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Method Article

# IS*26* mediated antimicrobial resistance gene shuffling from the chromosome to a mosaic conjugative FII plasmid

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ABSTRACT

In the present study we report the identification of a *sul3*-associated class 1 integron containing the *dfrA12*-orfF-*aadA2-cmlA1-aadA1-qacH* array embedded in a Tn*21*-derived element that is part of a conjugative FII plasmid named pST1007-1A. The plasmid was identified in the *Salmonella* Typhimurium strain ST1007, a member of a clinically relevant clonal MDR lineage diffuse in Italy. ST1007 exhibited resistance to ampicillin, chloramphenicol, streptomycin, sulphamethoxazole, tetracycline and trimethoprim encoded by $bla_{TEM-1}$, *cmlA1*, (*aadA1*, *aadA2*, *strAB*), (*sul2*, *sul3*), *tet*(B) and *dfrA12* genes, respectively. Apart from pST1007-1A, ST1007 also harbours two chromosome-integrated resistance units RU1 ($bla_{TEM-1}$-*sul2-strAB*) and RU2 (*tet*(B)), flanked by IS*26* elements. RU1 and RU2 were able to move as translocatable units, respectively TU1 and TU2, and integrate via IS*26* mediated recombination into pST1007-1A. A family of conjugative plasmids, harbouring different sets of antimicrobial resistance genes (ARG) was then generated: pST1007-1B (*dfrA12-aadA2-cmlA1-aadA1-sul3- tet*(B)), pST1007-1C (*dfrA12-aadA2-cmlA1-aadA1-sul3-*$bla_{TEM-1}$*-sul2-strAB*), pST1007-1D ($bla_{TEM-1}$-*sul2-strAB*), pST1007-1E (*tet*(B)) and pST1007-1F (*dfrA12-aadA2-cmlA1-aadA1-sul3- tet*(B) -$bla_{TEM-1}$*-sul2-strAB*). pST1007-1A is also a mosaic plasmid containing two distinct DNA fragments acquired from I1 plasmids through recombination within the *repA4*, *rfsF* and *repeat*-3 sites. This study further highlights the role played by IS*26* in intracellular ARGs shuffling. Moreover, attention has been focused on recombination hot spots that might play a key role in generating mosaic plasmids.

## 1. Introduction

Dissemination of antimicrobial resistance genes greatly contributes to the appearance of multidrug resistant (MDR) bacteria. This process is mainly mediated by genetic elements such as plasmids, integrons, transposons and insertion sequences (ISs).

The insertion sequence IS*26* is 820 bp long. It is bounded by 14 bp perfect inverted repeats and contains a single gene, *tnp26*, that encodes the 234-amino acid Tnp26 transposase. IS*26* is a member of the IS*6* family and, in Gram-negative bacteria, it has been found associated with many different antimicrobial resistance genes (ARGs) (Harmer et al., 2014; He et al., 2015; Mollet et al., 1985). IS*26* is able to move by a replicative mechanism which, when causing deletion of sequences immediately adjacent to it, produces a circular product composed of a single copy of IS*26* and the deleted DNA segment (Harmer and Hall, 2016). This product has been called a translocatable unit (TU). TUs may integrate randomly into target DNA molecules (untargeted replicative mechanism) or specifically target IS*26* elements. (Harmer and Hall, 2017). This last mode of cointegrate formation (conservative) involves a Tnp26-dependent reaction between two IS*26* and it occurs with a frequency over 50-fold higher than the replicative mechanism (Harmer et al., 2014). TUs have also been invoked as the units of movement for IS*26*-flanked transposons and for IS*26*-flanked DNA segments in a multimeric array (e.g. formation of overlapping IS*26*-bounded transposons). The origin of this last configuration is only consistent with the conservative mechanism of cointegrate formation (Harmer et al., 2014).

Tn*21* is a large transposon that confers resistance to mercuric ions and to more than one antibiotic (resistance mediated by the presence of

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class 1 integron) (Liebert et al., 1999). Class 1 integrons are generally composed of two conserved regions (5'-CS and 3'-CS) flanking one variable segment which contains the acquired gene cassette(s) often encoding for antimicrobial resistance (Stokes and Hall, 1989). Some class 1 integrons lack the 3'-CS (*qacEΔ1-sul1*-orf5) region as reported for the *sul3*-associated class 1 integrons where the 3'-CS has been replaced by a *sul3* segment (*tnp440-sul3*-orf1-IS*26*) (Antunes et al., 2007; Hall et al., 1994; Moran et al., 2016). In most class1 integrons harboured by transposons related to Tn*21*, an IS*26* is found between the truncated *mefB* and *tniA* genes (Curiao et al., 2011).

Tn*21*-derived sequences and IS*26* are frequently reported to reside in resistance plasmids (He et al., 2015; Partridge et al., 2018; Moran and Hall, 2018). In *Salmonella*, an important foodborne pathogen, IS*26* associated with MDR loci has extensively been reported for FI, FII, HI1 and I1 plasmids as well as in hybrid plasmids (Cain and Hall, 2012; Herrero et al., 2008). Hybrid plasmids contain backbone sequences derived from two or more different plasmid types (e.g. I1 and

FII) and may originate from fusion processes mediated by IS*26* or by site-specific genetic recombination. Some sites in plasmid backbones are thought to play an important role in recombination. For instance, it has been suggested the *rfsF*-ResD system plays a role in the resolution of cointegrated I1 plasmid dimers from which the diversity of genomes related to R64 may have arisen (Sampei et al., 2010). Likewise, in FII plasmids the *repA4* (that is part of the replication region) has been proposed as a site of recombination between FII- and I1-molecules (Fiett et al., 2014).

In this study we report the characterisation of a mosaic, conjugative FII plasmid (pST1007-1A) harboured by a clinical MDR *Salmonella* Typhimurium (ST) strain (ST1007). *S*. Typhimurium ST1007 is part of an epidemiologically relevant clonal lineage that has frequently been detected in MDR clinical isolates of ST and its monophasic variant (STMV) serovar 4,[5],12:i:- that are both widely distributed in Italy (Lucarelli et al., 2012). pST1007-1A contains a Tn*21*-derived transposon with an embedded *sul3*-associated class 1 integron harbouring the *dfrA12*-orfF-*aadA2-cmlA1-aadA1-qacH* array. The IS*26* present in the *sul3* segment (*tnp440-sul3*-orf1-IS*26*) was the target of two translocatable units (TU1 and TU2) originating from the respective chromosome-integrated resistance units (RU1 and RU2) flanked by IS*26*. RU1 and RU2 harboured the resistance genes $bla_{TEM-1}$-*sul2-strAB* and *tet* (B), respectively. This enabled ST1007 to act as donor to transfer different sets of ARGs through conjugation.

