Plasmid-like replication of a minimal streptococcal integrative and conjugative element

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INTRODUCTION

The horizontal transfer of mobile genetic elements (MGEs), such as conjugative plasmids, prophages and transposons, is a key mechanism of bacterial evolution allowing the acquisition of new adaptive traits (Brown-Jaque et al., 2015; Goessweiner-Mohr et al., 2013; Novick et al., 2010; Roberts et al., 2014; Smillie et al., 2010). Conjugation appears to be the preponderant transfer mechanism of adaptation genes in bacteria (Brochet et al., 2008; Clewell et al., 2014; de la Cruz et al., 2010; Guglielmini et al., 2011; Ochman et al., 2000). Integrative and conjugative elements (ICEs) were primarily defined as MGEs able to excise from a replicon of their bacterial host, self-transfer by conjugation and stably maintain by integration into a replicon of the recipient bacterium (Burrus et al., 2002a). Other integrated MGEs, called mobilizable elements, are not autonomous for their mobility and require a helper functional conjugative element to regain mobility by conjugation (see Bellanger et al., 2014 for review; Carraro et al., 2014a; Daccord et al., 2013; Doublet et al., 2005). Among these, the cis-mobilizable elements (CIMEs) are derived by deletion of conjugation and recombination modules from ICEs, but retain their recombination sites. These elements hijack the recombination and conjugation machinery of related tandem integrated ICEs to excise, transfer and integrate (Bellanger et al., 2011; Puymège et al., 2013).

As other bacterial MGEs, ICEs are a combination of functional modules, each of which includes all the sequences involved in a specific biological function (Toussaint & Merlin, 2002). The modules needed for transfer, integration and regulation ensure the basic biology of ICEs, and thus can be defined as the ‘core region’ (Carraro et al., 2011; Gaillard et al., 2010). The recombination module catalyses the site-specific integration of the ICE into the host genome, as well as the reverse reaction that leads to its excision as a circular form. The conjugation mechanism of adaptation genes in bacteria (Brochet et al., 2008). The horizontal transfer of mobile genetic elements (MGEs), such as conjugative plasmids, prophages and transposons, is a key mechanism of bacterial evolution allowing the acquisition of new adaptive traits (Brown-Jaque et al., 2015; Goessweiner-Mohr et al., 2013; Novick et al., 2010; Roberts et al., 2014; Smillie et al., 2010). Conjugation appears to be the preponderant transfer mechanism of adaptation genes in bacteria (Brochet et al., 2008; Clewell et al., 2014; de la Cruz et al., 2010; Guglielmini et al., 2011; Ochman et al., 2000). Integrative and conjugative elements (ICEs) were primarily defined as MGEs able to excise from a replicon of their bacterial host, self-transfer by conjugation and stably maintain by integration into a replicon of the recipient bacterium (Burrus et al., 2002a). Other integrated MGEs, called mobilizable elements, are not autonomous for their mobility and require a helper functional conjugative element to regain mobility by conjugation (see Bellanger et al., 2014 for review; Carraro et al., 2014a; Daccord et al., 2013; Doublet et al., 2005). Among these, the cis-mobilizable elements (CIMEs) are derived by deletion of conjugation and recombination modules from ICEs, but retain their recombination sites. These elements hijack the recombination and conjugation machinery of related tandem integrated ICEs to excise, transfer and integrate (Bellanger et al., 2011; Puymège et al., 2013).

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Material.

One supplementary table is available with the online Supplementary Material.

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Abbreviations: CCC, covalently closed circular; CIME, cis-mobilizable element; ICE, integrative and conjugative element; IME, integrative and mobilizable element; MGE, mobile genetic element; qPCR, quantitative PCR; RCR, rolling-circle replication.
module encodes the relaxase, which recognizes its origin of transfer (oriT) and nicks a specific site (nic) to initiate the ssDNA transfer (for review, see Bellanger et al., 2014) using its type IV secretion system (Cabezon et al., 2015; Goessweiner-Mohr et al., 2013). Finally, the regulation module controls ICE excision and transfer (Carraro et al., 2011; Pradervand et al., 2014; see Wozniak & Waldor, 2010, for review).

In addition to their core region, ICEs also carry modules that encode accessory functions involved in their maintenance in the population or in adaptation of their host to specific conditions (Burrus & Waldor, 2004). In streptococci, various ICEs were found to encode a wide range of adaptive functions, such as antibiotic or heavy metal resistance, restriction-modification systems, lactose catabolism, bacteriocin synthesis and pathogenicity (Bellanger et al., 2014; Benciani et al., 2014; Chaffanel et al., 2015; Chuzelle et al., 2012, 2015). ICEs with related core regions can carry very different cargo genes, thus defining a variable region.

