New Insights into the Classification and Integration Specificity of Streptococcus Integrative Conjugative Elements through Extensive Genome Exploration

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Recent genome analyses suggest that integrative and conjugative elements (ICEs) are widespread in bacterial genomes and therefore play an essential role in horizontal transfer. However, only a few of these elements are precisely characterized and correctly delineated within sequenced bacterial genomes. Even though previous analysis showed the presence of ICEs in some species of Streptococci, the global prevalence and diversity of ICEs was not analyzed in this genus. In this study, we searched for ICEs in the completely sequenced genomes of 124 strains belonging to 27 streptococcal species. These exhaustive analyses revealed 105 putative ICEs and 26 slightly decayed elements whose limits were assessed and whose insertion site was identified. These ICEs were grouped in seven distinct unrelated or distantly related families, according to their conjugation modules. Integration of these streptococcal ICEs is catalyzed either by a site-specific tyrosine integrase, a low-specificity tyrosine integrase, a site-specific single serine integrase, a triplet of site-specific serine integrases or a DDE transposase. Analysis of their integration site led to the detection of 18 target-genes for streptococcal ICE insertion including eight that had not been identified previously (ftsK, guaA, lysS, mutT, rpmG, rpsI, traG, and ebfC). It also suggests that all specificities have evolved to minimize the impact of the insertion on the host. This overall analysis of streptococcal ICEs emphasizes their prevalence and diversity and demonstrates that exchanges or acquisitions of conjugation and recombination modules are frequent.

Keywords: integrative and conjugative elements, T4SS, integrase, integration site, Streptococcus

INTRODUCTION

Streptococci are Gram positive bacteria belonging to the phylum of Firmicutes. This genus comprises 110 recognized species (*July 24, 2015). Almost all streptococci are commensal or pathogen of humans and/or animals. Numerous streptococci, such as Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus mutans or Streptococcus agalactiae, are responsible for a wide range of diseases in humans and animals. The streptococci are able to acquire DNA from their environment in order to establish new virulence traits and adapt to the environment in which they are hosted. This process relies on their ability to mediate recombination, transformation and conjugation. Conjugation is performed by a large family of plasmids, called conjugative elements (CEs), that transfer DNA from one bacterium to another within a short time. This process requires the presence of two modules: an integrase and an extracytoplasmic pilus that allows the two bacteria to come into close contact. This study focused on the classification and specificity of Streptococcus ICEs to better understand the diversity of these elements and their impact on the host.
variety of diseases worldwide, ranging from mild to invasive infections that have a severe impact on human and animal health and lead to significant morbidity and mortality (Mitchell, 2003; Kohler, 2007). Streptococci are also ubiquitously present as commensal inhabitants of the gastro-intestinal tracts of healthy adults and/or newborns. *Streptococcus salivarius*, in particular, is one of the first colonizers of the human oral cavity (Park et al., 2005; Nakajima et al., 2013) and is also a dominant part of the early life human intestinal microbiota (Arrieta et al., 2014). At last, two species deriving from commensal streptococci, *S. thermophilus* and *S. macedonicus* are used as starters in dairy industry to transform milk in yogurt and/or cheese (Franciosi et al., 2009).

During the last 20 years, it has become increasingly apparent that horizontal gene transfer (HGT) of genomic islands plays a key role in bacterial evolution and adaptation (Hacker and Kaper, 2000; Hacker and Carniel, 2001; Dobrindt et al., 2004; Juhas et al., 2009). In essence, genomic islands are chromosomal segments acquired by HGT that carry gene sets enhancing the fitness of their hosts. Recent data suggest that numerous genomic islands correspond to non-canonical classes of mobile genetic elements (MGEs) that can transfer by conjugation or are non-mobile elements deriving from such MGEs (Bellanger et al., 2014). Among them, the integrative and conjugative elements (ICEs) are mobile elements integrated in bacterial chromosomes or plasmids which encode their own excision, their transfer by conjugation, and their integration (Burrus et al., 2002b; Bellanger et al., 2014).

Integration of ICEs is catalyzed by three phylogenetically and structurally unrelated families of enzymes: tyrosine integrases, serine integrases, and DDE transposases (Wozniak and Waldor, 2010; Bellanger et al., 2014). Both tyrosine and serine integrases usually catalyze site-specific recombination between small (2–60 bp) similar or identical sequences included in the *attL* site of the circular form of the ICE and the *attB* site of the bacterial genome. This leads to the formation of *attL* and *attR* sites flanking the integrated elements; as a consequence, the integrated ICE is flanked by direct repeats (DR). Usually, tyrosine integrases catalyze ICE integration in a large array of specific sites, including the 3′ end of tRNA genes and the 3′ or 5′ end of genes encoding various housekeeping proteins (Bellanger et al., 2014). One exception is the tyrosine integrase of Tn916 that shows low integration specificity; as a consequence, Tn916 and its relatives are not flanked by DRs. Knowledge of the integration specificity of serine integrases from ICE is scarce. The third family of enzymes, DDE transposases, catalyzes transposition of DNA segments. Binding of the enzyme to terminal inverted repeats (IRs) at the extremities of elements enables strand cleavage required for the transposition reaction. DDE transposases have a low specificity of integration and catalyze the duplication of the target sequence (2–13 bp). Up to now, only one subfamily of DDE transposases was described for streptococcal ICEs. These DDE integrases catalyze the integration 15–16 bp upstream of the −35 box of the promoter region of various genes (Brochet et al., 2009; Guérillot et al., 2013).

The first step of the conjugative transfer is the excision of the ICE as a circular form that is ensured by the same enzyme as for integration. In general, tyrosine integrases need additional co-factors to carry out the reverse excision reaction (Groth and Calos, 2004); these are encoded by the element. So far, most ICEs (including all ICEs from Firmicutes) transfer as single-strand DNA. The transfer of the excised ICE would be similar to the transfer of conjugative plasmids that is well-known in Gram negative bacteria (Smailie et al., 2010; Low et al., 2014). The circularized ICE DNA is taken over by a relaxosome, a complex that includes a relaxase. A relaxase is a trans-esterase, acting as a dimer, that catalyzes a site and strand-specific cleavage at the nic site of the origin of transfer (*oriT*) of its cognate ICE. The relaxase, covalently bound to the 5′ end of the single-stranded DNA, is then recognized by the membrane-associated coupling protein (CP) that interacts with a type IV secretion system (T4SS). T4SSs are ATP-powered and multi-protein complexes that span the cellular envelopes of the donor cell in Gram negative bacteria. CP and T4SS translocate the DNA-relaxase complex through membranes and cell walls into the recipient cell. A rolling-circle replication of the element is likely concomitant to its transfer so that the ICE is not lost in the donor cell. Finally, the relaxase achieves the transfer by recircularizing the ICE (for a review see (Bellanger et al., 2014)). Although the conjugative transfer of DNA in Firmicutes is poorly known, it relies on relaxase, CP and T4SS (Goessweiner-Mohr et al., 2013; Guglielmini et al., 2014). Recent analyses suggest that T4SSs of Firmicutes include an homolog or analog of most T4SS membrane-spanning proteins found in inner membranes of Gram negative bacteria including the ATPase VirB4, VirB3, VirB6, VirB8 and the cell-wall degrading enzyme VirB1 (Goessweiner-Mohr et al., 2013; Guglielmini et al., 2014; Leonetti et al., 2015).

