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Nucleotide Sequence Analysis of Integrative Conjugative Element Tn*5253* of *Streptococcus pneumoniae*

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Conjugative transposon Tn*5253*, an integrative conjugative element (ICE) of *Streptococcus pneumoniae* carrying the *cat* and *tet*(M) genes, was shown to be 64,528 bp in size and to contain 79 open reading frames, of which only 38 could be annotated. Two distinct genetic elements were found integrated into Tn*5253*: Tn*5251* (18,033 bp), of the Tn*916*-Tn*1545* family of ICEs, and Ω*cat*(pC194) (7,627 bp), which could not conjugate but was capable of intracellular mobility by excision, circularization, and integration by homologous recombination. The highest conjugation frequency of Tn*5253* was observed when *Streptococcus pyogenes* was the donor (6.7×10^{-3} transconjugants/donor).

Comparative analysis of sequenced *Streptococcus pneumoniae* genomes indicates that many genes clustered in integrative and conjugative elements (ICEs) are responsible for pneumococcal genome evolution (1). ICEs, which comprise conjugative transposons, are found integrated into bacterial genomes and can be excised to form covalently closed circular intermediates that can either integrate within the cell at a different genomic site or move to a new bacterial host upon conjugative transfer (2). One of the first ICEs to be identified was Tn*5253*, originally called Ω(*cat-tet*) BM6001, which is a conjugative transposon found in the chromosome of a serogroup 19 clinical isolate of *S. pneumoniae* (3, 4). Tn*5253* was shown to be a genetic element conferring resistance to chloramphenicol and tetracycline that is capable of conjugal transfer and site-specific chromosomal integration in different Gram-positive bacteria belonging to the genera *Streptococcus* and *Enterococcus* (5–7). Tn*5253* was described as a composite conjugative transposon since it carried a complete copy of another ICE,

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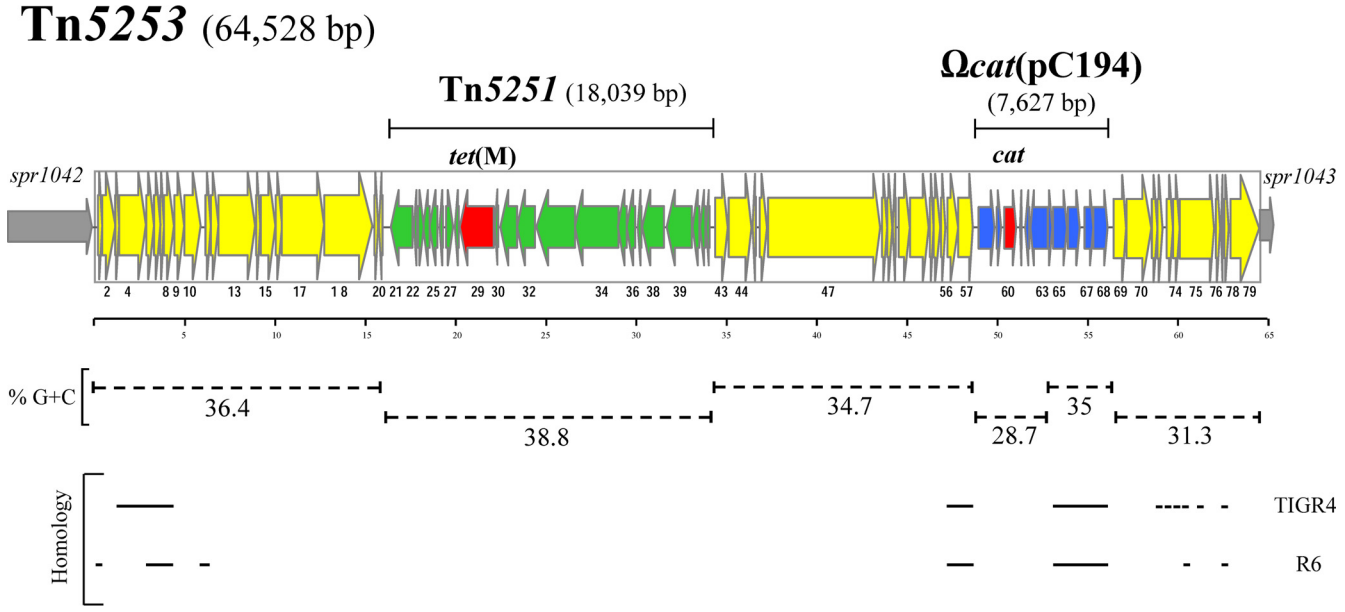