## 2. Materials and methods

### 2.1. Bacterial isolates and antimicrobial susceptibility testing

ST1007 is part of a collection of 113 MDR *S*. Typhimurium strains isolated from clinical cases in Southern Italy (De Vito et al., 2015). Classification and antimicrobial susceptibility tests were performed as reported previously (Oliva et al., 2017). The antimicrobial resistances reported in this study were: ampicillin (Ap), chloramphenicol (Cm), streptomycin (Sm), sulphamethoxazole (Su), tetracycline (Tc) and trimethoprim (Tp).

### 2.2. Strain molecular typing by PFGE

Genomic restriction was performed according to the standardised *Salmonella* protocol of the CDC PulseNet (Ribot et al., 2006). Digital images of the ST1007-PFGE profile were analysed using algorithms available in the BioNumerics software package (Applied Maths, Sint-Martens-Latem, Belgium). The PFGE profile was compared with those included in the PulseNet-Europe international database and named with a six-letter code followed by a four-digit numerical identifier. The ST1007-PFGE profile was assigned to the pulsotype STYMXB.0079.

### 2.3. Bacterial conjugation, gene detection and plasmid analysis

Conjugation experiments were performed at 37 °C as described previously (Oliva et al., 2017). Antimicrobial concentrations were: Ap 100 μg/mL, Cm 25 μg/mL, nalidixic acid (Nx) 50 μg/mL, rifampicin (Rf) 100 μg/mL, Sm 100 μg/mL, Su 600 μg/mL, Tc 20 μg/mL, Tp 30 μg/mL. Transconjugants were selected on Luria Bertani Agar (LBA) plates supplemented with Nx or Rf (selecting for CSH26 Nx or DH5α Rf strains, respectively as recipients) and Ap, Cm, Sm, Tc or Tp (selecting for antimicrobial resistance exhibited by donors). The frequency of transfer was determined as the number of transconjugants per donor. An average of 500 (unless otherwise stated) well isolated transconjugant colonies, from each mating type, were replicated on LBA supplemented with suitable antimicrobials (e.g. in mating between ST1007 and CSH26 Nx, transconjugants selected on LBA supplemented with Nx and Ap were replicated on LBA supplemented with Nx and Ap, Cm, Sm, Tc or Tp). The antibiotic concentration was as mentioned above. Plasmids (detected in donors and transconjugants) were typed by the PCR Based Replicon Typing protocol (PBRT) using positive controls kindly supplied by A. Carattoli (Carattoli et al., 2005). Detection of ARGs, genetic elements and gene organization were performed by PCRs on ST1007 and transconjugants. Primers used in this study were as reported previously (Camarda et al., 2013; Oliva et al., 2017) or, if newly designed, as reported in Table S1.

### 2.4. Plasmid isolation

Plasmid isolation was performed following the procedure described by Kado and Liu (Kado and Liu, 1981) modified as follows: cells were grown in 50 mL of LB broth overnight at 37 °C and pelleted by centrifugation (12,000 *g*, at 4 °C for 10 min). The cell pellet was resuspended in 5 mL of buffer A (50 mM Tris, 1 mM EDTA, adjusted to pH 8.0 with 37% HCl). The cells were gently lysed by adding 10 mL of the lysing solution L (3% SDS, 50 mM Tris, pH 12.6 adjusted with 5 N NaOH) and incubated at 65 °C for 1 h. After incubation, 10 mL of buffer N (3 M $CH_3COOK$, 2 M $CH_3COOH$) were added to the cell-lysed solution, gently mixed, incubated on ice for 5 min and centrifuged (27,000 *g*, at 4 °C for 30 min). The supernatant was recovered avoiding the white precipitate (if necessary centrifugation was performed twice), 10 mL of solution P (30% polyethylene glycol, 1.7 M NaCl) was added, gently mixed and incubated overnight at 4 °C. Following centrifugation (12,000 *g* at 4 °C for 30 min) the DNA pellet was washed with absolute ethanol, dried and gently resuspended in 300 μL of sterile $dH_2O$ (10 μL were generally sufficient for enzyme restriction). Restriction products were separated by 1% agarose gel (Pulsed Field Certified, BioRad) through a field inverted gel electrophoresis: programme 1 (Switch time ramp: 0.1–0.4 s, linear shape; Forward voltage: 180 V; Reverse voltage: 120 V; at 16 °C for 18 h); progamme 2 (Switch time ramp: 0.1–0.8 s, linear shape; Forward voltage: 180 V; Reverse voltage: 120 V; at 16 °C for 18 h).

### 2.5. DNA sequencing, assembly and annotation

Genomic DNA of ST1007 was extracted by the CTAB method (Murray and Thompson, 1980). Plasmids were purified as reported in section *2.4*. One ng of ST1007 genomic DNA or DNA plasmid from pST1007-1A to pST1007-1D was used for sequencing library construction (Nextera XT DNA Library Preparation Kit Illumina Inc.). Libraries were indexed using the Nextera XT Index Kit (96 Index, Illumina Inc.). Equimolar quantities of each library were pooled and subjected to 2 × 250 bp paired-end sequencing on the Illumina MiSeq platform. In order to increase the sample's genetic diversity, the phage PhiX genomic DNA library was added as a spike-in. Sequences were trimmed using Trimmomatic and assembled using SPAdes v 3.51 (Nurk et al., 2013). Contigs were assembled by specific PCRs and analysis of *EcoRI* profiles (Fig. 1, Fig. 5 and Fig. S1; Table S2). The genetic organization of

