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Diversity of integrative and conjugative elements of *Streptococcus salivarius* and their intra- and interspecies transfer

Running title: Diversity and functionality of ICEs in *S. salivarius*

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22 **ABSTRACT:**

23 Integrative and Conjugative Elements (ICEs) are widespread chromosomal mobile genetic elements,
24 which can transfer autonomously by conjugation in bacteria. Thirteen ICEs with a conjugation
25 module closely related to that of ICE*St3* of *Streptococcus thermophilus* were characterized in
26 *Streptococcus salivarius* by whole genome sequencing. Sequence comparison highlighted ICE
27 evolution by shuffling of 3 different integration/excision modules (for integration in the 3' end of *fda*,
28 *rpsI* or *rpmG* genes) with the conjugation module of the ICE*St3* subfamily. Sequence analyses also
29 pointed out a recombination occurring at *oriT* (likely mediated by the relaxase) as a mechanism of
30 ICE evolution. Despite a similar organization in 2 operons including three conserved genes, the
31 regulation modules show a high diversity (about 50% of amino acid sequence divergence for the
32 encoded regulators and presence of unrelated additional genes) with a probable impact on the
33 regulation of ICE activity.

34 Concerning the accessory genes, ICEs of the ICE*St3* subfamily appear particularly rich in restriction-
35 modification systems and orphan methyltransferase genes. Other cargo genes that could confer a
36 selective advantage to the cell hosting the ICE were identified, in particular genes for bacteriocin
37 synthesis and cadmium resistance.

38 The functionality of 2 ICEs of *S. salivarius* was investigated. Autonomous conjugative transfer to
39 other *S. salivarius* strains, to *S. thermophilus* and to *Enterococcus faecalis* was obtained. The
40 analysis of the ICE-*fda* border sequence in these transconjugants allowed the localization of the
41 DNA cutting site of the ICE integrase.

42

43 **IMPORTANCE**

44 The ICE*St3* subfamily of ICEs appears to be widespread in streptococci and targets diverse
45 chromosomal integration sites. These ICEs carry diverse cargo genes that can confer a selective

46 advantage to the host strain. The maintenance of these mobile genetic elements likely relies in part
47 on self-encoded restriction-modification systems.

48 Here intra and interspecies transfer were demonstrated for 2 ICEs of *S. salivarius*. Closely related
49 ICEs were also detected *in silico* in other *Streptococcus* species (*S. pneumoniae* and
50 *S. parasanguinis*) thus indicating that diffusion of ICES_{St3}-related elements probably plays a
51 significant role in HGT occurring in the oral cavity but also in the digestive tract where *S. salivarius*
52 is present.

53

54

Accepted version

55 INTRODUCTION

56 Acquisition of genes by horizontal gene transfer (HGT) is a major driving force for evolution of
57 bacterial genomes (1, 2). The main mechanism is conjugation, a process that enables transfer of large
58 DNA fragments without requiring any similarity between sequences thus being naturally broad host
59 range (3). Besides conjugative transfer of extrachromosomal elements (plasmids), recent analyses
60 revealed that many integrated mobile elements, called Integrative and Conjugative Elements (ICEs),
61 also encode their own transfer by conjugation (4, 5). Like all other mobile genetic elements (MGEs)
62 (6), ICEs have a modular structure. Three modules, a recombination module, a conjugation module
63 and a regulation module, altogether control and ensure the excision and transfer of the element (7).
64 ICEs are able to excise from the bacterial chromosome generally by site-specific recombination, to
65 transfer using their own conjugative machinery and to integrate in the chromosome of a recipient cell
66 (4, 5). Moreover, various ICEs can promote the transfer of large fragments of the bacterial
67 chromosome by an Hfr-like mechanism (8). They can also mobilize non autonomous integrated
68 transferable elements such as (i) Integrative and Mobilizable Elements (IMEs), genetic elements
69 unrelated to ICEs that can excise/integrate from the chromosome but need to hijack the transfer
70 machinery of a conjugative element or (ii) Cis Mobilizable Elements (CIMEs), elements that derive
71 from ICEs and IMEs by deletion of the conjugation/mobilization and recombination modules but
72 retain recombination sites *att* (4). In this latter case, an ICE that integrates in a recombination site of
73 a CIME can mobilize the non autonomous element by mediating the excision of the whole composite
74 element (process of accretion-mobilization) (9, 10). Multiplication of bacterial genome sequencing
75 projects in the last few years provides a remarkable opportunity to explore the pool of bacterial
76 genetic mobile elements ("mobilome"). These *in silico* analyses revealed the high abundance of ICEs
77 in bacteria (11-13). In addition to the genes involved or controlling their mobility, ICEs also carry

78 cargo genes, which can provide new properties (virulence, antibiotic resistance for example) to the
79 recipient cell (7).

80 *Streptococcus salivarius* is a Firmicute that is a major constituent of the human oral cavity
81 microbiota (14) and is commonly detected in the human gastrointestinal tract of healthy individuals
82 (15, 16). Few strains have also been associated with opportunistic infections in particular in cases of
83 meningitis (17), endocarditis (18) and bacteremia in immunocompromised patients (19, 20).
84 Analyses of *S. salivarius* genomes pointed out the considerable variability of gene content and the
85 differences in adaptive traits (21). Evidence of widespread HGT was obtained in link with the
86 presence of diverse MGEs (21) and the competence for natural transformation of the species (22).

87 We recently screened a collection of 138 strains of *S. salivarius* for the presence of MGEs (23). This
88 led to the identification of 60 strains (belonging to 39 MLST groups) with a positive PCR signal for
89 the relaxase gene of ICE*St3*, an ICE previously characterized in the closely related species
90 *Streptococcus thermophilus* (24-26). This indicated the presence in these strains of putative ICEs
91 belonging to the ICE*St3* subfamily. However, not all these strains showed a positive PCR signal for
92 the ICE*St3* integrase gene that catalyzes the integration into the *fda* site. This suggested the presence
93 of other recombination modules associated with the ICE*St3* conjugation module in *S. salivarius*
94 ICEs.

95 In this work, we selected 13 strains with different MLST patterns (including 6 strains with a negative
96 PCR signal for the ICE*St3* integrase gene) and sequenced their genome in order to gain access to
97 their ICE sequences. ICEs with a conjugation module closely related to the one of ICE*St3* were
98 searched in other NCBI available genomes of *S. salivarius* and in other genomes of Firmicutes. ICE
99 sequences were compared and the putative function encoded by their genes was analyzed *in silico*.

100 Two putative ICEs from *S. salivarius* were analyzed experimentally to test their excision and their
101 autonomous intraspecies and interspecies conjugative transfer.

102 MATERIAL AND METHODS

103 Bacterial strains, plasmids and culture conditions

104 The strains and plasmids used in the experimental part of this study are listed in Table 1.

105 Thirteen *S. salivarius* strains including 4 commensal (F1-4, F1-8, F4-2 and F6-1) and 9 clinical (B35,
106 B57, L22, L50, L60, L64, N5, N20 and T93) were selected on the basis of a previous work (23).

107 These strains were chosen according to 3 criteria. Firstly, the selected strains likely harbor an ICE
108 with a conjugation module related to ICES_{t3} (as suggested by a positive PCR signal for the relaxase
109 and coupling protein genes of ICES_{t3}). Secondly, to increase the diversity of ICEs included in the
110 analysis, half of the strains were selected according to the absence of a PCR signal for the integrase
111 gene of ICES_{t3} thus suggesting the presence of a different recombination module. Lastly, the
112 selected strains are distributed all over the phylogenetic tree built from MLST data (23). They belong
113 to different MLST groups and differ by at least 3 MLST alleles (out of 6 alleles analyzed) and can
114 thus be considered as unrelated, except two of them (strains L22 and L64) which differ by only 1
115 allele (23). These 13 strains are available upon request.

116 Modified strains were named according to the modification. For example, *S. salivarius* F1-8
117 (pMG36e) corresponds to a derivative of *S. salivarius* F1-8 carrying the plasmid pMG36e. pMG36e
118 is a small plasmid (3611 pb) that carries only genes required for its replication and an erythromycin
119 resistance gene. It is a non conjugative plasmid derived from pWV01 that is a broad spectrum
120 plasmid replicating in *E. coli* and in Firmicutes (27) and has been successfully used for labeling
121 recipient strains in conjugation experiments with *S. thermophilus* (24). *S. salivarius*, *E. faecalis* and
122 *S. thermophilus* were grown in M17 broth supplemented with 0.5% lactose (LM17) at 37°C
123 (*S. salivarius*, *E. faecalis*) or 42°C (*S. thermophilus*) without shaking. Selective mitis salivarius agar
124 (MSA; Difco) containing a 1% (wt/vol) tellurite solution, brain heart infusion (BHI; Difco) and

125 reconstituted skim milk broths were also used for mating assays. Solid cultures were made in
126 oxygen-free environment induced by GasPak utilization (BioMérieux).

127 When required, cultures were supplemented with the following antibiotics: chloramphenicol (5 μ g
128 ml⁻¹ for *S. thermophilus* or 8 μ g ml⁻¹ for *S. salivarius* and *E. faecalis*); erythromycin (5 μ g ml⁻¹ for
129 *S. thermophilus* or 10 μ g ml⁻¹ for *S. salivarius* and *E. faecalis*).

130 **DNA sequencing and sequence analysis**

131 The 13 selected *S. salivarius* strains were subjected to whole genome sequencing. Sequencing was
132 made using Illumina HiSeq2000 sequencer by Beckman Coulter genomics (2×100 pb after paired-
133 end library construction, expected at least 60 × coverage). Observed sequencing depth was higher
134 than 200 × for all the 13 genomes. *De novo* assembly was performed using CLC Genomics
135 Workbench (CLC Bio) using default parameters. Scaffold of the genomes was built by using the
136 Genome Finishing module of CLC Genomics Workbench with *S. salivarius* JIM8777 genome as
137 reference. Some assembly gaps were filled by doing PCR and sequencing. This enabled to obtain
138 genomes fragmented in less than 75 contigs (less than 30 contigs for two thirds of the genomes;
139 median size of the contigs higher than 40 kb for half of them). Contigs were first annotated using the
140 RAST annotation server (<http://rast.nmpdr.org/>) (28, 29). Then, contig(s) containing genes closely
141 related to conjugation genes of ICES_{St3} were identified by BlastN analysis (using *orfO-orfA* genes as
142 query by megaBlast analysis with default parameters and filter disabled) and annotation was
143 completed manually.

144 In NCBI sequence databanks, ICEs closely related to ICES_{St3} were searched by using Microbial
145 nucleotide blast (using *orfO-orfA* genes as query by megaBlast analysis with default parameters and
146 filter disabled) on complete (n=816, last accessed 2016/07/29) and draft (n=9573, last accessed
147 2016/07/29) genomes of Firmicutes. Hits with more than 90% of identity with the whole query
148 sequence were further analyzed.

149 Pairwise comparisons of elements were performed with Artemis Comparison Tool provided by the
150 Sanger Centre using comparison files generated by Double Act (available at: [http://www.hpa-
151 bioinfotools.org.uk/pise/double_act.html](http://www.hpa-bioinfotools.org.uk/pise/double_act.html)) (30). Manual editing of comparison figures was performed
152 using Inkscape.