Like all other bacterial MGEs (Toussaint and Merlin, 2002), ICEs have a modular structure, i.e., the genes involved in the same biological function (such as conjugation or integration/excision) are physically linked. In addition to the integrase and recombination directionality factor genes, the integration/excision module includes the recombination site *attI*. It is thus generally located at one end of the integrated element. The conjugation module includes genes coding for the T4SS, the CP, the relaxase (and eventually accessory proteins of the relaxase) and *oriT*. The regulation module encodes all the genes involved in the regulation of ICE dissemination and maintenance. In addition to modules dedicated to gene transfer, all ICEs also carry at least one adaptation module that encodes adaptive traits that might be beneficial for bacteria under certain growth or environmental conditions. Adaptation modules are highly variable and include genes involved in antimicrobial resistance, virulence or alternative metabolic pathways (Burrus et al., 2002a,b; van der Meer and Sentchilo, 2003; Schubert et al., 2004; Heather et al., 2008; Croucher et al., 2009; Chuzeville et al., 2012).

Although ICEs have a major impact on gene flow and genome dynamics in bacteria, their prevalence and diversity remain largely underscored (Bellanger et al., 2014).

In this work, we took advantage of the increase of publicly available genomic sequences (124 complete genomes of *Streptococcus* available at the beginning of this work) to
search for ICEs using the combined presence of signature proteins (from conjugation and integration/excision modules). Coding sequences (CDSs) encoding these signature proteins were localized on the chromosomes and a strategy was developed to search for ICEs boundaries and to identify their integration site. This work (i) gives a general overview of the high prevalence and diversity of ICEs within *Streptococcus* species, (ii) identifies their numerous sites of insertion, and (iii) sheds light on their phylogenetic relationships and on their modular evolution.

**MATERIALS AND METHODS**

**Genomes Examined and Database of Reference Proteins**

The dataset of the 124 complete chromosomes from *Streptococcus* species available at the beginning of this work was taken from GenBank (last accessed December 2013).

The initial database of reference proteins contains signature proteins from ICEs reported for Firmicutes in the literature at the beginning of this study. It includes protein sequences from 50 tyrosine integrases, 13 serine integrases, two DDE transposases, 50 relaxases, 37 CP, and 26 VirB4 proteins.

**Search Strategy**

The overall workflow of our search strategy to detect and characterize ICEs in streptococcal chromosomes is depicted in Figure 1.

**Detection of Signature Sequences in the Genomes of Streptococci**

The first step of our workflow consists in the search for signature proteins (tyrosine integrase, serine recombinase or DDE transposase, CP, relaxase, and VirB4 proteins). It was performed by BlastP comparison, using the accelerated BlastP version implemented in the ngKlast software (Nguyen and Lavrien, 2009; with default parameters except for disabled low-complexity filter). The queries were the sequences of all reference ICE proteins and the target was the set of multifasta files (one per genome) representing all translated CDSs of the studied genomes. Expert filters were designed in the ngKlast system to remove hits corresponding to the translation of pseudogenes and to related proteins not involved in transfer or integration of conjugative elements (i.e., recombinases involved in inversion of DNA segments or in resolution of DNA molecule multimers, transcriptional regulators carrying a HTH DNA binding domain, DNA translocase FtsK involved in cell division, some toxins, etc.). These filters include a percentage cover threshold (>25%), an E-value threshold (<1.10^{-4}) for relaxases and integrases and <1.10^{-5} for CPs and VirB4) and a length threshold (>320 amino acids for integrases, >180 amino acids for relaxases, between 180 and 700 aa and between 1000 and 1200 aa for CPs and >500 amino acids for VirB4). Related proteins with biological function other than conjugation (i.e., XerS) passing through filters were manually removed. An iterative search was performed with the reference protein database enlarged with the newly found proteins and the same parameters until no new hit was found. Thus, only elements lacking significant sequence similarity with all signature proteins of ICE or carried by a plasmid could be missed. Sequence redundancy was eliminated using the BlastClust program with an identity threshold of 90%. The final reference database for ICE signature proteins contains non-redundant sequences from 106 tyrosine integrases, 43 serine integrases, 10 DDE transposases, 72 relaxases, 45 CPs, and 36 VirB4 proteins.

The relative positions of the CDSs corresponding to the detected signature proteins were visualized using Artemis (Rutherford et al., 2000). This step allowed checking if the CDSs co-localize in the genome and can thus be part of the same ICE. The detection of VirB4 guarantees the retrieval of ICEs rather than IMEs (integrative and mobilizable elements) that never encode this protein.

**Detection of Insertion Sites and Delimitation of Putative ICEs**

In the second step of the workflow (Figure 1), CDSs known to be potential insertion sites for ICE encoding site-specific integrases were searched and retained as potential candidates for insertion sites if located close to the CDSs of signature proteins. In their absence, insertion site was examined by comparing synteny with other genomes.

Putative ICEs were delimited by searching DRs on both sides of the putative ICEs by BLASTn analysis with either the 3’ end or the 5’ end of the potential insertion CDS or tRNA genes as a “query.” If no such potential insertion CDS was detected, the intergenic sequence downstream from the integrase gene was used as a query. ICEs closely related to Tn916 were delimited by BLASTn with the ends of Tn916 from *Enterococcus faecalis* DS16 as queries.

**ICE/Decayed ICE (dICE) Counting**

All the elements delimited with DRs and containing CDSs for the four complete signature proteins (integrase, relaxase, CP, and VirB4) were considered as ICEs. When some signature CDSs were missing or were incomplete, the complete CDS encoded by the closest ICE was compared to the putative defective one by BLASTn in order to detect possible genome annotation errors (e.g., mis-identification of an authentic gene as a pseudogene most frequently due to the presence of a type II intron within the gene or mis-identification of START codon suggesting truncated genes). Elements that carry one or two defective signature CDSs, or that lack one of its extremity were considered as decayed ICEs (dICEs).

**Domain Composition Analysis**

The third step of our workflow (Figure 1) involved retrieving domain composition of all ICE signature proteins from Uniprot annotations using the BioMart Central Portal. De novo CD-search for conserved domains was performed when no data was available through BioMart.

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^3http://central.biomart.org/

^4http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi
FIGURE 1 | Procedure for identifying candidate ICEs in sequenced genomes. The amino-acid sequences encoded by a chromosome are collected as multifasta files and processed as follows (see Materials and Methods). ① Signature proteins are identified by BlastP search using our reference sequences as query and the set of multifasta files as search database. Resulting hits are filtered and validated. ② The location of genes encoding the validated signature proteins are visualized using Artemis and ICEs are delimited. ③ Domain composition of signature proteins are searched using Biomart and the signature proteins are grouped into classes. ④ Multiple alignments of signature proteins in each class are performed using Clustal Omega and their phylogeny is analyzed using maximum likelihood (ML) methods and BioNJ.

Tree Construction
In this step (Step 4 on Figure 1), signature proteins were aligned using Clustal Omega with default parameters (Sievers et al., 2011). Trees of ICE signature proteins were built with MEGA (Tamura et al., 2013) using both maximum likelihood (ML; tree shown) based on JTT with Freqs (+F) model (partial deletion of gaps and missing data (80% cutoff), Gamma distributed with Invariant sites G+I (five categories)) and BioNJ methods with the Poisson model (Gouy et al., 2010). Branch support of the groupings was estimated using bootstrap (100 replicates for the ML method and 1,000 replicates for BioNJ).

ICE Annotation and Comparative Analysis
The comparative analysis of the conserved CDSs within a given ICE family was performed only for those that were non- or mis-characterized and displayed a significant number of ICEs. Functional annotation of ICEs was performed using Agmial (Bryson et al., 2006). Protein product, gene name and EC_number were assigned using similarity with Uniprot databank.

