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# Resistance Genes and Genetic Elements Associated with Antibiotic Resistance in Clinical and Commensal Isolates of *Streptococcus salivarius*

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The diversity of clinical ( $n = 92$ ) and oral and digestive commensal ( $n = 120$ ) isolates of *Streptococcus salivarius* was analyzed by multilocus sequence typing (MLST). No clustering of clinical or commensal strains can be observed in the phylogenetic tree. Selected strains (92 clinical and 46 commensal strains) were then examined for their susceptibilities to tetracyclines, macrolides, lincosamides, aminoglycosides, and phenicol antibiotics. The presence of resistance genes *tet(M)*, *tet(O)*, *erm(A)*, *erm(B)*, *mef(A/E)*, and *catQ* and associated genetic elements was investigated by PCR, as was the genetic linkage of resistance genes. High rates of erythromycin and tetracycline resistance were observed among the strains. Clinical strains displayed either the *erm(B)* (macrolide-lincosamide-streptogramin B [MLS<sub>B</sub>] phenotype) or *mef(A/E)* (M phenotype) resistance determinant, whereas almost all the commensal strains harbored the *mef(A/E)* resistance gene, carried by a macrolide efflux genetic assembly (MEGA) element. A genetic linkage between a macrolide resistance gene and genes of Tn916 was detected in 23 clinical strains and 5 commensal strains, with a predominance of Tn3872 elements ( $n = 13$ ), followed by Tn6002 ( $n = 11$ ) and Tn2009 ( $n = 4$ ) elements. Four strains harboring a *mef(A/E)* gene were also resistant to chloramphenicol and carried a *catQ* gene. Sequencing of the genome of one of these strains revealed that these genes colocalized on an IQ-like element, as already described for other viridans group streptococci. ICES<sub>3</sub>-related elements were also detected in half of the isolates. This work highlights the potential role of *S. salivarius* in the spread of antibiotic resistance genes both in the oral sphere and in the gut.

*Streptococcus salivarius* is a Gram-positive commensal bacterium that is a major bacterial constituent of saliva and of biofilms found on the human tongue dorsum (1). It belongs to the early microbial community that colonizes the oral cavities of newborns (2) and persists in the adult oral flora (3). This species is considered to contribute to oral health by preventing the colonization of mucosal surfaces with pathogens involved in upper respiratory tract infections (4). For instance, *S. salivarius* strains have been reported to inhibit biofilm formation by *Streptococcus pyogenes* (5) and *Streptococcus mutans* (6) as well as adherence by *Candida albicans* (7). *S. salivarius* is also commonly detected as an inhabitant of the gastrointestinal tracts of healthy humans and has been described as a typical small intestine commensal species (8). However, some strains have been associated with opportunistic infections: a growing number of meningitis cases (9, 10), several cases of endocarditis (11), bacteremia in immunocompromised patients (12, 13), and invasion of the guts of patients with liver cirrhosis (14).

Macrolides and tetracyclines are commonly used for the treatment of streptococcal infections, but high rates of resistance to these antibiotics have been observed among pathogenic and commensal streptococci in the past decade (15–18). Two main mechanisms of macrolide resistance in viridans group streptococci have been described: target site modification and macrolide efflux systems. Target site modification occurs by methylation of 23S rRNA mediated by rRNA methylases, leading to a conformational change that reduces the binding of macrolides, lincosamides, and streptogramin B (MLS<sub>B</sub>) to ribosomes, conferring coresistance to these antibiotics (19). This MLS<sub>B</sub> phenotype may be expressed constitutively (cMLS<sub>B</sub>) or inducibly (iMLS<sub>B</sub>) and is encoded by *erm* (erythromycin ribosome methylation) genes, of which *erm(B)* is the most common

(15, 20). In contrast, the M phenotype mechanism is characterized by a lower level of resistance to erythromycin, and only macrolides with 14 or 15 carbons are expelled from the cell through efflux pumps encoded by *mef(A/E)* (transmembrane domain) and *msr(D)* (ATP-binding domain) genes (21). In viridans group streptococci, resistance to tetracycline is conferred mainly by ribosome protection genes [such as *tet(M)* and *tet(O)*], but efflux pumps encoded by various genes [such as *tet(K)* and *tet(L)*] have also been reported (15, 22).

Mobile genetic elements (MGE) contribute largely to the evolution and diversity of bacterial genomes through horizontal gene transfer (23, 24). Integrative and conjugative elements (ICEs) are MGE that promote their own excision, transfer by conjugation, and integration into a replicon of the recipient cell (25, 26). MGE usually encode adaptive functions that may cause drastic changes in the ecological and/or pathogenic properties of bacterial species. A variety of MGE conferring antibiotic resistance, particularly el-

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ements of the Tn916 family, have been described for streptococci, especially for the two species *Streptococcus pneumoniae* and *S. pyogenes*, which have been studied intensively (27, 28). In contrast, little is known about the prevalence of MGE in commensal bacteria (15, 29), even if they likely act as a reservoir of antibiotic resistance genes transferable to pathogenic bacteria (30–32).

