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IS*26*-Associated In4-Type Integrons Forming Multiresistance Loci in Enterobacterial Plasmids

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Three distinct multiresistant loci from enterobacterial plasmids each comprised an integron and an IS*26*-associated sequence. Sequence comparison suggested a common ancestral structure that derived from an IS*26* insertion into the 5′ conserved segment of an In4-type integron and evolved through acquisition of gene cassettes and IS*26*-mediated recruitment of additional resistance genes of diverse origin.

Resistance to multiple antibiotics in enterobacteria is largely attributed to acquisition of multiresistance plasmids (MRPs) (20). Sequencing data from MRPs have shown that resistance genes often occur in clusters carried by class 1 integrons. The latter, though not mobile themselves, are commonly associated with various transposons, such as Tn*21*, Tn*1696*, and Tn*1412* of the Tn*3* family (1, 13, 17).

We have previously reported on three MRPs: the SHV-5-encoding pSEM from *Salmonella enterica* serovar Typhimurium that belonged to IncL/M and carried In-t3 (18), the IBC-1-encoding pAK33 from *Klebsiella pneumoniae*, assigned to the IncL/M incompatibility group (data not shown), carrying the In-111 integron (19), and the VIM-1-encoding p541, an IncN plasmid from *Escherichia coli* including In-e541 (9, 10). Notwithstanding the different variable regions (Fig. 1), in each integron the first 113 bp of the common 5′ conserved segment (5′CS) (89 bp from the stop codon of *intI*) had been deleted due to an IS*26* insertion (IS*26*/Δ5′CS). Also, the sequences adjacent to this IS*26* (IS*26-1*) included additional resistance genes (*aphA* in pAK33, $bla_{\text{CMY-13}}$ in p541, and $bla_{\text{SHV-5}}$ in pSEM). These similarities prompted us to further investigate these structures by sequencing and comparison of the integron flanking regions.

Sequences flanking the 3′ end of integrons. To determine DNA sequences flanking the 3′ end of In-t3, In-111, and In-e541, the respective plasmids were partially digested with various endonucleases and the fragments were ligated into the chloramphenicol-resistant phagemid pBC-SK(+) (Stratagene, La Jolla, Calif.). *E. coli* DH5α was used as a host of recombinant plasmids. Recombinant clones were screened by PCR assays using various combinations of oligonucleotide primers specific for class 1 integrons (7), IS*26* (11), and gene cassettes carried by In-t3, In-111, and In-e541 (9, 18, 19). Sequencing was performed at both strands of selected clones and PCR products using an ABI PRISM 377 sequencer (Applied Biosystems, Foster City, Calif.).

Intact 3′CS sequences, including orf5, orf6, and the terminal inverted repeat (IRt) typical of many class 1 integrons, were identified in In-t3 and In-111. Both integrons also carried a complete copy of IS*6100* adjacent to the 3′CS (Fig. 1). The 3′CS of In-e541 included a truncated orf5 (orf5Δ1) and an IS*26* in opposite orientation with respect to IS*26-1*. This element was juxtaposed to codon 40 of orf5, and its inverted terminal repeat on the left (ITR_L) was deleted due to insertion of an IS*1* that included *insA* and a truncated *insB* gene. A 6.0-kb sequence comprising three genes encoding an EcoRII cytocine methylase (GenBank X05050), an EcoRII endonuclease (GenBank M26404), and a Uvp1 invertase-resolvase (GenBank X16119) as well as an additional IS*26* copy and the remaining 3′CS of In-e541 (3′ end of orf5, orf6, the internal copy of IRt, and an IS*6100*) was found downstream IS*1*. Identical 8-bp sequences, apparently representing the target site duplication generated by IS*26*-mediated transposition, were identified at the boundaries of orf5Δ1 and orf5Δ2 (Fig. 1). These experiments showed that the three integrons were associated with IS*6100* and, therefore, belonged to a lineage of class 1 integrons related to In4 from Tn*1696* (14).

Sequences flanking the 5′ end of integrons. To determine the sequences flanking the IS*26*/Δ5′CS region of the three integrons, PCR assays and cloning procedures as above were applied. In In-111 (pAK33), an *aphA* variant (816 bp) encoding an aminoglycoside phosphotransferase was adjacent to IS*26*/Δ5′CS. The 3′ end of *aphA* was bounded by a complete copy of IS*26* in direct orientation with IS*26-1* (Fig. 1). A similar structure, resembling the kanamycin-resistant transposon Tn*4352* (21), was also identified at the left-hand end of the *Citrobacter freundii* chromosomal fragment containing $bla_{\text{CMY-13}}$, located in the p541 plasmid adjacent to the VIM-1-encoding In-e541 (Fig. 1). In pSEM, a second IS*26*, occurring as a direct repeat copy of IS*26-1*, was identified downstream of $bla_{\text{SHV-5}}$. The IS*26*-bounded sequence (7,996 bp) comprised eight open reading frames: truncated putative endonuclease (Δ*ygbM*), fuculose-1-phosphate aldolase (*fucA*), putative tRNA synthase (*ygbK*), putative oxidoreductase (*ygbJ*), putative transcriptional regulator (*ygbI*), $bla_{\text{SHV-5}}$, *recF*, and a truncated lactose transport gene (Δ*lacY*) (Fig. 1). It exhibited >90% homology with a chromosomal segment of *K. pneumoniae* including the intrinsic bla_{SHV} gene (www.genome.wustl.edu/projects/bacterial/kpneumoniae/). Sequences upstream the extreme left IS*26* (at least 60 bp in each locus), compared with the relevant flanks of the other

