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Different Pathways to Acquiring Resistance Genes Illustrated by the Recent Evolution of IncW Plasmids▽†

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DNA sequence analysis of five IncW plasmids (R388, pSa, R7K, pIE321, and pIE522) demonstrated that they share a considerable portion of their genomes and allowed us to define the IncW backbone. Among these plasmids, the backbone is stable and seems to have diverged recently, since the overall identity among its members is higher than 95%. The only gene in which significant variation was observed was *trwA*; the changes in the coding sequence correlated with parallel changes in the corresponding TrwA binding sites at *oriT*, suggesting a functional connection between both sets of changes. The present IncW plasmid diversity is shaped by the acquisition of antibiotic resistance genes as a consequence of the pressure exerted by antibiotic usage. Sequence comparisons pinpointed the insertion events that differentiated the five plasmids analyzed. Of greatest interest is that a single acquisition of a class I integron platform, into which different gene cassettes were later incorporated, gave rise to plasmids R388, pIE522, and pSa, while plasmids R7K and pIE321 do not contain the integron platform and arose in the antibiotic world because of the insertion of several antibiotic resistance transposons.

Naturally occurring plasmids tend to fall into coherent genetic clusters commonly known as incompatibility groups (21). This is in contrast to the genetic organization of bacteriophages, which show extreme modularity, generating great diversity by the permutation of modules (82). Plasmids of a given incompatibility group normally share the mechanisms of replication (1), copy number control (25), and other maintenance functions (27; for reviews see references 5, 26, and 55). They thus function as a single pool in the bacterial cell and due to random selection for replication or partitioning will segregate cells with one or the other plasmid but not both. They are thus said to be incompatible. From the many incompatibility groups described, a few have been analyzed in detail. Some of the best-known incompatibility groups are those within the IncF complex (IncFI and IncFII) (13, 58), the IncPs (2, 9), the IncQs (62, 63), and the IncH group (33). Although incompatibility is not always associated with sequence identity, some incompatibility groups show a conserved genetic backbone. Their members generally share an essential gene set for plasmid survival, differing in the presence of mobile genetic elements (MGEs) harbored in their genomes. Plasmids can thus be considered vehicles carrying cargos of transposons, integrons, and insertion sequences that move across bacterial populations. IncP, IncN, and IncW groups have a broad host range, so they might serve as MGE shuttles between different species. The role of horizontal gene transfer in bacterial evolution is well documented (23, 56). A planetary-scale experiment in bacterial evolution took place when antibiotics were introduced by humans around 1950 (49). The result was that antibiotic-sensitive bacteria were rapidly replaced by bacteria that had acquired mobile pieces of DNA containing antibiotic resistance genes. Among them, transposons and integrons account for a large portion of the antibiotic resistance genes carried by plasmids and other MGEs (31).

The IncW group takes its name from T. Watanabe, who described the first member of the group, pSa (81). The IncW group includes three "classical" members: pSa (81), R388 (22), and R7K (17). Electron microscope heteroduplex studies (34) and restriction enzyme maps of these plasmids (6, 45, 78, 80) underscored the high DNA sequence homology between them. Physical and genetic maps of these plasmids were drawn based on information from many authors (79). R388 has been studied in the most detail, and its complete sequence has been used to infer many features of the family (28). In this work, we analyzed the DNA sequences of five IncW plasmids in order to ascertain their diversity and evolution. These are pSa, R388, R7K, and two more recent isolates, pIE321 and pIE522 (35). IncW plasmids are less than 40 kb in size (Table 1) and have been isolated from different species of *Enterobacteriaceae*; pSa was obtained from *Shigella* (81), R388 from *Escherichia coli* (41), R7K from *Providencia rettgeri* (17), pIE321 from *Salmonella dublin*, and pIE552 from *Klebsiella pneumoniae* (35). The host range of IncW plasmids, under laboratory conditions, has

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TABLE 1. General features of IncW plasmids

Plasmid	Original host (year of isolation) (reference)	Size (bp)	GC content (%)	No. of genes	No. of indels in the backbone	Antibiotic resistance profile[a]
R388	*E. coli* (1972) (22)	33,920	57.5	43	1	Tp^r, Su^r
pIE321	*Salmonella enterica* (1996) (35)	38,151	58.4	46	2	Tc^r, Sm^r
R7K	*Providencia rettgeri* (1972) (17)	39,792	56.7	42	3	Ap^r, Sm^r, Sp^r
pIE522	*Klebsiella pneumoniae* (1996) (35)	~38,000		42	~1[b]	Gm^r, Km^r, Su^r, Tob^r
pSa	*Shigella* sp. (1968) (82)	~39,000		47	~1[b]	Cm^r, Gm^r, Km^r, Sm^r, Sp^r, Su^r

[a] Tp^r, resistance to trimethoprim (20 μg/ml); Su^r, resistance to sulfonamide (10 μg/ml); Tc^r, resistance to tetracycline (10 μg/ml); Sm^r, resistance to streptomycin (20 μg/ml); Ap^r, resistance to ampicillin (100 μg/ml); Sp^r, resistance to spectinomycin (100 μg/ml); Gm^r, resistance to gentamicin (10 μg/ml); Km^r, resistance to kanamycin (50 μg/ml); Cm^r, resistance to chloramphenicol (25 μg/ml); Tob^r, resistance to tobramycin (10 μg/ml).

[b] Since these plasmids have not been completely sequenced, it is possible (but not likely according to the array results and calculated overall size of the plasmids) that they contain additional indels.

been shown to comprise most of the species from the phylum *Proteobacteria* that have been tested so far (for a review, see reference 28).

