

Characterization of a Self-Transferable Plasmid from *Salmonella enterica* Serotype Typhimurium Clinical Isolates Carrying Two Integron-Borne Gene Cassettes Together with Virulence and Drug Resistance Genes

Beatriz Guerra,^{1,2} Sara Soto,¹ Reiner Helmuth,² and M. Carmen Mendoza^{1,*}

Departamento de Biología Funcional, Área de Microbiología, Universidad de Oviedo, 33006 Oviedo, Principality of Asturias, Spain,¹ and Federal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV), National Salmonella Reference Laboratory, 12254 Berlin, Germany²

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An unusual self-transferable virulence-resistance plasmid (pUO-StVR2) was found in nine multidrug-resistant (ACSSuT phenotype) *Salmonella enterica* serotype Typhimurium clinical isolates that were assigned to four different phage types and a single and distinctive *XbaI* pulsed-field gel electrophoresis profile. pUO-StVR2 is an IncFII plasmid of about 140 kb in length carrying the *spvA*, *spvB*, and *spvC* (*Salmonella* plasmid virulence) and *rck* (resistance to complement killing) genes. It also carries the *oxa1/aadA1a* (ampicillin resistance and streptomycin-spectinomycin resistance) gene cassette configuration located within a class 1 integron with *qacEΔ1/sul1* (ammonium antiseptics resistance and sulfadiazine resistance); the transposon genes *merA*, *tnpA*, and *tnpR* (mercury resistance, transposase, and resolvase of Tn21, respectively); and the *catA1* (chloramphenicol resistance) and *tet(B)* (tetracycline resistance) genes. The insertion of resistance genes into a *Salmonella* virulence plasmid constitutes a new and interesting example of plasmid evolution and presents a serious public health problem.

Several *Salmonella enterica* serotypes harbor plasmids which are essential for virulence. Although virulence plasmids from different serotypes are not identical, the sequences required for virulence are highly conserved (5, 16, 20, 21, 23, 33). Most of the *Salmonella enterica* serotype Typhimurium isolates carry a serovar-specific 90-kb virulence plasmid which belongs to the incompatibility group IncFII. However, irrespective of their plasmid content, serotype Typhimurium isolates have been assigned to different clones, which are usually defined by phage typing (2) and DNA fingerprinting (14, 20, 22, 31, 39). A particular serotype Typhimurium clonal group which includes phage type 104 (DT104) strains that are, at the least, resistant to ampicillin, chloramphenicol-florfenicol, streptomycin-spectinomycin, sulfonamides, and tetracycline (ACSSuT phenotype) has emerged worldwide and presents a global health problem (12, 22, 26, 31, 34). Recently, the emergence of other multidrug-resistant serotype Typhimurium clones in which the resistance is plasmid mediated has been reported (7, 16, 21, 38, 39). In these clones, the resistance genes are often part of transposons and/or integrons, elements that facilitate the intracellular movement of resistance genes. Among the different classes of integrons described, class 1 integrons are the most frequently found in *Salmonella* as well as in other gram-negative bacteria and they can be found within transposons and be chromosome and/or plasmid located (3–7, 11, 13–17, 19, 28–30, 38).

In the present work, we report an unusual self-transferable virulence plasmid responsible for multidrug resistance (ACSSuT) in serotype Typhimurium clinical isolates of various phage types sharing a common *XbaI* pulsed-field gel electrophoresis (PFGE) profile.

MATERIALS AND METHODS

Bacterial strains. In previous works (reference 15 and unpublished data), 83 *Salmonella enterica* serotype Typhimurium isolates carrying class 1 integrons were identified. Nine of these isolates were selected for the study presented here. They originated from human feces and were collected at regional hospitals. All were received throughout 1993 to 1999 at the Laboratorio de Salud Pública (LSP) of the Principality of Asturias. The isolates were phage typed in the Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid, Spain, by using Anderson's scheme (2). As controls in different experiments, the following serotype Typhimurium strains carrying the 90-kb virulence plasmid were tested: LT2 (pSLT90) (Bayer AG Collection), ATCC 14028 (pSLT90) (American Type Culture Collection), and LSP14/92 (pUO-StV1), which is a DT104 (subtype I)-ACSSuT clinical isolate (14–16). For mating experiments, serotype Typhimurium LSP31/93 was used as plasmid donor and the rifampin-resistant *Escherichia coli* K-12 strain J53 and nalidixic acid-resistant *Salmonella enterica* serotype Panama LSP291/98 were used as recipients. These strains were tested for susceptibility to 15 antimicrobial agents (15) by disk diffusion (24) and to florfenicol by broth microdilution (25) by using a Sensititre semiautomatic system (Trek Diagnostics, East Grinstead, United Kingdom). Results were analyzed according to standards set by NCCLS (24, 25).

