

Mosaic Structure of p1658/97, a 125-Kilobase Plasmid Harboring an Active Amplicon with the Extended-Spectrum β -Lactamase Gene *bla*_{SHV-5}[∇]

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Escherichia coli isolates recovered from patients during a clonal outbreak in a Warsaw, Poland, hospital in 1997 produced different levels of an extended-spectrum β -lactamase (ESBL) of the SHV type. The β -lactamase hyperproduction correlated with the multiplication of ESBL gene copies within a plasmid. Here, we present the complete nucleotide sequence of plasmid p1658/97 carried by the isolates recovered during the outbreak. The plasmid is 125,491 bp and shows a mosaic structure in which all modules constituting the plasmid core are homologous to those found in plasmids F and R100 and are separated by segments of homology to other known regions (plasmid R64, *Providencia rettgeri* genomic island R391, *Vibrio cholerae* STX transposon, *Klebsiella pneumoniae* or *E. coli* chromosomes). Plasmid p1658/97 bears two replication systems, IncFII and IncFIB; we demonstrated that both are active in *E. coli*. The presence of an active partition system (*sopABC* locus) and two postsegregational killing systems (*pemIK* and *hok/sok*) indicates that the plasmid should be stably maintained in *E. coli* populations. The conjugative transfer is ensured by the operons of the *tra* and *trb* genes. We also demonstrate that the plasmidic segment undergoing amplification contains the *bla*_{SHV-5} gene and is homologous to a 7.9-kb fragment of the *K. pneumoniae* chromosome. The amplicon displays the structure of a composite transposon of type I.

Bacteria have an amazing ability to adapt to changes in their environment. They exchange genetic material via horizontal gene transfer, which is considerably enhanced by mobile genetic elements, such as plasmids, phages, transposons (Tn), insertion sequences (ISs), and gene cassettes. The majority of known gene cassettes and transposons, as well as numerous plasmids (R plasmids), contain antibiotic resistance determinants (38). Many of these have been demonstrated to originate from bacterial chromosomes, as is the case for several β -lactamase gene families, including those coding for the SHV-type enzymes. The spread of the *bla*_{SHV} genes throughout the bacterial world has probably been initiated by repeated events of the IS26-mediated transposition that mobilizes these genes to plasmids from the chromosome of *Klebsiella pneumoniae* (6, 7, 13). The plasmid-encoded SHV β -lactamases are frequently found in most of the clinically important enterobacterial species, but they have also been observed in other gram-negative organisms, like *Pseudomonas aeruginosa* (12, 18, 26, 32). One of the SHV enzymes, SHV-5, belongs to the most commonly encountered extended-spectrum β -lactamases (ESBLs) that hydrolyze the vast majority of β -lactam antibiotics, including the newer cephalosporins and monobactams. It has been iden-

tified worldwide in almost all countries in which ESBLs have been studied at the molecular level (12, 18).

The plasmidic *bla*_{SHV-5} gene was identified in 12 *Escherichia coli* isolates recovered during a clonal outbreak in a Warsaw, Poland, hospital in 1997 (25). All of the isolates exhibited identical levels of resistance to non- β -lactam antibiotics (aminoglycosides); but many of the isolates differed in their levels of resistance to expanded-spectrum cephalosporins and could be separated into two groups, “resistant” and “susceptible,” for which the MICs of ceftazidime were >128 μ g/ml and 4 μ g/ml, respectively. PstI restriction analysis of plasmids isolated from the “resistant” and “susceptible” groups revealed identical restriction patterns except for some fragments present in non-equimolar amounts in the “resistant” plasmid (25). Two of these fragments were subsequently found by hybridization with the gene-specific probe to contain the *bla*_{SHV} gene. These facts indicated that the “resistant” phenotype correlated with the multiplication of a plasmid region comprising *bla*_{SHV}. A study was commenced in order to understand the phenomenon of the plasmid fragment amplification. The first step of the project was to reveal the complete sequence of one of plasmids of the “susceptible” group, p1658/97, which is a presumed initial molecule in the amplification process. The results of the nucleotide sequence analysis and identification of the region within the *bla*_{SHV-5} gene that undergoes amplification are presented in this paper.