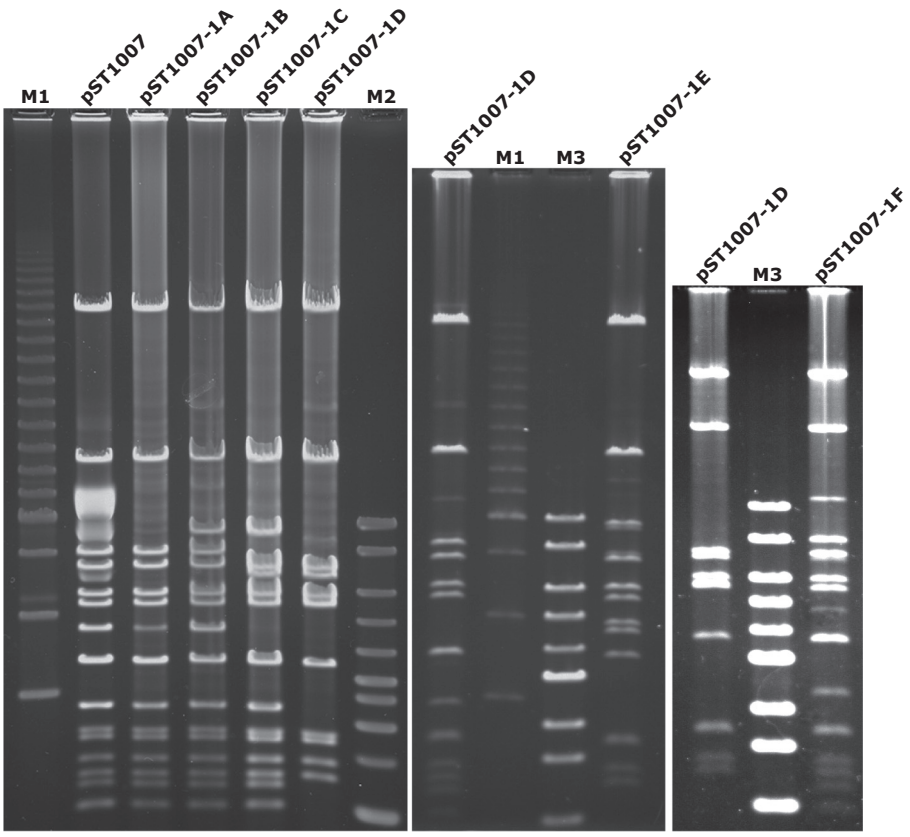


**Fig. 1.** *EcoRI* restrictions of pST1007-1A and its derivatives.
Plasmid names are reported above each line. pST1007 refers to plasmid content recovered from the donor strain ST1007. pST1007-1A to pST1007-1F refers to plasmids recovered from transconjugants BA1A to BA1F, respectively. M1: 2.5 kb Molecular Ruler (BioRad); M2: Bench Top 1 kb Ladder (Promega); M3: Quick Load 1 kb DNA Ladder (New England Biolabs).

pST1007-1E and pST1007-1F was deduced by comparing their Eco*RI* profiles with those of pST1007-1A to pST1007-1D, and by specific PCRs. DNA sequences of pST1007-1A, pST1007-1B, pST1007-1C, pST1007-1D and the chromosomal region spanning from STM2749 to STM2760 (where the RU1 and RU2 were located) were deposited in GenBank under accession numbers MH257753, MH626558, MH626559, MH648141 and MH257754, respectively. STM refers to chromosomal genes as reported in *S*. Typhimurium referring strain LT2. DNA sequences of pST1007-1A derivatives can be obtained by insertion of the translocatable unit TU1 and/or TU2 (for details see section 3.2.2).

Annotation was automatically performed using the Rapid Annotations using Subsystems Technology (RAST) (Aziz et al., 2008; Overbeek et al., 2014) and edited on the basis of its comparison with the well-characterised IncFII plasmid R100 (NC_002134.1) and IncI1 plasmid R64 (AP005147.1).

### 2.6. Bioinformatics analysis

Similarity searches were performed using the BLASTN algorithm of the NCBI Web BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using the pST1007-1A sequence (that does not hold the -like) as query. Results were graphically depicted by SnapGene (http://www.snapgene.com/) and Adobe Illustrator (https://www.adobe.com/it/).

## 3. Results

### 3.1. Resistance genes analysis

ST1007 is part of a collection of 113 clinical MDR *S*.Typhimurium strains isolated in Italy between 2006 and 2012. ST1007 was first characterised by PFGE (pulsotype STYMXB.0179) and ARGs. The exhibited ApCmSmSuTcTp resistance pattern was encoded by the $bla_{\text{TEM-1}}$, *cmlA1*, (*aadA1*, *aadA2*, *strAB*), (*sul2*, *sul3*), *tet*(B) and *dfrA12* genes, respectively. *sul3* was found as part of a *sul3*-associated class 1 integron (Antunes et al., 2007). ST1007 harboured a conjugative FII plasmid, and mating type experiments highlighted the HGT (horizontal gene transfer) of six different antimicrobial resistance patterns (Table 1). The CmSmSuTp resistance pattern (transconjugant BA1A) was transferred with a frequency of $6 \times 10^{-2}$. The CmSmSuTcTp (transconjugant BA1B), ApCmSmSuTp (transconjugant BA1C) ApSmSu (transconjugant BA1D) patterns were transferred with lower frequencies: $3.7 \times 10^{-6}$, $1.9 \times 10^{-6}$, $7.2 \times 10^{-7}$, respectively. The Tc (Transconjugant BA1E) and ApCmSmSuTcTp (transconjugant BA1F) patterns were rarely transferred, being detected with a frequency $\leq 5 \times 10^{-8}$. Each resistance pattern was linked to the transfer of a FII plasmid named: pST1007-1A (profile CmSmSuTp), pST1007-1B (profile CmSmSuTcTp), pST1007-1C (profile ApCmSmSuTp), pST1007-1D (profile ApSmSu), pST1007-1E (profile Tc) and pST1007-1F (profile ApCmSmSuTcTp). In the transconjugants BA1B, BA1E and BA1F resistance to tetracycline was due to acquisition of *tet*(B) while, in transconjugants BA1C, BA1D and BA1F resistance to ampicillin was due to acquisition of $bla_{\text{TEM-1}}$. Acquisition of $bla_{\text{TEM-1}}$ was associated with that of *strAB* and *sul2*. Transconjugants BA1A-BA1F were also used as donors in conjugation experiments with *E. coli* DH5α Rif as recipient strain. ARGs harboured by plasmids pST1007-1A, pST1007-1B, pST1007-1C, pST1007-1D and pST1007-1E were transferred with an order of magnitude comparable to that of pST1007-1A (Table 1). ARGs carried by pST1007-1F were also transferred with an order of magnitude comparable to that of pST1007-1A. However, some transconjugants did not exhibit (by replica plating) resistance to either ampicillin (≤1%) or tetracycline (≤1%) or both (≤5%).

All plasmids were purified by a protocol developed in this study. Analysis of *EcoRI* restriction patterns showed the presence of 12 common fragments shared by all plasmids (Fig. 1). Thus, based on restriction profiles and data obtained from conjugation frequencies, we supposed that the main plasmid harboured by ST1007 was pST1007-1A. The other identified plasmids were most likely due to

