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Sequence of pR3521, an IncB Plasmid from *Escherichia coli* Encoding ACC-4, SCO-1, and TEM-1 β-Lactamases▽

C. C. Papagiannitsis,[1] L. S. Tzouvelekis,[1,2] S. D. Kotsakis,[1] E. Tzelepi,[1] and V. Miriagou[1]*

Laboratory of Bacteriology, Hellenic Pasteur Institute,[1] and Department of Microbiology, Medical School, University of Athens,[2] Athens, Greece

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The sequence of pR3521, a self-transmissible plasmid from *Escherichia coli*, was determined. pR3521 (110,416 bp) comprised a contiguous IncB sequence (84,034 bp) sharing extensive similarities with IncI replicons and an acquired region (26,382 bp) carrying sequences of diverse origin, containing bla_{ACC-4}, bla_{SCO-1}, bla_{TEM-1b} (two copies), *strA*, *strB*, *sul2*, and *aacC2*.

Multidrug resistance among enterobacteria is commonly due to acquisition of plasmids carrying various resistance determinants (4). We have previously identified pR3521, a self-transferable and multiresistant plasmid from an *Escherichia coli* isolate from a hospitalized patient in Greece (18). Work had been largely focused on the characterization of loci containing bla_{ACC-4}, an *ampC* gene encoding ACC-4, an extended-spectrum variant of the ACC-1 cephalosporinase (19), and bla_{SCO-1}, a novel gene of unknown origin coding for an RTG-type carbenicillinase (18, 21). bla_{ACC-4} was included in a sequence derived from the chromosome of *Hafnia alvei* and linked with IS*Ecp1*. bla_{SCO-1} was included in a segment of likely chromosomal origin that was associated with IS*26* (18, 19). Plasmid-mediated production of SCO-1 and ACC-1 has also been described for *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Salmonella enterica* serovar Livingstone in Europe and North Africa, but detailed characteristics of the respective plasmids were not reported (6, 13).

In this study, the complete nucleotide sequence of pR3521 is presented. pR3521 belonged to incompatibility (Inc) group B, which is closely related to the IncI family of replicons (5, 17). A hypothesis as to the mechanisms of accumulation of diverse resistance genes in pR3521 is also discussed.

General features of pR3521. An *E. coli* K-12 transconjugant clone was used as a source of pR3521 (18). Plasmid pR3521, purified by CsCl gradient ultracentrifugation, was partially digested with Sau3A, and the fragments were ligated into the chloramphenicol-resistant vector pBCSK(+) (Stratagene, La Jolla, CA). The recombinant plasmids were used to transform *E. coli* DH5α. Transformants were selected with chloramphenicol (20 μg/ml). Recombinant plasmids were purified with a Qiagen plasmid midi kit (Qiagen, Hilden, Germany), and the nucleotide sequences of the inserts were determined using an ABI 377 sequencer (Applied Biosystems, Foster City, CA). Sequence gaps were filled by primer walking and sequencing of PCR products using primers hybridizing to known regions (18, 19). Contigs were assembled using the Laser Gene software program (DNASTAR, Madison, WI). For sequence analysis and annotation, the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST/), an insertion sequence (IS) finder (www-is.biotoul.fr/), an open reading frame (ORF) finder (www.bioinformatics.org/sms/), and the Artemis software program (www.sanger.ac.uk/) were utilized.

The sequence of pR3521 comprised 110,416 bp (G+C content, 52.6%) and included 124 coding sequences (119 complete and 5 truncated). The ORFs and their characteristics are presented in Table 1. A circular map of pR3521 is shown in Fig. 1. The plasmid was composed of two distinct parts: a contiguous plasmidic segment of 84,034 kb (G+C content, 53%), sharing similarities with replicons of complex I, and an acquired sequence of 26,382 bp (G+C content, 51.3%), containing eight antibiotic resistance genes (bla_{ACC-4}, bla_{SCO-1}, bla_{TEM-1b} [two copies], *strA*, Δ*strB*, *aacC2*, and *sul2*), intact ($n = 7$) and defective ($n = 1$) mobile elements (including four IS*26* elements, IS*26*-1 to IS*26*-4), single copies of IS*Kpn11* and IS*Kpn12*, a ΔIS*EcpI* element, and one Tn*2* transposon as well as sequences of diverse chromosomal origins.

