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Diversity and Evolution of the Tn*5801-tet*(M)-Like Integrative and Conjugative Elements among *Enterococcus*, *Streptococcus*, and *Staphylococcus*

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This work describes the diversity and evolution of Tn*5801* among enterococci, staphylococci, and streptococci based on analysis of the 5,073 genomes of these bacterial groups available in gene databases. We also examined 610 isolates of *Enterococcus* (from 10 countries, 1987 to 2010) for the presence of this and other known CTn-*tet*(M) elements due to the scarcity of data about Tn*5801* among enterococci. Genome location (by I*Ceu*-I–pulsed-field gel electrophoresis [PFGE] hybridization/integration site identification), conjugation and fitness (by standard methods), Tn*5801* characterization (by long-PCR mapping/sequencing), and clonality (by PFGE/multilocus sequence typing [MLST]) were studied. Twenty-three Tn*5801* variants (17 unpublished) clustered in two groups, designated "A" (25 kb; *n* = 14; predominant in *Staphylococcus aureus*) and "B" (20 kb; *n* = 9; predominant in *Streptococcus agalactiae*). The percent GC content of the common backbone suggests a streptococcal origin of Tn*5801* group B, with further acquisition of a 5-kb fragment that resulted in group A. Deep sequence analysis allowed identification of variants associated with clonal lineages of *S. aureus* (clonal complex 8 [CC8], sequence type 239 [ST239]), *S. agalactiae* (CC17), *Enterococcus faecium* (ST17/ST18), or *Enterococcus faecalis* (ST8), local variants, or variants located in different species and geographical areas. All Tn*5801* elements were chromosomally located upstream of the *guaA* gene, which serves as an integration hot spot. Transferability was demonstrated only for Tn*5801* type B among *E. faecalis* clonal backgrounds, which eventually harbored another Tn*5801* copy. The study documents early acquisition of Tn*5801* by *Enterococcus*, *Staphylococcus*, and *Streptococcus*. Clonal waves of these pathogens seem to have contributed to the geographical spread and local evolution of the transposon. Horizontal transfer, also demonstrated, could explain the variability observed, with the isolates often containing sequences of different origins.

The extended use of tetracycline since its introduction into the therapeutic arsenal in 1948 seems to have resulted in an evolutionary bottleneck in the population structure of some genera of *Firmicutes* that are of interest in human health. Recent studies have associated the acquisition and further fixation of tetracycline resistance (Tet[r]) by pathogenic clones of group B *Streptococcus* (GBS) with the global increase of high-mortality GBS neonatal infections in the last decades (1, 2). Similarly, some *Staphylococcus aureus* lineages are enriched in Tet[r] elements (3–5).

Tetracycline resistance in major Gram-positive human opportunistic pathogens is caused mainly by the acquisition of integrative and conjugative elements (ICE) of the Tn*916* family carrying the *tet*(M) gene (5–7). These elements display a common synteny but differ in the integrase (*int*) and the excisionase (*xis*) sequences, insertion site specificity, and host range (8, 9). Variants of some Tn*916-tet*(M) members, namely Tn*916*, Tn*5397*, Tn*6000*, or Tn*5801*, are widely spread among several genera of *Firmicutes*, apparently suggesting a successful multihost dissemination of these elements (7).

Tn*5801* has a site-specific tyrosine recombinase that recognizes the 3′ end of the GMP synthase gene (*guaA*) (10, 11), an insertion hot spot of genomic islands coding for pathogenicity or antibiotic resistance in different *Firmicutes* (12–14). Tn*5801* was originally detected in the vancomycin-resistant *Staphylococcus aureus* (VRSA) clinical strain Mu50 recovered in Japan in 1997 (15), but several studies have documented the presence of platforms highly similar to Tn*5801* in early isolates of *Streptococcus agalactiae*

(France, 1953) (1), *S. aureus* (Denmark, 1963; designated Tn*6014*) (3), and *Clostridium perfringens* (United States, 1977; a truncated element designated CW459*tet*(M)] (16). The diversity of Tn*5801*-like backbones in a few isolates of different species (3, 17–20) collected throughout more than 50 years suggests the evolutionary interplay of selective events, horizontal dissemination, and the genetic plasticity of this transposon, allowing its spread in different clonal backgrounds and environments. However, little is known about Tn*5801*, at either the molecular or the epidemiological level.

In this study, we created a data set comprising all the genomes of *Firmicutes* with the presence of a Tn*5801*-like ICE. Due to the relevance of enterococci in the spread of Tet[r] and the low number

of sequenced genomes in public databases at the time this study was started, we also analyzed the presence and genetic background of Tn*5801* in a large collection of enterococcal strains from different geographical areas as well as its transferability. This comprehensive phylogenetic and genomic analysis allowed documentation of the genetic variability of Tn*5801*-like elements among genomes of enterococci, staphylococci, streptococci, and other bacterial species through years and across continents. The results highlight the relevance of comprehensive analysis at local and global levels to accurately establish the transmission and evolvability of elements at different scales. It also proved the transferability of the element in different genetic contexts.

MATERIALS AND METHODS

Screening of Tn*5801* in GenBank databases and creation of data sets. The DNA sequence from *S. aureus* Mu50 (GenBank accession number BA000017) was used as a reference to screen for the presence of Tn*5801* among 5,073 draft and complete genomes from *Firmicutes* (4,130 *S. aureus*, 345 *Enterococcus faecalis*, 301 *S. agalactiae*, 254 *Enterococcus faecium*, 23 *Streptococcus mitis*, 14 *Lactococcus garvieae*, 4 *Staphylococcus pseudintermedius*, 2 *Enterococcus villorum*, and 1 *Streptococcus thoraltensis*) obtained from the NCBI archive (last updated January 2015). BLAST searches identified different sequences showing homology with Tn*5801*-like elements. They were further stored and analyzed by using the MUSCLE (multiple-sequence comparison by log expectation) algorithm (http://www.ebi.ac.uk/Tools/msa/muscle/) and Artemis comparison tool (21) (http://www.sanger.ac.uk/resources/software/act/). ICE maps were created with Vector NTI (ThermoFisher Scientific) and Inkscape (http://inkscape.org/) software.

Phylogenetic analyses were accomplished using MEGA version 6 (22). A common region of 13,895 bp was identified among all the sequences with homology with Tn*5801* (here called "Tn*5801* variant sequences"). The region comprises 16 open reading frames (ORFs) (int_{Tn5801}-*orf*24; *orf*22, *orf*18, *orf*16, *orf*15, *orf*12-10, and *orf*8-4), which were trimmed and concatenated to be further analyzed. A maximum-likelihood phylogenetic tree was constructed with 1,000 bootstrap replications in order to infer the relationship among all Tn*5801* variants. Due to the identification of modules with different GC contents and with recombination landmarks, the Tn*5801* variants were further split into five core fragments (int_{Tn5801}, *orf*26-19, *orf*18-14, *orf*12-8, and *orf*7-4), with each fragment being separately analyzed as mentioned before. The *orf*26-19 fragment was excluded from this analysis because of the variability observed among variants due to frequent indels and recombinatorial events. An arbitrary number was attributed to different sequences of each core fragment, and Tn*5801* sequences with unique number profiles were used to calculate a consensus tree. The data set created included epidemiological information for isolates (host/source, year of isolation, country, and sequence type [ST]). To identify the STs of the isolates, we used the MLST 1.8 software of the Center of Genomic Epidemiology server (23).