**Table 1**
HGT of ARGs harbouring by pST1007-1A and its derivatives.

| Donor strain | Resistance(s)[a] | Resistance genes[b] | Genomic localization | Transconjugant strains (FII plasmid) | Resistance genes transferred by conjugation | Frequency of conjugation |
|---|---|---|---|---|---|---|
| ST1007 | ApCmSmSuTcTp | *dfrA12-aadA2-cmlA1-aadA1-sul3*[c]; $bla_{TEM1}$-*strAB-sul2*[d]; *tet*(B) [e] | FII plasmid Chromosome | BA1A (pST1007-1A) | *dfrA12-aadA2-cmlA1-aadA1-sul3* | $6 \times 10^{-2}$ |
| | | | | BA1B (pST1007-1B) | *dfrA12-aadA2-cmlA1-aadA1-sul3*; *tet*(B) | $3.7 \times 10^{-6}$ |
| | | | | BA1C (pST1007-1C) | *dfrA12-aadA2-cmlA1-aadA1-sul3*; $bla_{TEM-1}$-*strAB-sul2* | $1.9 \times 10^{-6}$ |
| | | | | BA1D (pST1007-1D) | $bla_{TEM-1}$-*strAB-sul2* | $7.2 \times 10^{-7}$ |
| | | | | BA1E (pST1007-1E) | *tet*(B) | $\leq 5 \times 10^{-8}$ |
| | | | | BA1F (pST1007-1F) | *dfrA12-aadA2-cmlA1-aadA1-sul3*; $bla_{TEM-1}$-*strAB-sul2*; *tet*(B) | $\leq 5 \times 10^{-8}$ |
| BA1A | CmSmSuTp | *dfrA12-aadA2-cmlA1-aadA1-sul3* | FII plasmid | BA1A1 (pST1007-1A) | *dfrA12-aadA2-cmlA1-aadA1-sul3* | $1.6 \times 10^{-2}$ |
| BA1B | CmSmSuTcTp | *dfrA12-aadA2-cmlA1-aadA1-sul3*; *tet*(B) | FII plasmid | BA1B1 (pST1007-1B) | *dfrA12-aadA2-cmlA1-aadA1-sul3*; *tet*(B) | $1.5 \times 10^{-2}$ |
| BA1C | ApCmSmSuTp | *dfrA12-aadA2-cmlA1-aadA1-sul3*; $bla_{TEM1}$-*strAB-sul2* | FII plasmid | BA1C1 (pST1007-1C) | *dfrA12-aadA2-cmlA1-aadA1-sul3*; $bla_{TEM-1}$-*strAB-sul2* | $1.2 \times 10^{-2}$ |
| BA1D | ApSmSu | $bla_{TEM1}$-*strAB-sul2* | FII plasmid | BA1D1 (pST1007-1D) | $bla_{TEM-1}$-*strAB-sul2* | $4.5 \times 10^{-3}$ |
| BA1E | Tc | *tet*(B) | FII plasmid | BA1E1 (pST1007-1E) | *tet*(B) | $3,2 \times 10^{-3}$ |
| BA1F | ApCmSmSuTcTp | *dfrA12-aadA2-cmlA1-aadA1-sul3*; $bla_{TEM1}$-*strAB-sul2*; *tet*(B) | FII plasmid | BA1F1 (p1007-1F)[f] | *dfrA12-aadA2-cmlA1-aadA1-sul3*; $bla_{TEM-1}$-*strAB-sul2*; *tet*(B) | $7.4 \times 10^{-2}$ |

[a] ampicillin (Ap), chloramphenicol (Cm), streptomycin (Sm), sulphamethoxazole (Su), tetracycline (Tc) and trimethoprim (Tp).
[b] resistance genes of different RU are separated by a semicolon.
[c] RU3.
[d] RU1.
[e] RU2.
[f] the resistance genes were not always transferred as a single cluster.

recombination events between pST1007-1A and DNA elements carrying either *tet*(B) or $bla_{TEM-1}$-*strAB-sul2*.

In a group of clonally related ST and STMV strains the $bla_{TEM-1}$-*strAB-sul2* and *tet*(B) genes are organised in two genomic resistance regions here reported as RU1 and RU2, respectively. RU1 (IS*26*-$bla_{TEM-1}$-IS*26*-*strBA-sul2*-IS*26*) is a Tn*6029*E (a variant of Tn*6029*) (Reid et al., 2015); RU2 (IS*26*-*tetR-tet*(B)-IS*1-mer* genes-IS*26*) is composed of regions derived from Tn*10* and Tn*2670*, (Cain and Hall, 2012; Partridge and Hall, 2004) (Fig. 3 and Fig. S1). These strains are widely distributed in Italy among clinical isolates characterised by the ApSmSuTc resistance profile and the pulsotype STYMXB.0079 (Lucarelli et al., 2012). RU1 and RU2 were inserted into the 5′ portion of STM2753 and the 3′ portion of STM2759 chromosomal genes, respectively. The region located between RU1 and RU2 included the 3′ portion of STM2753, the chromosomal genes from STM2754 to STM2758, and the 5′ portion of STM2759, but inversely oriented with respect to the STM LT2 genome (reference STM strain, GenBank accession number NC_003197). We went on to check whether such genetic organization was also shared by ST1007. Specific PCRs, targeting the chromosomal region from STM2752 to STM2759, the $bla_{TEM-1}$, *strAB, sul2*, *tet*(B), and *mer* genes demonstrated that RU1 and RU2 were chromosomally located and organised in a structure similar to that reported by Lucarelli et al. However, the chromosomal region between RU1 and RU2 was not inversely oriented (Fig. 3 and Fig. S1). Moreover, in silico analysis of the ST1007 genome sequence highlighted that the region spanning from STM2752 to STM2759 had different features to those reported by Lucarelli et al.: in our case the integration of RU1 into STM2753 was linked to a deletion of 332 bp of this gene, while that of RU2 into STM2759 was flanked by a duplication of 8 bp with a detectable single mismatch (Fig. 3). By IS*26* recombination RU1 and RU2 excised from the chromosome, thus generating the translocatable units TU1 and TU2 respectively, and it was their insertion into pST1007-1A that accounted for the formation of pST1007-1A derivatives (see below). Excision of RU1 and RU2 was demonstrated by specific PCRs and enzyme restriction of the generated amplicons (Fig. S1).

### 3.2. Plasmid pST1007-1A

The fully assembled sequence of pST1007-1A consisted of 95,629 bp with an average G + C content of 52.4% (Fig. 2). BLAST search was performed using the BLASTN algorithm (http://ncbi.nlm.nih.gov/Blast.cgi), against the non-redundant nucleotide database (nr/nt) and the whole-genome shotgun contigs (wgs) database, limited by Bacteria (taxid:2). No plasmid identical to pST1007-1A was found.

pST1007-1A contained one region harbouring the conjugative plasmid backbone, two DNA fragments (named B and C) acquired from I1 plasmids and a Tn*21*-derived transposon inserted within fragment C (Fig. 2 and Fig. 3). The sequences of well-characterised plasmids R100 (IncFII), F (IncFIA, IncFIB, IncFII) and R64 (IncI1) were used to guide annotation of the backbone of pST1007-1A.

#### 3.2.1. Plasmid replication region

The replication region of pST1007-1A included *repA2* (negative regulation of *repA1* expression), *repA6* (positive regulation of *repA1* expression), *repA1* (promoting replication initiation), the origin of vegetative replication *oriV* and the antisense Inc-RNA encoded by the *inc* gene. The replication region of pST1007-1A showed similar organization to R100 with an average nucleotide identity of 90% (Kato and Mizobuchi, 1994; Womble et al., 1985).

#### 3.2.2. tra operon

The presence of conserved motifs within the 300 N-terminal residues of TraI and the fertility inhibition system *traJ/finO* leds us to assign pST1007-1A to the IncF/$MOB_{F12}$ group A (Fernandez-Lopez et al., 2016; Garcillan-Barcia et al., 2009).

