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Dissemination of *sul3*-Containing Elements Linked to Class 1 Integrons with an Unusual 3′ Conserved Sequence Region among *Salmonella* Isolates▽

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A *sul3* domain (IS*440*-*sul3*-*orf1*-IS*26*) was found linked to an unusual 3′ conserved sequence region (*qacH*) of class 1 integrons and detected among nontyphoid *Salmonella* isolates ($n = 47$) from different sources. Three types of integrons differing in the gene cassette array (*dfrA12*-*orfF*-*aadA2*-*cmlA1*-*aadA1*, *dfrA12*-*orfF*-*aadA2*/*1*, and *estX*-*psp*-*aadA2*-*cmlA1*-*aadA1*) were found associated with this *sul3* domain. They were associated with particular clones and specific high-molecular-weight plasmids.

Antimicrobial agents of the sulfonamide group have been widely used in the treatment of human infections and also administrated to food animals (14, 17), a practice which has been argued to contribute to the maintenance or emergence of resistance (4). Sulfonamide resistance in gram-negative bacilli generally arises from the acquisition of one of the three genes, *sul1*, *sul2*, and *sul3*, encoding forms of dihydropteroate synthase that are not inhibited by the drug (14, 17). The *sul3* gene is the most recently described gene conferring resistance to sulfonamides (14) and has been increasingly identified in humans and principally among food animal isolates in the family *Enterobacteriaceae*. Several studies have demonstrated the dissemination of the *sul3* gene among *Escherichia coli* isolates from different origins and countries (5, 6, 7, 10, 14) and among *Salmonella* isolates from different sources and of different serotypes and clones (1, 8). The association of the genetic determinants for sulfonamide resistance with horizontally transferable capture genetic units may facilitate their dissemination. The spread of the *sul1* gene seems to be related to class 1 integrons, and the *sul2* gene is incorporated in plasmids (17). The dissemination and genetic background of the *sul3* gene have not been completely characterized. The *sul3* gene found in a Swiss *E. coli* isolate was flanked by transposable elements (*sul3* domain) inserted into a conjugative plasmid (14). Other studies have reported that *sul3* could be found on different large plasmids (6, 8). Only one recent report described the linkage of *sul3* to other resistance gene cassettes as part of an integron in swine *E. coli* isolates from the United States (5). Also in a previous study, we observed that all of the *Salmonella* isolates carrying the *sul3* gene contained a class 1 integrase, and in several of these isolates, *sul3* was the only sulfonamide resistance gene detected (1). The objective of this study was to characterize the genetic background of the *sul3* gene, including its association with integron structures, in nontyphoid *Salmonella* isolates in order to understand the dissemination of this sulfonamide resistance gene.

This study included 45 *sul3*-carrying *Salmonella* isolates from different sources (humans, food products, and the environment) and of different serotypes detected among the sulfonamide-resistant isolates ($n = 331$) of a collection of 1,511 Portuguese nontyphoid *Salmonella* isolates recovered between 2002 and 2004. These 331 isolates were recovered from human clinical sources ($n = 204$), food products ($n = 114$), the environment ($n = 7$), and unknown sources ($n = 6$). Two isolates previously collected in a central hospital in 2000 were also included in the present study. Characterization of the sulfonamide-resistant isolates was performed as previously described (1, 2). The characteristics of the *sul3*-producing *Salmonella* isolates are presented in the Table 1. Sixteen of the *sul3*-positive isolates were from food products, principally pork products ($n = 10$), collected from distinct locations in Portugal. The 27 human *Salmonella* isolates were recovered from diverse sources (predominantly feces) and 14 hospitals in geographically dispersed regions. Three isolates were obtained from environmental sources (bathing water). The isolates carrying *sul3* belonged to four serotypes and eight PFGE clones, with most of them identified as *S. enterica* serotype Typhimurium (five clones) and *S. enterica* serotype Rissen (one clone). It is of note that isolates from two clones of *S. enterica* serotype Typhimurium were the predominant ones carrying the *sul3* gene. All of the *sul3*-positive isolates showed resistance to several antimicrobials; coresistance to streptomycin (*aadA*), chloramphenicol (*cmlA*, *catA*), tetracycline (*tetA tetB*), and trimethoprim (*dfrA12*) (Table 1) was observed in 60% of the isolates. The *cmlA* gene was observed in all of the chloramphenicol-resistant isolates and in one of them with *catA*.

