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Characterization and Localization of Drug Resistance Determinants in Multidrug-Resistant, Integron-Carrying *Salmonella enterica* Serotype Typhimurium Strains

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ABSTRACT

The genetic background of the antimicrobial resistance of 10 selected multiresistant *Salmonella* serotype Typhimurium (*S.* Typhimurium) strains (including the emerging monophasic variant [4,5,12:i:−]) was investigated. All strains shared class 1 integrons (with seven types of variable regions) and belonged to different lineages (L1–L6) according to their phage types, DNA polymorphisms by *Xba*I-pulsed-field gel electrophoresis (PFGE), integrons, and/or resistance patterns. The strains were screened for the presence and localization (chromosomal or plasmid) of 32 DNA sequences representing integron-, Tn*21*-like transposon-, resistance-, and virulence-plasmid genes. Strains belonging to lineage L1 (definitive phage type DT104) carried the 90-kb *Salmonella* virulence plasmid together with the complete or partial chromosomally located *Salmonella* Genomic Island 1 (SGI1). All strains belonging to the other five lineages carried their resistance determinants on various resistance plasmids. Two of these strains showed complex plasmid profiles, which included a 95-kb virulence plasmid together with two or four resistance plasmids. Two strains carried a resistance plasmid that lacked the virulence-plasmid-encoding sequences. The remaining two strains carried two different hybrid virulence-resistance plasmids. Twenty-three of the DNA sequences could be assigned to distinct *Xba*I genomic restriction patterns (PFGE profiles). In this way, the influence of the resistance and virulence plasmids on the PFGE profiles was determined, and several groups of resistance genes could be identified. The data obtained represent a useful epidemiological tool for tracing the emergence and distribution of multiresistant *S.* Typhimurium worldwide.

INTRODUCTION

RESISTANCE TO ANTIMICROBIALS is one of the best-known examples for the rapid response of bacterial populations to a selective pressure. The selective advantage of resistant strains can be explained by the acquisition of resistance genes by horizontal gene transfer and/or by the accumulation of point mutations in the target genes. Especially in the case of the horizontal spread of resistance determinants between bacteria, mobile genetic elements such as plasmids, phages, transposons, and integrons/gene cassettes are usually implicated.[7,30,34] Five classes of integrons carrying antibiotic resistance gene cassettes have been reported so far,[10] but class 1 integrons are most prominent in the Enterobacteriaceae, including Salmonellae.

Salmonella enterica is a zoonotic species that can acquire its resistance in livestock. The resulting animal food products are important vectors for the transfer of resistant bacteria from animals to humans.[36] The pandemic *S. enterica* subspecies *enterica* serotype Typhimurium (*S.* Typhimurium, antigenic formula [4,5,12:i:1,2]) is one of the most frequent serotypes causing human and animal salmonellosis. Many strains of *S.* Typhimurium are multidrug-resistant (MDR) to four or more antimicrobial agents, and their public health importance has led to many investigations on their emergence and prevalence.[36]

S. Typhimurium isolates are usually differentiated by phage typing, denoting the phage types as definitive types (DT),[2] and DNA fingerprinting methods. One of the most discriminatory and widely accepted molecular typing method is DNA restric-

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tion analysis by pulsed-field gel electrophoresis (PFGE).[3,15,27,29] The analysis of the relationships between PFGE profiles, alone or in combination with other typing methods, allows the grouping of these MDR organisms into clonal lines. Many strains belonging to these lineages carry class 1 integrons and have caused outbreaks in various countries.[7,19,36] Over the last decade, one *S.* Typhimurium lineage, defined as *S.* Typhimurium-DT104, with resistance to ampicillin, chloramphenicol, streptomycin-spectinomycin, sulfonamides, and tetracyclines (AMP-CHL-STR-SPE-SUL-TET-type) has widely spread, and can be considered pandemic.[16,36] The five resistance determinants (pentaresistance) form part of the chromosomally located *Salmonella* Genomic Island 1 (SGI1), which contains two class 1 integrons, designated InC and InD, that carry the *pse1* and *aadA2* gene cassettes,[5,6,8,11,31,35] respectively. Another *S.* Typhimurium clonal lineage is monophasic [4,5,12:i:−], has the phage type DTU302, and carries, in general, gentamicin and trimethoprim resistance in addition to the DT104 pentaresistance. This lineage is emerging in Spain and Portugal since 1997 (R. Vieira, 2004, personal communication).[12,19] In this clonal lineage, the resistance determinants are located on plasmids that also carry class 1 integrons.[20] Variants of this lineage have been found in England[38] and have caused outbreaks in the United States[1] and Denmark.[36]

The aim of this study is to elucidate the genetic basis and location of multidrug resistance in a set of 10 representative serotype *S.* Typhimurium strains, which carry different class 1 integrons and belong to six distinct clonal lineages. This was achieved by screening for the presence of 32 sequences representing resistance-, integron-, Tn*21*-like transposon- and virulence-plasmid genes, and chromosomal mutations conferring resistance. The genetic elements were localized on plasmids or the chromosome by hybridization, and 23 of the characterized DNA sequences were mapped in the *Xba*I genomic restriction patterns (PFGE profiles) of the strains. The results obtained are useful tools to trace the epidemiological spread of these six MDR *S.* Typhimurium lineages.

