RESEARCH LETTER

Nucleotide sequence and functional analysis of the tet(M)-carrying conjugative transposon Tn5251 of Streptococcus pneumoniae

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Streptococcus pneumoniae; Tn5251; conjugative transposon; ICE; MGE.

Abstract
The Tn916-like genetic element Tn5251 is part of the composite conjugative transposon (CTn) Tn5253 of Streptococcus pneumoniae, a 64.5-kb chromosomal element originally called Ω(cat-tet) BM6001. DNA sequence analysis showed that Tn5251 is 18,033-bp long and contains 22 ORFs, 20 of which have the same direction of transcription. Annotation was possible for 11 out of 22 ORFs, including the tet(M) tetracycline resistance gene and int and xis involved in the integration/excision process. Autonomous copies of Tn5251 were generated during matings of Tn5253-containing donors with S. pneumoniae and Enterococcus faecalis. Tn5251 was shown to integrate at different sites in the bacterial chromosome. It behaves as a fully functional CTn capable of independent conjugal transfer to a variety of bacterial species including S. pneumoniae, Streptococcus gordonii, Strep toneoccus pyogenes, Streptococcus agalactiae, E. faecalis and Bacillus subtilis. The excision of Tn5251 produces a circular intermediate and a deletion in Tn5253 at a level of 1.2 copies per 105 chromosomes.

Introduction
A large proportion of clinical isolates of Streptococcus pneumoniae (pneumococcus) contain the tet(M) gene conferring resistance to tetracycline antibiotics by ribosomal protection (Pożzi et al., 1986). The tet(M) gene is usually carried by genetic elements of the Tn916–Tn1545 family of conjugative transposons (CTns) (Clewell et al., 1995; Rice, 1998), and eight out of the 36 pneumococcal genomes available in public databases contain this element.

CTns, also called integrative and conjugative elements (ICEs), are mobile genetic elements (MGEs) that can integrate into the bacterial genome, excise from it and form a covalently closed circular intermediate (CI) that can integrate into a new position of the genome (intracellular transposition) and/or can be transferred by conjugation from a donor to a recipient cell, where it integrates into the genome (intercellular transposition) (Churchward, 2002; Mullany et al., 2002). CTns enable horizontal transfer of genes among distantly related bacteria playing an important role in the molecular evolution of many bacterial genomes (Frost et al., 2005). CTns contribute to the dissemination of antibiotic resistance determinants among pathogenic bacteria and their association is responsible for the spread of multiple antibiotic resistance determinants (Clewell et al., 1995; Rice, 2002; Roberts & Mullany, 2009). Among the best-studied CTns are (1) Tn916, originally found in the Enterococcus faecalis DS16 clinical strain, 18,032 bp in size and carrying the tet(M) tetracycline resistance gene (Franke & Clewell, 1981; Flannagan et al., 1994), and (2) Tn1545, found in the S. pneumoniae BM4200 clinical isolate, about 25.3 kb in length (GenBank X04388, X61025, X05577, X52632, AM903082, AM889142), related to Tn916, but carrying, in addition to tet(M), the aphA-3 and ermAM genes conferring resistance to kanamycin and erythromycin (Courvalin & Carlier, 1986; Cochetti et al., 2008).

Tn916-like CTns are found integrated at different sites in the pneumococcal chromosome, and in many cases, they do not exist as individual CTns, but are part of other genetic elements (Fig. 1). The Tn916-like CTn Tn5251 was shown to be part of the composite pneumococcal CTn Tn5253 (Shoemaker et al., 1979; Ayoubi et al., 1991; Provvedi et al., 1996), a chromosomal genetic element originally called Ω(cat-tet) BM6001 (Shoemaker et al., 1979). Tn5253-related elements have been reported to be common in antibiotic-resistant pandemic S. pneumoniae clones (Henderson-Begg et al., 2008). In our previous paper, we demonstrated that Tn5251 is able to excise from Tn5253 and form CIs...
Tn5251 of Streptococcus pneumoniae