ICEs were primarily assumed to be non-replicative elements. Nevertheless, the intracellular replication of the excised form of ICEs was recently proposed to be involved in their maintenance in both Gram-negative and Gram-positive bacteria (Carraro & Burrus, 2015; Lee et al., 2010; Wright et al., 2015). Notably, after induction, ICEBs1 of Bacillus subtilis is able to replicate using rolling-circle replication (RCR); this replication involves the relaxase as initiation protein and the orfI as origin of replication (Carraro & Burrus, 2014; Lee et al., 2010).

ICESt3, a 28.1 kbp ICE, was found to be integrated into the 3’ end of the fda gene in the chromosome of the lactic acid bacterium Streptococcus thermophilus CNRZ385 (Pavlovic et al., 2004). Recombination between two 27 bp direct repeats, included in its two flanking sites attL and attR, generates an attl site carried by the excised circular element, whereas the chromosome harbours an attB site (Bellanger et al., 2009). ICESt3 is able to transfer between S. thermophilus strains as well as towards other lactic acid bacteria such as Enterococcus faecalis (Bellanger et al., 2009). The gene content and organization of the conjugation modules of ICESt3, ICEBs1 and Tn916 is highly similar although their conjugation proteins are distantly or very distantly related (Burrus et al., 2002b). The members of the ICESt1/ICESt3 family carry closely related conjugation modules found in various streptococci and harbour a peculiar regulation module. This module encodes two regulatory systems related to lambdoid prophage cI repressor (Beaber et al., 2004) and related to ImmA/ImmR regulatory systems (Bose & Grossman, 2011), suggesting a particularly complex regulation (Carraro & Burrus, 2014). Exposure of S. thermophilus to the DNA-damaging agent mitomycin C was found to promote not only excision and transfer of ICESt3 (Bellanger et al., 2009; Carraro et al., 2011), but also its adoption of a multicopy state, suggesting its capacity to replicate (Carraro et al., 2011). Moreover, the recombination and the conjugation modules of the ICESt3-like elements are co-transcribed, which coordinates excision and transfer (Carraro et al., 2011).

Here, we constructed and characterized MiniICESt3, a functional ICE harbouring only the core region of ICESt3 and a spectinomycin resistance gene. This element was sufficient for its stable maintenance, as well as for its intra- and intercellular self-mobility. Whilst ICESt3 was previously found to conditionally replicate upon activation, MiniICESt3 was found to constitutively replicate using a plasmid-like rolling-circle mechanism. Further experiments using digestion and hybridization assays allowed us to clearly visualize the different replicative forms of MiniICESt3. To the best of our knowledge, this study provides the first visual evidence of replicative intermediates of plasmid-like forms of an ICE.

**METHODS**

**Bacterial strains, plasmids and culture conditions.** The bacterial strains and plasmids are listed in Table 1. Escherichia coli DH5α, used for plasmid propagation and cloning experiments, was routinely grown in LB medium (Sambrook & Russell, 2006) at 37 °C in aero-biosis, supplemented with 100 μg ampicillin ml⁻¹ and 50 μg spectinomycin ml⁻¹ if suitable. S. thermophilus strains were grown in 10% (w/v) reconstituted skimmed milk, Hogg–Jago broth (Stingle et al., 1996) supplemented with 1% (w/v) lactose (HJL) or M17 broth (Oxoid) supplemented with 0.5% (w/v) lactose (LM17) at 42 °C under anaerobiosis. Agar plates were prepared by adding 2% (w/v) agar to the media. Antibiotics were added to LM17 at the following concentrations: 4 μg chloramphenicol ml⁻¹, 5 μg erythromycin ml⁻¹, 500 μg spectinomycin ml⁻¹ and 500 μg kanamycin ml⁻¹. OD₆00 0.6 and 1.5 (GENESYS 20; Thermo Scientific) corresponded to the exponential and stationary phases, respectively.

