J Antimicrob Chemother 2012; **67**: 111–114
doi:10.1093/jac/dkr391 Advance Access publication 11 October 2011

Journal of Antimicrobial Chemotherapy

Nucleotide sequence of the chromosomal region conferring multidrug resistance (R-type ASSuT) in *Salmonella* Typhimurium and monophasic *Salmonella* Typhimurium strains

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Received 24 May 2011; returned 28 June 2011; revised 24 August 2011; accepted 24 August 2011

Objectives: The aim of this study was to sequence the chromosomal region conferring resistance to ampicillin, streptomycin, sulphonamides and tetracycline (R-type ASSuT) in a *Salmonella* Typhimurium (STM) monophasic strain (4,[5],12:i:−) belonging to the PFGE profile STYMXB.0079. The presence of this resistance region and the analysis of its genetic environment was investigated in a selection of strains.

Methods: A Sau3A1 genomic library was used to determine the nucleotide sequence of the genomic resistance region. PCRs were performed on 10 epidemiologically unrelated *Salmonella* strains, both STM and monophasic STM, with R-type ASSuT and PFGE profile STYMXB.0079, in order to investigate the presence of the resistance genes, the left and right junctions and the internal regions of the resistance region, as well as the genetic environment.

Results: The genomic resistance region consisted of two regions, resistance region 1 (RR1), conferring resistance to ampicillin, streptomycin and sulphonamides, and resistance region 2 (RR2), conferring tetracycline resistance. These resistance regions were both surrounded by IS*26* elements and sequence comparative analysis showed 99% sequence identity with a region of plasmid pO111_1 from an *Escherichia coli* strain. All 10 strains were positive for the four resistance genes, the left and right junctions and the internal regions of RR1 and RR2. Concerning the genetic environment, all the strains lacked the STM1053-1997 and STM2694 genes, while only monophasic STM strains showed deletion of the *fljA-fljB* operon.

Conclusions: This study describes two resistance regions localized on the bacterial chromosome of a clonal lineage of STM and monophasic STM that are widespread in Italy.

Keywords: *Salmonella* spp., MDR, resistance regions

Introduction

Antimicrobial resistance is quite common in *Salmonella* spp., especially in *Salmonella enterica* serovar Typhimurium (STM) and *Salmonella enterica* 4,[5],12:i:− (monophasic STM), an emerging serovar antigenically related to STM but lacking the second-phase flagellar antigen encoded by the *fljB* gene.[1]

In Italy, STM and monophasic STM have accounted for approximately 60% of all *Salmonella* serovars isolated from human infections in recent years, with a high percentage of multidrug-resistant strains (resistant to four or more drugs). Among these two serovars, most strains show the R-type ASSuT (i.e. resistance to ampicillin, streptomycin, sulphonamides and tetracycline) and belong to a unique clonal lineage, mainly represented by the PFGE profile STYMXB.0079.[2]

It has been demonstrated that ASSuT strains isolated in different European countries harbour the same resistance genes [bla_{TEM-1}, *strA-strB*, *sul*2 and *tetA*(B)][3–5] and that these are located on the bacterial chromosome, thus suggesting the presence of a genomic resistance cluster.[3]

In order to clarify its organization, the entire chromosomal resistance region has been sequenced and its presence investigated in a selection of STM and monophasic STM strains with R-type ASSuT. In addition, analysis of the genetic environment of the region has been performed.

