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The IncP-6 Plasmid Rms149 Consists of a Small Mobilizable Backbone with Multiple Large Insertions

Anthony S. Haines, Karen Jones, Martin Cheung, and Christopher M. Thomas*

School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom

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Plasmid Rms149, the archetype of *Pseudomonas* plasmid incompatibility group IncP-6, was identified in *Pseudomonas aeruginosa* as an agent conferring resistance to streptomycin, sulfanilamide, gentamicin, and carbenicillin in 1975. It has been classed as a broad-host-range plasmid due to its ability to replicate in both *Escherichia coli* (where it is designated IncG) and *Pseudomonas* species, although both species are γ-proteobacteria. To provide reference information on this Inc group, we have determined the complete sequence of Rms149 and found that, although the genome comprises 57,121 bp, it is essentially a small mobilizable plasmid carrying multiple mobile elements, which make up 79% (>45 kb) of its genome. A replicon has been identified which encodes a single polypeptide with moderate identity to other replication proteins. The region encoding this protein can replicate in *Pseudomonas putida* and *E. coli*. This sequence is directly downstream of a putative partitioning region highly similar to that of pRA2. A functional IncQ-type mobilization region is also present. Thus, the backbone appears to be a novel combination of modules already identified in other plasmid systems. Analysis of the segments that fall outside this core of stable inheritance and transfer functions show that this plasmid has been subject to multiple insertion events and that the plasmid appears to carry a considerable load of DNA that no longer should be phenotypically advantageous. The plasmid therefore functions not just as a vehicle for spread of selective traits but also as a store for DNA that is not currently under selection.

The key features of a plasmid are the ability to replicate autonomously and to be maintained in a cell lineage without a high rate of segregational loss. An optional but frequently encountered property is the ability to transfer or to be mobilized from one bacterium to another. From these properties it follows that genes that become associated with a plasmid may spread rapidly from one genetic background to another. Some plasmids appear to consist of nothing more than functions that confer the above core abilities, and these have been termed cryptic plasmids.

To succeed as such a selfish element, a plasmid must be stable and confer minimal burden on its host, or at least overcome competitive losses by horizontal transfer (42). Conversely, a plasmid carrying genes that promote the growth of its host, relative to competitor bacteria, will benefit by increased propagation. However, since maintenance of a plasmid is generally found to place a metabolic or phenotypic load on the cell, universally useful genes will be selectively reacquired by the chromosome over evolutionary time. Therefore, successful plasmids typically carry payload genes, favorable in some environments but not others (12), or genes with a transient plasmid association that are spreading through a microbial community. In passing through different strains or species, plasmids are exposed to different genetic contexts and provide the opportunity for combinations of genes to form new patterns of selective benefit. Thus, plasmids are key genetic elements in the diversity and adaptability of bacteria.

The genus *Pseudomonas* consists of a range of species that are found in diverse habitats. Many plasmids, conferring a range of phenotypes of both clinical and environmental importance, have been identified in members of this genus (43). To characterize the genomes of *Pseudomonas* species, it is therefore important to include a knowledge of the plasmid content of different strains of the species as well as the plasmids to which the species has access. Rapid classification of plasmids depends on the existence of DNA sequence information that can be exploited through hybridization or PCR to establish the relationship to previously studied plasmids (11). A key requirement is to establish the nature of the replication functions, since these will indicate the lineage and possible linked properties of the plasmid. While some *Pseudomonas* plasmids have been studied in detail (43, 45), little is known about many others, and consequently, existing probes have often been singularly unsuccessful in classifying new *Pseudomonas* plasmids from strain collections (26). A major purpose of our current work is to provide information to help rectify this defect. We have therefore set out to sequence the archetypes of the various *Pseudomonas* plasmid groups (7, 24).

Plasmid Rms149 was discovered in a clinical strain, *Pseudomonas aeruginosa* Ps142, from Frankfurt in Germany (37); it confers multiple antibiotic resistances. It has since been shown to replicate in *Escherichia coli* after mobilization by an IncP-1 plasmid (21, 22). Although tested against many plasmids, it remains the sole member of its designated incompatibility group (24). This may simply reflect the absence of intense work to classify plasmids by incompatibility testing and the absence of DNA sequence information for rapid screening. In this paper we report the complete sequence of Rms149. This should allow us to test for the distribution of this replicon.

* Corresponding author. Mailing address: School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom. Phone: 44 121 414 5903. Fax: 44 121 414 5925. E-mail: c.m.thomas@bham.ac.uk.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. For *E. coli* K-12 the strains used were as follows: DH5α [F^- *endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 recA1 gyrA96 relA1 deoR* Δ(*lacZYA-argF*)*U169* ϕ80*lacZ*ΔM15 λ^- *phoA*] (19) and

TABLE 1. Plasmids used in this study for functional analysis.

Plasmid	Relevant features[a]	Reference or source
Rms149	IncP-6, Cb^r Gm^r Sm^r Sp^r Su^r Mob$^+$	37
pUB307	IncP-1, deletion derivative of RP1, Km^r Tc^r Tra$^+$	4
pGEM-T Easy	Cloning vector for PCR products, received linearized Pn^r	Promega
pCR-4Blunt-TOPO	Cloning vector for shotgun sequencing, received linearized, Km^r Pn^r	Invitrogen
pAH149-1	Clone of Rms149 bases 54514 to 56668 (*repA*) in pCR-4Blunt-TOPO, Km^r Pn^r Rep$^+$	This work
pAH149-2	Clone of Rms149 bases 55315 to 349 (*parABC*) in pCR-4Blunt-TOPO, Km^r Pn^r Rep$^-$	This work
pAH149-3	Clone of Rms149 bases 55923 to 753 (*parABC*) in pCR-4Blunt-TOPO, Km^r Pn^r Rep$^-$	This work
pAH149-4	Clone of Rms149 bases 53432 to 55556 (′*repA*) in pCR-4Blunt-TOPO, Km^r Pn^r Rep$^-$	This work
pAH149-5	Clone of Rms149 bases 53922 to 56224 (*repA*) in pCR-4Blunt-TOPO, insert orientation is opposite pAH149-1 to 4, Km^r Pn^r Rep$^+$	This work
pAH149-6	Clone of Rms149 bases 4961 to 7198 [*int*I1 *aac*(3)-I] in pCR-4Blunt-TOPO, Gm^r Km^r Pn^r	This work
pAH149-7	Clone of Rms149 bases 4125 to 6601 [*int*I1′ *aac*(3)-I *aad*A5] in pCR-4Blunt-TOPO, Gm^r Km^r Pn^r Sm^r Sp^r	This work
pAH149-12	Derivative of pAH149-1 and pAH149-2 carrying Rms149 bases 54514 to 349, Km^r Pn^r	This work
pAH149-13	Derivative of pAH149-1 and pAH149-3 carrying Rms149 bases 54514 to 753, Km^r Pn^r	This work
pAH149-1r	Deletion derivative of pAH149-1 without vector replicon or penicillin resistance, Km^r	This work
pAH149-12r	Deletion derivative of pAH149-12 without vector replicon or penicillin resistance, Km^r	This work
pAH149-13r	Deletion derivative of pAH149-13 without vector replicon or penicillin resistance, Km^r	This work
pGEM-149mob	PCR product of mobilisation region, bases 30665 to 35361 cloned into pGEM-T Easy, Pn^r Mob$^+$	This work