Structure of the *sul3*-associated integrons. Several methodologies were used to determine the structure of the *sul3*-genetic elements: (i) PCR was used to screen isolates for the *sul3* gene, with plasmid pVP440 (14) used as a positive control. (ii) PCR amplification was then used to determine the organizational structure of *sul3*-associated integrons as described pre-

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TABLE 1. Characteristics of the *sul3*-producing *Salmonella* isolates used in this study

Integron type, serotype (no. of isolates)	PFGE[b]	Source[c] (no. of isolates)	Isolation: yr/location (no. of isolates)	Resistance phenotype[d] and gene profile	Integron size (bp) and/or gene cassette (5′CS-3′CS)[f]	Size(s) (kb)[e] of plasmid(s) carrying-*sul3*-associated integrons (no. of isolates)
I						
Rissen (2)	N	S (1), U (1)	2002 (1), 2004 (1)/north (1), south (1)	Str^r Chl^r Tet^r Sul^r Tmp^r *aadA2-aadA1 cmlA1 tetA sul3 dfrA12*	*int1*	100 (2)
Typhimurium (7)	Q	H (6), S (1)	2000 (1), 2003 (2), 2004 (4)/north (7)	Chl^r Sul^r Tmp^r (Str^r Kan^r Tet^r) *cmlA1 sul3 dfrA12* (*aadA2-aadA1 aphA1 tetB*	*int1*	100 (1), 160 (1), 165 (1)
IIIb:65:lv:enxz15 (1)	S	H (1)	2003 (1)/south (1)	Str^r Chl^r Sul^r Tmp^r *aadA2-aadA1 cmlA1 sul3 dfrA12*	*int1*	100 (1)
Haifa (1)	Z	H (1)	2004 (1)/north (1)	Str^r Amp^r Chl^r Tet^r Sul^r Tmp^r *aadA2-aadA1 bla$_{TEM}$ cmlA1 tetA sul2-sul3 dfrA12*	*int1*	165 (1)
Typhimurium DT104 (1)[a]	A	U (1)	2004 (1)/north (1)	Str^r Amp^r Chl^r Tet^r Sul^r Tmp^r *aadA2-aadA1 bla$_{TEM}$ cmlA1 tetA sul1 sul3 dfrA12*	1,000; *aadA2*	135 (1)
II						
Rissen (3)	N	S (3)	2002 (2), 2003 (1)/ north (3)	Str^r Amp^r Tet^r Sul^r Tmp^r *aadA2-aadA2/1 bla$_{TEM}$ tetA sul1-sul3 dfrA12*	2,000; *dfrA12 orfF aadA2*	70 (2)
III						
Typhimurium (1)	J	S (1)	2003 (1)/south (1)	Str^r Kan^r Amp^r Chl^r Tet^r Sul^r Tmp^r *aadA2-aadA1 aphA1 bla$_{TEM}$ cmlA1-catA tetB sul1-sul3 dfrA1*	1,700; *dfrA1 aadA1*	240 (1)
Typhimurium DT104 (2)[a]	X	H (2)	2004 (2)/south (2)	Str^r Gen^r Amp^r Chl^r Tet^r Sul^r Tmp^r *aadA2-aadA1 aac(3)-IV bla$_{TEM}$ cmlA1 tetA sul1-sul2-sul3 dfrA12*	2,000; *dfrA12 orfF aadA2*	220 (1)
Typhimurium DT104 (29)[a]	O	H (17), S (4), P (1), C (2), U (1), E (3), UN (1)	2000 (1), 2002 (3), 2003 (12), 2004 (13)/north (19), center (1), south (8), UN (1)	Str^r Chl^r Sul^r (Gen^r Kan^r Amp^r Tet^r Tmp^r Nal^r) *aadA2-aadA1 cmlA1 sul1-sul2-sul3* [*floR aac(3)-IV bla$_{TEM}$ tetA tetB dfrA12*]	2,000; *dfrA12 orfF aadA2*	150 (1), 170 (5), 220 (3), 150 + 170 (1)