In spite of their common genetic backbone, IncW plasmids confer different antibiotic resistance profiles, indicating that these plasmids differ in the antibiotic resistance determinants they carry. Based on the comparison of the DNA sequences of pSa, R388, R7K, pIE321, and pIE522, this work analyzes the recent evolution and diversification of the IncW backbone.

MATERIALS AND METHODS

Standard DNA techniques. For cloning, plasmid DNA was extracted using a Qiaprep spin miniprep kit (Qiagen). For DNA array, plasmid DNA was extracted using a GenElute plasmid miniprep kit (Sigma), and total genomic *E. coli* DNA was obtained according to the method described in reference 61. DNA was extracted from agarose gels with a Qiaquick gel extraction kit (Qiagen). Restriction enzyme digestions, agarose gel electrophoresis, DNA cloning, and the transformation of *E. coli* were all carried out according to the methods described in reference 65.

Conjugation assays. Matings were carried out using derivatives of strain DH5α [F^- *endA1 recA1 gyrA96 thi-1 hsdR17 supE44 relA1* Δ(*argF-lacZYA*)*U169* ϕ*80dlacZ*Δ*M15*] (38) carrying various plasmids as donors and UB1637 (F^- λ^- *lys his trp rpsL recA56*) (24) as a recipient strain. Saturated cultures of donor and recipient strains were mixed in a 1:1 ratio and mated on an LB agar surface for 1 h at 37°C as described previously (48). Transconjugants were selected on trimethoprim (20 μg/ml) for R388, tetracycline (10 μg/ml) for pIE321, chloramphenicol (25 μg/ml) for pSa, ampicillin (100 μg/ml) for R7K, and gentamicin (10 μg/ml) for pIE522. Donors were counterselected on streptomycin (300 μg/ml).

DNA macroarray hybridization. (i) Probe DNA labeling by random priming. Plasmid DNA (25 ng) was diluted to a 45-μl final volume in Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). DNA was denatured by heating at 95°C for 5 min and placed on ice for an additional 5 min. Denatured DNA was incubated in a reaction solution containing Rediprime II random prime labeling system (Amersham) and 50 μCi of [^{32}P]dCTP (Redivue Amersham) for 10 min at 37°C. The reaction was stopped by adding 0.2 M EDTA (5 μl), heating at 95°C for 5 min, and cooling on ice for an additional 5 min.

(ii) Membrane spotting. Target DNAs were obtained by PCR amplification of the relevant DNA fragments from either R388 or *E. coli* DNA. A total of 2.5 μg of each amplified DNA was denatured in alkali and spotted onto a nylon membrane (Zeta-Probe GT; Bio-Rad) by using the high-density replicating tool (HDRT-96) of robot Biomek 2000 (Beckman) and 0.045-in.-wide metal pins. The spotted membrane was neutralized by washing it in Tris-HCl (20 mM, pH 7.0) for 20 min and UV-cross-linking it at 1,200 mJ in a UV Stratalinker 1800 (Stratagene).

(iii) Array hybridization. The spotted membrane was prehybridized for 2 h with 5 ml buffer (0.25 M sodium phosphate, pH 7.2, 7% sodium dodecyl sulfate [SDS]) at 65°C. Then, 55 μl labeled probe DNA was added and hybridized for 20 h under the same conditions. After hybridization, the membrane was washed twice with 5 ml buffer A (sodium phosphate, 20 mM, pH 7.2) plus 5% SDS for 30 min at 65°C and washed twice again with 5 ml buffer A plus 1% SDS in the same conditions. Treated membranes were scanned using a Molecular Imager FX (Bio-Rad).

DNA sequencing, sequence analysis, and annotation. The R388 and pIE321 plasmid DNAs, as well as fragments of plasmids pIE522 and pSa, were sequenced by progressive DNA walking, using amplified DNA fragments. The BigDye Terminator v3.0 kit (Applied Biosystems) was used in sequencing reactions. Sequencing products were analyzed in an automatic multicapillary 3700 DNA sequencer with >4-fold DNA coverage. The program suite Vector NTI 5.5 was used for analyzing and annotating the plasmid sequences. ContigExpress was used for assembling the sequences. GC content analysis was carried out with BioPlot. Multiple alignments were done with AlignX. Open reading frames (ORFs) encoding at least 50 amino acids after a translation start codon were identified. DNA and deduced protein sequences were searched by BLAST analysis. ORFs were manually annotated using evidence from BLAST analyses (4) and experimental or other evidence from previous literature. For R7K, the DNA was fragmented by sonication and size fractionated before constructing libraries in pUC19. The sequence was generated from paired-end reads from two pUC19 libraries with insert sizes of 2 to 4 kb and 4 to 6 kb. These sequence reads were performed with ABI BigDye Terminator chemistry on ABI3730 sequencing machines and gave a total coverage for the plasmid of approximately eightfold. All identified repeats were bridged with read pairs or end-sequenced PCR products. Error checking and the finishing of the sequence were performed according to standard criteria using Phrap to assemble the sequence and Gap4 for visualization.

Nucleotide sequence accession numbers. Complete DNA sequences for R7K and pIE321 are available at EMBL-EBI Bank and GenBank, respectively, under accession numbers AM901564 (R7K) and EF633507 (pIE321). Partial sequences of pIE522 and pSa are available under GenBank accession numbers EU247928 and EU419764, respectively.

RESULTS AND DISCUSSION

A DNA array for the analysis of IncW plasmids. An array of 79 spots was prepared on nylon membranes by including each of the 43 R388 genes, 14 intergenic regions, 12 *E. coli* chromosomal genes, and total genomic DNA from *E. coli* and R388 (see Fig. S1 in the supplemental material). Among other projected applications, this array is useful as a first characterization tool to analyze the diversity of IncW plasmids.