[a] Antibiotic resistances: Cb, carbenicillin; Gm, gentamicin; Km, kanamycin; Pn, penicillin; Sm, streptomycin; Sp, spectinomycin; Su, sulfonamide. Other phenotypic traits: Mob$^+$, mobilizable; Tra$^+$, conjugative. For pAH149-1 to 5, Rep$^+$ and Rep$^-$ indicate ability to replicate in *P. putida* from a replicon on the Rms149 insert.

HB101 [F$^-$ Δ(*gpt-proA)62 leuB6 supE44 hsdS20*(r_B^- m_B^-) *ara-14 galK2 lacY1* Δ(*mcrC-mrr) rpsL20* (St^r) *xyl-5 mtl-1 recA13*] (8). For *E. coli* C, the strain used was: C2110 (*polA1 his rha* P2^S), obtained from D. R. Helinski, University of California, San Diego. For *Pseudomonas putida* the strain used was KT2440 (*hsdR1 hsdM*$^+$) (2). For *P. aeruginosa* the strain used was PU21 (FP$^-$ *ilvB112 leu-1 str^r-1 rif^r*) (23). Plasmids used or constructed during this work are described in Table 1.

In general standard microbiological techniques were used for growth and manipulation of bacteria. The standard medium was LB. Blood plates used were 40 g/liter tryptone soy agar with 50 ml/liter human blood. Minimal plates were 1.5% agar with M9 salts (6 g/liter NaH_2PO_4, 3 g/liter KH_2PO_4, 1 g/liter NH_4Cl, 0.5 g/liter NaCl) supplemented with 2 g/liter glucose, 0.1 μM $CaCl_2$, 1 mM $MgSO_4$ and 5 mg/liter thiamine. Antibiotics used at standard concentrations for work with *E. coli* were as follows: kanamycin, 50 μg/ml; penicillin, 150 μg/ml (broth) and 300 μg/ml (agar); and streptomycin, 30 μg/ml.

DNA isolation, manipulation, and sequencing. Large-scale purification of plasmid DNA from *P. aeruginosa* strain PU21 carrying Rms149 was performed using a slightly modified Birnboim and Doly technique followed by cesium chloride gradient purification from chromosomal DNA (39). Crude, small-scale purification of plasmid DNA during plasmid construction work used the modified Birnboim and Doly preparation only. The purified DNA was sheared and fragments were cloned using the TOPO shotgun subcloning kit from Invitrogen.

DNA preparation for sequencing used the QIAGEN MagAttract 96 miniprep system or in some cases Promega Wizard plus SV miniprep kits. Clones were initially sequenced using universal primers from both insert ends on an ABI 3700 sequencer. Finishing reads used custom primers on clones or PCR products. A few PCR products for sequencing or further study were cloned using the Promega pGEM-T Easy vector system.

The sequence was produced and assembled using the Phred/Phrap/Consed package (13, 14, 17, 18). Analysis and annotation of the sequence used Artemis and ACT (36) and WU-BLAST2 (16). Analysis of codon usage used the *Pseudomonas aeruginosa* PAO1 table (accessed from http://www.kazusa.or.jp/codon/).

Functional assays. Conjugative transfer was performed by mixing loops picked from selective plates of donor and recipient onto L agar plates with parental control patches spread singly alongside. After incubation overnight, a loop of the mating mixture and parental strains were streaked to single colonies on individual and combined selection plates for the desired plasmid and recipient bacterium.

Estimations of plasmid loss rates were performed as described previously (5). Transformants were restreaked onto selective agar before growth overnight with selection and then diluted into nonselective L-broth. Viable cell counts were made using selective and nonselective agar both before and after growth. The number of generations is given by $G = \log_2$(CFU per unit volume after growth/CFU per unit volume before growth). If the proportions of cells carrying plasmid before and after growth are given by P_0 and P_E, then the apparent loss rate (as the percentage of plasmid-free daughter-cells per division) is $L = (1 - (P_E/P_0)^{1/G}) \times 100$.

Nucleotide sequence accession number. The nucleotide sequence of Rms149 has been submitted to the EMBL database (accession number AJ877225).

RESULTS AND DISCUSSION

Overview. The 57,121-base-pair sequence of Rms149 was determined by sequencing a combination of cloned random fragments and PCR products. The entire range is covered by more than one clone or PCR and sequenced on both strands. Consed reports 0.0015 expected errors for this sequence. The average GC content is 59.5%, but using a 500-bp window, the content varies from 39 to 75%. The sequence was recursively analyzed for biologically relevant structures. Table 2 lists the 53 protein coding sequences identified, of which 10 appear to be inactivated by internal or upstream deletions or mobile element insertion. A map showing the locations of these coding regions is presented in Fig. 1. The functions of each open reading frame (ORF) were predicted by BLAST search of the EMBL databases: the products of 34 ORFs showed a predicted function by similarity to known proteins. Of these, five are predicted to be involved in replication and stable inheritance, six in transfer/mobilization, 14 in transposition, and nine in phenotypic determinants such as antibiotic resistance or virulence. The mosaic structure of Rms149 we have determined is shown in Fig. 2.