In this work, a large collection of *S. salivarius* strains comprising 120 commensal strains (half isolated from feces and half from saliva) and 92 clinical strains was analyzed by multilocus sequence typing (MLST). One hundred thirty-eight strains (46 commensal strains and the 92 clinical strains) were then selected to reflect the diversity of the collection and were analyzed for their susceptibilities to macrolides, lincosamides, tetracyclines, aminoglycosides, and phenicol antibiotics. PCR was then carried out to search for antibiotic resistance genes and MGE in these strains. For MGE, the focus was on elements described in other streptococci: Tn916 [and variants carrying the *erm*(B) and *mef*(A/E) resistance genes] (27, 28), ICES<sub>St3</sub> (33, 34), the macrolide efflux genetic assembly (MEGA) element (28), and the IQ element (15, 35). In addition, some elements were tested by PCR for excision in order to evaluate their functionality.

## MATERIALS AND METHODS

**Bacterial strains.** Commensal bacterial strains were isolated from the oral cavities and feces of 5 healthy human individuals who had not received antibiotic treatment in the preceding 2 months (four children 15 to 33 months old whose parents gave written consent and one 38-year-old adult from whom written consent was obtained). An aliquot of fresh saliva was diluted in 5 ml M17 broth with 1% glucose (36) and was incubated overnight at 37°C under an anaerobic atmosphere. Feces (0.2 to 0.5 g) was diluted in 5 ml M17 broth with 1% glucose. Dilutions of both samples were then spread on selective mitis salivarius agar (MSA; Difco) plates containing a 1% (wt/vol) tellurite solution. Bacteria were grown at 37°C for 24 to 48 h under an anaerobic atmosphere. A total of 1,000 (100 from the saliva and 100 from the feces of each individual) presumptive *S. salivarius* colonies (i.e., smooth blue “gumdrop” colonies on the selective medium) were picked up and were subcultured 3 times on M17 agar supplemented with 1% glucose before being stored at –80°C. The first letter in the name of each commensal strain stands for the biological material from which it was collected (F for feces and S for saliva), and the first number identifies the human subject (see Table S1 in the supplemental material).

In addition, 92 human clinical strains of *S. salivarius* isolated from different body locations (blood, aortic valve, bones, etc.) were collected from 4 different French university hospitals: Besançon (*n* = 8), Limoges (*n* = 60), Nancy (*n* = 18), and Tours (*n* = 6) (see Table S1 in the supplemental material). The first letter in the name of each clinical strain indicates its geographical origin (B for Besançon, L for Limoges, N for Nancy, and T for Tours).

**Multiplex PCR analysis of isolates.** All strains (both clinical and commensal isolates) were tested by multiplex PCR, with a 492-bp fragment for the *ilvC* gene (encoding ketol-acid reductoisomerase and specific for *S. salivarius*), a 620-bp fragment for the 23S ribosomal gene (specific for *Streptococcus*), and a 100-bp fragment for the 16S ribosomal gene (positive control for PCRs) (C. Delorme, personal communication). The primers used are listed in Table S2 in the supplemental material. PCR amplifications were carried out in a 25- $\mu$ l volume containing 1  $\mu$ l of overnight bacterial culture, 0.4  $\mu$ M each deoxynucleoside triphosphate (dNTP) (Thermo Scientific, France), 0.8  $\mu$ M 16S rRNA-specific primers, 0.32  $\mu$ M 23S rRNA-specific primers, 0.4  $\mu$ M *ilvC*-specific primers, and 2.5 U of DreamTaq DNA polymerase in an appropriate buffer (Thermo Scientific, France). PCR amplifications were performed using the following cycling parameters: 10 min at 95°C (extended time to ensure proper lysis of bac-

terial cells and DNA release), followed by 30 cycles of 30 s at 95°C, 30 s at 53°C, and 1 min at 72°C, with a final 5-min extension at 72°C. Amplified products were analyzed by electrophoresis on a 1.5% agarose gel.

**MLST analysis of isolates.** One hundred twenty commensal isolates (12 oral and 12 fecal isolates per individual) identified as *S. salivarius* by multiplex PCR were selected by visual inspection of the broth cultures (for a phenotype of flocculation or sedimentation) to maximize the diversity of the strains. In total, 212 strains (120 commensal and 92 clinical strains) were analyzed by multilocus sequence typing (MLST). MLST indexes the diversity of the nucleotide sequences of internal fragments of a set of housekeeping genes. Analysis was performed using the *ilvC* (encoding ketol-acid reductoisomerase), *sodA* (encoding superoxide dismutase), *ddlA* (encoding D-alanine ligase), *thrS* (encoding threonyl-tRNA synthetase), *pyrE* (encoding orotate phosphoribosyltransferase), and *dnaE* (encoding DNA polymerase III) genes with the amplification conditions described previously by Delorme et al. (37). The primers used are listed in Table S2 in the supplemental material. The six PCR products (404 to 519 bp of internal gene fragments), obtained independently for each isolate, were examined on a 1.5% agarose gel before sequencing. For each locus, all the sequences were compared using the BioEdit program and Vector NTI software, and new allele numbers were arbitrarily assigned to the sequences if they had not already been described by Delorme et al. (37). For each strain, the combination of alleles at each locus defined an allelic profile, or sequence type (ST).

Phylogenetic analysis of the nucleotide sequences of each housekeeping gene both separately and in a concatenated sequence of 2,299 bp (excluding the *ilvC* gene) was performed using the neighbor-joining (NJ) method and SeaView software (38). A model of the Kimura 2-parameter distance was used to estimate distances for nucleotide sequences. The significance of the groups observed in constructed trees was determined by a bootstrap analysis with 1,000 replicates.