When R388 DNA was hybridized against the array, the intensity of the signals correlated well with the size of the DNA in the spot (data not shown), indicating that association kinetics was the limiting step. No hybridization signal appeared with spots from *E. coli* ORFs, except an unexplained signal in gene *rpoB*. When the same array was hybridized against each of the four IncW plasmids shown in Table 1, hybridization to most R388 probes (ORFs and intergenic regions) was observed (see Fig. S1 in the supplemental material). Differences in the hybridization profiles of pIE522 and pSa concerned just the gene cassettes carried by the respective integrons, a consequence of their different antibiotic resistance profiles. Besides, plasmids pIE321 and R7K lacked the six spots (see Fig. S1, 9E to 10D,

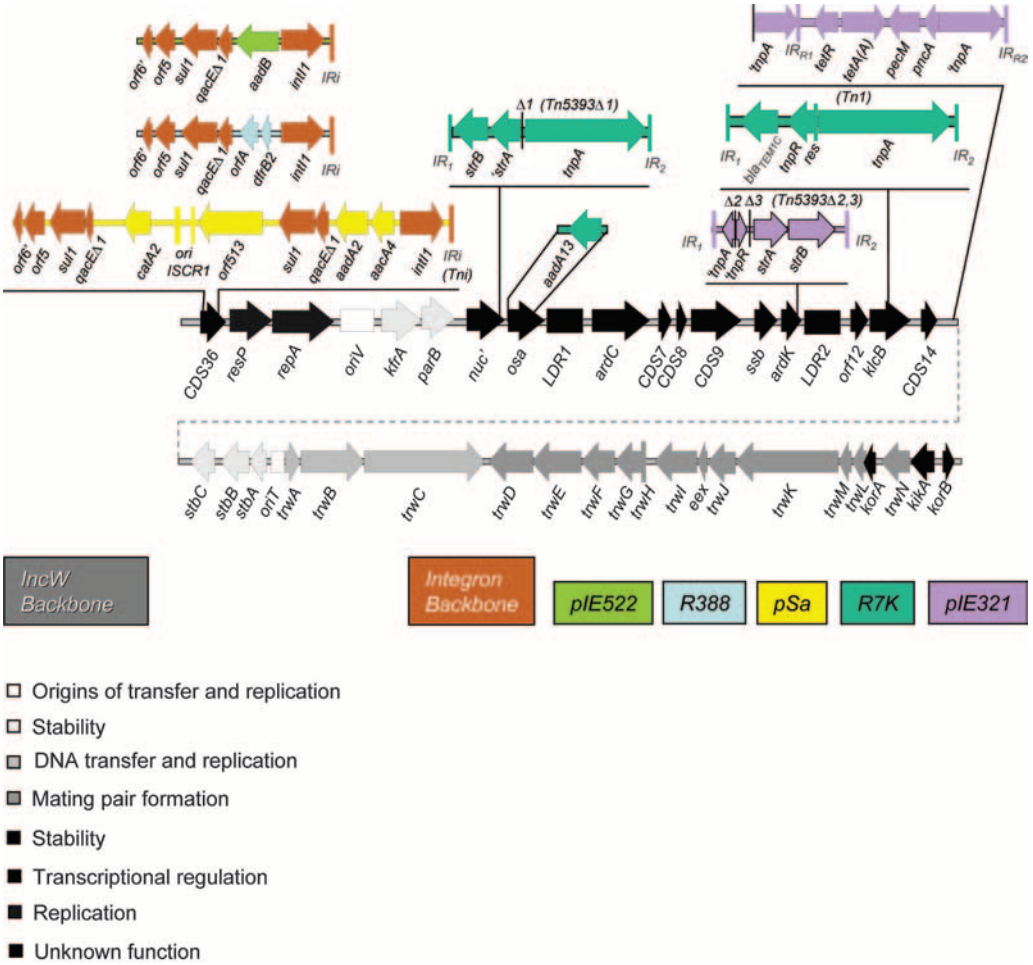

FIG. 1. Gene load in the IncW backbone. The insertions of the different MGEs in the IncW backbone that originated the five plasmid isolates are represented. Coding sequences (CDSs) and other sequence features belonging to the IncW backbone are gray, white, or gray-white combinations, depending on their assignment to functional modules. Plasmid-specific genes are represented by the color scheme indicated in the row of rectangles (at right) with inscribed plasmid names. The 5′ and 3′ conserved sequences of the integron platform are brown. When the 5′ or 3′ portion of a given CDS has been deleted, it is indicated by a prime (′) before or after the CDS name, respectively. The deletion sites within transposons are marked by vertical lines.

in the supplemental material) related to the integron, indicating that these two plasmids lack an integron platform. Finally, gene *osa* was absent in plasmid R7K. The use of the array allowed us to include plasmids pIE321 and pIE522 (from which nearly nothing was known) within the realm of IncW plasmids. Furthermore, based on the array results, we decided to sequence plasmids pIE321 and R7K, which showed the most interesting differences with respect to R388. We did not complete the sequences of the other two because they are practically identical to the R388 backbone.