153 **Nucleotide sequence accession numbers**

154 The sequences of ICEs have been deposited in the EMBL Nucleotide Sequence Database under
155 accession numbers LT622825-LT622837.

156 **Phylogenetic tree construction**

157 Proteins of twenty-seven ICEs were included in the analysis. These ICEs correspond to (i) the 19
158 ICEs of the ICE*St3* subfamily with closely related conjugation modules and (ii) 8 additional ones
159 previously reported to belong to the same ICE subfamily but showing more distantly related
160 conjugation modules (11, 26). The sequences of signature proteins were aligned using Clustal omega
161 with default parameters (31). The trees were built with MEGA (32) using (i) maximum likelihood
162 (ML) based on JTT (Jones-Taylor-Thornton) model including amino acid empirical frequencies
163 (partial deletion of gaps and missing data at 80% cutoff, Gamma distribution in 5 categories,
164 allowance for invariant sites), and (ii) BioNJ methods with the Poisson model (33). The branch
165 support of the groupings was estimated using bootstrap (100 replicates).

166 **ICE tagging**

167 ICE_*SsaF1-4_fda* and ICE_*SsaF4-2_fda* were tagged by a chloramphenicol resistance cassette
168 originating from pSET5s plasmid. The resistance cassette was inserted in an intergenic region
169 located between convergent coding sequences in the adaptive module to avoid impacting ICE
170 functionality. Two DNA fragments of about 1,000 bp corresponding to the upstream and the
171 downstream regions of the integration locus were amplified by PCR using specific primers that
172 present an extended sequence matching with the 5' and the 3' ends of the chloramphenicol resistance

173 cassette. A second PCR amplification was carried out using these two PCR fragments and the
174 resistance gene as a template to synthesize a fragment carrying the antibiotic resistance cassette
175 flanked by the upstream and downstream chromosomal regions of the gene. Natural competence of
176 *S. salivarius* cells was induced by addition of the synthetic peptide (H₂N – LPYFTGCL – COOH)
177 (22) and the overlap PCR product was then added for transformation. The cross-over events,
178 upstream and downstream from the tagged region, were positively selected by the newly acquired
179 antibiotic resistance of the transformed clones. The integrity of the regions flanking the antibiotic
180 cassette was confirmed by PCR.

181 PCRs were done using the Phusion high-fidelity DNA polymerase (Thermo Scientific). PCRs were
182 performed with 50 ng of genomic DNA, 200 μM of each deoxynucleotide triphosphate (dNTP), 0.5
183 μM of each primer (for primer sequences, see Table 2) and 0.02 U μl⁻¹ of Phusion DNA polymerase
184 in appropriate buffer per 50 μl reaction volume. Cycling conditions for the overlap PCR were: 3 min
185 at 98°C, 30 s at annealing temperature (with 1°C of incrementation at each cycle), 30 s/kb at 72°C,
186 followed by 30 additional cycles with an annealing temperature of 55°C and a final extension of 10
187 min at 72°C.

188 **Excision tests with or without mitomycin C (MMC)**

189 PCR amplifications of *attB*, *attI*, *attR* and *attL* fragments were carried out in a 25 μl volume
190 containing 1 μl of overnight culture, 200 μM of each dNTP (Thermo Scientific, France), 0.5 μM of
191 each primer (for primer sequences, see Table 2), and 0.025 U μl⁻¹ of DreamTaq DNA polymerase in
192 an appropriate buffer (Thermo Scientific, France). PCR amplifications were performed using the
193 following cycling parameters: 10 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 50°C,
194 and 1 min/kb at 72°C, with a final 5-min extension at 72°C. Amplified products were analyzed by
195 electrophoresis on a 1.5% agarose gel.

196 To test the impact of mitomycin C on the ICE excision, PCR amplifications of the attachment sites
197 were done after a 2.5 h-treatment of the cells with MMC. To select the MMC concentration that
198 gives the highest induction of excision, a range of MMC concentrations (0.025 to 0.4 mg ml⁻¹) was
199 first tested using 1 ng of genomic DNA as template. Semi-quantitative PCR (performed with 30
200 cycles) were then done at the selected concentration of MMC (0.05 mg ml⁻¹) using different genomic
201 DNA quantities (0.1 µg to 1 pg). Amplifications of the *fda* gene were done in parallel as controls.

202 **Mating experiments**

203 Donor and recipient strains were grown overnight with an appropriate antibiotic. 15 ml of broth
204 medium were inoculated with 150 µl of donor or recipient stationary phase cultures. Cultures were
205 grown until mid-exponential phase (optical density at 600 nm of 0.4), then were mixed and
206 centrifuged for 15 min in a prewarmed centrifuge at 4,500 × g to pellet cells. The pellet was
207 resuspended in 1 ml of LM17 broth and 150 µl were spread on 0.45 µm pore-size cellulose nitrate
208 filters (Sartorius stedim biotech) deposited on LM17 soft agar (0.8 %) plates. Plates were then
209 incubated at 37°C (for *S. salivarius*/*S. salivarius* and *S. salivarius*/*E. faecalis* mating pairs) or at
210 39°C (for *S. salivarius*/*S. thermophilus* mating pairs). After an overnight incubation, the filters were
211 removed from the agar plates and placed into 10 ml of LM17 liquid media. Bacteria were recovered
212 by vortexing for 30 s. The suspension was then directly spread on agar plates supplemented with the
213 appropriate antibiotics or concentrated 10 times by centrifugation at 4,500 × g for 15 min to enable
214 counting the CFUs of the donor, the recipient, and the transconjugants cells after a 24h-incubation.
215 Transconjugants clones obtained after *S. salivarius* intraspecies mating were typed by DNA
216 sequencing of PCR products corresponding to the *ddlA* gene (for primer sequences, see Table 2).

217 Treatment of donor cells with mitomycin C (MMC) was done as follows: cells carrying
218 ICE_*SsaF1-4_fda* were grown in LM17 liquid medium at 37°C to an optical density at 600 nm of
219 0.4. The culture was then diluted 10-fold in 15 ml of prewarmed LM17 containing MMC at the

220 concentration that showed the maximum ICE excision level (0.05 mg ml^{-1}). A 10-fold dilution
221 without MMC was used as a control. After 1 hour of culture, the cells were harvested by
222 centrifugation in a prewarmed centrifuge and washed once with 15 ml of prewarmed LM17. The
223 donor cells treated with MMC were mixed with recipient cells grown at optical density at 600 nm of
224 0.4 and centrifuged for 15 min in a prewarmed centrifuge at $4,500 \times g$ to pellet cells. The pellet was
225 resuspended in 1 ml of LM17 broth, and 150 μl were spread on nitrocellulose filter on LM17 soft
226 agar plates before incubation for 4h or overnight at appropriate temperatures. The filters were then
227 treated as previously described.

228 Mating frequencies were calculated by dividing the number of transconjugants by the number of
229 donor cells, except in the case of donor cells treated with MMC, where mating frequencies were
230 calculated relative to recipients. At least three independent biological replicates were done.

231 **Plasmid curing**

232 Transconjugants carrying plasmid pMG36e were cultured overnight without erythromycin, and were
233 then spread on LM17 plates at different dilutions. One hundred isolated clones were then streaked on
234 LM17 plates with or without erythromycin. Erythromycin sensitive clones were then confirmed by
235 PCR for the absence of plasmid (see Table 2 for primer sequences).

236 **Random Amplification of Polymorphic DNA (RAPD)**

237 RAPD (34) was carried out with DreamTaq enzyme (for primer sequences see Table 2). Cycling
238 conditions were: 40 cycles consisting of 94°C for 1 min, 31°C for 1 min, and 72°C for 2 min; the
239 final extension was continued for 7 min at 72°C . 1 μl of liquid culture was used as DNA template.
240 The PCR products were separated by electrophoresis on a 1.5% agarose gel.

241

242 RESULTS AND DISCUSSION

243

244 Diversity of Integrative and Conjugative Elements of the ICESt3 subfamily identified in 245 *S. salivarius* and in other streptococci

246 Conjugation modules

247 To explore the diversity of the ICEs of the ICESt3 subfamily found in *S. salivarius*, 13 strains (F1-4,
248 F1-8, F4-2, F6-1, B35, B57, L22, L50, L60, L64, N5, N20 and T93) with different MLST patterns
249 were selected from a previous work (23) (Table 1). These strains likely encode an ICE of the ICESt3
250 subfamily as suggested by a positive PCR signal for the ICESt3 relaxase gene (23). Their genome
251 was sequenced and assembled in order to gain access to their ICE sequences. Except for 2 ICEs
252 (ICE_*SsaF6-1_rpsI* and ICE_*SsaB57_fda*) split into 2 contigs, the sequences of the ICEs appear on a
253 single contig (Fig. 1). As shown in Fig. 1, ICEs found in these 13 *S. salivarius* strains all display a
254 conjugation module that is closely related to that of ICESt3 from *S. thermophilus* (>90% of nucleic
255 sequence identity). ICEs with a closely related full conjugation module were also detected in 2
256 genomes of *S. salivarius* available in NCBI (those of strains JF and 1270) (Fig. 1). The element of
257 *S. salivarius* NCTC 8618 (GenBank accession number: CP009913.1) also has a closely related full
258 conjugation module but is not shown in the figure since it harbors a truncated integrase gene, and is
259 therefore defective. Elements with a closely related conjugation module (partial in most of the
260 genomes due to gaps in the assembly) were also detected in the genomes of strains GED7778A
261 (GenBank accession number LRQS00000000), 140 (GenBank accession number: JVSQ01000000),
262 20-02 S1 (GenBank accession number: LXMB00000000), 20-12 S2 (GenBank accession number:
263 LXMC00000000) and UC3162 (GenBank accession number: JYOY01000000). These elements are
264 not shown in Fig. 1 because their sequence is incomplete. ICEs with a closely related conjugation
265 module (92-94% of nucleic sequence identity with that of ICESt3) were also identified in the genome

266 of *Streptococcus pneumoniae* 2842STDY5753514 (mitis group) and *Streptococcus parasanguinis*
267 DD19 (sanguinis group) (Fig. 1). Phylogenetic analysis of the relaxase OrfJ (Fig. 2) and the coupling
268 protein OrfK (Fig. 3) of these ICEs indicates that they group with OrfJ and OrfK from *S. salivarius*
269 ICEs. Similar results were obtained for the other proteins of the conjugation module (data not
270 shown). Taken as a whole, these phylogenetic analyses and sequence comparisons clearly showed
271 that transfers of ICEs closely related to ICE*St3* have occurred between distinct species from different
272 streptococcal groups.

