30 nm). Data were analyzed by using CellQuest Pro (BD Biosciences).

Transfer of PAPI-1. Both PAPI-1 donor and recipient strains were grown at 37°C overnight. Antibiotics used were 75 μg/ml gentamicin for PAPI-1 carrying strains; 75 μg/ml carbenicillin for PAO1Cb^R*lacZ*, CF6–1Cb^R*lacZ*, and M5C1Cb^R*lacZ*; and 75 μg/ml tetracycline for strain PAO1Tn1150. For plate mating, 50 μl of the donor strain was dropped on the LB agar plate and incubated at 37°C for 2 h followed by dropping 50 μl of recipient strains on top of the donor strain. When subjected to heat treatment, recipients were incubated with shaking at 42°C for 2 h before mating. After incubating the mating mixture at 37°C for 24 h, it was scraped off and suspended in 1 ml of PBS. For mating between strains PA14 and PAO1, transconjugants were selected on LB agar plates containing 75 μg/ml gentamicin, 150 μg/ml carbenicillin, and 40 μg/ml X-Gal. For mating between PAO1 strains, the same selection media were used, except carbenicillin was replaced with 75 μg/ml tetracycline. To confirm the transfer of PAPI-1, ≈10–20 transconjugants from each mating were randomly selected for PCR analysis by using primer pairs described in Fig. 1*A*. For mating in static liquid culture, 50 μl of the donor strain (≈4.0 at OD_{600}) was diluted into 1 ml of LB and incubated at 37°C for 2 h, followed by addition of 50 μl of recipient strain. After incubating at 37°C for 48 h, 1 ml of LB was added in the mating tube, and the culture was vigorously shaken before plating. The number of recipients in the final mating mixtures was determined by plate counting. The transfer efficiency was calculated by using the total number of transconjugants divided by the total recipients in the final mating mixture.

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