MATERIALS AND METHODS

Plasmids and bacterial strains. Plasmids p1658/97 and p1657/97 were purified from “susceptible” and “resistant” *E. coli* isolates 1658/97 and 1657/97, respectively, which were recovered from patients in the neonatal ward in a Warsaw

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hospital in October 1997 (25). pSGMU2 is a cloning vector and is a derivative of pUC13, which carries the *cat* gene. The *cat* gene confers resistance to ampicillin and chloramphenicol (8). pACYC184 is a cloning vector with *ori* P15A and genes causing resistance to tetracycline and chloramphenicol (5). Plasmids pFBAMP and pR were constructed in this study and are derivatives of p1658/97. pFBAMP (23,870 bp; positions 85091 to 108960 of p1658/97) was obtained after *Bcu*I digestion of p1658/97 and subsequent religation, and it contains the IncFIB replicon and the *bla*_{SHV-5} gene. pR (13,590 bp) was obtained upon *Stu*I digestion of p1658/97, followed by ligation of its fragment (positions 29356 to 42120) with the PCR-generated *cat* gene of pACYC184. It contains the IncFII replicon. Experiments in this study were performed with *E. coli* strains DH5 α and A15 (3, 25).

Conjugation assay. One-milliliter volumes of cultures of the donor and recipient strains (10^9 CFU of each strain per ml) grown in Luria-Bertani (LB) medium (Biocorp, Warsaw, Poland) were mixed and incubated for 30 min at 37°C. *E. coli* A15 or DH5 α , both of which are resistant to rifampin, was used as the recipient strain. Transconjugants were selected on LB agar (Biocorp) supplemented with ceftazidime (10 μ g/ml; GlaxoSmithKline, Stevenage, United Kingdom) and rifampin (100 μ g/ml; Polfa Tarchomin, Warsaw, Poland).

Selection of *E. coli* “resistant” clones carrying p1658/97 with the amplified *bla*_{SHV-5} gene. One-hundred microliters of the *E. coli* DH5 α (p1658/97) overnight culture or its appropriate dilution was plated on LB agar, and then a sterile disk (diameter, 10 mm; Whatman 3MM paper) with 50 μ g of ceftazidime was placed on it. The plates were incubated overnight at 37°C. The *E. coli* clones able to form colonies inside the zone of growth inhibition were collected as “resistant” colonies.

DNA cloning and sequencing. The vectors were purified by using a Plasmid Midi AX DNA purification kit (A&A Biotechnology, Gdańsk, Poland). A large amount of p1658/97 DNA for random library construction, direct sequencing, and digestions was prepared by the alkaline lysis procedure (2), followed by CsCl-ethidium bromide gradient ultracentrifugation (36). The library of random fragments of p1658/97 was prepared by sonication by the procedure used for sequencing of the *Haemophilus influenzae* Rd genome (42). Fragments of between 1.6 and 2.0 kb were cloned into a unique *Sma*I site of the pSGMU2 vector (8). The plasmid DNA from the resulting subclones was purified prior to sequencing by using DNA purification kits (Macherey & Nagel, Düren, Germany; QIAGEN, Hilden, Germany). Cycle sequencing was performed with the use of BigDye (version 1.0 or 2.0; Applied Biosystems, Foster City, CA) in a GeneAmp 9700 PCR system (Applied Biosystems). Samples were subsequently purified on CentriSep columns (Applied Biosystems) or subjected to sodium acetate-ethanol precipitation, according to the protocol of the BigDye kit manufacturer. Extension products were separated on an ABI 377 automated sequencer (Applied Biosystems). Sequencing was performed with random library clones, the PCR products, and p1658/97 as templates. In the last case, a BAC sequencing protocol was used (Applied Biosystems). All enzymes used for DNA manipulations were from MBI Fermentas, Vilnius, Lithuania.

PCR amplification of the *cat* cassette. Primers chlL (5'-ATCCGCTTATTATCACTATTACAGG-3') and chlP (5'-GGTGTCCCTGTGATACCGG-3') were used for amplification of the *cat* gene of pACYC184 (5). The reaction conditions were as follows: 3 min at 95°C; 25 cycles of 30 s at 94°C, 30 s at 48°C, and 30 s at 72°C; and finally, 7 min at 72°C. The resulting PCR products were run in 1% agarose gels (SeaKem; FMC Bioproducts, Rockland, ME) and purified for cloning by using a DNA Gel-Out gel extraction kit (A&A Biotechnology).

Plasmid sequence analysis. The resulting nucleotide sequences were assembled by using the DNA Lasergene 99 package (DNASTAR, Madison, WI); and analysis and annotation of the sequence were performed by additionally using Glimmer 2.0 (The Institute for Genomic Research, 1999 [35]; RBSFinder.pl [The Institute for Genomic Research]; SignalP [CBS; www.cbs.dtu.dk/services/SignalP-2.0] [24]), InterScan (Expasy; www.ebi.ac.uk/InterScan/ [45]), and TMHMM Server (version 2.0; CBS; www.cbs.dtu.dk/services/) software.