Plasmid and PFGE analysis. Plasmid profiling, plasmid curing, and transfer of antibiotic resistance by conjugation were performed as described in references 14 and 16. Plasmid relationships were tested by restriction analysis using 5 U of *HindIII*, *ClaI*, and *EcoRI* (Amersham Pharmacia Biotech, Barcelona, Spain) separately and by hybridization using gene-specific probes (16). PFGE analysis was performed with *XbaI* as described in reference 14.

PCR amplification, purification, and sequencing of DNA. The detection of *Salmonella* virulence plasmid genes, resistance genes, and transposon- and/or integron-related genes was performed with the PCR conditions and primers described previously (10, 14–16, 18) or designed for this work: *repFIIA-F/B*, CTGTCGTAAGCTGATGGC/CTCTGCCACAACCTTCAGC; *traT-F/B*, GAT

* Corresponding author. Mailing address: Departamento de Biología Funcional, Área de Microbiología, Facultad de Medicina, Universidad de Oviedo, c/Julián Clavería 6, 33006 Oviedo, Spain. Phone: 34-985103560. Fax: 34-985103148. E-mail: cmendoza@correo.uniovi.es.

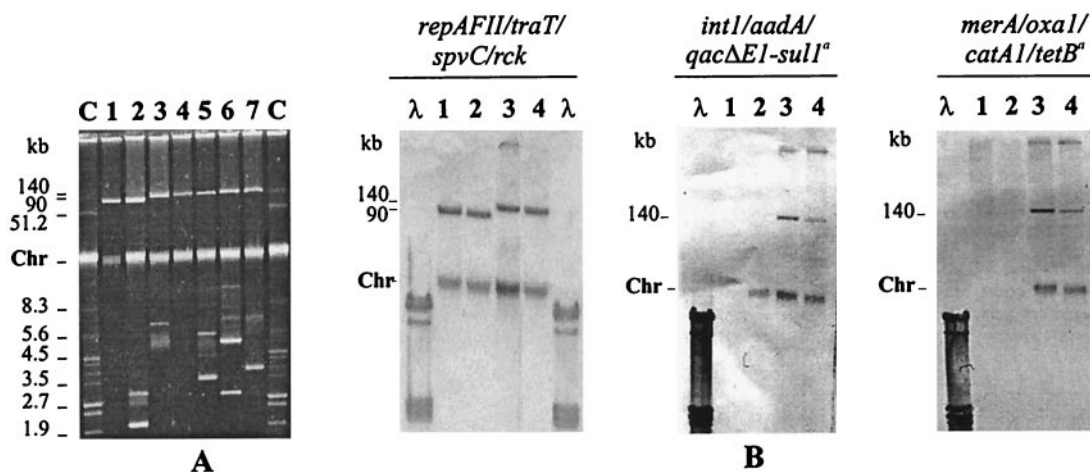


FIG. 1. Plasmid analysis of representative serotype Typhimurium clinical isolates and control strains. (A) Plasmid profiles. Lanes: C, molecular size standard plasmids; 1 through 7, profiles generated by LT2 (lane 1), LSP14/92-DT104 (subtype 1) (lane 2), LSP31/93-DT120 (lane 3), *E. coli* CT31 (lane 4), LSP28/98-DT104 (subtype b-low) (lane 5), LSP106/94-NT, LSP233/98-NT, LSP349/98-NT; 362/98-NT, LSP436/99-NT, and LSP559/99-NT (lane 6), and LSP21/99-RDNC (lane 7). (B) Lanes: 1 through 4, successive hybridizations of lanes 1 through 4 from panel A, with the probes cited at the top of the figure; λ , phage lambda DNA digested with *Pst*I. Chr, chromosomal DNA.