MATERIALS AND METHODS

Bacterial strains and plasmids

Table 1 shows the phenotypic and molecular properties of 10 selected MDR *S.* Typhimurium strains. They were chosen from 100 *S.* Typhimurium [4,5,12:i:1,2] and 50 monophasic variant [4,5,12:i:−] class 1 integron-carrying isolates that had previously been analyzed by PFGE and phage typing. These 150 isolates stemmed from Spanish and German human clinical, food, and animal origins, and some of their properties have previously been published.[18–21] From the results obtained, representative strains carrying integrons with different gene cassettes, or carrying the same integrons but showing different *Xba*I-PFGE profiles, were selected for the present study. Nine of the strains (SUO1-9, *Salmonella* University of Oviedo) had been collected from clinical samples in the Pricipality of Asturias, Spain. The other strain (NRL1) was collected from pork meat in the National *Salmonella* Reference Laboratory (NRL-Salm), Berlin, Germany. Strains were serotyped and phage typed[2] in the Centro Nacional de Microbiología (CNM, Instituto de Salud Carlos III, Madrid, Spain) or in the NRL-Salm. The susceptible strain *S.* Typhimurium LT2 (Bayer AG. Pharma Research Center Collection, Wuppertal, Germany) was used as a control. *Escherichia coli* K12 J53, which was rifampicin- and nalidixic acid-resistant, was used as plasmid recipient in mating experiments.

PCR amplification, purification, and sequencing of DNA

The detection of genes (Table 2) implicated in the drug resistance (*pse1*, *oxa1*, *tem1-like*, *cmlA1-like*, *catA1*, *floR*, *tet*(A), *tet*(B), *tet*(G), *aadA1*, *aadA2*, *strA*, *strB*, *aphA1*, *aac*(3)-*1V*, *dfrA1-like*, *dfrA12*, *dfrA7*, *dfrA17*, *dfrA14*, *sul1*, and *sul2*), class 1 integrons, their related genes (*intI1*, *qacEΔ1*), Tn*21*-like transposon-related genes (*tnpA*, *tnpR*, and *merA*), as well as *S.* Typhimurium virulence-plasmid genes (*spvC*, *rck*, *pefA*, *traT*), was performed by PCR amplification and sequencing as previously described.[19–21] Information about sequences and primers is given in Table 2. The sequences obtained were compared to those registered in GenBank. The probes used for hybridization experiments were obtained by PCR-amplification using DIG-labeled dNTPs (PCR DIG labeling mix, Roche Applied Science, Mannheim, Germany), followed by gel extraction and purification with the GFX™ DNA and Gel Band Purification Kit (Amersham Biosciences, Freiburg, Germany).

Plasmid analysis, PFGE analysis, and Southern hybridization

Plasmid profiling of supercoiled DNA was performed by the alkaline denaturation method of Kado and Liu.[23] Plasmids were labeled as pUO-*St*-V/R (plasmid of the University of Oviedo, *S. typhimurium,* virulence and/or resistance) followed by an ordinal number. Transfer of antibiotic resistance by mating experiments was performed at 37°C and 22°C in liquid and solid (filter) media, as previously described.[17,20] To estimate in a reliable way the molecular sizes of the large bacterial plasmids, plasmid profiles were also established by using nuclease S1 treatment followed by PFGE.[4] This procedure allows the conversion of cc-plasmid DNA to the linear form. Total DNA was embedded in agarose plugs,[19] and slices were treated with 8 units of nuclease S1 (Amersham Biosciences) at 37°C for 45 min. The preparation of the plugs and PFGE running conditions used were the same as the ones used for PFGE fingerprinting. Fingerprinting was performed by PFGE analysis using *Xba*I as previously described.[19] The *Xba*I PFGE profiles (PFGE-X) were defined considering well-visualized fragments larger than 18 kb. Lambda Ladder, MidRange-I, and Low Range PFG markers (New England Biolabs, Schwalbach, Germany) were used as molecular markers. The location of 23 specific DNA sequences (18 from resistance genes, *intI1* and *qacEΔ1* from the class 1 integrons, *merA* from Tn*21*-like transposon loci and the virulence gene *spvC*) was achieved by Southern hybridization of chromosomal (*Xba*I-PFGE patterns) and plasmid DNA as previously described.[20] The membranes were hybridized and rehybridized using a nonradioactive DNA labeling and detection kit (Roche Applied Science) following the manufacturer's recommendations. Under this assay conditions, only hybridization fragments larger than 10 kb could be detected.