Nucleotide sequence accession number. The complete nucleotide sequence of plasmid p1658/97 has been submitted to the GenBank database under accession no. AF550679.

RESULTS AND DISCUSSION

Preliminary plasmid analysis. *E. coli* 1658/97, one of the ESBL-producing outbreak isolates of the “susceptible” group (25), was chosen as a donor of plasmid p1658/97 for conjugal transfer to *E. coli* A15 and then to DH5 α . The transconjugants of A15 (RecA⁺), as well as those of DH5 α (RecA⁻), were

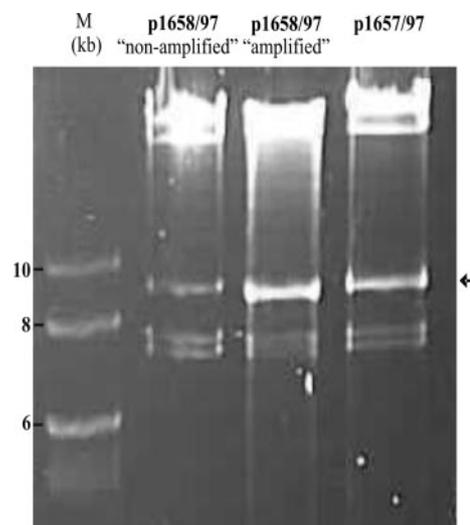


FIG. 1. *Swa*I digestion of p1658/97 and p1657/97. The results of *Swa*I digestion of p1658/97 DNA with the “nonamplified” and the “amplified” regions isolated from *E. coli* colonies collected from outside and inside the growth inhibition zone (“susceptible” and “resistant,” respectively) and p1657/97 isolated from DH5 α cultured with the presence of 128 μ g/ml ceftazidime are shown. Equal amounts of all plasmids were loaded. The lanes are described at the top. The arrow shows the amplified fragment of 8,817 bp. Lane M, GeneRuler DNA Ladder Mix (MBI Fermentas). Numbers on the left are in base pairs.

obtained; and subsequently, 10 of them were subjected to the ceftazidime disk test. All clones tested were able to form colonies inside the growth inhibition zone. Restriction analysis of the p1658/97 plasmids recovered from these colonies with the rare cutting enzyme, *Swa*I, revealed that one band was non-proportionally more intense when compared with p1658/97 isolated from the “susceptible” colony taken from outside the inhibition zone (Fig. 1). Although similar data were previously shown for plasmids p1657/97 and p1658/97 (extracted from “resistant” and “susceptible” clinical isolates, respectively) (25), this result indicated that acquisition of the “resistance” phenotype can proceed in a RecA-independent manner. Moreover, as shown in Fig. 1, comparison of the *Swa*I digestion of p1657/97 isolated from the *E. coli* DH5 α strain cultured in the presence of 128 μ g/ml ceftazidime with that of p1658/97 isolated from the “resistant” DH5 α colony revealed similar restriction patterns, with the overrepresented DNA fragments isolated from the plasmids being of identical sizes and similar intensities. This indicated that in both p1658/97 from the “resistant” DH5 α colony and p1657/97 the same region underwent amplification, pointing to the identical mechanism of this event. Therefore, the p1658/97 plasmid isolated from the “susceptible” DH5 α clone was subjected to DNA sequence analysis.

Overall gene content and G+C content of p1658/97. The complete nucleotide sequence of plasmid p1658/97 was determined by sequencing of its random fragments cloned into the pSGMU2 vector. It was found to be a circular molecule of 125,491 bp. Bioinformatic analysis of the sequence has revealed the presence of 142 putative genes, 7 of which (4.93%) had no homologs in public databases and thus could be considered p1658/97 specific, while 52 (36.62%) and 83 open read-

flanked by two *IS1* copies, suggesting that it was acquired by p1658/97 via an *IS1*-mediated recombination event.

