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Multidrug Resistance Is Mediated by Large Plasmids Carrying a Class 1 Integron in the Emergent *Salmonella enterica* Serotype [4,5,12:i:−]

BEATRIZ GUERRA, SARA M. SOTO, JOSE M. ARGÜELLES, AND M. CARMEN MENDOZA*

Área de Microbiología, Departamento de Biología Funcional, Universidad de Oviedo, 33006 Oviedo, Principado de Asturias, Spain

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A multidrug-resistant *Salmonella enterica* serotype [4,5,12:i:−] clone carried a class 1 integron harboring *dfrA12* and *aadA2* gene cassettes and $bla_{\text{TEM-1}}$, *aac(3)-IV*, *cmlA1*, and *tetA* genes located in large plasmids of about 140 kb (carrying *spv*) or 120 kb (lacking *spv*). Several segregants, lacking multidrug resistance, contained a plasmid smaller than the parental one and no longer hybridized with probes for the lost resistances. The genes mediating resistance to ampicillin, chloramphenicol, and tetracycline in the [4,5,12:i:−] clone are different from those found in the pentadrug-resistant serotype Typhimurium DT104 clone.

Multidrug-resistant *Salmonella enterica* serotype [4,5,12:i:−] isolates emerged in 1997 in Spain (5). DNA fingerprinting analysis of 29 isolates (27 collected in the Principality of Asturias between May 1997 and April 2000 and the reference strains CNM4IC and CNM9IC) had shown that they fall into a single genetic lineage or clone which seems to be closely related to some contemporary serotype Typhimurium lineages causing human salmonellosis (reference 10 and unpublished data). All [4,5,12:i:−] isolates expressed resistance to ampicillin (AMP), chloramphenicol (CHL), gentamicin (GEN), streptomycin (STR), sulfadiazine (SUL), tetracycline (TET), and trimethoprim (TMP) except two isolates which were TET susceptible. These isolates harbored two or three small cryptic plasmids and one large plasmid carrying (pUO-SVR3 [plasmid of University of Oviedo-*Salmonella* virulence resistance]) or lacking (pUO-SR4) *spv* loci (10) (Table 1). The objective of this study was to investigate the molecular basis of the multidrug resistance in serotype [4,5,12:i:−] and ascertain the relations between resistance genes, integrons, and plasmids. For this, several experimental approaches were made, and the results were compared with those obtained for Typhimurium DT104 LSP14/92 (9), used as the type strain of a pentadrug-resistance epidemic clone of concern in many countries (3, 7, 8, 9, 11, 15).

Determination of resistance genes. To determine the genes for AMP, GEN, and STR resistance, the [4,5,12:i:−] isolates were tested, by disk diffusion assay (13) and taking into account results described elsewhere (2, 16, 17), with (i) amoxicillin-clavulanic acid (AMC; 30 μg), carbenicillin (CAR; 100 μg), cephalothin (30 μg), ceftazidime (30 μg), cefotaxime (30 μg), imipenem (10 μg), oxacillin (OXA; 1 μg), and piperacillin (PIP; 100 μg) and (ii) amikacin (30 μg), apramycin (APR; 100 μg), 5-episisomycin (5EPI; 10 μg), fortimycin (100 μg), kanamycin (30 μg), netilmicin (NET) and its derivatives 2′-NET- and 6′-NET (100 μg), tobramycin (TOB; 10 μg), and spectinomycin (SPT; 10 μg). PCR amplication was performed using specific primers (Table 2). All [4,5,12:i:−] isolates showed resistance to AMP-CAR-OXA, GEN-APR-5EPI-NET-2′NET-6′NET-TOB, and STR-SPT and generated amplification products with the bla_{TEM}, *aac(3)-IV*, and *aadA* primers. Typhimurium LSP14/92 showed resistance to AMP-CAR-PIP-OXA-AMC and STR-SPT and generated the expected amplification products with the bla_{CARB} and *aadA* primers. PCR for other resistance genes using [4,5,12:i:−] isolates generated amplification products with the *cmlA, dfrA12, tetA*, and *sul1* primers, while Typhimurium LSP14/92 generated products with the *floR, tetG*, and *sul1* primers. Sequencing of the 460-bp bla_{TEM} and 435-bp *cmlA* amplicons generated by LSP389/97, conducted described elsewhere (9), confirmed the presence of $bla_{\text{TEM-1}}$-like and *cmlA1* genes (accession numbers AF126482.1 and U12338, respectively). These data support the finding that the genes implicated in AMP, CHL, and TET resistance in the [4,5,12:i:−] clone differ from those reported for the pentadrug-resistant Typhimurium DT104 clone.