Replication and partition region. The putative genes for partitioning of Rms149 were identified by high sequence similarity (98% identity) to a 1.6-kb region of the plasmid pRA2, encoding an operon of three genes, *parABC*. In pRA2, the *parAB* segment confers segregational stability on low-copy-number plasmids (27). In Rms149 there is an additional gene downstream of these *par* genes. This encodes a product with weak similarity to several hypothetical replication proteins and strong similarity (71% identity) to the putative Rep protein of the recently sequenced IncU plasmid pFBAOT6 from *Aero-*

TABLE 2. Biologically relevant reading frames

Gene name	Coordinates and direction[a]	Size (no. of residues)	GC (%)	Most closely related gene product (EMBL accession no. of sequence)[b]	Product identity (%)	Proposed function
istA-1	434–1438	334	54	IstA of IS1326, e.g., on Tn*21* (AF071413)	100	Transposition, 5′ truncated, presumed inactive
istB-1	1425–2210	261	50	IstB of IS1326, e.g., on Tn*21* (AF071413)	100	Transposition, presumed inactive
orf5	2386–2886 c	166	65	Orf5 in 3′ conserved region of integron, e.g., In2 on Tn*21* (AF071413)	100	Not known; conserved in integrons
sul1	3014–3853 c	279	62	Sul1 in 3′ conserved region of integron, e.g., In2 on Tn*21* (AF071413)	100	Sulfonamide resistance, conserved in integrons
qacEΔ1	2847–4194 c	115	50	QacEΔ1 in 3′ conserved region of integron, e.g., In2 on Tn*21* (AF071413)	100	Minimal resistance to quaternary ammonium compounds
aadA5	4400–5188 c	262	58	AadA5, integron cassette (e.g., AF137361)	100	Streptomycin and spectinomycin resistance
aac(3)-I	5293–5757 c	154	55	Aac(3)-I, integron cassette (AF318077)	95	Gentamicin resistance
intI1	5909–6922	337	61	IntI1 in 5′ conserved region of integron, e.g., In2 on Tn*21* (AF071413)	100	Gene cassette integration
tnpA-1	7270–7836 c	188	67	DNA recombinase of composite transposon on pFBAOT6 (CR376602)[c]	100	Transposition
ofn10	7935–8186	83	63	Unannotated open reading frame on pFBAOT6 (CR376602)	100	Unknown
ofn11	8347–8769 c	140	63	No significant protein matches		Unknown
ofn12	8766–9248 c	160	56	72% DNA identity to region of *Xylella fastidiosa* (AE003901)	-	Unknown
repB	9366–10238, 30756–30944 c	353	65	C-terminus of polyprotein of pRAS3.2 (AY043299); equivalent to RepB in other IncQ-like plasmids	97	Primase of IncQ replicon; not required by Rms149, interrupted by Tn*1011*
istB-2	10370–11110 c	246	57	IstB on pB4 (AJ431260)	52	Transposition
istA-2	11107–12657 c	516	59	IstA on pB4 (AJ431260)	45	Transposition
ofn15	13350–14351	333	58	No significant matches		Unknown
tnpA-2	14727–15707 c	326	60	Transposases, e.g., TnpA3 of ISPre2 on pCAR-1 (AB088420)	95	Transposition
hlyB	14559–14579, 15776–17914	719	61	Hemolysin export, ABC transporter protein of *Photorhabdus luminescens* subsp. *laumondii* (BX571861)	80	Toxin secretion ABC transporter protein-ISPa*16* insertion, presumed inactive
hlyD	17911–19326	471	63	Hemolysin export, membrane fusion protein of *Photorhabdus luminescens* subsp. *laumondii* (BX571861)	64	Toxin secretion, presumed inactive
hlyH	19362–20669	435	56	Probable glycosyltransferase of *Chromobacterium violaceum* (AE016911)	62	Possible glycosyltransferase, presumed inactive
hlyI	20656–21429	257	43	No significant matches		Toxin pathway related? presumed inactive
hlyJ	21414–30608	3064	58	Putative hemolysin, *Ralstonia solanacearum* (AL646058) (after removing annotated IS)	41	Putative hemolysin, presumed inactive
mobA	30687–32492 c	601	61	N-terminus of polyprotein of pRAS3.2 (AY043299); equivalent to MobA in other IncQ-like plasmids	87	Mobilization presumed functional; reads in to Tn*1011*
mobB	32542–32853 c	103	58	MobB, IncQ-like plasmids, e.g. pTF-FC2 (M57717) (unannotated in this plasmid)	92	Mobilization
mobC	33165–33521	118	55	MobC, IncQ-like plasmids, e.g., pRAS3.2 (AY043299)	98	Mobilization
mobD	33534–34217	227	53	MobD, IncQ-like plasmids, e.g., pRAS3.2 (AY043299)	99	Mobilization
mobE	34210–34851	213	60	MobE, IncQ-like plasmids, e.g., pTF-FC2 (M57717)	93	Mobilization
orfX	34848–35300, 44177–44408	228	55	OrfX, IncQ-like plasmids, e.g., pRAS3.2 (AY043299) (unannotated in this plasmid)	97	Mobilization?, possibly inactive due to Tn*1012* insertion
tnpA-3	35336–38305 c	989	65	Transposase of Tn3 family transposon on pFBAOT6 (CR376602)	95	Transposition
ofn30	38541–39077 c	178	63	Transposase-like protein of apparent insertion into mobile genome island TNCP23 on plasmid pKLC102 from *P. aeruginosa* (AY257539)	100	Transposition?
ofn31	39089–39484 c	131	62	Hypothetical protein of apparent insertion into mobile genome island TNCP23 on plasmid pKLC102 from *P. aeruginosa* (AY257539)	100	Unknown

Continued on following page

TABLE 2—*Continued*

Gene name	Coordinates and direction[a]	Size (no. of residues)	GC (%)	Most closely related gene product (EMBL accession no. of sequence)[b]	Product identity (%)	Proposed function
ofn32	39481–39732 c	83	62	Hypothetical protein of apparent insertion into mobile genome island TNCP23 on plasmid pKLC102 from *P. aeruginosa* (AY257539)	100	Unknown
tnpA-4	39914–40480	188	67	Recombinase of apparent insertion into mobile genome island TNCP23 on plasmid pKLC102 from *P. aeruginosa* (AY257539)[c]	100	Transposition
ofn34	40615–40905	96	63	Putative nucleotidyltransferase of Tn*3* family transposon on pFBAOT6 (CR376602)	98	Unknown
ofn35	40902–41339	145	61	Hypothetical protein of Tn*3* family transposon on pFBAOT6 (CR376602) (C-terminus affected by insertion/deletion, majority is 92% identical)	87	Unknown
invA	41333–41890	185	63	Recombinase of Tn*3* family transposon on pFBAOT6 (CR376602)	95	Transposition
gipA	41987–43105 c	372	55	Peyer's patch-specific virulence factor of lysogenic phage Gifsy-1, *Salmonella enterica* (AF246666)	72	Transposition
blaA	43233–44114	293	60	β-Lactamase, *Aeromonas hydrophila* (U14748)	58	Carbenicillin resistance
tnpA-5	44471–45298 c	275	64	Hypothetical exonuclease in bacteriophage 186 (U32222)	45	Transposition (putative)
kfrA	45684–46574 c	296	73	KfrA of IncP-1 plasmids, e.g., pB4 (AJ431260)	38	Replication/partition associated
ofn41	47109–48023 c	304	62	C-terminal (major) part of hypothetical proteins, e.g., of *Pseudomonas aeruginosa* (AE004827)	58	Unknown
ofn42	48062–48352 c	96	60	N-terminal (minor) part of hypothetical proteins, e.g., of *Pseudomonas aeruginosa* (AE004827)	41	Unknown
ofn43	48470–48850 c	126	64	Conserved hypothetical protein of Tn*3* family transposon on pFBAOT6 (CR376602)[d]	84	Unknown
ofn44	48847–49173 c	108	63	Conserved hypothetical protein of Tn*3* family transposon on pFBAOT6 (CR376602)[d]	94	Unknown
ofn45	49197–49532 c	111	59	Putative transposase repressor of unmarked transposon on plasmid pND6-1 (AY208917)	100	Unknown
ofn46	49547–49882 c	111	58	Hypothetical protein of unmarked transposon on plasmid pND6-1 (AY208917)	100	Unknown
ofn47	49863–50186 c	107	59	Hypothetical protein of unmarked transposon on plasmid pND6-1 (AY208917)	100	Unknown
tnpR-6	50399–51034	211	64	Resolvase of unmarked transposon on plasmid pND6-1 (AY208917)	100	Transposition
tnpA-6	51018–54047	1009	61	Transposase of unmarked transposon on plasmid pND6-1 (AY208917)	100	Transposition
repA	54559–55920 c	453	66	Putative replication protein of broad-host-range IncU plasmid pFBAOT6 (CR376602)	71	Replication
parC	56086–56472 c	128	54	Hypothetical protein of pRA2, *Pseudomonas alcaligenes* (U88088)	98	Partitioning
parB	56509–56727 c	73	61	Partitioning protein ParB of pRA2, *Pseudomonas alcaligenes* (U88088)	100	Partitioning
parA	56749–57121, 1–266 c	211	64	Partitioning protein ParA of pRA2, *Pseudomonas alcaligenes* (U88088)	100	Partitioning