Plasmid scaffold. pR3521 possessed a single replication region of 1,368 bp (positions 5133 to 6500) identical to that of the IncB plasmid pMU707 (GenBank accession no. M93062) (23). It also exhibited significant similarities, ranging from 92 to 99%, with the replication regions of the IncI1 plasmids R64, pO113, pSERB1, and pEK204 (GenBank accession no. AP005147, AY258503, AY686591, and EU935740, respectively). The replication region of pR3521 included, apart from *repA*, two segments, RNAI and RNAII, that control copy number by inhibiting RepA translation through an antisense-RNA-mediated mechanism (27). The origin of replication (*ori*) located downstream of *repA* and the *cis* regulatory region between *repA* and *ori* were identical to those of pMU707 (22).

The relatively large transfer region of pR3521 (>50 kb; positions 6866 to 59455) was upstream of the RepA-encoding sequence and shared extensive structural as well as sequence similarities (71 to 98%) with the transfer regions of the IncI1 plasmids pO113, pSERB1, and pEK204 (7, 14, 28). The transfer region was organized in two blocks, *tra* and *trb*. *tra* (positions 6866 to 47247) included 12 *pil* genes (*pilI* and *pilL* to *pilV*), encoding thin pili required for liquid matings (11), as well as 22 additional genes, namely, *traB*, *traC*, *traE*, *traF*, *traH*

* Corresponding author. Mailing address: Laboratory of Bacteriology, Hellenic Pasteur Institute, Vas. Sofias 127, Athens 11521, Greece. Phone: 30-210-6478810. Fax: 30-210-6426323. E-mail: miriagou@pasteur.gr.

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TABLE 1. Names, coordinates, and putative functions of ORFs identified in the IncB plasmid pR3521[a]

Gene name	Coordinates[b]	Putative function(s)
yagA	Compl. 533–1627	Hypothetical protein
yafB	1981–2583	Hypothetical protein
orf1	Compl. 3279–3560	Hypothetical protein
orf2	Compl. 3666–3941	Plasmid stabilization protein (RelE/ParE family)
orf3	Compl. 3941–4219	CopG family protein
repA	Compl. 5133–6209	Replication initiation protein
traB	6866–7507	F pilus assembly
traC	7648–8310	F pilus assembly
yqiJ	8577–9197	Putative adhesin
yqiK	9224–10918	Putative adhesin
pilI	10999–11241	Type IV prepilin cluster
pilL	11942–13012	Type IV prepilin cluster; lipoprotein
pilM	13016–13453	Type IV prepilin cluster
pilN	13485–15104	Type IV prepilin cluster; secretin protein
pilO	15125–16420	Type IV prepilin cluster
pilP	16410–16868	Type IV prepilin cluster
pilQ	16971–18479	Type IV prepilin cluster; ATP-binding protein
pilR	18481–19575	Type IV prepilin cluster; membrane protein
pilS	19703–20173	Type IV prepilin cluster; prepilin
pilT	20220–20705	Type IV prepilin cluster
pilU	20721–21347	Type IV prepilin cluster; prepilin peptidase
pilV	21364–22725	Type IV prepilin cluster
orf4	23183–23473	Hypothetical protein
orf5	Compl. 23565–23885	Hypothetical protein
traE	24147–24968	F pilus assembly
traF	25070–26272	F pilus assembly
traH	26376–26834	F pilus assembly
traI	26831–27667	DNA helicase
traJ	27651–28799	ATP-binding protein
traK	28796–29086	F pilus assembly
sogL	29150–33211	DNA primase
sogS	29339–33211	Regulator of SogL
traL	33228–33578	F pilus assembly
traM	33590–34285	Mating signal
traN	34296–35255	Aggregate stability
traO	35259–36590	Hypothetical protein
traP	36587–37300	Conjugal transfer protein
traQ	37297–37827	Conjugal transfer protein
traR	37874–38272	Hypothetical protein
traS	38329–38580	Surface exclusion
traT	38687–39313	Surface exclusion
traU	39608–42652	F pilus assembly
traV	42652–43272	F pilus assembly
traW	43395–44435	F pilus assembly
traX	44432–45001	F pilin acetylation
traY	45076–47247	Integral membrane protein
excA	47545–47982	Surface exclusion
orf6	48256–49842	Hypothetical protein
pndA	Compl. 49980–50132	Postsegregation killing
pndC	Compl. 49987–50280	Counter protein for PndA
orf7	50384–50704	Hypothetical protein
orf8	Compl. 50749–51066	Hypothetical protein
orf9	51035–51361	Hypothetical protein
orf10	51365–51580	Hypothetical protein
orf11	Compl. 51778–52332	Hypothetical protein
yeaH	52712–53029	Putative nuclease
orf12	Compl. 53419–53715	Hypothetical protein
orf13	Compl. 54250–54357	Hypothetical protein
trbA	54734–56050	Conjugal transfer protein
trbB	56047–57171	Conjugal transfer protein
trbC	57152–59455	Conjugal transfer protein
*orfA*1*	59569–59735	Hypothetical protein
sul2	60319–61134	Dihydropteroate synthase
strA	61195–61998	Streptomycin phosphotransferase
*strB*1*	61998–62106	Streptomycin phosphotransferase (nonfunctional)
$bla_{\text{TEM-1}}$	Compl. 62254–63114	TEM-1 β-lactamase precursor
tnpA	Compl. 63253–63957	TnpA transposase of IS*26*-1
*orfA*2*	Compl. 64047–64182	Hypothetical protein