Data mining of the orthologs and the conserved syntenies was performed using Insyght. Sequence alignments were carried out at the protein level (using BLASTp) to achieve the pairwise comparisons of all the CDSs of ICEs belonging to the same family. Two genes were considered orthologous if they gave rise to a bi-directional best hit (BDBH) of the corresponding ICE genomic regions and if the sequence alignment included more than 50% of the total proteins with an e-value less than 0.01. Two CDSs for which the E-value of the sequence alignment was less than 0.01 were considered homologous but were not analyzed unless they belong to a synteny. Syntenies were computed with a dynamic programming algorithm that determines the highest scoring paths amongst the chains of colinear homologs. The scores and penalties used were as follows: minimum synteny score: 8; ortholog BBDH: 4; homolog non BBDH: 2; mismatch: −3; gap creation: −4; gap extension: −2. This setting allows the insertion of small gaps within the conserved synteny. The “Orthologs table” view in Insyght was used to identify the conserved and idiosyncratic loci within ICEs.

RESULTS
Prevalence of ICEs and dICEs within Streptococcal Chromosomal Genomes
A total of 105 ICEs and 26 dICEs were identified among the 124 streptococcal genomes analyzed in this work (Supplementary Table S1). About half (63/124) of the examined strains contain at least one element and among those strains 61% (39/63) harbor several ICEs or dICEs. ICE denomination indicates whether the element is an ICE or a dICE, followed by letters and numbers allowing species and strain identification.
When ICE/dICE encodes a site-specific integrase, its denomination also specifies the name of the target-gene. Otherwise, it indicates the integrase family (Tn916 or DDE). For elements already well-characterized and named, the correspondence between names is indicated in Supplementary Table S1.

Some streptococcal species show relatively few ICEs and dICEs even if a significant number of genomes were analyzed (Table 1). In particular, in the salivarius group, only one element was found in the six analyzed genomes of S. thermophilus analyzed and no element in the three genomes of S. salivarius. By contrast, in the anginosus and bovis groups, there is an average of more than two ICEs or dICEs per genome, with extreme situations such as 13 and 11 elements found in the three genomes studied in S. anginosus and S. galloyticus, respectively. The occurrence of ICEs and dICEs per species or strain can vary within a group. For example in the pyogenic group, there were only six elements found in the 19 S. pyogenes genomes analyzed but as many as 10 elements in the five S. dysgalactiae genomes analyzed.

Diversity of ICE and dICE Relaxases, and VirB4 in Streptococci

A total of 121 relaxases, encoded by ICEs or dICEs, were detected. They can be classified in three distinct classes on the basis of their domains (Table 2). The ‘Rel-I’ regroups 52 relaxases that contain a C-terminal catalytic “Rep_trans” domain (PF02486) associated with an N-terminal Helix-Turn-Helix (PF01381) DNA binding domain. According to the CONJscan-T4SSscan server (Guglielmini et al., 2011), these 52 relaxases belong to the MOB T family that is related to initiators of rolling-circle replication of some plasmids and prophages (Guglielmini et al., 2014). The ‘Rel-II’ class regroups 62 relaxases sharing a common N terminal “relaxase” PF03432 catalytic domain and belonging to the MOBP family. Among them, 20 relaxases also carry a C terminal “Lantibiotic streptin immunity” PF11083 domain of unknown function. The ‘Rel-III’ class of relaxases contains seven proteins with no identified domains according to CD-search. These proteins are classified

5http://mobyle.pasteur.fr/cgi-bin/portal.py#forms::CONJscan-T4SSscan

| Group of species | Streptococcus species or strains | Number of strains | Total number of ICEs/dICEs per species | Minimum number of ICEs/dICEs per genome | Maximal number of ICEs/dICEs per genome | Average number of ICEs/dICEs per genome |
|------------------|--------------------------------|------------------|-------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|
| anginosus        | S. anginosus                   | 3                | 13                                  | 2                                      | 7                                      | 4.3                                    |
| anginosus        | S. intermedius                 | 3                | 7                                   | 1                                      | 3                                      | 2.3                                    |
| anginosus        | S. constellatus                | 3                | 6                                   | 0                                      | 4                                      | 2.0                                    |
| bovis            | S. galloyticus                 | 3                | 11                                  | 3                                      | 5                                      | 3.7                                    |
| bovis            | S. pasteurianus                | 1                | 3                                   | 3                                      | 3                                      | NA                                     |
| bovis            | S. infantarius                 | 1                | 2                                   | 2                                      | 2                                      | NA                                     |
| bovis            | S. lutetialis                  | 1                | 2                                   | 2                                      | 2                                      | NA                                     |
| bovis            | S. macedonicus                 | 1                | 2                                   | 2                                      | 2                                      | NA                                     |
| mitis            | S. pneumoniae                  | 28               | 18                                  | 0                                      | 4                                      | 0.6                                    |
| mitis            | S. olghofermentans             | 1                | 2                                   | 2                                      | 2                                      | NA                                     |
| mitis            | S. mitis                      | 1                | 1                                   | 1                                      | 1                                      | NA                                     |
| mitis            | S. oralis                     | 1                | 1                                   | 1                                      | 1                                      | NA                                     |
| mitis            | S. pseudopneumoniae            | 1                | 1                                   | 1                                      | 1                                      | NA                                     |
| mutans           | S. mutans                     | 4                | 1                                   | 1                                      | 1                                      | 0.3                                    |
| pyogenic         | S. agalactiae                 | 8                | 13                                  | 0                                      | 7                                      | 1.6                                    |
| pyogenic         | S. dysgalactiae               | 5                | 10                                  | 0                                      | 5                                      | 2.0                                    |
| pyogenic         | S. pyogenes                   | 19               | 6                                   | 0                                      | 3                                      | 0.3                                    |
| pyogenic         | S. equi                       | 4                | 4                                   | 0                                      | 2                                      | 1.0                                    |
| pyogenic         | S. parauberis                 | 1                | 2                                   | 2                                      | 2                                      | NA                                     |
| pyogenic         | S. iniae                      | 1                | 0                                   | 0                                      | 0                                      | NA                                     |
| pyogenic         | S. uberis                     | 1                | 1                                   | 0                                      | 0                                      | NA                                     |
| sanguinis        | S. parasanguinis              | 2                | 3                                   | 1                                      | 2                                      | 1.5                                    |
| sanguinis        | S. gordonii                   | 1                | 0                                   | 0                                      | 0                                      | NA                                     |
| sanguinis        | S. sanguinis                  | 1                | 0                                   | 0                                      | 0                                      | NA                                     |
| suis             | S. suis                       | 18               | 21                                  | 0                                      | 5                                      | 1.2                                    |
| salivarius       | S. thermophilus               | 6                | 1                                   | 0                                      | 1                                      | 0.2                                    |
| salivarius       | S. salivarius                 | 3                | 0                                   | 0                                      | 0                                      | 0.0                                    |
| ND               | S. sp I-G2                    | 1                | 1                                   | 1                                      | 1                                      | NA                                     |
| ND               | S. sp I-P16                   | 1                | 0                                   | 0                                      | 0                                      | NA                                     |

NA, not applicable.
**Classification of Conjugative Modules in Superfamilies and Families**

Table 2 summarizes the co-occurrence of the different classes of signature proteins within the conjugation modules of ICEs and dICEs. This allowed classification of the conjugation modules into three distinct superfamilies, named according to the first characterized element of each superfamily: **ConjTn**$_{916}$, **ConjTn**$_{5252}$, and **ConjTn**$_{GBS1}$.

**The ConjTn**$_{916}$ Superfamily of Conjugation Modules**

The **ConjTn**$_{916}$ superfamily groups together 44 ICEs and nine dICEs. All encode a ‘Rel-I’ relaxase associated with a ‘CP-I’ and a ‘VirB4-1a’ or ‘-1b’ VirB4 protein.