Epidemiological background of bacterial strains and identification of *tet*(M) transposons among enterococci. A collection of 610 enterococcal isolates from humans (*n* = 320; hospitalized patients [HP], *n* = 195; healthy human volunteers [HV], *n* = 125); animals (*n* = 236; poultry [P], *n* = 164; swine/piggeries [SP], *n* = 72), and hospital/urban sewage (SW) (*n* = 54) collected in different countries over a 23-year period (1987 to 2010) was included (9, 24). All isolates were tested for susceptibility to tetracycline by the disk diffusion and/or agar dilution method following CLSI guidelines (25). The presence of genes coding for tetracycline resistance [*tet*(M), *tet*(L), *tet*(S), *tet*(K), and *tet*(O)] was assayed by PCR as previously described (26). The presence of the main transposons of the Tn*916* family (Tn*5801*, Tn*916*, Tn*5397*, Tn*5386*, and Tn*6000*) was investigated with a multiplex PCR assay designed here for detecting integrases, excisionases, and other specific sequences of known transposons. The susceptibility and specificity of the assay were validated using appropriate controls. Primers and conditions are listed in Table S1 in the supplemental material. The clonal relationship among *E. faecium* and *E. faecalis* isolates harboring the integrase of Tn*5801* (int_{Tn5801}) was established by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) (http://pubmlst.org/) (27, 28). Population genetic analysis was performed using BAPS software with *E. faecium* isolates as previously described (29, 30).

PCR characterization of Tn*5801* backbones in *Enterococcus*. The Tn*5801* backbone was fully characterized by a PCR mapping assay based on the Tn*5801* sequence of *S. aureus* Mu50 (GenBank accession number BA000017). Amplified fragments obtained from positive int_{Tn5801} strains were further sequenced (Table 1; see Fig. S1 in the supplemental material). Genomic location of Tn*5801* was assessed by hybridization of I-*Ceu*I (TaKaRa Biotechnology, Dalian, China) digested genomic DNA using specific int_{Tn5801} and 23S rRNA probes (31). Amplification of the int_{Tn5801}-*guaA* region was performed in order to characterize the integration site of Tn*5801* (Table 1; see Fig. S1 in the supplemental material) (14).

Transferability of Tn*5801*. Transferability of Tn*5801* was screened by filter mating using different recipient strains of *E. faecalis* (JH2-2, OG1RF, and OG1SSp) and *E. faecium* (64/3, BM4105RF, and BM4105SS), all being resistant to rifampin and fusidic acid (designated RF) or streptomycin and spectinomycin (designated SS) and negative for the presence of the *tet*(M) gene (32). The mating assays were performed at 37°C for 36 h using aliquots of broth cultures of donors and recipients grown up to the exponential phase (McFarland 0.5) and mixed at a ratio of 1:1 (32). Transconjugants were selected on brain heart infusion (BHI) agar (Pronadisa, Laboratorios Conda, SA, Madrid, Spain) plates supplemented with tetracycline (10 mg/liter) plus rifampin (30 mg/liter) and fusidic acid (25 mg/liter) or streptomycin (250 mg/liter) and spectinomycin (250 mg/liter) (Sigma-Aldrich, Inc., St. Louis, MO). Unexpectedly, we identified a Tn*5801* Δ*tet*(M) variant in the laboratory recipient *E. faecalis* strain JH2-2, which is an emblematic receptor strain (see below). Secondary filter mating assays were performed using *E. faecalis* JH2-2 transconjugants as donors and the *E. faecalis* OG1SS and *E. faecium* BM4105SS strains as recipients. Transconjugants were characterized according to the susceptibility to tetracycline (Etest; bioMérieux SA, France), the presence of Tn*5801* [int_{Tn5801} and *tet*(M)], the transposon backbone (PCR overlapping), and comparison of SmaI-digested DNA PFGE profiles of transconjugants with those of recipients and wild-type strains. The location of Tn*5801* in the recipient genomes was assessed by hybridization of SmaI-digested DNA with specific *guaA*, int_{Tn5801}, and *tet*(M) probes. PCR of the *guaA*-int_{5801} fragment was performed in all transconjugants.

Fitness cost assays. Transconjugants and the corresponding recipient strain were cultured at 37°C in BHI broth overnight. Grown cultures were diluted 1:1,000 into BHI broth to obtain an inoculum of approximately 10^5 CFU/ml. An aliquot (300 μl) of this dilution was transferred into a multiwell plate and incubated at 37°C, and the growth rate was estimated from the interval of optical density at 600 nm estimated to be exponential (0.01 to 0.1) by using the GrowthRates 2.1 program (33). The measurement was performed every 15 min for 22 h in a Microbiology Workstation Bioscreen C (ThermoLabSystems, Helsinki, Finland). To guarantee culture optical homogeneity, the plates were shaken for 10 s before all measurements. Three biological and technical replicates were assayed for each strain. Relative values for each parameter were calculated by dividing the average corresponding growth rate of a given transconjugant by the average corresponding parameter of the donor in the same experiment (34).

Nucleotide sequence accession number. The A4 variant of Tn*5801* detected in this study is available in the GenBank database with accession number KP001176.

RESULTS

Genetic diversity of Tn*5801* in the genomes of four major Gram-positive genera. Twenty-three Tn*5801* variants were identified among the 5,073 genomes screened, 17 of which had not been previously published. The 23 distinct Tn*5801* backbones corre-

