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Stability of a *Pseudomonas putida* KT2440 Bacteriophage-Carried Genomic Island and Its Impact on Rhizosphere Fitness

Jose M. Quesada, María Isabel Soriano, and Manuel Espinosa-Urgel

Department of Environmental Protection, Estación Experimental del Zaidín, CSIC, Granada, Spain

The stability of seven genomic islands of *Pseudomonas putida* KT2440 with predicted potential for mobilization was studied in bacterial populations associated with the rhizosphere of corn plants by multiplex PCR. DNA rearrangements were detected for only one of them (GI28), which was lost at high frequency. This genomic island of 39.4 kb, with 53 open reading frames, shows the characteristic organization of genes belonging to tailed phages. We present evidence indicating that it corresponds to the lysogenic state of a functional bacteriophage that we have designated Pspu28. Integrated and rarely excised forms of Pspu28 coexist in KT2440 populations. Pspu28 is self-transmissible, and an excisionase is essential for its removal from the bacterial chromosome. The excised Pspu28 forms a circular element that can integrate into the chromosome at a specific location, *att* sites containing a 17-bp direct repeat sequence. Excision/insertion of Pspu28 alters the promoter sequence and changes the expression level of PP_1531, which encodes a predicted arsenate reductase. Finally, we show that the presence of Pspu28 in the lysogenic state has a negative effect on bacterial fitness in the rhizosphere under conditions of intraspecific competition, thus explaining why clones having lost this mobile element are recovered from that environment.

Bacterial chromosomes are dynamic and undergo rearrangements, which are an important source of variability that may cause new favorable mutations and eventually increase bacterial fitness (21). Several pieces of evidence point to a role for chromosomal DNA rearrangements in the establishment and fitness of root-associated *Pseudomonas* populations (17). A transposase was identified in a screen for genes involved in colonization of corn seeds by *P. putida* KT2440 (19). This insertion element, ISPpu*10*, has a specific target sequence and was shown to mediate DNA rearrangements (51). A site-specific recombinase has also been found to be relevant for competitive colonization of tomato root tips by *P. fluorescens* (13), and the generation of phenotypic variants mediated by site-specific recombinases has been demonstrated in rhizospheric bacteria (59). These proteins belong to a family whose members participate in various processes involving DNA recombination, such as the biosynthesis of the siderophore pyoverdine in a plant growth-promoting *P. aeruginosa* strain (31).

Genomic islands (GIs) are discrete segments of DNA, generally between 10 and 200 kb in length, that have been acquired by horizontal gene transfer and are typically found only in some isolates from a given species or strain (27, 36). Common features of GIs include a sequence composition different from that of the core backbone, their insertion is usually adjacent to RNA genes, often flanked by direct repeats, and they frequently include mobility functions (transposases, integrases, or recombinases) (65). Langille et al. (36) described two main bioinformatic approaches for identifying GIs: sequence composition-based and comparative genomics-based methods. Analysis of the complete genome sequence of *Pseudomonas putida* KT2440 with the first approach showed that 105 DNA regions larger than 4 kb with atypical sequence features are interspersed in the genome of this microorganism (67). Recently, Wu et al. (70) identified 61 putative genomic islands in the KT2440 genome using methods based on sequence composition and comparative genomics.

GIs have an important role in bacterial evolution, promoting microbial adaptations that have consequences of medical and environmental interest (15, 26). Biological advantages provided by horizontally transferred DNA favor its retention in the host genome. For example, several newly acquired prophages and GIs provide an advantage to a virulent *Pseudomonas aeruginosa* strain (69), and a GI that increases the fitness of a *P. putida* strain degrading phenolic compounds has been identified (54). However, beyond the symbiosis islands of rhizobia (15), the potential mobility and role of GIs in root-associated bacterial populations are unknown. On the other hand, bias toward deletion and gene loss has been postulated as a major driving force in the evolution of bacterial genomes, particularly under circumstances where no selection pressure exists to maintain those functions (43). This is particularly evident in obligate symbionts (42), where extreme genome reduction is observed.

In this study, we have evaluated the potential for mobilization of the 25 DNA regions with atypical sequence features identified by all three sequence composition-based criteria at once in *Pseudomonas putida* KT2440 (67). The presence of genes related to DNA rearrangements like integrases, recombinases, or transposases was analyzed. In those cases where mobilization activities were predicted, a study was conducted to define the functions encoded by these genetic islands and their potential relevance for rhizosphere persistence. A bioinformatic analysis with comparative genomics-based methods using the genome sequences of related strains or species provided clues about more or less recent acquisitions of these islands. Finally, we show that the loss of one of these elements, a lysogenic bacteriophage, occurs in part of the population after prolonged maintenance of KT2440 in the corn rhizosphere, resulting in a fitness increase in this environment.

Received 20 March 2012 **Accepted** 18 July 2012

Published ahead of print 27 July 2012

Address correspondence to Manuel Espinosa-Urgel, manuel.espinosa@eez.csic.es.

Supplemental material for this article may be found at http://aem.asm.org/.

doi:10.1128/AEM.00901-12

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. *P. putida* KT2440 is a plasmid-free derivative of strain mt-2 (45, 55). *Escherichia coli* DH5α was used as the host for cloning experiments. *P. putida* cultures were grown at 30°C in LB medium or M9 minimal medium (58) supplemented with 1 mM $MgSO_4$, 50 μM $FeCl_3$ or iron citrate, and trace metals, with citrate (15 mM) as a carbon source. *E. coli* cultures were grown in LB medium at 37°C. When appropriate, antibiotics were added at the following concentrations (μg/ml): kanamycin (Km), 25; tetracycline (Tc), 15; ampicillin (Amp), 50; streptomycin (Sm), 100; rifampin (Rif), 25; and gentamicin (Gm), 10 (for *E. coli*) or 100 (for *P. putida*).

***In silico* determination and analysis of genomic islands in KT2440.** All analyzed DNA sequences were retrieved from GenBank (http://www.ncbi.nlm.nih.gov) or from the *Pseudomonas* Genome Database V2 (http://www.pseudomonas.com/). Public databases were searched for homologous sequences by BLASTN algorithms (http://www.ncbi.nlm.nih.gov/blast/) with default settings.

Limits of the 25 DNA regions in KT2440 with the most atypical sequence features detected by Weinel et al. (67) and identification of KT2440-specific open reading frames (ORFs) were determined using mGenomeSubtractor (http://bioinfo-mml.sjtu.edu.cn/mGS/), which allows identification and exploration of the most dynamic regions of bacterial genomes (62). Results obtained by Wu et al. (70), as well as information obtained by the computational identification of GIs with the interface IslandViewer (http://www.pathogenomics.sfu.ca/islandviewer) and the data obtained from the GI database Islander (http://cbs.ym.edu.tw/services/islander/about.cgi), were taken into account in the determination of limits of these DNA regions (35, 40). Each nucleotide sequence of the ORFs assigned in the 25 DNA regions of the KT2440 strain was used as a query for homology search with BLASTN against the genome sequence of *Pseudomonas putida* F1, GB1, and W619, *P. entomophila* L48, *P. fluorescens* Pf0-1, Pf-5, and SBW25, *P. syringae* pv. *syringae* B728a, *P. syringae* pv. *phaseolicola* 1448A, *P. syringae* pv. *tomato* DC3000, and *P. mendocina* ymp (accession numbers NC_009512, NC_010322, NC_010501, NC_008027, NC_007492, NC_004129, NC_012660, NC_007005, NC_005773, NC_004578, and NC_009439, respectively) with the default parameters used in the NCBI database. The *H* value (homology value) from genes in the 25 DNA regions of KT2440 compared to each of 11 pseudomonads was calculated based on the length of match and degree of identity (24) as follows: $H = iX(l_m/l_q)$, where *i* is the level of identity of the region, with the highest bit score expressed as a frequency between 0 and 1, l_m is the length of the highest scoring matching sequence (including gaps), and l_q is the query length. If no matching sequences had a BLASTN E value of <0.01, the *H* value assigned to that query sequence was zero (24). Therefore, *H* belonged to the set $H \in [0, 1]$.