Analysis of the DNA sequences of five IncW plasmids. The complete sequences of plasmids pIE321 (accession number EF633507) and R7K (accession number AM901564) were obtained. Plasmid R388 (accession number BR000038) was resequenced, and the 50+ sequence differences were submitted to GenBank for amending. The revised R388 sequence was used in all later analyses. In addition, we used the following pSa sequences available in the databases: L06822 (18, 40), AF143206 (10), X00060 (71), X68227 (11), U04277 and U04278 (68, 77), U30471 (16, 57), and M11444 (72). Additionally, we sequenced 5,600 bp of plasmid pIE522, comprising the backbone region *oriT-trwA* and the integron insertion (accession number EU247928) as well as the *stbA-oriT-trwA* region of pSa (accession number EU419764).

Both the DNA sequences and the previous array data indicate that all five IncW plasmids show extensive genetic conservation. Using the sequence information, the IncW genetic backbone was extrapolated as the genomic sequence in common between its five members and is represented in Fig. 1. The IncW backbone is 29,684 bp long and contains 37 ORFs. Some comparative data among backbones are shown in Table 2. Plasmids R388, pIE321, and R7K diverged significantly (pIE321 and R7K were 97.0 and 97.5% identical to R388, respectively) in comparison to pSa and pIE522 (both had almost 100% identity to R388 in the available backbone sequences). The five IncW plasmids contain one or more indels containing antibiotic resistance genes in an otherwise practically identical backbone (Table 1). They are

TABLE 2. Percent nucleotide identity between five IncW backbones[a]

Plasmid	Overall DNA sequence identity (%)				
	R388	pIE321	R7K	pSa[b]	pIE522
R388	100	97 (91.2)	97.5 (87.4)	99.9 (96.8)	(100)
pIE321		100	97.3 (83.2)	98 (94.2)	(94)
R7K			100	97.9 (86.8)	(91.7)
pSa[b]				100	(97.8)
pIE522					(100)

[a] The values in parentheses refer to DNA sequence identities (%) in the *oriT-trwA* region.
[b] For pSa, since the complete DNA sequence is not known, comparisons were made using the region between *resP* and *ardC* (8,384 bp).

depicted in Fig. 1. Since all IncW plasmid isolations were carried through biparental matings, based on direct detection of antibiotic resistance markers, IncW cryptic plasmids devoid of transposable elements or integrons have yet to be found.

Sequence divergence in the IncW backbone. The high degree of sequence conservation and the evidence of recent MGE insertions indicate that IncW is a homogeneous and relatively "young" plasmid cluster. This assertion can be confirmed by using relaxase protein domains as a molecular clock for estimating divergence rates in plasmid backbones, following the seminal work of Francia et al. (30). For instance, IncP plasmids also share an extensive backbone (73). Relaxases of IncP subgroup β are 89% identical. Identity drops to 64% if the relaxases of subgroup α plasmids are considered. Meanwhile, IncW relaxase domains share 100% amino acid identity. The only alterations in the IncW backbone are indels caused by the MGEs described above. No evidence of backbone disruption was observed (see Table S1 in the supplemental material), contrary to what happened in the IncP-β plasmid pB10 (66). It can be assumed that the described IncW plasmids diverged from a common ancestor more recently than IncP plasmids. Perhaps due to their recent origin, IncW plasmids are found less frequently in natural isolates than IncPs (35, 36).

However, <95% identity was observed in four individual genes (see Table S1 in the supplemental material), of which *trwA*, encoding a relaxosomal protein, shows the highest variation (74 to 87% amino acid identity). These differences explain the lack of PCR and hybridization signals when using *trwA* probes in several IncW isolates that were positive using *oriT* and *oriV* probes (35). *trwA* sequence divergence indicates that this gene suffered a substitution rate higher than that of other backbone genes. The nonsynonymous-to-synonymous substitution ratio (K_a/K_s) between all pairs of *trwA* sequences is lower than 1 (R388 versus pIE321, 0.1601; R388 versus R7K, 0.3773; and R7K versus pIE321, 0.2956; calculated with the K-Estimator 6.1 program [http://www.biology.uiowa.edu/comeron/index_files/Page432.htm]) (19, 20). That is, *trwA*, as well as the remaining IncW genes, is under purifying selection.

As can be seen in the alignment depicted in Fig. 2A, amino acid differences between $TrwA_{R388}$ and $TrwA_{pIE321}$ are distributed along the protein lengths. Conversely, $TrwA_{R388}$ and $TrwA_{R7K}$ exhibit their amino acid differences mainly in the N-terminal part of the protein (K_a = 0.15038 for the 72 N-terminal residues; K_a = 0.00861 for the 48 C-terminal residues). All three TrwA variants retain residue R10, proved to be the most important amino acid for TrwA binding to *oriT* (51). Interestingly, $TrwA_{R7K}$ shows mutations in two apparently important residues, Q8 and S12, that are involved in DNA recognition (50). Perhaps even more interesting, the *oriT* region also shows striking differences among these plasmids (Fig. 2B). The sequence recognized by the relaxase domain of TrwC (identical for all these plasmids), comprising the inverted repeat IR_2 and the *nic* site, remains unchanged. Variations fundamentally affect the regions recognized by the proteins integration host factor (IHF) and TrwA (51, 52), as well as the *trwA* promoter sequence. $oriT_{R7K}$ exhibits the highest variation. IHF binding inhibits *nic* cleavage catalyzed by TrwC (52), while TrwA binding performs a dual function: stimulation of conjugation by enhancing TrwC nicking and transcriptional repression of the *trwABC* operon (51). No significant differences were found in conjugation frequencies of these plasmids. It is likely that *oriT* and TrwA coevolved to conform plasmid-specific interactions. As a result, they could influence *nic* cleavage and *trwABC* transcription rates, modeling differences in the conjugation properties in different environments.