273 The comparison of the clustering groups obtained for the relaxase (OrfJ) and the coupling protein
274 (OrfK) indicated that OrfJ of ICE_*Sma33MO_fda* is closely related to the relaxases of ICE*St3* and
275 *S. salivarius* ICEs whereas OrfK is clearly different from the coupling proteins of these ICEs, but is
276 closer to that of ICE_*SparauNCFD2020_rpsI* (see phylogenetic trees in Fig. 2 and 3). Nucleic
277 sequence alignments indicated that the left extremity of the conjugation module (including *orfN* to
278 *orfK* genes) of ICE_*Sma33MO_fda* is closely related to the corresponding sequence of
279 ICE_*SparauNCFD2020_rpsI* (86% of nucleic sequence identity compared to 64% of nucleic
280 sequence identity with the corresponding genes of ICE*St3*) whereas the right extremity of the
281 conjugation module of ICE_*Sma33MO_fda* (corresponding to *orfJ* to *orfA* genes) is closely related to
282 those of ICE*St3* (96% of nucleic sequence identity compared to 64% of nucleic sequence identity
283 with the corresponding genes of ICE_*SparauNCFD2020_rpsI*) and *S. salivarius* ICEs. The putative
284 *nic* site of the transfer origin of ICEs of the ICE*St3* subfamily, predicted by comparison with the *oriT*
285 characterized for ICE*Bs1* of *Bacillus subtilis* (35), is located between *orfK* and *orfJ* genes
286 (supplementary Fig. S1). Sequence alignment of this region indicated that the drop in nucleic
287 sequence identity between ICE_*Sma33MO_fda* and ICE_*SparauNCFD2020_rpsI* occurs at the *nic*
288 site (supplementary Fig. S1). This suggests that a recombination event occurred at *oriT* in
289 ICE_*Sma33MO_fda*. This *oriT* recombination was likely mediated by the relaxase of the ICE that is

290 able to recognize and nick this sequence. Such *oriT* site-specific recombination has already been
291 demonstrated for several canonical relaxases of plasmids (belonging to MobF, MobP, MobC and
292 MobM families) (4, 36, 37) and has been suggested to occur in ICEs of the SXT/R391 family and for
293 ICE*clc* (both encoding canonical relaxases of the MobH family) (38, 39). This mechanism that
294 enables to create hybrid ICEs could thus also be mediated by relaxases of the non-canonical MobT
295 family, which are related to rolling circle replication initiators and are found in ICEs of the
296 Tn916/ICE*St3*/ICE*Bs1* family, a family of ICEs widespread in streptococci (11) and other Firmicutes
297 (5).

298

299 *Integration modules*

300 ICE*St3* conjugation modules found in *S. salivarius* ICEs are associated with 3 different
301 recombination modules (see supplemental Figure S2) enabling integration of the corresponding ICEs
302 in the 3' end of three different genes: *fda* (encoding the fructose-1-6-diphosphate aldolase, as for
303 ICE*St3*), *rpsI* (encoding the S9 ribosomal protein) and *rpmG* (encoding the L33 ribosomal protein).
304 This confirms our previous results showing a positive PCR signal for ICE*St3* relaxase gene but not
305 for ICE*St3* integrase gene for 22 strains (23). Closely related integrases were found in ICEs of
306 *S. salivarius* strains that are distant in the phylogenetic tree built on the basis of MLST data (for
307 example in strains F1-4, L22 and N5 or in strains B35/L50) (23) but also in ICEs found in other
308 species (for example in *S. pneumoniae* 2842STDY5753514 or in *S. parasanguinis* DD19 and
309 *S. macedonicus* 33MO) (supplemental Figure S2). This is in agreement with the previously reported
310 exchanges of recombination modules in ICE*St3* subfamily (11, 26).

311

312 *Regulation modules*

313 Comparison of the regulation modules of all ICEs of the ICE $St3$ subfamily showed their high
314 diversity (Fig. 1). The only common characteristic is the presence, in most of the ICEs, of homologs
315 of *arp1*, *orfQ* and *arp2* genes. This strongly suggests that these genes participate to the same
316 regulation cascade and likely interact/interfere with each other. Exceptions are ICE_*SsaL60_rpsI*
317 devoid of *arp1* gene, ICE_*SsaB35_rpsI* lacking the *orfQ* gene and ICE_*SpaDD19_fda* carrying
318 truncated *arp1* and *orfQ* genes. ICE_*SsaN20_rpsI* also displays a peculiar regulation organization,
319 with full copies of *arp1* and *orfQ* genes separated by truncated copies of *orfQ* and *arp1* genes. This
320 is due to an insertion of genes (including one encoding a transposase) in this region of the ICE (Fig.
321 1). The *arp1*, *orfQ* and *arp2* genes encode respectively a cI-related repressor, a ImmA-related
322 putative protease (40) and a putative transcriptional regulator (41) that are related to those of ICE $St3$
323 from *S. thermophilus* (26, 42). Arp1 and Arp2 homologs all share an N-terminal helix-turn helix
324 PF01381 domain that is found in several regulatory proteins including Cro and cI regulators of the λ
325 phage. Arp1 proteins also display a C-terminal PF00717 peptidase S24-like domain involved in the
326 autocleavage of the cI repressor induced by DNA damage. OrfQ homologs are characterized by a
327 COG2856 Zn-dependent peptidase ImmA domain, also found in the ImmA protease of ICE $Bs1$ of
328 *B. subtilis* that cleaves the ImmR repressor in response to DNA damage (40). A high diversity is
329 observed in the nucleotide (Fig. 1) and amino acid primary sequences of the encoded regulators. Up
330 to 45%, 58% and 53% of divergence was observed between the sequences of Arp1, OrfQ and Arp2
331 proteins encoded by ICEs of *S. salivarius*. If considering only the ICEs that harbor the *arp1*, *orfQ*
332 and *arp2* genes, the sequence divergence goes from 5 to 37% for Arp1, 7 to 58% for OrfQ and 7 to
333 37% for Arp2.

334 In support of a modular evolution of ICEs that was already noticeable from the exchanges in the
335 recombination modules mentioned previously, some ICEs harbor regulators which are distantly
336 related to those found in the other ICEs of *S. salivarius* despite having a closely related conjugation

337 module (deduced from the phylogenetic trees of relaxases and coupling proteins). This is the case of
338 ICE_*SsaF4-2_fda* that has a conjugation module closely related to the one of ICE*St3* (94% of nucleic
339 sequence identity over the whole conjugation module) but encode more distant regulators (with 63,
340 46 and 73% of amino acid identity with those encoded by ICE*St3* for Arp1, OrfQ and Arp2
341 respectively).

342 Furthermore, all the regulation modules include additional genes (1 to 3 depending on the ICE)
343 between the *arp2* and *orfQ* genes. These genes that encode proteins of unknown function contribute
344 to the diversity of the regulation modules.

345

346 *Accretions*

347 Accretion of ICEs with other genetic elements was observed: ICEs of strains F1-8 and L60 are in
348 accretion with a putative IME, whereas those of strains F6-1 and L50 are adjacent to 1 or 2 CIMEs
349 (data not shown).

350 Evidences of accretion or recombination events were also found in the sequence of several ICEs of
351 *S. salivarius*. A truncated supplementary copy of the *orfA* gene was found between *orfA* and the
352 excisionase genes in *S. salivarius* L22 and B57 ICEs and in the closely related ICE found in
353 *S. parasanguinis* DD19. The presence of supplementary truncated *arp1* and *orfQ* genes and of a
354 transposase gene was also detected in the regulation module of *S. salivarius* N20 ICE. These
355 structures could result from (i) the integration of an ICE within an *att* site flanking a resident element
356 (accretion) or (ii) by recombination between ICEs, followed by internal deletion(s) in both cases.
357 These mechanisms contribute to the plasticity and evolution of ICEs and potentiate gene transfer
358 mediated by these MGE.

359

360 **Cargo genes found on ICEs of the ICE*St3* subfamily in *S. salivarius* and other streptococci**

361 A diversity of cargo genes was found in the left part of the ICEs of the *ICES_{St3}* subfamily explaining
362 the variation of their size ranges: from 25.8 kb for the smallest one (*ICE_SsaL64_fda*) to 37.3 kb for
363 the largest one (*ICE_SsaN20_rpsI*) (Fig. 1). The 2 closely related strains included in the analysis
364 (L22 and L64) harbor ICEs with unrelated cargo genes (Fig. 1). In addition, ICEs found in unrelated
365 strains of *S. salivarius* (for example in strains JF and L22 or F4-2 and F1-8) or in different species (in
366 *S. salivarius* T93 and *S. parasanguinis* DD19) harbor the same cargo genes (Fig. 1). This distribution
367 is due to the horizontal transfer of these genes (exchange of module between ICEs or transfer of the
368 whole ICE).

369 Two thirds (13/19 if including *ICES_{St3}* (43)) of the ICEs analyzed in this work carry a restriction-
370 modification (RM) system (n=6) or an orphan methyltransferase (n=7) (Fig. 1). This is in accordance
371 with a previous study indicating that the abundance of RM systems correlates with the presence of
372 MGEs in small genomes (44). RM systems were first described as bacterial innate immune systems
373 allowing protection against foreign unmethylated DNA (45). Unmethylated incoming DNA will be
374 degraded by restriction enzymes produced by the cell while the genome of the host remains protected
375 due to methylation by the cognate methyltransferase. RM systems carried by ICEs of *S. salivarius*
376 could protect their host from invasion by other genetic elements such as phages, thus playing a role
377 in “cellular defense” as described for other bacteria (46-48). RM systems also turn out to be selfish
378 mobile elements themselves (49). RM systems that carry the restriction enzyme activity and
379 modification enzyme activity on separate proteins (as observed for the ICEs of the *ICES_{St3}*
380 subfamily) can have an impact on the maintenance of the MGE carrying them (mechanism of genetic
381 addiction). The MGE encodes the poison endonuclease activity and its “antidote”, the
382 methyltransferase (45). Post-segregational killing would occur if the whole RM system is lost.
383 Dilution of the modification enzyme by cell division will lead to the exposure of unmethylated
384 recognition sites on newly replicated chromosomes that will be targeted by the restriction enzyme.

385 Only a few remaining molecules of restriction enzymes are sufficient to kill the cell. Concerning
386 orphan methyltransferases (encoded by 7 of the ICEs of the ICES_{St3} subfamily analyzed in this work),
387 if these enzymes target the same DNA sequence as a resident RM system, they could protect the host
388 from post-segregational killing thus participating in the displacement of the resident MGE (50).

389 Two ICEs found in *S. salivarius* L60 and N20 carry a cadmium resistance cluster (*cadD/cadX*) (Fig.
390 1). Cadmium is a widespread heavy-metal air pollutant which is commonly released into the
391 environment from industrial processes (in particular glass manufacturing) and urban activities, as
392 well as from the widespread application of fertilizers, manures and sewage sludge (51). The
393 *cadD/cadX* genes also appear in a CIME in accretion with the ICE from strains F6-1 and L50 (data
394 not shown). In these latter ones, full recombination sites are still present and thus the CIME and the
395 ICE are considered separately. In these strains, the whole CIME-ICE composite element could excise
396 and transfer by a process of accretion-mobilization already demonstrated with other ICEs of this
397 family (9, 10). Sequence comparison revealed 98% of nucleic identity over 2683 bp between the
398 CIME located upstream of the ICE in strain L50 and the region that includes *cadD/cadX* genes in
399 ICE_*SsaL60_rpsI*. This suggests that in strain L60 the cadmium resistance cluster was acquired by
400 the ICE after deletion of the recombination site delimiting the ICE and the adjacent CIME. This
401 mechanism contributes to the plasticity and evolution of ICEs as previously suggested (4).