Multiplex PCR assays. The stability and evolution of GIs 28, 34, 42, 76, 80, 92, and 104 were studied in root-associated populations of *P. putida* KT2440 using four multiplex PCR assays according to standard methodology (16, 28). For each GI, four oligonucleotides were synthesized (see Table S1 in the supplemental material), two outward-facing primers internal to the GI (primers 2 and 3) and two inward-facing primers in the flanking core genome adjacent to the island (primers 1 and 4), at suitable distances for short extension periods. If the GI was maintained, amplification would take place with the pairs of primers 1 and 2 as well as 3 and 4 (see Fig. S1 in the supplemental material), whereas the pair of primers 1 and 4 would not result in amplification because of the great distance between them. In contrast, the loss of the island would prevent PCR amplification with primers 1 and 2 as well as 3 and 4 and allow PCR amplification with the pair 1 and 4. This method allows the analysis of a large number of clones for the presence/absence of these genetic elements. Amplifications were performed by adding 2.5 μl of bacterial suspension (10^5 CFU/ml) as the template to a 22.5-μl reaction mixture containing PCR buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), and 0.01% Tween 20], 2 mM $MgCl_2$, 100 μM deoxynucleoside triphosphates (dNTPs), 18.75 pmol of primers, and 0.75 U of DNA polymerase (Eurotaq, Milan, Italy). Amplification was performed in a MyCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA). An initial cycle of 95°C for 5 min was followed by 30 cycles of 95°C for 30 s, 59°C for 30 s, and 72°C for 3 min. The final extension was at 72°C for 10 min. Bands of amplified products were visualized under UV light after electrophoresis on 0.8% agarose gels and staining with GelRed (Biotium, Hayward, CA). Positive (KT2440 suspension) and negative controls (reaction mixture without DNA) were included in each PCR run.

Stability and evolution of GIs in rhizosphere colonization assays. For rhizosphere colonization experiments, surface-disinfected corn seeds were allowed to germinate at 30°C in the dark for 48 h. Overnight cultures were diluted in M9 salts (58) to an optical density at 600 nm (OD_{600}) of 1, and 10 μl of the suspension (about 5×10^6 CFU/ml) was added to 10 ml of M9 and poured into tubes containing 40 g of sterile washed silica sand, where germinated seeds were then sown. Plants were maintained in a controlled chamber at 24°C and 55 to 65% humidity with a daily light period of 16 h. Four plants were collected at days 7, 14, 21, 28, and 35, shoots were discarded, and the roots were placed in tubes containing 20 ml of M9 salts and 4 g of glass beads (diameter, 3 mm). Tubes were vortexed for 2 min, and dilutions were plated on M9 with citrate (15 mM) as a carbon source. Plates were incubated at 30°C overnight, and 50 KT2440 colonies isolated from each washing root solution were analyzed by multiplex PCR methods I, II, III, and IV. The band profile obtained with the KT2440 colonies recovered from the rhizosphere was compared to the band profile of the initially inoculated bacteria. A procedure to enrich for efficient competitive root colonizers after bacterization of seeds also was applied to isolate efficient competitive root colonizers (34). This enrichment procedure was performed using KT2440-Sm^r, an otherwise isogenic KT2440 derivative carrying a streptomycin resistance gene in single copy in the chromosome (71). Six plants were inoculated with KT2440-Sm^r as described above. Plants were collected after 14 days, and 1 ml of bacterial suspension recovered from washed roots was used to inoculate another batch of germinated corn seeds. These rounds of enrichment were carried out nine times, corresponding to days 14, 28, 46, 61, 75, 89, 103, and 117 after the initial inoculation. Fifty colonies isolated in the last four enrichment cycles from washed roots by serial dilution and plating on selective medium were analyzed by the four multiplex PCR methods.

Identification of clones showing GI28 loss. The identity of clones recovered from the rhizosphere as KT2440 was confirmed by PCR with KT2440-specific oligonucleotides (51). A PCR method was used to determine excision of GI28 using primers 28I-UP and 28D-DOWN (see Table S1 in the supplemental material), designed in the KT2440 core genome flanking GI28. Amplifications were performed as described above. The loss of GI28 was further confirmed by Southern analysis. Samples of genomic DNA were digested with PstI or EcoRI prior to electrophoresis and DNA transfer to a nylon membrane (Biodyne Plus; Pall Corporation) as described by Sambrook et al. (58). Southern hybridizations were done using as the probe a 473-bp-long DNA fragment internal to GI28, obtained by PCR amplification with primers 28son-UP and 28son-DOWN (see Table S2 in the supplemental material) and labeled with digoxigenin (DIG)-11-dUTP according to the manufacturer's protocol. Prehybridization, hybridization, and colorimetric detection were performed using the DIG detection kit (Roche).

Bacteriophage purification. Bacteriophage production and purification were performed using previously described methods (60). KT2440 was grown in LB at 30°C with vigorous agitation (225 rpm) to an OD_{600} of 0.25. At this point, lysis was induced by adding 500 μl of KT2440 supernatant obtained from an overnight culture grown with 50 μM 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* quinolone signal, or PQS), which was previously shown to promote phage-mediated lysis (20). The culture was incubated for 2.5 h. After an additional incubation of 30 min, 5 μl of DNase I (10 U/μl; Roche) was added per 200 ml culture. Lysates were collected, and the debris was removed by centrifugation at 22,000 × *g* at 4°C for 1 h. Saturated ammonium sulfate (65 ml per 100 ml of superna-

tant) was slowly added at a rate of 1 ml/min while stirring on ice. The suspension was stored at 4°C overnight. The ammonium sulfate precipitate was sedimented at 22,000 × *g* for 1 h, and the pellet was resuspended in 5 ml of 50 mM NaCl-10 mM Tris-HCl, pH 7.5 (TN50 buffer). Bacteriophage particles were then sedimented at 61,000 × *g* for 1 h at 4°C and resuspended in 500 μl of TN50 buffer.

TEM. Phage suspensions were adsorbed on Formvar-carbon-coated grids (diameter, 3 mm; 300 mesh), negatively stained with 2% (wt/vol) potassium phosphotungstate (pH 6.6) for 2 min, and air dried. The grids were observed under a JEOL JEM-1011 transmission electron microscope (TEM) at 80 kV. The diameter size of capsid-like particles was obtained as the mean of 10 measurements of different viral particles.

Detection of circularized DNA forms of GI28 in KT2440 supernatants. Two ml of overnight cultures in LB medium were centrifuged, and the recovered supernatants were mixed (1:1) with ice-cold ethanol, followed by incubation at −20°C for 30 min. The mix was then centrifuged for DNA precipitation, and the pellet was washed with ice-cold 70% ethanol, air dried, and resuspended in distilled water. DNA was pretreated with Plasmid-Safe ATP-dependent DNase (Epicentre Biotechnologies, Wisconsin) or BAL-31 nuclease (New England BioLabs, Inc., Ipswich, MA), which specifically digests linear double-stranded DNA. Treated samples were used as the template for two PCR methods: the method described above to identify when excision of GI28 takes place, and a second PCR method specific for amplification of circularized forms of GI28, using oligonucleotides OFA28U and OFA28D (see Fig. 4A; also see Table S2 in the supplemental material). Positive controls containing untreated DNA from KT2440 supernatants were included.

Transfer of GI28. Transfer of GI28 via liquid mating was performed as previously described, with modifications (8). Briefly, the GI28 donor and recipient strains were grown overnight in LB broth supplemented with the appropriate antibiotics at 30°C on a shaker (200 rpm). The donor cells (OD_{600} of 0.8) were then mixed with the recipient cells (OD_{600} of 0.4), collected by spinning at 8,000 × *g* for 3 min, and resuspended in 1 ml of LB. The cell suspension was transferred to a 15-ml culture tube and incubated at 30°C statically for 24 h prior to plating. Recipient clones acquiring the prophage were selected on LB agar plates supplemented with kanamycin and streptomycin. The transfer efficiency was calculated using the total number of new recipient clones containing the prophage divided by the total number of recipients in the mating mixture.