Fig. 1. Integration sites of Tn5251 and Tn5251-like elements in the Streptococcus pneumoniae chromosome. Bacterial chromosome is represented by a circle, and the origin of replication (oriC) is reported; nucleotides indicated on the map refer to the R6 strain (Hoskins et al., 2001). The solid triangles (\(\bigtriangledown\)) on the circle indicate the insertion sites of Tn5251 in transconjugants obtained in this work. Tn5251 insertions were mapped to spr0357 at position 357 477, to three intergenic regions at positions 96 694, 122 345 and at position 1 175 526 in two transconjugants obtained from different matings. Tn5251-like CTn insertion sites (empty triangles, \(\bigtriangleup\)) obtained from published nucleotide sequences map to four sites in the chromosome. Composite CTNs (empty squares, \(\Box\)) containing a copy of a Tn5251-like CT integrate only at two sites that represent two hotspots for insertion. Elements are designed with their name or, when not annotated, with the name of the strain harbouring them.

Here, we report the complete annotated sequence of Tn5251, describe how autonomous copies of this element are generated upon conjugal transfer and show that Tn5251 is in fact a fully functional CTn capable of independent conjugal transfer to a variety of bacterial species.

Materials and methods

Bacterial strains, media and growth condition

The bacterial strains used in this work and their relevant properties are reported in Table 1. Streptococci and enterococci were routinely grown in tryptic soy broth or tryptic soy agar (Difco) supplemented with 3% horse blood and, where appropriate, with antibiotics (Iannelli & Pozzi, 2007). Bacillus subtilis was grown in Luria–Bertani broth (LB) or LB agar.

DNA purification

Bacterial cells were harvested by centrifugation at the end of exponential phase growth. Pneumococcal cells were lysed for 15 min at 37 °C in sodium dodecyl sulphate (SDS) 0.008% and sodium deoxycholate (DOC) 0.1% (lysis solution), whereas enterococcal cells were lysed according to the protocol already described (Manganelli et al., 1995). DNA was purified using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer’s instructions. Purified DNA was quantified by both electrophoretic and spectrophotometric analysis.

PCR and sequencing

Long PCRs were carried out using the Expand High Fidelity PCR System (Roche) essentially according to the protocol already described (Iannelli et al., 1998). Briefly, the 25 µL reaction mixture was in 1 × Expand High Fidelity buffer and contained (1) 1.5 mM MgCl₂, (2) 100 µM dNTPs, (3) 10 pmol of each primer, (4) 0.2 U of Expand High Fidelity Enzyme Mix and (5) 1 µL of liquid bacterial culture (Iannelli et al., 1998). Amplification was performed using the following cyclic thermal profile: 1 cycle at 92 °C for 2 min, then 30 cycles at 50 °C for 10 s, 68 °C for 10 min, 92 °C for 10 s, and 1 cycle at 50 °C for 1 min and 68 °C for 20 min. The direct automated sequencing of the PCR fragments was performed using a primer walking strategy as described (Iannelli et al., 1998). Two primer pairs IF487/IF393 and IF394/IF488 were used to amplify two fragments 5518 and 13 743 bp in length, respectively. Primers are directed to the already sequenced tet(M) and Tn5251 flanking regions (Provvedi et al., 1996): IF487 (5'-TTC GCT GAA GAC CTT TAT TCG-3') is complementary to nucleotides 358 through 378 of the Tn5251 left junction (GenBank X90940); IF488 (5'-TCC TGA TTA CAG GTA ATG TAT CA-3') corresponds to nucleotides 52 through 71 of the Tn5251 right junction (GenBank X90941); and IF393 (5'-TTC TGG CGA AAT ATG TGT CA-3') corresponds to nucleotides 2541 through 2560 and IF394 (5'-GCT GAA GTA TTA GCC ATC AAT CA-3') is complementary to nucleotides 3602 through 3621 of Tn5251 tet(M) (GenBank X90939). To confirm the sequence on the other strand, fragments about 1000 bp in size were produced by PCR and used as sequencing starting templates.