The second major part of the p1658/97 sequence emphasizes its mosaic character; it is composed of six subregions displaying homology to molecules other than F and R100: (i) two subregions homologous to plasmid R64 (GenBank accession no. AP005147; *Salmonella enterica* serovar Typhimurium); (ii) a subregion homologous to the R391 genomic island (GenBank accession no. U136331; *Providencia rettgeri*) and the STX transposon (GenBank accession no. AY055428; *Vibrio cholerae*); (iii) a subregion with the *bla*_{SHV-5} gene homologous to a fragment of the *K. pneumoniae* chromosome (<http://genome.wustl.edu/projects/bacterial/kpneumoniae>), as well as plasmids pSEM (GenBank accession no. AJ245670; *S. enterica* serovar Typhimurium), pACM1 (GenBank accession no. AY081221; *Klebsiella oxytoca*), and pHNM1 (GenBank accession no. AY532647; *Enterobacter cloacae*); (iv) a subregion with an integron shared with pSEM (GenBank accession nos. AJ245670 and AJ009820), pACM1 (GenBank accession nos. U90945, AY309067, and AY309066), and some other plasmids; and (v) a subregion homologous to the chromosomes of *E. coli* and *S. enterica* serovar Typhimurium (GenBank accession nos. AF155222 and AL627276, respectively). This part of the p1658/97 molecule also contains two small p1658/97-specific subregions and two other regions that display homology to DNA molecules different from those mentioned above.

The mean G+C content of the p1658/97 nucleotide sequence is 50.5%, which is consistent with the average amount of G+C found in the genomes of members of the family *Enterobacteriaceae*. However, the G+C content among the particular sequence blocks of p1658/97 varies from 30% to 69%. Four blocks with mean G+C contents different from the average by more than 10% are marked in Fig. 2, and their borders are superimposed with some of the mosaic subregions homologous to DNA molecules other than F and R100.

Plasmid backbone. The functional modules of the plasmid backbone coding for replication, conjugative transfer, and the stable maintenance of p1658/97 have homologs in either F or R100, or both, suggesting that all three of these plasmids evolved from a common ancestor.

The functionalities of two replication systems found in p1658/97, IncFIB (positions 99420 to 101435) and IncFII (positions 37402 to 39441), were tested in *E. coli*. The replicons were separated by construction of two p1658/97 derivatives, pR and pFBAMP, which contain the IncFII and IncFIB replication systems, respectively. These plasmids were introduced into *E. coli* DH5 α cells. In both cases transformants were obtained, and the reisolation of the introduced plasmids (data not shown) demonstrated that the two replication systems of p1658/97 are functional in *E. coli*.

The stability of p1658/97 in a bacterial population might be ensured by two mechanisms: active partition determined by the *sopABC* operon (positions 109127 to 111994) and homologous to that of the plasmid F (11, 15) and two postsegregational killing systems, *pemI/pemK* (downstream of the IncFII replication module; positions 40555 to 41158) (40) and *hok/sok* (positions 124314 to 124583) (37). Both of these systems are identical to those in their R100 counterparts.

The conjugative transfer of plasmid p1658/97 is ensured by the products of the *tra* and *trb* genes. These genes are orga-

nized in a 33.3-kb operon (positions 2103 to 35307) that is almost identical at the nucleotide level to the *tra* operon found in the F plasmid (U01159) and that consists of 36 genes: 24 *tra* genes, 10 *trb* genes, *artA*, and *finO* (10). Nevertheless, two differences between these *tra* operons have been found. The first one is a frame shift in the *trbF* gene, creating the stop codon that results in shortening of the TrbF protein of p1658/97 by 25 amino acids. The TrbF function is unknown, but it is predicted to be an integral membrane protein (10). The second difference concerns the *finO* sequence, which codes for a conjugation regulatory function. In the F plasmid, this gene is interrupted by *IS3a*, resulting in an increase in transfer frequency in broth, evaluated as one transconjugant per donor cell (10, 38). In contrast to the sequence of the F plasmid, no IS(s) has been found within *finO* of p1658/97. The conjugative transfer frequency of p1658/97 in liquid medium was measured to be $4.5 \times 10^{-3} \pm 4.1 \times 10^{-4}$ transconjugants per donor cell. This value seemed to be significantly lower than the transfer frequency of the F plasmid and could have been caused by the presence of the functional *finO* sequence (10, 38).

Mobile elements. Nineteen mobile elements identified in p1658/97 constituted about 14% of the plasmid sequence and are listed in Table 1. All of them (e.g., *IS26*, *IS1*, and *IS100*), except for the remnant of *Tn21*, are situated within the region of similarity to other than F or R100 molecules. The fragment of *Tn21* is localized downstream of the IncFII replicon; thus, it has the same localization as that in the R100 plasmid, in which its complete version is found. The mobile elements of p1658/97 separate subregions of different homologies, which suggests their involvement in the mobilization of these subregions to the plasmid molecule.