Construction of ΔPP_1533 mutant. A mutant derivative of *P. putida* KT2440 was generated by replacing the entire excisionase gene (PP_1533) with a kanamycin resistance cassette. Two fragments of 618 and 580 bp, corresponding to the upstream and downstream regions of PP_1533, respectively, were PCR amplified (primer sequences are available in Table S2 in the supplemental material), digested with HindIII and KpnI (upstream fragment) or KpnI and EcoRI (downstream fragment), and cloned sequentially into pUC18Not (29), resulting in plasmid pJMQ1. A KpnI fragment containing the kanamycin resistance cassette from p34S-Km3, which does not have terminators but produces nonpolar mutations (14), was then cloned in pJMQ1. The resulting plasmid, pJMQ2, was electroporated into *P. putida* KT2440, and kanamycin-resistant clones were selected. Since the ColE1-based vector does not replicate in *P. putida*, these clones were the result of either the integration of the plasmid into the chromosome by a single recombination event or the replacement of PP_1533 by double recombination. The clones were selected after screening by PCR amplification. One clone (EU197) was chosen, and the corresponding chromosomal region was sequenced to confirm the mutation.

RNA extraction and preparation of cDNA. *P. putida* KT2440 and the clones 2.43 and EU197 were grown overnight at 30°C in LB medium with shaking at 200 rpm. The cultures were then subcultured 1:100 into 25 ml LB liquid and incubated at 30°C with shaking at 200 rpm. The cultures were grown to early stationary phase (OD_{600} of 1.7 to 1.8), and the cells were collected in precooled tubes by centrifugation at 6,500 × *g* (4°C) for 8 min. The resulting pellets were immediately frozen in liquid nitrogen and preserved at −80°C. Each bacterial culture was performed in triplicate. Total RNA from frozen pellets of each bacterial culture was extracted with TRI Reagent (Ambion) as recommended by the manufacturer, except that the TriPure isolation reagent was preheated at 70°C. The RNA was pretreated with RNase-free DNase I (10 U) (Roche) plus RNaseOUT (40 U) (Invitrogen), followed by purification with RNeasy columns (Qiagen) and a second DNase I treatment with the Turbo DNA-free kit (Ambion). Reverse transcription reactions on 0.5 μg RNA to generate the corresponding cDNA were performed by means of SuperScript II reverse transcriptase (Invitrogen) with random hexamers as primers according to the protocol supplied.

Quantitative real-time PCR. The primers used for real-time PCR analyses were ArsC-UP and ArsC-DOWN for the gene PP_1531 and 13-RNAr 16S and 14-RNAr 16S for the gene encoding 16S rRNA (primer sequences are available in Table S2 in the supplemental material). Real-time PCR amplification was carried out on a MyiQ2 system (Bio-Rad) associated with iQ5 optical system software (version 2.1.97.1001). Each 25-μl reaction mixture contained 12.5 μl iQ SYBR green Supermix (100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 μM each dNTP, iTaq DNA polymerase [50 U ml^{-1}], 6 mM $MgCl_2$, SYBR green I, 20 nM fluorescein, and stabilizers [Bio-Rad] [0.4 μM for each primer]) and 2 μl template cDNA (diluted 10- or 1,000-fold). Thermal cycling conditions were the following: one cycle at 95°C for 10 min and then 40 cycles at 95°C for 15 s, 68°C for 30 s, and 72°C for 20 s, with a single fluorescence measurement per cycle according to the manufacturers' recommendations. A final extension cycle (72°C for 1 min) was performed. The PCR products were 156 and 300 bp for the PP_1531 and 16S rRNA genes, respectively. Melting curve analysis was performed by gradually heating the PCR mixture from 55 to 95°C at a rate of 0.5°C per 10 s for 80 cycles. The relative expression of PP_1531 was normalized to that of 16S rRNA, and the results were analyzed by means of the comparative cycle threshold ($\Delta\Delta C_T$) method (49).

Rhizosphere colonization assays with KT2440 clones without GI28 and EU197. Rhizosphere colonization experiments with individually inoculated strains were similar to those described above, except that six germinated seeds were inoculated with each strain and bacteria were recovered after 1 week. For competitive colonization assays, an otherwise isogenic KT2440 derivative carrying a gentamicin resistance gene in single copy in the chromosome (KT2440-Gm^r), generated by a site-specific insertion of mini-Tn*7*-ΩGm1 (33) at an extragenic location near *glmS*, was used. Strains were grown in LB medium, diluted in M9 salt to an OD_{660} of 1, and mixed in a 1:1 proportion (5 × 10^6 CFU of each strain) before proceeding as described above. Plants were collected after 1 week, and the numbers of each strain were determined by plating dilutions on selective medium (LB with Sm or Gm, respectively). All experiments were repeated three times.

RESULTS

Analysis of GIs with potential for mobilization in KT2440 DNA regions with atypical sequence features. Bioinformatic analysis of DNA regions in the whole genome of *Pseudomonas putida* KT2440 was undertaken to identify regions with potential for mobilization. Weinel et al. (67) distinguished 105 DNA regions from the core genome of KT2440 using local G+C content and di- and tetranucleotide frequencies as measures of variability in this genome. We only consider 25 of these 105 DNA regions (Tables 1 and 2), because they showed the most atypical sequence features detected by all three variability criteria used by Weinel et al. (67). We assessed the specificity of these 25 DNA regions for KT2440 using the BLAST-based procedure mGenomeSubtractor with 11 previously sequenced pseudomonad genomes with different phylogenetic distances from the KT2440 genome. This information provided some clues about their more or less recent acquisition or their mobilization potential. The boundaries of the 25 DNA regions were also redefined. Signatures of mobile elements were not

TABLE 1 *Pseudomonas putida* KT2440 atypical genomic regions without mobile element signatures[a]

DNA region	Position		Size (bp)	No. of genes[b]				Main functional role(s)
	5′ End	3′ End		Total in KT2440	KT2440 specific ($H < 0.42$)	Conserved in *P. putida* strains ($H \geq 0.81$)	Conserved in all strains ($H \geq 0.81$)	
2	171387	182009	10,622	7	0	7	7	Ribosomal operons *rrnA* and *rrnA′*
11	524947	529901	4,954	3	0	3	3	Ribosomal operon *rrnB*
12	530085	564346	34,261	44	0	40	23	Ribosomal proteins
14	697822	703001	5,179	3	0	3	3	Ribosomal operon *rrnC*
24	1325501	1330467	4,966	3	0	3	3	Ribosomal operon *rrnD*
31	1981629	2040234	58,605	44	26	1	0	Lipopolysaccharide biosynthesis genes
41	2548687	2553866	5,179	3	0	3	3	Ribosomal operon *rrnE*
45	2819189	2822312	3,123	4	4	0	0	Glutathione *S*-transferase and regulator
58	3446684	3453443	6,759	7	3	1	0	End of pyocin R2 cluster
64	3748952	3754058	5,106	6	4	0	0	Heat shock proteins
74	4108175	4116581	8,406	5	0	1	0	Hypothetical proteins
95	5307554	5311162	3,608	3	0	3	3	Ribosomal operon *rrnF*
103	5969161	5977681	8,520	7	2	1	0	SciM homologue

[a] DNA regions with atypical sequence features of *Pseudomonas putida* KT2440 characterized by a high dinucleotide bias, low G+C content, and low variance of tetranucleotide frequencies (67). The nomenclature of DNA regions is the same as that used by Weinel et al. (67). The size of genomic regions and the positions of the 5′ and 3′ ends are indicated.
[b] Number of genes within genomic regions, specific to KT2440, conserved in *P. putida* strains KT2440, F1, GB-1, and W619, and conserved in the four *P. putida* strains plus *P. entomophila* L48, *P. fluorescens* Pf0-1, Pf-5, and SBW25, *P. syringae* DC3000, 1448A, and B728a, and *P. mendocina* ymp are indicated using the *H* value (24).

found in 13 of them (Table 1). Six (regions 2, 11, 14, 24, 41, and 95) of these 13 DNA regions corresponded to *rrn* operons, and another (region 12) resembled a cluster of ribosomal proteins. The remaining six regions without a mobile element signature (regions 31, 45, 58, 64, 74, and 103) included a very small number of conserved genes in *P. putida* strains F1, GB-1, and W619 (4 of 73 genes included in the six DNA regions) and a high number of KT2440 strain-specific genes with *H* values lower than 0.42 (39 of 73 genes) (Table 1). Five of these regions (regions 31, 45, 58, 64, and 74) were previously predicted as GIs (70). Region number 58 was also identified as a prophage (70).