402 ICE_*SsaF6-1_rpsI* carries a cluster of genes (10.8 kb) that could be involved in membrane lipid
403 synthesis. It includes in particular a *fabF*-like gene encoding a beta-ketoacyl-[acyl-carrier-protein]
404 synthase II (KASI/IIcd00834 domain with E=1.4e-144) and a KBL_like gene encoding a serine
405 palmitoyltransferase involved in sphingolipid synthesis (cd06454 domain with E=3.7e-151).

406 Two ICEs (found in the unrelated strains *S. salivarius* JF and L22) harbor a closely related cluster of
407 genes (*slv* cluster, 15.7 to 15.9 kb) involved in the biosynthesis of a bacteriocin (salivaricin D). These
408 clusters differ from the one previously described in the commensal strain 5M6c of *S. salivarius*

409 isolated from a healthy infant (52) by the number of copies of the *slvD* structural gene (2 and 3
410 identical copies respectively compared to 1 in strain 5M6c). Salivaricin D is a nisin-like lantibiotic
411 with a broad-spectrum that includes a large array of Gram positive bacteria in particular the
412 important pathogens *S. pneumoniae* and *S. pyogenes* (52). Thus, it could be used as a potential
413 weapon, for commensal species of the oral cavity such as *S. salivarius*, to compete with
414 oronasopharynx-colonizing streptococci including pathogens (52-54). Bacteriocins could also play a
415 role of addiction system by killing neighboring cells that do not encode resistance to the bacteriocin
416 and thus contribute to the maintenance of the MGE in the population (55).
417 The ICE found in *S. macedonicus* 33MO carries genes that encode cell-envelope proteins: one with a
418 PF03780 domain with cell envelope-related function ($E=7.93e-23$), and 3 membrane proteins. Lastly,
419 ICEs of *S. salivarius* T93 and *S. parasanguinis* DD19 harbor the same cargo genes, in particular (i) a
420 two-component system with a signal transduction histidine kinase (nitrate/nitrite-specific COG3850
421 NarQ domain, $E=5.3e-13$) and a NarL DNA-binding response regulator (COG2197 domain, $E=6.1e-$
422 48) and (ii) a cluster of genes encoding a putative LolCDE complex which catalyzes the release of
423 lipoproteins from the cytoplasmic membrane (LolC PF13521 AAA_28 domain, $E=1.2e-09$; LolD
424 cd03255 ABC_MJ0796_LolCDE_FtsE domain, $E=1.2e-108$ and LolE COG4591 domain, $E=4.4e-$
425 14) (Fig. 1).

426

427 **Excision tests of 2 putative ICEs: ICE_*SsaF1-4_fda* and ICE_*SsaF4-2_fda***

428 Experiments were carried out to test the functionality of 2 putative ICEs of *S. salivarius*. The first
429 one, ICE_*SsaF1-4_fda*, has a conjugation module showing the highest percentage of identity with
430 that of ICE*St3* of *S. thermophilus*, an ICE whose transfer was previously demonstrated (24) (Fig. 1).
431 Arp1, OrfQ and Arp2 regulators encoded by this ICE are also closely related to those of ICE*St3* (85,
432 71 and 84% of amino acid identity respectively). As mentioned before, the second one, ICE_*SsaF4-*

433 *2_fda*, has a conjugation module closely related to ICE_*SsaF1-4_fda* and ICE*St3* but harbors a
434 distantly related regulation module.

435 Excision of these 2 ICEs was tested by PCR. Amplifications were obtained for *attI* and *attB*
436 corresponding to the circular excised form and the empty chromosomal site respectively, indicating
437 the functionality of their recombination module. Recombination sites (*attL* and *attR*) flanking the
438 ICEs were also amplified (data are shown only for ICE_*SsaF1-4_fda*, Fig. 4).

439

440 **Test of intraspecies conjugative transfer of 2 putative ICEs of *S. salivarius***

441 Both ICE_*SsaF1-4_fda* and ICE_*SsaF4-2_fda* were first tagged by a chloramphenicol resistance
442 cassette (Table 1). Donor strains were then used in filter mating experiments with two *S. salivarius*
443 recipient strains: JIM8777 and F1-8, displaying different genotypes (Table 1 and 3). Both strains
444 carry an empty *fda* integration site and strain F1-8 carries an ICE belonging to the ICE*St3* subfamily
445 (Fig. 1) in accretion with an IME integrated in the 3' end of *rpmG*. Some putative transconjugants
446 were recovered when JIM8777 or F1-8 *S. salivarius* strains were used as recipients (Table 3). After
447 sub-culturing, transconjugants were confirmed by detection of the integrase gene (data are shown
448 only for ICE_*SsaF1-4_fda*, Fig. 5a, 5b). For JIM8777 derivative clones, this screen was combined
449 with detection of the pMG36e plasmid and RAPD (Fig. 5a). F1-8 transconjugants were confirmed by
450 sequencing of an MLST locus (*ddlA* gene). The site specific insertion in the *fda* gene and the
451 excision of the newly acquired ICEs were also confirmed by the PCR detection of *attL*, *attR*, *attI* and
452 *attB* sites for both F1-8 (data are shown only for ICE_*SsaF1-4_fda*, Fig. 5c), and JIM8777 (data not
453 shown). This suggests that the elements are still active in these transconjugants (at least for excision).
454 Mating experiments were repeated with at least 3 different cultures of donor and recipient cells
455 (biological repetitions) but very few transconjugants (2 clones for the whole experiment) were
456 obtained thus preventing any calculation of transfer frequency. Attempts to increase ICE_*SsaF1-*

457 *4_fda* transfer frequency were made: (i) test of different media (milk, BHI, mitis, M17 broth with 1%
458 final glucose instead of 0.5% final lactose), (ii) test of different donor/recipient ratio ranging from
459 1:1, 2:1, 10:1, 50:100 to 100:1, (iii) mix of donor and recipient at the end of exponential growth
460 phase or stationary phase instead of mid-exponential phase. None of the tested conditions enabled to
461 increase transfer frequency which remained inferior to 10^{-8} transconjugants per donor cells.

462

463 **Test of interspecies conjugative transfer of ICEs of *S. salivarius***

464 To evaluate ICE interspecies transfer, mating assays were carried out with two other *Firmicutes* as
465 recipients: the closely related *S. thermophilus* and *Enterococcus faecalis*. Recipient strains were
466 *S. thermophilus* LMG18311 and *E. faecalis* JH2-2 both carrying pMG36e and described as recipients
467 strains of ICE*St3* from *S. thermophilus* (transfer frequency of $3.4 \times 10^{-6} \pm 0.5 \times 10^{-6}$ and $3.9 \times 10^{-7} \pm$
468 0.9×10^{-7} respectively (24)). Some putative transconjugants (2 clones for the whole experiment)
469 were recovered when using strains F1-4 or F4-2 as donors in mating experiments (Table 3) and
470 confirmed by PCR detection of the ICE integrase gene (data are shown only for ICE_*SsaF1-4_fda*,
471 Fig. 6). LMG18311 genetic background was confirmed by PCR amplification of the internal
472 transcribed spacer (ITS) followed by *Hae*III DNA digestion, which allows discriminating
473 *S. salivarius* strains and *S. thermophilus* LMG18311 (data are shown only for ICE_*SsaF1-4_fda*, Fig.
474 6a). JH2-2 genetic background was confirmed by the PCR amplification of the ITS and by the
475 amplification of *fda* gene with primers specific to *E. faecalis* (data are shown only for
476 ICE_*SsaF1-4_fda*, Fig. 6b).

477 ICE_*SsaF1-4_fda* carries 27 bp showing identity with the 3' end region of the *fda* gene of
478 *S. salivarius* (Fig. 7). This sequence (present in *attI*) enables specific recombination with the
479 chromosomal integration site of the recipient cell (*attB* site) and generates a 27 bp-imperfect direct
480 repeat (DR) in intraspecies transconjugants (Fig. 7). Sequencing of the *attR* site obtained after

481 integration of ICE_*SsaF1-4_fda* in *E. faecalis* JH2-2 recipient cells provided relevant information on
482 the DNA localization of one cutting site of *fda* integrase. This *attR* site is a hybrid between the
483 sequence found in the *attI* of ICE_*SsaF1-4_fda* and the *attB* of *E. faecalis* JH2-2 recipient cells.
484 Since the *attB* sequence in the recipient strains is different from the *attI* of ICE_*SsaF1-4_fda* (Fig. 7),
485 the presence of a T nucleotide at position 20 of the DR in the *attR* of the transconjugants, as in
486 ICE_*SsaF1-4_fda*, indicates that integrase cuts downstream of this nucleotide to allow strand
487 exchange during recombination. A previous work aiming at studying CIME-ICE accretion also
488 identified the same cutting position as well as the position of a second staggered cutting site located
489 6 bp downstream (Fig. 7) (56).

490 Intra- and interspecies conjugative transfer of two different ICEs found in *S. salivarius* was obtained
491 in laboratory conditions. These ICEs could thus play a significant role in HGT occurring both in the
492 oral cavity (as exemplified by the presence of a closely related ICE in *S. parasanguinis*) and in the
493 digestive tract.

494

495 **Impact of mitomycin C on excision and transfer of ICE_*SsaF1-4_fda***

496 A previous work demonstrated that excision and transfer of ICE*St3* of *S. thermophilus* can be
497 increased by treating donor cells with mitomycin C (42). Hence we examined the impact of such
498 treatment on excision and transfer of an ICE from *S. salivarius* (ICE_*SsaF1-4_fda*). Induction of ICE
499 excision by mitomycin C was tested by PCR amplification of *attI* and *attB* fragments using different
500 amounts of genomic DNA (0.1 µg to 1 pg) after MMC treatment or not of the cells (Fig. 8). The
501 minimal quantity of DNA producing a positive result for *attI* and *attB* was 0.1 ng for non-treated
502 cells whereas 10 pg of DNA were sufficient to detect a signal after MMC treatment of the cells (Fig.
503 8). Furthermore, when cells were treated with MMC, a higher intensity of the PCR signal was
504 observed for *attI* and *attB* using 0.1, 1 and 10 ng of DNA compared to cells without MMC treatment

505 (Fig. 8). Such difference was not observed for the *fda* control (Fig. 8). Thus, as for ICE*St3* (42),
506 MMC treatment leads to an increase of ICE_*SsaF1-4_fda* excision.

507 To evaluate the effect of MMC on ICE transfer, strain F1-4 was treated with MMC (at the
508 concentration inducing the maximum level of ICE excision) and used in filter mating experiments
509 with *S. salivarius* strain F1-8 as recipient. No difference of ICE transfer was detectable between cells
510 treated or not with MMC. This is in contrast to what was observed for ICE*St3* (42). Thus, it appears
511 that for ICE_*SsaF1-4_fda* excision is not a limiting step for transfer.