The genetic organization of the *tra* operon of pST1007-1A was

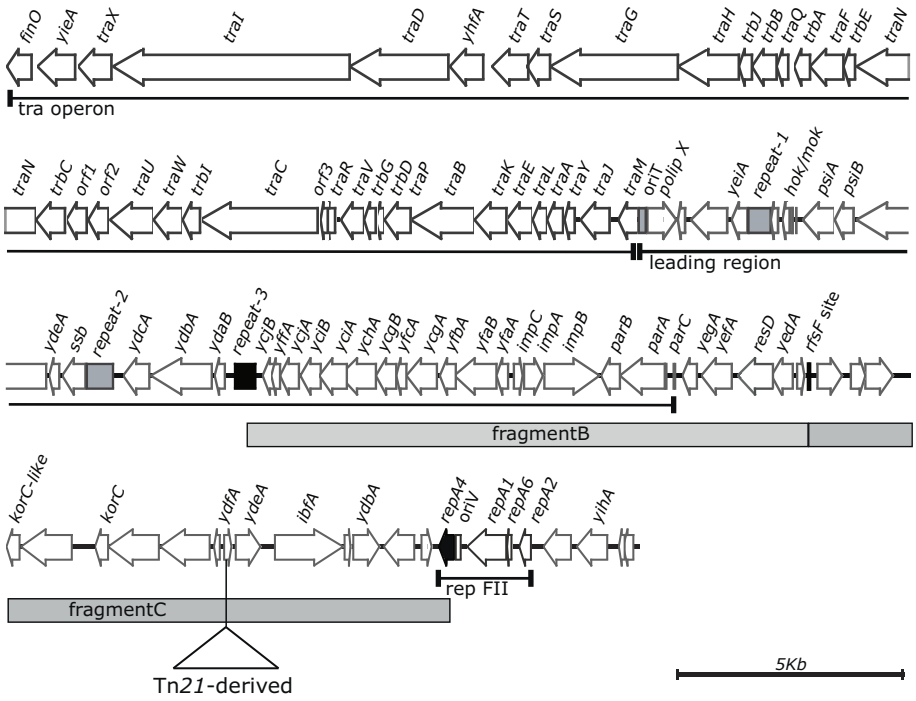


**Fig. 2.** Linear map of pST1007-1A.
Genes and open reading frames are represented by arrow boxes pointing in the direction of transcription. Features names are labelled above arrow boxes. The locations of the *tra* operon and the leading and replication regions are marked by thin black lines below the arrow boxes. The Tn*21*-derived element (Fig. 3) inserted into *ydfA* is indicated with a triangle. The length of fragment B and fragment C is shown as dark grey and pale grey rectangles, respectively. *Repeat-3*, *rfsR* and *repA4*, corresponding to the hotspots, are in black.

similar to that of R100 and F (94 and 97% nucleotide identity, respectively). The most notable differences with R100 were: the presence (as in F) of distinct *trbG* and *trbD* alleles (in R100 *trbG* is absent); moreover, TrbD of pST1007-1A (65aa) was shorter than TrbD of R100 (122aa). Additionally, in pST1007-1A *trbF* was not present and three hypothetical genes named *hp1-hp2* and *hp3* were found instead of *ygeA* and *yfiC-yfiB-yfiA-yfhA*, respectively. With respect to the *tra* operon of F, that of pST1007-1A lacked *artA* and *trbH*. Of particular interest was the 99% amino acid identity with TraN (with TraN of R100 the identity was only 71%). TraN recognises lipopolysaccharide in the recipient cell. In matings mediated by F, but not for those mediated by R100, TraN is also supposed to interact with OmpA thereby increasing the efficiency of conjugation (Klimke and Frost, 1998; Klimke et al., 2005). The specificity of TraN for OmpA maps on the central region (aa 164–333) and this differs significantly from that of R100. The specific central region of TraN of pST1007-1A is identical to that encoded by F. It is also worth mentioning that the gene products of two regulatory genes (*traY* and *traJ*) exhibited the lowest % of identity (lower than 61%) with the respective gene products of F and R100.

### 3.2.3. Leading region and DNA fragment from I1 plasmid

The leading region (the first DNA transferred to the recipient cell) is

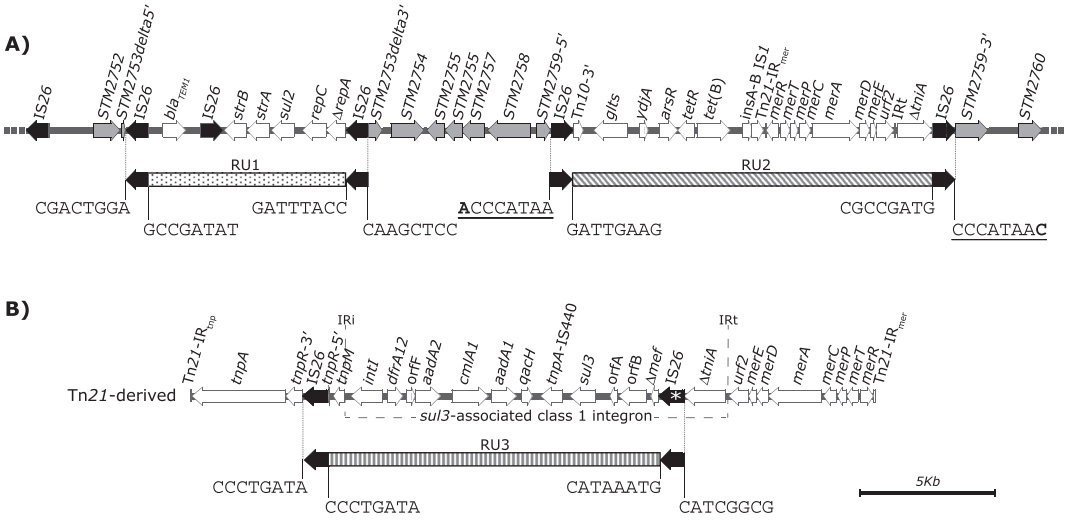


**Fig. 3.** Schematic representation of the resistance units RU1, RU2 and RU3 flanked by IS*26*.
Genes and genetic elements are represented by arrow boxes pointing in the direction of transcription. IS*26* elements are in black. The 8-bp flanking sequences of IS*26* elements are shown. (A) Location of RU1 and RU2 in the chromosome of ST1007. Chromosomal genes are indicated in grey boxes. The resistance unit RU1 is reported as a dotted rectangle, RU2 is reported as a downward diagonal rectangle. The 8-bp target site duplication caused by insertion of RU2 is shown as underlined sequences and the mismatch now detectable (most likely following an event of single bp deletion) is highlighted in bold. (B) Location of RU3 in the Tn*21*-derived element. The resistance unit RU3 is reported as a narrow vertical rectangle. The IS*26* element highlighted with an asterisk (*) marks where RU1 and RU2 have been found inserted. Dashed lines define the *sul3*-associated class 1 integron. $IR_i$ and $IR_t$ represent the 25-bp imperfect inverted repeats of In2. orfA-orfB correspond to the orf1 as reported by Curiao et al., 2011.