**Molecular biology methods.** The restriction enzymes were used according to the manufacturer’s instructions (New England Biolabs). Plasmid DNA was prepared by alkaline lysis using a Qiagen MiniPrep plasmid kit and genomic DNA was extracted according to Colmin et al. (1991). The oligonucleotide primers used for PCRs are listed in Table S1 (available in the online Supplementary Material). PCR and high-fidelity PCR were carried out according to the instructions of the ThermoPol PCR kit (New England Biolabs) and of the Triple Master PCR System (Eppendorf), respectively. When needed, PCR products were purified using a QIAquick PCR purification kit (Qiagen) according to the manufacturer’s instructions. Sequencing reactions were performed by Cogenics. Quantitative PCR (qPCR) experiments were performed as described previously (Carraro et al., 2011). Experiments were performed in duplicate and repeated at least three times using three independently derived clones. Data were expressed as mean ± SD. Statistical analysis was performed with Student’s t-test, P < 0.05 was considered significant.

**Construction of the LMG18311 CIMEVar–MiniICESt3 strain.** Primer sequences and their use are described in Table S2. All cloning steps were verified by sequencing. The pNC1 plasmid was obtained by cloning a 478 bp PCR fragment including the attl site from ICESt3 between XhoI and AvrII restriction sites of the pSL1180 vector (Table 1). The spectinomycin resistance gene (spc) was isolated by SpeI digestion of the pSc44s vector (Table 1) and cloned in pNC1 digested with XhoI. The resulting plasmid was named pNC2. The pNC5 plasmid was obtained by cloning a 702 bp PCR fragment, corresponding to the left end of the regulation module of ICESt3.
**Table 1. Strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant phenotype or genotype</th>
<th>Reference</th>
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<tr>
<td><strong>Strains</strong></td>
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<td><em>Streptococcus thermophilus</em></td>
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<tr>
<td>LMG18311 pMG36e</td>
<td>WT strain carrying the erythromycin resistance plasmid pMG36e, Ery&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Bellanger et al. (2009)</td>
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<tr>
<td>LMG18311kan</td>
<td>WT strain with an insertion of the kanamycin resistance gene aphA3 carried by IS1191, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Unpublished data</td>
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<tr>
<td>LMG18311 ICES3cat</td>
<td>WT strain carrying ICES3 tagged with the chloramphenicol resistance gene cat inserted in the pseudogene Porf385J, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Bellanger et al. (2009)</td>
</tr>
<tr>
<td>LMG18311 CIMEVar–MiniICESt3</td>
<td>WT strain carrying a composite element derived from ICES3cat by insertion of an attL site and a spectinomycin resistance gene spc between the right end of the variable region and the left end of the regulation module of ICES3, Cm&lt;sup&gt;R&lt;/sup&gt; and Spc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
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<td>LMG18311 MiniICESt3</td>
<td>WT strain carrying MiniICESt3, i.e. an element carrying the core region of ICES3 and a spc gene, Spc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
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<tr>
<td>LMG18311kan MiniICESt3</td>
<td>Strain LMG18311kan carrying MiniICESt3, Kan&lt;sup&gt;R&lt;/sup&gt; and Spc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
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<td>DH5α</td>
<td>supE44 lacU169 (q80 lacZ M15) hsdR17 endA1 gyrA96 thi-1 relA1</td>
<td>Taylor et al. (1993)</td>
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<td><strong>Plasmids</strong></td>
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<td>pMG36e</td>
<td>3.4 kbp, replication origin from pWV01, Ery&lt;sup&gt;R&lt;/sup&gt;</td>
<td>van de Guchte et al. (1989)</td>
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<tr>
<td>pSet4s</td>
<td>4.5 kbp, pWV01-type thermosensitive replication origin from pVE6002, LacZ&lt;sup&gt;+&lt;/sup&gt;, Spc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Takamatsu et al. (2001)</td>
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<tr>
<td>pSL1180</td>
<td>3.4 kbp, replication origin from pBR322, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Amersham</td>
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<td>pNC1</td>
<td>pNC180 carrying the attL site of ICES3, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
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<td>pNC2</td>
<td>pNC1 carrying a spectinomycin resistance gene (attL–spc cassette), Amp&lt;sup&gt;R&lt;/sup&gt; and Spc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
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<td>pNC5</td>
<td>pNC180 carrying the 702 bp left end of the regulation module of ICES3, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pNC6</td>
<td>pNC5 carrying the attL–spc cassette from pNC2, Amp&lt;sup&gt;R&lt;/sup&gt; and Spc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
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<td>pNC7</td>
<td>pNC180 carrying the attL–spc cassette inserted between the 710 bp right end of the variable region and the 702 bp left end of the regulation module of ICES3, Amp&lt;sup&gt;R&lt;/sup&gt; and Spc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
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in pSL1180 digested by NheI and EcoRI. A SpeI/NheI fragment of pNC2 including the attL–spc cassette was cloned in pNC5 digested by the same enzymes. The resulting pNC6 plasmid was digested by SfiI and SpeI to clone a 710 bp PCR fragment, corresponding to the right end of the variable region of ICES3. The resulting pNC7 plasmid was digested by BsrBI. The 2788 bp BsrBI fragment of pNC7, including the right end of the ICE<sup>St3</sup> variable region, the attL–spc cassette and the left end of the ICES3 regulation module (Fig. 1), was purified and introduced by natural transformation in the *S. thermophilus* LMG18311 ICES3cat strain (Bellanger et al., 2011) according to Gardan et al. (2009). Putative transformants were selected on LM17 plates supplemented with spectinomycin. The presence of the attL–spc cassette between the variable region and the regulation module of ICES3 was validated by PCR (Fig. 2d). This insertion yielded to the CIMEVar–MiniICESt3 composite element schematized in Fig. 1.