TABLE 1. PHENOTYPIC AND MOLECULAR PROPERTIES OF INTEGRON-CARRYING *S.* TYPHIMURIUM STRAINS

Line/	*Strain*	*Phage type*	*PFGE*	*Antimicrobial resistance-pattern/ resistance genes profile*[a]	*Integron(bp)*[b]*/gene cassettes Other In-*[c]*; Tn-*[d] *genes*	*Integron location*
L1/	SUO1	DT104	X1	AMP-CHL-[STR-SPE]-SUL-TET /*pse1-floR-aadA2-sul1-tet*(G)	InC:1,200/*pse1*; InD:1,000/*aadA2 intI1, qacEΔ1*	SGI1
	SUO2	DT104	X2	AMP-CHL-[STR-SPE]-SUL-TET /*pse1-floR-aadA2-sul1-tet*(G)	InC:1,200/*pse1*; InD:1,000/*aadA2 intI1, qacEΔ1*	SGI1
	NRL1	DT104	X1	AMP-CHL-[CIP-NAL]-[STR-SPE]-SUL-TET-TMP-SXT /*[tem1 like-pse1]-floR-[aadA2-aadA5]-sul1-tet*(G)-*dfrA17-gyrA*^{Asn87}	InC:1,200/*pse1*; InD:1,000/*aadA2* InE:1,600/*dfrA17-aadA5 intI1, qacEΔ1; tnpA, tnpR, merA*	SGI1 pUO-*St*-R5
	SUO3	DT104	X1	AMP-SUL /*pse1-sul1*	InC:1,200/*pse1 intI1, qacEΔ1*	SGI1-B
L2/	SUO4	RDNC	X3	[STR-SPE]-SUL /*aadA1a-sul1*	In2:1,000/*aadA1a intI1, qacEΔ1; tnpA, tnpR*	pUO-*St*-R6
L3/	SUO5	DT120	X4	AMP-CHL-[STR-SPE]-SUL-TET /*oxa1-catA1-[strA/B-aadA1a]-[sul1-sul2]-tet*(B)	InH:2,000/*oxa1-aadA1a intI1, qacEΔ1; tnpA, tnpR, merA*	pUO-*St*-VR2
L4/	SUO6	RDNC	X5	AMP-CHL-[KAN-NEO]-[STR-SPE]-SUL-TET-TMP-SXT /*tem1 like-catA1-aphA1-aadA1a-sul1-tet*(B)-*dfrA14*	InF:1,600/*dfrA14-aadA1a intI1, qacEΔ1; tnpA, tnpR, merA*	pUO-*St*-R7
L5/	SUO7	204c	X6	AMP-CHL-[KAN-NEO]-[STR-SPE]-SUL-TET-TMP-SXT /*tem1 like-catA1-aphA1-strA/B-tet*(A)-*[sul1-sul2]-dfrA7*	InG:700/*dfrA7 intI1, qacEΔ1; tnpA, tnpR, merA*	pUO-*St*-R11
L6/	SUO8	U302	X7	AMP-CHL-GEN-[STR-SPE]-SUL-TET-TMP-SXT /*tem1 like-cmlA1-aac(3)-IV-aadA1a-[sul1-sul2]-tet*(A)-*dfrA12*	InI:1,900/*dfrA12-aadA2*; In0:150/*none intI1, qacEΔ1; merA*	pUO-*St*-VR3
	SUO9	U302	X8	AMP-CHL-GEN-[STR-SPE]-SUL-TMP-SXT /*tem1 like-cmlA1-aac(3)-IV-aadA1a-[sul1-sul2]-dfrA12*	InI:1,900/*dfrA12-aadA2*; In0:150/*none intI1, qacEΔ1; merA*	pUO-*St*-R4

SUO, *Salmonella* of University of Oviedo (Spain); NRL, National Reference Laboratory (Berlin, Germany); RNDC, reaction that does not conform; SGI, *Salmonella* Genomic Island; pUO-*St*, Plasmid of University of Oviedo-*Salmonella* Typhimurium, V: virulence, R: resistance, VR: hybrid.

[a]Antimicrobials abbreviations, resistance genes; ampicillin (AMP), *pse1, tem1-like, oxa1;* chloramphenicol (CHL), *floR, catA1, cmlA1*; tetracycline (TET), *tet*(G)*, tet*(A)*, tet*(B); streptomycin (STR)-spectinomycin (SPE), *aadA1a, aadA2, aadA5*; sulfamethoxazol (SUL), *sul1, sul2*; trimethoprim (TMP), *dfrA17, dfrA14, dfrA12, dfrA7*; kanamycin (KAN)-neomycin (NEO), *aphA1*; gentamicin (GEN), *aac*(*3*)*-IV*; nalidixic acid (NAL), *gyrA*^{Asn87}.

[b]Variable region size. Amplicons generated using the 5′CS-3′Cs primers.[24]

[c]Class 1 integron-related genes: *intI1*, class 1 integrase; *qacEΔ1*, quaternary ammonium compounds resistance.

[d]Tn*21*-related genes: *tnpA*, transposase; *tnpR*, resolvase; *merA*, mercury resistance.