The remaining 12 of these 25 DNA regions (1, 15, 28, 34, 42, 59, 76, 79, 80, 83, 92, and 104) presented signatures of mobile elements (Table 2). These regions, here called genomic islands (GIs), could be mobilizable. They included a very small number of conserved genes in *P. putida* strains F1, GB-1, and W619 (51 of 563 genes included in the 12 DNA regions) and a high number of KT440 strain-specific genes (388 of 563 genes) (Table 2). The ex-

TABLE 2 *Pseudomonas putida* KT2440 atypical genomic regions with signatures of mobile elements[a]

GI	Position		Size (bp)	% Coverage in GI prediction[b]			No. of genes[c]				tRNA[d] (coordinates)
	5′ end	3′ end		IslandViewer	MaGe	Islander (GI)	Total in KT2440	KT2440 specific ($H < 0.42$)	Conserved in *P. putida* strains ($H \geq 0.81$)	Conserved in all strains ($H \geq 0.81$)	
1	16589	59184	42,595	26			41	4	36	0	
15	741898	757185	15,287	89	100		17	15	0	0	Thr (741323)-X
28	1738082	1776894	39,415	43	100		53	31	0	0	
34	2163137	2227398	64,261	100	100		57	52	0	0	
42	2586633	2625819	39,186	98	100	100 (Ppu40L)	33	33	0	0	Arg, His, and Leu (2585761–2586088)-X-His (2626119)
59	3492379	3525146	32,767	43	71		32	17	5	0	
76	4175034	4232789	57,755	100	100		48	46	0	0	
79	4362271	4427678	65,407	47	100	85 (Ppu56C)	87	68	2	0	Leu (4361679)-X-Cys (4427589)
80	4454806	4501060	46,254	51	100	35 (Ppu17S)	41	16	2	0	X-Ser (4501402)
83	4596179	4632391	36,212	73	100		31	19	0	0	X-Gly (4632625)
92	4997100	5076538	79,438	88	99		75	59	0	0	X-Arg (5075625–5075760)
104	6117294	6170284	52,990	29	58		48	28	6	0	

[a] DNA regions with atypical sequence features of *Pseudomonas putida* KT2440 characterized by a high dinucleotide bias, low G+C content, and low variance of tetranucleotide frequencies (67). These regions were named genomic islands (GIs) because they contain signatures of mobile elements. The GI nomenclature is the same as that used by Weinel et al. (67). Sizes and positions of 5′ and 3′ ends of GIs are indicated.
[b] Information obtained by computational identification of GIs with IslandViewer (35), the MaGe annotation system (70), and the data from the GI database Islander (40) were taken into account to determine the GI edges and, within each GI, percent GI identified with these predictions.
[c] Number of genes within GIs, specific to KT2440, conserved in *P. putida* strains KT2440, F1, GB-1, and W619, and conserved in the four *P. putida* strains plus *P. entomophila* L48, *P. fluorescens* Pf0-1, Pf-5, and SBW25, *P. syringae* DC3000, 1448A, and B728a, and *P. mendocina* ymp are indicated using the *H* value (24).
[d] The presence of tRNA on GI edges is indicated with the name of the specific amino acid for tRNA. X represents the position of GI with respect to tRNA position.

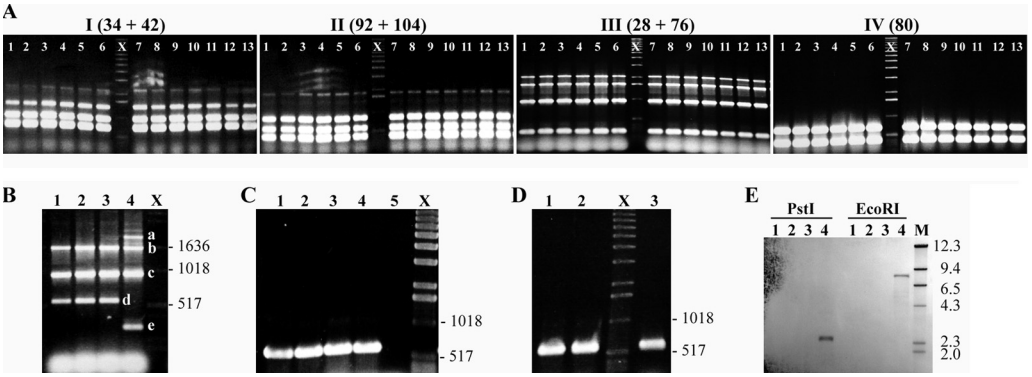

FIG 1 Analysis of stability and evolution of genomic islands (GIs) 28, 34, 42, 76, 80, 92, and 104 of *P. putida* KT2440 in the corn rhizosphere. (A) Band profiles obtained by four multiplex PCR assays of representative clones recovered from root colonization procedures without (lanes 1 to 6) and with enrichment (lanes 8 to 13). Clones shown in lanes 1 to 3 and 4 to 6 were recovered 28 and 35 days after inoculation, respectively. Lane 7 corresponds to KT2440 initially inoculated in the root colonization procedure without enrichment. Clones of lanes 8 to 10 and 11 to 13 were recovered 103 and 117 days after inoculation, respectively. (B) Band profiles obtained by multiplex PCR assay III (GI28, bands a and e, and GI76, bands b and c) of clones recovered from root colonization procedures without (16.35; lane 1) and with enrichment (2.43 and 4.34; lanes 2 and 3) which differed (band d) from the profile of KT2440 (lane 4). Lane X, molecular size marker. Three fragment sizes are indicated. (C) PCR amplification with KT2440-specific primers to confirm the identity of obtained clones. Lane 1, 16.35; lane 2, 2.43; lane 3, 4.34; lane 4, KT2440; lane 5, negative control; and lane X, molecular size marker. Two fragment sizes are indicated. (D) PCR amplification with oligonucleotides corresponding to the core genome regions flanking GI28 (28I-UP and 28D-DOWN) to confirm its loss. Lane 1, 16.35; lane 2, 2.43; lane 3, 4.34; and lane X, molecular size marker. Two fragment sizes are indicated. (E) Southern blot analysis using a GI28-specific probe to discard the possible translocation of GI28 to another chromosomal region. Lane 1, 16.35; lane 2, 2.43; lane 3, 4.34; lane 4, KT2440; and lane M, molecular size marker (sizes are indicated).

ception is GI1, which is mainly formed by genes conserved in *P. putida* strains but absent from other *Pseudomonas* species. Only four genes were KT2440 strain specific (Table 2). This indicates that GI1 was acquired prior to the divergence of the present strains from a common ancestor.

Other supporting pieces of evidence for GI identification are the presence of neighboring tRNA genes and direct repeats (25). tRNA genes were found in one edge of 4 GIs (GIs 15, 80, 83, and 92) and in both edges of GIs 42 and 79 (Table 2). Direct repeats with sizes of 52, 67, and 57 bp were also described by Mantri and Williams (40) in GIs 42 (Ppu40L), 79 (Ppu56C), and 80 (Ppu17S), respectively (Table 2). In these three cases the repeated portion corresponded to the 3′ edge of each GI (40).

Signatures of mobile elements were carried by 48 genes in the 12 GIs. These included 37 genes encoding predicted transposases, 8 encoding predicted integrases, and 3 encoding predicted group II introns (see Table S3 in the supplemental material). Predicted group II introns found in GIs 15 and 79 correspond to copies of those described for intron P.p.I3, while the predicted group II intron found in GI92 has been previously described as an intron fragment (12). Finally, three genes predicted as *rhs* (rearrangement hot spot) (39) family proteins are included in GIs 59 (PP_3108) and 83 (PP_4085 and PP_4093.2).