GGTTACTGCTGAGATCTGTACGTCG; *rck*-F/B, TCGTTCTGTCCTCACTGC/TCATAGCCCAGATCGATG; and *pefA*-F/B, GCACACGCTGCCAATGAA/CACAGACTTGAAGTCACC (accession number AE006471); and [*tnpR*-F/B], [GGCGACACCGTGGTGGTCATAGC/CGGTAAGCCCCGCTTGCTTGGC] (accession number AL513383). Restriction and sequencing of the PCR products were carried out as described in references 15 and 16, and the sequences obtained were compared to those registered in GenBank.

RESULTS AND DISCUSSION

Characterization of a self-transferable virulence-resistance plasmid of *Salmonella enterica* serotype Typhimurium. In previous works (reference 15 and unpublished data), nine serotype Typhimurium isolates were identified which carried a class 1 *qacEΔ1/sulI* (encoding ammonium antiseptics resistance and sulfadiazine resistance) integron, generating PCR products of 2,000 bp with 5' CS and 3' CS primers. By using *Pvu*I, *Taq*I, and *Bgl*II endonucleases, the 2,000-bp PCR products yielded the same restriction pattern as the one described previously (15) for an integron-borne *oxa1/aadA1a* (encoding ampicillin resistance and streptomycin-spectinomycin resistance) cassette array. PCR amplification confirmed the presence of both *oxa1* and *aadA1a* within the 2,000-bp amplicons. The *oxa1/aadA1a* cassette configuration is characteristic for the transposon Tn2603 (27, 40). The presence of transposon genes *tnpA*, *tnpR*, and *merA* (encoding the transposase, resolvase, and mercury reductase, respectively) was confirmed by PCR amplification (amplicons of about 327, 240, and 1,232 bp, respectively, were generated) in the nine isolates, but long PCR amplification (data not shown) failed to confirm a link between transposon and integron genes. On the other hand, amplification and sequencing also revealed the presence of the *catA1* (encoding chloramphenicol resistance) and *tet(B)* (encoding tetracycline resistance) genes (accession numbers AP000342 and Y19113, respectively) in the nine isolates.

Plasmid analysis showed that the nine serotype Typhimurium isolates carried a large plasmid of about 140 kb to-

gether with other small plasmids but not the serotype Typhimurium 90-kb virulence plasmid (Fig. 1). However, when these isolates were tested by PCR for virulence plasmid genes (*repFIIA* [plasmid incompatibility group FIIA replicons]; *traT* [conjugative transfer]; *spvA*, *spvB*, and *spvC* [*Salmonella* plasmid virulence]; *rck* [resistance to complement killing]; and *pefA* [plasmid-encoded fimbria]), the expected amplicons (about 288, 483, 600, 1,060, 424, 474, and 442 bp, respectively) were generated with all except the *pefA* primers.

To ascertain if the virulence and resistance genes were linked and whether they were located in the plasmid or chromosome, the isolate LSP31/93 was tested by curing and conjugation experiments. By curing, no segregants were found among the approximately 300 CFU tested. Moreover, the 140-kb plasmid was self-transferable to *E. coli* K-12 J53 and more effectively transferable to Panama LSP291/98. The transconjugants expressed the same resistance phenotype (AC-SSuT) as the parental strain, and their plasmid DNAs generated the expected PCR products and hybridized with probes for the virulence plasmid, transposon and/or integron, and resistance genes (Fig. 1 and 2). This plasmid was labeled pUO-StVR2 (plasmid University of Oviedo-*Salmonella enterica* serotype Typhimurium virulence and resistance).