Table 2. Region and Genes Screened and PCR Primers Used

Region or gene[a]	*Primers*		*GenBank accession no.*	*Reference*[b]	*Expected amplicon*
	Name	*Sequence (5′ to 3′)*			
Integron	[5′CS/3′CS]	[GGCATCCAAGCAGCAAGC/AAGCAGACTTGACCTGAT]	U12338	24	Variable
Integrase1	[Int1-F/B]	[GCCTTGCTGTTCTTCTAC/GATGCCTGCTTGTTCTAC]	X12870	20	558 bp
qacE Δ1	[*qacEΔ1*-F/B]	[ATCGCAATAGTTGGCGAAGT/CAAGCTTTTGCCCATGAAGC]	X15370	20	250 bp
merA	[merA-F/B]	[ACCATCGGCGGCACCTGCGT/ACCATCGTCAGGTAGGGGAACAA]	K03089	25	1232 bp
tnpA	[tnpA-F/B]	[AGAAAGTTCGTCCTGGGCTG/GGCCAAGGACAAGAACCTGT]	AF071413	21	327 bp
tnpR	[tnpR-F/B]:	[GGCGACACCGTGGTGGTGCATAGC/CGGTAAGCCCCGCGTTGCTTGGC]	AF071413	21	240 bp
sul1	[sul1-F/B]	[CTTCGATGAGAGCCGGCGGC/GCAAGGCGGAAACCCGCGCC]	X12869	20	436 bp
sul2	[sul-II-F/B]	[TCAACATAACCTCGGACAGT/GATGAAGTCAGCTCCACCT]	M36657	9	707 bp
tem1-like	[OT-1/2]	[TTGGGTGCACGAGTGGGT/TAATTGTTGCCGGGAAGC]	AF126482.1	20	503 bp
pse-1	[pse1-F/B]	[CGCTTCCCGTTAACAAGTAC/CTGGTTCATTTCAGATAGCG]	M69058	35	419 bp
oxa1-like	[oxa1-F/B]	[AGCAGCGCCAGTGCATCA/ATTCGACCCCAAGTTTCC]	AJ009819	18	708 bp
aac(3)-IV	[aac(3)-IV-F/B]	[GTTACACCGGACCTTGGA/AACGGCATTGAGCGTCAG]	X01385	20	674 bp
aadA1-like	[aadA1a-F/B]	[GTGGATGGCGGCCTGAAGCC/ATTGCCCAGTCGGCAGCG]	M10241	20	526 bp
aadA2	[aadA2-F/B]	[TGTTGGTTACTGTGGCCGTA/GATCTCGCCTTTCACAAAGC]	AF071555	38	623 bp
strA	[strA-F/B]	[CCTGGTGATAACGGCAATTC/CCAATCGCAGATAGAAGG]	M28829	26	548 bp
strB	[strB-F/B]	[ATCGTCAAGGGATTGAAACC/GGATCGTAGAACATATTGGC]	M28829	26	509 bp
aphAl	[aphAI-IAB-F/B]	[AAACGTCTTGCTCGAGGC/CAAACCGTTATTCATTCGTGA]	AF024666	13	461 bp
dfrA1-like	[dfrA15-F/B]	[GTGAAACTATCACTAATGG/CCCTTTTGCCAGATTTGG]	Z83311	18	473 bp
dfrA12	[dfrA12-F/B]	[ACTCGGAATCAGTACGCA/GTGTACGGAATTACAGCT]	AF175203	20	462 bp
dfrA5-14	[dfrA4/A14-F/B]	[GATTGGTTGCGGTCCA/CTCAAAAACAACTTCGAAGG]	—	14	379 bp
dfrA7-dfrA17	[dfrA7/A17-F/B]	[CAGAAAATGGCGTAATCG/TCACCTTCAACCTCAACG]	—	14	345 bp
dfrA17	[dfrA17-F/B]	[GATTTCTGCAGTGTCAGA/CTCAGGCATTATAGGGAA]	AF220757	22	384 bp
catA1	[cat-F/B]	[CCACCGTTGATATATCCC/CCTGCCACTCATCGCAGT]	U46780	20	623 bp
cmlA1-like	[cmlA-F/B]	[TGTCATTTACGGCATACTCG/ATCAGGCATCCCATTCCCAT]	M64556	20	435 bp
floR	[paspp-flo-F/B]	[CACGTTGAGCCTCTATAT/ATGCAGAAGTAGAACGCG]	AF071555	20	868 bp
tet(A)	[tetA-F/B]	[GCTACATCCTGCTTGCCT/CATAGATCGCCGTGAAGA]	X61367	20	210 bp
tet(G)	[tetG-F/B]	[GCTCGGTGGTATCTCTGC/AGCAACAGAATCGGGAAC]	S52437	20	500 bp
tet(B)	[tetB-F/B]	[TTGGTTAGGGGCAAGTTTTG/GTAATGGGCCAATAACACCG]	J01830	28	600 bb
spvC	[spvC-F/B]	[ACTCCTTGCACAACCAAATGCGGA/TGTCTTCTGCATTTCGCCACCATCA]	517162	20	424 bp
pefA	[pefA-F/B]	[GCACACGCTGCCAATGAA/CACAGACTTGAAGTCACC]	AE006471	21	442 bp
traT	[traT-F/B]	[GATGGTTACACTGGTCAG/TCTGAGATCTGTACGTCG]	AE006471	21	483 bp
rck	[rck-F/B]	[TCGTTCTGTCCTCACTGC/TCATAGCCCAGATCGATG]	AE006471	21	474 bp

[a]Function of the genes shown in Tables 1 and 3. *tem1-like*, the primers used amplify several *tem* genes; *oxa1-like*, the primers amplify at least *oxa1* and *oxa31* genes; *cmlA1-like*; the primers amplify *cmlA1, A4, A5, A6* genes; *aadA1a-like*, the primers amplify *aadA1a, A2, A8* genes; *dfrA1-like*, the primers amplify *dfrA1, 15, 15b* genes.

[b]Original references can be found in the cited articles.

RESULTS

Characterization of integron-carrying S. *Typhimurium strains*

On the basis of phenotypic (phage type) and genotypic markers (*Xba*I-PFGE profile, integron type, and/or similar genetic background of resistance), the 10 selected integron-carrying strains could be assigned to six lineages, L1–L6 (Tables 1 and 3). L1 is represented by four DT104 strains (showing two PFGE profiles), which carry the resistance determinants of a complete or partial *Salmonella* Genomic Island I (SGI). Only two strains, SUO1 and SUO2, showed the typical pentaresistance and InC and InD integrons. The third strain, NRL-1, showed in addition trimethoprim and nalidixic acid resistance together with a reduced susceptibility to ciprofloxacin (MIC 0.25 μg/ml). It carried a self-transferrable plasmid containing a third integron with the *dfrA17-aadA5* gene cassette configuration, some Tn*21*-related genes, and a *tem1-like* gene. The fourth strain (SUO3) presented a defective resistance pattern (AMP-SUL/*pse1-sul1*) associated with the presence of InC.