Quantitative nested PCR

Quantitative nested PCR was performed essentially as reported previously (Manganelli et al., 1995). The 25 µL reaction mixture was in 1 × DreamTaq buffer and contained (1) 2 mM MgCl₂, (2) 75 µM dNTPs and (3) 0.4 U of DreamTaq DNA Polymerase (Fermentas). DNA was denatured at 92 °C for 2 min, and then the cyclic thermal profile was as follows: annealing at 50 °C for 10 s, extension at 72 °C for 30 s and denaturation at 92 °C for 10 s, followed by a final step at 50 °C for 1 min and 72 °C for 5 min. In the first 25 cycles of PCR, 5 pmol of each outer primer was used with serial dilutions of the chromosomal DNA as the

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starting templates. The second 30 cycles of PCR were performed with 10 pmol of each inner primer and 1 μL of the first PCR product as a template. The primers used to produce the 357-bp outer fragment were IF485 (5′-GGA TAA ATC GTC GTA-3′) and IF486 (5′-AGA CCA TTC-3′), whereas the 141-bp inner fragment was obtained with IF487 and IF488.

**Inverse PCR**

In a final volume of 50 μL, 1 μg of chromosomal DNA was incubated with 10 μL of Sau3A (Roche) at 37 °C for 2 h. One microlitre of digested DNA (20 ng) was circularized in a 20-μL reaction mix containing 10 μL of T4 DNA Ligase (Roche) at 16 °C for 2.5 h. Ligated DNA was extracted with phenol and phenol–chloroform, precipitated in ethanol and resuspended in 15 μL of water. Five microlitres of purified ligated DNA were used as a template in PCR experiments carried out with the divergent primers IF505 (5′-CTG GTA TCT TCC TAG ATG-3′) and IF452 (5′-ACT CAT TCT AAT AGC CCA TTC-3′) or with IF433 (5′-GGT GGA ACT TAT CAA TCC CAT-3′) and IF506 (5′-GGA TAA ATC GTC GTA TCA AAG-3′).

**DNA sequence analysis**

DNA sequence analysis including coding sequence identification was carried out using the software ARTEMIS ver. 11 available for download at http://www.sanger.ac.uk/Software/Artemis/website. Manual gene annotation was carried out by conducting BLAST homology searches of the databases available at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/sites/sgquery) and at the S. pneumoniae Sybil website (http://strept pneumo-sybil.igs.umaryland.edu/). Protein domains were identified by searching the protein family database Pfam available at the Wellcome Trust Sanger Institute (http://pfam.sanger.ac.uk). Multiple sequence alignments were performed using the CLUSTALW2 tool at the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/clustalw2/).

**Mating experiments**

Plate mating experiments were performed essentially as already described (Iannelli & Pozzi, 2007). Donor and recipient cells were grown separately in TSB in the presence of appropriate antibiotics at 37 °C, until the end of the exponential phase (OD900 nm = 0.5). Cells were mixed at a 1 : 10 ratio, harvested by centrifugation for 15 min at 3000 g, resuspended in 0.1 mL of TSB and plated on TSA enriched with 5% horse blood. Following 4 h of incubation in 5% CO2 at 37 °C, cells were harvested by scraping the plates with a sterile plain swab and resuspended in 1 mL of TSB containing 10% glycerol. Selection of transconjugants was carried out with the multilayer plating. Briefly, 2 mL of TSB/10% horse blood containing the appropriately diluted mating reactions were combined with 6 mL of melted TSA and