Predicted functional roles within potentially mobilizable GIs. A bioinformatic study was conducted to define the functions encoded by the 12 GIs pertaining to mobilization activities, which could influence bacterial persistence in the environment. The classification of genes within these islands into four broad groups and 25 functional categories, based on the definitions produced by the J. Craig Venter Institute (formerly The Institute of Genomic Research) (48), was assessed, and the proportions of group and category distribution compared to the averages found in the entire genome are presented in Fig. S2 in the supplemental material. As expected for laterally transferred genes, the most represented genes within the islands are those involved in genomic mobility and recombination (see Fig. S2). In contrast, the least represented functional categories correspond to the groups metabolism and cellular processes and signaling (see Fig. S2). Genes in the 12 GIs potentially involved in environmental adaptation are listed in Table S4 in the supplemental material.

Stability of GIs in populations associated with the corn rhizosphere. We decided to analyze the stability of potentially mobilizable GIs in populations of *P. putida* KT2440 after prolonged persistence in the rhizosphere. Seven GIs were selected for this analysis (GIs 28, 34, 42, 76, 80, 92, and 104). Their stability and evolution was monitored by four multiplex PCRs under two experimental situations, as described in Materials and Methods. The first situation corresponded to bacterial populations recovered at different times after inoculation of corn seedlings, and the second used an enrichment method by successive passage through the rhizosphere. Band profiles of 1,000 clones recovered from the first set of experiments after amplification with multiplex PCR method I (GIs 34 and 42), II (GIs 92 and 104), and IV (GI80) were identical to the initial band profile of KT2440 (Fig. 1A). The same result was obtained with clones recovered from the enrichment procedure and amplified with multiplex PCR I, II, and IV. However, multiplex PCR III band profiles (GIs 28 and 76) of 1 out of 1,000 clones recovered from the nonenrichment procedure (clone 16.35) and 2 out of 733 clones recovered from the enrichment procedure (clones 2.43 and 4.34) were different from the band profile of KT2440 (Fig. 1B). The identity of the three clones was confirmed by PCR analysis using *P. putida* KT2440-specific primers, discarding a possible contamination (Fig. 1C). The multiplex PCR III band profiles of these three clones were identical, showing two bands common to the profile of KT2440 initially inoculated (Fig. 1B), which correspond to amplicons on the left and right edges of GI76 (bands b and c, respectively). On the contrary, the bands corresponding to amplicons on the left and right edges of GI28 were absent (bands a and e, respectively). Instead, a new band of 538 bp was present (band d). This band was identical to

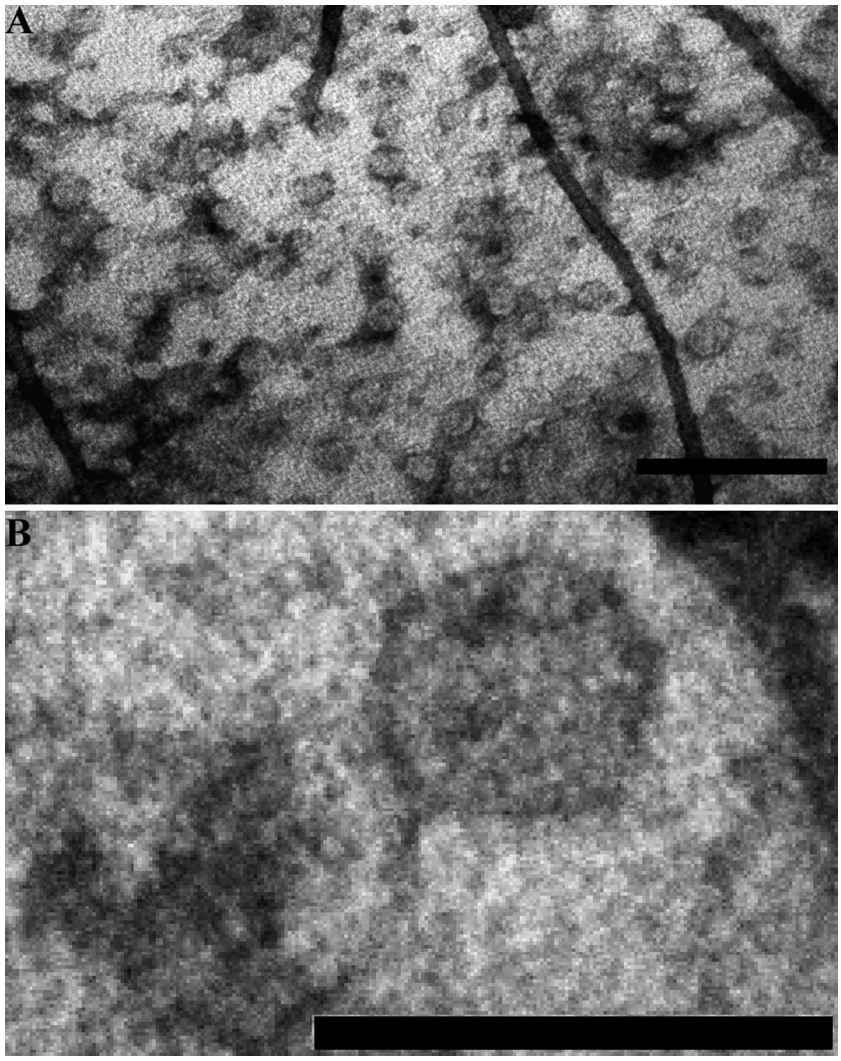

FIG 2 Transmission electron microscopy of negatively stained capsid-like particles. Suspensions of purified phages from KT2440 were adsorbed on Formvar-coated grids, stained with 2% phosphotungstic acid for 2 min, and air dried. The grids were observed under a JEOL JEM-1011 transmission electron microscope at 80 kV. Bars: 200 nm (A) and 100 nm (B).

the amplicon generated by PCR amplification with 28I-UP and 28D-DOWN primers designed in the KT2440 core genome flanking GI28 (Fig. 1D). Thus, GI28 was lost at least from its insertion site in clones 16.35, 2.43, and 4.34. Southern blot analysis using a GI28-specific probe discarded its possible translocation to another chromosomal region by recombination, since none of the three clones showed hybridization (Fig. 1E), thus confirming the loss of GI28.

GI28 corresponds to the lysogenic state of a filamentous bacteriophage (Pspu28). GI28 (here called Pspu28) was previously described as a prophage (67, 70). The genome of phage Pspu28 is 39.4 kb, and its GC content (61%) is similar to that of the KT2440 core genome (61.6%). Pspu28 contains 53 ORFs, from PP1532 to PP1584 (Table 2; also see Data Set S1 in the supplemental material), which share significant sequence similarity to known protein sequences. However, putative functions were assigned to only 28 ORFs (53%), joining data from GenBank annotation by Nelson et al. (46) and from the ACLAME database (38). Pspu28 ORFs are organized into two groups, early genes (DNA replication) and late genes (head, tail, and lysis), and they are located on the complementary strand (see Data Set S1). This prophage shows the typical organization of gene systems as listed in order of transcription by Casjens (9): terminase, portal, protease, scaffold, major head shell (coat) protein, head/tail-joining proteins, tail shaft protein, tape measure protein, tail tip/base plate proteins, and tail fiber.

Transmission electron microscopy using negative staining revealed numerous phage-like particles with a hexagonal structure (size, 47.41 $\pm$ 2.36 nm) after induction of lysis with 2-heptyl-3-hydroxy-4-quinolone (PQS) followed by a precipitation step, as described in Materials and Methods (Fig. 2). Phage tails were slightly visible in a small number of phage-like particles, probably due to their density limiting observation after negative staining (1). These structures and the genome size of Pspu28 were compatible with a *Siphoviridae* phage (6, 7).