**Conjugation procedure.** Filter mating assays were performed as described previously by Bellanger et al. (2009). LMG18311 CIMEVar–MiniICESt3 and LMG18311 carrying the erythromycin resistance plasmid pMG36e (Table 1) were used as donor and recipient cells, respectively. Transconjugants carrying MiniICESt3 or complete composite element were recovered on LM17 medium supplemented with erythromycin combined with spectinomycin or chloramphenicol and spectinomycin, respectively. In total, 24 clones, carrying spectinomycin resistance, were tested by PCR using at least 10 primer pairs to characterize the elements carried. The resulting strain LMG18311 carrying the plasmid pMG36e and MiniICESt3 was used as the donor strain for the next conjugation assay. In this experiment, the recipient cells were kanamycin-resistant LMG18311kan (unpublished data), and putative transconjugants were recovered on LM17 plates supplemented with kanamycin and spectinomycin. PCR-based characterizations were performed on 24 clones to verify MiniICESt3 integration.

**Stability assays.** The stability assays were performed as described previously (Bellanger et al., 2011). Briefly, *S. thermophilus* LMG18311 clones carrying ICESt3cat or MiniICESt3 were grown over ~100 generations in liquid HJL medium without antibiotic by successive 10⁻³ dilutions. The presence of ICESt3cat or MiniICESt3, carrying the cat or the spc resistance genes, respectively, was monitored before and after dilutions in 200 clones based on their susceptibility to chloramphenicol or spectinomycin. Under these conditions, the loss is irreversible (no transfer in liquid medium) and genetic drift is negligible. Considering the element as a single copy, the rate (Lₙ) of the population which should have lost the element after n generations was calculated: Lₙ = 1 - (1 - e/2)ⁿ, where e is the relative excision rate.
Southern blot procedure. The DNAs from exponential cultures of *S. thermophilus* were extracted as described by Colmin *et al.* (1991). A minor modification consisted of the addition of 5 mg lysozyme ml⁻¹ to weaken the cell wall of streptococci prior to lysis. The extracted DNA was digested with *Sst*I or *Pst*I and *Bgl*I. Total DNAs were separated on 0.6% (w/v) agarose gel by electrophoresis and stained with ethidium bromide prior to transfer under denaturing conditions onto a positively charged nylon membrane (Boehringer Mannheim). The *fda* and ICE probes were generated by PCR and labelled with a DIG DNA Labeling kit (Roche). Probes chosen were of similar length and G+C content in order to allow rough relative quantification. Imaging was acquired by a Molecular Imager ChemiDoc XRS System (Bio-Rad) with a non-saturated exposure, and relative quantification analysis was performed with Quantity One software (Bio-Rad).

RESULTS

Organization of ICESt3

ICESt3 is a functional ICE that shares a core region with related elements from streptococci (Carraro *et al.*, 2011) (Fig. 1). This region was defined as genes and sequences involved in the basic biology of the element, i.e. the recombination, conjugation and regulation modules. In addition to the core region, a highly variable region carries cargo genes (Fig. 1). The variable region of ICESt3 was previously reported to encode two putative methyltransferases (*orf385G* and *orf385F*), and to carry pseudogenes and genes encoding unknown hypothetical proteins (Pavlovic *et al.*, 2004). A reanalysis of this region by BLASTP performed on REBASE (http://rebase.neb.com/rebase/rebase.html) predicted thatorf385E encodes an endonuclease related to GCSGC-recognizing endonucleases. Together with *orf385G* and *orf385F* that encode putative cytosine-C5 DNA methyltransferases, this gene is part of a module encoding a putative type II restriction-modification system. Modules identified in *Streptococcus anginosus*, *Streptococcus lutetiensis* and *Candidatus Arthromitus* spp. exhibit identical organization and high nucleotide identity (>65%), strongly suggesting that they are functional. To validate this variable region and core region organization, we attempted to obtain a minimal element MiniICESt3 harbouring only the core region of ICESt3.

