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Characterization of Tn*5801.Sag*, a Variant of *Staphylococcus aureus* Tn*916* Family Transposon Tn*5801* That Is Widespread in Clinical Isolates of *Streptococcus agalactiae*

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Tn*5801*, originally detected in *Staphylococcus aureus* Mu50, is a Tn*916* family element in which a unique *int* gene (int_{5801}) replaces the *int* and *xis* genes in Tn*916* (int_{916} and xis_{916}). Among 62 *tet*(M)-positive tetracycline-resistant *Streptococcus agalactiae* isolates, 43 harbored Tn*916*, whereas 19 harbored a Tn*5801*-like element (Tn*5801.Sag*, ~20.6 kb). Tn*5801.Sag* was characterized (PCR mapping, partial sequencing, and chromosomal integration) and compared to other Tn*5801*-like elements. Similar to Tn*5801* from *S. aureus* Mu50, tested in parallel, Tn*5801.Sag* was unable to undergo circularization and conjugal transfer.

Tn*916* family elements (1, 2) are broad-host-range elements, widespread in Gram-positive bacteria, that mostly exhibit the distinctive properties of integrative and conjugative elements (ICEs) (3). Their open reading frames (ORFs) are organized into functional modules (conjugation, recombination, transcriptional regulation, and accessory functions): albeit with well-known variations (1, 2), the recombination module mostly consists of an integrase (int_{916}) gene and an excisionase (xis_{916}) gene, and the accessory gene is typically the tetracycline (TET) resistance determinant *tet*(M).

The best-known Tn*916* family element from *Staphylococcus aureus* is Tn*5801* (~25.8 kb) (2), which was detected in the genome of Mu50 (DDBJ accession no. BA000017) (4), a well-established methicillin-resistant and vancomycin-intermediate Japanese clinical isolate (5). Tn*5801*, regarded as one of the nine genomic islands in the Mu50 genome (6), shows a modular organization similar to that of Tn*916* and has several similar ORFs. However, besides the presence of additional ORFs, whose functions are largely unknown, DNA identities are rather low except in the case of *tet*(M) (97.7%). In particular, the recombination module differs from that of Tn*916*, as it lacks the *xis* gene and shows very low DNA identity (38.6%) between int_{5801} and int_{916}. This organization closely resembles that found in CW459*tet*(M), a genetic element from *Clostridium perfringens* CW459 (GenBank accession no. AF329848) (7).

Tn*5801*-like transposons have been detected in other human isolates of *S. aureus* (8, 9); in one case the element, Tn*6014* from *S. aureus* 1680, was able to transfer, at low frequency, to *S. aureus* recipients (8). Among streptococci, a Tn*5801*-like element has been described for *Streptococcus mitis* B6 (EMBL accession no. FN568063) (10).

In the present study, we showed that a Tn*5801*-like transposon, designated Tn*5801.Sag*, is found in about 30% of TET-resistant clinical isolates of *Streptococcus agalactiae*, a species in which TET resistance is around 90% worldwide. The genetic organization of Tn*5801.Sag* was determined and compared with that of other Tn*5801*-like elements, and the putative core site was identified. Similar to Tn*5801* from *S. aureus* Mu50, which was tested in parallel in this study, Tn*5801.Sag* was unable to undergo circularization and conjugal transfer.

All PCR primers used are shown in Table 1.

Characterization of TET-resistant *S. agalactiae* isolates. Sixty-nine clinical isolates of *S. agalactiae*, recovered in laboratories of central Italy in 2010–2011 and confirmed as being Lancefield group B using Slidex Strepto Plus (bioMérieux, Marcy l'Étoile, France), were used. Of them, 64 (93%) were TET resistant (MICs, ≥8 μg/ml). PCR assays demonstrated that *tet*(M) and *tet*(O) were the sole *tet* genes in 58 and 2 isolates, respectively; 4 isolates carried both determinants. Among the 62 *tet*(M)-positive isolates, 43 yielded positive PCRs for int_{916} and xis_{916}; the remaining 19 were negative for both genes as well as for three additional regions of the transposon. However, sequence analysis of the *tet*(M) amplicon, performed in 3/19 randomly selected isolates, showed 100% DNA identity to the corresponding *tet*(M) portion of Tn*5801* from *S. aureus* Mu50 (4). This finding prompted us to look for int_{5801}, the integrase gene of Tn*5801*, which was found in all 19 isolates. The latter fell into several serotypes and pulsotypes (data not shown), thus excluding that they represented a clonal population.