Continued on following page

TABLE 1—*Continued*

Gene name	Coordinates[b]	Putative function(s)
*tnpB*1* (*rcr2*)	64299–64771	Putative transposase (nonfunctional)
tnpA	Compl. 64824–65528	TnpA transposase of IS*26*-2
Δ*tnpR*	65642–66003	Resolvase (nonfunctional)
$bla_{\text{SCO-1}}$	Compl. 66139–67005	SCO-1 carbenicillinase precursor
Glycosidase-like gene	67167–68366	Cellulase-like protein
umuC	Compl. 69483–70760	UV protection
umuD	Compl. 70766–71194	UV protection
dbp	Compl. 71290–71562	Putative DNA-binding protein
tnpA	71687–72037	Putative transposase of IS*Kpn12*
tnpA	72058–72447	Putative transposase of IS*Kpn12*
σ′70 gene	72515–73066	σ′70 factor-like protein
tnpA	Compl. 73591–74421	Putative transposase of IS*Kpn11*
tnpA	Compl. 74418–74750	Putative transposase of IS*Kpn11*
aacC2	Compl. 75088–75948	Gentamicin-(3)-N-acetyl-transferase
$bla_{\text{TEM-1}}$	Compl. 76090–76950	TEM-1 β-lactamase precursor
tnpR	Compl. 77133–77726	TnpR resolvase of Tn*2*
tnpA	77695–80859	TnpA transposase of Tn*2*
*strB*2*	80893–81625	Streptomycin phosphotransferase (nonfunctional)
*tnpB*2*	Compl. 81597–81668	Putative transposase (nonfunctional)
tnpA	81732–82436	TnpA transposase of IS*26*-3
Δ*tnpA*	82489–82669	TnpA transposase of IS*Ecp1* (nonfunctional)
$bla_{\text{ACC-4}}$	82963–84123	ACC-4 cephalosporinase precursor
gdhA	Compl. 84186–85100	Glutamate dehydrogenase
Δ*tnpA*	Compl. 85123–85297	TnpA transposase of Tn*2* (nonfunctional)
tnpA	85361–86065	TnpA transposase of IS*26*-4
orfB	Compl. 86177–86626	Hypothetical protein
nikB	Compl. 86694–89405	Relaxase
nikA	Compl. 89417–89836	Relaxosome component protein
yggA	89976–90317	Hypothetical protein
ydiA	Compl. 90403–91254	Hypothetical protein
ydhA	Compl. 91376–91747	Hypothetical protein
ygeA	91831–92082	Hypothetical protein
orf14	Compl. 92113–92295	Hypothetical protein
ydgA	Compl. 92358–93272	Hypothetical protein
ccgAII	Compl. 93269–93658	Prevention of RecA overproduction
ygdA	Compl. 93835–94197	Hypothetical protein
ygcA	Compl. 94194–94628	Hypothetical protein
orf15	94747–95244	Hypothetical protein
ardA	Compl. 95360–95860	Anti-restriction protein
ygaA	Compl. 96330–96926	Hypothetical protein
psiA	Compl. 96923–97642	Plasmid SOS inhibition protein A
psiB	Compl. 97639–98073	Plasmid SOS inhibition protein B
yfhA	Compl. 98128–100086	Hypothetical protein
orf16	100145–100378	Hypothetical protein
ssb	Compl. 100436–100963	Single-stranded DNA-binding protein
orf17	101084–101557	Hypothetical protein
yffA	Compl. 101735–101926	Hypothetical protein
yfeC	Compl. 101926–102345	Hypothetical protein
yfeB	Compl. 102392–102817	Hypothetical protein
yfdA	Compl. 103234–104004	Hypothetical protein
yfcB	Compl. 103961–104482	Hypothetical protein
yfcA	Compl. 104496–104717	Hypothetical protein
yfbB	Compl. 104714–105400	Hypothetical protein
yfbA	Compl. 105474–105779	Hypothetical protein
yfaB	Compl. 105783–106709	Hypothetical protein
yfaA	Compl. 106723–106992	Hypothetical protein
impC	107104–107352	UV protection
impA	107349–107786	UV protection
impB	107786–109057	UV protection
parA-like gene	109258–110184	ParA-like partitioning protein