Natural history of each MGE insertion. The IncW backbone was loaded with different MGEs in each of the five plasmids (Fig. 1). The integron platform was inserted in R388, pSa, and pIE522. Class II transposons were inserted in R7K (Tn*1* and Tn*5393*Δ1) and pIE321 (Tn*1721*Δ1 and Tn*5393*Δ2,3). Interestingly, an integron gene cassette, *aadA13*, was inserted in a secondary site of plasmid R7K. All insertions occurred outside the conjugation modules. MGEs in IncP plasmids show significant hot spots in *oriV-klcA* and *tra-trb* (44, 74). Judging from the representations in Fig. 1 and 3, there are not preferred regions for MGE insertion in the IncW backbone. It may also be an indication of the recent origin of IncW plasmids that all MGE insertions can be explained in one or a few steps. The inserted MGEs themselves are not composite transposons. Figure 1 shows that insertions did not produce extensive reorganizations in the IncW backbone and synteny was maintained.

Transposon and cassette insertions in plasmid R7K. In R7K, a 4,949-bp insertion (Fig. 1) disrupts the repressor gene *klcB* (28). The inserted element is 99.8% identical to Tn*1*, located in Birmingham IncP-α plasmids RP1/RP4 (accession number L27758) (59), 98.2% to Tn*2* (accession numbers X54607 and AY123253) (60), and 98% to Tn*3* (accession number V00613). Flanking the inserted element are duplications of the target pentanucleotide AAATT (Fig. 3, panel A), pointing to a clean transposon insertion. Tn*1* insertion in plasmid RP4 occurs also within gene *klcB*, although the insertion site is not identical. $Tn1_{R7K}$ shows perfect 38-bp terminal IRs and retains its three functional genes: *tnpA* (transposase), *tnpR* (resolvase), and $bla_{TEM\text{-}1c}$ (encoding TEM-1 β-lactamase), as well as the resolution *res* site. $Tn1_{RP4}$ and $Tn1_{R7K}$ differ in only 10 nucleotides, resulting in a three-residue change in their TnpAs (A457R, D797G, and D801N) and one residue mutation in TEM proteins (K37Q). Gene $bla_{TEM\text{-}1c}$, as present in R7K, constitutes a new allele, and differs from $bla_{TEM\text{-}1a}$, $bla_{TEM\text{-}1b}$, and $bla_{TEM\text{-}2}$, present in transposons Tn*3*, Tn*2*, and Tn*1*, respectively, by the mutations shown in Table S2 in the supplemental material. Two regions are delimited: one comprising the promoter and the coding region up to position 345, which is identical to that in Tn*3*, and the other spanning from position 346 to the stop codon, which is identical to that in Tn*1*.