FIG 1 Structure of *S. pneumoniae* Tn*5253*. Sequence analysis of Tn*5253* showed that it is 64,528 bp in size and contains 79 ORFs. Tn*5253* contains two distinct integrated genetic elements, the 18,033-bp-long ICE Tn*5251* carrying *tet*(M) and the 7,627-bp-long element Ω*cat*(pC194) containing *cat*. ORFs and their directions of transcription are represented by arrows, and the annotated ORFs are indicated only by their numbers. The regions corresponding to Tn*5251* and Ω*cat*(pC194) are indicated by solid bars, and their ORFs are represented by thinner arrows. Tetracycline and chloramphenicol resistance genes are represented by red arrows. Chromosomal genes flanking the Tn*5253* insertion site are represented by thin gray arrows. The different GC contents of the various regions are indicated by dotted bars. Homologies with pneumococcal chromosomal regions are marked by solid lines. The scale is in kilobases.

TABLE 1 Annotated ORFs of Tn*5253*

ORF (no. of amino acids)[a,b]	Annotation and comments; reference	Pfam domain(s)[c] (amino acids) [E value(s)]	Homologous protein ID/origin	No. of amino acids identical/total (%)-no. similar/total (%)	E value[d]
repA/*orf2* (259)	Plasmid replication initiator protein A, N terminal; 20	RepA_N (13-88) [4.1e-28]			
orf4 (452)	DNA cytosine methyltransferase; 21				
orf8 (195)	Protease, putative	Abi-CAAX protease self-immunity (103-188) [1.6e-13]			
orf9 (196)	Abortive infection protein AbiEi; 22	DUF4095 (29-146) [2.1e-23]	AAB52382.1/pNP40 *L. lactis*	53/203 (26)-91/203 (44)	6e-06
orf10 (278)	Abortive infection protein AbiEii; 22	DUF1814 (37-260) [2.1e-19]	AAB52383.1/pNP40 *L. lactis*	81/279 (29)-136/279 (48)	3e-18
traG/*orf13* (625)	TraG-like protein, involved in bacterial conjugal transfer; 23	T4SS-DNA_transf (133-574) [1.1e-66]			
orf15 (284)	Membrane protein, putative	TrbL (123-269) [0.00026]	YP_195781.1 (*prgH*)/pCF10 *E. faecalis*	88/261 (33)-150/261 (57)	5e-24
orf16 (119)	Type IV secretion system protein	PrgI (4-95) [2.1e-22]			
orf17 (785)	Type IV secretion protein, VirB4, ATPase	AAA_10 (449-736) [3.8e-44]	YP_195783.1 (*prgJ*)/pCF10 *E. faecalis*	284/750 (37)-448/750 (59)	5e-134
orf18 (937)	Peptidoglycan hydrolase, putative	CHAP (805-926) [8.1e-30]			
orf20 (64)	ISSth5, transposase, *IS110* family, truncated		YP_139721.1/ *S. thermophilus*	43/64 (67)-47/64 (73)	1e-13
orf43 (243)	ABC transporter: ATP-binding protein, putative	ABC_tran (27-173) [2.8e-30]			
orf44 (438)	ABC transporter: permease (10 transmembrane helices predicted)	FtsX (55-176) [4.7e-07]			
orf47 (2028)	Restriction-modification protein, putative	Methyltransf_26 (504-615) [1e-17], SNF2_N (1253-1600) [2.3e-14], Helicase_C (1698-1768) [2.2e-05]			
orf52 (361)	DNA primase, putative	DUF3991 (113-198) [2e-13], Toprim_2 (203-329) [4.7e-10]			
pezA/*orf56* (158)	Antitoxin (TA system); 18				
pezT/*orf57* (252)	Toxin (TA system); 18				
cat/*orf60* (216)	CAT[e]	CAT (1-205) [2.8e-95]	NP_040437.1/pC194 *S. aureus*	216/216 (100)	
orf63 (281)	Plasmid replication protein	Rep_1 (54-250) [1e-48]	NP_040435.3/pC194 *S. aureus*	194/195 (99)-195/195 (100)	6e-111
nplT/*orf65* (245)	Truncated neopullulanase (α-amylase); 24	Alpha-amylase (2-141) [5.5e-25]			
pezA/*orf67* (158)	Antitoxin (TA system); 18				
pezT/*orf68* (256)	Toxin (TA system); 18				
umuD/*orf69* (227)	UmuD MucA homolog; 25	HTH_3 (7-61) [2.3e-17], Peptidase_S24 (143-205) [8e-12]			
umuC/*orf70* (471)	SOS response UmuC protein; 25	IMS (17-207) [2.2e-17], IMS_HHH (223-255) [5.1e-06], IMS_C (268-382) [5.9e-12]			
orf74 (121)	Bacterial mobilization protein, putative; 26	MobC (60-107) [5.4e-15]			
orf75 (609)	Relaxase; 27	Relaxase (12-264) [1.6e-73]			
orf76 (75)	Transcriptional regulator, putative; 28	HTH_3 (5-60) [2.1e-15]			
xis/*orf78* (77)	DNA excisionase; 29		AAA72427.1/Tn*5276* *L. lactis*	26/60(43)-43/60 (71)	5e-06
int/*orf79* (502)	DNA integrase; 30	Phage_integrase (188-394) [2.3e-24]	NP_150133.1/bacteriophage MM1 *S. pneumoniae*	121/413 (29)-193/413 (46)	4e-26