Construction of MiniICESt3

Considering the impossibility of deleting the variable region that contains the addiction module, we used an alternative multi-step strategy approach. Briefly, a composite structure was generated by introducing a copy of the left end of ICESt3 (including the attachment site *attL*) between the variable and core regions of ICESt3cat, an ICESt3 derivative labelled with a chloramphenicol resistance gene (Fig. 1). For this purpose, a DNA fragment carrying the *attL* site of ICESt3 and a spectinomycin resistance gene (*attL–spc* cassette; Fig. 1) inserted between the right end of the variable region and the left end of the regulation module was used to transform LMG18311 ICESt3cat (Fig. 1). Homologous recombination led to a composite structure that consisted of two elements in tandem: (i) a putative CIME (CIMEVar) that carried the variable region of ICESt3 and the *cat* gene flanked by two identical *attL* sites (*attL* and *attL3*), and (ii) a minimal ICE (MiniICESt3) flanked by *attL* and *attR*, carrying the core region of ICESt3 and the *spc* gene (Fig. 1).

In the resulting transformants, the structure of the composite element and its excision capacity were monitored by PCR amplification of the various *att* sites carried by the integrated element or resulting from its excision and compared to those of ICESt3 (Fig. 2). All the specific *att* sites of both integrated or excised forms of the three elements CIMEVar, MiniICESt3 and the whole composite structure CIMEVar–MiniICESt3 were observed (Fig. 2a–d). Detection of a circular form of MiniICESt3 demonstrated that MiniICESt3 harbouring only the core region of ICESt3 was able to excise. The circular composite structure was also detected, highlighting the intracellular cis-mobilization of the CIME by the adjacent ICE. Detection of a circular form of CIMEVar revealed that the integrase and excisionase encoded by MiniICESt3 were able to act in trans. This circular form could have resulted from either the excision of CIMEVar by recombination between *attL* and *attR* sites carried by the integrated composite structure (shown in
Excision, conjugation and integration of MiniICESt3

LMG18311 clones carrying MiniICESt3 allowed the detection of attLM and attB sites (Fig. 2d) as expected upon excision of the element (Fig. 2c). To further assess the functionality of MiniICESt3, its excision rate was measured by qPCR as described previously (Carraro et al., 2011). Briefly, the excision rate corresponds to the ratio of chromosomal attB sites resulting from excision of the ICE per copy of the adjacent chromosomal locus fda. The MiniICESt3 excision rate (3.1 ± 0.8%) during exponential growth phase was very similar to that of ICESt3cat (3.7 ± 0.3%; Carraro et al., 2011).
Conjugation experiments were performed using LMG18311 carrying MiniICESt3 as donor strain and a kanamycin-resistant derivative from LMG18311 as recipient. In total, 24 putative transconjugants were analysed by PCR to confirm the site-specific integration of MiniICESt3 into the 3' end of fda (Fig. 2c, d). The transfer frequency of MiniICESt3 was similar to that of ICESt3cat in the same strain, i.e. $10 \pm 0.2 \times 10^{-6}$ and $14 \pm 3 \times 10^{-6}$ transconjugants per donor cell, respectively (Bellanger et al., 2011). Therefore, the functionality of MiniICESt3 demonstrated that the core region of ICESt3-like ICEs carried the entire recombination, conjugation and regulatory functions that are necessary for the transfer of ICESt3.