[a] PCR assay for the identification of *S. enterica* serotype Typhimurium DT104 and U302 phage types (15) positive.
[b] Clones are designated by capital letters as previously described (2).
[c] H, humans; S, pork products; P, poultry products; C, beef products; U, unknown food product; E, environment; UN, unknown source.
[d] Antibiotic resistances and resistance genes transferred to transconjugants are underlined. Variable antibiotic resistance and resistance gene among isolates of the same PFGE are in parentheses. The following genes implicated in antimicrobial resistance were detected by PCR (1, 2, 9): *bla$_{PSE-1}$*, *bla$_{OXA-30}$*, and *bla$_{TEM}$* encoding β-lactam resistance; *floR*, *cmlA1*, and *catA* encoding chloramphenicol resistance; *tetA*, *tetB* and *tetG* encoding tetracycline resistance; *sul1*, *sul2* and *sul3* encoding sulfonamide resistance; *aac(3)-IV* and *aphA1* encoding, respectively, gentamicin and kanamycin resistance. The characterization of genes associated with streptomycin (*aadA*) and trimethoprim (*dfrA*) resistance was done by PCR amplification and DNA sequencing in isolates carrying those genes inserted into integrons, as described previously (2). Antimicrobial agents were tested as previously described (2).Abbreviations: Str^r, streptomycin resistance; Gen^r, gentamicin resistance; Kan^r, kanamycin resistance; Amp^r, ampicillin resistance; Nal^r, nalidixic acid resistance; Chl^r, chloramphenicol resistance; Tet^r, tetracycline resistance; Sul^r, sulfamethoxazole resistance; Tmp^r, trimethoprim resistance.
[e] Plasmid profiles obtained by S1 PFGE in the isolates selected for testing; the plasmids transferred to transconjugants are underlined.
[f] *int1* gene was detected, as previously described (2) in isolates without a positive 5′CS-3′CS PCR assay.

viously (5), with TripleMaster enzyme mix (Eppendorf, Hamburg, Germany) and the primer combinations 5′CS (13) and cmlA-B (9), cmlA-F (9) and sul3F (14), and INT/5CS (16) and sul3F (14). (iii) Typing of *sul3*-carrying integrons was performed by a PCR-restriction fragment length polymorphism analysis in which PCR products corresponding to the amplification of the 5′ conserved sequence (5′CS)-*cmlA* region and of the *cmlA-sul3* region were purified and further digested with TaqI endonuclease (New England BioLabs, Ipswich, MA). PCR products representing the two different amplicons or the complete integron containing the *sul3* gene were sequenced through a primer-walking strategy with specific designed primers. Sequence comparisons were made with the BLAST program available at the National Center for Biotechnology Information website, and the sequence data for each integron type were deposited in the GenBank database.

The following three *sul3*-associated integrons presenting distinct gene cassette organizations were observed (Fig. 1): type I, 5′CS-*dfrA12-orfF-aadA2-cmlA1-aadA1-qacH*-IS*440-sul3* (7,085 bp); type II, 5′CS-*dfrA12-orfF-aadA2*/*1-qacH*-IS*440-sul3* (4,525 bp); type III, 5′CS-*estX-psp-aadA2-cmlA1-aadA1-qacH*-IS*440-sul3* (7,304 bp). All of the types presented genes coding for strep-