Detection of integrons. Detection of class 1 integrons and the resistance genes located therein was performed by PCR amplification with specific primers (Table 2). All [4,5,12:i:−] isolates carried two integrons defined by PCR products of 1,900 and 150 bp, sizes indicating that only in the first could gene cassettes carrying resistance genes be inserted. Class 1 integrons in their conserved regions, in addition to the integrase (*intI1*) gene, usually contain genes encoding resistance to quaternary ammonium compounds and ethidium bromide (*qacE*Δ) and sulfonamides (*sul1*) (6, 12, 14). The presence of these genes was confirmed in all [4,5,12:i:−] isolates by PCR. Sequencing of the 1,900-bp amplicon from LSP389/97 confirmed the presence of the *dfrA12 (dhfrXII)* gene cassette near the 5′ conserved region (5′CS), an open reading frame with an unknown function and a 59-bp element, and the *aadA2* gene cassette near the 3′CS (accession number AF284063). Intragenic primers for *dfrA12* were used to confirm that this gene, together with *aadA2*, was carried in the 1,900-bp amplicons

* Corresponding author. Mailing address: Área de Microbiología. Departamento de Biología Funcional, Facultad de Medicina, Universidad de Oviedo, Calle Julián Clavería 6, 33006 Oviedo, Spain. Phone: 34-985-103560. Fax: 34-985-103148. E-mail: camf@sauron.quimica.uniovi.es.

TABLE 1. Features of multidrug-resistant *Salmonella* serotype [4,5,12:i:−] isolates used in curing experiments and their segregants

Strain/PP segregant type[a]	AMP[b] *tem1*-like[c]	CHL *cmlA1*	GEN *aac(3)-IV*	STR *aadA2*[d]	SUL *sul1*[e]	TMP *dfrA12*[d]	TET *tetA*	*qacEΔ1*[e]	*spvABC*	Integron	
										1,900 bp	150 bp
LSP389/97 PPI	+	+	+	+	+	+	+				
	+	+	+	+	+	+	+	+	+	+	+
s1 (1[f])	+	+	+	+	+	+	−				
	+	+	+	+	+	+	−	+	+	+	+
s2 (10)	+	+	−	+	+	−	+				
	+	+	−	+	+	−	+	+	+	−	+
s3 (13)	−	+	−	+	+	−	+				
	−	+	−	+	+	−	+	+	+	−	+
s4 (2)	−	−	−	−	+	−	+				
	−	−	−	−	+	−	+	+	+	−	+
LSP272/98 PPII	+	+	+	+	+	+	−				
	+	+	+	+	+	+	−	+	−	+	+
s5 (1)	−	−	−	+	+	+	−				
	−	−	−	+	+	+	−	+	−	+	+
s6 (13)	−	+	−	+	+	−	−				
	−	+	−	+	+	−	−	+	−	−	+
s7 (1)	−	+	−	−	+	−	−				
	−	+	−	−	+	−	−	+	−	−	+
LSP132/98 PPIII	+	+	+	+	+	+	+				
	+	+	+	+	+	+	+	+	+	+	+
s8 (17)	−	−	−	−	+	−	+				
	−	−	−	−	+	−	+	+	+	−	+
LSP457/98 PPIV	+	+	+	+	+	+	+				
	+	+	+	+	+	+	+	+	+	+	+
s9 (1)	+	−	−	+	+	+	+				
	+	−	−	+	+	+	+	+	+	+	+
s10 (1)	−	−	−	+	+	+	+				
	−	−	−	+	+	+	+	+	+	+	+

[a] PP, plasmid profiles described in reference 10. PPI is represented by 20 clinical and 2 pork isolates (including CNM4IC); PPII, PPIII, and PPIV are represented by 4 (including CNM9IC), 1, and 2, clinical isolates, respectively. PPI, PPIII, and PPIV include pUO-SVR3, while PPII includes pUO-SR4.
[b] Resistance.
[c] PCR product generated with the primers compiled in Table 2.
[d] Located in variable region of class 1 integron defined by 1,900-bp amplicon.
[e] Located in 3′CS of class 1 integrons.
[f] Number of segregants, out of 100 colonies tested, presenting the same resistance phenotype as indicated PCR product.

from all [4,5,12:i:−] isolates studied. Typhimurium LSP14/92 carried two other integrons, with variable regions previously defined as 1,200-bp *pse1* and 1,000-bp *aadA2* (9).

Determination of relationships between drug resistance and plasmids. In a previous work, we found that when two representative [4,5,12:i:−] isolates were grown in the presence of sodium dodecyl sulfate (1%), some cells (segregants) lost AMP, CHL, GEN, and TMP resistance and their large plasmids appeared to be smaller (10). To confirm the apparent relationship between drug resistance and plasmids, as well as the possible relationship between integrons and plasmids, we used the following approaches.