**Stability of MiniICESt3**

As reported previously, the loss of ICESt3 was undetectable (Bellanger et al., 2011) while it can excise from the chromosome. This led us to consider the presence of a maintenance mechanism for ICESt3. A good candidate was the putative type II restriction-modification system encoded by the variable region of ICESt3 (orf385G and orf385F encoding putative methyltransferases, orf385E encoding a putative endonuclease). Type II restriction-modification systems are known to act as addiction modules that enhance plasmid maintenance (Kobayashi, 2001). The restriction-modification system encoded by ICESt3 could thus be involved in its stability, as proposed for sth368IR/sth368IM in the variable region of ICESt1 (Burrus et al., 2001). Therefore, MiniICESt3 stability was tested in the strain LMG18311 after 100 generations without selective pressure, as previously done for ICESt3 (Bellanger et al., 2009). Once excised from the chromosome as a single-copy element, MiniICESt3 may be lost by dilution during cell division. Considering that MiniICESt3 excises in 3.1% of the cell population, as calculated above and similar to that of ICESt3, 78% of the population should have lost MiniICESt3 after 100 generations in the absence of selective pressure (see Methods for calculation). Nevertheless, no loss was detected, suggesting that MiniICESt3 loss was infrequent. As MiniICESt3 is devoid of the variable region, and consequently of its addiction module, MiniICESt3 likely benefits from another mechanism encoded by the core region that favours its maintenance through generations.

**MiniICESt3 is a multi-copy element**

Replication of the element after excision is a plausible explanation for the stable inheritance of MiniICESt3. The copy number of MiniICESt3, as well as ICESt3cat and CIMEVar–MiniICESt3, was monitored in exponential-phase cultures of LMG18311 derivative strains. The copy number corresponds to the ratio between the copy number of an internal fragment of the conjugation module (orfM/orfL junction; Fig. 3a) per copy of the adjacent chromosomal locus fda. While ICESt3cat was found as a single-copy element, MiniICESt3 was present at $8.4 \pm 1.3$ copies per chromosome without any specific treatment (Fig. 3b). Similar measurements were performed for CIMEVar (internal fragment of orf385I, Fig. 3a) and MiniICESt3 in a strain harbouring the CIMEVar–MiniICESt3 structure. Whilst CIMEVar appeared in a single copy, MiniICESt3 was found at $11.7 \pm 1.5$ copies per chromosome (Fig. 3b). Taken together, these data revealed that MiniICESt3 behaves as a multi-copy element in the cell population whilst ICESt3cat was present in a single copy. Deregulation of the MiniICESt3 copy number control could have been the cause of this multi-copy state. The regulation module of three MiniICESt3 clones was then fully sequenced and revealed the absence of any mutation when compared to ICESt3 (data not shown). However, the variable region and the MiniICESt3 in a multi-copy state are present simultaneously in the...
strain containing the ClMEVar–MiniICEST3 composite element, demonstrating that the repressor effect of the variable region was not effective in trans. Therefore, the variable region has a strong cis-impact on ICESt3 replication.

**MiniICEST3 produces ssDNA and concatemeric DNA species**

The total genomic DNA of *S. thermophilus* strains harbouring ICESt3cat, MiniICEST3 or no ICE was analysed.

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**Fig. 4.** Analysis of the different forms of ICEs. (a) Chromosomal and excised forms of ICESt3cat. The symbols for the core and variable regions, the attachment sites, and the antibiotic resistance genes are identical to those used in Fig. 2(b). The restriction enzyme recognition sites are labelled with black diamonds. The thick black and grey lines indicate the regions hybridizing with the probes specific to the chromosomal *fda* locus and ICE core region, respectively. The arrows indicate the expected fragment sizes after enzymatic digestion. Stars represent the location of putative oriT. (b) Chromosomal and excised forms of MiniICEST3. The symbols and representation are identical to those used in (a) and Fig. 2(c). (c) LMG18311 and LMG18311 ICESt3cat genomic DNA analysis. Left: ethidium bromide-stained agarose gel; right: corresponding Southern blot using *fda* and ICE probes. Genomic DNAs were digested by *PstI* or *PstI* and *BglI*. ICESt3cat-specific signals are indicated by grey-filled triangles for ICESt3cat integrated into the chromosome (Chr), ssDNA or ICESt3cat inner fragment (Inner). Close to triangles, the numerical values correspond to the expected fragment sizes (kbp). Digested λ DNA and LMG18311 genomic DNA stained with ethidium bromide or revealed with λ and *fda* probe were used as DNA ladders. Some relevant fragment sizes (kbp) are indicated. (d) LMG18311 MiniICEST3 genomic DNA analysis as in (c). MiniICEST3-specific signals are indicated by empty triangles for detection of the chromosomal complete MinSt3 (Chr) or the *attL*M-containing fragment and different MiniICEST3 forms: CCC DNA, ssDNA, linear monomer (L), and high- and very-high-molecular-mass DNA (hDNA and vhDNA). Fragment sizes (kbp) are indicated. Grey-filled triangles, ICESt3cat; empty triangles, MiniICEST3.
by restriction and hybridization with ICE-specific and insertion site-matching probes (Fig. 4). According to the ICE dynamics model (Burrus et al., 2002a), ICEs could be found either integrated into the chromosome or excised as monomeric circular forms, as depicted in Fig. 4(a, b).