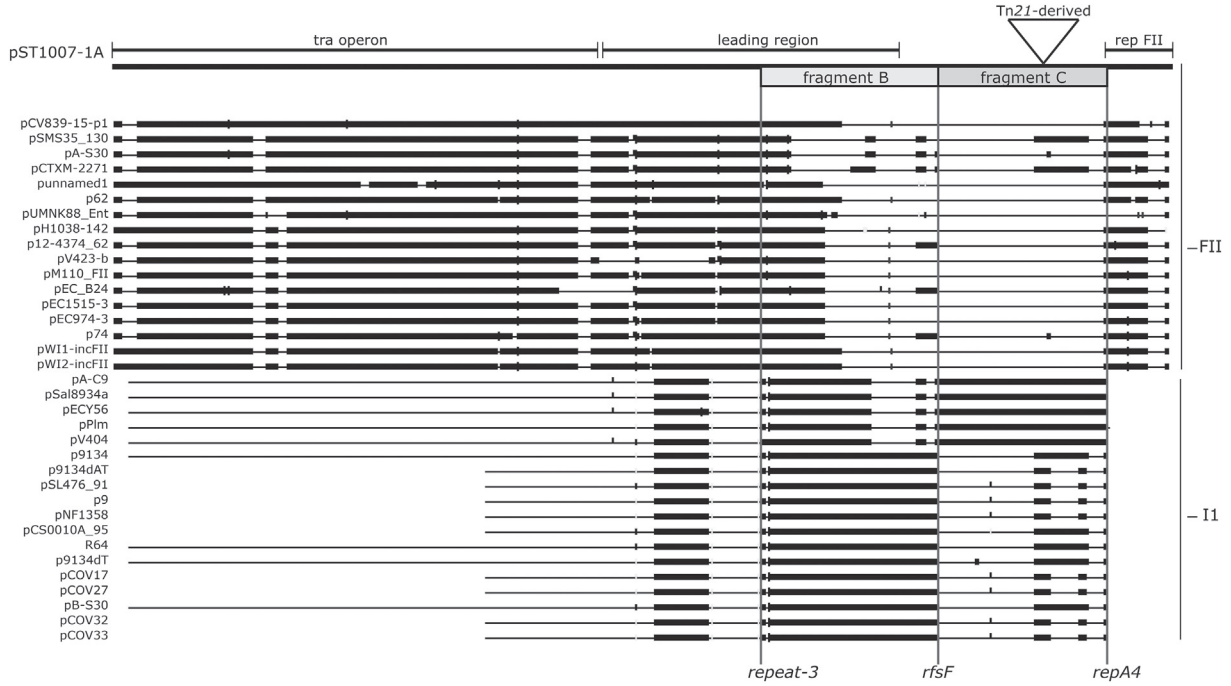


**Fig. 4.** Hits of BLAST search using pST1007-1A sequence as query.
pST1007-1A is reported on the top as a thick black line. Insertion of the Tn*21*-derived element is indicated with a triangle. The location of the *tra* operon, leading and replication regions are marked by thin black lines above pST1007-1A. The lengths of fragment B and fragment C are shown (below pST1007-1A) as grey and light grey rectangles, respectively. FII and I1 plasmids are grouped by vertical lines on the right side. The three hot spots (*repeat-3*, *rfsF*, *repA4*) are reported on the bottom and their position in pST1007-1A is linked by vertical lines.

assumed to start at the origin of transfer *oriT* and, in F, to end at the *EcoRI* site downstream of the *sopABC* partitioning locus (Cox and Schildbach, 2017). The leading region of pST1007-1A (Fig. 2) (assumed to span from *oriT* to the *parABC*) included two domains: one (fragment A, spanning from *oriT* to *yfbA*) was homologous (nucleotide identity ranging from 89 to 94%) with the corresponding leading region of R100; the other one, named fragment B that extends beyond the *parABC*, spanned from *repeat*-3 (one of the three repeats present in the leading region of FII plasmids) to *rfsF*. Homology (nucleotide identity ranging from 97 to 99%) was detected between fragment B and I1 plasmids (GenBank accession numbers CP010831; KF787110; LN890525; CP019215; CP010317; CP012627; CP024471; KM409652) isolated from *E. coli*, *Salmonella* strains serovar Weltevreden, and *Shigella* species *flexneri* and *sonnei* (Fig. 4; Table S3). Fragment B overlapped A over a region of 4532 bp.

Genes encoding the toxin-antitoxin system *hok-sok* were present in fragment A. This system is conserved between R100 and F, the toxin and antitoxin proteins showing 90% and 100% identity, respectively. The hok-sok system of pST1007-1A showed lower amino acid identity with the *hok-sok* of R100 (84% and 61%) and *flmA-flmC* of F (84% and 54%). As in R100, fragment A also contained the three repeat regions (here named *repeat*-1 to *repeat*-3) homologous to the two *Frpo* sequences found in the leading region of plasmid F. The F*rpo* sequences are presumed to act as a promoter in single-stranded DNA (Bates et al., 1999; Masai and Arai, 1997). The *oriT* of pST1007-1A was identical to that reported for plasmid F (Thompson et al., 1984).

Fragment B contained the *impCAB* operon which is homologous to the *umuDC* operon encoding an error-prone DNA repair system and the *rfsF*-ResD system involved in the resolution of cointegrated dimers resulting from genetic recombination (Dowden et al., 1984; Sampei et al., 2010). The leading region of pST1007-1A harbours *parABC*, a type Ib segregation system composed of a *cis*-acting centromere-like site (*parC*) and two genes encoding a motor protein (ParA) and a DNA-binding adaptor protein (ParB) (Pinto et al., 2012).

The leading region of I1 plasmids contains repeats that are partially homologous to repeats present in FII plasmids. In particular, repeat 4 of I1 plasmids and *repeat*-3 of pST1007-1A had a nearly identical 61 bp fragment (1 or 2 nucleotide of difference,) that corresponded to the left extremity of fragment B.

pST1007-1A contained an additional segment (termed C; Fig. 2) 99% identical to DNA sequences harboured by I1 plasmids (GenBank accession numbers CP010130, JF274993, KU043116, JQ901381, LM651376) (Fig. 4; Table S3). This fragment spanned from *rfsF* to *repA4* and contained a Tn*21*-derived element as well as hypothetical genes. It should be highlighted that the I1 plasmids harbouring a complete segment B did not contain a complete segment C and vice versa.