Materials and methods

The nucleotide sequence of the genomic resistance region was determined, using a Sau3A1 genomic library, in a monophasic STM strain (105/7/03, R-type ASSuT, PFGE profile STYMXB.0079 and phage type

Table 1. Primers used in this study

Primer	Target region	DNA sequence (5′ to 3′)	Melting temperature (°C)	Extension time (s)	Expected amplicon (bp)	Position in RR1/RR2
STM2753Δ′	RR1LJ	TATTGATATCGTCTCGGTGG	62	120	1658	1191–1210
TEM		GGATCTTACCGCTGTTGAG				2831–2849
REPA	RR1RJ	CCATGATGTTGGGCAGGC	62	120	1200	9053–9070
STM2759Δ′		TATCGCAATCTGGATCCGC				10235–10253
STM2753Δ″	RR2LJ	CAAAATCATTGATCGGGTCG	62	120	1680	16279–16298
lysR		GACATCTCGATATCTTTTATGC				17948–17969
tniAΔ	RR2RJ	TTCACGACGAACTGCCGG	62	120	1400	30102–30119
STM2759Δ″		GTGCTGGTAATCAAACAGC				31481–31499
TEM2	*tnpB*	AGTTGGGTGCACGAGTGGGT	62	60	1173	2796–2815
tnpB		AAACGCAGCTGAAACGGG				3950–3968
repAΔ	*repC*	TCTGCTGGAGGTGGGCG	62	60	796	8422–8440
repC		CGCTGAACTCGATCCGCTT				9218–9233
IS10	IS*10*	TTGAAAAGTCCTGCCTACG	62	60	736	18280–18297
glts		CGTTCCTATGGTCGGTGC				17527–17544
tetC(B)Δ	*tetC*(B)Δ	GGCATCACTTCTTGGATAGG	62	60	1337	23503–23522
merR		AACCTGACCATTGGCGTTTTT				24820–24840

LJ, left junction; RJ, right junction.
The target regions are indicated in Figure 1(a).

U311) isolated in Italy in 2003 from a human case of gastroenteritis. In addition, 10 epidemiologically unrelated *Salmonella* strains, comprising 7 STM and 3 monophasic STM [R-type ASSuT, PFGE profile STYMXB.0079 and various phage types (U302, U311, 32 and NT)], isolated in Italy between 2003 and 2010, were tested by PCR for bla_{TEM}, *strA-strB*, *sul2* and *tetA*(B) genes, for the left and right junctions and for the internal region of the resistance cluster (Table 1). Moreover, the surrounding genetic environment of the resistance region, consisting of the *fljA-fljB* operon, including the genes *fljA*, *fljB* and *hin*, and the genes STM1053-1997, STM2692, STM2694, STM2740, STM2741, STM2757, STM2758, *iroB* and STM2774, was analysed using the primers described by Soyer *et al.*[1]

Results and discussion

The nucleotide sequence (Figure 1a; GenBank accession no. HQ331538) harbours the ASSuT resistance genes, which are clustered into two regions [resistance region 1 (RR1) and resistance region 2 (RR2)] inserted in two adjacent loci of the chromosome, involved in polysaccharide transport and metabolism. In particular RR1 (8761 bp), conferring resistance to ampicillin, streptomycin and sulphonamides, was inserted in gene STM2753, causing a deletion of 190 bp, while RR2 (14587 bp), conferring tetracycline resistance, was inserted in gene STM2759, causing a deletion of 6 bp. The chromosomal fragment located between the two regions included the genes from STM2753Δ″ to STM2759Δ′ (6543 bp), but inversely orientated. Comparison with the sequence database revealed that the chromosomal regions involved in the insertion were well conserved; 100% sequence identity was observed for the chromosomal segment upstream of RR1 (STM2752 and STM2753Δ′) while the region between RR1 and RR2 (STM2759Δ′-STM2753Δ″) and the region downstream of RR2 (STM2759Δ″ and STM2760) showed 99% sequence identity with the complete STM LT2 genome (GenBank accession no. AE006468).

RR1 interrupted the STM2753 gene and was composed of an IS*26* element, a truncated Tn*3* transposon containing the partial resolvase gene (*tnp3R*Δ) and the bla_{TEM-1} gene, followed by a *tnpB* gene and an IS*26* element in the opposite orientation. Downstream, *strB*, *strA* and *sul2* resistance genes, *repC* and *repA*Δ, partially encoding replication proteins of the RSF1010 plasmid, and, at the end, another IS*26* element, were found. When compared with multidrug resistance clusters described previously, RR1 showed 99% sequence identity with a region of the plasmid pO111_1 harboured in an *Escherichia coli* strain isolated in Japan in 2001 (GenBank accession no. AP010961; nt 107040–113069). However, in RR1 the resistance gene bla_{TEM-1}, between two IS*26* elements, was located upstream of IS*26*-*repA*Δ and inversely orientated (Figure 1a).