512

513 **Retransfer of ICE_*SsaF1-4_fda* from transconjugants**

514 To test whether ICE_*SsaF1-4_fda* transfers autonomously, retransfer was tested by using
515 transconjugants (obtained as described above) as donor cells. Plasmid pMG36e was removed by
516 curing transconjugants prior to the retransfer assays. F1-8 (ICE_*SsaF1-4_fda*) was used as donor
517 cells in filter mating experiments with either F1-8 (pMG36e) or LMG18311 (pMG36e) as recipient
518 cells. LMG18311 (ICE_*SsaF1-4_fda*) and JH2-2 (ICE_*SsaF1-4_fda*) were used as donor cells with
519 LMG18311 (pMG36e) and JH2-2 (pMG36e) respectively (Table 3). For each mating assay,
520 transconjugants were recovered and confirmed by PCR detection of both the ICE integrase gene and
521 the plasmid pMG36e present in the recipients (data not shown). A small number of transconjugant
522 colonies (as for intraspecies transfer of ICE_*SsaF1-4_fda* i.e. 2 clones for the whole experiment) was
523 observed when using *S. salivarius* as donor, and strains with the same genetic background as
524 recipients (F1-8/F1-8 mating pairs) or *S. thermophilus* LMG18311 as recipient cells. By contrast,
525 more transconjugant colonies (at least 20 clones for the whole experiment) were obtained using
526 *S. thermophilus* LMG18311 (ICE_*SsaF1-4_fda*) and *E. faecalis* JH2-2 (ICE_*SsaF1-4_fda*) as donors
527 and LMG18311 (pMG36e) and JH2-2 (pMG36e) as recipient cells. These results confirm that
528 ICE_*SsaF1-4_fda* is able to transfer autonomously but also suggest that (i) host factors could impact

529 ICE replication or/and assembly of the conjugation machinery in *S. salivarius* as already suggested
530 for some strains of *S. thermophilus* (26), (ii) molecules displayed at the cell surface of *S. salivarius*
531 could impact the donor/recipient cell contacts and thus the assembly of the conjugative machinery.
532 These hypotheses are consistent with the fact that no transconjugant was recovered when
533 *S. thermophilus* CNRZ368 donor cells carrying ICE $St3$ were mated with *S. salivarius* JIM8777 or
534 F1-8 recipient cells (data not shown in this study) whereas transconjugants were obtained using
535 *S. thermophilus* LMG18311 as recipient cells as already reported (24).
536 It should be kept in mind that *S. salivarius* encounters different physiological conditions in the oral
537 cavity and along the digestive tract. The impact on ICE regulation of these complex interactions
538 between bacteria and of changing environment in these ecological niches should be explored in close
539 future.

540

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549

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- 725
726

727 **LEGENDS OF FIGURES**

728

729 FIG 1 Comparison of the Integrative and Conjugative Elements (ICEs) found in *S. salivarius* and in
730 other streptococci.

731 ICEs are named according to their host strain and integration site. ICEs of *S. salivarius* are indicated
732 in bold. For more clarity, elements in accretion with ICEs are not shown. Nucleic sequence identity
733 higher than 80% between sequences is indicated in light grey and higher than 90% in dark grey.

734 Direct repeats (DR) delimiting ICEs are shown as a triangle, circle or square depending on their
735 sequences. ORFs appear as arrows (truncated genes are indicated by Δ). Modules of recombination
736 (integrase *int* and excisionase *xis* genes), conjugation (*orfA-orfO* genes) and regulation (including
737 *arp1*, *arp2* and *orfQ* genes) appear in red, blue and green respectively. The three different integration
738 genes (*fda*, *rpsI* or *rpmG*) targeted by the integrase are indicated by distinct symbols in the integrase
739 gene and is stated in the ICE name. Genes from the adaptation module encoding proteins with
740 putative function inferred from *in silico* analysis are indicated in color: in pink for RM systems and
741 orphan methyltransferase genes, in dark grey for cadmium resistance genes (*cadD/X*), in yellow for
742 the membrane lipid synthesis cluster, in orange for the bacteriocin synthesis cluster, in light green for
743 genes encoding cell envelop proteins, in light pink for the cluster of genes for putative lipoprotein
744 transport, in brown for the two component system (TCS), in black for the *tnp* transposase gene(s).

745 Sequences used for this analysis are: ICE*St3* of *S. thermophilus* [AJ586568] and sequenced genomes
746 of *S. pneumoniae* 2842STDY5753514 [FDNK01000013], *S. macedonicus* 33MO [JNCV01000015]
747 and *S. parasanguinis* DD19 [LQNY01000339-LQNY01000340]. Gaps in the assembly found in
748 ICE_*SsaF6-1_rpsI*, ICE_*SsaB57_fda* and ICE_*SpaDD19_fda* are indicated by a double slash.

749

750 FIG 2 Phylogenetic BioNJ tree obtained for relaxases (OrfJ) of ICEs belonging to the ICESt3
751 subfamily. The relaxase protein sequence of twenty-seven ICEs (the 19 ICEs of the ICESt3
752 subfamily with closely related conjugation modules and 8 additional ones previously reported to
753 belong to the same ICE subfamily but showing more distantly related conjugation modules) were
754 included in the analysis. ICEs of *S. salivarius* are indicated in bold and *ICE_Sma33MO_fda* is
755 indicated by a star. Bootstrap values supporting main branches are given for BioNJ/ML respectively.

756

757 FIG 3 Phylogenetic BioNJ tree obtained for coupling proteins (OrfK) of ICEs of the ICESt3
758 subfamily. The sequence of the coupling protein of twenty-seven ICEs (the 19 ICEs of the ICESt3
759 subfamily with closely related conjugation modules and 8 additional ones previously reported to
760 belong to the same ICE subfamily but showing more distantly related conjugation modules) were
761 included in the analysis. ICEs of *S. salivarius* are indicated in bold and *ICE_Sma33MO_fda* is
762 indicated by a star. Bootstrap values supporting main branches are given for BioNJ/ML respectively.

763

764 FIG 4 PCR detection of integrated and excised forms of *ICE_SsaF1-4_fda*.

765 The sizes of the PCR fragments obtained for the amplification of the *attB*, *attI*, *attR* and *attL* sites
766 (586 bp, 887 bp, 900 and 523 bp respectively) were confirmed by parallel migration of a DNA
767 ladder. The primer pairs used for these amplifications are indicated in Table 2.

768

769 FIG 5 Characterization of transconjugants carrying *ICE_SsaF1-4_fda* after intraspecies transfer.

770 F1-4 (*ICE_SsaF1-4_fda*) was used as donor in mating experiments with (a) JIM8777 (pMG36e) and
771 (b, c) F1-8 (pMG36e) as recipients. Tc. indicates transconjugants; integrase, amplification of the
772 integrase gene of *ICE_SsaF1-4_fda*; pMG36e, amplification of an internal fragment of the pMG36e
773 plasmid; RAPD, random amplification of polymorphic DNA; *attL*, *attR*, *attI* and *attB*, amplification

774 of fragments carrying these attachments sites in F1-8 transconjugants (c). The primer pairs used for
775 these amplifications are indicated in Table 2.

776

777 FIG 6 Characterization of transconjugants carrying ICE_*SsaF1-4_fda* after interspecies transfer.

778 F1-4 (ICE_*SsaF1-4_fda*) was used as donor in mating experiments with (a) LMG18311 (pMG36e)
779 and (b) JH2-2 (pMG36e) as recipients. Tc. indicates transconjugants; integrase, amplification of the
780 integrase gene of ICE_*SsaF1-4_fda*; ITS16S/23S, amplification of the internal transcribed spacer;
781 ITS16S/23S+*Hae*III digestion, amplification of the internal transcribed spacer followed by *Hae*III
782 digestion of the obtained fragment; EF *fda*, amplification of *fda* fragment using *E. faecalis* specific
783 primers.

784

785 FIG 7 Localization of the DNA cutting site of integrase by sequencing of *attR* site in *E. faecalis* JH2-
786 2 transconjugants.

787 ICE_*SsaF1-4_fda* is shown in green in its integrated form in donor *S. salivarius* strain F1-4 (upper
788 part of the figure), its excised form (middle part of the figure) and its integrated form in *E. faecalis*
789 JH2-2 recipient strain after transfer and integration (lower part of the figure). Nucleotide differences
790 in *att* sequences are indicated in green for ICE_*SsaF1-4_fda* and in red for *E. faecalis* JH2-2. The
791 position of the DNA cutting site of integrase deduced from this analysis is indicated by a black
792 arrow. A previous work aiming at studying CIME-ICE accretion also identified the same cutting
793 position as well as the position of a second staggered cutting site located 6 bp from the first one
794 (indicated in grey in the figure) (56).

795

796 FIG 8 Impact of mitomycin C on ICE_*SsaF1-4_fda* excision.

797 Fragments corresponding to the recombination sites *attB* and *attI* of non-treated (MMC-) or treated
798 (MMC+) cells were amplified by PCR using template DNA quantities ranging from 0.1 µg to 1 pg.
799 The amplification of the *fda* gene was used as a control.

800

Accepted version

TABLE 1 Bacterial strains and plasmids used in this study (to be continued)

Strains and plasmids	Relevant phenotype or genotype	Source or reference
Strains		
<i>S. salivarius</i>		
B35	Wild-type (WT) strain carrying a putative ICE of the ICES <i>t3</i> subfamily	(23)
B57	WT strain carrying a putative ICE of the ICES <i>t3</i> subfamily	(23)
F1-4	Wild-type (WT) strain carrying a putative ICE of the ICES <i>t3</i> subfamily (ICE_ <i>SsaF1-4_fda</i>)	(23)
F1-4(ICE_ <i>SsaF1-4_fda</i>) Cm ^r	F1-4 carrying ICE_ <i>SsaF1-4_fda</i> tagged with a Cm ^r cassette	This work
F1-8	WT strain carrying a putative ICE in accretion with an IME integrated in the 3' end of <i>rpmG</i> , no element in <i>fda</i>	(23)
F1-8(pMG36e)	F1-8 carrying pMG36e, a plasmid conferring erythromycin resistance	This work
F4-2	WT strain carrying a putative ICE of the ICES <i>t3</i> subfamily	(23)
F6-1	WT strain carrying a putative ICE of the ICES <i>t3</i> subfamily	(23)
JIM8777	WT strain carrying a putative IME in the 3' end of <i>rpmG</i> and a putative CIME in the 3' end of <i>rpsI</i> , no element integrated in the <i>fda</i> gene	(57)
JIM8777(pMG36e)	JIM8777 carrying pMG36e, a plasmid conferring erythromycin resistance	This work
L22	WT strain carrying a putative ICE of the ICES <i>t3</i> subfamily	(23)
L50	WT strain carrying a putative ICE of the ICES <i>t3</i> subfamily	(23)
L60	WT strain carrying a putative ICE of the ICES <i>t3</i> subfamily	(23)
L64	WT strain carrying a putative ICE of the ICES <i>t3</i> subfamily	(23)
N5	WT strain carrying a putative ICE of the ICES <i>t3</i> subfamily	(23)
N20	WT strain carrying a putative ICE of the ICES <i>t3</i> subfamily	(23)
T93	WT strain carrying a putative ICE of the ICES <i>t3</i> subfamily	(23)
<i>S. thermophilus</i>		
CNRZ385 ICES <i>t3cat</i>	CNRZ385 carrying ICES <i>t3</i> with <i>cat</i> gene inserted in <i>orf385J</i> pseudogene, Cm ^r	(24)
LMG18311	WT strain with no element in <i>fda</i>	BCCM/LMG
LMG18311(pMG36e)	LMG18311 carrying pMG36e, a plasmid conferring erythromycin resistance	(24)

TABLE 1 Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant phenotype or genotype	Source or reference
<i>E. faecalis</i>		
JH2-2	WT strain with no element in <i>fda</i>	(58)
JH2-2(pMG36e)	JH2-2 carrying the plasmid pMG36e, conferring erythromycin resistance	(24)
Plasmids		
pSET5s	pWV01-type thermosensitive replication origin from pVE6002, LacZ, Cm ^r	(59)
pMG36e	3.6 kb, replication origin from pWV01, Ery ^r	(49)

TABLE 2. Primers used in this work (to be continued).