In the strain harbouring ICESt3cat, hybridization signals (ICE probe) corresponding to DNA fragments of 24.5 (‘Chr’ signal) and 10.8 kbp (‘Inner’ signal) were detected with the simple PstI and the double PstI/BglI digestions, respectively (Fig. 4c). The presence of these signals and the absence of the 28 kbp linearized ICESt3cat were in agreement with the detection of the sole integrated form of the ICE into the attB site (Fig. 4a). The intensities of ICESt3cat and fda signals were similar, which was consistent with qPCR experiments revealing the presence of a unique copy of ICESt3cat per chromosome. Furthermore, two specific additional forms of ICESt3cat exhibited a delayed mobility relative to the double-stranded fragments mentioned earlier; the faster showed a mobility equivalent to a 44 kbp ssDNA fragment whilst the apparent size of the slower one could not be estimated using DNA markers. These two DNA bands were insensitive to restriction enzyme treatment (i.e. BglI) (Fig. 4c) and were consequently assumed to correspond to single-stranded species of ICESt3cat (ssDNA signals).

In contrast, the simultaneous presence of integrated and circular forms of MiniICESt3 was observed in the strain harbouring MiniICESt3. The single PstI restriction enzyme analysis showed the presence of the integrated form as a 28.5 kbp fragment (Fig. 4d) in addition to four additional signals. As the PstI treatment preserved the native forms of the MiniICESt3, their fate after the double PstI/BglII digest analysis gave an insight into their molecular nature: three of them were sensitive to the second restriction enzyme treatment and therefore contained dsDNA. Two fragments had a low mobility (high- and very-high-molecular-mass DNA) and the third exhibited fast mobility and was assumed to correspond to covalently closed circular DNA (CCC DNA). In contrast, the fourth fragment was resistant to restriction enzyme exposure and was interpreted as ssDNA. In addition, double digestion revealed two fragments hybridizing with the ICE probe with sizes of ~13.2 (attL3M signal) and 19 kbp (L signal). The former corresponded to the chromosome-integrated form of the ICE, whilst the latter should include the excised and/or multimeric forms (very-high-molecular-mass DNA, high-molecular-mass DNA and CCC DNA) after MiniICESt3 linearization as 19 kbp monomers. Moreover, their relative intensities were consistent with the extrachromosomal replication of the element suggested by the qPCR copy number quantification (Fig. 3). The very-high-molecular-mass DNA and high-molecular-mass DNA forms, revealed by PstI digestion, strongly hybridized with the ICE probe. Considering their relative intensities in hybridization signals and with ethidium bromide staining, which intercalates into dsDNA, high-molecular-mass DNA likely contained a large amount of ssDNA and might correspond to concatemeric forms of the element, explaining why the intensity of the 19 kbp signal was not the summation of the DNA concatemers.

In conclusion, whilst both ICESt3cat and MiniICESt3 generated ssDNA species, excised forms including dsDNA could be detected only for MiniICESt3. This was consistent with the higher copy number of MiniICESt3 compared with ICESt3. The presence of concatemeric forms as well as ssDNA species, together with CCC DNA forms of the element, indicated that MiniICESt3 replicates autonomously using a RCR mechanism.

**DISCUSSION**

The ICESt3 subfamily has been defined based on its regulation and conjugation modules, as well as its bipartite organization with a variable region carrying cargo genes and a core region carrying genes controlling the ICE’s behaviour (Carraro et al., 2011). This structure is particularly appropriate to elaborate an autonomous mini-element-based strategy as was done for other MGEs (miniF, Hayakawa & Matsubara, 1979; mini-mu, Faelen et al., 1979; mini-Tn4001, Pour-El et al., 2002; MiniP1, Wickner & Chattoraj, 1987; pVCR94AX, Carraro et al., 2014a), but not for ICEs to date. To study the essential functions that characterize the ICESt3 subfamily, we engineered and characterized MiniICESt3, an autonomous mini-element containing only the functional core region of ICESt3, from *S. thermophilus* strain CNRZ385.