TABLE 1 Characterization of Tn*5801*: PCR strategy and conditions

Primer	Sequence (5′→3′)	Position in Tn*5801* (bp)	PCR	Sequence amplified	Amplicon size (bp)	Amplification conditions
gmp1	CCTGGTCTTGGTATTCGTGT	25763–25783[a]	gmp1-gmp2	*guaA*	170	1 cycle of 95°C for 10 min; 25 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 30 s; 1 cycle of 72°C for 10 min
gmp2	GTAGTCTCCCATAACACC	25609–25627[a]				
Int1	CTGTTTCCGATATTGAGC	24490–24473[a]	Int1-gmp1	*int*Tn*5801*-*guaA*	1,311	35 cycles of 96°C for 30 s, 45–59°C (according to primer pair) for 1 min, 72°C for 7 min; 1 cycle of 72°C for 10 min
P1	GTTTCGCAAGTAGTCTACAG	25310–25329[a]	P1-P2	*orf25*-*int*Tn*5801*	2,085	
P2	GTAAAGGCGACAGATGG	23244–23260[a]				
P3	CTTAGAGATGAGTTTCGTTTC	23391–23411[a]	P3-P4	*orf22*-*orf25*	1,455	
P4	GGATAGTTCTTTGTCTGTAAAG	21956–21977[a]				
P5	CGATTTTAGAGCCGTTGGTTTAG	22210–22232[a]	P5-P6	*tet*(M)-*orf22*	2,448	
P6	GAACGTCAGAGAGGAATTAC	19784–19803[a]				
P7	GGGAATCCCCATTTTCCTAA	19986–20005[a]	P7-P8	*orf15*-*tet*(M)	6,498	
P8	ACCCACTCGCTGTTTAATCG	13507–13526[a]				
P9	CTTGCAAGGCTAGGTTGGAG	14162–14181[a]	P9-P10	*orf7*-*orf15*	5,993	
P10	ATGTCTGAAATGGGCTTTGG	8188–8207[a]				
P11	CTTTCACTTCGTGCGGTACA	8382–8401[a]	P11-P12	*orf3*-*orf7*	4,631	
P12	GGGTGGGGACAATACATCAG	3770–3789[a]				
P13	CAGCCATGTAGCGTCTTTGA	4315–4334[a]	P13-P14	*orf1*-*orf3*	4,228	
P14	ACGGAGTTAACGGCTTTCCT	106–125[a]				
P15	CCGTATGTTCTTTCAACCACT	1030–1051[b]	P15-P11	*orf5*-*orf7*	2,675	
P16	GCTGACAGTTCCAGTATCC	18096–18115[c]	P16-P5	*orf23′*-*orf22*	1,209	
P17	CAGAACCAGCCATTACC	18561–18578[c]	P17-P18	*orf23′*-*orf19′*	3,938	
P18	GAAGATACGAGAAACCAATAG	14602–14623[c]				
P19	GGTGGCAATTCAAGTGTTCC	14859–14878[c]	P19-P20	*orf19′*-*orf17*	1,957	
P20	GGCGTATGACAAAGCTGG	12921–12939[c]				

[a] Reference sequence, *Staphylococcus aureus* Mu50 (GenBank accession number BA000017).
[b] Reference sequence, *Streptococcus agalactiae* COH1 (GenBank accession number AAJR01000021.1).
[c] Reference sequence, *Enterococcus faecalis* JH2-2 (GenBank accession number AXOI01000011.1).

sponded to 225 strains (139 *S. aureus*, 43 *Streptococcus agalactiae*, 24 *E. faecium*, 15 *E. faecalis*, 1 *Streptococcus mitis*, 1 *Enterococcus villorum*, 1 *Streptococcus thoraltensis*, and 1 *Lactococcus garvieae*). Table 2 and Table S2 in the supplemental material show the epidemiological data and the data set of the analyzed genomes.

The Tn*5801* backbones were classified in two main groups arbitrarily designated by capital letters as "group A" (14 types, A1 to A14) and "group B" (9 types, B15 to B23), which are shown in Fig. 1. These two groups differ in length (20 to 25 kb, due to the presence of an extra 5-kb fragment at the left arm of the group A transposons), nucleotide sequence, and the presence of indels and rearrangements in different regions. Variants within each group were designated by the corresponding capital letter followed by a number. The detailed analysis of the core sequences facilitated

TABLE 2 Presence of Tn*5801* types A and B in *Firmicutes* sequences deposited in the GenBank genome database as of January 2015

Species	No. of genomes studied	No. with Tn*5801* type: A	B
Streptococcus agalactiae	301	1	42
Streptococcus mitis	23	1	
Streptococcus thoraltensis	1		1
Enterococcus faecalis	344	1	13
Enterococcus faecium	254	16	7
Enterococcus villorum	2	2	
Staphylococcus aureus	4,130	139	
Staphylococcus pseudintermedius	4	1	
Lactococcus garvieae	14	1	

detection of differences and common features within a given type (Fig. 2; see Table S2 in the supplemental material). The GC percentage of the common Tn*5801* backbone (36%) contrasted with that of the 5-kb fragment found in type A sequences (32%) (see Fig. S2 in the supplemental material). An alignment of selected variants is shown in Fig. 3.

Below we analyze in detail the features of variants of these clusters.

(i) Tn*5801* group A. Fourteen variants (A1 to A14) were identified among the available sequences (Fig. 1 and Table 3; see Table S2 in the supplemental material). Phylogenetic analysis allowed us to group them in different subgroups of highly related variants (Fig. 2).

Tn*5801* type A1 was considered the paradigm of the group. It was detected mostly in *Staphylococcus* (96%; n = 107 *S. aureus/S. pseudintermedius* isolates out of 112 isolates with Tn*5801* type A1) and was also the predominant Tn*5801* variant within this genus (76%; n = 107/140 *Staphylococcus* isolates carrying Tn*5801*). Variant A1 was also present in clonally unrelated *E. faecium* and *E. faecalis* isolates and species of *Streptococcus* (*S. agalactiae* and *S. mitis*), as reported previously (19). It is of note that most Tn*5801* type A1 isolates showing particular mutations were associated with specific geographical locations and/or from specific clonal backgrounds (Fig. 2; see Tables S2 and S3 in the supplemental material) (see below). For example, most *S. aureus* isolates (83%; n = 88/106) carrying Tn*5801* A1 belonged to CC8 (ST8, ST507, and ST609; many of them were recovered in the United States), and only a few isolates were associated with other clonal lineages (ST5 and ST664).

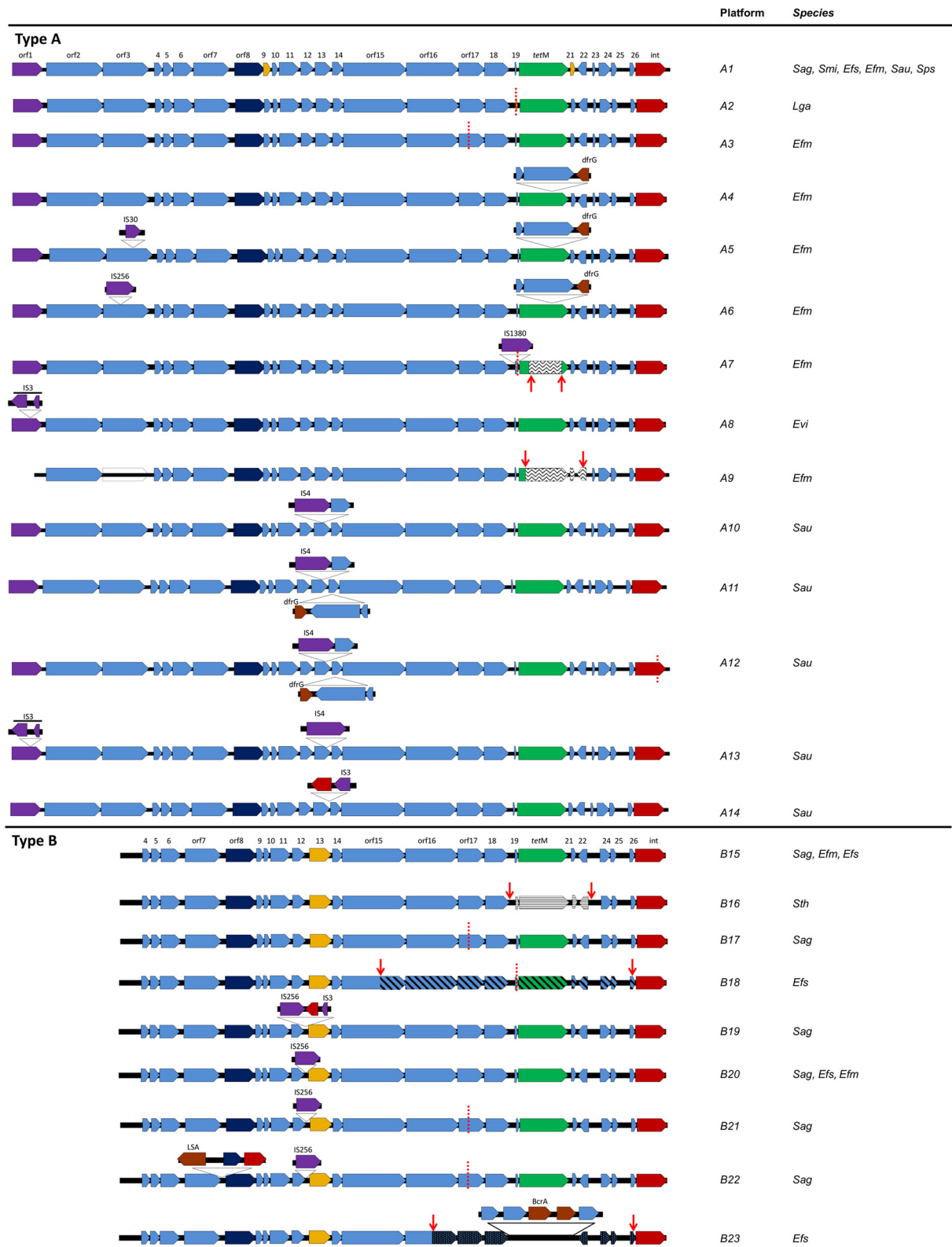Variant A4 contained a 3,285-bp insertion within the *tet*(M)