[a] c indicates the ORF is present on the complementary strand.
[b] Where similar strength matches are found in multiple genera, organism names are not given.
[c] TnpA-1 and TnpA-4 are identical. Different matches are given to represent the different contexts.
[d] The DNA sequence covering *ofn43* and *ofn44* is highly conserved in an unmarked transposon on plasmid pND6-1, but these reading frames are not annotated in favor of an open reading frame in the opposite direction.

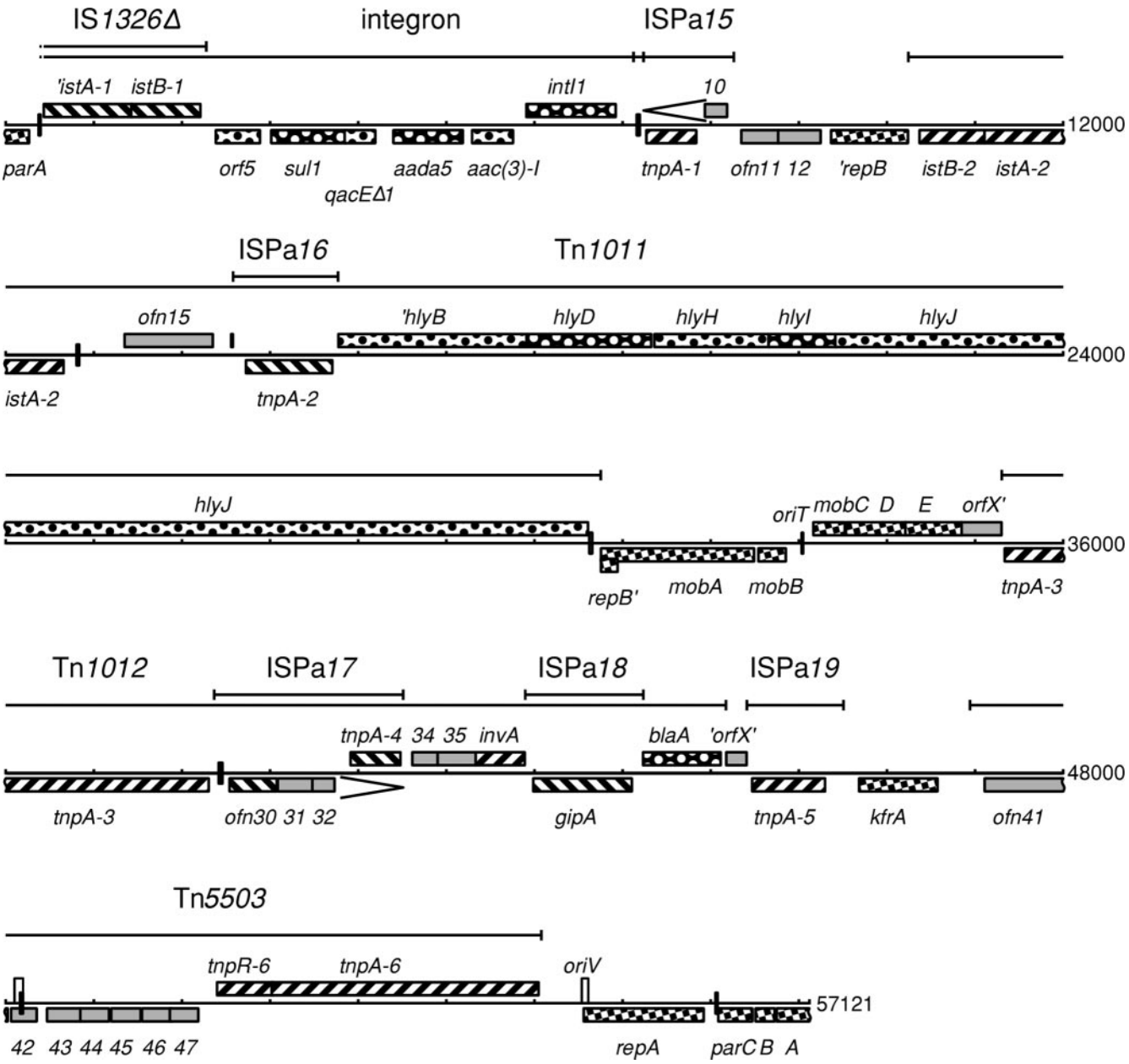


FIG. 1. Linear map of Rms149. Bars above the map indicate the extent of mobile elements. Genes are shown above (sense strand) or below (complementary strand) the DNA line and filled according to known or putative function. Genes involved with plasmid functions are patterned with alternating squares, and phenotypic load genes are dotted. Genes involved with transposition are striped, with the direction being reversed for secondary insertions. Open reading frames of unknown function are shown in grey. Stem-loops are shown as black boxes over the DNA. The series of repeats in *ofn42* is shown as a white box. The repeat between *tnpA-1* and *tnpA-4* is shown by chevrons.

monas caviae (34). In pRA2 this gene is absent and the region is bordered by a transposon downstream of *parC*.