RR2 started with an IS*26* element and a defective Tn*10* transposon. This transposon included a truncated IS*10*-left transposase, *lysR*Δ, *gltS2*, *gltS1*, *ydjA*, *ydjB* and *jemC* genes, with hypothetical functions, *tetR*(B) and *tetA*(B) genes encoding the tetracycline repressor and resistance proteins, respectively, and a *tetC*(B)Δ gene. The Tn*10* transposon was interrupted by the insertion of an IS*1* element, which caused the deletion of the *tetD*(B) gene and IS*10*-right. Downstream, a partial Tn*21* transposon included IR_{mer} (where IR stands for inverted repeat), the mercury resistance operon, and *urf2* and *tniA*Δ*1* genes, encoding a hypothetical protein and a putative transposase, respectively. The Tn*21* transposon was interrupted, at its right side, by an IS*26* element.

RR2 also showed 99% sequence identity with a region of the pO111_1 plasmid (nt 92230–105999). Moreover, the Tn*10* transposon of RR2 lacked the right terminal (IS*10*-right), as in the pO111_1 plasmid, but our Tn*10* was also truncated at the left terminal (Figure 1a).

Both RR1 and RR2, as expected, lacked the *aph* gene, conferring resistance to kanamycin, present in the pO111_1 plasmid.

Overall, the comparison of the entire sequence harbouring RR1 and RR2 with sequences of the pO111_1 plasmid and of