FIG 1 Diversity of Tn*5801*-like elements, types A and B, based on strains used in this study and sequences deposited in the GenBank genome database. *In silico* comparative analysis was made using BLAST and MUSCLE software available at the BLAST (http://www.ncbi.nlm.nih.gov/blast//) and MUSCLE (http://www .ebi.ac.uk/Tools/msa/muscle/) websites. Dashed red lines, deletion sequences; red ORFs, ORFs encoding integrase-like proteins; dark blue ORFs, ORFs encoding replication proteins; light blue ORFs, ORFs encoding hypothetical proteins; green ORFs, tetracycline resistance gene *tet*(M); yellow ORFs, *orf*9 and *orf*21 are absent in *Staphylococcus aureus* Mu50 (reference genome) and *orf*13 in type B differs from *orf*13 in type A; brown ORFs, other resistance genes; purple ORFs, insertion sequences (IS). Wavy lines, recombination with Tn*5397*; straight lines, recombination with Tn*916*; diagonal lines, recombination with type A variant. ORFs with spots, B23 sequence differing from type B variants. *dfrG*, dihydrofolate reductase; LSA, lincosamides and streptogramins A; BcrA, bacitracin transport; *Sag*, *Streptococcus agalactiae*; *Smi*, *Streptococcus mitis*; *Efs*, *Enterococcus faecalis*; *Efm*, *Enterococcus faecium*; *Sau*, *Staphylococcus aureus*; *Sps*, *Staphylococcus pseudintermedius.* All IS correspond to IS families: IS*1062* (IS30 family, A5), IS*1542* (IS*256* family, A6), IS*1678* (IS*1380* family, A7), IS*Efa8* (IS*3* family, A8), IS*1542* (IS*256* family; B19, B20, B21, and B22), IS*Lgar5*/ISE*fm2* (IS*256* family, *orf*1).

gene that was flanked by direct and inverted repeats that comprised *dfrG* (encoding a dihydrofolate reductase) and two hypothetical protein sequences. Such an element confers resistance to trimethoprim, and it was also detected within *orf*14 of Tn*5801* types A11 and A12 (see below), in the Tn*916*-like transposon (Tn*6198*) of *Listeria monocytogenes*, in the plasmid pMG1 from *E. faecium*, and also in *Streptococcus pyogenes* isolates from India (35–37). The backbone of A4 was identical to that of Tn*5801* types A5 and A6, which also showed additional insertion sequences (IS) interrupting the *uvrD* (*orf*3) gene (IS*30* or IS*256*, respectively). All A4, A5, and A6 variants were found only among *E. faecium* ST18 isolates. Interestingly, the core sequences of A4, A5, and A6 variants clustered with type A1 sequences from human isolates of *E. faecium* and *S. aureus* from Japan and different European countries and shared specific mutations (G2336A_*orf*17, A3918T_*orf*16, A6574T_*orf*15, and T13303C_*orf*6), suggesting a common origin for them (Fig. 2; see Table S3 in the supplemental material).

Types A2, A7, A8, A13, and A14 and some A1 subvariants also clustered together (Fig. 2). Types A7 and A8 from enterococci and A2 from *L. garvieae* were highly similar according to the backbone consensus sequence (only 1 nucleotide [nt] of difference, G2644A_*orf*17). Types A2 and A7 (and also B18, an "A+B" Tn*5801* hybrid [see below]) shared a 125-bp deletion in *orf*19 (*orf*12$_{Tn916-like}$) that also appeared in the Tn*5801*-like element originally called CW459*tet*(M) from *C. perfringens* (16). In A7, such a deletion is upstream of *tet*(M) and next to an IS*1380*/IS*1678* insertion. A7 also showed a mosaic *tet*(M) gene with Tn*5397* *tet*(M) (GenBank accession number NG_034213.1). Variant A8 identified in a genome of *E. villorum* had the *orf*1 (identified as IS*Lgar5*/IS*Efm2* belonging to the IS*256* family) truncated by a fragment comprising two ORFs identified as IS*Efa8* (IS*3* family). Variants A13 (ST5) and A14 (ST247) from *S. aureus* isolates exhibited insertions of partial and complete copies of the IS*4* family and IS*Efa8* (IS3 family) sequences in *orf*14 and *orf*1 (variant A13) or an insertion with an integrase core domain within *orf*14 (variant A14). A2, A7, A8, A13, A14, and A1 shared 5 mutations, namely, G1457A and T1804C in *orf*18, G3043C_*orf*17, G70008T_*orf*15, and C13758T_*orf*4. Some isolates collected in common geographical areas also had specific additional amino acid changes (see Table S3 in the supplemental material).

Variants A10, A11, and A12 clustered together (Fig. 2) and contained insertions within *orf*13 and *orf*14 at different positions, which include an ATP binding protein and a hypothetical protein encoded within *orf*13 (A10, A11, and A12) or the cassette comprising *dfrG* mentioned above in *orf*14 (A11 and A12). A12 also had a 107-bp deletion in the integrase. They were mostly observed in *S. aureus* belonging to ST239 (84%; *n* = 26/31) and shared four nucleotide change mutations (T7199C_*orf*15, G7884A_*orf*14, T9310A_*orf*11, and C12423T_*orf*7) (see Table S3 in the supplemental material). Specific mutations were detected among isolates from specific geographical areas (variant A10 from China had an A4479G_*orf*16 mutation which was absent in A10 from the United States or Australia).

