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Characterization of pUO-StVR2, a Virulence-Resistance Plasmid Evolved from the pSLT Virulence Plasmid of *Salmonella enterica* Serovar Typhimurium▽

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pUO-StVR2 is a virulence-resistance plasmid which originated from pSLT of *Salmonella enterica* serovar Typhimurium through acquisition of a complex resistance island, flanked by regions that provide a toxin-antitoxin system and an iron uptake system. The presence of resistance and virulence determinants on the same plasmid allows coselection of both properties, potentially increasing health risks.

pUO-StVR2 is a hybrid virulence-resistance plasmid that is widely distributed among isolates of *Salmonella enterica* serovar Typhimurium, recovered in Spain not only from clinical samples but also from food and food-producing animals (2, 9, 11, 12). Similar isolates have recently been detected in the United Kingdom (A. Herrero, unpublished results), and indirect evidence suggests their presence in other European countries (1, 14). pUO-StVR2 confers resistance to ampicillin, chloramphenicol, streptomycin-spectinomycin, sulfonamides, and tetracycline, encoded by the bla_{OXA-1} (also called bla_{OXA-30}) (3), *catA1*, *aadA1*, *sul1*, and *tetA*(B) genes, with two of them (bla_{OXA-1} and *aadA1*) located in the variable region of an integron termed InH. It also contains the *spvC*, *rck*, *samA*, *traT*, *traX*, *repA*, and *parA*/*B* genes of pSLT, the virulence plasmid specific for *S. enterica* serovar Typhimurium (17, 18), but it lacks the InFIB/*repA2* replication region, including *rsk* (a 66-bp sequence that corresponds to the B, C, and D iterons of the replicon), and most of the *pef* operon (9, 11, 12).

The possibility that pUO-StVR2 originated from pSLT was investigated by hybridization experiments. For these experiments, pSLT and pUO-StVR2 were extracted from *S. enterica* serovar Typhimurium LT2 and *S. enterica* serovar Typhimurium LSP 146/02, respectively (11), and digested with different endonucleases (Fig. 1). After separation by conventional agarose gel electrophoresis, fragments were transferred onto a nylon membrane and hybridized with a probe comprising the entire pSLT (19). As shown in Fig. 1, many fragments of pUO-StVR2 have a counterpart in pSLT, as revealed by both coincidence in size and hybridization, hence demonstrating the close relationship between the two plasmids. Other fragments generated from pUO-StVR2 were absent in the lanes corresponding to pSLT and failed to hybridize with the probe. They could be internal to DNA newly gained by pSLT, and this was in fact confirmed by identification of the junction regions as the fragments generated from pUO-StVR2 which hybridized with the probe but lacked a pSLT counterpart.

The genetic organization of the DNA acquired by pSLT to build pUO-StVR2 was further established by cloning and sequencing of the differential DNA and the junction regions. The hybrid plasmid was subjected to single and double digestions with the endonucleases used in the hybridization experiment (described above). The generated fragments were ligated into the corresponding sites of pUK1921, a cloning vector that contains a kanamycin resistance gene as a selectable marker (10). The obtained libraries were transformed into *Escherichia coli* DH5α (Invitrogen) (19). Recombinant plasmids were selected for acquisition of resistance properties associated with pUO-StVR2 or for the presence of fragments with sizes matching internal or junction regions of the differential DNA (described above). Relevant insertions were sequenced at the Servicio de Secuenciación de DNA, Centro de Investigaciones Biológicas (CSIC [Madrid, Spain]). Overlapping fragments were assembled to generate a contiguous sequence of 49,507 bp, which was analyzed online at the European Bioinformatics Institute web site (http://www.ebi.ac.uk/).

The sequence obtained (Fig. 2A) consists of 47,606 bp of foreign DNA and 1,901 bp of flanking pSLT DNA (525 and 1,376 bp at the conventional left and right ends, respectively). Within the latter, the new DNA is located between the noncoding region upstream of the *ccdA* gene and the 3′ end of open reading frame 6 (ORF6) upstream of *pefI*. The intermediate segment of pSLT (ca. 12 kb), which includes IncFIB/*repA2*, the overlapping *rsk* sequence (described above), and most of the *pef* operon (*pefBACD*), appears to have been replaced by the acquired DNA (Fig. 2B).