To determine whether the region containing this gene is indeed capable of replication, several shotgun library clones were tested for their ability to transform *P. putida* strain KT2440, in which vector replicon is nonfunctional. It was found that clones carrying the downstream gene (and some fraction of *parC*) produced colonies, while control clones carrying *parABC* or other segments of Rms149 did not. Although small-scale plasmid DNA preparation did not yield enough DNA to visualize by agarose gel electrophoresis, the extracted DNA could be used to generate further transformants with competent bacteria. This confirms that free plasmid DNA was present even if the copy number was very low. We named this gene *repA*.

Since both orientations of the *repA* insert within the vector were found to allow growth in KT2440, it seems that replication ability is independent of transcription from the vector, and it is therefore likely that a promoter exists upstream of *repA*. We were unable to identify any close matches to promoter

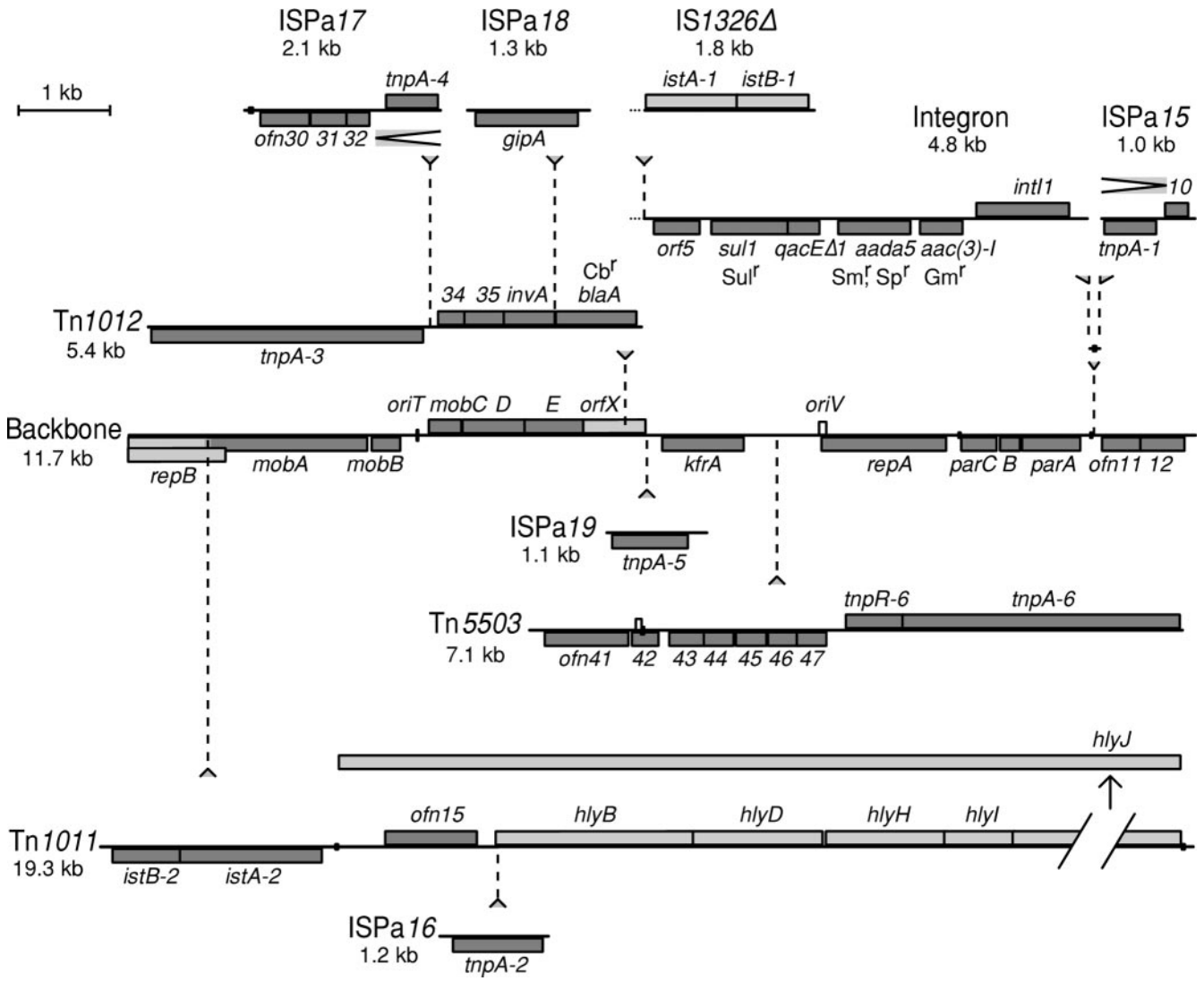
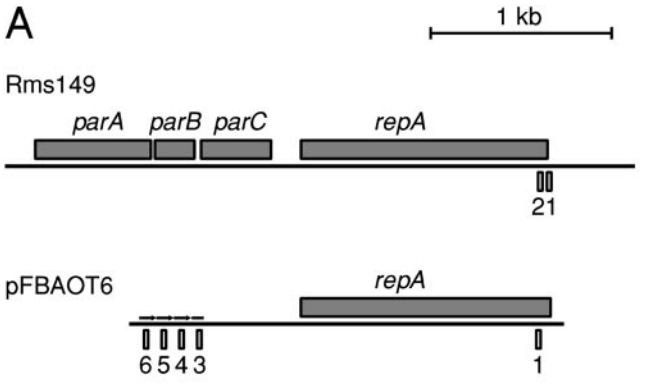


FIG. 2. Map of Rms149, showing insertions into the plasmid backbone. DNA is represented by black horizontal lines, with identified open reading frames as grey boxes above (sense strand) or below (complementary strand). Light grey boxes indicate apparently nonfunctional genes. Insertions of mobile elements are indicated by vertical dotted lines leading to the element. Stem-loops are shown as black boxes centered on the DNA. The tandem repeats in Tn*5503* and the putative *oriV* are shown as white boxes. Terminal repeats of mobile elements are not shown. The deletion from inside IS*1326* is indicated by a dotted DNA strand. The gene *hlyJ* has been shown as an outgroup because of its size. The large repeat between ISPa*15* and ISPa*17* is shown by chevrons.

consensus boxes in this sequence, although we did find a putative promoter as a weak match. We did not find an obvious candidate for a replication origin. However, overlapping the 3′ end of the *repA* protein coding sequence are two repeats of the sequence GTGAGACA(G/A)GT(T/C)CCTCCCCTATTAC, which may be part of the origin of replication. In addition, although there is only one copy of the repeat at the 3′ end of the homologous gene in pFBAOT6 (34), there are three copies separated by 73-bp spacers 548 bases upstream of the start codon, followed by a copy with a single-base-pair deletion (Fig. 3).