Antibiotic resistance determinants in p1658/97. Apart from the *bla*_{SHV-5} gene (see below), all other antimicrobial resistance determinants of p1658/97 were found to be located in a class 1 integron (Fig. 3). These are genes *aadA1*, *aacC1*, and *aacA4*, which altogether confer resistance to the entire spectrum of clinically used aminoglycosides. The integron (positions 78683 to 85188) is flanked by one complete *IS26* sequence and one incomplete *IS26* sequence in the direct orientation (*IS26cp2* and Δ *IS26*, respectively). The *aadA1*, *aacC1*, and *aacA4* gene cassettes form the variable region, together with two ORFs, *orfX* and *orfX'*, which code for polypeptides of unknown function. The identical composition of the gene cassette array has been found in integron *INTCZD37* from *K. pneumoniae* (GenBank accession no. AF282595); integron *In-t3*, which is present in plasmid pSEM (20, 39); an integron from plasmid pACM1 (20, 29, 30, 31); and an integron from *Acinetobacter baumannii* (GenBank accession no. DQ370505). The sequence identities of the variable regions of these integrons vary between 99 and 100%. The p1658/97 integron contains the complete 3' conserved sequence (3'-CS) with the left inverted repeat (IRL), whereas its 5'-CS is truncated by 113 bp at the 5' end (89 bp from the stop codon of *intI1*) due to the *IS26* insertion (*IS26*/ Δ 5'-CS). The insertion produced the incomplete copy of the element (Δ *IS26*) and caused the lack of the integron's right inverted repeat (IRI). An identical *IS26*/ Δ 5'-CS has been reported in integrons in three plasmids belonging to the IncL/M group, the aforementioned plasmids pSEM (20, 39), pACM1 (20, 29, 30,

TABLE 1. Mobile elements found in p1658/97^a

Element type and name	Components	Component(s) in p1658/97	Component(s) in prototype	No. of copies	Coordinates	Remarks
Complete						
IS1	IRL, <i>insA</i> , <i>insB</i> , IRR			5	42726 to 43493; 68116 to 68834; 102972 to 103739; 108084 to 108851; 112952 to 113719	
IS26	IRL, <i>tnpA</i> , IRR			4	47137 to 47956; 77747 to 78556; 85735 to 86554; 94552 to 95371	
IS903	IRL, <i>tnpA</i> , IRR			1	69339 to 70395	
IS100	IRL, <i>orf27</i> (codes for ATP-binding protein), <i>tnpA</i> , IRR			1	70661 to 72614	
Incomplete						
IS26		<i>tnpA</i> , IRR	IR-L, <i>tnpA</i> , IR-R		85189 to 85734	
Tn21		IRL, <i>merR</i>	IRL, <i>merR</i> , <i>merT</i> , <i>merP</i> , <i>merC</i> , <i>merA</i> , <i>merD</i> , <i>merE</i> , <i>urf2</i> , <i>In2</i> , <i>tnpM</i> , <i>res</i> , <i>tn</i> , <i>tnpA</i> , IRR		43552 to 44032	
Tn1721		IRL, <i>orf33</i> (<i>orf1</i>)	IRL, <i>orf1</i> , <i>res</i> , <i>tnpR</i> , <i>tnpA</i> , IRR, <i>tetR</i> , <i>tetA</i> , IRL, <i>tnpA</i> , IRR		76040 to 77626	
Tn1		IRL, <i>tnpA</i>	IRL, <i>tnpA</i> , <i>tnpR</i> , <i>bla_{TEM}</i> , IRR		44181 to 47141	
IS21		IRL, <i>orf45</i> , <i>orf46</i> (incomplete <i>istA</i>), IRR	IRL, <i>istA</i> , <i>istB</i> , IRR		101703 to 102292	
Putative						
<i>tnpA1</i>		IRL, <i>tnpA1</i> , IRR			50833 to 51636	Product of <i>tnpA1</i> has 84% identity to putative TnpA from pEA29 of <i>Erwinia amylovora</i> (GenBank accession no. AF264951)
<i>orf17</i>		<i>orf17</i>			50501 to 50785	Product of <i>orf17</i> has 86% identity to unknown protein of pEA29 of <i>E. amylovora</i> (GenBank accession no. AF264951)
<i>orf54</i>		<i>orf54</i>			113715 to 114494	Product of <i>orf54</i> has 62% identity to putative transposase of <i>K. pneumoniae</i> (GenBank accession no. NP943536)

^a IRL and IRR, left and right inverted repeats, respectively; *tnpA*, transposase gene; *tnpR*, resolvase gene.