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**Table 1. Bacterial strains and relevant properties**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant properties*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>D39</td>
<td><em>S. pneumoniae</em> type 2 Avery’s strain</td>
<td>Pearce et al. (2002), Lanie et al. (2007)</td>
</tr>
<tr>
<td>TiGR4</td>
<td><em>S. pneumoniae</em> type 4</td>
<td>Tettelin et al. (2001)</td>
</tr>
<tr>
<td>Rx1</td>
<td>Unencapsulated derivative of type 2 D39 strain</td>
<td>Shoemaker &amp; Guild (1974)</td>
</tr>
<tr>
<td>DP1002</td>
<td>Rx1 but nov-1; Nov&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Guild &amp; Shoemaker (1976), Munoz et al. (1995)</td>
</tr>
<tr>
<td>DP1004</td>
<td>Rx1 but str-41; Sm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Guild &amp; Shoemaker (1976), Salles et al. (1992)</td>
</tr>
<tr>
<td>DP1322</td>
<td>Rx1 carrying Tn5253; Cm&lt;sup&gt;R&lt;/sup&gt;, Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Smith et al. (1991)</td>
</tr>
<tr>
<td>F5</td>
<td>Rx1, but ΔcomC; Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Iannelli et al. (2005)</td>
</tr>
<tr>
<td>FP10</td>
<td>FP5, but str-41 (by transformation with DP1004 chromosomal DNA); Cm&lt;sup&gt;R&lt;/sup&gt;, Sm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Iannelli &amp; Pozzi (2007)</td>
</tr>
<tr>
<td>FP11</td>
<td>FP5, but nov-1 (by transformation with DP1002 chromosomal DNA); Cm&lt;sup&gt;R&lt;/sup&gt;, Nov&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Iannelli &amp; Pozzi (2007)</td>
</tr>
<tr>
<td>FP22</td>
<td>Unencapsulated derivative of D39; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Pearce et al. (2002)</td>
</tr>
<tr>
<td>FP23</td>
<td>Unencapsulated derivative of TiGR4; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Pearce et al. (2002), Oggoni et al. (2003)</td>
</tr>
<tr>
<td>FP47</td>
<td>TiGR4, but nov-1 (by transformation with DP1002 chromosomal DNA); Nov&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>FP22</td>
<td>FP10 carrying Tn5253 (by conjugation with DP1322); Cm&lt;sup&gt;R&lt;/sup&gt;, Sm&lt;sup&gt;R&lt;/sup&gt;, Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Pozzi et al. (1988)</td>
</tr>
<tr>
<td>GP204</td>
<td><em>S. gordonii</em> Challis V288, but str-204; Sm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Ferretti et al. (2001)</td>
</tr>
<tr>
<td>SF370</td>
<td><em>S. pyogenes</em> serotype M1</td>
<td>Tettelin et al. (2005)</td>
</tr>
<tr>
<td>H36B</td>
<td><em>S. agalactiae</em> serotype Ib</td>
<td>Bourgogne et al. (2008)</td>
</tr>
<tr>
<td>OG1RF</td>
<td><em>E. faecalis</em> Fus&lt;sup&gt;R&lt;/sup&gt;, Rif&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Dunny et al. (1979)</td>
</tr>
<tr>
<td>OG1SS</td>
<td><em>E. faecalis</em> Spe&lt;sup&gt;R&lt;/sup&gt;, Sm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Jacob &amp; Hobbs (1974)</td>
</tr>
<tr>
<td>JH2-2</td>
<td><em>E. faecalis</em> Fus&lt;sup&gt;R&lt;/sup&gt;, Rif&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Spizizen (1958)</td>
</tr>
<tr>
<td>168</td>
<td><em>B. subtilis</em></td>
<td><a href="mailto:enquiries@igs.umaryland.edu">enquiries@igs.umaryland.edu</a> (2012)</td>
</tr>
</tbody>
</table>