Integrated and excised forms of prophage Pspu28 coexist in KT2440 populations. The loss of Pspu28 from the KT2440 genome in rhizosphere populations suggests it is an unstable genetic element under these environmental conditions. We decided to

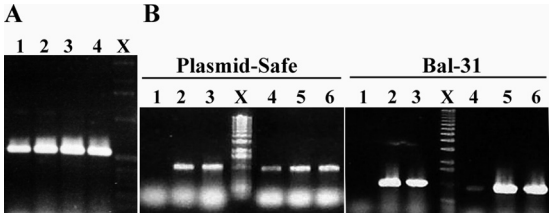

FIG 3 Detection of excised forms of Pspu28. (A) PCR amplification with 28I-UP and 28D-DOWN oligonucleotides corresponding to the core genome region of KT2440 flanking GI28. The result was positive only when GI28 excision takes place, given the long distance between the primers when the island is present. Lane 1, 16.35; lane 2, 2.43; lane 3, 4.34; lane 4, KT2440; and lane X, molecular size marker. Two fragment sizes are indicated. (B) Detection of circularized Pspu28. DNA from supernatants of KT2440 cultures in LB was pretreated with nucleases Plasmid-Safe ATP-dependent DNase or BAL-31, specific for linear double-stranded DNA (lanes 1 and 4), prior to PCR amplification with primers OFA28U and OFA28D (lanes 4, 5, and 6) or with primers 28I-UP2 and 28D-DOWN (lanes 1, 2, and 3) to check the efficiency of DNA digestion. PCR amplification with primers OFA28U and OFA28D only takes place with circularized DNA of GI28. Positive controls (DNA from KT2440 supernatants not treated with nucleases) were also included (lanes 2, 3, 5, and 6). Positive controls of lanes 2 and 5 contained all of the components of the Plasmid-Safe or BAL-31 kit except nuclease. Diagrams indicating where the primer pairs OFA28U and OFA28D as well as 28I-UP and 28D-DOWN anneal are shown in Fig. 4.

analyze the stability of this GI in KT2440 under standard laboratory conditions by PCR amplification with 28I-UP and 28D-DOWN primers. Interestingly, a band of the same size as that observed with clones 16.35, 2.43, and 4.34 could be observed using DNA obtained from overnight cultures as the template (Fig. 3A). This suggests the loss of Pspu28 in part of the population. The excision of prophage DNA often results in circularized forms that can be detected in cell lysates or supernatants. Two specific oligonucleotides (OFA28U and OFA28D) (Fig. 4A) were used to detect circularized GI28 by PCR amplification. DNA was extracted from supernatants of KT2440 cultures in LB medium and pretreated with Plasmid-Safe ATP-dependent DNase or BAL-31 nuclease, each of which specifically digests linear double-stranded DNA. Afterwards, PCR for the specific amplification of linear DNA template in the KT2440 core genome regions flanking Pspu28 and of the circularized form of the GI were performed. No amplification was observed with the first PCR method when supernatants were treated with nucleases (Fig. 3B), but a band of 1,082 bp was observed with OFA28U and OFA28D, confirming the presence of an excised and circular form of Pspu28. All of these data support the natural coexistence of the prophage in both forms in *P. putida* populations.

The excisionase encoded by PP_1533 is essential for mobilization of Pspu28. The gene PP_1533 encodes a putative excisionase enzyme which could play a role in the release of the prophage by a site-specific recombination reaction (11, 57). A ΔPP_1533 mutant (EU197) was generated by replacing the entire gene with a kanamycin resistance cassette, as described in Materials and Methods. Mutant EU197 grew normally in all media tested and did not show any differences with respect to the wild type in colony and cell morphology, swimming motility, or antibiotic resistance (data not shown). When DNA of EU197 was used as the template, no amplification was obtained with PCR methods designed for detection of prophage excision from bacterial genome or for detection of the circularized form of Pspu28 (data not shown). The same result was obtained even when EU197 was exposed to PQS, which can induce the transition from the lysogenic state to lytic growth (22). These results indicate that PP_1533 encodes an excisionase required for phage Pspu28 to enter the lytic cycle.

Identification of the insertion site of prophage Pspu28. Many temperate bacteriophages integrate into the host genome by site-specific recombination using unique attachment sites in both the phage and host genomes (*attP* and *attB*, respectively). The inserted prophage is flanked by two reciprocally recombinant sites, *attL* and *attR*. The sequence of the four *att* sites was identified in prophage Pspu28 after sequencing the *attB* and *attP* amplicons obtained by PCR when prophage excision takes place and a DNA circle of GI28 is formed, respectively. Sequences identified in the four *att* sites show differences in base composition. The *attL* site is included in part of the PP_1532 and PP_1531 genes, while the *attR* site is included in a wide intergenic region, 658 bp in length, between PP_1584 and PP_1585 (Fig. 4A). A 17-bp direct repeat (5′-TACGGCATCAAAGCCTG-3′) was identified in *attL* and *attR* sites. Direct repeats of DNA sequence play a major role in determining the probability of deletion events (2). During excision, a crossover could occur between *attL* and *attR* (Fig. 4B). After excision, *attB* (formed in the chromosome) and *attP* (formed in circularized Pspu28) sites retained more than 75% of the sequence identified in *attR* and *attL*, respectively (Fig. 4C).

We analyzed whether Pspu28 excision causes a change in neighboring genes by comparing the sequences near the *att* sites before and after excision. Although there is no change at the protein level, the promoter of PP_1531, encoding a predicted arsenate reductase, is significantly altered when Pspu28 is absent from the chromosome. The impact of the promoter changes in the expression levels of PP_1531 were analyzed by quantitative real-time PCR, comparing clone 2.43 (Pspu28-free) to KT2440 and EU197 (Pspu28 locked in the lysogenic state). The relative expression level of PP_1531 (normalized internally to that of 16S rRNA) showed an increase of 1.62 ± 0.2 and 2.55 ± 0.68 in clone 2.43 with respect to KT2440 and EU197, respectively.

Pspu28 transfer to prophage-free cells. Strain KTR1542 was used to determine if Pspu28 could be transferred to clones devoid of the prophage. This strain is a Rif^r KT2440 derivative (18) containing a mini-Tn*5*[Km1] insertion in PP_1542, which encodes a hypothetical protein of unknown function and is contained within Pspu18. Overnight cultures of KTR1542 and clone 2.43 were mixed and coinoculated into LB medium. Numerous Km^r Sm^r Rif^s colonies were obtained after 24 h, indicating transfer of Pspu28 from KTR1542 to 2.43. PCR amplification with specific primers was used to confirm that these clones were not spontaneous Sm^r mutants of the donor (see Fig. S3 in the supplemental material). The presence of Pspu28 in these clones was also confirmed by PCR amplification of the left edge of Pspu28 and of part of PP_1561 and PP_1562 within Pspu28. Transfer frequency (number of new recipients with Pspu28 divided by the total number of recipients) was calculated to be $1.08 \times 10^{-7} \pm 0.48 \times 10^{-7}$.

Loss of GI28 or blockage of the lytic cycle of Pspu28 increase intraspecific fitness in the rhizosphere. Since clones having lost the genetic island corresponding to Pspu28 were obtained from rhizosphere populations, we decided to establish how this loss influenced bacterial fitness in this environment as well as in the laboratory. The parent strain *P. putida* KT2440, clones 16.35, 2.43, 4.34, and excisionase mutant EU197 were indistinguishable in