Characterization and comparative analysis of Tn*5801* from *S. agalactiae* (Tn*5801.Sag*). The 19 int_{5801}-positive isolates underwent PCR mapping using the primers and strategies summarized in Table 1 and Fig. 1A. All isolates yielded comparable results, with positive PCRs and amplicons of the expected sizes obtained with all but six of the relevant primer pairs. Specifically, negative reactions were obtained with those pairs in which at least one primer targeted one of the last three ORFs of Tn*5801* from *S. aureus* Mu50 (*sav413*, *sav414*, and *sav415*), which thus appeared not to be found in Tn*5801* from *S. agalactiae* (designated Tn*5801.Sag*).

One of the 19 isolates (strain 14774) was used in DNA sequencing experiments, performed as described elsewhere (16). Two amplicons, yielded by primer pairs 1812/400F [7,593 bp, encompassing the *tet*(M) gene and most int_{5801}] and 408R/411F (3,814 bp), were sequenced (EMBL accession no. HF930766). The two se-

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TABLE 1 Oligonucleotide primer pairs used

Procedure and gene/amplicon	Primer designation	Sequence (5′–3′)	Reference or source	Product size (bp)
Detection of TET resistance genes				
tet(M)	TETM3	ATGGAAGCCCAGAAAGGAT	11	740
	TETM2	GAACTCGAACAAGAGGAAAGC	11	
tet(O)	TETO1	AACTTAGGCATTCTGGCTCAC	11	519
	TETO2	TCCCACTGTTCCATATCGTCA	11	
PCR evidence of Tn*916*				
int_{916}	int-for	GCGTGATTGTATCTCACT	12	1,046
	int-rev	GACGCTCCTGTTGCTTCT	12	
xis_{916}	xis-for	AAGCAGACTGAGATTCCTA	13	194
	xis-rev	GCGTCCAATGTATCTATAA	13	
orf7-orf8[a]	O15	GTACGTCCACCAATGTGG	14	902
	O16	GCACGCTTCCACGAAAGGAG	14	
orf20-$IR_{18–19}$[a]	J12	CCCATTGAAGACGCAGAAGT	15	801
	J11	AAAAATCCCTACCGCACT	15	
orf24-orf20[a]	TN6-rev	CCATCAAACATTCATTCAGC	15	3,358
	J13	GGTTTTGTGGTTAGTTTT	15	
PCR mapping of Tn*5801.Sag*[b]				
int_{5801}	1812	GTCCATACGTTCCTAAAGTCGTC	8	726
	1811	CCGATATTGAGCCTATTGATGTG	8	
sav400	400R	TCGTATTTCAAGGCTTCGTC	This study	369
	400F	TACCGAAGAGTCCATCAAAC	This study	
sav408	408R	AATGTAGGGGCGACTTGATG	This study	1,005
	408F	ACTGGCTTATGGCGTTTCTC	This study	
sav409	409R	GCAGACAAACCAAGATAAGC	This study	940
	409F	GAGAGCGAATCAAAGCCAAC	This study	
sav413	413R	AACACCGTTGTCGTCTCCAC	This study	743
	413F	TTGCTAGTAATATAAGGGCGA	This study	
sav414	414R	ATTAGATACACAACATCCTCATC	This study	579
	414F	ACAGGCAATCCCATCAGAAC	This study	
sav415	415R	TAGATGAGGCTTGATACACC	This study	677
	415F	TTCTCGTAACGGCTCCTATG	This study	
int_{5801}-*tet*(M)	1812	GTCCATACGTTCCTAAAGTCGTC	8	4,971
	TETM2	GAACTCGAACAAGAGGAAAGC	11	
tet(M)-*sav400*	TETM3	ATGGAAGCCCAGAAAGGAT	11	3,362
	400F	TACCGAAGAGTCCATCAAAC	This study	
sav400-sav408	400R	TCGTATTTCAAGGCTTCGTC	This study	9,331
	408F	ACTGGCTTATGGCGTTTCTC	This study	
sav408-sav409	408R	AATGTAGGGGCGACTTGATG	This study	2,612
	409F	GAGAGCGAATCAAAGCCAAC	This study	
sav409-sav411	409R	GCAGACAAACCAAGATAAGC	This study	2,142
	411F	GAGATTAGCAGAAGGTATTGTG	This study	
sav409-sav413	409R	GCAGACAAACCAAGATAAGC	This study	3,874
	413F	TTGCTAGTAATATAAGGGCGA	This study	
sav413-sav414	413R	AACACCGTTGTCGTCTCCAC	This study	1,968
	414F	ACAGGCAATCCCATCAGAAC	This study	
sav414-sav415	414R	ATTAGATACACAACATCCTCATC	This study	3,295
	415F	TTCTCGTAACGGCTCCTATG	This study	
Tn*5801.Sag* chromosomal integration site[c]				
SAG967 (*guaA*)	LJ967	CGTGAAGAAATCGCTAAAG	This study	1,228
int_{5801}	1811	CCGATATTGAGCCTATTGATGTG	8	
sav411	CF1	TTCAAAGGAACAGAAGCGGG	This study	1,414
SAG964	RJ964	GAAGTAGAAGAGAGCCATAG	This study	
Search for circular form				
int_{5801}	1811	CCGATATTGAGCCTATTGATGTG	8	
sav411	CF1	TTCAAAGGAACAGAAGCGGG	This study	