*Cm, chloramphenicol; Fus, fusidic acid; Km, kanamycin; Nov, novobiocin; Rif, rifampicin; Spe, spectinomycin; Sm, streptomycin; Tc, tetracycline.*
poured into a Petri dish containing a base layer of TSA. After 90 min of incubation at 37 °C for phenotypic expression, an 8 mL TSA layer containing the appropriate antibiotics, for the resistance marker of the donor genetic element and for the chromosomal resistance marker of recipient strain (where available), was added. The antibiotic concentrations were as follows: chloramphenicol 5 μg mL⁻¹, fusidic acid 25 μg mL⁻¹, novobiocin 10 μg mL⁻¹, rifampicin 25 μg mL⁻¹, spectinomycin 400 μg mL⁻¹, streptomycin 1000 μg mL⁻¹ and tetracycline 5 μg mL⁻¹. Conjugation frequencies were determined by plating each parent strain alone. At this stage, we carefully performed genetic analysis of the transconjugants in order to exclude isolation of spontaneous mutants or colonies that might grow even in the absence of any genotype conferring resistance. Colonies were transferred to four different plates: (1) a plate containing both antibiotics for the resistance marker of Tn5251 and for the chromosomal resistance marker of the recipient strain; (2) a plate containing the antibiotic for the resistance marker of Tn5251; (3) a plate containing the antibiotic for the chromosomal resistance marker of the recipient strain; and (4) a plate containing no antibiotics. To rule out the contribution of transformation to the genetic exchange during conjugation, we used as S. pneumoniae recipients strains FP10 and FP11 impaired in natural competence for genetic transformation (Table 1) (Iannelli & Pozzi, 2007), while matings with other bacterial species as recipients were carried out in presence of 10 μg mL⁻¹ DNase I. In mating assays where S. pneumoniae was the donor and the recipient strains lacked the chromosomal resistance marker, selection of transconjugants was obtained after addition of 0.05% DOC to the mating mixture, followed by incubation at 37 °C for 15 min. Selection of B. subtilis transconjugants was obtained on LB agar plates.

**Nucleotide sequence accession number**

Tn5251 sequence was assigned GenBank accession no. FJ711160.

**Results and discussion**

**Nucleotide sequence of Tn5251**

The complete nucleotide sequence of Tn5251 was determined in S. pneumoniae strain DP1322 (Smith et al., 1981) using as sequencing templates two long PCR fragments spanning the element, and confirmed using smaller PCR fragments as templates. DNA sequence analysis showed that Tn5251 is 18 033 bp in length, with an overall GC content of 38.8%. It contains 22 ORFs, 20 of which have the same direction of transcription (Fig. 2a). Sequence alignment showed that Tn5251 is essentially identical to CTns of the Tn916–Tn1545 family. The Tn5251 DNA sequence differs from Tn916 in only 79 nucleotide (nt) changes, of which 69 are clustered in the tet(M) coding sequence (Provvedi et al., 1996). Comparison of the Tn5251 DNA sequence with 10 pneumococcal Tn5251-like elements, whose complete sequences are available in the public database, indicates that eight out 10 have insertions of either the ‘mega’ element carrying the macrolide efflux gene mef(E) (Del Grosso et al., 2006) or three different erm(B)-carrying elements, each at a specific site (Fig. 2b). Two more strains carry defective Tn5251-like elements. Strain G54 (GenBank CP001015) contains a truncated form of a Tn5251-like element. Truncation occurs downstream of tet(M) at nt 3869, which is the site of insertion of mega in other elements. Furthermore, the erm(B)-carrying element inserts into orf20 at nt 14 194. Strain CGSP14 (GenBank CP001033) contains a Tn5251-like element where the insertion of a 9-kb genetic element carrying sat4, aphIII and two erm(B) genes produces a deletion spanning from orf6 to orf19 (Ding et al., 2009).

In Tn5251, 46 nt changes are clustered in orf16 and orf20, resulting only in two amino acid substitutions in Orf6. Tn6003 (Genbank AM410044) has 339 nt changes including two deletions that introduce frameshifts in tet(M) and orf24, and two changes causing premature stop codons in orf5 and orf14. The same orf14 premature stop codon is present in the Tn5253-like element (GenBank FM201786). The element of strain ATCC 700669 (GenBank FM211187) has a deletion of 1 nt in the int coding sequence that determines a frameshift. Completely sequenced Tn916/Tn5251-like elements are also present in many other bacterial species including Streptococcus pyogenes, Streptococcus agalactiae, Streptococcus parauberis, Streptococcus suis, Streptococcus cristaus, Streptococcus intermedius, E. faecalis and Ureaplasma urealyticum. Noteworthy, as found in S. pneumoniae, many of these elements are inserted into other chromosomal genetic elements, such as the 89 K pathogenicity island present in recently sequenced strains of S. suis (Chen et al., 2007).