*repA4* is a DNA region of 297 bp located immediately downstream of *oriV* and is conserved in FII plasmids (Jiang et al., 1993). *repA4* contains inverted repeat sequences that are homologous to the *terC* inverted repeats located in the replication terminus of *E. coli* chromosome. The inverted repeats of *repA4* prevent replication from proceeding further and play an important role in plasmid stability. *repA4* has also been proposed as the site of recombination between FII and I1 plasmids (Fiett et al., 2014). Interestingly, while the *repA4* of pST1007-1A had an average and uniformly distributed nucleotide identity of 89% with the *repA4* of R100, a higher nucleotide identity (97%) was found between the 182 bp 3'end of *repA4* and the region (next to *repZ*) involved in replication termination of some I1 plasmids (GenBank accession numbers CP010130, JF274993, KU043116, JQ901381, LM651376).

The *rfsF*-ResD system of I1 plasmids plays a role both in the resolution of cointegrated dimers and in generating diversity in R64-related plasmids via recombination (Sampei et al., 2010). The *rfsF*-ResD system is also conserved between FII and I1 plasmids; thus, it may conceivably play a similar role in FII plasmids. However, if this were the case, pST1007-1A would have acquired fragment C through the two recombination sites, *repA4* and *rfsF*, while acquisition of fragment B would not only have involved *rfsF*, but also recombination between identical fragments (61 bp) shared by the *repeat*-3 of pST1007-1A and repeats within the leading regions of I1 plasmids.

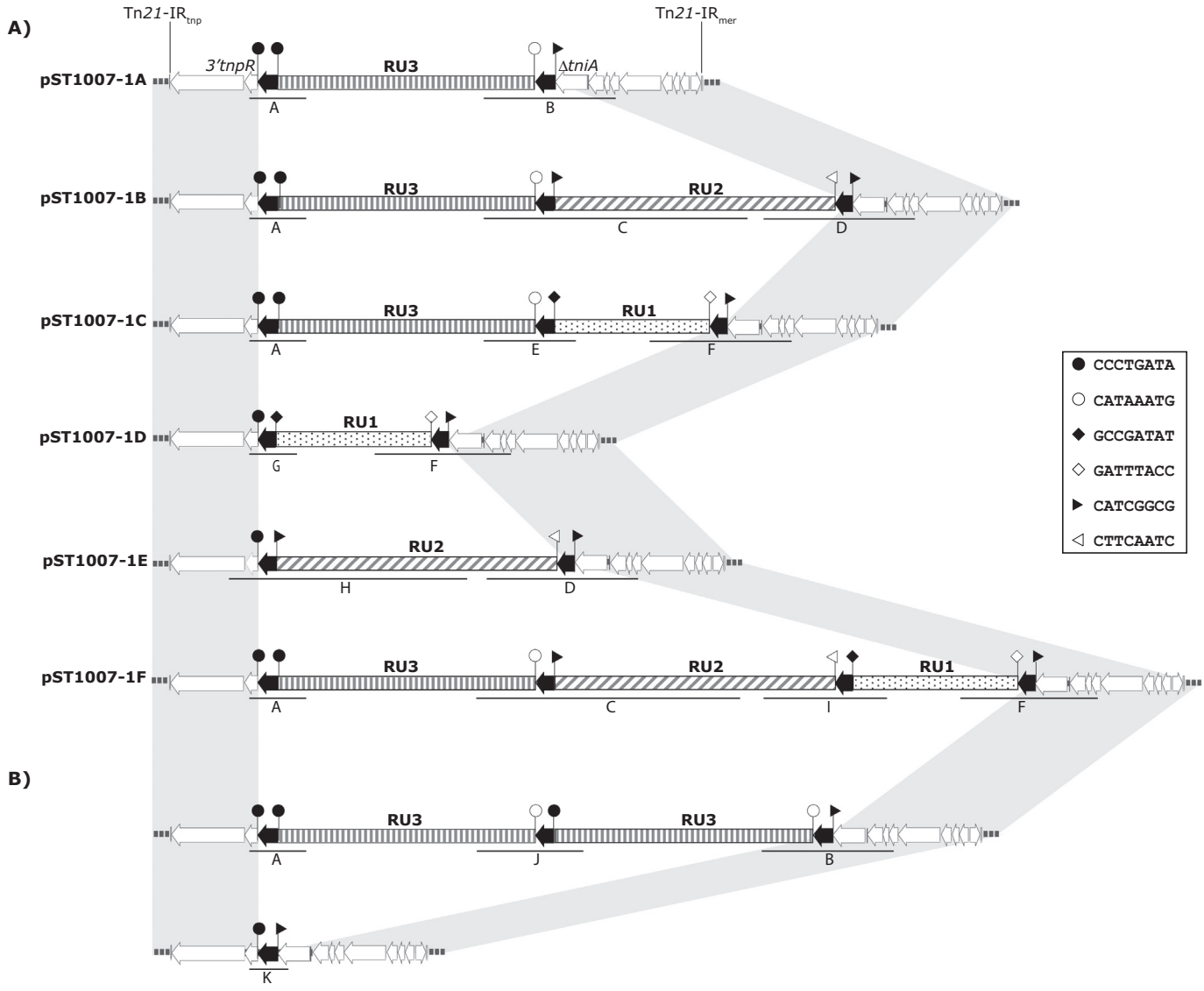


**Fig. 5.** Schematic representation of pST1007-1A and its derivatives.
(A) pST1007-1A and its derivatives originated by insertion of RU1 and/or RU2. (B) Plasmids derived from nonreciprocal recombination followed by unequal cross-over of sister plasmids. Genes and genetic elements are represented by arrow boxes pointing in the direction of transcription. IS*26* elements are in black. Flanking 8-bp sequences of IS*26* are indicated by flags (legend on the right side). The resistance units RU1, RU2 and RU3 are reported as dotted, upward diagonal and narrow vertical rectangles, respectively. The upward diagonal representation (in Fig. 3 represented with downward diagonal) highlights the inverted orientation of RU2 required by the IS*26* mediated recombination. The same TSD (triangle black flags) showed twice in pST1007-1B, −1E and —1F are due to identical regions (from Δ*tniA* to Tn*21* $IR_{mer}$, see Fig.3) present in RU2 (Tn*2670*) and in the Tn*21*-derived transposon of pST1007-1A. Thin black lines labelled with letters from A to K show the extent of PCR products (Table S2).