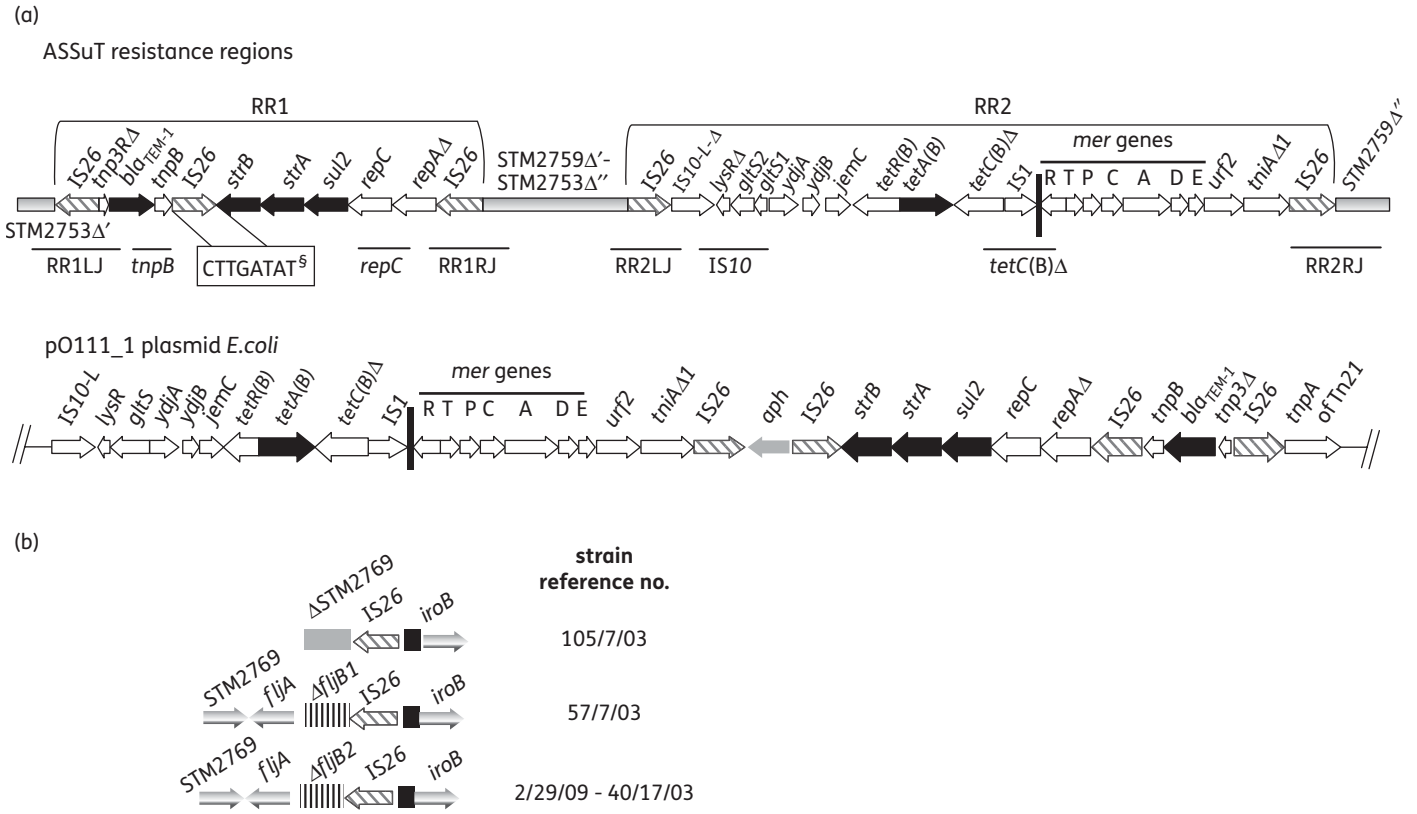

Figure 1. (a) Comparison of the genetic organization of the resistance regions of the 105/7/03 strain and the pO111_1 plasmid. Antibiotic resistance genes and IS elements are indicated by black arrows and grey hatched arrows, respectively. Grey boxes indicate chromosomal DNA. Vertical black bars indicate 38 bp terminal imperfect IRs of transposon Tn*21*. STM2753Δ′ and STM2759Δ′ indicate the 3′ deleted portions of STM2753 and STM2759, respectively. STM2753Δ″ and STM2759Δ″ indicate the 5′ deleted portions of STM2753 and STM2759, respectively. §DRs of IS*26*. (b) Genetic organization of the *fljA-fljB* operon in the four monophasic strains. Black boxes indicate the intergenic region between the *hin* and *iroB* genes.

the complete STM LT2 genome, showed seven single-nucleotide polymorphisms (SNPs) and one frameshift, caused by a nucleotide deletion. Only two SNPs were non-synonymous substitutions, one in *strA* (Arg175Gly) and one in the STM2756 gene (Gly107Asp). The frameshift, present in the *gltS* gene, led to the formation of a stop codon and a second start codon responsible for the formation of two hypothetical proteins, *gltS1* and *gltS2*. This frameshift has been reported previously in pHCM1, a plasmid found in a *Salmonella* Typhi strain isolated in Vietnam in 1993 (GenBank accession no. AL513383), which showed a resistance cluster highly similar to an IncHI1 plasmid of *Salmonella* Paratyphi A, pAKU_1, isolated from a Pakistani patient in 2002 (GenBank accession no. AM412236) and to a 120 kb IncF plasmid pRSB107, isolated from activated sludge bacteria in a sewage treatment plant in Germany (GenBank accession no. AJ851089). In addition, the 9 kb sequence, incorporating bla_{TEM-1}, *sul2* and *strA-strB*, flanked by IS*26* elements, has been described in other STM strains, integrated in the chromosome (GenBank accession no. AY524415) or on an IncF-like plasmid pU302L (GenBank accession no. AY333434). Finally, parts of RR1 and RR2, such as the *mer* resistance operon, the bla_{TEM} gene flanked by two IS*26* elements and the *strA-strB* gene, have also been described in variants of the *Salmonella* genomic island 1 ('SGI1').[6,7]