**Pulsed-field gel electrophoresis.** Pulsed-field gel electrophoresis experiments were carried out using a CHEF-DR III system (Bio-Rad) with SmaI as the restriction enzyme as described previously (39).

**Susceptibility to antimicrobial agents and double-disk induction test (D-test).** Clinical strains (*n* = 92) and commensal strains belonging to different STs (26 feces isolates and 20 saliva isolates, with a maximum of 2 strains with the same ST per sampling site per individual) were analyzed for their susceptibilities to five families of antibiotics, including tetracyclines (tetracycline, 30  $\mu$ g per disk), macrolides (erythromycin, 15  $\mu$ g per disk; spiramycin, 100  $\mu$ g per disk), lincosamides (lincomycin, 15  $\mu$ g per disk), aminoglycosides (kanamycin, 1,000  $\mu$ g per disk), and phenicol antibiotics (chloramphenicol, 30  $\mu$ g per disk). Antibiotic susceptibility was determined in accordance with the guidelines of the Antibiogram Committee of the French Society of Microbiology (CA-SFM) ([http://www.sfm-microbiologie.org/page/page/showpage/page\\_id/90.html](http://www.sfm-microbiologie.org/page/page/showpage/page_id/90.html)) by a standard disk diffusion assay on Mueller-Hinton agar supplemented with 5% horse blood. *S. pneumoniae* ATCC 49619 was used as the quality control in each assay. The zone diameters were measured to evaluate the susceptibility of each strain to each antibiotic. The 2014 CA-SFM breakpoints used were as follows: erythromycin, <19 mm; chloramphenicol, <23 mm; tetracycline, <21 mm; spiramycin, <19 mm; lincomycin, <19 mm; kanamycin, <11 mm.

D-tests were performed to determine the phenotypes of macrolide-lincosamide-streptogramin resistance (40). A bacterial suspension was adjusted to match the turbidity of a 0.5 McFarland standard and was streaked onto Mueller-Hinton agar supplemented with 5% horse blood. An erythromycin disk (15  $\mu$ g) and a lincomycin disk (15  $\mu$ g) were placed on the agar surface 15 mm apart. The plate was incubated for 18 h at 37°C. A “D”-shaped area of inhibition around the lincomycin disk was interpreted as indicating inducible resistance to lincosamides (40).

**Detection of antibiotic resistance genes by PCR.** The macrolide resistance genes *erm*(A), *erm*(B), and *mef*(A/E) and the tetracycline resistance genes *tet*(M) and *tet*(O) were detected by PCR amplification using the primers listed in Table S2 in the supplemental material and the con-

ditions previously described by Malhotra-Kumar et al. (41) for multiplex PCR [except for *erm(B)* primers, used at 0.8  $\mu$ M instead of 0.5  $\mu$ M]. The chloramphenicol resistance gene *catQ* was detected as described by Del Grosso et al. (42).

**Detection of genetic elements and tests of excision.** The integrase, relaxase, and coupling protein genes of ICES<sub>St3</sub> from *Streptococcus thermophilus* (33) and the relaxase gene of Tn916 from *Enterococcus faecalis* (43) were detected by PCR amplification using the primers listed in Table S2 in the supplemental material. The linkage of *erm(B)* and *mef(A/E)* with genes of genetic elements (Tn3872, Tn6002, Tn2009, the MEGA element, and the IQ element) was also examined (for the primers used, see Table S2 in the supplemental material).

Tests of excision consisted of the detection of the *attI* site of the circular form of ICEs (ICES<sub>St3</sub>, Tn916, and Tn916-related elements) (see Table S2 in the supplemental material for the primers used). For ICES<sub>St3</sub> elements, detection of an empty integration site (*attB*) was also performed.

PCR amplifications were carried out in a 25- $\mu$ l volume containing 1  $\mu$ l of overnight bacterial culture, 0.4  $\mu$ M each dNTP (Thermo Scientific, France), 0.5  $\mu$ M each primer, and 2.5 U of DreamTaq DNA polymerase in an appropriate buffer (Thermo Scientific, France). PCR amplifications were performed by using the following cycling parameters: 10 min at 95°C, followed by 30 to 35 cycles of 30 s at 95°C, 30 s at 50°C, and 1 min/kb at 72°C, with a final 5-min extension at 72°C. Amplified products were analyzed by electrophoresis on a 1.5% agarose gel.

**DNA sequencing and sequence analysis.** PCR products for the MLST analysis were sequenced by Beckman Coulter Genomics. The genome of *S. salivarius* strain F4-20 was sequenced using an Illumina HiSeq 2000 sequencer by Beckman Coulter Genomics (read length, 2  $\times$  100 bp after paired-end library construction; 60 $\times$  coverage). *De novo* assembly was performed by using CLC Genomics Workbench (CLC bio) with default parameters. Contigs were annotated using the RAST annotation server (<http://rast.nmpdr.org/>) (44, 45). Pairwise comparisons of elements were performed with the Artemis Comparison Tool (46), provided by the Sanger Centre, using comparison files generated by Double Act ([http://www.hpa-bioinfotools.org.uk/pise/double\\_act.html](http://www.hpa-bioinfotools.org.uk/pise/double_act.html)). Comparison figures were manually edited using Inkscape.