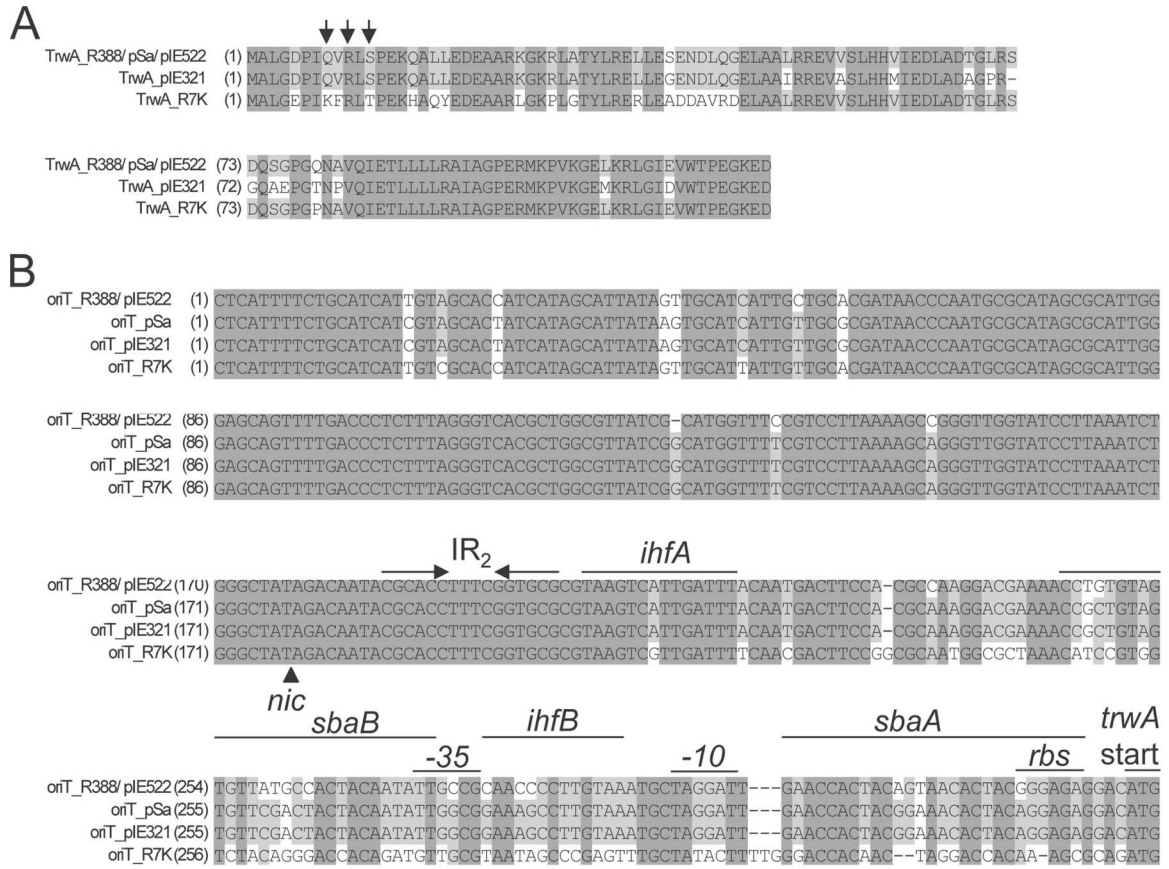

FIG. 2. Alignments of the TrwA and *oriT* sequences. Alignments of the TrwA (panel A) and *oriT* (panel B) sequences of R388/pIE522/pSa, pIE321, and R7K are shown. Invariant nucleotides or amino acids in all plasmids are shadowed in dark gray, while those identical in most plasmids are shadowed in light gray. (A) Residues 8, 10, and 12, which are important for *oriT* recognition by $TrwA_{R388}$, are indicated by vertical arrows. (B) The *oriT* DNA strand complementary to that cleaved by the relaxase is shown. The *nic* site is represented by a black triangle. Structural motif IR_2 is represented by convergent horizontal arrows. The sites of interaction of $oriT_{R388}$ with proteins IHF (*ihfA* and *ihfB*) and TrwA (*sbaA* and *sbaB*) are marked by horizontal lines. The start codon of gene *trwA* (*trwA* start), its putative ribosome binding site (*rbs*), and the *trwA* promoter −10 and −35 sequences are indicated.

This fact suggests homologous recombination between $bla_{TEM\text{-}1a}$ and $bla_{TEM\text{-}2}$, originating $bla_{TEM\text{-}1c}$. The resulting protein, as well as its putative weak promoter, is identical to those encoded by Tn*2* and Tn*3* (37).

A second transposon, Tn*5393*Δ1, is inserted in gene *nuc*, leaving its last 14 codons contiguous to the transposon 3′ end (Fig. 1 and see Fig. S2 in the supplemental material). Tn*5393*Δ1 is 99% identical to Tn*5393* from plasmid pEa34 (accession number M95402) (21) but has an internal deletion (1,982 bp) comprising the resolvase gene, insertion element IS*1133*, and the first 50 codons of *strA*. The deletion is clean, without relics of other transposable elements. The transposon is flanked by imperfect IRs (77 nucleotides out of 81) and a direct repeat of GACAG, corresponding to the target duplication at the insertion site (Fig. 3, panel B).

Gene *aadA13*, coding for aminoglycoside-3′-adenylyltransferase that confers resistance to streptomycin and spectinomycin, is located proximal to Tn*5393*Δ2 in plasmid R7K (Fig. 1). The presence of 14 codons of the 3′ region of *nuc* between the insertions of Tn*5393*Δ1 and *aadA13* demonstrates that both elements inserted independently (see Fig. S2 in the supplemental material). Protein $AadA13_{R7K}$ shows 98% identity to reported versions of AadA13 (accession numbers AAV49321, AAY18576, ABG76948, ABG76949, and BAF73713). The AadA family alignment (from AadA1 to AadA15), which contains the N-terminal nucleotidyltransferase domain, shows 33.5% consensus identity. AadA1 is most related to AadA13 (85.3% identity), while AadA14 is the least similar (54.3%).

The *aadA13* insertion looks like an integron gene cassette (855 bp long), containing a 3′ *attC* element as well as a recombination site (G/TTAGAC) upstream of the *aadA13* start codon (Fig. 3, panel C). It lacks *attI* or any other integron component. The *attC* sequence of the hypothetical circularized cassette (60 bp long) is 100% identical to previously reported *aadA13*-related *attC* sequences (accession numbers AY940492, DQ779001, DQ779002, and AB332415) (43). All these homologues are contained in class I integrons. Thus, we assume that the *aadA13* cassette integrated in an IntI1 secondary site (G/TTAGCG; the consensus being GWTMW [29]) located in the complementary strand of gene *nuc*, just downstream of its stop codon (see Fig. 3, panel C). Since the *aadB* cassette was shown to excise from a secondary site with a completely conserved GTT triplet (67), the composite core site of cassette *aadA13* may also be active in IntI1-mediated recombination.