[a] GenBank accession no. GU256641.
[b] "Compl." indicates that the gene is the reverse complement of the positions shown.

to *traK*, and *traL* to *traY*, implicated in conjugal transfer (12). Between *traC* and *pilI*, the adhesin-encoding *yqiJ* and *yqiK* genes were identified (2). The transfer region also contained a *sog* gene upstream of *traL*. SogL is a SogS-regulated DNA primase suppressing *dnaG* mutations (16). *trb* (positions 54734 to 59455) comprised the *trbABC* operon, whose products are also involved in conjugation (9). In the region intervening between *tra* and *trb*, a segment including *excA*, analogous to the

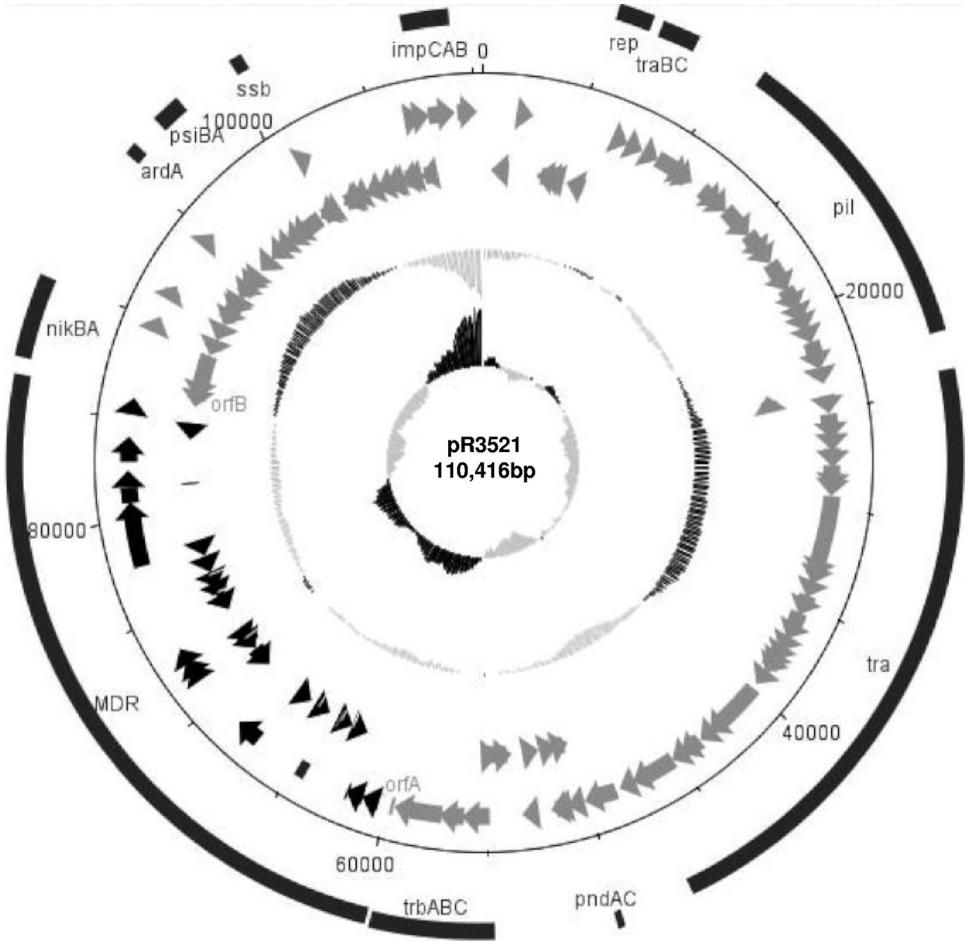

FIG. 1. Overview of the IncB plasmid pR3521. The main regions as well as the indicative genes are shown in the outer circle (a complete list of genes can be found in Table 1). The next circle shows ORFs in the plus orientation and the 3rd-circle ORFs in the minus orientation. The innermost circle shows the G+C content plotted against the average G+C content of the entire plasmid sequence (52.6%). The inner circle plots the G+C skew. MDR, multidrug resistant.

exc gene of IncI1 plasmids required for surface exclusion (8), along with *pndA* and *pndC* was identified. The products of the *pnd* genes contribute to plasmid maintenance (9). Also, 10 ORFs of unknown function were scattered throughout *tra*. The *oriT* operon, which included the origin of transfer and the *nikA* and *nikB* genes (whose products form a relaxation complex at the *oriT* site [10]), was distantly located from the *trbABC* operon due to insertion of a 26.4-kb acquired region in the sequence intervening between *trbC* and *nikB*. However, the *oriT* operon was apparently functional, as indicated by the self-transfer capability of pR3521 (18).