**Nucleotide sequence accession numbers.** The sequences of all MLST alleles have been deposited in the European Nucleotide Archive under accession numbers LN811537 to LN811552 (*ilvC* fragment), LN811553 to LN811570 (*sodA* fragment), LN811571 to LN811614 (*ddlA* fragment), LN811615 to LN811648 (*dnaE* fragment), LN811649 to LN811675 (*pyrE* fragment), and LN811676 to LN811704 (*thrS* fragment). The sequence of the IQ-like element of *S. salivarius* strain F4-20 has been deposited in the European Nucleotide Archive and assigned accession number LN812955.

## RESULTS

**MLST analysis of *S. salivarius* isolates.** Two hundred twelve isolates (60 fecal isolates, 60 oral isolates, and 92 clinical strains) were subjected to MLST analysis. The number of alleles detected was 20 for the *ilvC* gene, 25 for the *sodA* gene, 34 each for the *thrS* and *pyrE* genes, 43 for the *dnaE* gene, and 51 for the *ddlA* gene. Two kinds of *ilvC* genes were detected; thus, this gene was not used in the construction of the phylogenetic tree. Ninety-six different STs, all different from the 27 STs described by Delorme et al. (37), were identified (see Table S1 in the supplemental material). Three STs (ST82, ST83, and ST117) share 4 common alleles, and 13 STs (ST32, ST35, ST51, ST62, ST63, ST71, ST72, ST84, ST85, ST95, ST102, ST111, and ST122) share 3 common alleles, with previously described STs (37) (see Table S1 in the supplemental material). A high diversity of strains was observed, without clear clustering of the strains on the phylogenetic tree (Fig. 1). In particular, commensal and clinical strains did not appear in separate clusters.

The most common STs were ST83 (1 clinical and 22 commensal strains), ST57 (15 commensal strains), ST94 (4 clinical and 8

commensal strains), ST44 (12 commensal strains), and ST114 (11 commensal strains).

Some STs were found in 2 clinical strains isolated in the same hospital but not in the same year (ST40 and ST68) or in two different hospitals (ST72). No clonal relationship was observed, even between strains isolated in the same hospital in the same year (except for strains L28 and L33, strains L19 and L23, and strains L38 and L41).

Most of the individuals harbored multiple oral and fecal strains with different STs (as many as 5 STs for 12 strains analyzed per sampling site per individual). All individuals (except for individual 1) harbored strains belonging to the same ST at the two sampling sites analyzed (saliva and feces) (see Table S1 in the supplemental material).

**Antimicrobial susceptibility and presence of antibiotic resistance genes.** Clinical strains ( $n = 92$ ) and commensal strains belonging to different STs (26 fecal isolates and 20 saliva isolates) were analyzed for their susceptibilities to 6 antibiotics (erythromycin, spiramycin, lincomycin, tetracycline, chloramphenicol, and kanamycin) (see Table S1 in the supplemental material).

The results indicated that only 41% of the clinical isolates and 24% of the commensal isolates were susceptible to all the antimicrobials tested (see Table S1 in the supplemental material). High rates of erythromycin resistance were found for both clinical isolates (56%;  $n = 52$ ) and commensal isolates (76%;  $n = 35$ ) (Table 1). No difference in erythromycin resistance was observed between strains isolated from the saliva or feces of healthy individuals. Among the 52 clinical strains that were resistant to erythromycin, 21 harbored only an *erm(B)* gene (cMLS<sub>B</sub> phenotype), except for strain L31, with an iMLS<sub>B</sub> phenotype, 29 carried only a *mef(A/E)* gene (M phenotype), and 2 (L29 and L57) carried both *erm(B)* and *mef(A/E)* genes (Table 1). Most of the erythromycin-resistant commensal strains harbored a *mef(A/E)* gene (M phenotype) ( $n = 32$ ), and only three carried an *erm(B)* gene (cMLS<sub>B</sub> phenotype) (Table 1). Eight strains (5 commensal and 3 clinical) were susceptible to spiramycin but resistant to lincomycin.

The second-highest resistance rates were those against tetracycline: 28% ( $n = 26$ ) for the clinical strains and 17% ( $n = 8$ ) for the commensal strains. Most of the tetracycline-resistant strains carried a *tet(M)* gene ( $n = 32$ ) (see Table S1 in the supplemental material). No *tet(M)* or *tet(O)* tetracycline resistance determinant was detected in 2 strains (L29 and T80). Three clinical strains (L25, L31, and L57) carried a *tet(M)* gene but were susceptible to tetracycline.

Two clinical strains (L20 and N11) and two commensal strains (F3-10 and F4-20) were resistant both to chloramphenicol and to erythromycin. These four strains were found to carry a *mef(A/E)* and a *catQ* gene. In addition, only two clinical strains (T00 and T80) were resistant to kanamycin.

Strains with different antibiotic resistance patterns were isolated from the two sampling sites (oral and fecal) of the same individual. In addition, some fecal and oral isolates of the same individual belonged to the same ST (i.e., ST27, ST32, ST57, ST83, ST94, ST105, and ST114) and harbored the same antibiotic resistance profile (see Table S1 in the supplemental material).