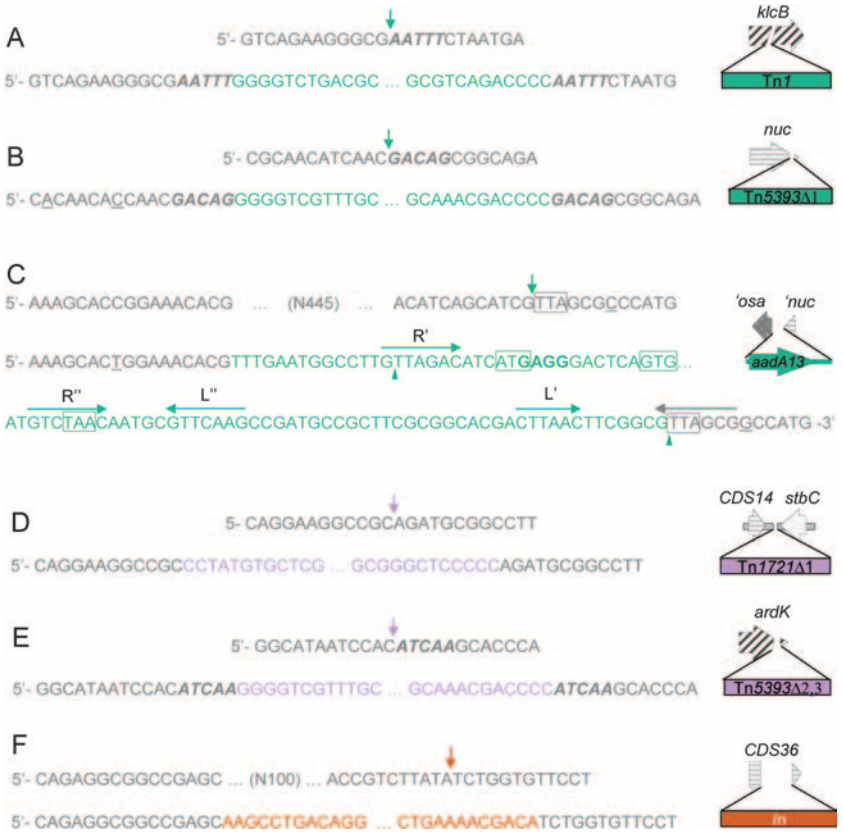

FIG. 3. Junction sequences of specific insertions. Each insertion is represented at the right by a diagram following the color scheme used in Fig. 1 and is preceded by two DNA sequences. The top one represents the backbone sequence (gray) where the insertion takes place. The exact point of insertion is depicted by a vertical arrow. The bottom sequence contains the corresponding insertion colored according to the same scheme. Target duplications of the backbone sequence are shown in bold italics. Individual base changes with respect to the backbone sequence are underlined. In panel C, two green triangles indicate suggested recombination crossover points flanking the *aadA13* integrated cassette. Gene *aadA13* start and stop codons as well as the complementary sequence of the *nuc* stop codon are boxed. A putative ribosome binding site is shown in bold. Core (R′), inverse core (R″), and internal IR (L′ and L″) sequences of the potentially recircularized *aadA13* cassette are signaled by horizontal green arrows, while the composite core site of the integrated cassette is indicated by a horizontal gray arrow. *′osa*, 5′ portion of *osa* has been deleted; *′nuc*, 5′ portion of *nuc* has been deleted.

The integration of cassette *aadA13* produced a deletion in the *osa* gene, leaving 114 bp of its 3′ region. Interestingly, this truncated segment shows only 83% identity to the functional osa_{R388}, perhaps underscoring the rapid shift of nonfunctional sequences (see Fig. S2 in the supplemental material). Osa impairs transport of Vir proteins (46) and DNA transfer (14) to plant cells. The repercussion of *osa* deletion in the ecology of plasmid R7K remains to be investigated.

Gene cassettes do not contain their own promoters and thus are transcribed from the P_{ant} promoter when inserted in the integron, or from a suitable oriented promoter when inserted in a secondary site (64). For example, cassette *aadB* was integrated in a secondary site of plasmid pRAY and is transcribed from a plasmid promoter (67). In R7K, *aadA13* is expressed since the host bacteria are resistant to spectinomycin, a phenotype that is not attributable to the *str* genes present in transposon Tn*5393*Δ1. A putative promoter is located upstream of *aadA13* in the *′osa* region (detected using the bacterial promoter prediction BPROM program at http://www.softberry.com) (see Fig. S2B in the supplemental material). Finally, a 13-bp string of DNA appears between cassette *aadA13* and *′osa* that is neither part of the gene cassette nor part of the IncW backbone.

Insertions in plasmid pIE321. Plasmid pIE321 contains insertions of truncated class II transposons Tn*1721* and Tn*5393*c (Fig. 1). The 6,295-bp-long Tn*1721*Δ1 element is inserted between coding sequence 14 (*CDS14*) and *stbC*. Compared to the canonical Tn*1721* (11,139 bp long) (accession number X61367) (3), this derivative shows a 4,844-bp deletion that includes the left terminal IR, the gene *orfI*, the resolution site *res*, the resolvase gene *tnpR*, and part of the transposase gene *tnpA*. No DNA target duplication was observed (Fig. 3, panel D). The transposon keeps the last 256 codons of *tnpA*, *tetR* (encoding a tetracycline resistance repressor), *tetA*(*A*) (encoding a tetracycline efflux protein that confers resistance to tetracycline), *pecM* (related to a permease repressor), *pncA* (a cystein-hydrolase), the second truncated copy of *tnpA* (encoding the 583 C-terminal residues), and the right IR (IR_{R2}), which appears duplicated in the middle of the element (IR_{R1}), as happens in the original transposon. A simple one-ended transposition event explains the Tn*1721*Δ1 insertion. Single-ended derivatives of Tn*3*-like transposons Tn*1721*, Tn*21*, and Tn*A* were

found to cleanly insert into a recipient plasmid (7, 42, 53). Insertion products contain the entire donor plasmid plus a duplication of the IR (7, 42, 53, 54), or only variable-length portions of the transposable derivative (8, 42, 53). So, transposition termination at a non-IR sequence can yield a simple insertion of a truncated transposon without duplication of the target sequence, like Tn*1721*Δ1 in plasmid pIE321. This is not an isolated example. A single-end Tn*1721* derivative found in the IncP plasmid pB10 (accession number AJ564903) has almost the same truncation (66). Given the fact that single-ended TnpA$^-$ Tn*21* and Tn*A* derivatives transpose when the cognate transposase is provided in *trans* (7, 42), the Tn*1721*Δ1 copy in pIE321 could potentially be mobilized.