Our strategy, based on the insertion of a new functional attL site between the variable and the core region of ICESt3cat, led us to generate a composite element composed of CIMEVar and MiniICESt3 (Fig. 1). We provided evidence for the third time of effective mobilization of a CIME that did not code for any protein involved in mobility (Bellanger et al., 2011; Puymége et al., 2013). There were 10-fold fewer transconjugants carrying CIMEVar–MiniICESt3 than MiniICESt3. The higher copy number of the MiniICESt3 part of the composite element compared with the full CIMEVar–MiniICESt3 element may have caused this difference (Fig. 3). The frequency of intraspecific transfer of the isolated MiniICESt3 was shown to be similar to that of the original ICESt3. These experiments demonstrated that the variable region is dispensable and the core region of ICESt3 is able to self-transfer by conjugation, with or without CIMEVar.

When integrated into their host genome ICEs are stably maintained and are replicated along with the chromosome (Burrus et al., 2002a). However, once excised from the host genome ICEs might be lost following cell division but the variable regions of various ICEs encode functional or putative addiction systems that may be involved in their stability (Carraro et al., 2015a; Carraro & Burrus, 2015b; Wozniak & Waldor, 2009). Without any mechanism preventing the growth of daughter cells lacking the element,
the loss of MiniICESt3 should occur whilst it is excised during cell division. Nevertheless, no loss of MiniICESt3 was observed, which is devoid of addiction systems. Alternatively to addiction systems, some conjugative plasmids are stably maintained in the population by transferring at high frequency (Bahl et al., 2007a, b). However, here, stability assays were performed under conditions that did not allow ICESt3 to transfer (Bellanger et al., 2009). Based on these observations, the core region of ICESt3 carries determinants required for its stability using a mechanism not based on an addiction system or transfer at high frequency. Recent studies on various ICEs of Firmicutes, carrying related conjugation modules, encoding a MOB1 relaxase related to RCR replicases of plasmids (Guglielmini et al., 2014), including ICESt3 (Carraro et al., 2011), RD2 from Streptococcus suis MGAS6180 (Sitkiewicz et al., 2011) and ICEBs1 (Lee et al., 2010), strongly supported their intracellular RCR. No replication module, per se, carried by these ICEs could be identified (Bellanger et al., 2014; Carraro & Burrus, 2014c). Nevertheless, studies on the replication of ICEBs1 revealed that it is conditional upon activation of the element and implies the relaxase as replication initiation protein with the oriT sequence as replication origin (Lee et al., 2010), which are both enclosed in the conjugation module. Similarly, ICESt3 was shown to replicate in cells where it was activated after mitomycin C treatment (Carraro et al., 2011). However, we found that the multi-copy state of MiniICESt3 did not require any induction (Fig. 3). Our hybridization experiments revealed typical replication intermediates and single-strand forms in both ICESt3cat and MiniICESt3 profiles (Fig. 4), as observed for RCR plasmids (Gregorova et al., 2004; Raha et al., 2006; Ruiz-Másò et al., 2006). The RCR mechanism is particularly widespread in Gram-positive bacteria and mainly concerns small plasmids, although RCR replicons >20 kbp have been identified (Lorenzo-Díaz et al., 2014). The 10 kbp reduction could have had a stimulative effect on MiniICESt3 replication. In the strain carrying MiniICESt3, the lack of proteins encoded by the variable region of ICESt3 does not seem to be a plausible cause for its intrinsic replication because, in the composite strain (Fig. 3), the replication of MiniICESt3 is also observed whilst CIMEVar is present. These proteins expressed from the variable region should be able to impact the activity of the oriT locus in the composite strain in trans. Therefore, the variable region seems to repress ICESt3 replication in cis. Transcripts overlapping the variable regions and the regulation module of the core region of ICESt3 were observed previously (Carraro et al., 2011). If the transcription was initiated in the variable region, this might have affected the expression level of regulators encoded by the nearby regulation module. The precise regulation and mechanism of ICESt3 and MiniICESt3 replication will be further explored.

Our prototype MiniICESt3 is functional in S. thermophilus, and was similar to ICESt3 in terms of its excision rate, conjugation frequency and chromosomal integration site. This work, together with other studies (Carraro & Burrus, 2015; Carraro et al., 2011, 2015; Lee et al., 2010; Puymége et al., 2013; Ramsay et al., 2006; Sitkiewicz et al., 2011; Wozniak & Waldor, 2010), breaks common stereotypes about ICEs. As the replication initiation protein is the relaxase and the origin of replication is oriT, the replication module is intrinsically encompassed in the conjugation module of ICEs (Carraro & Burrus, 2014, 2015; Lee et al., 2010; Menard & Grossman, 2013). Therefore, conditional plasmid-like RCR could be a common capacity of many ICEs that likely promotes their stability amongst bacterial populations.

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