(i) New curing experiments using representative isolates of the four plasmid profiles. For each experiment we analyzed about 100 colonies, finding that some colonies had lost some resistances and could be grouped in segregant types. A strong correlation between phenotype and genotype was found in all segregants, and one colony of each type was selected for the following experiments (Table 1).

(ii) Integron analysis. Using *qacEΔ1, sul1*, and 5′CS/3′CS primers, all segregant types generated amplification products of about 250, 430, and 150 bp, respectively, but only some of them generated products of 1,900 bp with *dfrA12* primers and expressed TMP resistance. Only some of the TMP-susceptible segregants were also STR susceptible indicating that more than one gene was implicated in STR resistance.

(iii) Plasmid analysis. None of the small cryptic plasmids was eliminated by curing, and all pUO-SVR3 segregants yielded an amplification product with *spv* primers. In segregants lacking the 1,900-bp *dfrA12-aadA2* integron and the other resistance genes, pUO-SVR3 or pUO-SR4 derivatives were smaller than the original plasmids.

(iv) Plasmid DNA hybridization. Hybridization using DNA from parents and representative segregants was performed with probes for the presumably lost genes and *spv*C. The results (Fig. 1) confirmed that 150- and 1,900-bp *dfrA12-aadA2* integrons, as well as $bla_{\text{TEM-1}}$-like, *aac(3)-IV, cmlA1, tetA*, and *spvC* genes, were plasmid located.

Plasmids, complete integrons, and gene cassettes are elements with potential for individual and joint horizontal transfer (4, 6, 14). The transfer of multidrug-resistant *Salmonella* plasmids, or the resistance gene cluster(s) therein, to other salmonellae or other pathogenic bacteria in animal reservoirs or human hosts could result in serious problems. The relative

TABLE 2. PCR primers and conditions used in this work

Region or gene (resistance)	Primer				Expected amplicon	PCR conditions[a]	
	Name[b]	Sequence (5′ to 3′)	EMBL GenBank accession no.	Reference		T_a (°C)	t_a (min:s)
Integron	5′CS/3′CS	GGCATCCAAGCAGCAAGC/AAGCAGACTTGACCTGAT	U12338	12	Variable	55	2:30
Integrase	*Int1*-F/B	GCCTTGCTGTTCTTCTAC/GATGCCTGCTTGTTCTAC	X12870	11	558 bp	55	0:30
qacEΔ1	*qacEΔ1*-F/B	ATCGCAATAGTTGGCGAAGT/CAAGCTTTTGCCCATGAAGC	X15370	15	250 bp	60–65	0:20
sul1 (SUL)	*sul1*-F/B	CTTCGATGAGAGCCGGCGGC/GCAAGGCGGAAACCCGCGCC	X12869	15	436 bp	65	0:30
bla_{CARB} (AMP)	OC-1/2[c]	AATGGCAATCAGCGCTTC/GGGGCTTGATGCTCACTC		1	566 bp	55–60	0:30
bla_{TEM} (AMP)	OT-1/2[c]	TTGGGTGCACGAGTGGGT/TAATTGTTGCCGGGAAGC		1	503 bp	55	0:30
bla_{OXA} (AMP)	OO-1/2[c]	ACCAGATTCAACTTTCAA/TCTTGGCTTTTATGCTTG		7	598 bp	50–55	0:30
aac(3)-IV (GEN)	*aac(3)-IV* F/B	GTTACACCGGACCTTGGA/AACGGCATTGAGCGTCAG	X01385	This work	674 bp	55–60	0:40
aadA (STR-SPT)	*aadA1a*-F/B	GTGGATGGCGGCCTGAAGCC/ATTGCCCAGTCGGCAGCG	M10241	15	526 bp	70	0:30
dfrA1-like (TMP)	*dfrA15*-F/B	GTGAAACTATCACTAATGG/CCCTTTTGCCAGATTTGG	Z83311	9	473 bp	55	0:30
dfrA12 (TMP)	*dfrA12*-F/B	ACTCGGAATCAGTACGCA/GTGTACGGAATTACAGCT	AF175203	This work	462 bp	55	0:30
cat (CHL)	*cat*-F/B	CCTGCCACTCATCGCAGT/CCACCGTTGATATATCCC	U46780	This work	623 bp	60	0:30
cmlA (CHL)	*cmlA*-F/B	TGTCATTTACGGCATACTCG/ATCAGGCATCCCATTCCCAT	M64556	J. Ruíz and J. Vila, personal communication	435 bp	55	0:30
floR (CHL)	paspp-*flo*-F/B	CACGTTGAGCCTCTATAT/ATGCAGAAGTAGAACGCG	AF071555	11	868 bp	55	0:40
tetA (TET)	*tetA*-F/B	GCTACATCCTGCTTGCCT/CATAGATCGCCGTGAAGA	X61367	11	210 bp	55–60	0:20
tetG (TET)	*tetG*-F/B	GCTCGGTGGTATCTCTGC/AGCAACAGAATCGGGAAC	S52437	11	500 bp	55	0:30
spvA[d]	*spvA*-F/B	GTCAGACCCGTAAACAGT/GCACGCAGAGTACCCGCA	517162	This work	604 bp	60	0:30
spvB[d]	*spvB*-F/B	ACGCCTCAGCGATCCGCA/GTACAACATCTCCGAGTA	517162	This work	1,063 bp	60	1:00
spvC[d]	*spvC*-F/B	ACTCCTTGCACAACCAAATGCGGA/TGTCTTCTGCATTTCGCCACCATCA	517162	10	424 bp	60	0:30