[a] ORFs numbered according to the reported organization of Tn*916* (GenBank accession no. U09422).
[b] Tn*5801* from the genome of *S. aureus* Mu50 (DDBJ accession no. BA000017; *sav* genes) was used as the reference sequence. In PCR assays, *S. aureus* Mu50 (ATCC 700699) was used as a positive control and *S. pneumoniae* BM4200 (Pasteur Institute Collection), harboring the Tn*916*-like transposon Tn*1545* (1), was used as a negative control.
[c] The genome of *S. agalactiae* strain 2603V/R (GenBank accession no. AE009948; SAG genes) was used as the reference sequence.

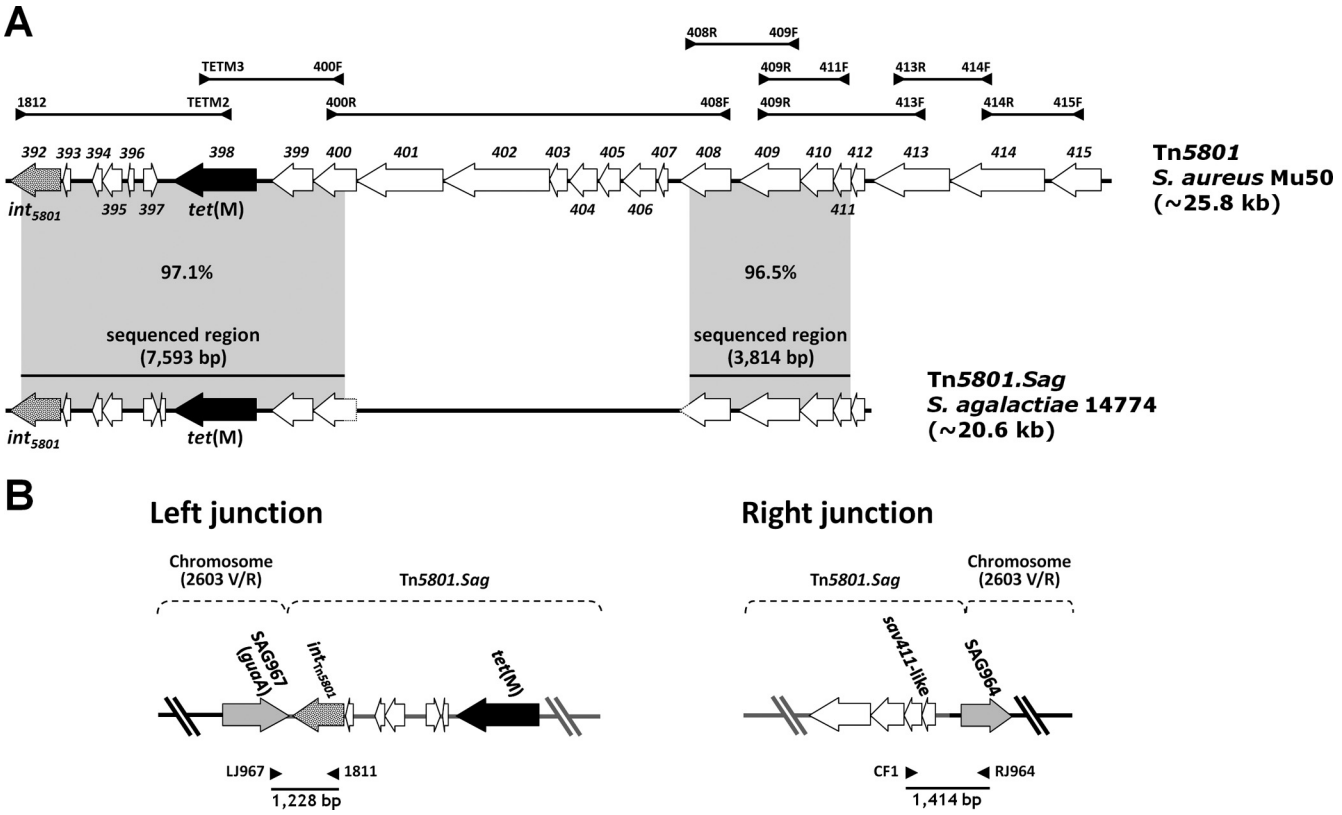

FIG 1 Schematic representation of Tn*5801.Sag* from *S. agalactiae* strain 14774 (A) and its chromosomal integration (left and right junctions) (B). (A) Tn*5801.Sag* was determined by PCR mapping and sequencing of two regions. The primers used are listed in Table 1. The mapping strategy is outlined in the upper portion (the amplicons used to detect individual ORFs, i.e., obtained by pairing two primers internal to the same ORF, are not shown). The two regions sequenced initially (7,593 bp, left, and 3,814 bp, right) are indicated by horizontal bars. Tn*5801.Sag* is compared to Tn*5801* from *S. aureus* Mu50, where ORFs are numbered *sav392* to *sav415* according to the original designations (DDBJ accession no. BA000017); percent DNA identities are reported in gray areas between sequenced regions. *tet*(M) and int_{5801} are represented as black and spotted arrows, respectively. (B) Tn*5801.Sag* was integrated at the 3′ end of the *guaA* gene. This gene, detected in all *S. agalactiae* genomes sequenced to date, corresponds to ORF967 from *S. agalactiae* 