Primer use	Primer name	Sequence (5'-3')	Reference
ICE labeling			This study
	F1-4 CDS7 F	GAGATTGAGCATATCCTTCC	
	F1-4 CDS8 R_Bis	GGTGACTAGTTATCTACACGCGAGATTCGTGGACTAACTT	
	F1-4 CDS9 F_Bis	CCATATCCTTCTTTTTCTGCTCACTATCTTGTTTCGTTTTGT	
	F1-4 CDS9 R	GGAGAGTTTAGCTGGGAGG	
	ICE F4-2 fragment I_F	GGAAATATCCTGTTGTCATC	
	ICE F4-2 fragtI_R-catbis	GGTGACTAGTTATCTACACGCCTATAAAGTTGTTAAGTTCACT	
	ICE F4-2 fragtII_F-catbis	CCATATCCTTCTTTTTCTGGCGTGTAATTGAAGAGTGA	
	ICE F4-2 fragment II_R	GTCTAAACTGAGCCAAGAAG	
	Cat_F	GCCTCCTAAATTCACTTTAG	
	Cat_R	GTAAAAAGTACAGTCGGCAT	
Detection of ICE integrated and excised forms			
<i>attB</i> amplification			(23)
	attBfdaSsal	GCCCAACCAAATAAACAATAAA	
	attB ST3 Rev	CTCTTCGACCCACGTAATTC	
<i>attI</i> amplification			(23)
	intST3 For	AGGGCTTTCTGACGAATTAG	
	attI ST3 Rev	CGGTGTAATGGGAAGTATGG	
<i>attL</i> amplification			
	attL Rv CDS gwIG	CGGTGTAATGGGAAGTATGG	This study
	attBfdaSsal	GCCCAACCAAATAAACAATAAA	(23)
<i>attR</i> amplification			
	intST3 for	AGGGCTTTCTGACGAATTAG	(23)
	intICEST3-fdaRev	ACCAGGTTTCGATGCTATTACAG	(43)
Integrase gene			(23)
	intST3 for	AGGGCTTTCTGACGAATTAG	
	intST3 Rev	GAGTTCTAATAACTGAGGCTA	

TABLE 2. Primers used in this work

Primer use	Primer name	Sequence (5'-3')	Reference
Semi-quantitative PCR			
<i>attI</i> amplification	intICEF1-4 For attIICEF1-4 Rev	AGGTCTTTCTGACGAATTAG CGGCGTAATGGGAAGTATGG	This study
<i>attB</i> amplification	attBfdaSsal attB ST3 Rev	GCCCAACCAAATAACACTAAA CTCTTCGACCCACGTAAATTC	(23)
<i>fda</i> amplification	Fda1 Fda2	TTCAAGAATTTACGTGGG AGATGCTAAAGCTATGGTTG	This study (34)
Donor / Recipient discrimination			
ITS16S/23S	16SITS 23SITS	TTGTACACACCGCCCGTCA GGTACCTTAGATGTTTCAGTTC	(24)
<i>ddlA</i> gene	ddlA up ddlA dn	TCAAGTGTGGCTATGGA GTAGATGGCTCCATCCTC	(60)
<i>E. faecalis fda</i> specific amplification	Efa fba1b Efa fba2	ATGTGTTCTTCTGCATCTTT CCCATTGATTACGATTTTT	(24)
RAPD	XD9	GAAGTCGTCC	(34)
pMG36e	pMG36e Fwd pMG36e_R	GCCTCCTCATCCTCTTCAT ACAGAACCGTTTCTACTCAATGAAC	(24) This study

TABLE 3 Mating pairs tested in filter experiments

	Donor cells with AB ^r ^a	Recipient cells with AB ^r ^a
Intraspecies transfer	F1-4(ICE_ <i>SsaF1-4_fda</i>) Cm ^r	JIM8777 (pMG36e) Ery ^r F1-8 (pMG36e) Ery ^r
	F4-2(ICE_ <i>SsaF4-2_fda</i>) Cm ^r	JIM8777 (pMG36e) Ery ^r F1-8 (pMG36e) Ery ^r
Interspecies transfer	F1-4(ICE_ <i>SsaF1-4_fda</i>) Cm ^r	LMG18311 (pMG36e) Ery ^r JH2-2 (pMG36e) Ery ^r
	F4-2(ICE_ <i>SsaF4-2_fda</i>) Cm ^r	LMG18311 (pMG36e) Ery ^r JH2-2 (pMG36e) Ery ^r
ICE retransfer from transconjugants	F1-8(ICE_ <i>SsaF1-4_fda</i>) Cm ^r	F1-8 (pMG36e) Ery ^r LMG18311 (pMG36e) Ery ^r
	LMG18311(ICE_ <i>SsaF1-4_fda</i>) Cm ^r	LMG18311 (pMG36e) Ery ^r
	JH2-2(ICE_ <i>SsaF1-4_fda</i>) Cm ^r	JH2-2 (pMG36e) Ery ^r