One reason for the very poor yield of plasmid DNA from clones with just *repA* in *P. putida* may be a high rate of segregational instability due to low copy number and lack of a partitioning system. Using clones from the random shotgun library digested with BstAPI and BstXI (which cut once in the insert and once in the vector, respectively), it was possible to construct plasmids carrying both *parABC* and *repA*. Surprisingly, the addition of the *parABC* region did not appear to improve the yield of plasmid DNA.

Hedges and Jacoby (21) reported that Rms149 could be mobilized to *E. coli*, implying that it was also capable of replication in this species. We wished to verify this property for the *rep* region identified. The shotgun library clones can replicate in *E. coli* under the control of the high-copy-number vector (pMB1) replicon, so we attempted to introduce these plasmids by transformation into C2110, a *polA* mutant strain of *E. coli* C in which the pMB1 replicon is known not to function. All attempts with plasmids carrying *repA* or *parABC-repA* of Rms149 proved negative. We concurrently transformed *E. coli*



B

Rms149

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1 agcgcaaagc gtgagacaagtccctcccctattac taatggcgaa
2 atgccagcct gtgagacaggttcctcccctattac agcctccaag
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pFBAOT6

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1 caacaaaaca gtgagacaagtccctcccctattac taatggcgaa
3 cctcaatgtt gtgagacaagtccctcccctattac agggcgggcc
4 cctcgatgcg gtgagacaagtccctcccctattac aggcggcagc
5 cctcgatgcg gtgagacaagtccctcccctattac aggcggcagc
6 cctcgatgcg gtgagacaagtccc.cccctattac aggcggcagc
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FIG. 3. Comparison of proposed replicons of Rms149 and pFBAOT6. A. Comparison of putative replicon structure. Sense-strand protein-coding regions are indicated by grey boxes above the black DNA line. Repeats putatively involved with replication are represented by numbered boxes below the DNA line. Repeats previously identified in pFBAOT6 (34) are shown as black arrows above the DNA line. The region of Rms149 shown is flanked by the truncated integron on the left and Tn*5503* on the right. The region of pFBAOT6 shown is flanked by Tn*1721* on the left and a further series of repeats on the right. B. Alignment of repeats proposed to be involved in replication. Ten surrounding bases are shown for each repeat. Repeat 2 of Rms149 has two base mismatches compared to the other repeats (underlined), while repeat 6 of pFBAOT6 has a single base deletion.

DH5α with samples of the same DNA and transformed the competent *E. coli* C2110 with a control plasmid. All these control transformations produced colonies. This suggests that this minimal IncP-6 replicon is either not functional in *E. coli* or is unable to function without DNA polymerase I.

To distinguish between the above possibilities, we deleted the pMB1 replicon from *repA* and *parABC-repA* plasmids in vitro and transformed *P. putida*, producing kanamycin-resistant but penicillin-sensitive clones. When plasmid DNA from these were used to transform *E. coli* DH5α, colonies were obtained. Plasmid DNA prepared from these transformants yielded faint bands with the expected restriction patterns. The mini replicon plasmids that we constructed could not be introduced into *E. coli* C2110. These results strongly suggest that the IncP-6 replicon depends on DNA polymerase I for replication.

Plasmid stability. To determine whether the *parABC* region is important for stabilization of the replicon, we compared the stability of plasmids carrying only *repA* with that of two plasmids carrying *parABC* and *repA*. Of the two plasmids with *par* regions, one carries all the sequences that appear to be involved with partitioning, while the other one has the *parABC* coding region and the consensus promoter boxes but lacks one of three ParB regulatory sites identified in pRA2 (27). In *E. coli*, all three plasmids appeared to be stable, with 100% retention after approximately 20 generations of growth in nonselective medium. In *P. putida*, however, all three plasmids appeared very unstable, with a very low proportion of cells carrying the plasmid even after preliminary growth in selective broth. All had loss rates of 22 to 25% per generation over the 20-generation experiment. This suggests that there may be additional factors encoded by the plasmid that normally are involved in plasmid replication or stability.

It may be that the regions we have cloned are not the full sequence required for efficient replication or partitioning in some species. Downstream of *repA*, a transposon has inserted (Tn*5503*). However, beyond this insertion there appears to be a further region of plasmid backbone, encoding a predicted protein with significant identity and similar general characteristics to KfrA encoded by IncP-1 plasmids, particularly a predicted high α-helical content and heptad repeats characteristic of coiled-coil proteins (10). Such genes are found close to replication or partition regions of plasmids from many incompatibility groups (1). Upstream of the *par* region, beyond an integron insertion, are two orphan genes. Either or both of these regions may be necessary for reliable replication or full stability in *Pseudomonas* species, or alternatively the IncP-6 replicon may not be fully functional in the *P. putida* strain we used.

Mobilization. A 5.6-kb region similar to the mobilization cassette of the IncQ-like plasmids pTF-FC2 (35) and pRAS3 (29) is present within Rms149. This places it in the MOB_P family of mobilization regions (15). Transposons have inserted close to both ends of the region, truncating the *mobA* and *repB* gene products at one end and an unnamed open reading frame at the other. Although Hedges and Jacoby reported mobilization of Rms149 (21), attempts here using the IncP-1-mobilizing plasmids pUB307 and R751 failed to reproduce this. As an alternative way to test this property, the putative *mob* region between the two transposons was amplified by PCR and ligated into pGEM-T Easy to produce pGEM-149mob. This plasmid could be mobilized from *E. coli* DH5α to *E. coli* HB101 using pUB307, an IncP-1α plasmid lacking Tn*1* and not conferring penicillin resistance. No mobilization was obtained with the negative control, a nonmobilizing pGEM derivative.

Insertions into the putative plasmid backbone and phenotypic determinants. Rms149 carries several mobile elements, as is apparent from the number of transposition-related genes (Fig. 1 and Table 2). Figure 2 presents the genetic map redrawn so as to make clear how the backbone components and the transposons and insertion sequences are integrated. The central core of the plasmid consists of the replication, stable inheritance, and mobilization functions. Into this are inserted three major transposons, a partial, composite transposon carrying an integron, and one putative insertion sequence. A further four insertion sequences are inserted into these transposons. These insertions into the backbone are described below, starting with the integron, which provides the majority of the known phenotypic determinants.

In2-related truncated integron. This integron is very similar to In2, a widespread element (9) originally discovered in Tn*21* (32), and the related In0 from pVS1 (6). It contains the insertion sequence IS*1326*, which is also present in In0 and In2.

However, a deletion has removed the integron's transposition gene-proximal terminus and part of this insertion sequence, which probably leaves both elements immobile.