**ORFs of Tn5251**

All ORFs, with the exception of orf10, orf20 and orf24, are preceded by a ribosome-binding site. The start codon of orf6 is missing; orf15 starts with the GTG codon and orf20 with TTG. By manual annotation, it was possible to attribute a putative function only to 11 out of 22 ORFs (Table 2). Predicted gene products were blasted against public protein databases and the Pfam protein family database, taking into account significant homologies with functionally characterized proteins or good matches with Pfam domains. As reported for Tn916, int and xis are known to be involved in
the integration/excision process (Rudy et al., 1997); Int also acts as an accessory protein in the cleavage process that initiates the conjugal transfer mediated by the relaxase Orf20 (Rocco & Churchward, 2006). The tet(M) gene codes for a ribosomal protection protein responsible for tetracycline resistance. orf7 and orf9 code for a putative sigma factor and a putative transcriptional regulator, respectively, and possibly play a role in the gene expression regulation of Tn5251 genes (Celli & Trieu-Cuot, 1998). orf21 is an FtsK-SpoIIE family protein that may play a role in segregating the replicated element between the donor and the recipient. Orf14, coding for a putative cell wall hydrolase, may help degrade the peptidoglycan during conjugal mating. Orf16 is homologous to TcpF of Clostridium perfringens pCW3, which has been demonstrated to be essential for the plasmid conjugal transfer. This protein has an ATP-binding motif and is homologous to the VirB4 type IV secretion protein of Gram-negative bacteria that use energy from ATP hydrolysis to pump transposon DNA into the recipient cell (Rabel et al., 2003). orf18 codes for an antirestriction protein that protects the transposon DNA from restriction inhibiting host restriction enzymes (Serfiotis-Mitsa et al., 2008).

**Tn5251 attB site**

Tn5251 is inserted into Tn5253, flanked by two different 6-bp-long coupling sequences (CS). Excision of Tn5251 from its attB site produces a CI and a deletion in Tn5253. In the CI, single-stranded overhangs, produced by staggered cleavages of CS, are joined into a heteroduplex (Provvedi et al., 1996). Deletion of Tn5251 occurs between nts 16293 and 34339 of the Tn5253 sequence (Iannelli et al., unpublished data), leaving a heteroduplex formed by the two 6-bp CS. Chromosomal DNA purified from DP1322 was subjected to nested PCR analysis using PCR primers directed at the regions flanking Tn5251. Tn5251 deletion was present at a level of 1.2 copies per 10⁵ chromosomes. Sequence analysis of attB showed the presence of two bacterial populations, each harbouring one of the two CS, as a result of heteroduplex resolution following chromosomal replication.

**Conjugal transfer of Tn5251**

Tn5253 was transferred by plate mating from DP1322 to our S. pneumoniae recipient FP10 and the resulting strain FR22 was used as a Tn5251 donor (Table 1). Until now, Tn5251 conjugal transfer was described only in association with the
whole Tn5253, whereas, here, we first report the autonomous transfer of Tn5251. Transfer of Tn5251 as an independent CTn was only obtained in *S. pneumoniae* and *E. faecalis* (Table 3). In *S. pneumoniae*, transfer occurred in a strain-dependent manner; in fact, it was possible to move Tn5251 into the TIGR4 derivative FP47, but not in the Rx1 derivative FP11. The representative transconjugant *E. faecalis* FR64 harbouring an autonomous copy of Tn5251 was used as a donor to determine the Tn5251 host range. For this purpose, *S. pneumoniae*, *Streptococcus gordonii*, *S.