Variant A3, detected in a few isolates of *E. faecium* (ST50) collected in France during the 1990s, clustered separately and showed an 81-bp deletion in *orf*17 (Fig. 2). Variant A9, previously described as Tn*6086* (GenBank accession no. HM636636.1), was a chimera of Tn*5397* [region *tet*(M)-*orf*21-*orf*22] and Tn*5801* and

also had an A/T mutation at nt 985 of the *uvrD* (*orf*3) gene that resulted in a stop codon and lack of *orf*1. It is of note that the A9 platform detected in *E. faecium* TC6 clustered with other A1 variants from *S. mitis* and *E. faecalis* isolated in Germany and Denmark, respectively, all sharing three distinct mutations (T3046A, T3586A, and C4861T). In fact, *E. faecium* TC6 is an *E. faecium* D344SRF (ST25) transconjugant from strain C68 that contains the same A9 variant (38), thus reflecting transferability of the element. Independent recombination events between Tn*5801* and Tn*5397* seem to have occurred in A9 and A7.

(ii) Tn*5801* group B. Group B comprises nine Tn*5801* elements identified among available sequences of streptococci and enterococci (B15 to B23) (Fig. 1 and Table 3; see Table S2 in the supplemental material). They split into three main clusters represented by platforms B15 to B22 (mainly in streptococci) and B18 and B23 (both in enterococci).

The B15 variant (also designated Tn*5801*.Sag) (20) was considered the paradigm of the Tn*5801* type and was detected among *E. faecalis* (this study), *E. faecium*, and *S. agalactiae* isolates. Some type B Tn*5801* elements shared a deletion of 81 bp in *orf*17 (B17, B21, and B22 from *S. agalactiae*; also detected in A3 from *E. faecium*) or an IS*256*-like insertion upstream of *orf*13 (B20, B21, and B22). The B19 sequence had this IS*256* insertion followed by an integrase core domain and an IS*3* family element. The B22 variant had an insertion of a 5,227 bp between *orf*7 and *orf*8, which comprises a phage-specific recombinase gene, a replication protein gene, and a *lsa*(C) gene encoding resistance to lincosamide, streptogramin A, and pleuromutilins (39). Such an insertion is identical to another found in *S. agalactiae* UCN70 (GenBank accession number HM990671.1) and similar to that of a Tn*916*-like element from *S. mitis*, although it lacks the *lsa*(C) gene (18, 40).

Type B23 has a region of five ORFs (encoding a transcriptional regulator, an histidine kinase, a bacitracin ABC transporter, a bacitracin transport permease, and an ABC transporter permease-like protein) instead of *orf*19-*tet*(M)-*orf*21. The sequence between *orf*26 and *orf*16 shared only a 92% similarity with the other type B sequences. This B23 variant was restricted to clonal complex 8 (CC8) (ST8, ST64, and ST90) *E. faecalis* isolates, one of the most ancient *E. faecalis* lineages (41). It included the oldest Tet^r strain described to date (an ST90 isolate from 1954, part of the historical collection of the Laboratory of Streptococcal Diseases at the National Institute of Allergy and Infectious Diseases in the United States) and ST8-related isolates of an emblematic strain widely used as a recipient (strains FA2 and JH2-2) which predate 1973 (41). The B18 variant is a mosaic of type A (region between *orf*26 and *orf*15) and type B (integrase, *orf*4 to part of *orf*15) platforms and exhibits the 125-bp deletion in *orf*19 also observed in type A variants (A2 and A7). The integrases of B18 and B23 share three unique nucleotide changes. Finally, variant B16, detected in an *S. thoraltensis* isolate (GenBank accession no. ARCI01000001.1), revealed a recombination event that resulted in the replacement of the *orf*19-*orf*22 region of Tn*5801* by that of Tn*916*.

Epidemiological background of Tn*5801* among enterococci. *int*$_{Tn5801}$ was detected in 10% of the enterococcal isolates analyzed (*n* = 61/610; 41 *E. faecium*, 13 *E. faecalis*, and 7 *Enterococcus* spp.) (Table 3). A large proportion of the *int*$_{Tn5801}$-positive isolates (84%; *n* = 51/61) were resistant to tetracycline, and most of them carried *tet*(M), *tet*(L), and/or *tet*(S) (77% [*n* = 47/61], 38% [*n* = 23/61], and 2% [*n* = 1/61], respectively). Specific transposon sequences such as *int/xis*$_{Tn916}$ (16%; *n* = 10/61), resolvase$_{Tn5397}$
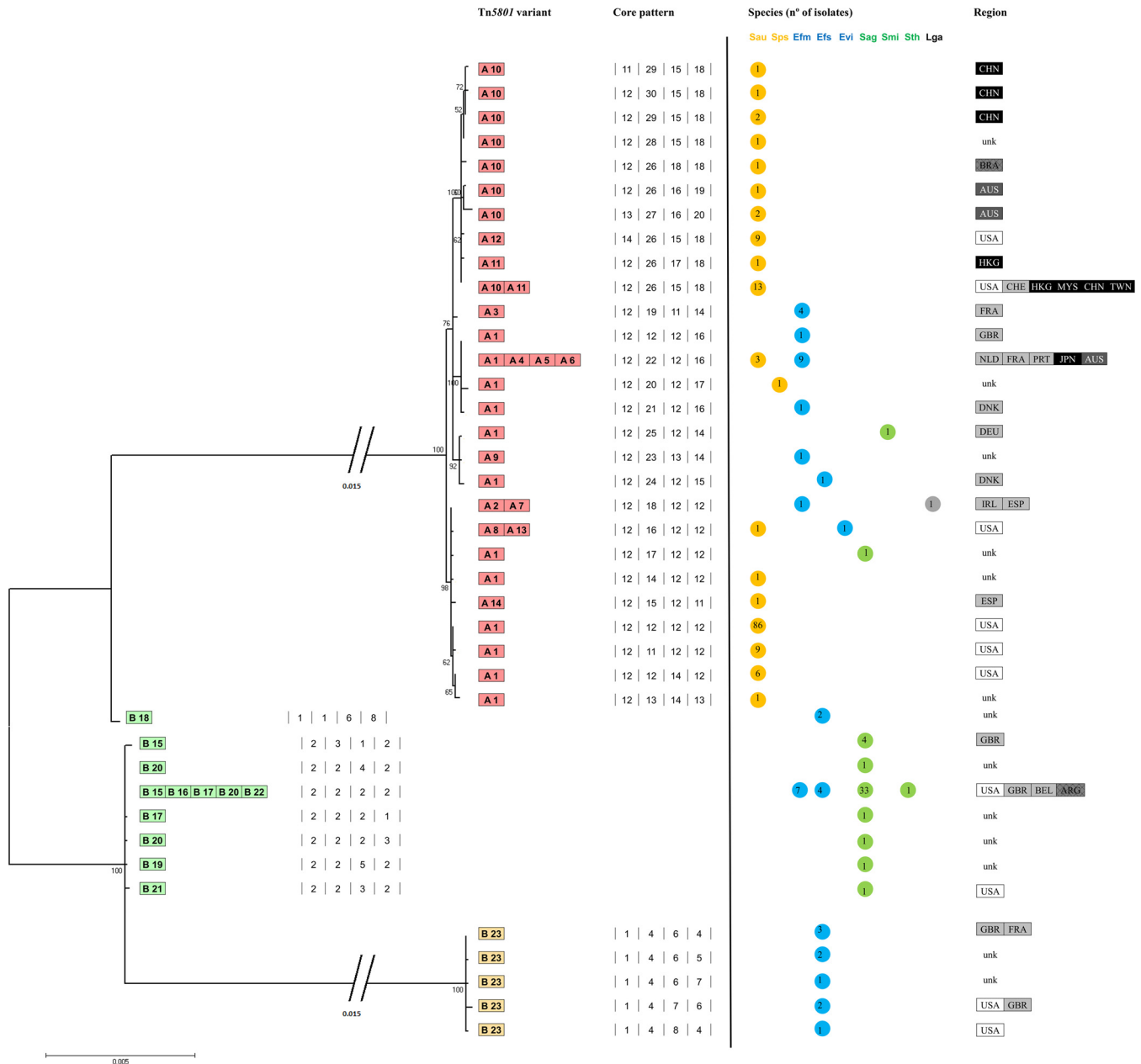
FIG 2 Phylogenetic analysis of Tn*5801* variants in *Firmicutes*. The 4 numbers separated by lines indicate the four core fragments (*int*Tn*5801*, *orf*18-14, *orf*12-8, and *orf*7-4) representing the common region in all Tn*5801* variants described in the GenBank database. These sequences were aligned, concatenated, trimmed, and further analyzed. A maximum-likelihood phylogenetic tree is represented, with 1,000 bootstrap replications. The *orf*26-19 fragment was excluded from this analysis because of its variability (resulting from both mutation and recombination events). The distribution of Tn*5801* variants by species and geographical regions is also represented. Countries within each continent are represented by different tones of gray, black and white. Sau, *Staphylococcus aureus*; Sps, *Staphylococcus pseudintermedius*; Efm, *Enterococcus faecium*; Efs, *Enterococcus faecalis*; Evi, *Enterococcus villorum*; Sag, *Streptococcus agalactiae*, Smi, *Streptococcus mitis*, Sth, *Streptococcus thoraltensis*; Lga, *Lactococcus garvieae*; ARG, Argentina; AUS, Australia; BEL, Belgium; BRA, Brazil; CHE, Switzerland; CHN, China; DEU, Germany; DNK, Denmark; FRA, France; GBR, Great Britain; HKG, Hong Kong; IRL, Ireland; JPN, Japan; MYS, Malaysia; NLD, The Netherlands; PRT, Portugal; ESP, Spain; TWN, Taiwan; USA, United States; unk, unknown.