2603V/R (GenBank accession no. AE009948), from which chromosomal ORF designations derive. The amplicons obtained by pairing primers LJ967/1811 (left junction) and CF1/RJ964 (right junction), whose sequencing extended the two portions of Tn*5801.Sag* sequenced initially, are shown as bars. *tet*(M) and int_{5801} are represented as black and spotted arrows, respectively, and other Tn*5801.Sag* ORFs as white arrows; chromosomal ORFs are depicted as gray arrows.

quenced regions of Tn*5801.Sag* displayed 97.1% [*tet*(M), 100%] and 96.5% DNA identities with the corresponding regions of Tn*5801* from *S. aureus* Mu50 (Fig. 1A) and 96.9% [*tet*(M), 99.5%] and 96.5% with those of *S. mitis* B6; the former sequence displayed 96.1% identity [*tet*(M), 100%] with the corresponding region (the only one that has been sequenced; GenBank accession no. EU918655) of Tn*6014* from *S. aureus* 1680. Greater identities [99.9% and 100%; *tet*(M), 100%] were recorded with the corresponding regions of *E. faecalis* 62, for which a "Tn*916* element" (not identified as Tn*5801*-like) was reported (17) in the sequenced genome (GenBank accession no. CP002491). The latter element (~20.6 kb) was very similar to Tn*5801.Sag* (~20.6 kb based on sequencing and PCR mapping data) also as to ORF organization: in particular, the two elements share the lack of the last three ORFs of Tn*5801* from *S. aureus* Mu50 (*sav413*, *sav414*, and *sav415*), located after the conjugation module and not present in Tn*916*. It is worth noting that the last ORF (*sav415*, a transposase gene) is also missing in the Tn*5801*-like element from *S. mitis* B6, in which *sav413* and *sav414* are present.