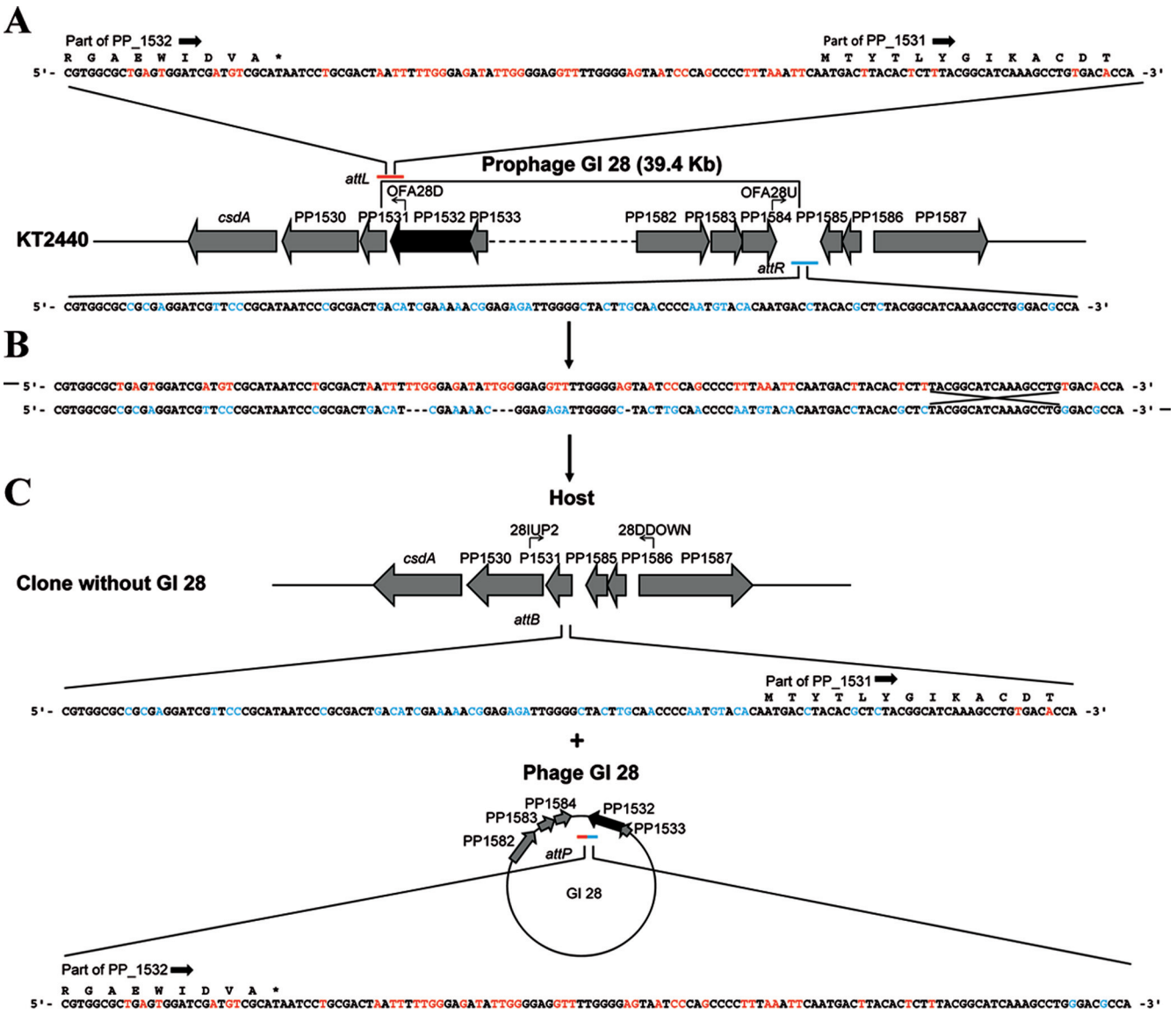

FIG 4 Diagram of Pspu28 excision. (A) The position, unique sequences, and partial translation of the two attachment sites in the chromosome of KT2440 are indicated. The red bar shows the left-hand attachment site (*attL*), and the blue bar shows the right-hand attachment site (*attR*). Letters differing in the sequences of the left and right attachment sites are indicated in red and blue, respectively. (B) During excision, a crossover occurs between the two *att* sites. (C) Structure of the chromosomal region in clones lacking the prophage (16.35, 2.43, and 4.34) and of the excised Pspu28 in its circular form. The *attB* site is retained in the chromosome, and *attP* is in the phage-like circle. The position of the primers used in Fig. 3 is indicated. OFA28U and OFA28D (A) amplify a PCR product only when a DNA circle is formed at the *attP* site (C). Primers 28I-UP2 and 28D-DOWN allow verification of the complete deletion of GI28 by PCR and sequencing.

terms of growth rate in LB and M9 media individually or in competition (data not shown). No differences were observed with respect to swimming and swarming motility or biofilm formation under static conditions in microtiter plates and glass tubes.

Colonization of corn roots in sterile sand by clones without Pspu28 and mutant EU197, individually inoculated, resulted in bacterial numbers comparable to those measured for the wild type after 7 days (Fig. 5A). However, when those clones were assayed under the same conditions in competition with the parent strain, all of them were able to displace significantly the wild-type strain, so that the wild type represented less than 30% of the total population after 7 days (Fig. 5B). This trend was maintained after longer periods of time, with the parental strain representing less than 10% of the total population beyond 21 days (Fig. 5C) when in competition with a clone lacking Pspu28. To exclude that this competitive advantage was not simply due to the different antibiotic resistance cassettes carried by each strain, a control experiment was performed in which KT2440-Sm^r was coinoculated with KT2440-Gm^r and competitive rhizosphere colonization was assayed. Although there was a slight increase in the proportion of Sm^r clones recovered after 7 days, this increase was significantly lower than that observed for clones devoid of Pspu28. An even more pronounced effect was observed when sterile soil was used instead of sand (Fig. 6). Clones without Pspu28 outcompeted the parental strain when either the whole root system or only the root

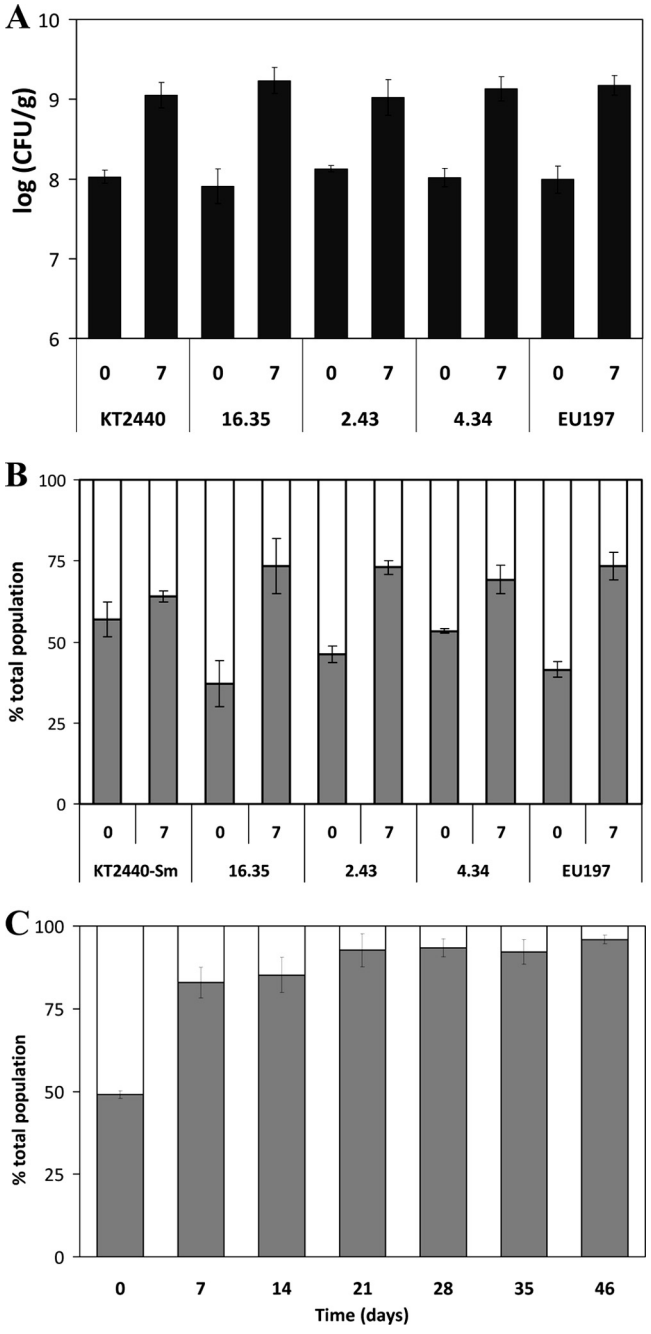

FIG 5 Quantitative analysis of corn rhizosphere colonization by KT2440 clones without GI28 (clones 16.35, 2.43, and 4.34), excisionase mutant EU197 (ΔPP_1533), and KT2440 in sterile sand. (A) Cells of each strain were inoculated individually, and the number recovered after 0 and 7 days is shown. (B) Clones without GI28 and excisionase mutant EU197 (gray bars) were inoculated in a 1:1 mix with KT2440-Gm (white bars) in a competitive colonization assay, and the proportion of each strain in the total population after 7 days was analyzed. A control in which KT2440-Gm was mixed with KT2440-Sm is also shown. (C) Rhizosphere colonization of clone 2.43 (gray bars), without GI28, was assayed in competition with KT2440-Gm (white bars) for a longer period of time. The proportion of each strain recovered after 7, 14, 21, 28, 35, and 46 days is shown. Data from B and C were analyzed with SPSS software for Windows (release 20.0.0; SPSS Inc., Chicago, IL). The analysis of variance (ANOVA) rendered significant differences for the clones without GI28 and the excisionase mutant ($P < 0.05$) compared to the wild type, and there were no significant differences between wild-type strains with the different antibiotic resistance markers. The error bars correspond to the standard deviations of the means.