The SGI could not be detected in the other six strains assigned to L2–L6 (all not DT104 and generating distinctive PFGE profiles), and the integron, transposon, and resistance genes were located on plasmids (Tables 1 and 3). Strain SUO4 (L2) showed only the STR-SPE-SUL resistance, and harbored a large plasmid, which carried an integron with the *aadA1a* gene cassette and some Tn*21* genes. The strains SUO6 (L4) and SUO7 (L5), in contrast, showed a more extended resistance pattern (seven resistance determinants), which also included kanamycin-neomycin. Both strains showed complex plasmid profiles: SUO6 carried one virulence and four resistance plasmids, and SUO7, one virulence and two resistance plasmids. The properties of these plasmids are shown in Table 3. In both strains, the largest plasmids (225 kb and 250 kb, respectively) carried the integrons (with *dfrA14-aadA1a* and *dfrA7* gene cassettes, respectively), Tn*21* genes, and other resistance genes. Strain SUO5 (L3) showed the pentaresistance phenotype, but a distinctive resistance genotype mediated by a hybrid self-transferrable virulence-resistance plasmid of about 140 kb, and two small plasmids. On the large plasmid, an integron with *oxa1-aadA1a* gene cassettes, all the transposon and most of the resistance genes (except *strA/B* and *sul2*, carried on small plasmids) were located. Strains SUO8 and SUO9 (L6) represented the emergent monophasic variant [4,5,12:i:−]-U302, and generated PFGE profiles that differed only in three bands <100 kb. Both strains showed extended-resistance patterns (seven and six resistance determinants), which included gentamicin resistance, and carried non-self-transferrable resistance plasmids with or without *spv* genes. All resistance genes, two integrons (one carrying *dfrA12-aadA2* and another lacking gene cassettes), and the *merA* gene were located on these plasmids.

TABLE 3. VIRULENCE-, RESISTANCE-, AND VIRULENCE/RESISTANCE PLASMIDS HARBORED BY INTEGRON-CARRYING *S.* TYPHIMURIUM STRAINS

	Plasmid						
Strain	*Name*	*Size (kb)*	*Integron*	*Tn-genes*[a]	*V genes*[b]	*Other R genes*[c]	*Self-transfer*[d]
SUO1,2,3	pUO-*St*-V1a	90	None	None	*spvC, rck, pefA, traT*	None	No
NRL1	pUO-*St*-V1a	90	None	None	*spvC, rck, pefA, traT*	None	No
	pUO-*St*-R5	50	InE	*tnpA, tnpR, merA*	None	*tem1-like*	Yes
SUO4	pUO-*St*-R6	100	In2	*tnpA, tnpR*	None	None	Unknown
SUO5	pUO-*St*-VR2	140	InH	*tnpA, tnpR, merA*	*spvC, rck, traT*	All	Yes
	Others	6 and 5.4	None	None	None	Both *strA/B, sul2*	Unknown
SO6	pUO-*St*-V1b	95	None	None	*spvC, rck, pefA, traT*	None	No
	pUO-*St*-R7	225	InF	*tnpA, tnpR, merA*	None	*catA1, tet*(B)	Yes
	pUO-*St*-R8	135	None	None	None	*tem1, aphA1*	No
	pUO-*St*-R9	70	None	None	None	*tem1, aphA1*	Yes
	pUO-*St*-R10	50	None	None	None	*tem1*	Co-transferrable
SO7	pUO-*St*-V1b	95	None	None	*spvC, rck, pefA, traT*	None	No
	pUO-*St*-R11	250	InG	*tnpA, tnpR, merA*	None	All	Yes
	Others	3.1	None	None	None	*sul2*	Unknown
SUO8	pUO-*St*-VR3	200	InI, In0	*merA*	*spvC*	All	No
SUO9	pUO-*St*-R11	150	InI, In0	*merA*	None	All	No
LT2	psLT90	90	None	None	*spvC, rck, pefA, traT*	None	No

SUO, *Salmonella* of University of Oviedo (Spain); NRL, National Reference Laboratory (Berlin, Germany); pUO-*St*-, Plasmid of University of Oviedo-*Salmonella typhimurium*; V, virulence; R, resistance; VR, hybrid.

[a]Tn*21*-related genes: *tnpA*, transposase; *tnpR*, resolvase; *merA*, mercury-resistance.

[b]V genes: *spvC, Salmonella* plasmid virulence; *rck*, resistance to complement killing; *pefA*, plasmid-encoded fimbriae; *traT*, transfer and surface exclusion.

[c]When all, shown in Table 1.

[d]Under the assay conditions used.