Table 2. Annotated ORFs of Tn5251

<table>
<thead>
<tr>
<th>ORF (aa)*</th>
<th>Annotation and comments (reference)</th>
<th>Homologous protein</th>
<th>Protein ID Origin</th>
<th>Amino acid identity</th>
<th>Amino acid similarity</th>
<th>Pfam domains [E value]*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>int</em> (405)</td>
<td>DNA integrase (Connolly et al., 1998)</td>
<td>YP_133692.1 Tn916 E. faecalis [0]</td>
<td>404/405 (99%)</td>
<td>405/405 (100%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>xis</em> (67)</td>
<td>DNA excisionase (Abbani et al., 2005)</td>
<td>YP_133691 Tn916 E. faecalis [0]</td>
<td>67/67 (100%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>orf9</em> (117)</td>
<td>Transcriptional regulator, putative (Brennan &amp; Matthews, 1989)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>tet(M)</em> (639)</td>
<td>Tetracycline resistance determinant (ribosomal protection protein) (Burdart, 1991)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>orf12</em> (28)</td>
<td>Tet(M) leader peptide (Su et al., 1992)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td><em>orf14</em> (333)</td>
<td>Cell wall hydrolase, putative</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>orf16</em> (815)</td>
<td>Type IV secretion protein, VirB4, putative</td>
<td>ABF47331.1 pCW3 C. perfringens [2e–68]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>arda/orf18</em> (165)</td>
<td>Antirestriction protein, ArdA (Serfiotis-Mitsa et al., 2008)</td>
<td>ArdA (5–165) [3.4e–81]</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td><em>orf20</em> (401)</td>
<td>Relaxase (Rocco &amp; Churchward, 2006)</td>
<td>HTH_3 (15–53) [1.6e–10]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>orf21</em> (461)</td>
<td>FtsK-SpoIIIE family protein, putative</td>
<td>FtsK_SpoIIIE (184–366) [1.5e–57]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Number of amino acids is shown in parentheses.
Method: compositional matrix adjust.
Position of Pfam domains is reported in parentheses.

Table 3. Conjugal transfer of Tn5253 from donor strain *Streptococcus pneumoniae* FR22

<table>
<thead>
<tr>
<th>Recipient strain</th>
<th>Transfer frequency*</th>
<th>%</th>
<th>Marker</th>
<th>Tn content</th>
<th>Representative transconjugant</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. pneumoniae</em> FP11</td>
<td>1.6 × 10^-4</td>
<td>100</td>
<td>Tc Crm</td>
<td>Tn5253</td>
<td>FR58</td>
</tr>
<tr>
<td><em>S. pneumoniae</em> FP47</td>
<td>3.6 × 10^-5</td>
<td>96</td>
<td>Tc Crm</td>
<td>Tn5253</td>
<td>FR54</td>
</tr>
<tr>
<td><em>S. pneumoniae</em> GP204</td>
<td>2.5 × 10^-5</td>
<td>100</td>
<td>Tc Crm</td>
<td>Tn5253</td>
<td>FR43</td>
</tr>
<tr>
<td><em>S. pyogenes</em> SF370</td>
<td>8.2 × 10^-6</td>
<td>100</td>
<td>Tc Crm</td>
<td>Tn5253</td>
<td>FR68</td>
</tr>
<tr>
<td><em>S. agalactiae</em> H36B</td>
<td>3.8 × 10^-6</td>
<td>100</td>
<td>Tc Crm</td>
<td>Tn5253</td>
<td>FR67</td>
</tr>
<tr>
<td><em>E. faecalis</em> OG1RF</td>
<td>5.4 × 10^-6</td>
<td>35</td>
<td>Tc Crm</td>
<td>Tn5253</td>
<td>FR66</td>
</tr>
<tr>
<td><em>E. faecalis</em> HJ2-2</td>
<td>8.6 × 10^-7</td>
<td>50</td>
<td>Tc Crm</td>
<td>Tn5253</td>
<td>FR50</td>
</tr>
</tbody>
</table>

*Conjugation frequency is expressed as CFU of transconjugants per CFU of donor. The results are presented as the mean of at least three mating experiments.