The 2,148-bp Tn*5393*Δ2,3 derivative is inserted between *ardK* and *LDR1* (Fig. 1), duplicating the target sequence ATCAA (Fig. 3, panel E). The element retains the Tn*5393* 81-bp terminal IRs and genes *strA* and *strB* encoding streptomycin resistance (aminoglycoside-3′-phosphotransferase and aminoglycoside-6-phosphotransferase) but has two internal deletions. The first (Δ2; 2,316 bp) includes the N-terminal portions of the transposase (*tnpA* and *tnpR*) and resolvase genes, leaving their last 77 and 37 codons, respectively. The second (Δ3; 1,233 bp) comprises IS*1133*. The three remaining portions, IR-′*tnpA*, ′*tnpR*, and *strA-strB*-IR, are juxtaposed, as happens in the R7K element, and are 100%, 98%, and 100% identical, respectively, to Tn*5393* (accession number M95402) (15). Blast analysis of Tn*5393*Δ2,3 showed 100% nucleotide identity and coverage with an element contained in plasmid pMBSF1, which has exactly the same deletions (accession number AJ518835). This plasmid was obtained from *E. coli* isolated from pigs and, as with pIE321, also contains a truncated Tn*1721* copy but inserted in a different location (12).

Insertion of the integron platform in plasmids R388, pSa, and pIE522. Complete class I integrons are present in R388, pSa, and pIE522 at exactly the same backbone location (Fig. 3, panel F). They contain the same 5′ and 3′ conserved regions. In all three cases, the integron disrupts orphan gene *CDS36*, present in integron-free plasmids R7K and pIE321. As a result of the integron insertion, an internal 112-nucleotide deletion was produced in *CDS36*, leaving 22 codons 5′ and 77 codons 3′ of the inserted integron. Thus, we assume the integron inserted just once in an ancestral IncW backbone, as proposed by Gorai et al. (34) and Stokes et al. (68), as will be discussed later.

Upon integration, gene *CDS36* is split in such a way that an in-frame GTG start codon near the integron border gives rise to a chimeric gene in plasmids containing the integron platform. We annotated it as *tnpM* in R388 (28) by analogy with *tnpM* of Tn*21* (also located after *intI1*, as shown previously [47]). However, there is practically no similarity with the Tn*21* gene, so it should not be called *tnpM*, to avoid confusion. The DNA sequence that *CDS36* and *tnpM* have in common is responsible for hybridization of the R388 *tnpM* probe to plasmids R7K and pIE321 in the DNA array (see Fig. S1 in the supplemental material).

The IncW integron platform does not retain the transposition functions responsible for integron movement but conserves the terminal sequence IRi (25 bp long; close to the 3′ end of *intI1*) of Tni, the vehicle transposon present in Tn*21* (47). The integron 3′ conserved region, besides the common *qacE*Δ*1* gene and *sul1*, the sulfonamide resistance gene, harbors *orf5* and a truncated orphan gene, *orf6* (coding for its first 57 residues). The integron variable regions contain different gene cassettes. In R388, cassette *dhfr* codes for dihydrofolate reductase that confers resistance to trimethoprim (70, 83), while cassette *orfA* (69, 70) has no assigned phenotype. In pIE522, cassette *aadB* encodes 2-aminoglycoside (2′) adenylyltransferase and confers resistance to gentamicin/kanamycin/tobramycin. In pSa, the inserted integron contains two gene cassettes, *aacA4* and *aadA2*, which confer resistance to gentamicin/kanamycin and streptomycin/spectinomycin, respectively (39, 72).

The existence of different gene cassettes as well as the high conservation of the integrase with that of Tn*21* (they differ only in residue 39: His in Int_{Tn21} and Asn in Int_{IncW} plasmids) suggests that the IncW integrase is active in the genesis of new IncW plasmids. According to theory, the first acquired cassette is located in the last position of the integron variable region. Consistent with this, the first inserted cassette in each integron contains the same sequence (GTTAGAT) in its *attC* core site, derived from the primary *attI* recombination site in the empty integron.

In the integron version of plasmid pSa, the 3′ conserved genes *qacE*Δ*1* and *sul1* have been duplicated (68). In between, there is a chloramphenicol resistance gene, *catA2*, which does not correspond to a cassette insertion, followed by an IS*CR1* element. This insertion sequence contains *orf513*, a gene found close to many integrons, which encodes a protein related to the IS*91* transposase (32, 76). Three copies of the putative 5′ end of the IS*CR1* element oriIS*CR1* appear downstream of *orf513*. The pSa integron distal 3′ conserved sequence is identical to that in R388. Based on the proposed three-step mechanism (75, 76), a possible scenario for the formation of the pSa integron includes rolling-circle transposition of IS*CR1* fused to the 3′ conserved region of a class I integron (containing *orf513*, *sul1*, and *qacE*Δ*1*) to the vicinity of gene *catA2*. Subsequent one-ended rolling-circle transposition of this element, now containing *catA2*, gives rise to circular intermediates that can recombine with the 3′ conserved region of a simple integron platform, like that present in R388.

Concluding remarks. Several horizontal gene transfer events shaped currently known IncW plasmids. The most parsimonious assumption is to consider that the integron platform was inserted only once in an ancestral IncW plasmid, since its genetic location is exactly the same for the three IncW plasmids harboring it. Different gene cassettes were then incorporated into the integron by integrase-catalyzed reactions, producing the present versions in R388, pIE522, and pSa. A subsequent acquisition of an IS*CR1* element in the pSa integron occurred. The diversifications in the integron arrangements must be very recent, considering the 100% nucleotide identity in the integron platform of the three plasmids. Independent events of MGEs loaded in the ancestral plasmid gave rise to the IncW variants pIE321 and R7K. Divergence of those plasmids is older than that of R388/pIE522/pSa, since pIE321 and R7K exhibit small but significant differences in the backbone (see Table S1 in the supplemental material). The IncW group thus provides an example of the recent evolution of a plasmid backbone which acquired different MGEs that were selected by the heavy use of antibiotics.

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