**Detection of genetic elements and tests of excision.** We searched for four families of genetic elements: (i) the integrative and conjugative element ICES<sub>St3</sub> (34) described for *S. thermophilus*, a species that is closely related to *S. salivarius*, (ii) the integrative and conjugative element Tn916 and its variants carrying either an



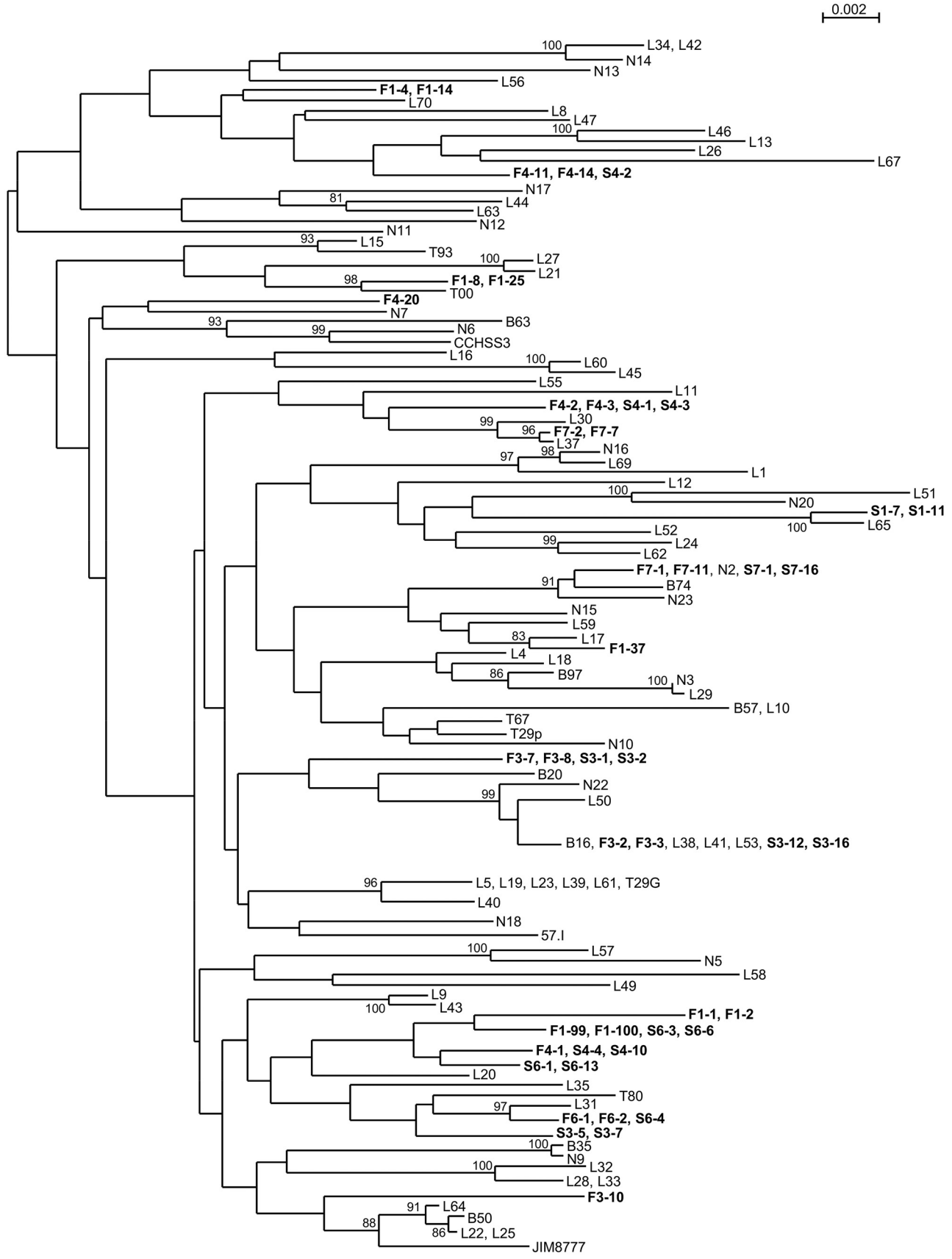


FIG 1 Phylogenetic tree based on the concatenated sequences of five housekeeping genes, *ddlA*, *dnaE*, *thrS*, *pyrE*, and *sodA*, obtained for the clinical and commensal isolates of *S. salivarius* included in this work ( $n = 138$ ). Three strains of *S. salivarius* (JIM8777, CCHSS3, and 57.1) whose genomes have been sequenced were added to the phylogenetic tree as references. Commensal isolates are indicated in boldface. Bootstrap values that are  $>80\%$  are given at the nodes.

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*erm(B)* (Tn3872 and Tn6002) or a *mef(A/E)* gene (Tn2009) (28), (iii) the MEGA element, which carries a *mef(A/E)* gene, and (iv) the IQ element, recently identified in viridans group streptococci (15).

More than one-third of the clinical strains ( $n = 36$ ) and half of the commensal strains ( $n = 24$ ) were positive for the relaxase gene of ICES $t3$  (Table 2; see also Table S1 in the supplemental material). Among these strains, 23 clinical and 15 commensal strains were also positive for the integrase gene of ICES $t3$  (specific for integration in the *fdA* gene, encoding fructose-1,6-diphosphate aldolase). Sixteen strains were tested for excision of ICES $t3$ , and in each case, a positive signal was obtained for both *attB* (empty integration site) and *attI* (circular form), indicating that these ICEs are functional for their excision (Table 2).