[a] The number of amino acids is shown in parentheses.
[b] *orf21* through *orf42* correspond to *orf1* to *orf22* of Tn*5251* and are annotated elsewhere (11). *orf58* through *orf68* belong to Ω*cat*(pC194).
[c] The numbers in parentheses represent the part of the protein homologous to the Pfam domain.
[d] Determined by compositional matrix adjustment.
[e] CAT, chloramphenicol acetyltransferase.

named Tn*5251*, integrated into its sequence (8, 9). Tn*5251* is a conjugative transposon of the Tn*916*-Tn*1545* family (10) that was shown to be a fully autonomous ICE capable of conjugal transfer to a variety of bacterial species, including *S. pneumoniae*, *Streptococcus gordonii*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Enterococcus faecalis*, and *Bacillus subtilis* (11). Here we report the complete annotated sequence of Tn*5253* and its phenotypic characterization, which allowed the identification of a new genetic element, Ω*cat*(pC194), integrated into the Tn*5253* sequence and containing a linearized copy of *cat*-carrying plasmid pC194 (12).

The nucleotide sequence of Tn*5253* (GenBank accession no. EU351020) was determined by direct sequencing of seven PCR fragments spanning the whole element and obtained from *S. pneumoniae* strain DP1322 (13). PCR primers (see Table S1 in the supplemental material) were designed on the basis of (i) available Tn*5253* sequences (8, 14), (ii) Tn*5253* chromosomal junction sequences (7), and (iii) the *cat* gene of staphylococcal plasmid pC194 (GenBank accession no. V01277), which is homologous to the *cat* gene of Tn*5253* (15). The DNA sequence was confirmed on the other strand by using short PCR fragments as sequencing templates. PCR, DNA sequencing, and sequence analysis were performed as already described (11).