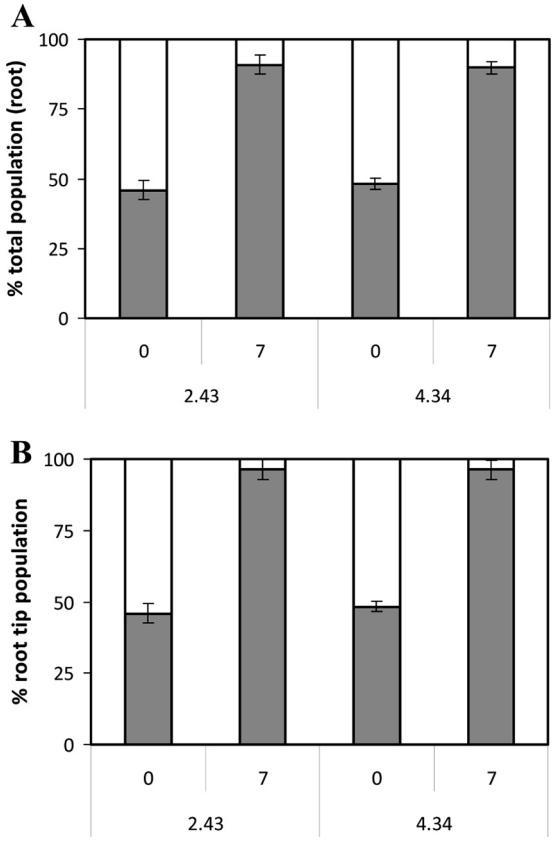

FIG 6 Competitive colonization of corn rhizosphere by two KT2440 clones without GI28 (clones 2.43 and 4.34) (gray bars) and KT2440-Gm (white bars) in sterile soil. The number of cells of each strain recovered after 7 days from root (A) and root tip (B) is shown. All data were analyzed with SPSS software. The analysis of variance (ANOVA) rendered significant differences for the clones without GI28 ($P < 0.05$) compared to the wild type. The error bars correspond to the standard deviations of the means.

tips were analyzed. These results are consistent with the relatively high frequency at which clones devoid of Pspu28 are obtained from the rhizosphere.

DISCUSSION

The discovery of GIs that differ from core genomes and can be acquired by horizontal gene transfer has been crucial to our understanding of microbial evolution. However, there is a relative lack of knowledge about those GIs that are not related to pathogenesis. The genome-sequencing era has led to the discovery of a variety of GIs providing diverse metabolic capacities in nonpathogenic bacterial strains, accentuating that horizontal gene transfer is not limited to virulence traits (15). We have begun to analyze the part that GIs play as driving forces for the evolution of root-associated *Pseudomonas putida* populations. In a list of 116 complete prokaryotic genomes classified by descending order according to their proportion of horizontally transferred genes, *P. putida* KT2440, with a value of 16.8%, is in position 29, being ahead of pathogenic strains like *Yersinia pestis* KIM, *Mycobacterium tuberculosis* CDC1551, and *Staphylococcus aureus* N315 (a methicillin-resistant strain) (44).

The signature of mobile elements in a DNA region indicates recent acquisition by horizontal gene transfer and that this region

has high potential for mobilization. However, sometimes the potential transmission mechanisms are not obvious due to mutations affecting the genes involved in transmission. Additionally, the composition of a sequence derived from a horizontal gene transfer event could adapt to that of the new host over time (36, 37). Biological functions of horizontally transferred genes in KT2440, except for mobile element genes, are quantitatively biased toward regulatory functions (44). Recently, it has been suggested that many of the resident GIs in a single prokaryotic genome originated from the same source, considering their similar genomic signature compositions (56). The identity of the donor microorganism(s) of the 12 GIs selected in this work is unknown, because the results from sequence alignments did not show homology with any sequence longer than 4 kb in the databases, including *P. putida* strains BIRD-1, S16, and B6-2 (41, 64, 72).

We selected seven GIs to determine if the functions they encode are dispensable in *P. putida* populations in the rhizosphere, in which case the lack of selective pressure could result, combined with their predicted potential for mobilization, in their rapid loss. Six of these GIs seemed to be stably maintained during the period of these experiments even after successive rounds of incubation in the rhizosphere, although the loss of some of them cannot be ruled out because of the low transfer frequencies of GIs previously demonstrated (5, 50, 52, 53, 61). However, GI28 was completely lost in 1 out of 1,000 clones recovered with the nonenrichment procedure and in 2 out of 733 clones recovered with the enrichment procedure. This island corresponds to the lysogenic state of Pspu28, a filamentous bacteriophage which can enter a lytic state, since capsid-like particles were observed under TEM in our work and phage-mediated lysis took place in KT2440 in the presence of PQS at a low frequency (22). A gene belonging to GI28, which encodes an integrase (PP_1532), has been identified as being preferentially expressed in populations of KT2440 in the rhizosphere soil of different plants using *in vivo* expression technology (J. L. Ramos and M. Fernández, personal communication). The excision of the prophage could allow the expression of lytic genes carried by phages, as was previously demonstrated with other prophages (9, 32). However, Pspu28 also seems to be excised from the KT2440 genome without causing cell lysis, thus giving rise to viable clones that have lost the whole genomic island. This event may also take place *in vitro* in cultures grown in LB medium. Pspu28 can also be transferred to a prophage-deficient KT2440 derivative with a frequency of approximately 10^{-7}. Attempts with other bacteriophages to identify prophage excision in various environments have shown very low frequencies of excision, indicating a stable residence of prophages under most conditions (63). Once integrated into the KT2440 genome, the temperate phage Pspu28 and its host may achieve a balanced equilibrium between the costs and benefits to both, as was previously described for *Escherichia coli* (10). However, under conditions of intraspecific competition in the rhizosphere, clones lacking the prophage show an advantage over those harboring it. These results are in agreement with an earlier work suggesting lysogens have a competitive disadvantage in nature (30), although this is not always the case (3). This effect cannot be explained simply by the reduction in genetic load resulting in increased growth rate, since no difference was observed in the growth of those clones and the parental strain, and the competitive advantage is not observed under regular laboratory conditions. Furthermore, a clone in which Pspu28 is locked in the lysogenic state due to deletion of the excisionase gene is also more competitive than the wild type in the rhizosphere. At this point, it is unclear why the loss of Pspu28 and its presence locked in the lysogenic state both result in competitive fitness gain in the rhizosphere. Roles of prophages in host physiology remain widely undefined, with few exceptions (66). Global transcriptional analyses revealed broad changes in host gene expression, although they were not very elevated in quantitative terms (22, 47), as well as DNA amplification during temperate phage induction. Escape replication strategies have also been described in some lambdoid phages prior to excision (23, 68) and in prophages with deleted excisionase (4, 22). Prophage Pspu28 is flanked by two reciprocally recombinant sites (*attL* and *attR*). Excision and subsequent formation of *attB* and *attP* sites causes a significant change in the promoter sequence of the adjacent gene PP_1531, encoding a predicted arsenate reductase. Excision of Pspu28 alters its transcriptional profile. Whether this change or other potential alterations in the gene expression profile of the host contribute in any way to the persistence of *P. putida* in the rhizosphere are possibilities that are currently being explored.

ACKNOWLEDGMENTS

We are grateful to M. I. Ramos for helpful comments on the manuscript and to E. Duque and J. de la Torre for providing strain KTR1542. We thank J. D. Alché and A. Rodríguez-Sánchez at the Microscopy Service of the Estación Experimental del Zaidín for assistance in sample preparation and observation by transmission electron microscopy.

This work was supported by grant P08-CVI-03869 from Junta de Andalucía and FEDER.

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