The foreign DNA has a mosaic structure and consists of a central region containing all of the resistance genes previously identified in pUO-StVR2 clustered within an antimicrobial resistance island of 28,756 bp and two flanking regions where additional virulence genes were identified (Fig. 2A). The left-hand region, at the *ccdA* end, comprises 7,102

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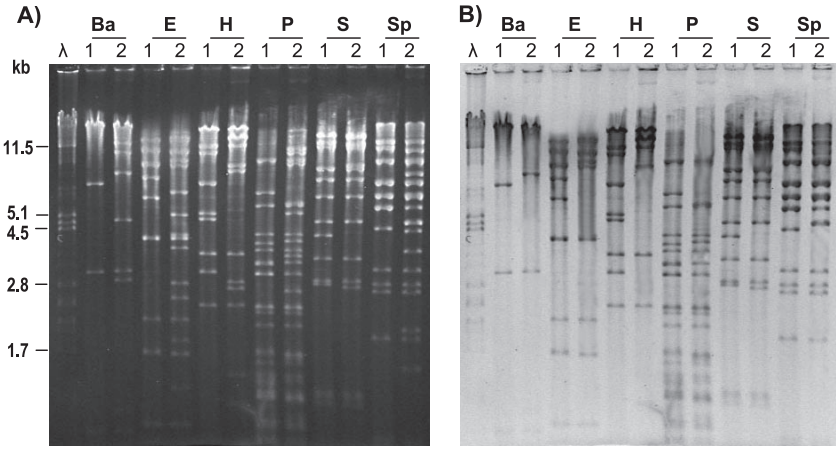

FIG. 1. Relationship between pSLT and pUO-StVR2. (A) Restriction analysis of pSLT and pUO-StVR2. (B) Hybridization with the entire pSLT plasmid. Ba, BamHI; E, EcoRI; H, HindIII; P, PstI; S, SalI; Sp, SphI; λ, DNA of phage lambda digested with PstI, used as a size standard; lanes 1 and 2, pSLT and pUO-StVR2, respectively.

bp with a GC content of 46.9%, which is nearly identical (99%) to sequences present in two multidrug-resistant plasmids, pRSB107 isolated from an uncultured bacterium in a sewage treatment plant (20) and pU302L from *S. enterica* serovar Typhimurium (5). It contains several ORFs of unknown function (here termed ORFL1 to ORFL5), and the *vagC* and *vagD* genes that encode a toxin-antitoxin system. The latter were originally detected in pSDV, the virulence plasmid specific for *S. enterica* serovar Dublin, and shown to be important for virulence (16), although the effect is now considered to be indirect, due to an enhancement of plasmid stability (8). In pUO-StVR2, the capture of a second toxin-antitoxin system, in addition to that encoded by the *ccdA*/*ccdB* genes already present in pSLT (8, 18), provides an extra level of security, further ensuring plasmid stability and therefore persistence of the resistance and virulence functions it confers.

The central resistance cluster includes a composite transposon almost identical to Tn*2670*, harbored by the NR1 (R100) plasmid of *Shigella flexneri*, which consists of Tn*21* inserted within Tn*9* and is thus delineated by the two copies of IS*1* in direct orientation (13). However, the InH integron carried by the Tn*21*-like transposon of pUO-StVR2 differs from the In*2* integron of Tn*21* by insertion of the bla_{OXA-1} gene cassette upstream of *aadA1*. A defective Tn*10* completes the resistance island of the hybrid plasmid. It carries *ydjB*, *yeaA*, *tetR*(B), *tetA*(B), *tetC*, and a truncated *tetD*. Yet, IS*10*-L, *ydhA* and *ydjA* are missing at one end, while most of *tetD* and part of *yedA* (which encodes the transposase of IS*10*-R) are replaced by a copy of IS*1* at the other end. The resulting structure comprises the *tetR*(B)-*tetA*(B) genes between two oppositely oriented copies of IS*1* and could therefore represent a new composite tetracycline transposon. It should further be noted that colinearity between a Tn*2670*-like transposon and a defective Tn*10* has also been found in pRSB107 as well as in a pathogenicity island of *Shigella flexneri* 2a (15). However, the complexity of the Tn*2670* derivative and/or the extent of the deletions affecting Tn*10* differ in the three resistance clusters.

The remaining DNA incorporated by pSLT (11,748 bp [54.9% GC]) is 100% identical to another region of pRSB107. It includes truncated and intact copies of IS*26* which flank the *scsC* (incomplete) and *scsD* genes (for *s*uppression of *c*opper *s*ensitivity), an *nqrC*-like gene (for Na^+ transport), and several ORFs designated here as ORF-R1 to ORF-R9. At least two of the latter, ORF-R1 and ORF-R2, which encode an Fe^{2+}/Pb^{2+} permease and an Fe^{2+} transport protein, respectively, and which are preceded by two potential Fur boxes, could be involved in iron uptake (20). The similarity of the right-hand region of pUO-StVR2 to the corresponding DNA of pRSB107 extends to include the entire IS*1* downstream of the truncated *tetD*, which is here considered as part of the central resistance island. The sequence from *scsC* to ORF-R9 is also found in pUTI89, a plasmid from the uropathogenic *E. coli* strain UTI89 (99% similarity) (6), as well as in the chromosome of *Citrobacter koseri* ATCC BAA-895 (accession no. CP000822 [98% similarity]). In the former, a copy of IS*26* is placed at the right-hand end but not at the *scsC* end, while no IS*26* flanked the equivalent segment of the *C. koseri* chromosome. Interestingly, the ORFs encoding the putative iron acquisition system are also present in a pathogenicity island of the highly invasive organism *Yersinia pestis* (accession no. AL031866).