The phylogenetic tree of relaxases (Figure 2A) of the **ConjTn**$_{916}$ superfamily indicates three well-supported groups: relaxases related to the one of **Tn**$_{916}$, to that of ICE$_{SmuA159}$ _tRNAleu_ and to that of ICE$_{ES3}$. However, the latter two groups are not supported by the phylogenetic trees of CPs and VirB4 (Figures 2B,C). Therefore, two families of conjugative modules can be distinguished in the **ConjTn**$_{916}$ superfamily: the **ConjTn**$_{916}$ family and the **ConjICE$_{ES3}$** family.

The **ConjTn**$_{916}$ family is well-supported by phylogenetic trees of the relaxase, CP and VirB4 protein sequences (Figures 2A–C). It clusters 27 ICEs (and five dICEs) whose signature proteins are almost identical to those of the well-described **Tn**$_{916}$ element originally isolated from the Firmicute _E. faecalis_ (Franke and Clewell, 1981). It also includes the more distant ICE$_{SmuB6}$ _guaA_ whose signature proteins shared only 67, 66, and 80% identity with the _E. faecalis_ **Tn**$_{916}$ relaxase, CP and VirB4 protein, respectively. All the 12 genes of the conjugation module of the prototype **Tn**$_{916}$ from _E. faecalis_ DS16 were found with a similar organization in the vast majority of ICEs by the CONJscan-T4SSscan server in the MOB$_p$ family of relaxases.

The 126 CPs encoded by ICEs or dICEs of streptococci are divided into two classes. The CP-I class groups 50 CPs sharing a unique central FtsK_SpoIIIE catalytic domain (PF01580; Table 2). According to the CONJscan-T4SSscan server, they belong to a particular class of CPs named TcpA, unrelated to all CPs of Gram-negative bacteria (Guglielmini et al., 2014). The ‘CP-II’ class contains 76 CPs with a central catalytic TraG/TraD domain (PF02534) and an additional C-terminal ‘TraM recognition site of TraD and TraG’ PF12696 domain. According to the CONJscan-T4SSscan server, these proteins belong to the main family of CPs named VirD4 (Guglielmini et al., 2014). Among them, 67 CPs, constituting the ‘CP-IIa’ class, are 598 aa to 688 aa-long. The nine remaining CPs are composed of about 1045 aa and are representatives of the ‘CP-IIb’ class (Table 2).

All 124 VirB4 proteins from ICEs and dICEs show a unique C-terminal PF12846 ‘AAA-10’ catalytic domain. Reconstruction of their phylogenetic relatedness (data not shown) suggested that they can be grouped in four classes designated ‘VirB4-1a’ (30 proteins), ‘VirB4-1b’ (20 proteins), ‘VirB4-1c’ (65 proteins), and ‘VirB4-1d’ (nine proteins; Table 2). These proteins are all identified as VirB4 using the CONJscan-T4SSscan server.
**FIGURE 2 | Continued**

A

**Conj**

Conj\textsubscript{Tr916}

Conj\textsubscript{ICE}\textsubscript{S3}

B

Conj\textsubscript{Tr916}

Conj\textsubscript{ICE}\textsubscript{S3}

C

Conj\textsubscript{Tr916}

Conj\textsubscript{ICE}\textsubscript{S3}
of the ConjTn916 family (data not shown) thus confirming their relationships. Others only differ by one or few deletions, pseudogenizations or insertions.

The ConjICESt family gathers together 21 elements whose signature proteins are much more variable in sequence than those of the ConjTn916 family. The sequence of relaxases, CPs and VirB4 proteins of the most distantly related elements displayed around 20, 30, and 60% of identity, respectively.

For a better characterization of the ConjICESt family, a search for conserved CDSs in all members of this family was undertaken. Orthologous CDSs were identified on the basis of the identity of their product using InSyght. The integration module of all elements of the ConjICESt family is composed of both a tyrosine integrase and an excisionase (Figure 3A). In addition to the relaxase, CP, VirB4 CDSs, tyrosine integrase and excisionase CDSs, they share seven other CDSs including CDSs involved in the T4SS formation (VirB1, VirB3, VirB6, and VirB8) and a CDS encoding a regulation protein (HTH regulator).

**The ConjTn5252 Superfamily of Conjugation Modules**

The ConjTn5252 superfamily gathers together 54 ICEs and 15 dICEs encoding a ‘Rel-II’ relaxase associated with a ‘CP-IIa’ CP...
FIGURE 4 | Phylogenetic analysis of ICEs and dICEs belonging to the Conj\textsubscript{5252} superfamily. The trees of CPs are shown in this figure. ML bootstrap values for relaxases/CPs/VirB4 (in this order) are given. X marks the nodes that are not validated with other proteins. Families are also supported by BioNJ analysis (data not shown). In mauve are ICEs/dICEs belonging to the Conj\textsubscript{vanG} family, in dark blue those belonging to the Conj\textsubscript{Tn1549} family, in light-blue those of the Conj\textsubscript{TnGBS} family and in purple that of the Conj\textsubscript{5252} family. Elements marked with an asterisk are not integrated in their primary sites but in secondary ones. Refer to Table 1 for ICE/dICE and strain details.
Thus, within streptococcal genomes, the Conj Tn superfamily indicated that they shared more than 56% of identity of the signature proteins of the most divergent ICEs of this superfamily. For instance, the CP VirB4 CDSs, they share three other CDSs probably involved in maintenance of excised ICEs. Many of these ICEs also encode a protein carrying a repA_N domain that could be involved in maintenance of the ICE after excision (segregation ATPase).

As for ICEs of the ConjTn5252 family, the 23 ICEs of the ConjTn5252 family do not share the same integration module. However, they display 24 conserved CDSs. In addition to the relaxase, and the CP VirB4 CDSs, they share three other CDSs probably involved in the T4SS formation (VirB1, VirB3, VirB6) and one encoding a replication initiator (repA_N; Figure 3C).

The ConjTnGBS1 Superfamily of Conjugation Module

The ConjTnGBS1 superfamily of conjugation modules is the least represented in streptococcal genomes with only seven ICEs and two dICEs exhibiting such a module. It is characterized by the presence of a 'Rel-III' relaxase co-occurring with a 'CP-IIb' CP and a 'VirB4-Ic' protein. This superfamily was not identified by Guérillot et al. (2013) for GBS1 superfamily. According to the presence of a 'Rel-III' relaxase co-occurring with a 'CP-IIb' CP and a 'VirB4-Ic' protein, this superfamily was not identified by Guérillot et al. (2013). As expected for the ConjTn5252 superfamily, whatever the signature protein used, all phylogenetic trees are congruent and therefore only the CP one is shown (Figure 5). Sequence comparison of the signature proteins of the most divergent ICEs of this superfamily indicated that they shared more than 56% of identity. Thus, within streptococcal genomes, the ConjTnGBS1 superfamily is represented by the unique ConjTnGBS1 family. As expected from Guérillot et al. (2013), systematic comparisons of the protein sequence of the members of the ConjTnGBS1 family with TnGBS1 CDSs confirmed that they are related to TnGBS1 (data not shown).

Prevalence of the Different Families of Conjugation Modules within Streptococcal Species

In summary, seven families of conjugation modules belonging to three superfamilies were identified in streptococcal ICEs/dICEs. Most ICEs of the ConjTn916 family are found in S. suis and S. pneumoniae, while S. dysgalactiae and S. galolyticus mainly harbor ICEs of the ConjICEs5252 family. ICEs of the ConjTn5252 family are frequently found in S. suis, and ICEs of the ConjTnGBS1 family are only found in S. agalactiae, S. anginosus, S. intermedius, and S. lutetiensis genomes.