[a] PCR assays were performed as described elsewhere (9). T_a, annealing temperature; t_e, elongation time.
[b] F: forward; B: backward.
[c] Shorter than original one primer.
[d] *Salmonella* plasmid virulence gene.

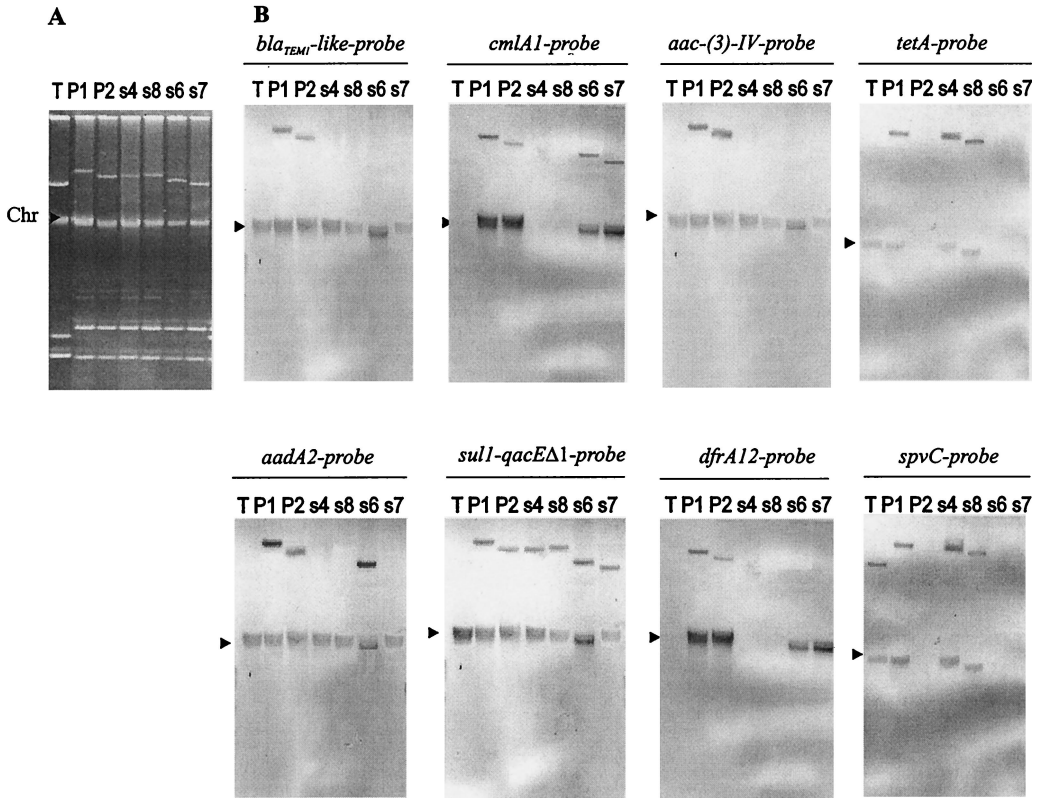

FIG. 1. Analysis of plasmids from representative *Salmonella* serotype [4,5,12:i:−] isolates used in curing experiments and from some of the segregates obtained. (A) Plasmid profiles; (B) hybridization of the plasmids shown in panel A with gene-specific probes. Lanes T, Typhimurium LSP14/92; lanes P1 and P2, [4,5,12:i:−] LSP389/97 and LSP272/98, respectively; lanes s4 to s8, segregant types. The features of the [4,5,12:i:−] organisms shown are compiled in Table 1. Arrowheads, chromosomal (Chr) DNA.

arrangement of the integrons with the other resistance genes and the *spv* loci in plasmids carried by [4,5,12:i:−] organisms remain to be determined.

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