(7%, *n* = 4/61), or *int/xis*$_{Tn6000}$ (2%; *n* = 1/61) were also observed. The Tn*5801* platforms identified in isolates of this collection correspond to A1, A4, and B15 variants. Epidemiological features of the *int*$_{Tn5801}$-positive isolates of *E. faecium* and *E. faecalis* are shown in Table 3.

Location of Tn*5801*. All Tn*5801* platforms identified in enterococci in this study were chromosomally located at the 3′ end

of *guaA*, as previously observed for three emblematic Tn*5801* elements of staphylococci, *Clostridium*, and *S. agalactiae* (13, 28). In the available sequences of the Tn*5801* variants was the 11-bp sequence described previously as direct repeats (DRs) situated within the 3′ end of *guaA* (13). All *guaA* sequences within the same species had high similarity (99 to 100%), which was somewhat lower (70 to 80%) when comparing isolates from different species.
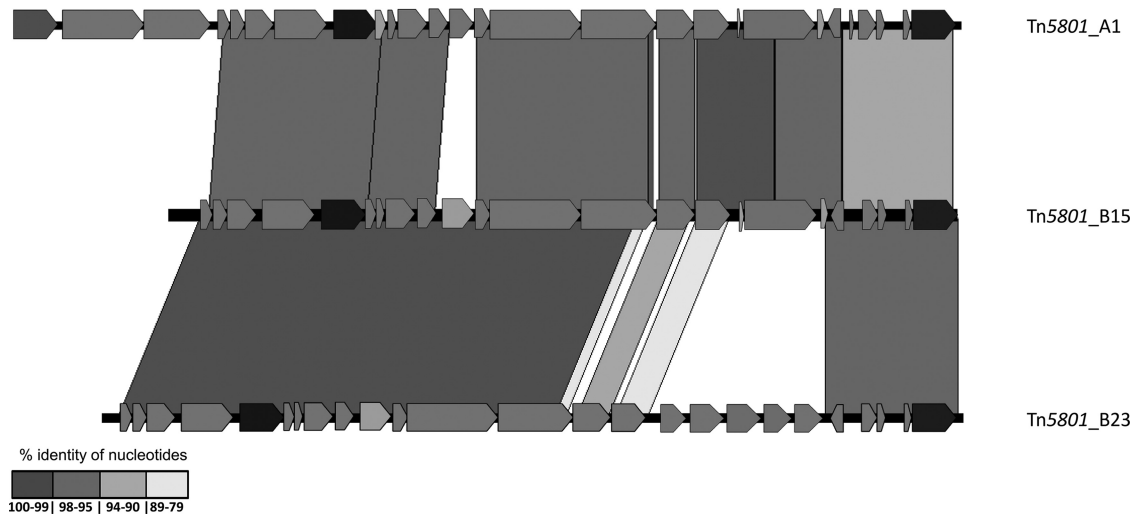
FIG 3 Alignment of selected transposons (Tn*5801*_A1, Tn*5801*_B15, and Tn*5801*_B23) by using the Artemis comparison tool (ACT). Only regions of >50 bp are represented. Gray areas mark matching regions of the same orientation among ICE from different species, indicating an identity between 79 and 100%. Only the areas with the highest percentages of similarity are shown.

Tn*5801* transfer in *Enterococcus*. The ability of Tn*5801* to be transferred was studied in mating assays using enterococcal isolates carrying the Tn*5801* variants A1, A4, and B15 as donors. Only Tn*5801* variant B15 was successfully transferred by conjugation to *E. faecalis* strains JH2-2 and OG1RF. The conjugation frequency was similar for the two receptors despite the presence of the B23 variant in the genome of the JH2-2 recipient (Table 4). Hybrid-ization of SmaI-digested DNA from transconjugants with specific probes for *guaA*, *int*$_{Tn5801}$, and *tet*(M) allowed us to infer the location and the size of the transferred region (see Fig. S3 in the supplemental material). A single band of 150 kb that hybridized with the three probes tested was observed for all *E. faecalis* OG1RF transconjugants obtained in primary mating experiments. For *E. faecalis* JH2-2 transconjugants, the sizes of the bands that hybrid-

TABLE 3 Epidemiological features of the enterococcal isolates carrying Tn*5801*-like ICE[a]