To demonstrate the genetic relationship between pUO-StVR2 from the *E. coli* CT31 transconjugant and pSLT90 from LT2, both plasmids were tested by restriction analysis (Fig. 2A) with three endonucleases (*Hind*III, *Cla*I, and *Eco*RI) and by hybridization with probes derived from pSLT90 virulence genes (Fig. 2B). Plasmid restriction analysis showed that each plasmid revealed a distinct restriction profile with some fragments banding at identical positions. The hybridizations made in order to locate the virulence plasmid genes in the *Hind*III, *Cla*I, and *Eco*RI restriction patterns showed that the *traT* probe always hybridized to matching fragments (18, 6.6, and 14.3 kb, with each endonuclease), the *spvC* probe also always hybridized to matching fragments (3.6, 6.6, and 3.8 kb), and the

ments and between 6 and 11 mismatching fragments regarding the ones from the control strains. These results support the fact that the nine isolates should be classified as members of a single clonal group, well differentiated from the control strains, one of which represented the prevalent DT104-ACSSuT clone (with PFGE2) (14, 22). Within this new clonal group, there is some heterogeneity with respect to phage type and plasmid content and a relationship between both markers seems to exist (Fig. 1).

Previous work has shown that changes in phage types can be the result of the loss or acquisition of plasmids (37). Since not all DT104 strains present the markers described for the so-called DT104-ACSSuT clone or clonal line (in our experience, NT strains could also be ascribed to this clone), it must be taken into account that phage typing alone is not a suitable method for the identification of a clone.

Hypothesis concerning the origin of the virulence-resistance plasmid pUO-*St*VR2. Some interesting facts regarding the dispersion of virulence and resistance plasmids, as well as of the genes harbored by pUO-*St*VR2, have previously been published. (i) Serovar-specific virulence plasmids of different sizes have been found in *Salmonella* subspecies I (23, 33), but it has also been reported that some serotypes belonging to subspecies II, IIIa, and IV carry *spv* genes on the chromosome (5). (ii) At least three other types of virulence-resistance plasmids, each of them encoding different resistance genes, have been detected in *Salmonella* collected in different countries (9, 14, 16, 21). (iii) The integron reported here is found forming part of transposon Tn2603 (27, 40), and a similar integron has been found in a 140-kb IncFI plasmid of serotype Typhimurium non-DT104-resistant isolates collected in Italy (38). (iv) Both the *catA1* and *tet(B)* genes are widely dispersed among *Enterobacteriaceae* and can be part of transposons (Tn9 and Tn10, respectively) and be located in plasmids (1, 8, 19, 32). These facts encourage us to suggest the following possible events leading to the generation of pUO-*St*VR2. First, in the animal reservoir, a serotype Typhimurium bacterium showing a distinctive PFGE profile and carrying a virulence plasmid (in association with other small plasmids) acquired one (or more) resistance plasmid(s). In a second step, recombination-transposition processes between the virulence and the resistance plasmid(s) could have taken place, generating the hybrid plasmid (pUO-*St*VR2). This hybrid plasmid belongs to the serotype Typhimurium virulence plasmid family (because it carries the *repFIIA*, *traT*, *spv*, and *rck* genes) but is of a larger size since it has gained several resistance genes [*oxa1*, *aadA1a*, *qacEΔ1*, *sull*, *merA*, *catA1*, and *tet(B)*], some of them integron and/or probably transposon related. Finally, pUO-*St*VR2-carrying *Salmonella enterica* serotype Typhimurium strains spread and established in their reservoir and sporadically reach humans through the food chain. The latter is supported by the isolation of such strains from human feces during the period from 1993 to 1999. In addition to the strains described above, we found in the year 2000 three new serotype Typhimurium clinical isolates carrying the same resistance genes, including the integron-borne *oxa1/aadA1a* cassette configuration. To date, we have found no other *Salmonella* isolate presenting this cassette array among about 2,000 isolates (isolated between 1989 and 2000) tested for integrons and/or resistance plasmids (15, 23, 35, 36; unpublished data).

The findings presented above support the fact that different serotype Typhimurium clones or clonal groups, with distinctive PFGE profiles, display similar resistance phenotypes based on different resistance genes which can be located on the chromosome and/or on plasmids. In each clone, some resistance genes and their gene arrays seem to be characteristic, frequently including one or more transposon and/or integron structures. Serotype Typhimurium has a wide animal reservoir, composed of mainly cattle and swine, which is linked to humans via the food chain. Consequently, the selection and maintenance of different resistance genes in different serotypes and clones like the one reported here constitute a serious public health problem.

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