TABLE 2 Conjugation frequencies of Tn*5253*

Donor strain (species)	Pneumococcal recipient	Transfer frequency[a]	Representative transconjugant
FR67 (*S. agalactiae*)	FP11 (type 2 rough)	1.1×10^{-6}	FR81
FR43 (*S. gordonii*)	FP11 (type 2 rough)	8.3×10^{-7}	FR61
FR40 (*S. pyogenes*)	FP11 (type 2 rough)	6.7×10^{-3}	FR59
FR49 (*E. faecalis*)	FP11 (type 2 rough)	$<1.8 \times 10^{-8}$	
FR50 (*E. faecalis*)	FP11 (type 2 rough)	$<2.7 \times 10^{-8}$	
FR22 (*S. pneumoniae*)	FP11 (type 2 rough)	1.6×10^{-4}	FR58
FR58 (*S. pneumoniae*)	FP58 (type 2)	2×10^{-5}	FR38
FR58 (*S. pneumoniae*)	HB565 (type 3)	4.4×10^{-7}	FR39
FR22 (*S. pneumoniae*)	FP47 (type 4)	3.2×10^{-5}	FR54
FR58 (*S. pneumoniae*)	FR55 (type 6)	1.3×10^{-5}	FR56

[a] The transfer frequency is expressed as the number of transconjugant CFU per donor CFU, and each result is the mean of at least three mating experiments.

Tn*5253* was found to be 64,528 bp in size and to contan 79 open reading frames (ORFs). Two distinct genetic elements were found integrated into Tn*5253*, (i) Tn*5251*, 18,033 bp long, belonging to the Tn*916*-Tn*1545* family of *tet*(M)-carrying ICEs, and (ii) Ω*cat*(pC194), 7,627 bp long, an element carrying *cat*-containing plasmid pC194 (Fig. 1). Insertion of Tn*5251* and Ω*cat*(pC194) identified three regions differing in GC content in Tn*5253*, (i) the left arm, containing ORFs 1 to 20, with a GC content of 36.4%; (ii), the central region, ORFs 43 to 57, with a GC content of 34.7%; and (iii) the right arm, ORFs 69 to 79, with a GC content of 31.3%. Tn*5251* showed the highest GC content (38.8%), the integrated copy of pC194 showed the lowest GC content (28.7%), and the GC content of the rest of Ω*cat*(pC194) was 35%. (Fig. 1). A further mark of separation between the left arm and the rest of Tn*5253* is the presence of *orf20*, coding for a truncated transposase of the *IS110* family, right before the *att* site of Tn*5251* (Table 1). Manual homology-based annotation (11) with functional prediction of the hypothetical gene product was possible for only 38 out of the 79 predicted ORFs, whereas 41 ORFs encoded hypothetical proteins that showed no homology to other characterized sequences (Table 1). Tn*5253* showed a typical ICE modular organization (16) with an "intercellular mobility module" spanning *orf13* to *orf18* and a "recombination module" spanning *orf74* to *orf79*.

Transconjugants of different bacterial species (*Streptococcus agalactiae*, *Streptococcus gordonii*, *Streptococcus pyogenes*, and *Enterococcus faecalis*) harboring Tn*5253* were used as donors in mating experiments with *S. pneumoniae* FP11 (17) as the recipient (Table 2). The frequency of transfer of the element from *S. pyogenes* transconjugant FR40 is, to our knowledge, the highest obtained for an ICE (6.7×10^{-3} transconjugants per donor), while we could not transfer Tn*5253* from the enterococcal donor FR49 ($<1.8 \times 10^{-8}$ transconjugants per donor). The frequency of Tn*5253* transfer was also investigated in capsulated *S. pneumoniae* recipients with different genetic backgrounds. Conjugal transfer