^a AB^r antimicrobial resistance of the strain

FIG 1

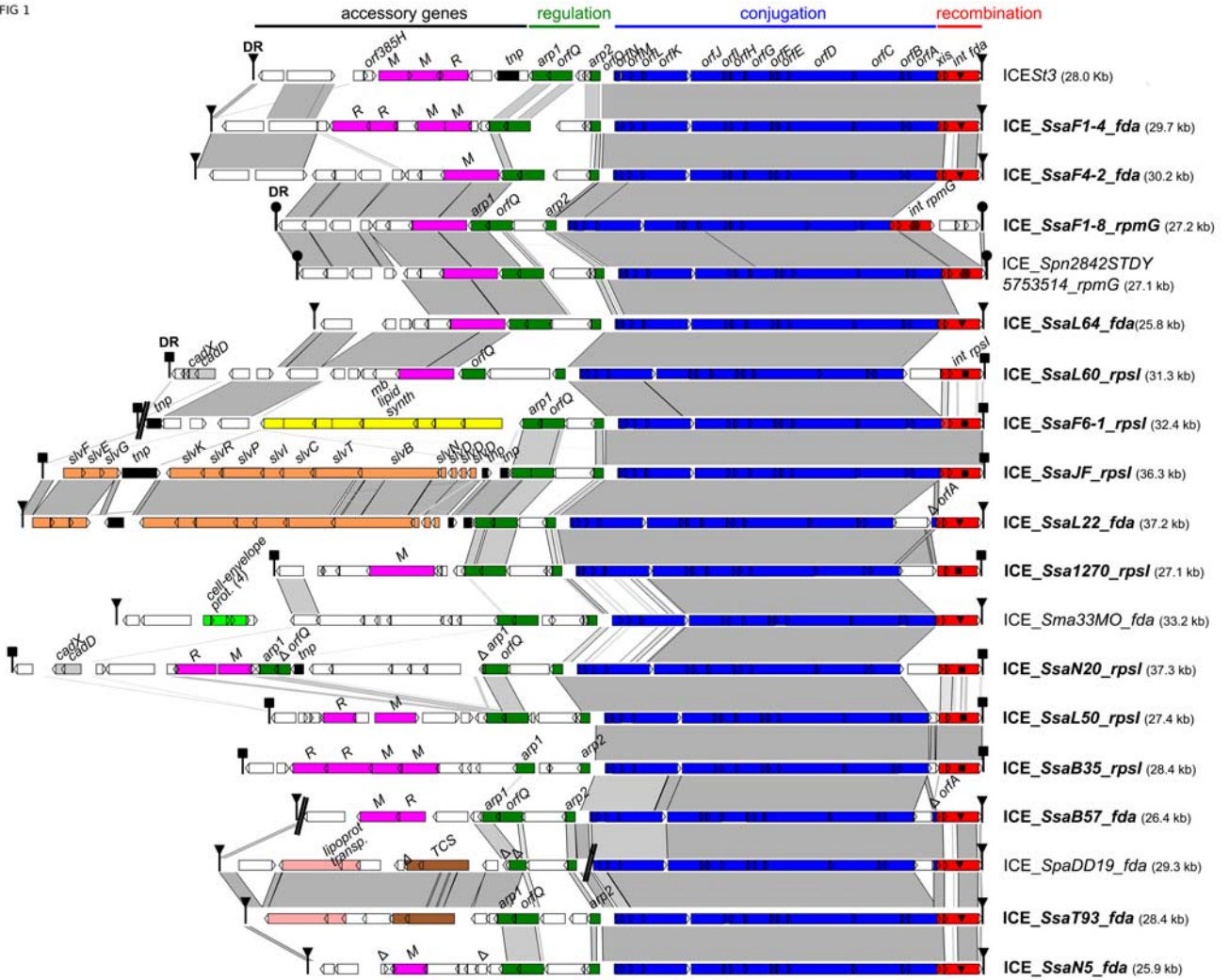


FIG 2

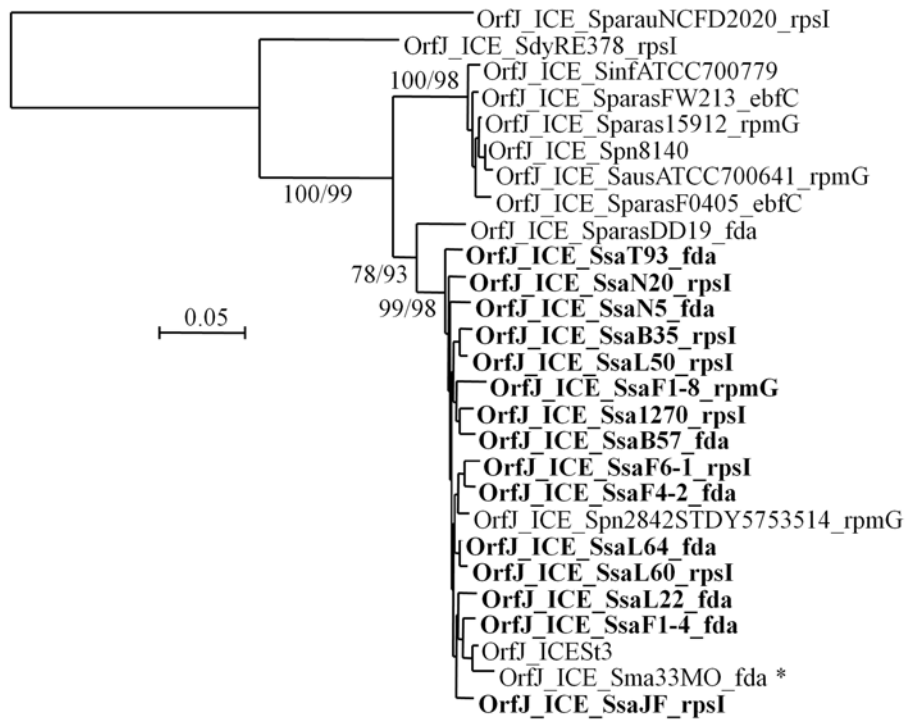


FIG3

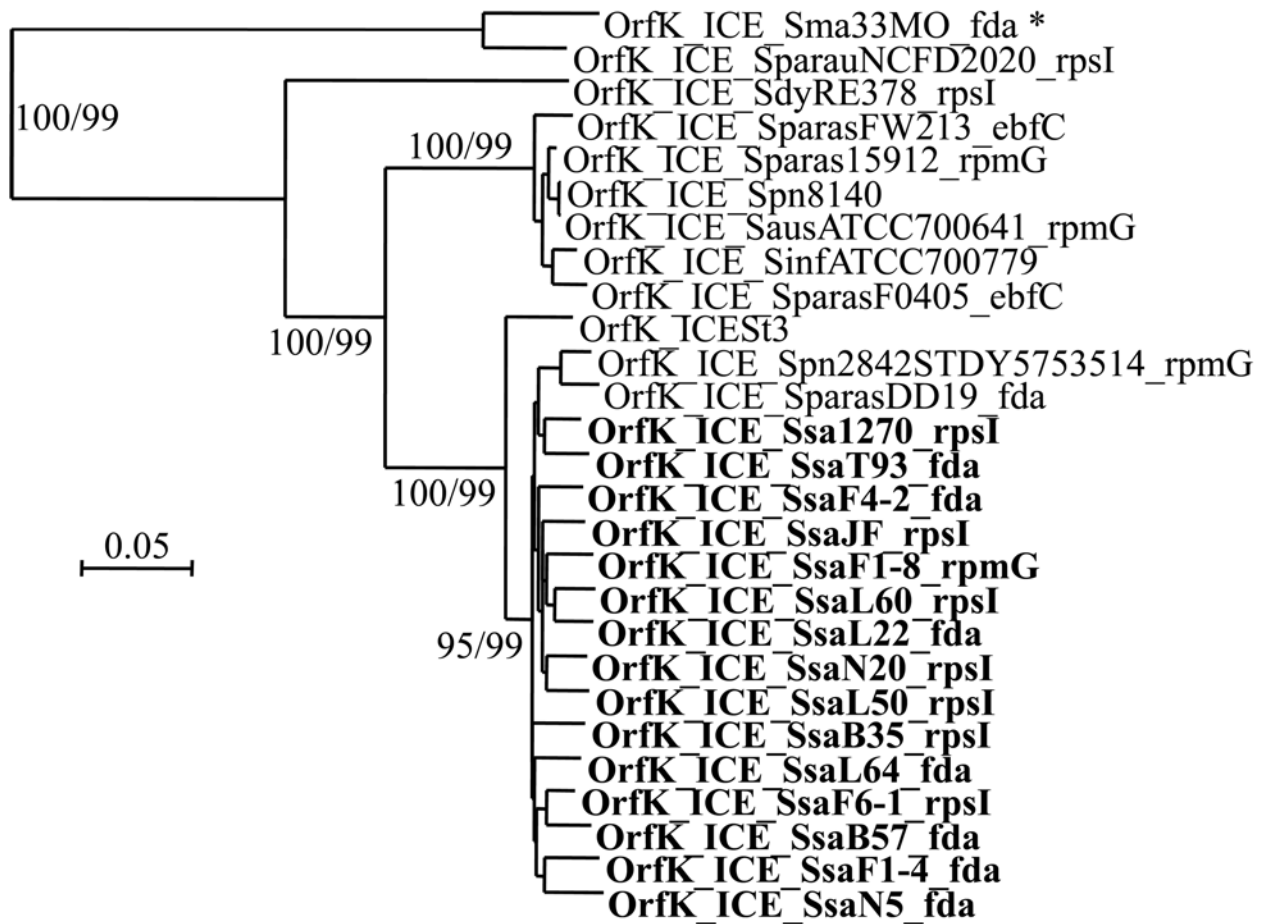
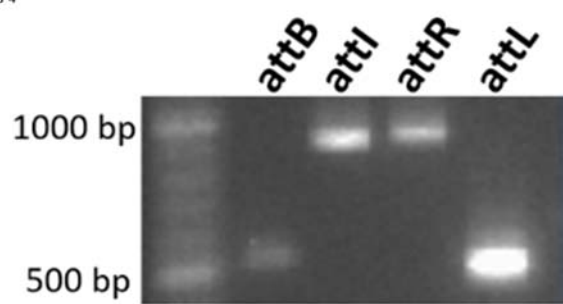
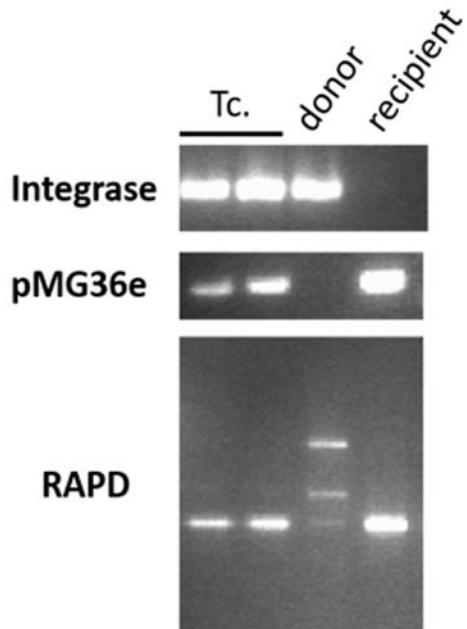


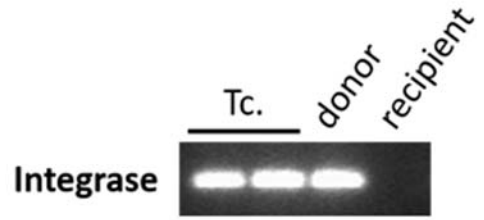
FIG 4



a



b



c

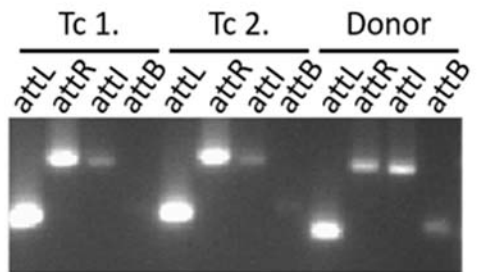
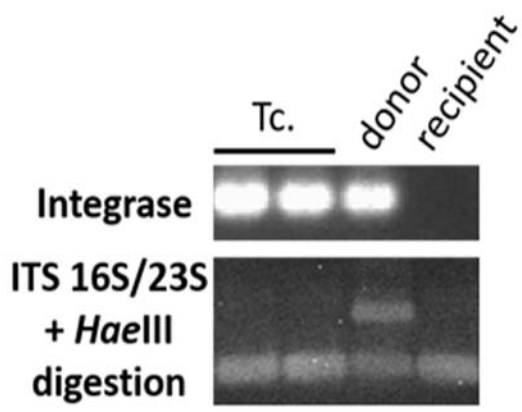


FIG6

a



b

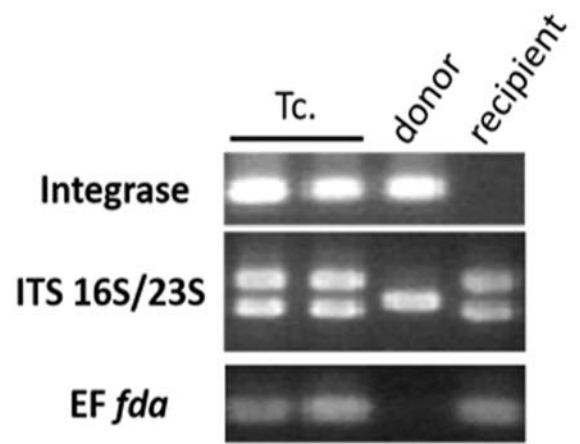


FIG7

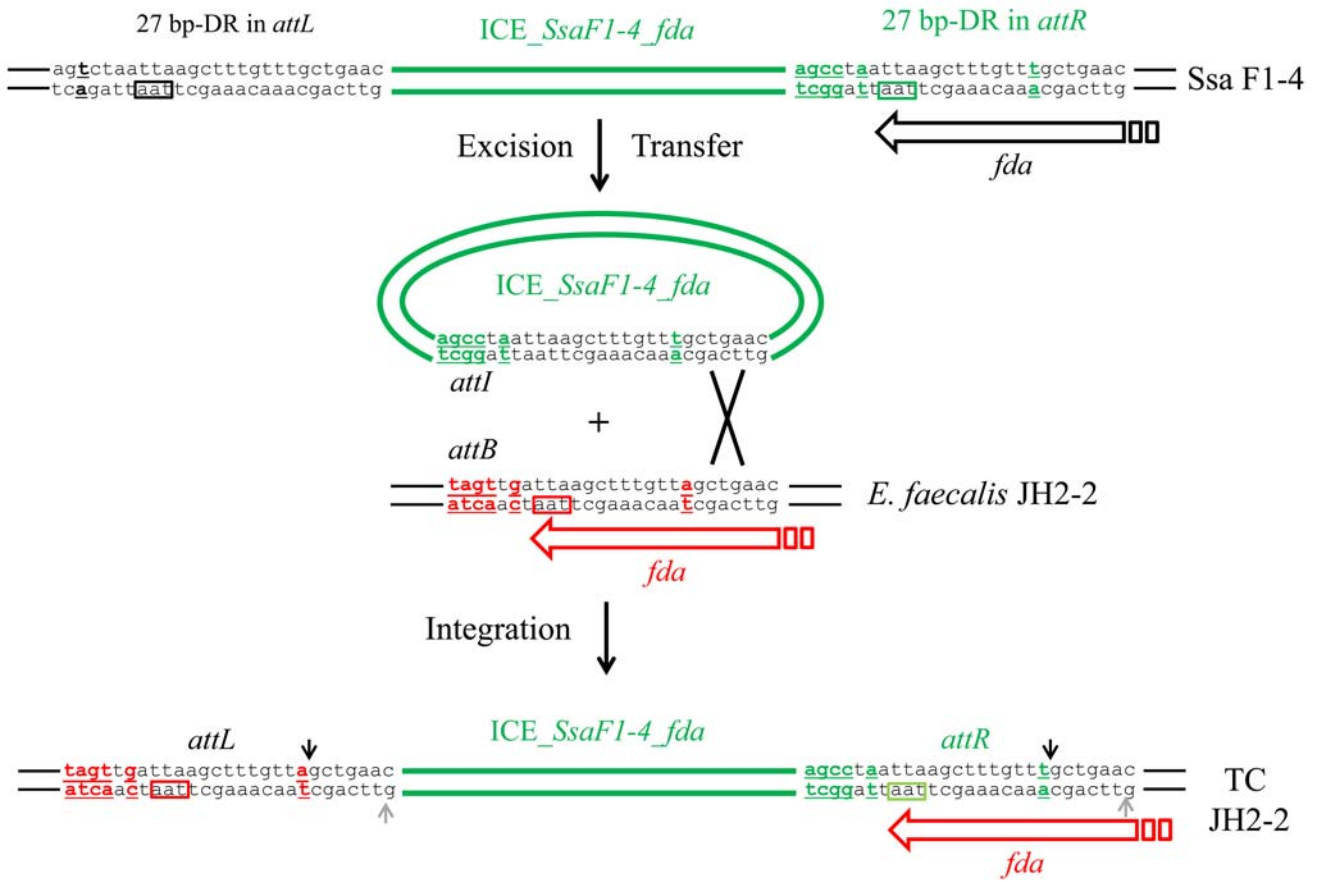
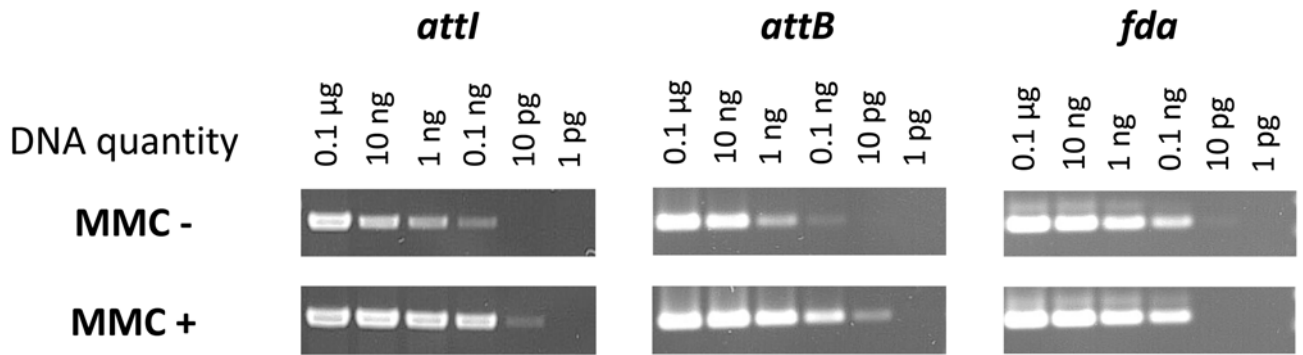