Transconjugants are selected for acquisition of tetracycline resistance. Transconjugants resistant to both tetracycline and chloramphenicol (Tc–Cm) contain the whole Tn5253, whereas transconjugants resistant to tetracycline (Tc) were found to contain either Tn5251 or defective forms of Tn5253 in which the cat gene was deleted.
**Tn5251 target sites**

Using inverse PCR on *S. pneumoniae* transconjugants, we found 4 Tn5251 integration sites in the pneumococcal chromosome (Fig. 1). Using the *S. pneumoniae* R6 genome (Hoskins et al., 2001) as a reference, the insertions occurred in *spr0357* at nt 357,477, in intergenic regions at nts 96,766 and 120,345 and in two transconjugants, obtained from different matings, at nt 117,225. It is worth noting that the insertion of Tn5251 into *spr0357* CDS occurs at the 3' end of the gene, probably not impairing the functionality of the encoded protein. Analysis of *E. faecalis* transconjugants showed that the Tn5251 insertion occurred in intergenic regions at nts 625,078, 789,261, 825,176 and 1830,021 of the OG1RF chromosome (Bourgogne et al., 2008). Tn5251 target sites are formed by a T-rich region separated from an A-rich region by a 6-bp CS and have short fragments of homology with the ends of the transposon. This has also been noted for Tn916 and Tn1545 insertion sites (Trieu-Cuot et al., 1993).

Genome-wide sequence analysis of both pneumococcal genomes and MGEs showed that there are 14 Tn5251-like CTns, seven of them being present in a composite CTn (Fig. 1). The seven Tn5251-like CTns integrate at four sites; the Tn3872-like elements present in strains CGSP14 (GenBank CP001033) and Hungary19A-6 (GenBank CP000936) integrate at nts 159,534 and 1,166,926, respectively, Tn2009 (Del Grosso et al., 2004) at nt 1,195,582, whereas Tn3872 (Del Grosso et al., 2006), Tn2010 (GenBank AB426620) and the elements of strains Taiwan19F-14 (GenBank CP000921) and TCH8431/19A (GenBank, NZ_ACJ)P00000000) integrate at nt 1,731,928. Composite elements integrate at two different sites: Tn5253 (Shoemaker et al., 1979; Ayoubi et al., 1991), Tn5253-like (GenBank FM201786) and the elements of strains 670-6B (http://strepneumo-sybil.igs.umaryland.edu/) and P1031 (GenBank CP000920) integrate at nt 1,036,330, whereas ICESp23FST81 (Croucher et al., 2008) (GenBank FM211187), Tn2008 of CGSP14 and the element of G54 (GenBank CP001015) integrate at nt 1,207,256. We reported the integration site positions referring to the R6 chromosome. It is worth noting that insertion of the Tn5251-like element within ICESp23FST81 and Tn2008 occurs at the same site, while in Tn5253, Tn5253-like and in the composite elements of strains 670-6B and P1031, insertion occurs at four different sites within the larger transposon (data not shown). Our analysis of genetic elements' integration into the *S. pneumoniae* chromosome clearly showed that three sites are 'preferred' targets for the integration of these elements and can be regarded as insertion hotspots.

**Conclusions**

In this work, we showed that pneumococcal Tn5251 belonging to the Tn916-Tn1545 family of CTns is an 18,033-bp-
long element containing 22 ORFs. In silico annotation was obtained for 11 ORFs including the tet(M) for ribosomal protection protein conferring tetracycline resistance. Here, we first demonstrate that Tn5251 excises from Tn5253 and is capable of autonomous transfer in S. pneumoniae and E. faecalis. Autonomous copies of Tn5251 can be independently moved into S. pneumoniae, S. gordonii, S. pyogenes, S. agalactiae, E. faecalis and B. subtilis. Analysis of Tn5251 and Tn5251-like elements’ insertion into S. pneumoniae and E. faecalis showed that besides the presence of hotspots, several integration sites are located in AT-rich regions of the bacterial chromosome and that generally element insertion does not disrupt coding sequences. Furthermore, Tn5251-like elements are highly capable of capturing other genetic elements carrying different antibiotic resistance determinants such as the mef(E) and erm genes conferring macrolide resistance, aadE, sat4 and aphA-3 conferring resistance to streptomycin, streptothricin and kanamycin, respectively. These features make these elements successful in disseminating multidrug resistance determinants among pathogenic bacterial species. In this context, characterization of Tn5251 contributes to the understanding of the mechanisms of the spread of antibiotic resistance.

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