Location of antimicrobial resistance-, integron-, transposon-, and virulence-plasmid-genes in the XbaI-restriction genomic fragments of S. *Typhimurium*

The genomic *Xba*I restriction fragments (Fig. 1) from the strains shown in Table 1 were hybridized with the suitable probes. Representative examples are shown in Fig. 2. The most relevant findings were: (1) In the strains belonging to L1, most of the molecular markers screened could be located in the visible PFGE fragments. The *spvC* probe mapped on a 90-kb fragment, which corresponds to the virulence plasmid. All chromosomally located resistance and/or integron genes mapped on one matching 11.7-kb and one mismatching 16-kb fragment of the L1 PFGE-profiles (X1, X2). The exceptions were the SUO3 strain, where no resistance probe mapped, and the NRL1 strain, where none of its resistance-plasmid-specific probes mapped. (2) No probe hybridized to the PFGE-X3 of SUO4, suggesting that *Xba*I digestion of pUO-*St*-R6 resulted in fragments that were smaller than 15 kb and not detectable under the PFGE conditions used, or that *Xba*I did not cut at all, and that the supercoiled DNA ran out of the gel. (3) In the PFGE-X4 of SUO5, the probes for the virulence-resistance plasmid mapped on two specific fragments (about 75 and 48 kb), which probably result from the *Xba*I digestion of this plasmid. (4) In the PFGE-X5 from SUO6, at least two fragments (80 and 48 kb) corresponding to pUO-*St*-R7 could be detected. The other three resistance plasmids of this strain could be recognized by the hybridization of the *tem1-like* (the first plasmid) and the *tem1-like* together with *aphA1* probes (the other two). The *spvC* probe mapped on a 95-kb fragment, which corresponds to the virulence plasmid. (5) In the PFGE-X6 of SUO7, at least two fragments (65 and 40 kb) could be recognized from the *Xba*I digestion of pUO-*St*-R11. The typical virulence plasmid, as in other lineages, was recognized as an ~95-kb fragment. (6) In the PFGE-X7 and X8 of SUO8 and SUO9, the plasmid probes mapped on multiple fragments of sizes <100 kb. Hybridization profiles support that these plasmids have several and different *Xba*I restriction sites, and that more than one class 1 integron was present in these strains. The *sul2* probe did not hybridize in PFGE-X8.

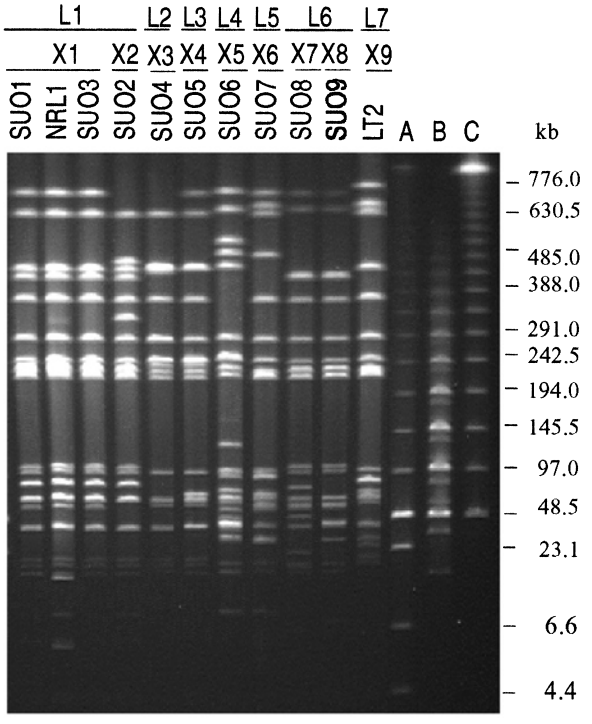

FIG. 1. *Xba*I-PFGE profiles of integron-carrying *S.* Typhimurium strains. (Lane A) Low-range PFGE marker; (lane B) Midrange PFGE marker I; (lane C) Lambda Ladder PFGE marker (New England Biolabs).

DISCUSSION

Since the early 1990s, a MDR clonal lineage of *S.* Typhimurium DT104 with the AMP-CHL-STR-SPE-SUL-TET/*pse1-floR-aadA2-sul1-tet*(G) pattern has gained increasing importance in industrialized countries.[3,15,16,28,36] Fifty-four of the 150 *S.* Typhimurium isolates tested were pentaresistant DT104 (represented by strains SUO1 and SUO2). Thirty-one additional isolates showed a related phage type or variations in the resistance phenotype compatible with this lineage. Thus, 85% of the *S.* Typhimurium [4,5,12:i:1,2] analyzed could be ascribed to the lineage designated in the present study as L1. Previous work has shown that the resistance genes and integrons (InC and InD) characteristic of these strains, are located on the SGI1.[5,8,11,31] SGI1 has been found mainly in *S.* Typhimurium (DT104 and DT120), but also in *S.* Agona and *S.* Paratyphi B strains.[5,6] The constant and dynamic evolution occurring within this lineage led to the emergence of new types and different genetic arrangements including deletions and insertions within the SGI1 generating new resistance phenotypes/genotypes.[6,8] One example for a deletion is strain SUO3 (AMP-SUL/*pse1-sul1*). This phenotype/genotype was only found in five of the 150 isolates (5% of the *S.* Typhimurium [4,5,12:i:1,2]). Similar strains have been described by other authors as well.[6,11,15,31] Although in the present and similar studies,[11,31] the location in the *Xba*I-PFGE profiles of the integron carried by these strains was not possible under the assay conditions used, Boyd *et al.*[5] demonstrated that the integron is located in *Xba*I fragments of 4.3 kb (*qacEΔ1/sul1-, pse1-* probes), and 2.5 kb (*pse1* probe) of the SGI1-B.