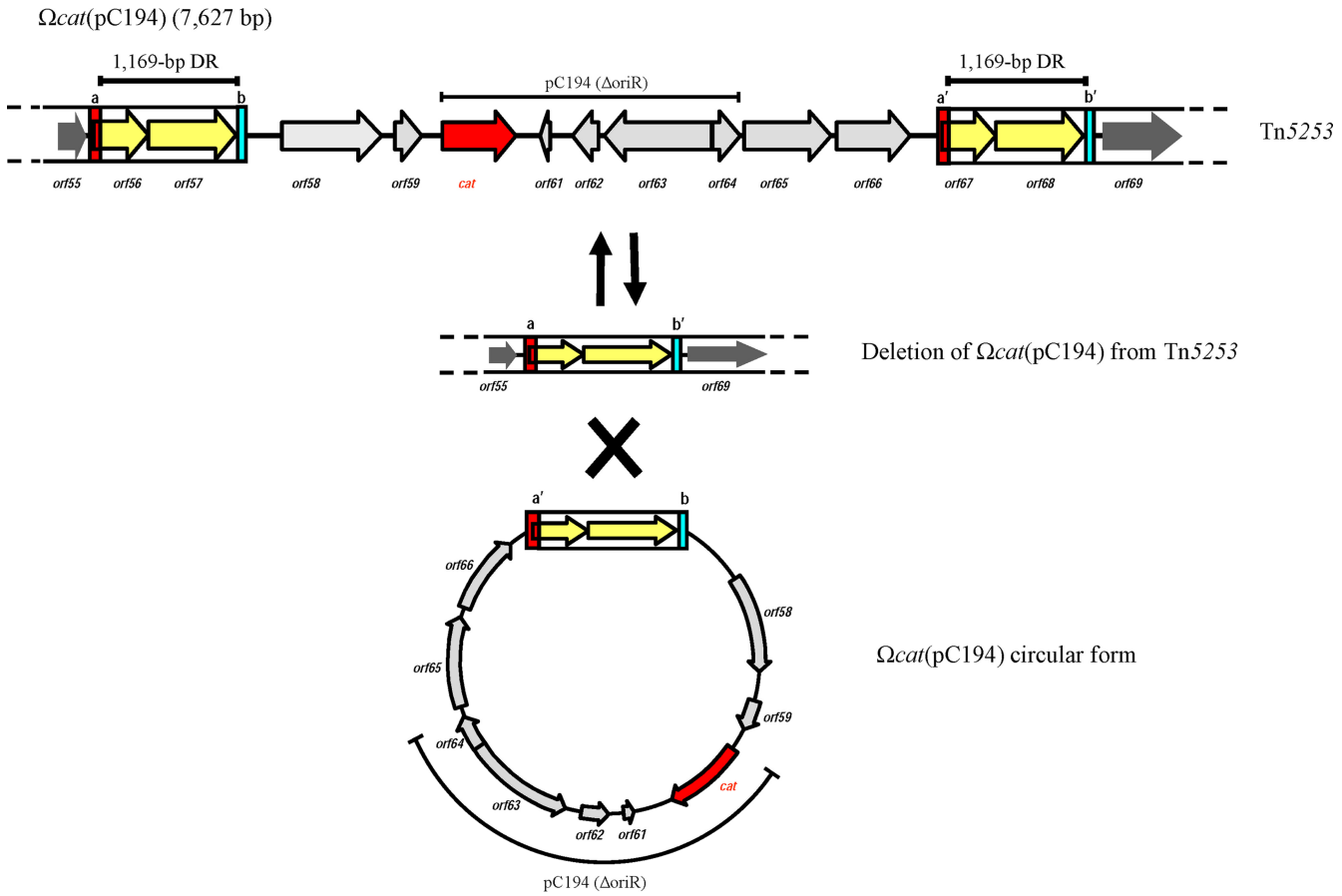

FIG 2 *S. pneumoniae* Ω*cat*(pC194) is a 7,627-bp-long element integrated into Tn*5253* and contains a copy of plasmid pC194 lacking its replication origin. It is flanked by two 1,169-bp DRs (boxed yellow arrows) that are part of two longer 1,357-bp imperfect DRs. The 5′ and 3′ ends of the imperfect DRs are represented by red (a and a′) and blue (b and b′) boxes. Ω*cat*(pC194) is excised from Tn*5253* and produces a circular form and a deletion in Tn*5253*. Excision of Ω*cat*(pC194) occurs by recombination between the flanking DRs and leaves in Tn*5253* a single DR flanked by a and b′, while the DR of the circular form is flanked by a′ and b.

of Tn*5253* from the standard rough donors to capsulated pneumococci occurred at frequencies ranging from 3.2×10^{-5} to 4.4×10^{-7} transconjugants per donor, while the frequency was 1.6×10^{-4} transconjugants per donor among the rough type 2 derivatives.

Ω*cat*(pC194) was found to be 7,627 bp long and to carry a copy of *cat*-containing plasmid pC194 harboring a 93-bp deletion involving the replication origin (Fig. 1 and 2). The element in its Tn*5253*-integrated form was flanked by two 1,169-bp direct repeats (DRs) corresponding to *orf56-orf57* and *orf67-orf68* (Fig. 2). The 1,169-bp DRs were part of two longer 1,357-bp imperfect DRs; 9 out of 13 mismatches (including two deletions) were located at the 5′ end, clustered in the first 146 nucleotides (designated a and a′ in Fig. 2), whereas 4 out of 13 are located at the 3′ end, clustered in the last 42 nucleotides (designated b and b′ in Fig. 2). PCR and sequencing analysis showed that the Ω*cat*(pC194) element excised from Tn*5253* produced a circular intermediate and a deletion in Tn*5253* (Fig. 2). The recombination event leading to the excision of Ω*cat*(pC194) from Tn*5253* occurred within the 1,169-bp segment, leaving in Tn*5253* a copy of the 1,169-bp segment flanked by imperfect DRs a and b′, whereas in the circular intermediate it was flanked by