### *3.3. Tn21-derived transposon and pST1007-1A derivatives*

The Tn*21*-derived element located within fragment C was inserted into the *ydfA* gene (Fig. 2) generating a 5-bp direct duplication of target site. The Tn*21*-derived element includes a *sul3*-associated class 1 integron containing the cassette array *dfrA12*-orfF-*aadA2*-*cmlA1*-*aadA1*-*qacH* similar to that identified in pCERC3, a ColV virulence-multidrug resistance plasmid (Moran et al., 2016). The *sul3*-associated class 1 integron was flanked by imperfect inverted repeats of 25-bp ($IR_i$ and $IR_t$), bounded by a 5-bp direct duplication of the target site (TSD) and inserted in the same position as In2 in Tn*21* (Liebert et al., 1999). Two copies of IS*26* were also detected: one present within the Δ*tniA* of the *sul3*-associated class 1 integron; the second within *res* of the Tn*21*-module (Fig. 3). This latter is likely to represent a recent insertion since it is flanked by an identical TSD. The two IS*26* elements generated a potential composite transposon (here reported as RU3) harbouring the *dfrA12*, *aadA2*, *cmlA1*, *aadA1* and *sul3* ARGs. Restriction analysis of pST1007-1A (Fig. 1) revealed the presence of a mixed population where some plasmids carried two copies of RU3. Duplication of RU3 was also confirmed by specific PCR reactions (Fig. 5). The presence of a mixed plasmid population might be explained by nonreciprocal recombination followed by unequal cross-over of the transposons carried by sister plasmids. This would lead to some plasmids containing tandem repeats of the composite transposon and other plasmids retaining just a single copy of IS*26* (He et al., 2015). The presence of plasmids harbouring a single copy of IS*26* was demonstrated by specific PCR reactions followed by restriction analysis of the PCR products (Fig. 5).

The IS*26* inserted within the *tniA* of the *sul3*-associated class 1 integron was also the recombination target for TU1 and TU2 (Fig. 3) that generated the plasmids pST1007-1B and pST1007-1C, respectively. In pST1007-1B, RU2 is inverted orientated since the conservative mechanism of integration requires the same orientation of IS*26*.

Subsequential insertions of both accounted for pST1007-1F. The variants pST1007-1D and pST100–1E may have been generated by respective insertion of TU1 and TU2 into a plasmid harbouring a single copy of IS*26*. Alternatively, pST1007-1D and pST100–1E may have resulted from the deletion of RU3 from pST1007-1C and pST1007-1B, respectively (Fig. 5). The fact we only found TU1 and TU2 in the IS*26* of *tniA* of the *sul3*-associated class 1 integron does not exclude the possibility that the IS*26* in *res* of the Tn*21*-module may also be a target for TU1 and TU2 insertion. Integration of TU1 and TU2 into pST1007-1A was consistent with the model proposed by Harmer et al., explaining how DNA regions (e.g. carrying ARGs) can translocate as a circular mobile unit composed of a single copy of IS*26* and the DNA fragment carrying ARGs (Harmer et al., 2014). The mobile unit can then target an existing copy of IS*26* and incorporate immediately adjacent to it.

Finally, it is worth mentioning that BLAST searches using DNA fragment C, harbouring the Tn*21*-derived element, revealed only one single match with 99% nucleotide identity (100% coverage) with an I1 plasmid isolated from the *E. coli* strain KV7 (GenBank accession number LT795504). However, the Tn*21*-derived transposon was inserted into a different position (*yafB*) and inversely oriented with respect to that harboured by pST1007-1A indicating that this region has acquired the transposon on at least two occasions.

## 4. Discussion

Plasmids are considered a major driving force in prokaryotic evolution. They considerably contribute to the intra- and inter-species HGT that provides new genetic information to host cells. Some of this information (e.g. genes encoding for antimicrobial resistance) may confer benefits, particularly where anthropogenic activities exert a strong selective pressure. The healthcare environment is a clear example in this respect where the extensive and sometime excessive use of antibiotics has been accounting for the selection and spread of both MDR bacteria and genetic elements harbouring ARGs (e.g. plasmids, IS elements, transposons, integrons, etc.). Among IS-elements, IS*26* has been reported to play a critical role in the dissemination of ARGs and to mediate genomic rearrangements and genetic exchange between chromosomes and plasmids (Garcia et al., 2016; Harmer et al., 2014; Lucarelli et al., 2012).

*S*. Typhimurium ST1007 is part of an epidemiologically relevant clonal lineage that has frequently been detected among MDR clinical ST and STMV isolates widely distributed in Italy (Lucarelli et al., 2012). This clonal lineage is characterised by the pulsotype STYMXB.0079 and the ApSmSuTc resistance pattern encoded by the chromosome-integrated resistance units RU1 ($bla_{TEM-1}$-*sul2-strAB*) and RU2 (*tet*(B)). Apart from RU1 and RU2 *S*. Typhimurium ST1007 harboured the conjugative FII plasmid pST1007-1A that conferred the supplementary resistances to chloramphenicol and trimethoprim encoded by *cmlA1* and *dfrA12*, respectively. *cmlA1* and *dfrA12* are part a composite transposon, named RU3 embedded into a Tn*21*-derived transposon.

In this study we demonstrated the intracellular transfer of both $bla_{TEM-1}$-*sul2-strAB* and *tet*(B), organised respectively as TU1 and TU2, from the chromosome into pST1007-1A. The transfer was IS*26* mediated and generated a family of plasmids encoding distinctive antimicrobial resistance patterns. Going on from this, ST1007 acted as a donor for the HGT of pST1007-1A (*dfrA12-aadA2-cmlA1-aadA1-sul3*) and its derivatives: pST1007-1B (*dfrA12-aadA2-cmlA1-aadA1-sul3- tet*(B)), pST1007-1C (*dfrA12-aadA2-cmlA1-aadA1-sul3-*$bla_{TEM-1}$*-sul2-strAB*), pST1007-1D ($bla_{TEM-1}$*-sul2-strAB*), pST1007-1E (*tet*(B)) and pST1007-1F (*dfrA12-aadA2-cmlA1-aadA1-sul3- tet*(B) -$bla_{TEM-1}$*-sul2-strAB*).

Plasmids can generally be classified, with respect to their host range, as narrow and broad (Suzuki et al., 2010). FII and I1 plasmids are narrow host range (NHR) and are only found in bacteria of the *Enterobacteriaceae* family (Partridge et al., 2018). Nevertheless, FII and I1 plasmids are widely isolated from human, animal and environmental sources (Rozwandowicz et al., 2018). In the present study we supply bioinformatic data on possible recombination events between the pST1007-1A ancestor and I1 plasmids. We identify three hot spots in pST1007-1A (*rfsF*, *repA4* and *repeat*-3) involved in the acquisition of DNA fragments from I1 plasmids. *rfsF* and *repA4*, which accounted for the acquisition of fragment C, have already been proposed as potential sites for genetic recombination and our data support this. The third hot spot (*repeat*-3), to the best of our knowledge, has never been reported as such: together with *rfsF* it accounted for the acquisition of fragment B.

The identification of plasmids with mosaic structures derived from the combinations of regions from different Inc. groups is a relevant topic with potential implications for plasmid host range, their persistence, and for host evolution. In this respect, the hot spots highlighted in the present study contribute to a better knowledge of the possible origin of conglomerate plasmids.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plasmid.2018.10.001.

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