FIG8



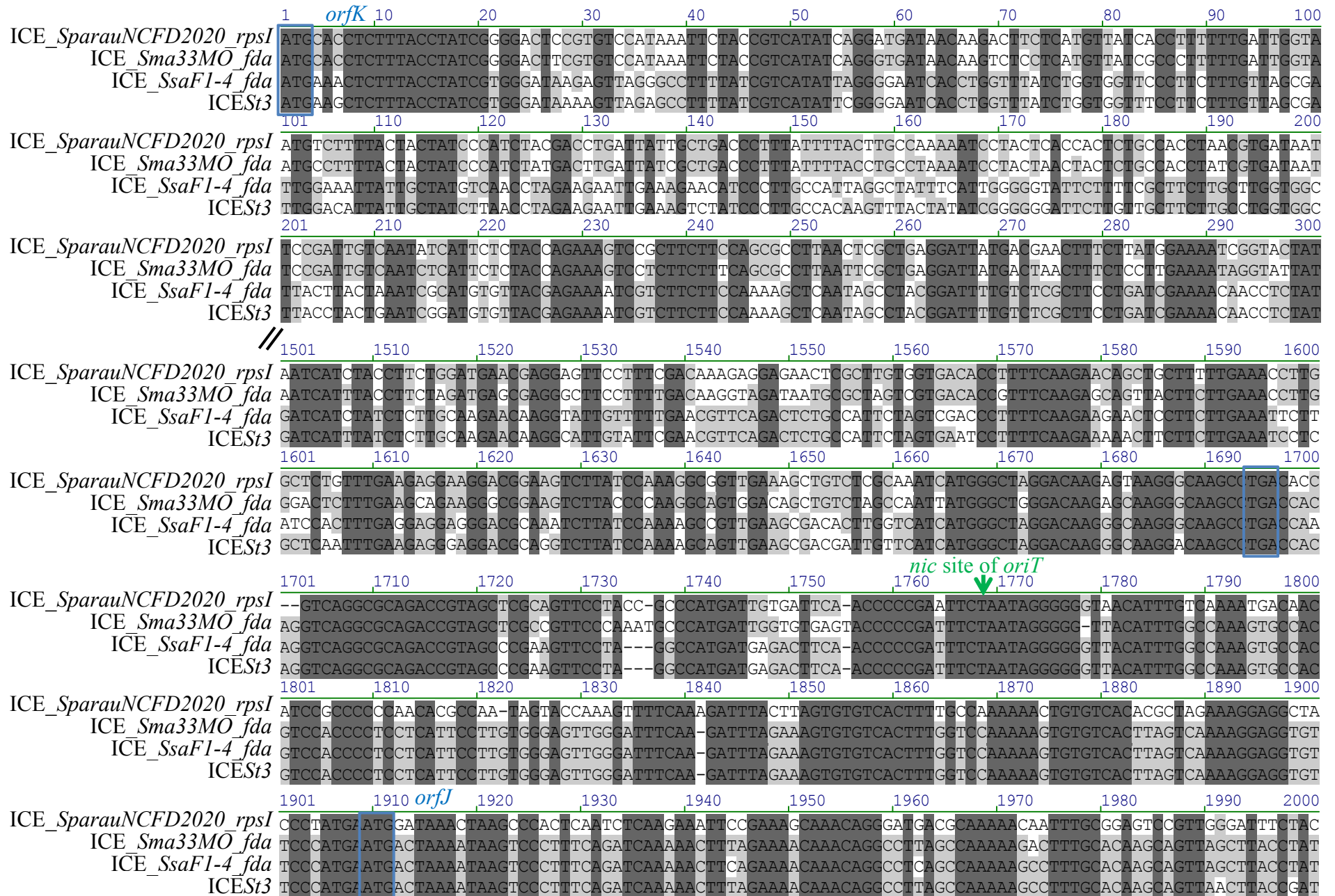


Fig. S1: sequence comparison of *orfK*, intergenic sequence *orfK-orfJ* and beginning of the *orfJ* gene in ICEs found in *S. parasanguinis* NCFD2020, *S. macedonicus* 33MO, *S. salivarius* F1-4 compared to ICESt3 of *S. thermophilus*. Start and stop codons are indicated as blue rectangles and the *nic* site of *oriT* is indicated as a green arrow.

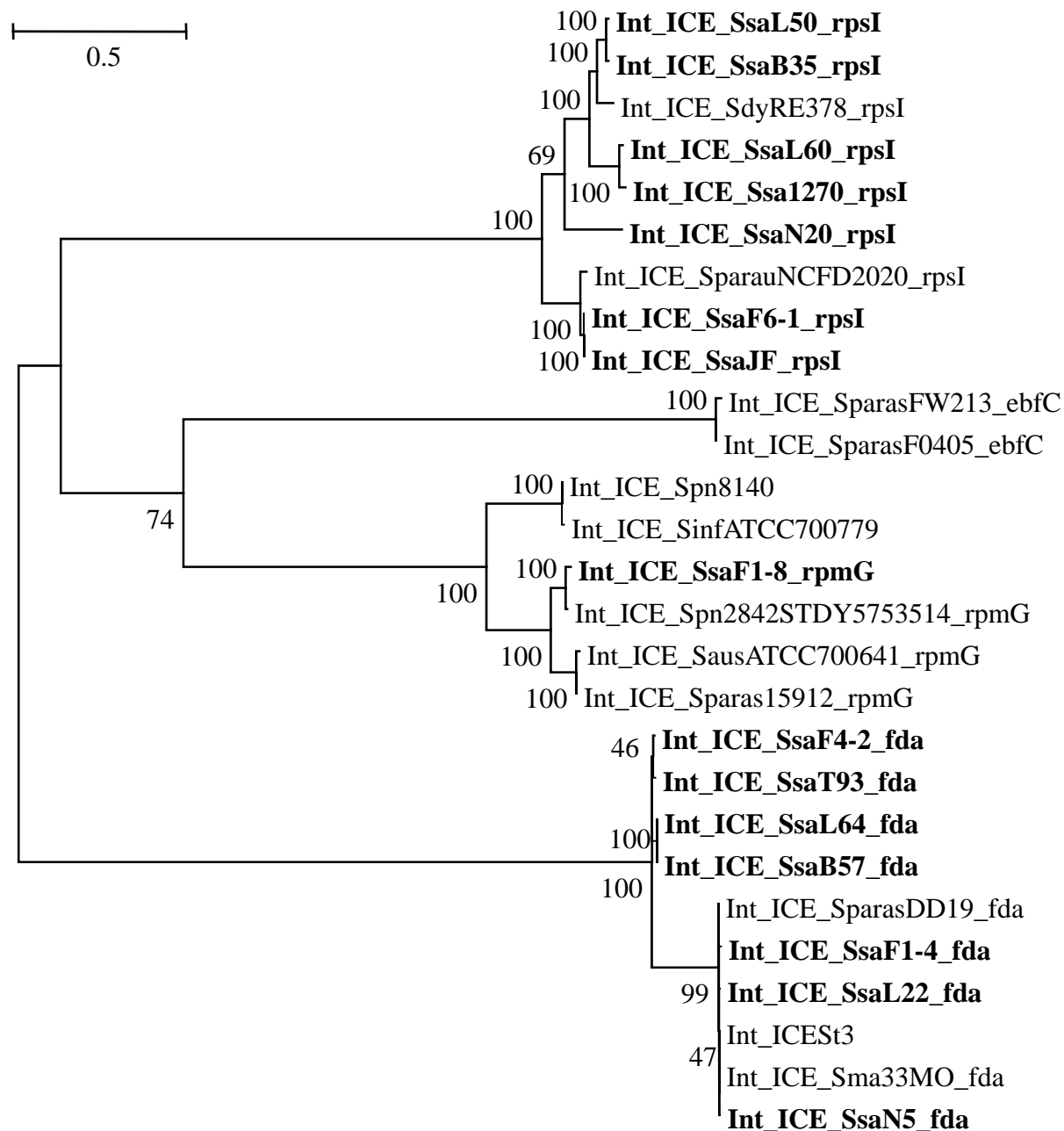


Fig. S2. Phylogenetic BioNJ tree obtained for integrases of ICEs belonging to the *ICES_{t3}* subfamily. The integrase protein sequence of twenty-seven ICEs (the 19 ICEs of the *ICES_{t3}* subfamily with closely related conjugation modules and 8 additional ones previously reported to belong to the same ICE subfamily but showing more distantly related conjugation modules) were included in the analysis. ICEs of *S. salivarius* are indicated in bold. Bootstrap values supporting main branches are given at nodes.