Species	Tn*5801* platform (*n*)[b]	MLST (BAPS)	No. of isolates	Yr	Source(s) (*n*)	Country (*n*)	Tet^r phenotype(s) (*n*)	Tet^r gene(s) (*n*)	Other Tn(s) (*n*)
E. faecalis	B15 (2)	ST9/CC9	3	2001	HP	ESP, ARG	+	*tet*(M) (3), *tet*(L) (3)	
	A1	ST30/CC30	1	1997	HP	AUS	+	*tet*(M)	
	B15 (1)	ST318/CC9	2	ND	HP	BRA	+	*tet*(M)	
	B15	ST55/CC55	1	2001	HV	PRT	+	*tet*(M)	Tn*916*, Tn*6000*
	A1	ST445	1	2001	SP	PRT	+	*tet*(M), *tet*(L), *tet*(S)	
	B15 (3)	ND/ND	5	2001/2002	HP (2), HV (2), SW (1)	BRA (2), PRT (3)	+	*tet*(M)	Tn*916* (3)
E. faecium	+	ST17/CC17 (3.32)	7	1998–2005	HP (5), SW (2)	ESP (3), AUS (1), POL (1), PRT (2)	+ (6), − (1)	*tet*(M) (5), *tet*(L) (3)	
	+	ST16/CC17 (3.32)	2	1999	HP	AUS (2)	+	*tet*(M)	
	A1 (1), A4 (2)	ST18/CC17 (3.31)	9	1996–2005	HP (7), SW (2)	PRT (8), SER (1)	+	*tet*(M) (7), *tet*(L) (7)	Tn*916* (2), Tn*5397* (2)
	+	ST64/CC17 (3.31)	4	2003–2004	HP	CHI	+	*tet*(M) (3)	
	+	ST173/CC17 (3.31)	1	2000	HP	AUS	+	*tet*(M), *tet*(L)	
	+	ST80/CC17 (2.1a)	1	2004	HP	HUN	+		
	+	ST132/CC17 (3.31)	1	2001	HP	PRT	+	*tet*(M), *tet*(L)	Tn*5397*
	A1	ST182/CC17 (7)	1	1992	HP	USA	+	*tet*(M)	
	+	ST202/CC17 (3.32)	1	2005	HP	POL	+	*tet*(M), *tet*(L)	
	A1	ST50/CC9 (2.1a)	1	NA	HP	BRA	+	*tet*(M)	
	+	ST366 (5)	1	2000	HP	PRT	+	*tet*(M)	Tn*916*
	+	ST368 (3.31)	1	2001	SW	PRT			
	+	ND/ND	11	2001–2002	HP (4), HV (1), SW (3), P (2), SP (1)	PRT (9), ESP (1), TUR (1)	+ (8), − (3)	*tet*(M) (8), *tet*(L) (5)	Tn*916* (2), Tn*5397* (1)
Enterococcus sp.	+	ND/ND	7	2001–2006	HV (4), SW (2), SP (1)	PRT	+ (3), − (4)	*tet*(M) (4), *tet*(L) (1)	Tn*916* (1)

[a] Abbreviations: ST, sequence type; BAPS, Bayesian analysis of population structure; HV, healthy volunteer; HP, hospital patient; SW, sewage; P, poultry; SP, swine/piggeries; ARG, Argentina; AUS, Australia; BRA, Brazil; CHI, China; HUN, Hungary; POL, Poland; PRT, Portugal; ESP, Spain; SRB, Serbia; TUR, Turkey; ND, not determined; Tet^r, tetracycline resistance; Tn(s), transposon(s).

[b] +, Tn*5801*-like platform which has not been characterized in the corresponding type.

TABLE 4 Results of filter mating assays with *Enterococcus faecalis* strains[a]

Transconjugant	Donor[b]	Recipient	No. of Tn5801 copies detected	Hybridized SmaI-digested DNA band size (added fragment size), kb	Hybridized probe			Transfer rate per donor	Growth rate, mean/SD	Relative growth rate, % (transconjugant/recipient[c])	Tet MIC, μg/ml
					int	*tet*	*gua*				
JH2-2::Tn5801_1	*E. faecalis* Ef1	JH2-2	1	200 (50)	+	+	+	1.6×10^{-8}	0.15437/0.01096	3.66385	48
JH2-2::Tn5801_2	*E. faecalis* Ef1	JH2-2	2	250 (100)	+	+	+	1.6×10^{-8}	0.15489/0.01568	3.34281	96
OG1SS::Tn5801_1.1	JH2-2::Tn5801_1	OG1SS	1	150 (20)	+	+	+	1.81×10^{-7}	0.16787/0.00576	3.16674	32
OG1SS::Tn5801_1.2	JH2-2::Tn5801_1	OG1SS	2	340 (20)	+	+	−	1.01×10^{-8}	0.16773/0.00321	3.24749	96
				150 (20)	+	+	+				
OG1RF::Tn5801_1	*E. faecalis* Ef1	OG1RF	1	150 (20)	+	+	+	3.3×10^{-8}	0.16087/0.00769	0.16068	24
OG1RF::Tn5801_2	*E. faecalis* Ef1	OG1RF	1	150 (20)	+	+	+	3.3×10^{-8}	ND[d]	ND	48
OG1RF::Tn5801_3	*E. faecalis* Ef1	OG1RF	1	150 (20)	+	+	+	3.3×10^{-8}	ND	ND	64
OG1SS::Tn5801_2.1	JH2-2::Tn5801_2	OG1SS	1	150 (20)	+	+	+	2×10^{-7}	0.17217/0.00709	0.68706	96
OG1SS::Tn5801_2.2	JH2-2::Tn5801_2	OG1SS	2	150 (20)	+	+	+	2×10^{-7}	0.17717/0.00661	−2.19576	96
				120 (20)	+	+	−				
OG1SS::Tn5801_2.3	JH2-2::Tn5801_2	OG1SS	2	200 (50)	+	+	−	1.6×10^{-5}	0.17283/0.00776	0.30828	96
				170 (50)	+	+	+				
OG1SS::Tn5801_2.4	JH2-2::Tn5801_2	OG1SS	1	150 (20)	+	+	+	1.6×10^{-5}	0.16903/0.00645	2.50019	32

[a] Following the order presented in Fig. S3 in the supplemental material.
[b] *Enterococcus faecalis* Ef1 tetracycline MIC, 48 μg/ml.
[c] Recipient growth rates (means/standard deviations): 0.16024/0.01179 (*E. faecalis* JH2-2), 0.17336/0.00362 (*E. faecalis* OG1SS), and 0.16112/0.00771 (*E. faecalis* OG1RF).
[d] ND, not determined.

ized with the probes were 200 and 250 kb (the estimated size of the transferred region was ca. 20 kb for OG1RF and ca. 50 or 100 kb for the two JH2-2 transconjugants). Mating assays using these *E. faecalis* JH2-2::Tn5801_1 (50 kb) and *E. faecalis* JH2-2::Tn5801_2 (100 kb) transconjugants as donors and *E. faecalis* OG1SS as the recipient yielded *E. faecalis* OG1SS::Tn5801 transconjugants with one or two copies of a ca. 20-kb transposable element. All transconjugants that contain two Tn5801 copies showed positive hybridization of two bands with the int$_{Tn5801}$ and *tet*(M) genes, but only one band hybridized with the *guaA* probe (see Fig. S3 in the supplemental material). Moreover, a PCR mapping assay demonstrated the presence of a B15 Tn5801-like element in all primary and secondary transconjugants, while amplification of specific B23 fragments was observed only for one primary and one secondary transconjugant, *E. faecalis* JH2-2::Tn5801_2 and *E. faecalis* OG1SS::Tn5801_2.3, respectively (Table 4; see Fig. S3 in the supplemental material).