Chromosomal integration of Tn*5801.Sag* and identification of the putative core site. The early study of CW459*tet*(M) (7) and later studies of genetic elements related to Tn*5801* (10, 18, 19) concur in describing an integration site just downstream of *guaA*, a chromosomal gene encoding a GMP synthase that is consistently found adjacent to int_{5801} in the sequenced genomes containing a Tn*5801* element. Using strategies refined in previous studies (16, 20–22), this site was thus explored in the 19 *S. agalactiae* isolates harboring Tn*5801.Sag*. The genome of *S. agalactiae* strain 2603V/R (GenBank accession no. AE009948) (23) was used as the reference sequence. As illustrated in Fig. 1B, pairing of primers LJ967/1811 (left junction) gave an ~1.2-kb amplicon from all 19 *S. agalactiae* isolates; by pairing primers CF1/RJ964 (right junction), an ~1.4-kb amplicon was obtained from all but one isolate, which yielded an ~1-kb-larger amplicon; this was subsequently shown to reflect the presence of SAG965 and SAG966, encoding insertion sequences in the *S. agalactiae* 2603V/R genome that were not found in the other 18 isolates. By analyzing and comparing the two amplicon sequences from strain 14774, it was possible to determine the chromosomal junctions of Tn*5801.Sag*. The putative core site was an almost completely overlapping 11-bp sequence identified on the left (GAGTGGG AGTA) and right (GAGTGGGAATA) ends of the transposon; the

latter sequence was identical to that found in both Tn*5801* junctions of *S. aureus* Mu50.

Transferability studies. Three isolates, including strain 14774, were used as donors in conjugal transfer experiments, performed as described elsewhere (24). No transconjugants were obtained with any of the three recipients used: *S. agalactiae* 1357RF (25), *S. pyogenes* 12RF (24), and *S. aureus* RN4220RF (26), used in the sole successful conjugative transfer of a Tn*5801* element (Tn*6014*) reported so far (8). Similar negative results were obtained using *S. aureus* Mu50 (ATCC 700699) as the donor.

The apparent nontransferability of Tn*5801.Sag* and of Tn*5801* from *S. aureus* Mu50 was consistent with the absence, in both cases, of an intermediate circular form, as resulting from the negative PCR obtained using the outward-directed primer pair 1811/CF1.

Conclusions. Among the so-called Tn*916*-like elements (1, 2), major differences are found in the recombination module, where the prevailing two-gene organization (int_{916} and xis_{916}) typical of Tn*916* may be replaced by a single gene. This is the cases of *tndX* in Tn*5397* from *Clostridium difficile* (7), a gene that in streptococci is commonly found in *S. pyogenes* in ICE*Sp1116* (22); of int_{459} in CW459*tet*(M) from *C. perfringens* (7); and of int_{5801} (identical to int_{459}) in Tn*5801* from *S. aureus* (4). Now, the finding that in no less than 30% of TET-resistant clinical isolates of *S. agalactiae*—a species for which TET resistance rates are around 90%—resistance was mediated by the *tet*(M) gene carried by a Tn*5801*-like transposon (Tn*5801.Sag*) is a major result of this study. Accordingly, a sizable proportion (about 50/250) of *S. agalactiae* scaffolds and contigs currently found in GenBank harbors Tn*5801.Sag*. The frequent occurrence of Tn*5801.Sag* in *S. agalactiae* strengthens the notion of a composite organization of the chromosome of this species (27–29).

Subsequent to the original detection of Tn*5801* in *S. aureus* Mu50, Tn*5801*-like transposons were detected in other human *S. aureus* isolates; one such transposon (Tn*6014*) was shown to be able to transfer to an *S. aureus* recipient (8). Conversely, Tn*5801.Sag* is apparently unable to transfer, like Tn*5801* from *S. aureus* Mu50, whose actual transferability had not been tested before the present study.

As to the genetic organization of Tn*5801.Sag*, differences from other Tn*5801*-like transposons mainly involved the right terminus of the element, with the last three ORFs of Tn*5801* from *S. aureus* Mu50 (*sav413*, *sav414*, and *sav415*, not present in Tn*916*) missing in Tn*5801.Sag*, while only the last one (*sav415*) is missing in Tn*5801*-like from *S. mitis* B6. In contrast, the left termini are very similar in all Tn*5801*-like transposons, and the adjacent chromosomal gene is unvaryingly *guaA*. Therefore, while Tn*916* preferentially integrates into A·T-rich targets in a broad range of hosts (2), int_{5801} and related genes appear to code for integrases leading to site-specific recombination at the 3′ end of *guaA*.

Nucleotide sequence accession number. Two new nucleotide sequences reported in this work have been deposited in the EMBL database under accession no. HF930766.

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