Although the inclusion of *dfrA* genes encoding trimethoprim resistance into the SGI has been described,[6] they were not detectable in our study. Members of the pentaresistant DT104 lineage can also gain resistance plasmids and/or mutations leading to the emergence of new and extended-resistance patterns. One example is the NRL1 strain, which carried a self-transferrable plasmid containing a *tem1-like* gene and the InE integron (*dfrA17-aadA5*). In this case the plasmid added the trimethoprim, as well as ampicillin and streptomycin resistance to the chromosomally located resistances of DT104. The InE integron has been found in *E. coli* (B. Guerra, 2003, unpublished),[22,24,39] but it is very rare in Salmonellae, because among 2000 isolates tested in our laboratories it could only be found in one *S.* Brandenburg isolate. The NRL1 strain also showed nalidixic acid resistance together with reduced susceptibility to ciprofloxacin associated with a mutation in the *gyrA* gene. The

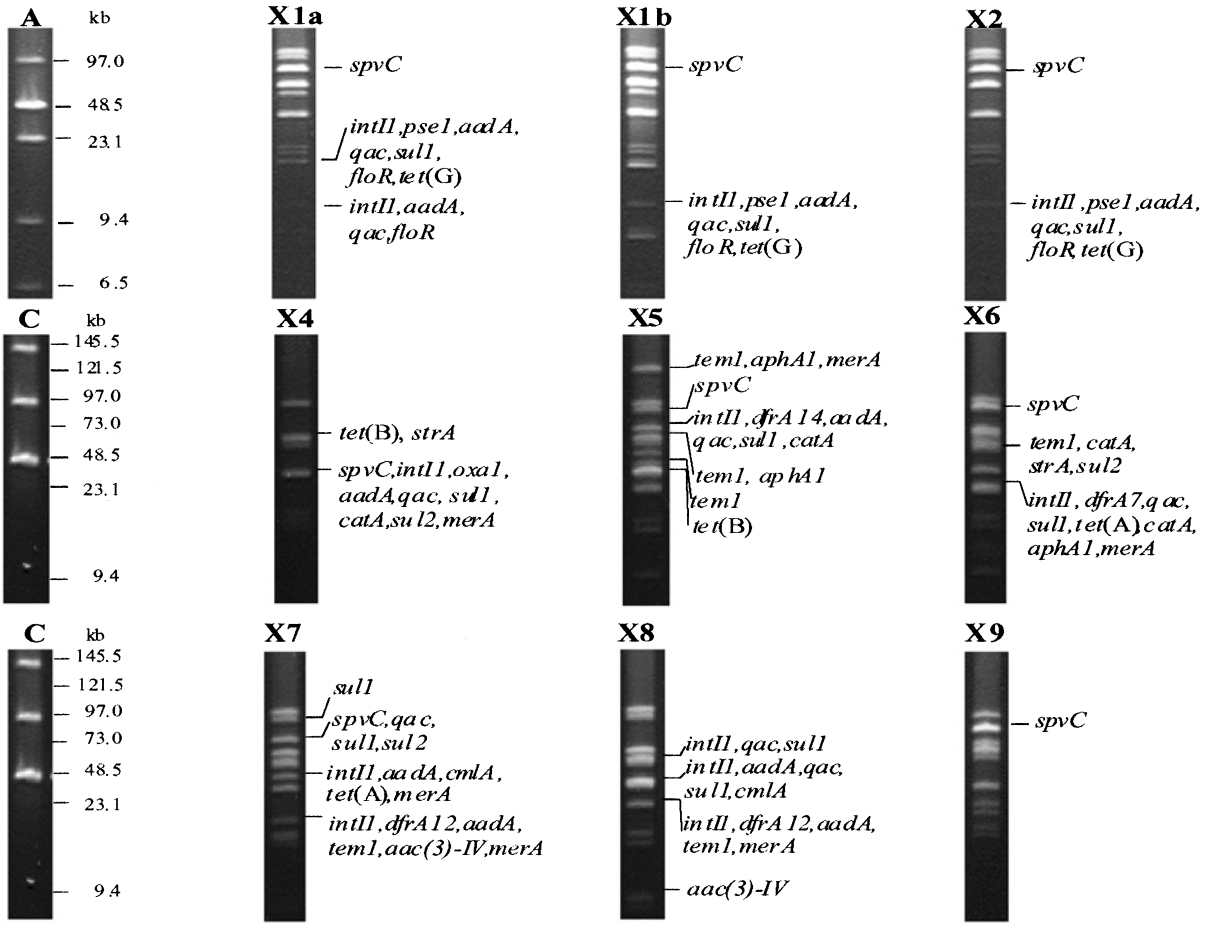

FIG. 2. Groups of resistance- and virulence-plasmid genes detected by hybridization of the PFGE profiles shown in Fig. 1. Probes for resistance (*pse1*, *oxa1*, *tem1-like=tem*, *cmlA1-like=cmlA*, *catA1=catA*, *floR*, *tet*(A), *tet*(B), *tet*(G), *aadA1-like=aadA*, *strA*, *aphA1*, *aac(3)-IV*, *dfrA12*, *dfrA7-A17=dfrA7* and *17*, *dfrA14*, *sul1*, *sul2*), integron (*intI1*, *qacEΔ1=qac*), Tn*21*-like transposon (*merA*) and virulence-plasmid (*spvC*) genes were used. (Lane A) Low-range PFGE marker; (lane C) Lambda Ladder PFGE Marker (New England Biolabs); (lane X1a) profile for SUO1 strain; (lane X1b) profile for NRL1 strain. None of the tested probes hybridized to X1c (profile for SUO3 strain) and X3.