As for the origin of pUO-StVR2, it is not known whether the distinct components of the foreign DNA have been sequentially acquired by pSLT or if the tripartite structure has been previously assembled in an as-yet-unidentified precursor plasmid. Nevertheless, the simultaneous presence of resistance and virulence determinants on the same extrachromosomal element will allow coselection of both properties, yielding potentially more dangerous strains that represent a hazard to human and animal health (7). The capture of an iron acquisition system by pSLT is of particular relevance, since successful competition for this essential nutrient, which is not freely available in the host, is of crucial importance for pathogens to establish infection (4).

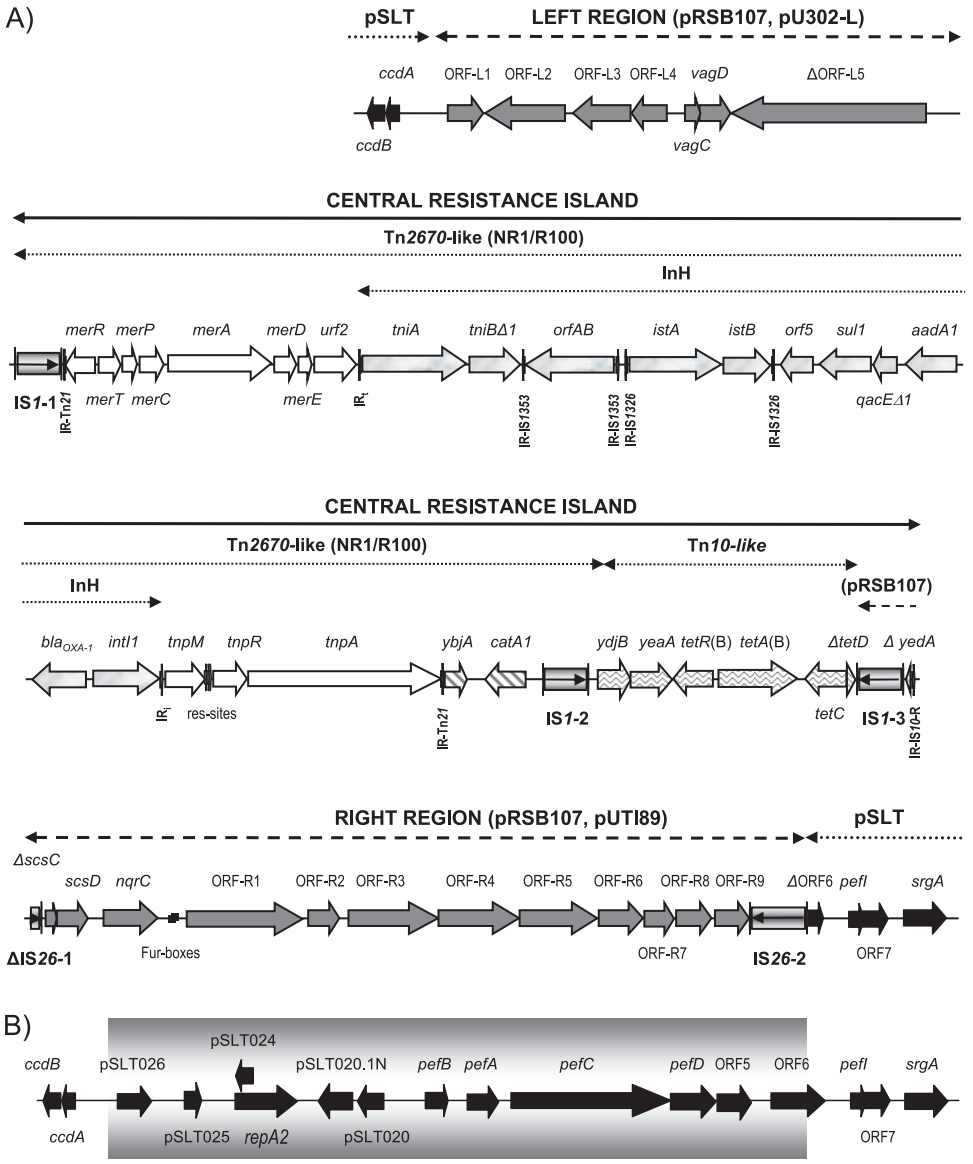

FIG. 2. (A) Schematic representation of the DNA acquired by pSLT to compose pUO-StVR2. Solid and dashed horizontal lines above the scheme are used to point out the distinct components of the represented DNA. Plasmids showing the highest similarity to the main regions are indicated in parentheses. (B) pSLT region where the foreign DNA was inserted (based on accession no. AE006471). The segment of pSLT absent in pUO-StVR2 is shown within a gray box.

Nucleotide sequence accession number. The sequence generated in this work has been deposited in the EMBL database under accession no. AM991977.

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