Fitness cost of the transconjugants. The growth rates of *E. faecium* transconjugants containing B15 variants ranged from a 2.2% increase to a 3.7% reduction in the growth rate of the recipient strain (growth rates and standard deviations are indicated in Table 4). Detailed analysis of the data revealed that the secondary transconjugants (using JH2-2 as donor and OG1SS as recipient) that apparently acquired two entire Tn5801 elements yielded different values of fitness increase or fitness reduction in comparison with the donor strain. They correspond to transconjugants that showed slightly different PFGE types, showed hybridization patterns that indicate insertion of the two acquired Tn5801 at different genomic locations, and had a high MIC of tetracycline (96 μg/ml) (Table 4).

DISCUSSION

This study represents the first description of Tn5801-like elements in enterococci and one of the first studies addressing the evolutionary changes of antibiotic resistance transposons throughout decades. To date, only scarce Tn5801-like backbones of a few *S.*

aureus and *Streptococcus* strains have been identified in separate studies (3, 15, 18–20), which did not analyze the clonal context of the isolates, thus limiting the understanding of the evolvability of this element.

Sequence analysis of type A and B variants revealed a common Tn5801 backbone compatible with an *S. agalactiae* origin on the basis of the GC content (36%) and suggested the further acquisition of a 5-kb extra fragment by staphylococci (GC content = 32%). Such acquisition might have occurred through an IS256 family insertion event (*orf*1 of the group A platforms) and would have resulted in the emergence of type A variants.

The apparent confinement of Tn5801 type A or B in particular clonal backgrounds is remarkable. A recent study suggests that the introduction and massive use of tetracycline since the 1950s could have contributed to a positive selection of neonatal hypervirulent clonal lineages (CC17, CC23, or CC10) of *S. agalactiae* carrying *tet*(M)-CTn, as Tn5801, which we here designated Tn5801 B type platforms (1). We were also able to detect Tn5801 in predominant antibiotic-resistant clonal lineages associated with human infections, such as *S. aureus* (CC8, CC5, and ST239) and *E. faecium* (ST17 and ST18) from the 1990s, or early lineages of *E. faecalis* (CC8). However, our data and available knowledge necessitate a carefully interpretation of the relationship between the emergence and persistence of antibiotic resistance and selective processes of particular clones due to antimicrobial treatments (42). In this respect, we should take into account therapeutic strategies and relevant bacterial features that could have facilitated the selective acquisition of certain elements by particular clones. For example, anti-restriction-modification (anti-RM) occurring in Tn916-like elements often influences selective horizontal gene transfer between staphylococcal populations and other species of *Firmicutes* (43, 44). Putative anti-RM detected in Tn5801 (*orf*12) acts against staphylococcal type I RM systems which are present in *S. aureus* CC5 and CC8 (the backgrounds in which Tn5801 seems to have initially been detected in the late 1950s) (3, 15) and ST239 (an emergent hybrid lineage of ST8 and ST30) (10, 17, 45).

Deep sequence analysis is a powerful tool to accurately establish transmission of pathogens (46). In this study, the comprehensive analysis of Tn*5801* variants identified some types associated with sympatric populations of major clonal lineages (as reflected by the presence of specific nucleotide changes among isolates from particular geographical areas). This seems clear for *S. aureus* ST239 (which carries highly related A10, A11, and A12 variants) and ST8/ST609 human isolates from the United States recovered from 1994 to date (carrying A1), for which differences in the genome sequences of regional endemic clones have also been reported (17, 44). Besides clonal spread of strains carrying Tn*5801*, the presence of some variants in isolates of different species recovered in different countries and, eventually, different hosts indicates that horizontal gene transfer has also contributed to the current occurrence of Tn*5801*. This study demonstrates the transference of Tn*5801* between different *E. faecalis* backgrounds, even if the strain already harbored a Tn*5801* element. Although the *guaA*-associated islands theoretically integrate site specifically, tyrosine recombinases can be functional at secondary integration sites (11, 12). This would explain the insertion of elements at different genomic locations (transconjugants *E. faecalis* OG1SS::Tn*5801*_1.2, OG1SS::Tn*5801*_2.2, and OG1SS::Tn*5801*_2.3). Moreover, integration at the same site cannot be discarded if a consensus hot spot remains available for new integration events after the first Tn*5801* acquisition. For example, two *E. faecalis* genomes (*E. faecalis* Com1 and *E. faecalis* Com2; GenBank accession no. AJES01000002.1 and AJBL01000002.1, respectively) harbored a composite structure that comprises Tn*5801* B15 and a putative transposable element (GC content of 27.3%, compatible with a clostridial origin [47]), both located upstream of the *guaA* gene. The clostridial element seems to have been inserted at the 11-bp hot spot of a *guaA*-int_{Tn5801} region. Finally, recombination cannot be discarded for some cases, as described for other elements in *E. faecalis* (48). Such events increase the number (if they include different genes) or the level of resistance (if the gene dosage is increased as we observed here), with a variable fitness cost to the cell, influencing the probability of being selected and stably maintained.

Although the classical JH2-2 lab strain was serendipitously useful for this work, as it allowed detection of the acquisition of more than one Tn*5801* element by a single strain, the presence of conjugative genetic elements that can interfere with the transferability of other elements under study opens discussion about the suitability of certain classic laboratory strains for use in transfer experiments.

CTn-*tet*(M) elements with the same genetic backbone may carry different determinants associated with resistance (e.g., to bacitracin, lincosamides/streptogramins, and trimethoprim in our study) and/or ISs (IS*3*, IS*4*, IS*30*, IS*256*, and IS*1380* families) which are frequently designated with different numbers in other studies (7, 20, 49). However, all these variants reflect the dynamics of a basic particular element circulating among bacterial communities. In this study, ISs with intact inverted repeats (IR) were observed within different genes (*orf*1, *orf*3, *orf*13, and *orf*19) of all Tn*5801* type A isolates of *S. aureus* and *E. faecium*, suggesting their recent acquisition. Among type B variants, only the mutator family IS*256* was consistently detected upstream of *orf*13 (types B20, B18, and B19), and this stability persisted even when some particular variants (e.g., B20) were disseminated across genera. Some ISs from the IS*256* family have been implicated as modulators of gene

expression, suggesting that future studies might reveal the role of this IS in the function of type B Tn*5801*-like elements (50–53).

In summary, this study shows that Tn*5801*-like variants have been present among *Firmicutes* since at least the 1950s, being predominant in microorganisms obtained from human hosts (54). Different waves of expansion in human populations of certain clones of *Staphylococcus*, *Enterococcus*, and *Streptococcus* seem to play an important role in their occurrence and evolvability, although transfer events between different clonal backgrounds would also contribute to their spread, as demonstrated experimentally here for *E. faecalis*. The detection of genetic rearrangements in the four functional regions highlights once more the plasticity of CTns, potentially influencing microevolutionary events eliciting adaptive functions in microbial populations and communities in common or heterogeneous hosts and environments (55–58).

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