The integron expression operon retains the conserved downstream-end genes *qacEΔ1*, *sul1*, and *orf5*. In addition, the first two integrated gene cassettes are highly related to genes known to confer gentamicin resistance (EMBL accession no. AF347074) and streptomycin-spectinomycin resistance (38). We have confirmed the resistances conferred by these two genes in *E. coli*, using shotgun-cloned DNA segments expressed from the integron promoter, by streaking to single colonies on selective media.

ISPa*15*. This 1.0-kb element lies adjacent to the In2-related integron and is separated from it by a 114-bp segment. The integron terminal repeats are similar to ISPa*15* termini: the remaining integron repeat in Rms149 matches 23 of 25 bp, in highly related integrons, the other one is identical. It therefore seems likely that ISPa*15* would be capable of mobilizing a complete integron as a composite transposon. This would be consistent with the absence of direct repeats of insertion for ISPa*15*.

The 114-bp fragment between ISPa*15* and the integron has several perfect matches in the EMBL nucleotide databases. Where neighboring sequence is available, these matching sequences themselves appear to be between an In2 family integron on one side and an ISPa*15*-related insertion sequence on the other. In particular, plasmid pFBAOT6 (34) appears to have a complete composite transposon, comprising the structure described above of an In2-like integron, a 114-bp spacer, and an ISPa*15*-like element, and in addition a different insertion sequence element on the other side of the integron. This opposing element has the same terminal repeats as ISPa*15*, and the whole structure is flanked by 5-bp direct repeats, indicating that it has moved as a unit. Therefore, we propose that in Rms149, a structure including at least ISPa*15* and the integron previously formed a composite transposon.

On Rms149, a 715-bp global inverted repeat of 99% identity is a match of the majority of ISPa*15* to ISPa*17*. A second small, divergently expressed open reading frame exists in the nonduplicated portion of ISPa*15*.

Tn*1011*. This transposon is the largest element (19.3 kb) in the Rms149 mosaic and is inserted in the *mobA-repB* region, presumably inactivating *repB* but leaving the Mob function of *mobA* intact. Its transposition appears to be directed by a two-component transposase. At the termini there are multiple adjacent, degenerate repeats (four on the left, three on the right). The repeats are extended by a few bases at the outside ends to uniquely define the termini. Outside these there are direct repeats of 6 base pairs.

Tn*1011* carries an operon of five genes, *hlyBDHIJ*, which we interpret to encode a pathway for the production and secretion of a large, repetitive hemolysin-like protein (HlyJ). The protein products of the first two genes, HlyB and HlyD, exhibit 80 and 64% sequence identity, respectively, across their entire length to known proteins involved in hemolysin export, while the fifth, HlyJ, shows a lower but still significant similarity (41% identity) to hemolysin proteins. The third encoded protein, HlyH, may be involved in glycosylation of one of the other proteins, as it is similar to several probable glycosyltransferases. The closest match found (62% identity) is expressed from an operon between the *hlyBD* and *hlyJ*-like genes. We could find no related proteins for the fourth encoded protein, HlyI.

The putative *hly* operon appears to have been rendered inactive by the simple insertion of the IS*5* family insertion sequence ISPa*16*, just after the start of *hlyB*. This insertion has created a direct repeat of the 4-base-pair target site CTAG, typical of elements in this family. *P. aeruginosa* PU21(Rms149) did not produce halos when streaked onto blood plates, which would have been expected if it were producing active hemolysin (data not shown).

The GC content of the end of *hlyH* and *hlyI* and the start of *hlyJ* is relatively low, averaging 43%, so this region may have been integrated from another source, or alternatively the high AT content may have a structural purpose. Because of the low G+C content there will be many stop codons on a random basis unless counterselected, as in a protein-coding sequence. However, it was striking that the extra stop codons in this region were predominantly in one of the two alternative reading frames over both *hlyI* and the start of *hlyJ*. The expected stop codon frequency given the GC content is 5.7% for *hlyI* and 5.5% for the first 1,500 bases of *hlyJ*. The plus-one reading frame over both regions matches quite well to this (5.0% for *hlyI*, 5.6% for *hlyJ*). However, the plus-two reading frames have two to three times more stop codons than expected (13.2% for *hlyI*, 14.8% for *hlyJ*). We do not have an explanation for this.

Tn*1012*. This transposon consists of a gene encoding a transposase divergently transcribed from an operon of three or four ORFs, including an invertase and a probable β-lactamase gene. It is also disrupted by two probable insertion sequences, one between the two transcriptional units and the other inserted just upstream of the β-lactamase. However, since the plasmid confers carbenicillin resistance and this is the only such gene, we conclude that the *bla* gene is still functional. The termini of the transposon are flanked by 5-bp direct repeats, suggesting that Tn*1012* was acquired as a simple insertion into *orfX* without loss of information.

The first of the two insertions in Tn*1012*, ISPa*17*, appears to encode a transposase divergently from an operon of three genes ending with a second transposase-like gene. This structure is similar to that of Tn*1012* prior to ISPa*17* insertion. ISPa*17* has a perfect, full-length bounded match in the sequence of transposon TNCP*23* (of plasmid pKLC102 from *P. aeruginosa*) (25). In TNCP*23*, unlike in Rms149, it does not have direct repeats beyond its termini, which is presumably why it was not identified as an autonomous unit.

The second element disrupting Tn*1012*, ISPa*18*, encodes a single protein most similar to GipA, a transposase-like protein from the lysogenic phage Gifsy-1. While in the bacteriophage this gene is reported to confer virulence (40), it is flanked by matching palindromic repeats which are similar to those of ISPa*18*. Further, this protein shows similarity to the transposase of the IS*605* family. This family does not have inverted repeats at its termini and does not generate direct repeats on insertion. Therefore, we identified the ends of this insertion sequence by comparing the alignment of several related sequences identified in a BLAST search.

Putative ISPa*19*. We have identified what we believe may be a new kind of insertion sequence element. This would contain one ORF (*tnpA-5*) and be bordered by three 20-bp imperfect

repeats at each end, the terminal repeats being extended by 9 bp at their outside ends. At the presumed promoter-proximal end, the repeats are separated, while at the other they are adjacent.

TnpA-5 has reasonable similarity to several putative exonucleases. There are no direct repeats bordering this sequence. However, the homology of Rms149 to pRAS3.2 stops at the insertion sequence border. This suggests that a deletion involving two insertion sequence copies in direct repeat may have occurred.

Tn*5503*. This Tn*3* family transposon shows 99% identity to a transposon on pND6-1 (31) and 97% to Tn*5501* (30), a plasmid-carried transposon of *P. putida*. However, after a partial terminal inverted repeat-like sequence, this transposon encodes an additional two genes followed by a full-length terminal repeat. This extended structure is similar in nature to that described for Tn*5502* (30). Outside the terminal repeats are 5-bp direct repeats, suggesting that these represent the ends of an element that was inserted as a single unit. The main part of the transposon encodes a two-component transposase system divergently from an operon of five small genes. Expression from these may continue into the extender region.