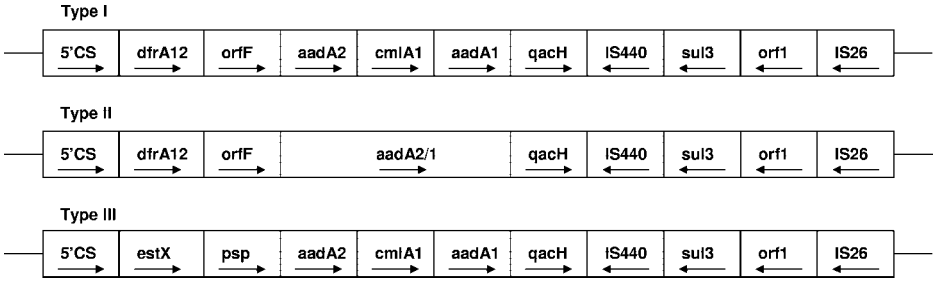

FIG. 1. Organization of the three types of *sul3*-carrying integrons in *Salmonella* (not to scale). Gene orientations are indicated by arrows.

tomycin resistance (*aadA*), including one new hybrid *aadA* gene, and two (types I and III) of them contained the *cmlA1* gene coding for chloramphenicol resistance. In all of the class 1 integron types described in this report, it seems that the *sul3* gene had replaced *sul1* in addition to the replacement of *qacE*Δ*1* by the *qacH* gene in the 3′CS region.

Two *sul3*-carrying class 1 integrons (types I and II), presenting an original gene cassette organization, were observed in multidrug-resistant (MDR) isolates of four serotypes (Rissen, Typhimurium, IIIb:65:lv:enxz15, and Haifa) corresponding to five clones. Interestingly, of the *S. enterica* serotype Rissen clone isolates, two contained the type I integron and three contained the type II integron. The structure of 7,085 bp (type I) contained the first three gene cassettes (*dfrA12-orfF-aadA2*) typical of a class 1 integron widely disseminated among members of the family *Enterobacteriaceae*, including *Salmonella* (2) and downstream an organization identical to part of the type III structure. In 10 of 12 isolates carrying the type I genetic structure, *sul3* was the only *sul* gene detected. The other new integron structure (type II), only described in the MDR *S. enterica* serotype Rissen clone, contained the first two gene cassettes (*dfrA12-orfF*) typical of class 1 integrons and also present in the type I integron described here, but with a hybrid *aadA* gene cassette as the third gene, suggesting recombination between the *dfrA12-orfF-aadA2* cassette array and an *aadA1* gene cassette (the crossover in the hybrid *aadA2/1* cassette was located between positions 289 and 299 in the cassette). This genetic event and the absence of the *cmlA* gene, before the *qacH-tnp-sul3* sequence, observed in isolates from the same clone, suggest that the type II integron may be a result of evolution (e.g., by an incorrect cassette excision event) from the *sul3*-carrying class 1 type I integron.

Class 1 integron structure type III, identical to that recently described by Bischoff et al. (5) in swine *E. coli* isolates, was observed in the majority ($n = 32$) of the *sul3*-producing *Salmonella* isolates (including the oldest strain from 2000). This genetic structure was located only in MDR *S. enterica* serotype Typhimurium isolates corresponding to three clones. Most of those isolates (24/32) also carried another class 1 integron (*dfrA12-orfF-aadA2* in 23 and *dfrA1-aadA1* in 1) with the *qacE*Δ*1-sul1* genes as part of the 3′CS. With the exception of one isolate, all presented the three types of *sul* gene simultaneously.

Finally, characterization of the *sul3* genetic context was also performed by PCR with several specific primer combinations (TNP-F-SUL3F and SUL3FR-TNP-R) and sequencing (PCR products and plasmids extracted from transconjugants). Characterization of the *sul3* genetic vicinity showed a gene cluster comprising *sul3* and transposase-like sequences (IS*440-sul3-orf1*-IS*26*) identical in all of the isolates, with the exception of one (deletion of ca. 500 bp in the *orf1*-IS*26* region). The *tnp* gene of IS*440* was identical to the one described in an *S. choleraesuis* plasmid (GenBank accession no. AY509004), to the *E. coli* plasmids characterized by Bischoff et al. (5), and also to the partial sequence of the truncated IS*440* sequence described by Perreten and Boerlin (14). The insertion sequence observed downstream of the *sul3* gene was identical to IS*26*, already observed flanking resistance genes, including in several MDR *S. enterica* plasmids (e.g., GenBank accession no. AY333434 and AJ628353). It is of note that the orientation of the IS*440-sul3-orf1*-IS*26* element is the opposite of that previously described by Perreten and Boerlin (14), suggesting a different *sul3* gene acquisition event.