The plasmidic backbone also included the following: an *ardA* gene (positions 95367 to 95867), encoding an antirestriction protein; a *psiBA* operon (positions 96930 to 98080), whose products, PsiB and PsiA, inhibit the SOS response (1); and an *ssb* locus (positions 100443 to 100970), encoding Ssb, a single-stranded DNA protein (25). At positions 107104 to 109057, an *impCAB* operon, implicated in survival and induction of mutagenesis under UV irradiation, was found (24). pR3521 also possessed a *parA*-like gene, involved in segregational stability.

The close relationship of the *rep* regions of the complex I plasmids (IncI, -B, -K, and -Z) has been established in earlier studies (5, 17). The structure of the backbone of pR3521, being the first fully characterized IncB plasmid, extends this similarity to additional regions, such as *tra*, further supporting the common origin of these replicons.

Multidrug resistance region. A 26.4-kb mosaic region was inserted into an ORF (here designated *orfA*) of the plasmidic backbone that corresponded to the LH0063 ORF of p0113 (GenBank accession no. AY258503) (Fig. 2). A remnant of the first 167 bp of *orfA* (*orfA*1*) was found at the boundary of the acquired region. A segment of 281 bp, exhibiting no significant homology with any known sequence, was adjacent to the remnant of *orfA*. This segment was followed by a noncoding sequence (302 bp), *sul2*, *strA*, and an *strB* gene with a truncation of the 3′ end (*strB*1*) similar to that described for plasmid RSF1010 (GenBank accession no. M28829). *strAB* could be a remnant of Tn*5393*, as suggested by the presence of an inverted repeat (IR) characteristic of this transposon (positions 64704 to 64758). The module comprising *trbC*, *orfA*1*, *sul2*, *strA*, and *strB*1* has also been described to occur in other partially characterized IncB plasmids from *E. coli* (3).

A fragment of a Tn*2*-like transposon including a 38-bp inverted repeat (IR*bla*) and bla_{TEM-1} was adjacent to *strB*1*.