Taken together, the two open reading frames of the extra sequence show similarity to almost the whole of a single ORF in the *P. aeruginosa* PAO1 genome. In the first of the two Rms149 ORFs, there is a region which is not present in the *P. aeruginosa* PAO1 sequence. It consists of a 16-bp-stem perfect stem-loop that overlaps the first in a series of repeats by 6 bp. The repeats consist of two 9-bp repeats of sequence CCCA GAGAG, followed by seven 7-bp repeats of sequence CCCA GAG. One possibility is that this region may be involved in programmed genetic variation; a mutation deleting one (or adding two) 7-bp repeats from the current set of seven would create a fusion protein of *ofn42* and *ofn41*.

Relationship of Rms149 sequences to Tn*21*. Rms149 has two regions of similarity to Tn*21*, a transposon that carries an integron between the transposition and mercury resistance cassettes (32). The similar regions are the integron (excluding two captured antibiotic resistance genes) and the transposase and terminus of Tn*1012*. Adjacent to these is a long inverted repeat from the related insertion sequences ISPa*15* and ISPa*17*. Therefore, a recombination event could rearrange these sequences into a conformation similar to that of Tn*21*. Conceivably, this process may have proceeded "in reverse"; a Tn*21*-like transposon could have been broken up. That this is not the case is clear from the direct repeats on either side of ISPa*17* and Tn*1012* itself, as well of the identification of an alternative, composite transposon carrying an integron on pFBAOT6. Indeed, the existence of this composite transposon in forms both with IS*1326* (Rms149) and without (pFBAOT6) suggests that the insertion sequence insertion occurred in this context. Subsequent transfer of the IS*1326*-carrying integron has yielded a family of integrons, including In0, In2, and In5, in a variety of locations (9).

Propagation and descent of Rms149. Given the large number of apparent insertion sequence function knockouts in this sequence, we were concerned that the plasmid may have evolved during subculture by removal of functions advantageous in the clinical context. However, Hedges and Jacoby (21) presented an EcoRI digest pattern for Rms149 which consists of four visible bands representing fragments with estimated sizes of 16, 12.6, 6, and 3 kb (after conversion from MDa). Within the degree of accuracy that is possible from the molecular weight markers available at the time and the relationship between size and mobility, this matches the predicted pattern from our DNA sequence. Were any of the detected mobile elements to have inserted after the initial restriction mapping, this would either modify the restriction pattern or require a larger error in estimation of fragment sizes. Similarly, the 3′ conserved fragment of the integron is 100% identical to that of In2, as far as the truncation point, and the integron of pFBAOT6 up to the IS*1326* insertion. If this conservation were to continue to the end of the integron (9) or the end of a composite transposon, as in pFBAOT6, then the restriction map would be clearly different prior to the deletion.

However, we cannot exclude the loss of DNA mediated by a direct duplication and resolution of the putative element ISPa*19*. Indeed, the neighboring mobilization region's similarity terminates at one border of the element, suggesting that just such a deletion has occurred. The restriction fragment in the digestion pattern of Hedges and Jacoby (21) that we interpreted as covering this region equates to 12.6 kb, while we determined a size of 11.7 kb. The removal of up to around 1.7 kb by ISPa*19* duplication and deletion by homologous recombination would increase the agreement between the two restriction patterns.

Conclusions. Plasmids can be divided into two general groups: small, high-copy-number plasmids and larger, low-copy-number plasmids. Large plasmids are often self-transmissible, encoding a system that enables their horizontal transmission to other bacteria. Small plasmids, on the other hand, are often mobilizable, parasitizing a conjugative plasmid to transfer if one is present in the cell. From the size of Rms149, we expected it to fall into the former category, but the sequence reveals it to have characteristics of both smaller mobilizable plasmids and larger low-copy-number plasmids.

The largest group of backbone genes (*mobA*, *mobB*, *mobC*, *mobD*, and *mobE*) are those related to the IncQ superfamily of plasmids, but rather than providing both replication and mobilization, they only appear to provide mobilization functions. This is significant because IncQ-like plasmids use a sequential single-strand displacement mechanism of replication, where the DNA strands are synthesized separately. This may result in structural instability when plasmid size grows too large (33), and would thus not be compatible with expansion to 57 kb. Also, as the IncQ family replicon is optimized for high copy number, it does not include a partitioning system, so the low copy number necessary to prevent a plasmid of this size from being a significant burden on its host would result in segregational instability (3). It thus makes sense that the plasmid carries a different replicon, linked to widely distributed active partitioning genes.

Accumulating studies on the backbone functions of plasmids suggest that generally, once maintenance functions have been acquired by a plasmid, there is optimization of clustering and/or coregulation of the genes (41). In the case of Rms149, the *mob* block comes from a long lineage, while the *rep-par* region, although showing suitable integration, is composed of segments that are also found with other partners, suggesting relatively recent mixing and matching of these functions. Un-

surprisingly, the relationship between the *mob* and *rep-par* systems does not reveal any evidence of optimization of organization or regulation. Plasmid pRA2, carrying the closest relative of the Rms149 *par* region, has a different *rep* system and distantly related *mob* and *kfrA*, although some of the linear organization of the plasmid is similar (28). Plasmid pFBAOT6, carrying the closest relative of the *rep* region, again has a distantly related *kfrA* gene and a different *par* and *mob*/*tra* system (34). It may be significant that although Rms149 has a novel combination of backbone functions, almost all are related to other known systems rather than representing novel replication, transfer, or partitioning genes. This is consistent with the view that a limited number of different plasmid module types are available for construction of new plasmids.

While we have emphasized that Rms149 carries a large amount of genetic baggage, this just represents a rather extreme version of a general tendency for plasmids to consist of a high proportion of mobile elements of one sort or another. We have previously noted how the multiple genetic layers of a plasmid can be uncovered by careful analysis of the DNA sequences present, as for example in our study of the TOL plasmid pWW0 (20). Our dissection of Rms149 illustrates this very clearly, although other plasmids have been found to have an even higher proportion, with 53% of identified genes encoding transposition functions (44). Apart from the antibiotic resistance determinants, the potential phenotypic determinants in Rms149 are probably nonfunctional. The putative secretory pathway on the large transposon Tn*1011* takes up 15 kb but is apparently rendered inactive by insertion sequence ISPa*16*. While we cannot exclude the possibility that this insertion occurred during laboratory culture prior to the restriction mapping, it may be that it is a sign of rapid evolution in the clinical context, with conferred phenotypes switching rapidly between advantageous and detrimental. These lessons from Rms149 should help us to add to the general rules that apply to plasmid genome evolution, an important part of bacterial genomics.

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