Diversity of Integration Modules and Integration Sites of Streptococcal ICEs and dICEs

Three unrelated families of integrases (tyrosine integrase, serine integrase, and DDE transposase) are encoded by streptococcal ICEs/dICEs. Most of these integrase genes are located at one extremity of the ICE and adjacent to the integration site.

Prevalence and Integration Site of ICEs/dICEs Encoding a Tyr Integrase

Tyrosine integrases are the most prevalent integrases detected as they are found in 53% of the elements. In total, 73 tyrosine integrases were identified (66 for ICEs and 7 for dICEs). Most of them are ~400 aa long, except for five that are composed of 502 aa. Despite a variable degree of identity between these proteins, all tyrosine recombinases share a "phage-integrase" PF00589 domain in their N-terminal region. Most of them carry an additional N-terminal binding domain being either: (i) a pfam02920 "DNA binding domain characteristic of Tn916 integrase" or (ii) a PF14659 "Phage integrase, N-terminal SAM-like domain" (iii) and/or more rarely a PF14657 "AP2-like DNA-binding integrase domain."

The phylogenetic tree of the tyrosine integrases reveals 11 well-supported groups (Figure 6). The overall finding is that almost all tyrosine integrases are grouped according to their insertion loci. Exceptions are the two non-grouped tyrosine
integrases catalyzing integration in the 3′ end of the rpmG gene that display two distinct orientations and locations relative to the rpmG gene (Figure 6) and share only 21% of identity. Interestingly, all tyrosine recombinases catalyzing integration in a tRNA gene are grouped together. Half of the elements encoding a tyrosine integrase are inserted in the 3′ end of either tRNA CDSs (9/73) or a well-conserved housekeeping genes (30/73) such as rplL (L7/L12 ribosomal protein), rpsI (S9 ribosomal protein), lysS (lysyl-tRNA synthetase), rpmG (L33 ribosomal protein), or guaA (GMP synthase). In rare cases (7/73), the integration sites are found in the 5′ end of genes such as ftsK (DNA translocase involved in cell division), rbgA (ribosomal biogenesis GTPase) and ebfC (nucleoid associated protein). The remaining tyrosine integrases (27/73) catalyze low-specificity integration as previously shown for the very closely related integrase of Tn916 (Scott et al., 1994).

All the ICEs/dICEs encoding a site-specific tyrosine integrase are flanked by DRs whose size ranges from 12 to 54 bp. DRs in rplL, guaA or in genes encoding tRNAthr only contain the exact 3′ end of the target genes. By contrast, DRs of elements integrated in...
the 5′ end of *ftsK* and *rbgA*, and in the 3′ end of genes of *tRNA*leu and *tRNA*s, overlap the flanking intergenic regions (Figure 7). Three elements are integrated in the *lysS* gene and are flanked by DRs with similar sequences. However, these DR sequences have distinct locations within *lysS* resulting from a slight difference in the length of this gene: one corresponds to the last 17 bp of the *lysS* gene while the others contain 13 bp and are more internal (Figure 7).

**Prevalence and Integration Sites of ICEs and dICEs Encoding a Serine Integrase**

A total of 42 serine integrases were identified: 32 from ICEs and 10 from dICEs. They all displayed an N-terminal catalytic and dimerization ‘Resolvase’ domain (PF00239) that contains a conserved serine residue. This domain is always associated with a ‘Recombinase’ PF07508 domain. All of them but 10 also contain a pfam13408 ‘Zinc ribbon recombinase’ domain that is likely to play a DNA-binding role.

Streptococcal conjugative elements encode either a single serine integrase (14 ICEs and four dICEs) or a triplet of serine integrase genes (6 ICEs and 3 dICEs). The phylogenetic tree of the serine integrases is compatible with the existence of two groups of integrases (Figure 8). One of the groups clustered all the single serine integrases that target integration in *rumA* [23S rRNA (uracil-5')-methyltransferase]. ICEs/dICEs encoding such serine integrase are flanked by 2–21 bp-DRs localized at one end in the *rumA* gene and at the other end in the serine integrase gene (Figure 7). Integration leads to a reciprocal exchange of the 3′ part of the *rumA* and serine integrase genes leading to a modification of sequence and length of the C-terminal end of the corresponding proteins. It should be noticed that the replacement changes the translation frame of both proteins.

The second group of serine integrases is composed of the 24 integrases that are organized in triplets within the elements (Figure 8). Interestingly, these serine integrases are clustered according to their position within the triplets: external (E), middle (M), or internal (I) with respect to the ICE/dICE extremity (Figure 8). This suggests that all these modules derive from an ancestral module that already encoded three serine recombinases and that the presence of triplet results from two successive duplications. Triplets of serine integrases catalyze sitespecific integration within several genes thus leading to their disruption: *mutT* (Nudix hydrolase), *traG* (CP of another ICE, dICE, or ICE remnant) and *hsdM* (methyltransferase subunit of
Insertion of ICEs/dICEs encoding triplets of serine integrases leads to very small 2–8 bp DRs (Figure 7). In some cases, one or several CDSs separated the triplets of serine integrases from the target gene (Figure 7). All serine integrases have the same orientation with respect to the target gene.

In several cases (element names marked with an asterisk in Figure 8), the comparison of the observed integration sites with the ones of elements with closely related integrases strongly suggests that these elements are not integrated in their primary sites but in secondary ones. ICE_SanMAS624_hsdM * disrupts a gene encoding a protein carrying the domain PF0267 (“Adenine nucleotide alpha hydrolase”). ICE_SsuBM407_mutT * disrupts a gene encoding a luciferase-like protein. dICE_SanMAS624_rumA * and ICE_SanC238_rumA * disrupts sstT, a gene encoding a serine/threonine transporter. The relaxase, CP and VirB4 of these last two elements are very closely related (Figure 4) as well as their integrases (Figure 8). This suggests that they were both inherited from the last common ancestor of their hosts.
Prevalence and Integration Sites of ICEs/dICEs Encoding a DDE Transposase

A total of 23 DDE transposases related to those encoded by Tn\textsuperscript{GBS1} and Tn\textsuperscript{GBS2} elements (Guérillot et al., 2013) were detected. All show an “Uncharacterized protein family” PF06782 conserved domain with unknown function. Alignment of the amino acid sequences of the most divergent DDE transposases revealed that they share 42% identity showing that all these transposases belong to the same family. However, DDE transposases encoded by ICEs carrying a Conj\textsuperscript{Tn\textsubscript{GBS1}} module are clustered together and are distinct from those encoded by ICEs with a Conj\textsuperscript{Tn\textsubscript{GBS2}} module (Figure 9).

Analysis of the junction sequence of ICEs encoding DDE transposases shows an 8-bp DR sequence that results from the duplication of the target sequence. Comparison of the insertion sites between all ICEs and dICEs of this group did not reveal any significant sequence similarity among the duplicated sequences but they are all located 15–16 pb upstream from −35 boxes of sigma A promoters as previously reported (Brochet et al., 2009).

Diversity and Evolution of ICEs/dICEs

The determination of the limits of each element allows the comparison of their size. If one excludes the Conj\textsubscript{vanG} ICE family (only two elements), the Conj\textsubscript{Tn\textsubscript{916}} and Conj\textsubscript{Tn\textsubscript{GBS1}} families are the most homogeneous in size with elements from 18 to 26 kb and from 40 to 53 kb, respectively. By contrast, the size of the Conj\textsubscript{ICE\textsubscript{St3}} elements is much more variable: most of them are 19- to 37-kb long and one exceeds 60 kb. The size of the Conj\textsubscript{Tn\textsubscript{1549}} elements can double (from 36 to 72 kb) and that of Conj\textsubscript{Tn\textsubscript{GBS2}} elements shows a very large disparity (from 25 to 82 kb). When ICEs/dICEs are present within \textit{Streptococcus} genomes, they contribute to 1–13% of chromosomal DNA.