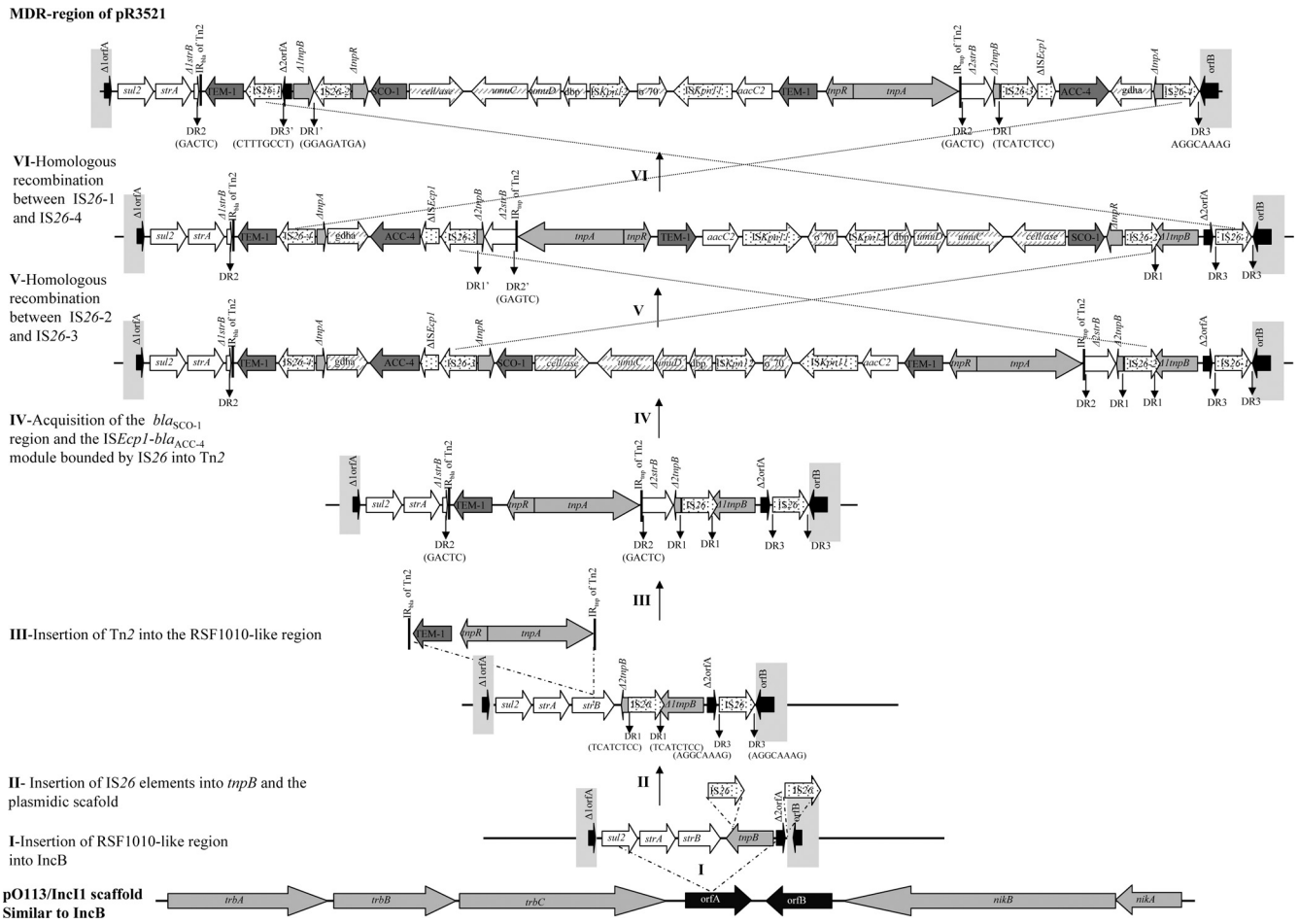

FIG. 2. Schematic diagram of the hypothetical successive steps (I to VI) leading to the evolution of the multiresistant acquired region of pR3521 (top of the figure). Sequences are drawn to scale. ORFs are shown by arrows indicating direction of transcription. The sequence depicted at the bottom of the figure is from the IncB-related pO113/IncI plasmid. Target site duplications (TSD) generated by transposition are indicated as DR1, DR2, and DR3. The complement and reverse sequences of DR1, DR2, and DR3 are indicated as DR1′, DR2′, and DR3′, respectively.