recently observed increase in the number of *Salmonella* DT104 strains with resistance to fluoroquinoles due to mutations in the genes encoding for gyrase A has been described.[36]

The other six resistant strains analyzed in the present study did not belong to phage type DT104 and lacked SGI1 and other chromosomal-located resistance islands. In these strains, different large plasmids (50–250 kb), most of them self-transferrable and carrying different class 1 integrons and transposons derived from Tn*21*, were implicated in the resistance. Interesting findings were: (1) pUO-*St*-R6: This plasmid encodes only STR-SPE-SUL-resistance mediated by the integron In2, carries *tnpA* and *tnpR* genes but lacks *merA* genes, and, among the strains investigated, appeared only in two other *S.* Typhimurium isolates. In2 forms part of the Tn*21* transposon,[25] and it is frequently found in other *Salmonella* serotypes[7,18,24] and Enterobacteriaceae.[7,22,24,25,39] (2) pUO-*St*-R7: This plasmid carries the InF integron (*dfrA14-aadA1a*) together with the *catA1* and *tet*(B) genes. The integron has also been found in other Enterobacteriaceae[22] but it is not very frequent in *Salmonella* (B. Guerra, 2004, unpublished).[19] (3) pUO-*St*-R11: This plasmid encodes AMP-CHL-KAN-NEO-STR-SPE-SUL-TMP-SXT resistance and carries the InG integron (*dfrA7*) and has only been found in the Spanish *S.* Typhimurium DT204c clinical isolate (SUO7). A similar plasmid has been associated with DT204c strains isolated from cattle in Germany[17] (S. Schwarz, 2002, personal communication) and England,[36] some of them have been implicated in a large outbreak in Middle Europe.[36] The integron (also labeled In8), has been found in *E. coli* and *Klebsiella* strains, forming part of the Tn*5086*[25] transposon.

In relation to the presence/absence of virulence-plasmids and/or hybrid (virulence and resistance)-plasmids, six of the ten strains analyzed carried pSLT-like[32] plasmids (*Inc*FII, 90–95 kb), two strains lacked a virulence plasmid or its sequences, and two strains carried interesting hybrid plasmids. The first, pUO-*St*-VR2 (about 140 kb), belongs to *Inc*FII and carries *spv*, *traT*, and *rck* but not *pefA* genes. It also contains the InH integron (*oxa1-aadA1a*) and the *merA* gene, both typical markers of the Tn*2603* transposon.[25] This transposon has also been found in an Italian *S.* Typhimurium *Inc*FI-plasmid.[37] In previous studies[21] (B. Guerra, 2003, unpublished), this hybrid plas-

mid was found in 10 *Salmonella* strains, all of them *S.* Typhimurium, belonging to different phage types, and presenting similar genetic traits. In a recent survey, we found other five Spanish *S.* Typhimurium isolates carrying pUO-*St*-VR2, but exhibiting different PFGE profiles. These data show the epidemiological spread of this plasmid among other *S.* Typhimurium lineages. The second hybrid plasmid, pUO-*St*-VR3 (about 200 kb), belongs to *Inc*N and carries *spv* but no *traT*, *rck*, or *pefA* genes[20,21] (B. Guerra, 2003, unpublished). It also contains the InI (*dfrA12-aadA2*) and In0 integrons and the *merA* gene, and has only been described in strains belonging to the emerging monophasic variant.[20] Most of the strains belonging to this lineage presented MDR and carried large resistance plasmids, not transferrable under the assay conditions used. Some of these strains (represented in the present study by SUO9) contain a related ~150-kb *Inc*N plasmid, pUO-*St*-R4, which lacks virulence genes. *S.* Typhimurium AMP-CHL-GEN-STR-SPE-TET-TMP-SXT U302 (not monophasic variant) strains analyzed in England, showed similar PFGE profiles to the Spanish strains and carried resistance plasmids of 200 kb, which could be transferred to *E. coli* strains using a mobilizing plasmid.[38] The InI integron has been found in strains belonging to other *Salmonella* serotypes strains and in other Enterobacteriaceae, including *E. coli*.[22,39]

In conclusion, the presented study gives new information on the genetic basis of MDR in *S.* Typhimurium strains that are members of different lineages. These strains carry a broad range of integrons described for *S.* Typhimurium isolates.[18,20,21] It could be shown that the integrons are associated to various resistance genes, apparently forming distinctive resistance groupings, located on the chromosome or plasmid. Furthermore, a strong relationship between some of these groupings and the *Xba*I-PFGE profiles exists. Experimental data about the association of resistance and transposons genes derived from Tn*21* to a *Salmonella spv* plasmid, and of *spv* genes to a resistance plasmid, are also given. In addition, it has been demonstrated that isolates with the same or very similar resistance phenotype can have a completely different genetic background. In fact, in these strains, several different genes conferring resistance to defined antimicrobials such as ampicillin (*tem1-like*, *pse1*, and *oxa1*), chloramphenicol (*floR*, *cmlA1*, and *catA1*), streptomycin (*aadA1a*, *aadA2*, *aadA5*), tetracycline (*tet*(A), *tet*(B), and *tet*(G)), and trimethoprim (*dfrA1*, *dfrA7*, *dfrA12*, *dfrA14*, *dfrA17*) are present. The findings presented here constitute common examples of multidrug resistance evolution in non-hospital environments, and could be used to trace the continuous emergence and spread of MDR zoonotic bacteria.

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