Thirty-one clinical strains and 30 commensal strains gave a positive signal for the MEGA element (Table 2; see also Table S1 in the supplemental material), and all were positive for the *mef(A/E)* gene. Two commensal strains (F3-10 and F4-20) carried a *mef(A/E)* gene but did not harbor a MEGA element. These strains were also resistant to chloramphenicol. The genome of strain F4-20 was sequenced in order to identify the chloramphenicol resistance gene and its genetic context. We found that this strain carries a *catQ* resistance gene, which colocalizes with a *mef(E)* gene on an IQ-like element of 12,038 bp (Fig. 2). This element differs from the IQ element described for *S. pneumoniae* strain 529 (47) in that it carries a *mef(E)* gene instead of a *mef(I)* gene. In addition, it lacks the right *tnp1* gene (and is thus a defective element) and 2 open reading frames (ORFs) located between the left *tnp1* gene and the trio of recombinase genes (Fig. 2). No other genetic element was detected in the vicinity of this genetic element. Two other chloramphenicol-resistant strains (L20 and N11) harbored a *mef(A/E)* and a *catQ* gene but also carried a MEGA element.

Forty-one clinical strains (44%) and 11 commensal strains (24%) carried the relaxase gene of Tn916 (Table 2; see also Table S1 in the supplemental material). Among these, 23 clinical and 6 commensal strains were also positive for the *tet(M)* gene. Twenty-three Tn916 elements were tested for excision, but no circular form was detected. A genetic linkage between the *erm(B)* gene and Tn916 genes was detected for 21 clinical strains (13 Tn3872 and 8 Tn6002 elements) and 3 commensal saliva strains (3 Tn6002 elements) (Table 2; see also Table S1 in the supplemental material). Two strains (L25 and L31) carried a Tn3872 element with a silent *tet(M)* gene. One clinical strain (T00) gave positive signals for both Tn3872 and Tn6002 elements. Thirteen Tn3872 and 11 Tn6002 elements were tested for excision, but no circular form was detected (Table 2; see also Table S1 in the supplemental material).

Among the 22 strains (12 clinical and 10 commensal) that carried a *mef(A/E)* gene and a Tn916 relaxase gene, 4 (2 clinical and 2 commensal) showed a genetic linkage between the *mef(A/E)* gene and the *tet(M)* gene, as in Tn2009 (Table 2; see also Table S1 in the supplemental material). These 4 Tn2009 elements were tested for excision, but no circular form was observed.

Thirty-two strains (13 clinical and 19 commensal) harbored both MEGA and ICES $t3$  elements; 22 strains (12 clinical and 10 commensal) carried both MEGA and Tn916 elements; and 8 strains (2 clinical and 6 commensal) carried all three of these genetic elements.

Strains with different genetic element contents were isolated from the two sampling sites (oral and fecal) of the same individual.

TABLE 1 Antimicrobial susceptibilities and resistance gene contents of *Streptococcus salivarius* isolates ( $n = 138$ )

Source	No. of isolates	No. (%) of isolates resistant to <sup>a</sup> :										No. of isolates positive for the following gene(s):						
		Ery	Spi	Lin	Tet	Ery + Tet	Chl	Kan	<i>erm(B)</i>	<i>mef(A/E)</i>	<i>tet(M)</i>	<i>catQ</i>	<i>erm(B)</i> + <i>mef(A/E)</i>	<i>erm(B)</i> + <i>tet(M)</i>	<i>mef(A/E)</i> + <i>tet(M)</i>	<i>erm(B)</i> + <i>mef(A/E)</i> + <i>tet(M)</i>	<i>mef(A/E)</i> + <i>catQ</i>	
Clinical	92	52 (56)	23 (25)	16 (17)	26 (28)	24 (26)	2 (2)	2 (2)	23 <sup>b</sup>	31 <sup>b</sup>	27	2	2	19	7	1	2	
Commensal																		
Feces	26	20 (77)	1 (4)	4 (15)	4 (15)	4 (15)	2 (8)	0 (0)	1	19	4	2	0	0	4	0	2	
Saliva	20	15 (75)	2 (10)	4 (20)	4 (20)	4 (20)	0 (0)	0 (0)	2	13	4	ND <sup>c</sup>	0	2	2	0	ND	

<sup>a</sup> Ery, erythromycin; Spi, spiramycin; Lin, lincomycin; Tet, tetracycline; Chl, chloramphenicol; Kan, kanamycin.

<sup>b</sup> Two strains carry a *mef(A/E)* gene and an *erm(B)* gene.

<sup>c</sup> ND, not determined. The *catQ* gene was searched for only in chloramphenicol-resistant strains.

TABLE 2 Genetic element contents of *Streptococcus salivarius* isolates ( $n = 138$ )

Source	No. of isolates	No. with the following genetic element (excision, <sup>a</sup> no. of isolates tested for excision):						
		ICES <sub>t3</sub> <i>rel</i> <sup>b</sup>	MEGA [ <i>mef</i> (A/E) element]	Tn916 <i>rel</i> <sup>b</sup>	Tn3872 [ <i>erm</i> (B) in <i>orf9</i> of Tn916]	Tn6002 [ <i>erm</i> (B) in <i>rel</i> of Tn916]	Tn2009 [ <i>mef</i> (A/E) in <i>orf6</i> of Tn916]	IQ-like element [ <i>mef</i> (A/E) and <i>catQ</i> linkage]
Clinical	92	36 (yes, 13)	31	41 (no, 23)	13 (no, 13)	8 (no, 8)	2 (no, 2)	2
Commensal								
Feces	26	13 (yes, 2)	17	7	0	1 (no, 1)	2 (no, 2)	2
Saliva	20	11 (yes, 1)	13	4	0	2 (no, 2)	0	0

<sup>a</sup> Occurrence (yes) or absence (no) of excision.