IS*26*-1, along with the remaining part of *orfA* (*orfA*2*), was located upstream of $bla_{\text{TEM-1}}$. However, *orfA*2* was in an orientation opposite to that expected, indicating an inversion event. The bounding sequence contained Δ*tnpB* (also designated *rcr2* in the IncB plasmid p838B-R; GenBank accession no. HM749967), IS*26*, Δ*tnpR*, $bla_{\text{SCO-1}}$, and a glycosidase-like gene (18). Downstream (1,118 bp) of the glycosidase gene, two probably chromosomal genes, *umuC* and *umuD* (needed for maximal SOS mutagenesis [26]), as well as *dbp*, encoding a putative DNA-binding protein (15), were identified. A mosaic sequence comprising IS*Kpn12*, IS*Kpn11*, *aacC2*, and a Tn*2* transposon was located upstream of *dbp*. This region was highly homologous to a sequence carried by *K. pneumoniae* 12836 (15). The IR*tnp* repeat of Tn*2* was adjacent to the remaining part of *strB* (*strB*2*). Direct repeats (DRs) of 5 bp (GACTC) (DR2 in Fig. 2) were found within the coding sequences of *strB*1* and *strB*2* at the boundaries of IRs of Tn*2*. Downstream of *strB*2*, there was the remaining part of *tnpB* (*tnpB*2*) adjoining IS*26*-3. Notably, 8-bp reverse and complement sequences were located at the boundaries of *tnpB*1* (*tnpB* with a truncation of the 3′ end) and IS*26*-2 (GGAGA TGA) (DR1′ in Fig. 2) and *tnpB*2* (*tnpB* with a truncation of the 5′ end) and IS*26*-3 (TCATCTCC) (DR1 in Fig. 2) as well as *orfA*2* and IS*26*-1 (CTTTGCCT) (DR3′ in Fig. 2) and *orfB* and IS*26*-4 (AGGCAAAG) (DR3 in Fig. 2). The reverse and complement orientation of these 8-bp sequences suggested IS*26*-mediated inversions. Upstream of the IS*26*-4 sequence was an ACC-4-encoding segment that has been described previously (19). This segment comprised an IS*Ecp1* element that was truncated (due to insertion of IS*26*-3), a fragment that originated from the chromosome of *H. alvei* ($bla_{\text{ACC-4}}$ and *gdhA*), and an IS*26*-4 sequence in parallel orientation with IS*26*-3, therefore forming a class I composite transposon.

Hypotheses on the formation of the acquired region. The multiresistant region apparently arose from multiple insertions and DNA rearrangements (Fig. 2). A possible initial event could be the insertion of the RSF1010-originated module comprising *sul2*, *strA*, *strB*, and *tnpB* within *orfA* of pR3521 (possible step I). This hypothesis is corroborated by sequencing data from other IncB plasmids, such as p99309, p99051, and p99171, showing insertion of similar modules in *orfA*-like genes (3). A subsequent event could be the insertion of two IS*26* elements, one disrupting the 3′ end of the *tnpB* gene and another inserted into the IncB scaffold in the sequence inter-

vening between *orfA*2* and *orfB*, thus creating 8-bp DRs at the boundaries of the elements (DR1 and DR3, respectively) (possible step II). Truncation of *strB* was likely due to transposition of a Tn*2*-like element, as indicated by the target site duplications (TSD) (DR2) flanking the IRs of the transposon (possible step III). Of note, the structure resulting from steps I, II, and III was similar to a sequence identified in the IncB plasmid p838B-R. A SCO-1-encoding segment (comprising bla_{SCO-1}, the glycosidase gene, *umuC*, and *umuD*) and a module comprising IS*Ecp1*, bla_{ACC-4}, and *gdhA*, both associated with IS*26* elements, could then have been inserted within Tn*2* (possible step IV). It was not clear whether IS*Kpn11* and IS*Kpn12* had been inserted independently or recruited in a single event along with Tn*2*. However, the latter notion is supported by the existence of a similar sequence (comprising *umuD*, *dbp*, IS*Kpn12*, the σ′70 gene, IS*Kpn11*, Tn*1000*, and the Tn*2** hybrid) in *K. pneumoniae* 12836 (15). Homologous recombination between IS*26*-2 and IS*26*-3 and then recombination between IS*26*-1 and IS*26*-3 could be proposed as the final events in the evolution of the acquired region (possible steps V and VI). This assumption is in line with the reverse and complement orientation of the 8-bp sequences found within *tnpB*1* (DR1′) and *orfA*2* (DR3′). The related sequences discussed by Doloy et al. (6) and Partridge (20) underscore the central role of IS*26* in the formation of this structure type.

Nucleotide sequence accession number. The complete nucleotide sequence of plasmid pR3521 has been assigned GenBank accession no. GU256641.

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