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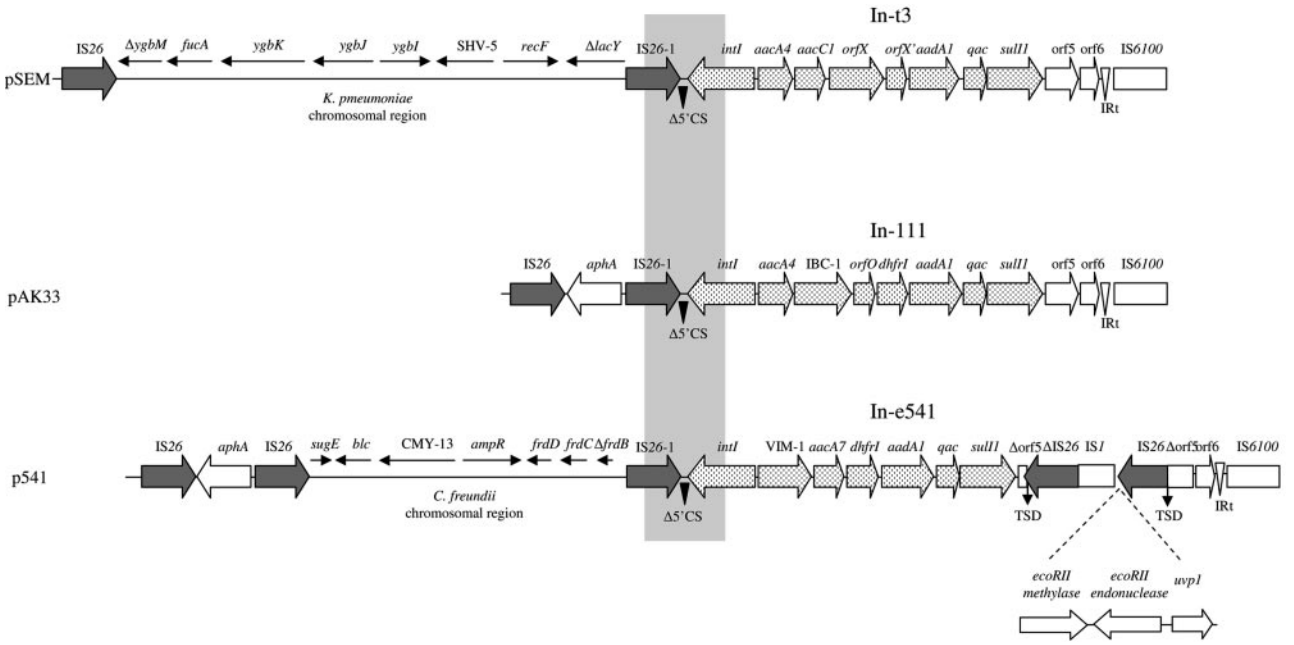

FIG. 1. Graphic presentation of the multiresistant loci found in the enterobacterial plasmids pSEM, pAK33, and p541. Inverted terminal repeats (IRt) at the 3′CS boundaries of the In4-like integrons and target site duplications (TSD) are indicated. The shaded parts represent the common IS*26*/Δ5′CS sequences.

IS*26* elements, did not include duplications characteristic of IS*26*-mediated transposition.

A GenBank search revealed two more MRPs resembling pSEM, the IncFII p1658/97 plasmid from *E. coli* (AF550679; unpublished data) and the IncL/M pACM1 from *Klebsiella oxytoca* (U90945, AY081221, AY309067, AY309066) (15, 16). They both carried In4-type integrons similar to In-t3 in which the 5′CS was truncated by an IS*26* at the same position as in the integrons discussed here. Additionally, these integrons were associated with SHV-5-encoding sequences flanked by IS*26* that, however, were differently oriented than those in pSEM.

Formation and spread of IS*26*/Δ5′CS-containing multiresistant loci. It is likely that the mobile elements of the IS*6* family, including IS*26*, do not exhibit any marked target site specificity (8). Therefore, the hypothesis of independent IS*26* insertions into the same "hot-spot" of different class 1 integrons that, additionally, all belonged to the In4 family was discarded. The key features of the loci described here were the following: (i) the occurrence of the IS*26*-In4 structure in distinct replicons and (ii) the presence of multiple copies of IS*26* in the sequences adjoining the 5′CS that were, most probably, inserted independently as indicated from the absence of target site duplications. Based also on the properties of IS*26* (2, 4, 5, 8), it can be proposed that the IS*26*/Δ5′CS-containing multiresistant loci derived from a common structure. The initial step would be the insertion in the 5′CS of a plasmid-borne In4-type integron of an IS*26-1* derived either by intramolecular transposition or by transposition of an element located on a different replicon. IS*26-1* probably facilitated recruitment of diverse IS*26*-flanked sequences, such as *aphA* and the β-lactamase-encoding chromosomal fragments observed here, by homologous recombination as suggested by the lack of target site duplications. Also, IS*26*-mediated cointegration of different replicons, subsequently resolved by RecA-dependent homologous recombination, can explain mobilization of IS*26*-In4 among distinct MRPs.

IS*26* is widely spread among plasmids (2, 6, 12, 21) and implicated in the dissemination of resistance genes in several ways. Compound IS*26* transposons carrying from one to nine resistance genes have been described previously (5, 12). Also, IS*26* elements seem to facilitate mobilization of chromosomal sequences containing resistance genes (3, 6). The findings of this study suggest that association of IS*26* with a class 1 integron of the In4 lineage was probably a critical step in the evolution of diverse multiresistance plasmids found in clinical enterobacteria.

Nucleotide sequence accession numbers. The GenBank accession numbers of the sequences presented here are AJ245670 and AJ009829 (pSEM), AY260546 (pAK33), and AY339625 and AY340637 (p541).

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