Analysis of streptococcal ICEs and dICEs also allowed determining their typical combination of conjugation and integration/excision modules (Table 3). Almost all conjugation modules of the Conj\textsubscript{Tn\textsubscript{916}} family are associated with a tyrosine...
integrate identical, or almost identical, to the one of Tn916, that is known to have a low specificity of integration (Scott et al., 1994). The only exception is ICE_SmiB6_guaA that encodes a conjugation module of the ConJ916 family but is site-specifically integrated in the 3′ end of guaA. Elements with a ConJICER module are associated with site-specific tyrosine integrases integrating conjugation in two distinct integration sites (3′ end of three types of tRNA encoding genes, rpsI, rpmG, and lysS as well as the 5′ end of ftsK or ebfC). The conjugation modules of ConJ916 superfamily can be associated with a tyrosine recombinase, a serine site specific recombinase or a DDE transposase. Thus, ICEs and dICEs carrying a ConJ916 module are associated with: (i) a single serine integrase catalyzing the insertion in rumA; (ii) a triplet of serine integrases catalyzing the insertion in hsdM or traG or (iii) a tyrosine integrase catalyzing the insertion in the 5′ end of rgbA. Those carrying a ConJ916 module can encode: (i) a tyrosine integrase catalyzing the integration in the 3′ end of rplL or the 5′ end of rgbA; (ii) a single serine integrase catalyzing the integration in rumA; (iii) or a triplet of serine integrases in the insertion in mutT. The two ICE (ICE_SmuG21_1savS and ICE_Spy2096_rumA) displaying a ConJwag module conjugation module are associated with a tyrosine or a serine integrase, respectively. All the ConJ916 and 14 of the 17 ConJ916 module conjugation modules are associated with a DDE transposase. However, three ICEs carrying a ConJ916 module are not associated with a DDE transposase: ICE_Sco1050_mutT encodes a tripeptide of serine recombinases and both ICE_SparauNCFD20202_rplL and ICE_SagILRI005_rplL encode a tyrosine integrase. Sequence comparison suggests that ICE_SagILRI005_rplL is composite (Figure 10). Its ConJ916 conjugation module is closely related to the one of ICE_SgaUCN34_TnGBS2. However, its left part is closely related to the left part of ICE_Sag018883_rplL, an ICE carrying a conjugation module belonging to ConJ916 family. It includes not only the recombination module but also a lactose utilization module and a pseudogene of relaxase typical of the ConJ916 family. The right end of ICE_SagILRI005_rplL carries a gene encoding a repA_N domain closely related to a gene located in the right of ICE_Sga018883_rplL and additionally a pseudogen, whose product also carry a repA_N domain.

DISCUSSION

Detection of ICEs and dICEs in Streptococcal Genomes

Burrus et al. (2002a), a precursor analysis of 24 genomes from various Firmicutes, led to the identification of 17 putative ICEs and suggested that these elements are widespread at least in this division of bacteria. Over the last decade, with the revolution of sequencing technology, the number of fully sequenced bacterial genomes greatly increased. By the same time, efforts were made to improve in silico analysis of the data sets and in particular those allowing the detection of genomic islands including ICEs. Almost all searches of ICEs in bacterial genomes were only performed with a strain-centric or an ICE family centric point of view. However, few recent studies also reported extensive characterization of these conjugative elements. The first extensive study identified 335 chromosomal conjugative modules by scanning 1124 genomes of prokaryotes for conjugative genes (using HMM profiles of conjugative proteins of essentially proteobacterial plasmids; Guglielmini et al., 2011). Soon after, Ghinet et al. (2011) reported the characterization of 161 ICEs within 275 genomes of Actinobacteria but did not identify their limits. More recently, Puyrmé et al. (2015) searched for genetic elements integrated in the tRNAlys CTT gene in 303 genomes of S. agalactiae, leading to the identification and delimitation of 108 putative ICEs or derivatives. It should be noticed that in 2012, Bi et al. (2012) developed a web database6, compiling information on ICEs from both Gram+ and Gram− bacteria. However, if this database has the merit to list a large number and a great diversity of elements, it was not updated since November 2012 and some studies describing novel streptococcal ICEs published before this date escaped to the attention of the authors (for example, Brochet et al., 2008, reporting 10 novel ICEs). ICEberg have limitations considering ICEs from Streptococcus since (i) about half of ICE/dICE boundaries are incorrectly delimited in ICEberg and (ii) the insertion site of many of them, although published, is not registered in this database. More widely, information on numerous elements from Firmicutes is inconsistent or wrong. Thus, while the authors indicated that a family should include only elements that carry both related integration and conjugation modules, they also included in the Tn916 family ICEs that carry Tn916-unrelated integration modules (such as Tn5397 or ICElm1), Tn916-unrelated conjugation modules (such as Tn1549) or elements completely unrelated to Tn916 but carrying conjugation modules related to the one of Tn1549 (such as CTn2 or CTn5). Furthermore, although ICElm1 and Tn5801 carry almost

### TABLE 3 | Various combinations of conjugation and integration modules in ICEs and dICEs.

| Conjugation module | Number of elements | Integrases or transposases |
|--------------------|--------------------|----------------------------|
| Superfamily | Family | Tyrosine | Serine | Serine | DDE |
| ConJ916 | ConJ916 | 32 | 28 | 0 | 0 |
| ConJ916 | ConICEF | 21 | 21 | 0 | 0 |
| ConJ916 | ConICEG | 29 | 19 | 4 | 3 |
| ConJ916 | ConICEH | 21 | 2 | 13 | 5 |
| ConJ916 | ConICEI | 17 | 2 | 0 | 14 |
| ConJ916 | ConICEJ | 2 | 1 | 1 | 0 |
| ConJ916 | ConICEK | 9 | 0 | 0 | 0 |

1 Four ConJ916 elements are deprived of an integrase CDS (absent or as a pseudogene).
2 Three ConJ916 elements are deprived of an integrase CDS (absent or as a pseudogene).
3 One ConJ916 element exhibits an integrase pseudogene.

6http://db-mml.sjtu.edu.cn/ICEberg/index.php

...
identical integration and conjugation modules (Burrus, plasmid 2002, cited in ICEberg for ICESlm1), they were included in different families, Tn916 and Tn5801, respectively. Even more problematically, the Tn1207.3 family and 10750-RD.1 family only contain elements unrelated toICEs, prophages for the first one and highly decayed derivates of integrative mobilizable elements for the second one. In general, these failures (and many others not mentioned here) make this database unreliable and very difficult to use for ICEs from Streptococci and other Firmicides.

Here, we present the results of ICE detection within 124 complete streptococcal genomes. Our search is based on an iterative search for genes encoding signature proteins from ICEs. The co-occurrence of an integrase and three proteins of the conjugation module guarantees the retrieval of ICEs or dICEs. When one or two signature CDSs appeared to be a pseudogene or to be absent, an analysis of the whole element was undertaken to confirm its nature.

This work led to the identification and characterization in 63 Streptococcus genomes of 131 ICEs/dICEs whose extremities were precisely mapped on the genome. Elements that were already precisely identified are marked by a reference in Supplementary Table S1.