Genetic locations of *sul3*-associated integrons. Several methods were used to determine the locations of *sul3*-containing genetic elements. (i) Conjugation assays with *E. coli* K802N (Nal^r Rif^r) as a recipient strain were attempted by mating in agar plates. Transconjugants were selected on Mueller-Hinton agar 2 (bioMérieux, Marcy l'Étoile, France) containing sulfamethoxazole (256 μg/ml) plus nalidixic acid (64 μg/ml) (or 100 μg/ml rifampin if the donor was nalidixic acid resistant). (ii) Plasmid DNA was isolated from donors and transconjugants by several methods (11, 12). The relatedness of plasmids harbored by the transconjugants was determined by single-restriction analysis with EcoRI. For better resolution and sizing of high-molecular-weight plasmids, extraction from selected isolates was also performed by nuclease S1 (Amersham Biosciences, Uppsala, Sweden) digestion prior to PFGE (3). (iii) Southern blot hybridizations of S1-PFGE patterns were performed by standard methods, by using a nonradioactive technique (Amersham Biosciences) with probes for *sul3* from pVP440 (14) and probes for class 1 integron- and gene cassette-specific sequences (5′CS-*dfrA12* and *estX-psp*).

The *sul3* gene was originally identified on a 54-kb conjugative plasmid (14), but it also appeared to be located on large plasmids of different sizes (6, 8, 14). Sulfonamide resistance and the *sul3* gene were transferred, by conjugation assays, in 5 of 12 isolates carrying genetic structure type I and in all of the isolates carrying genetic structure type II, with cotransference of resistance to trimethoprim and streptomycin. Plasmid characterization by S1-PFGE, followed by Southern hybridizations, demonstrated that plasmids carrying the *sul3* gene cohybridized with specific probes for class 1 integrons and gene cassette-specific sequences. The *sul3* gene and *sul3*-associated

type I and II integrons were located on large plasmids of different sizes (≥100 kb) and on identical conjugative plasmids of ca. 70 kb, respectively (Table 1). The conjugative plasmid of ca. 100 kb was the most disseminated among the different clones; although the RFLP with EcoRI showed different profiles between isolates from different clones, several bands were shared between them, suggesting that they are highly related (data not shown). Conjugation experiments failed to demonstrate the occurrence of conjugative transfer of resistance determinants, including *sul3*, from all of the isolates of *S. enterica* serovar Typhimurium DT104 carrying *sul3*-associated integron type III. However, Southern hybridizations after S1-PFGE experiments demonstrated that the *sul3* gene and *sul3*-associated integron type III were located on large plasmids of different sizes (between 150 and 220 kb) (Table 1). One isolate from the environment presented two copies of the *sul3* gene on plasmids of different sizes (150 and 170 kb), both also detected in human and food isolates of the same clone. The conjugative plasmid of ca. 240 kb carrying *sul3* integron type III was only detected on a non-DT104 *S. enterica* serotype Typhimurium isolate (Table 1).

We describe the dissemination of *sul3* associated with plasmid-borne class 1 integrons containing an unusual 3′CS site. The presence of similar *sul3*-integron platforms containing different gene cassette arrays or hybrid genes suggests evolution of the genetic background by different recombinatorial events. The association with epidemic plasmids and particular MDR clones of *Salmonella* might contribute to the maintenance and further spread of modular antibiotic resistance elements from food animals to hospitalized humans, as reported for other *Salmonella* genetic elements (2).

Nucleotide sequence accession numbers. The nucleotide sequences of the *sul3*-carrying integrons reported in this study have been submitted to the EMBL/GenBank sequence databases and assigned accession numbers EF051037 (type I), EF051038 (type II), and EF051039 (type III).

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