31), and pAK33 from *K. pneumoniae* (In111; GenBank accession no. AY260546) (20, 44), and also in plasmid p541 from *E. coli*, which belongs to the IncN group (In-e541; GenBank accession no. AY340637) (20, 21, 43). The integrons of p1658/97,

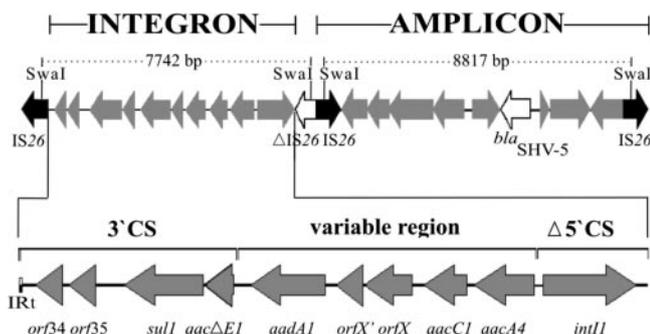


FIG. 3. Schematic representation of the multiresistance locus of p1658/97 with the amplicon and the integron. Black arrows indicate IS26, gray arrows represent genes localized within the integron and the amplicon, and white arrows indicate the remnant of IS26 (Δ IS26) and the *bla_{SHV-5}* gene. The position of SwaI and the sizes of the restriction fragments are indicated.

pSEM, and pACM1 are associated with the almost identical region encompassing the *bla_{SHV-5}* gene and are flanked by two IS26 copies (IS26cp3 and IS26cp4; see below); however, the mutual localization of the integron and this region is different in all three molecules (20, 29). In p1658/97 and pACM1, the integron is localized downstream of the *bla_{SHV-5}* gene-containing region, but in pSEM it is inverted and placed upstream. In both pACM1 and pSEM, the complete IS26 copy was found at the 5'-CS of the integrons, whereas in p1658/97 it is truncated (Δ IS26). Moreover, in pACM1 the integron and the *bla_{SHV-5}*-containing region are separated by another unrelated integron that is adjacent to IS1, and the IS6100 element was identified in the 3'-CS of the pSEM integron.

Evolutionary history of the p1658/97 structure. A region flanked by identical copies of an IS(s) can be considered a composite transposon type I that, as a consequence, should be able to transpose (38). Most of the transposons duplicate their target sequence of a specific size during transposition (19), and such duplicated sequences (direct repeats [DRs]) that flank segments of more complex structures allow these to be identified as integrated mobile elements; e.g., IS100 generates 5-bp DRs (1), whereas IS26 produces 8-bp DRs (22). In p1658/97,