**Distribution of ICEs and dICEs in the Different Streptococcal Species**

Among the 27 streptococcal species analyzed, all, except 5 (S. iniae, S. gordonii, S. iberis, S. sanguinis, for which only one complete genome is available and S. salivarius for which three genome sequences exist), contain ICEs/dICEs showing the ubiquity of these elements in Streptococcus. *S. suis* appears as the species containing the highest number of ICEs/dICEs since 61% of the strains carry at least one ICE/dICE. However, the high prevalence of ICEs/dICEs in this species might be due to strain sampling since many strains carrying an ICE are related. This is also the case for *S. pneumoniae* genomes of which 40% (11/28) encode at least one ICE. Among them, seven are derived from clinical isolates known to be resistant to one or multiple antibiotics. All of them carry at least one ICE/dICE suggesting a possible correlation between the presence of ICEs and resistance to antibiotics. Indeed among the families detected, Tn916 (Roberts and Mullany, 2011), Tn5252 (Korona-Glowniak et al., 2015), and Tn1549 (Garnier et al., 2000) are known vectors of antibiotic resistance genes.

**Definition of Different Families of Elements on the Basis of their Conjugative Module**

These ICEs were classified into seven distinct families belonging to three superfamilies on the basis of their conjugation modules: (i) ConjTn916 and ConjICEs3 belonging to the ConjTn916 superfamily, (ii) ConjANG, ConjTn5252, ConjTn1549, and ConjTnGBSI belonging to the ConjTn5252 superfamily, and the ConjTnGBSI family. The ConjTn916 and the ConjTn5252 superfamilies of conjugation modules belong respectively to the MPFE4 and to the MPF FATA classes of T4SS involved in bacterial conjugation as defined by Guglielmini et al. (2014). No match was found for the ConjTnGBSI family.

**Modular Evolution**

The phylogenetic trees obtained for relaxase, CP and VirB4 encoded by elements belonging to the ConjTn916 superfamily (Figure 2), the ConjTn5252 superfamily (Figure 4), and the ConjTnGBSI family (Figure 5) are highly similar, suggesting that genes exchanges or replacements within the conjugation modules of these families have not occurred or are rare. However, incongruences were found for relaxases, CPs and VirB4 proteins of some elements belonging to ICEs3 family, suggesting that some gene exchanges or replacements have occurred within the conjugation modules belonging to this family.

Unrelated or very distantly related integrases were found to be encoded by at least some of the ICEs belonging to the same family (except for TnGBSI family) and frequently by closely or very related elements (for example in the Tn5252 family). Furthermore, related site-specific integrases were found in unrelated or distantly related ICEs. Such incongruences are due to multiple exchanges of integration/excision and/or conjugation modules between and/or within ICE families. For most cases, the data do not allow to determine what precisely happened. However, it was previously reported that the last common ancestor of TnGBSI family acquired its DDE transposase from an insertion sequence and that the last common ancestor of TnGBSI family acquired its DDE transposase from an ICE belonging to Tn5252 family (Guérrilot et al., 2013). Here, the comparison of phylogenetic trees obtained for ConjtTn5252 superfamily, serine integrases and tyrosine integrases clearly shows three independent replacements of the DDE transposases by unrelated integrases. One of these ICEs, ICE_Sag1LR1005_rplP, probably results from: (i) the integration of an ICE belonging to Tn5252 family (encoding a low specificity/site-preferential DDE transposase and a RepA_N protein) into an ICE belonging to Tn5252 family (encoding a tyrosine integrase specific of rplP and another RepA_N protein), and (ii) the loss of the conjugation and replication modules of the Tn5252-related element and the deletion of the DDE transposase gene of the Tn5252-related ICE.

**Integration Specificity of ICEs in Streptococcal Genomes: Impact on Host Fitness and on the Evolution of Elements**

In this work, efforts were made to identify the boundaries of the ICEs and therefore to identify the insertion site of each of them. This analysis of integration/excision modules and site specificity is the first one carried for a large array of ICEs encoding their transfer as single-stranded DNA. The ICEs and dICEs of Streptococci carry diverse integration/excision modules (75 encoding a tyrosine recombinase, 20 encoding a unique serine recombinase, nine encoding three serine recombinases, and 23 a DDE transposase) and have a large array of integration specificity (low or preferential integration, 18 different site-specific integrations). This work led to the detection of eight new
target-genes for streptococcal ICE insertion that have not been identified previously (ftsK, guaA, lysS, mutT, rpmG, rpsL, traG, and ebfC).

It should be noticed that, among the 131 ICEs/dICEs identified, only nine (restricted to one family) were found to be integrated into the 3′ end of genes encoding tRNAs. This contrasts with the results of the analysis of actinobacterial ICEs (most of these ICEs carry conjugation modules unrelated to the ones of streptococcal ICEs and transfer as double-stranded DNA). Among the 144 actinobacterial ICEs analyzed, 100 were found integrated in the 3′ end of a tRNA gene (Ghinet et al., 2011).

In most streptococcal ICEs, as for almost all other known ICEs, the attI site is located in the vicinity of the integrase gene. However, it should also be noticed that for all streptococcal ICEs/dICEs integrated in rumA, the attR site is located within the serine integrase gene and consequently the integrase gene carried by the excised ICE has a different length and C terminus. att sites are found within the genes of their cognate integrase in very few integrative elements, such as the prophage Mx8 from Myxococcus xanthus for which the phage attachment site, attP, is located within the tyrosine integrase gene (Magrini et al., 1999). Site-specific integration of Mx8 leads to the replacement of the 112-residue C-terminal sequence by a 13-residue C terminus. This modified integrase is less active than the integrase encoded by the excised element. Therefore, it seems probable that the differences between the integrases encoded by the ICEs integrated in rumA and the integrases encoded by the excised elements can lead to differences in the function of the two forms of the integrase.

Besides mechanistic constraints leading to integration in conserved palindromic sequences for many tyrosine integrases (Williams, 2002), one would expect that selection criteria for ICE integration in evolution would be (i) to have the least effect on host fitness and, (ii) since many have a large host range (Bellanger et al., 2014), to allow integration into a wide range of strains and species. Numerous ICEs encoding a tyrosine integrase were found to be site-specifically integrated in the conserved 3′ end of essential conserved genes that are isolated or are the last gene of an operon. The insertion does not modify the gene product (tRNA, ribosomal proteins) or leads to very little modification of the 3′ end of the protein (lysyl-tRNA synthetase). Hence, such integrations would have no effect on host fitness and can occur in a large array of species.

All (except one) the streptococcal ICEs belonging to the Tn916 family detected in this study encode a tyrosine integrase identical or almost identical to the Tn916 integrase.
Analyses of a high number of insertion sites in various hosts showed that Tn916, despite having a low specificity of integration, still has a preference for AT rich regions (consensus TTTTnnnnnnAAAAA; Hosking et al., 1998; Cookson et al., 2011). Furthermore, the analyses of insertion sites after conjugal transfer to *Butyrivibrio proteoclasticus* B316™, whose genome has a similar GC percent as the ones of *Streptococci* (39%), showed that only 34% of the 123 analyzed insertions disrupt annotated ORFs even if 90% of this genome is made of ORFs (Cookson et al., 2011). This may be due to lower GC ratio (34.7%) of intergenic regions. Therefore, the AT-rich region preference of Tn916 probably leads to a null or low impact of most Tn916 insertion events on host fitness. Since MGEs have generally a lower G+C percent than their host genome (Rocha and Danchin, 2002), this preference could also explain the frequent presence of Tn916 or Tn916-related elements in plasmids or Tn5252-related elements (Clewell and Gawron-Burke, 1986; Ayoubi et al., 1991; Ding et al., 2009; Mingoa et al., 2011; Chancey et al., 2015). This putative preference for MGEs would also lead to a lower impact on the fitness of the bacterial host. It was previously shown that a Tn916 element carried by a Tn5252-related element can be transferred alone (Santoro et al., 2010) or as a part of the Tn5252 element (Ayoubi et al., 1991). Therefore, besides a low impact on host fitness, this A+T rich preference could increase the transfer ability of Tn916 (either autonomously or by mobilization in cis).