Analysis of the sequences flanking all the IS*26* elements allowed us to identify direct repeats (DRs) only upstream and downstream of the second IS*26* (CTTGATAT; Figure 1a), suggesting a reminiscent transpositional event. Despite RR1 and RR2 being bracketed by two IS*26* elements in direct orientation, the lack of DR elements suggested that their insertion occurred by multiple illegitimate recombination events, rather than through a transpositional mechanism, as previously described.[6,7] In addition, the presence of IS*26* elements may have contributed to the intrinsic instability of the resistance regions.

All 10 strains investigated for the presence of bla_{TEM}, *strA-strB*, *sul2* and *tetA*(B) genes, RR1 and RR2 (left, right and internal regions; Figure 1a) were positive for all the genes/regions tested, regardless of whether they were STM or monophasic STM. These results suggest that the genetic organization of the ASSuT chromosomal resistance regions is well conserved among *Salmonella* strains with PFGE profile STYMXB.0079.

The genetic environment of the RR1 and RR2 insertion regions has been reported to be frequently subjected to rearrangements and insertions.[1] The lack of the chromosomal regions in STM2694-STM2740, STM2758-STM2773 and STM2694-STM2771, including the *fljA-fljB* operon, responsible for the expression of the second flagellar phase, has been reported in

strains isolated in Spain and the USA.[1] In contrast, when tested by PCR according to Soyer *et al*.,[1] all ASSuT strains of this study were found to be positive for the genes contained in the described regions, except for STM1053-1997, an insertion region described only in US strains and STM2694, a gene of the Fels-2 prophage. As expected, all the monophasic strains were found to lack *fljB* and *hin* genes, and strain 105/7/03 also lacked the *fljA* gene. A similar deletion genotype has been described only in one US strain, which seems quite different from all the other strains analysed by Soyer *et al*.[1]

An analysis of the *fljA-fljB* operon in the monophasic STM strains showed that the insertion of IS*26* elements, as previously described,[8,9] is responsible for deletions in the *fljA-fljB* operon (Figure 1b); in particular strain 57/7/03 lacked 727 bp, while the strains 40/17/03 and 2/29/09 lacked 694 bp, at the 5′ end of the *fljB* gene, as a consequence of the IS*26* insertion.

As far as the sequenced strain, 105/7/03, is concerned, the STM2769 gene and *fljA-fljB-hin* genes were, respectively, partially and completely deleted by an IS*26* insertion. The chromosomal region downstream of the IS*26* element was found to be conserved; it restarted at the same nucleotide position of the intergenic region followed by the *iroB* gene in all strains.

Overall, our study shows that ASSuT strains, which are widespread in Italy, both STM and monophasic STM, belonging to a unique clonal lineage, harbour two new genomic resistance regions that are highly conserved. The chromosomal integration of these regions, probably by illegitimate recombination, is of great concern since antimicrobial resistance in ASSuT strains could be maintained without selective pressure and could contribute to their spread in humans and animals. Finally, in strains belonging to PFGE profile STYMXB.0079 we observed a high degree of conservation in the genomic resistance regions but greater variability in the *fljA-fljB* operon in the monophasic STM strains. These observations suggest that this operon could be an integration hotspot of acquired DNA.

Further studies are needed to assess the presence of these resistance regions in a larger number of ASSuT strains belonging to the same or different clonal lineages circulating both in Italy and in other European countries.

Acknowledgements

We would like to thank Mr Ildo Benedetti and Mr Sergio Arena for their technical assistance.

Funding

This work was supported by a grant from the Italian Ministry of Health—Centro Nazionale per la Prevenzione ed il Controllo delle Malattie (CCM; project 'Sorveglianza dell'antibiotico resistenza in comunità nelle infezioni trasmesse da alimenti e in quelle di origine zoonosica').

Transparency declarations

None to declare.

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