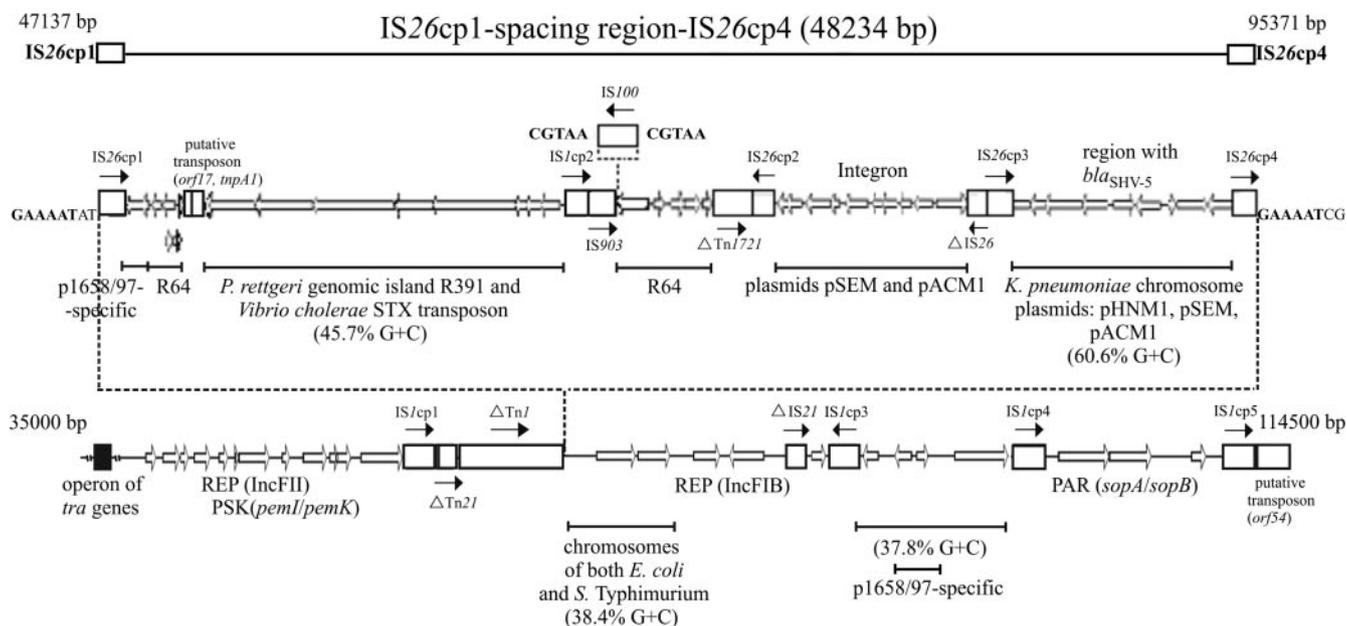


FIG. 4. Hypothetical events leading to the present structure of p1658/97. The partial p1658/97 sequence (positions 35000 to 114500) is drawn to scale; the remaining part (including the *tra* region) is out of scale. The module “IS26cp1-spacing region-IS26cp4” (positions 47137 to 95371) is marked at the top. Open boxes represent the mobile elements, and arrows represent ORFs. The orientations of the mobile elements are marked with black arrows; incomplete mobile elements are marked with a Δ symbol. Dotted lines show hypothetical mobilization events that implant different submodules. The regions of the plasmid backbone are marked: REP, replication; PAR, partition; PSK, postsegregational killing. DRs, when they have been identified, are shown. Regions of over 90% similarity (at the nucleotide level) to DNA molecules other than F and R100 are shown.

the perfect duplication of the target site was observed only in the case of IS100 (Fig. 2). The sequences surrounding other potentially mobile elements are different; however, IS26cp1 and IS26cp4 are each flanked at one end by DR-like sequences that are identical in six of eight nucleotides (underlined): GAAAATAT and GAAAATCG, respectively (Fig. 4). The 48-kb “IS26cp1-spacing sequence-IS26cp4” segment encompasses almost all of the mosaic subregions of the plasmid, e.g., IS100, the “IS26cp2-integron- Δ IS26” region, and the “IS26cp3-*bla*_{SHV-5}-IS26cp4” region. This finding suggests that the 48-kb segment of p1658/97 could have originated from the IS26-mediated transposition of a composite element from an unidentified molecule into the site adjacent to the incomplete TnI copy (Fig. 4). On the other hand, the structure of this segment itself probably arose from a variety of separate integration events that implanted other mobile elements present within this region. The “IS26cp2-integron- Δ IS26” and “IS26cp3-*bla*_{SHV-5}-IS26cp4” regions that are localized within the 48-kb segment of p1658/97 have the structure of a composite transposon type I themselves. However, the lack of DRs at their ends suggests, rather, recombination as a mechanism of their acquisition by p1658/97, similar to that for the IS26-flanked DNA fragments observed in plasmids pSEM, pAK33, p541, pACM1 (20), and pHNM1. It is impossible to elucidate whether the full structure of the 48-kb segment was formed before or after the recruitment of the putative composite transposon into p1658/97.

Identification of the region that undergoes amplification. In order to determine the DNA region that undergoes amplification in plasmid p1658/97, the results of restriction analysis

obtained in silico were compared with those obtained in vitro. The SmaI restriction enzyme recognizes only seven sites within the plasmid molecule, and five of them are localized within the IS26 copies, one site per each copy (Fig. 3). The increased intensity of one band of approximately 9 kb in the lane representing plasmid DNA from the “resistant” colony indicated the amplification of that region (Fig. 1). The only SmaI fragment corresponding in size to the observed band was that of 8,817 bp, which contained the *bla*_{SHV-5} region flanked by the parts of IS26cp3 and IS26cp4 delimited by the SmaI sites. Numerous reports concerning DNA amplification showed that amplifying segments are most frequently flanked by homologous sequences (repeats) at their ends (27, 28, 33, 34). The two IS26 elements, each of which is 820 bp, probably played the role of such long homologous repeats. Therefore, the region of 9,637 bp, including the complete IS26cp3 and IS26cp4 sequences, was identified as an amplicon. Restriction analysis of plasmid p1658/97 isolated from the “resistant” colony performed with other enzymes confirmed that amplification was limited only to the region of the amplicon (data not shown). The amplification of the *bla*_{SHV} gene copy number in association with the presence of IS26 has also been observed in *K. pneumoniae* clinical isolates by Hammond et al. (14).