Some ICEs integrate in the conserved 5’ end of the first gene of an operon or of an isolated gene that encodes an essential protein (the DNA translocase FtsK that coordinates cell division and chromosome segregation; the nucleoid-associated protein EbfC; the ribosome assembly GTPase RbgA). Importantly, the insertion does not modify the N-terminus of the protein encoded by the target gene. Moreover, for three ICEs integrated in *rbgA* and two ICEs integrated in *ftsK*, the integration does not change the 15–64 bp sequence located upstream from the START codon. However, in the two other ICEs integrated in *rbgA* and in the one integrated in *ebfC*, the sequence upstream from the gene, including its promoter, is completely different, suggesting that the expression of the gene is impacted by the integration of the element. This situation is reminiscent of the integration of the putative satellite prophage SpyCI from *S. pyogenes*. In stationary phase, SpyCI is integrated into the 5’ end of the DNA mismatch repair gene *mutL*, disrupting its expression and that of three other genes located downstream (Nguyen and McShan, 2014). During early exponential growth, SpyCI excises from the bacterial chromosome and replicates as an episome, thus allowing the expression of *mutL* and of downstream genes. Concerning the *ebfC* gene, it is known that in the spirochaete *Borrelia burgdorferi*, it is highly expressed in rapidly growing bacteria but mRNA is undetectable in stationary phase (Jutras et al., 2012). Thus, ICE integration in the promoter of this gene in *S. parasanguinis* FW213 may not alter host fitness at all: expression of *ebfC* gene would not be required in the stationary phase when the element is integrated and excision of the element in the exponential phase restores the expression of this gene.

TnGBS1 and TnGBS2 are two known elements from *S. agalactiae* encoding DDE transposases that integrate in various intergenic regions located 15 or 16 bp upstream from the 35 box of sigma A promoters (Brochet et al., 2009; Guérillot et al., 2013). In this study, all elements belonging to TnGBS1 and TnGBS2 families (except three TnGBS2 that encode serine or tyrosine recombinases) are also integrated in such location. Insertion into intergenic regions is expected to minimize the effects caused by the transposon insertion on host fitness. However, such insertions may interfere with the transcription level of the downstream gene. However, it should be noticed that the insertion of TnGBS2 does not seem to affect significantly the transcription level of the gene located downstream from the preferred insertion site.

All insertions of the 29 ICEs encoding serine integrase disrupt genes encoding proteins. A large majority are site-specific. Most of these genes are widespread but are not essential for the strain (*rumA, hsdM, mutT*). The *rumA* gene encodes a widespread RNA methyltransferase. In *Escherichia coli*, the deletion of this gene has little effect on growth or on the fidelity of translation, but alters the sensitivity of the ribosomes to fusidic acid and capreomycin (Persaud et al., 2010). The hsdM gene encodes the methyltransferase subunit of type I restriction-modification systems (Murray, 2000). The *mutT* gene encodes a Nudix hydrolase that removes oxidized nucleotide precursors so that they cannot be incorporated in DNA during replication (Lu et al., 2001). One element, dICE_SanC238_traG is site-specifically integrated into the *traG* gene that encodes the CP of an ICE remnant (not detailed in this report because this remnant is too much decayed) belonging to Tn1549 family.

The consequences of the integration/excision balance of ICE encoding serine recombinases on the expression of the target genes encoding proteins have never been studied. However, several examples can be cited for prophages or prophage-related elements (Stragier et al., 1989; Kunkel et al., 1990; Rabinovich et al., 2012). Thus, the DNA uptake competence system of the intracellular bacterial pathogen *Listeria monocytogenes* serovar 1/2 was considered non-functional because the competence master activator gene, *comK*, is disrupted by the insertion of the temperate prophage A118 encoding a serine recombinase (Rabinovich et al., 2012). However, the prophage excises not only during the activation of lytic phase but also during intracellular growth, primarily within phagosomes of macrophages, without any production of progeny virions, thus allowing expression of the *comK* gene (Rabinovich et al., 2012). In the same way, ICEs integrated within specific genes and disrupting them may excise when these genes are useful for the host cell (rumA, hsdM, mutT) or for the host ICE (traG) to reduce the impact on host fitness and guarantee their maintenance in the cell. Four elements encoding serine integrases are integrated in secondary sites within protein-encoding genes. As for primary integration sites, if the elements are still able to excise, expression of the target gene might not be impacted. The integration of CTn5, an ICE belonging to the Tn1549 family and encoding a serine integrase occurs in an adhesin gene in *Clostridium difficile* 630 (Sebaihia et al., 2006). However, comparison of this genome with that of the derived strain 630Δerm showed that CTn5 has excised from its original location and has inserted in *rumA* (CD3393) of 630Δerm (van Eijk et al., 2015). This suggests that an ICE integrated in a secondary site is able to excise and reintegrate in its primary site and conversely.
Globally, the impact of the integration upstream from promoters, in the 5' end of CDSs or within CDSs could be reduced if the ICE excises when the targeted genes are expressed. It was initially thought that ICEs do not replicate autonomously in the cell, although conjugative transfer can be seen as an intercellular replication (Burrus et al., 2002b). Therefore, although the excision could be advantageous for the host, if the cell divides when the ICE is excised, the ICE would be lost in one of the daughter cell. Nevertheless, several recent studies showed or strongly suggested that various single-strand DNA transferring ICEs are capable of extrachromosomal replication in both Gram-positive and Gram-negative bacteria (Carraro et al., 2015 and references therein). In particular, replication was found to be involved in maintenance of TnGBS1 and TnGBS2 in transconjugants before their integration (Guérillot et al., 2013). These elements encode a protein that carries a RepA_N domain and is related to the protein controlling the θ replication of various plasmids and a protein related to ParA, a protein involved in maintenance of some plasmids (Guérillot et al., 2013). We found genes encoding a protein with a RepA_N domain in all ICE belonging to the Tn5252 family and numerous ICEs belonging to Tn1549 family. We also found ParA segregation ATPase CDSs in all ICEs belonging to Tn1549 family and vanG family suggesting that all these elements can replicate as episomes. Evidence of intracellular extrachromosomal replication was also recently obtained for ICESt3 (Carraro et al., 2011) and another element belonging to ICESt3 family, RD2 (i.e., ICE_Spy6180, tRNAThr) of S. pyogenes (Sitkiewicz et al., 2011) although these elements do not carry any replication module. Moreover, extrachromosomal replication of ICEBs1 from Bacillus subtilis, an element belonging to the Tn916 superfamily (Burrus et al., 2002a) was found to be involved in the stability of the element. This intracellular replication is initiated from the ICEBs1 oriT and required the ICEBs1-encoded relaxase (Lee et al., 2010). At last, all ICEs belonging to the Tn916 superfamily encode a peculiar relaxase (MOBp) related to rolling circle replication initiators involved in maintenance of various plasmids (Guglielmini et al., 2014), suggesting that all these elements are also able to replicate as episomes.

**CONCLUSION**

This study greatly enriches our understanding of the classification and integration sites of ICEs/dICEs in streptococci genomes. In the future, it will be updated and further extended to take into account newly sequenced genomes and to confirm all the trends proposed here. An automated bioinformatics procedure will be developed to keep pace with the constantly growing number of available genomes. Extension to other species of Firmicutes and to the search for IMEs is also envisaged.

**AUTHOR CONTRIBUTIONS**

GG and SP conceived the reference database of signature proteins. GG, SP, NL-B, and M-DD contributed to the conception of the work. CA, CC, GG, NL-B, M-DD, SP, VL, and TL were involved in the acquisition and/or the analysis of the data. NL-B, GG, CC, and SP contribute to the drafting of the manuscript. NL-B and CC elaborated the figures and tables. All authors criticized and finally approved this final version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01483

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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