<sup>b</sup> A positive signal in the PCR detecting the relaxase (*rel*) gene of the ICES<sub>t3</sub> or Tn916 element was interpreted as the presence of this genetic element.

In addition, some fecal and oral isolates of the same individual that belonged to the same ST (i.e., ST27, ST32, ST57, ST83, ST94, ST105, and ST114) harbored the same genetic elements (see Table S1 in the supplemental material). These strains were analyzed by pulsed-field gel electrophoresis and were found to harbor identical electrophoresis patterns (data not shown).

## DISCUSSION

To our knowledge, this is the first study to examine the antimicrobial susceptibilities, antibiotic resistance genes, and genetic elements of *S. salivarius* strains recovered in human infections (clinical strains). This is also the first comparison of commensal strains isolated from different sampling sites (oral and fecal) of the same healthy individuals.

MLST of these isolates revealed a high diversity of strains irrespective of clinical or commensal origin. No clear clustering of the strains could be observed in the phylogenetic tree. Strains isolated in the same hospital were not usually clonal. In addition, clinical and commensal strains are distributed all over the branches of the phylogenetic tree. This is consistent with the occurrence of opportunistic infections following accidental introduction of *S. salivarius* into the patient's body (after surgery or another medical procedure), as reported in a previous study conducted by Delorme et al. (37). It is thus probable that strains found in the microbial flora of healthy individuals (referred to as "commensal strains" in this work) could cause disease symptoms if the opportunity arose (after injury, decrease of host defenses, etc.).

A high rate of erythromycin resistance was observed for clinical isolates and an even higher rate for commensal isolates ( $\geq 75\%$  and  $56\%$  erythromycin resistance in commensal and clinical

strains, respectively). This is consistent with earlier studies reporting high pharyngeal carriage of erythromycin-resistant viridans group streptococci in healthy individuals (20, 48). Almost equal numbers of *erm*(B) (MLS<sub>B</sub> phenotype) and *mef*(A/E) (M phenotype) erythromycin resistance determinants were detected in clinical strains, whereas commensal strains harbored the *mef*(A/E) gene almost exclusively. Previous studies conducted on commensal *S. salivarius* strains reported a dominance of either the *erm*(B) determinant (20, 49) or the *mef*(A/E) determinant (15, 48) in this species. One hypothesis to explain the difference in *erm*(B) and *mef*(A/E) distribution between clinical and commensal strains could be that strains isolated in cases of infection in hospitals are more exposed to high-level erythromycin [and thus to selection of the *erm*(B) determinant] than commensal strains. Except for one, all strains carrying an *erm*(B) gene displayed a constitutive MLS<sub>B</sub> phenotype, as observed by other authors for viridans group streptococci (15).

Most of the strains that carried an *erm*(B) gene were positive for the Tn916 relaxase and *tet*(M) genes and were thus resistant to both erythromycin and tetracycline. A physical linkage between the *erm*(B) gene and Tn916 genes was proved for a large proportion of these strains. The most common insertion of the *erm*(B) gene occurs in the *orf9* gene of Tn916 (Tn3872 element), but a few insertions in the relaxase gene of Tn916 (Tn6002 elements) have also been detected. One clinical strain gave positive signals for both Tn3872 and Tn6002. The predominance of Tn3872 elements in *S. salivarius* was also noticed by Ciric et al. (49), but this is the first description of the presence of both elements in the same strain. To date, conjugal transfer of Tn3872 has been observed at

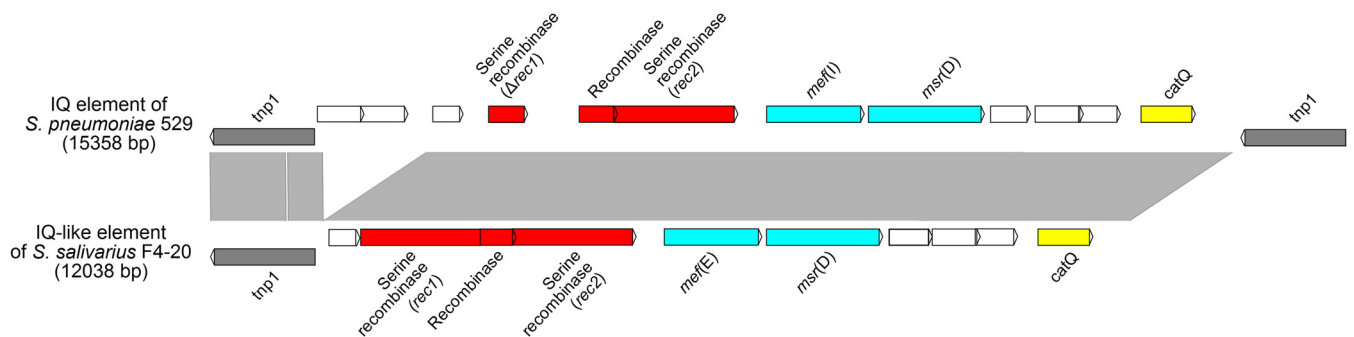


FIG 2 Schematic representation of the IQ-like element found in *S. salivarius* strain F4-20 compared to the IQ element described for *S. pneumoniae* 529 (47). ORFs appear as arrows. Genes are color-coded as follows: dark gray, transposase genes; red, recombinase genes; blue, erythromycin resistance genes; yellow, the *catQ* gene. Nucleic acid identity higher than 80% between sequences is indicated by light shading.