imperfect DRs a′ and b. The element contained 11 ORFs (*orf58* to *orf68*), 5 out of 11 belonging to plasmid pC194, while 4 out of 11, *orf65* to *orf68*, showed a high level of homology (>90%) to region *spr0948* to *spr0952* of R6 *S. pneumoniae* (Table 1). When investigating Ω*cat*(pC194) mobility, we found that under our experimental conditions, the element could not be transferred by conjugation ($<4.1 \times 10^7$ transconjugants per donor), but it was capable of intracellular mobility. In fact, we found and sequenced a copy of Ω*cat*(pC194) integrated into the chromosomal *spr0948*-to-*spr0952* region (GenBank accession no. GU808561). DNA sequence analysis of three different transconjugants containing Tn*5253* indicated that ectopic integration of Ω*cat*(pC194) occurred by insertion duplication at different positions between *spr0948* and *spr0952* (data not shown), as expected for homologous recombination. This ectopic integration generated two long DRs that flank the element and are homologous to the long DRs that also flank Ω*cat*(pC194) when it is inserted into Tn*5253*. It is interesting that the long DRs at both sites each contain a complete copy of the genes coding for a toxin-antitoxin system (18), the presence of which may contribute to the stable maintenance of these genetic elements in the bacterial host.

In conclusion, sequencing of the whole element allowed us to clearly define the composite nature of Tn*5253*, which was shown to contain two independent genetic elements, (i) fully functional conjugative transposon Tn*5251* (11) and (ii) the newly recognized *cat*-containing element Ω*cat*(pC194). It is noteworthy that a large fraction (52%) of the Tn*5253* ORFs code for putative proteins of unknown function, confirming that previously undescribed genes are preferentially carried by mobile genetic elements and reinforcing the notion that investigation of the mobilome is essential for understanding bacterial genomes (19).

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