The details of gene amplification in plasmid p1658/97 are the subject of a further study. Preliminary results, which have shown no difference in the amplification frequencies of RecA-proficient and RecA-deficient *E. coli* isogenic strains bearing p1658/97 (tested on medium with 30 μ g/ml ceftazidime), suggest, rather, the RecA independence of the process studied. However, when high antibiotic pressure was applied (128

μg/ml ceftazidime), approximately 100-fold more “resistant” colonies were obtained for the RecA-proficient strain. Simultaneously, “resistant” clones selected with 128 μg/ml ceftazidime harbored plasmids with significantly more copies of the amplified *bla*_{SHV-5} region than those isolated with 30 μg/ml ceftazidime, irrespective of the RecA content (data not shown). These results led to the speculation that the RecA protein could be involved in amplification only subsequent to the RecA-independent amplification, when several amplicon copies are available. Similar results have been demonstrated for plasmid NR1, in which the initial duplications of the amplifiable region flanked by *IS1* occurred in the RecA-independent but *IS1*-mediated manner, and these duplications were a limiting step for further RecA-mediated multiplication (28).

Amplicon structure. The organization of the amplicon is shown in Fig. 3. Apart from *bla*_{SHV-5}, the region contains an incomplete operon involved in sugar metabolism (truncated *ygbM* and genes *fucA*, *ygbK*, and *ygbJ*) and its regulatory gene (*ygbI*) in p1658/97, named ORFs 36 to 39 and 40, respectively (41, 46); a gene coding for a putative RecF protein with the incomplete catalytic domain (*orf42*) (9); and a truncated *lacY* gene, which encodes the lactose transport protein. As mentioned above, the DNA fragment located between the two *IS26* elements was found to be a derivative of the *K. pneumoniae* chromosome (20, 39), which could be expected, considering the earlier data indicating that this species is a source of *bla*_{SHV} genes (4, 6, 7, 13, 16). The presence of the same segment of *K. pneumoniae* DNA flanked by two *IS26* elements in p1658/97, pSEM, pACM1, and pHNM1 indicates that it probably arose from a single escape from the *K. pneumoniae* chromosome and later became disseminated into a variety of plasmid molecules. Interestingly, as already mentioned, pHNM1, pSEM, and also pACM1 belong to the IncL/M incompatibility group and are able to replicate in a broad range of hosts (38). This fact emphasizes the possibility of the spread of the amplicon among various DNA replicons and bacterial species. Similar fragments of the *K. pneumoniae* chromosome with *bla*_{SHV} genes have also been found within other partially sequenced plasmids: pZMP1 (GenBank accession no. X53817; *K. pneumoniae*), pPa-1 (GenBank accession no. AF074954; *P. aeruginosa*), pNSF-1 (GenBank accession no. AF282921; *Shigella flexneri*) (9), and pPAG-KE (GenBank accession no. AF096930; *P. aeruginosa*). The structures of plasmids pZMP1 and pPa-1 show the association of SHV-type β-lactamase genes with *IS26* as well. The results presented and discussed above strongly support the hypothesis that *IS26* plays a major role in mobilization of the *bla*_{SHV} genes from the *K. pneumoniae* genome and their further dissemination to a wide range of enterobacterial strains (6).

Conclusions. We report here the complete nucleotide sequence of a plasmid in which ESBL gene *bla*_{SHV-5} undergoes amplification. This process results in the high-level resistance of clinical isolates to β-lactam antibiotics, including expanded-spectrum cephalosporins. The amplicon originated by mobilization from the chromosome of *K. pneumoniae* and was later spread into several different plasmids and, by means of their horizontal transfer, to a variety of bacterial species over a wide geographic area. Although the amplicon is flanked by two *IS26* elements and thus has a structure of a type I composite transposon, the major way of its dissemination among DNA replicons is probably mediated by recombination by *IS26* copies,

which is a subject of our further studies. This work, together with several other investigations (7, 14, 17, 20, 23), strongly underlines the important role of *IS26* in the spread of β-lactamase genes acquired by clinical bacterial populations, especially those coding for the SHV-type enzymes.

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