very low frequencies and only between *S. pyogenes* strains (28, 29). Tn6002 was found to be able to transfer by conjugation between *S. pneumoniae* and *S. pyogenes*, albeit at a low frequency and in a recipient-dependent manner (50). Hence, conjugative transfer of these elements cannot be excluded if propitious conditions are encountered (adequate bacterial recipient cells in particular). Under the conditions tested in this work, no excision of these elements was detected, precluding any transfer of these elements by conjugation.

Most of the erythromycin-resistant strains that carried a *mef(A/E)* gene were also positive for the MEGA element. This genetic element has been reported by Brenciani et al. to be frequent in viridans group streptococci (15). It can contribute to the dissemination of erythromycin resistance genes, since conjugative mobilization of MEGA from viridans group streptococci to *S. pyogenes* and *S. pneumoniae* has been reported (51, 52). Forty-six strains that harbor a MEGA element also carry a conjugative element related to ICES<sub>t3</sub> or Tn916 that could participate in the conjugative mobilization of MEGA. A physical linkage between *mef(A/E)* and *tet(M)* genes (Tn2009 element [28]) was detected in 4 strains (2 clinical and 2 commensal). To our knowledge, this is the first report of an association of the *mef(A/E)* and *tet(M)* genes on the same genetic element in *S. salivarius*. No conjugative transfer of Tn2009 has been observed to date, but its transfer was obtained by transformation in *S. pneumoniae* (29). Since many streptococcal species (in particular *S. pneumoniae*, *S. mutans*, *S. thermophilus*, and *S. salivarius*) can acquire genes by natural transformation (53, 54), this mechanism of lateral gene transfer likely also plays a role in the dissemination of antibiotic resistance.

Four chloramphenicol-resistant strains harbored *mef(A/E)* and *catQ* genes. Sequencing of the genome of one of these strains revealed that erythromycin [*mef(E)* gene] and chloramphenicol (*catQ* gene) resistance determinants colocalized on a genetic element related to the IQ element described for *S. pneumoniae* (47) and *S. pyogenes* (42). The IQ<sub>F4-20</sub> element is similar to those reported very recently for other viridans group streptococcal species (15) except for the deletion of the 2 ORFs downstream of the left *tnp1* gene. This is the first description of this variant and the first description of an IQ-like element in *S. salivarius*. In *S. pneumoniae* and *S. pyogenes*, the IQ element is carried by larger genetic elements that harbor a conjugative module of the ICE Tn5252 family and either a recombination module similar to that of ICESp23FST81 (integration in the *rplL* gene for ICESpn529IQ and ICESpy029IQ) or the recombination module of Tn5252 (for ICESpy005IQ) (35). ICESpn529IQ and ICESpy029IQ also carry a Tn916 fragment with a silent *tet(M)* gene. Only ICESpy005IQ was found to excise itself and transfer by conjugation (35). No ICE was detected in the vicinity of the *mef(E)* and *catQ* genes in the genome of the commensal strain F4-20 that was analyzed, ruling out acquisition of this genetic element by conjugation.

Tetracycline resistance correlated in most cases with the presence of a *tet(M)* gene carried by Tn916 or Tn916-related elements. This is consistent with previous studies that also described the predominance of this tetracycline resistance determinant in viridans group streptococci (15, 20). No *tet(M)* or *tet(O)* gene was identified in three of the tetracycline-resistant strains. These strains thus harbor an uncommon tetracycline resistance determinant that remains to be identified. A similar observation was made by Brenciani et al. in their recent survey of antibiotic resistance determinants in viridans group streptococci (15). Furthermore,

three silent *tet(M)* genes were detected in clinical strains, two of them carried by Tn3872 elements. This has already been reported for other streptococci (15, 28).

A large proportion of the clinical and commensal isolates of *S. salivarius* also harbored elements related to *S. thermophilus* ICES<sub>t3</sub> (34). This is the first report of the presence of such elements in *S. salivarius*. One-third of these elements were negative for the *fda* integrase, suggesting that the conjugative module of this family of elements can be associated with other recombination modules. It is likely that these elements participate in gene transfers (by conjugation or mobilization of other elements) between *S. salivarius* and pathogenic streptococci or streptococci used in the food industry.

High erythromycin and tetracycline resistance rates were observed in the clinical and commensal strains of *S. salivarius* analyzed in this study. In addition, most of the individuals harbored multiple oral and fecal strains of *S. salivarius* with different antibiotic resistance patterns and genetic element contents. This suggests that this species is a reservoir of antibiotic resistance genes and participates in lateral gene transfers with other species in both the oral and fecal spheres. This further confirms the role of commensal bacteria as a reservoir of antibiotic resistance genes (30–32).

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