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ICE*Sp1116*, the Genetic Element Responsible for *erm*(B)-Mediated, Inducible Resistance to Erythromycin in *Streptococcus pyogenes*

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ICE*Sp1116*, responsible for *erm*(B)-mediated, inducible erythromycin resistance in *Streptococcus pyogenes*, was comprehensively characterized, and its chromosomal integration site was determined. It displayed a unique mosaic organization consisting of a scaffold, related to Tn*Gallo*1 from *Streptococcus gallolyticus*, with two inserted fragments separated by IS*1216*. One fragment, containing *erm*(B), displayed high-level identity to a portion of the *S. pyogenes* plasmid pSM19035; the other, containing a truncated *tet*(M) gene, displayed high-level identity to the right-hand portion of *Clostridium difficile* Tn*5397*.

Three erythromycin resistance methylase genes have so far been described in *Streptococcus pyogenes*: the classic, long-established *erm*(B) determinant, an *erm*(A) subclass commonly referred to as *erm*(TR), and the much less common *erm*(T) determinant (30). Whereas *erm*(TR) and *erm*(T) normally encode inducible erythromycin resistance, the *erm*(B) gene may encode constitutive resistance in some *S. pyogenes* isolates and inducible resistance in others.

Consistent with such heterogeneity of *erm* gene-encoded erythromycin resistance, a variety of *erm*-carrying genetic elements have been found and characterized in *S. pyogenes*, including diverse integrative and conjugative elements (ICEs) carrying *erm*(TR) (3, 9, 12) and closely related plasmids carrying *erm*(T) (11, 33). As regards *erm*(B), it is carried by different elements depending on whether it is expressed constitutively or inducibly. Tn*916* family elements, such as Tn*6002* (~21 kb) (32) or Tn*3872* (~24 kb) (17), aside from rare plasmid locations, are primarily involved in the former case (7). When inducibly expressed, *erm*(B) has been shown to be carried by a different element, called Tn*1116*, of which only an ~7-kb region—containing *erm*(B) and a fragment highly homologous to the right-hand portion of *Clostridium difficile* Tn*5397* (18) — has so far been characterized (7). Tn*5397* (~21 kb; accession no. AF333235) is a unique Tn*916* family element where the *tndX* (resolvase) gene replaces *int* (integrase) and *xis* (excisionase) (31).

Within the erythromycin-resistant *S. pyogenes* population circulating in Italy, isolates bearing inducible *erm*(B) represent a major subpopulation (13, 21, 27, 34) clearly prevailing among *erm*(B)-positive isolates (7, 24) and typically characterized by uniform susceptibility to tetracycline (7, 13, 21). In this study, Tn*1116*, renamed ICE*Sp1116* (23), was comprehensively characterized (accession no. HE802677). ICE*Sp1116* was uniformly distributed in *S. pyogenes* isolates with *erm*(B)-mediated, inducible erythromycin resistance.

ICE*Sp1116* was investigated in *S. pyogenes* strain A-3 [erythromycin MIC > 128 μg/ml, inducible phenotype, *erm*(B) genotype; tetracycline MIC of 0.25 μg/ml, *tet*(M) genotype] (7). The principal oligonucleotide primer pairs used in PCR experiments are listed in Table 1. Most oligonucleotides for long PCR experiments were designed from the broad-host-range Gram-positive plasmid pSM19035 (~29 kb; accession no. AY357120) (16) and from transposon Tn*Gallo*1 of *Streptococcus gallolyticus* UCN34 (~41 kb; accession no. FN597254) (25). DNA sequencing and sequence analysis were performed as described elsewhere (9). A reported percentage of amino acid identity represents the mean value resulting from the comparison of deduced proteins encoded by individual open reading frames (ORFs) in the DNA fragments considered.

Genetic organization of ICE*Sp1116*. Most ORFs of ICE*Sp1116* encoded proteins with various degrees of amino acid identity to those encoded by the corresponding ORFs of Tn*Gallo*1. Two fragments, separated by IS*1216*, were found to be inserted in a scaffold largely including Tn*Gallo*1-like sequences. One fragment contained *erm*(B) and displayed high-level identity to a portion of the *S. pyogenes* plasmid pSM19035 (16); the other contained a truncated *tet*(M) gene and displayed high-level identity to the right-hand portion of *C. difficile* transposon Tn*5397* (18). ICE*Sp1116* (size, 48,174 bp; G+C content, 35%; 45 ORFs) is shown in Fig. 1, where its ORF map is aligned with the ORF maps of Tn*Gallo*1, pSM19035, and Tn*5397*; the characteristics of the ORFs are detailed in the supplemental material (see Table S1). The organization of ICE*Sp1116* is summarized below.

(i) *orf1-orf28* (bp 1 to 32,827; 36% G+C). Most ORFs in this region encoded proteins with various degrees of amino acid identity (41% to 85%) to Tn*Gallo*1. The specific functions associated with some such ORFs (*orf2*, *orf13*, *orf16*, *orf20*, *orf23*, and *orf27*) (see Table S1 in the supplemental material) are presumably involved in ICE transfer. Among Tn*Gallo*1-unrelated ORFs, *orf5-orf7* is a cluster that does not occur in Tn*Gallo*1 but does occur in the genomes of closely related *Streptococcus* species which, like *S. gallolyticus*, result from the recent reclassification of *Streptococcus bovis* (26); the highest degree of amino acid identity (96%) was with the proteins encoded by the corresponding three-ORF cluster of *Streptococcus macedonicus* (accession no. HE613569). Spe-

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TABLE 1 Principal oligonucleotide primer pairs used

Category and gene[a]	Primer designation	Sequence (5′–3′)	Source or reference	Product size (bp)
Resistance genes				
erm(B)	ERMB1	GAAAAGGTACTCAACCAAATA	28	639
	ERMB2	AGTAACGGTACTTAAATTGTTTAC	28	
tet(M)	TETM2	GAACTCGAACAAGAGGAAAGC	19	740
	TETM3	ATGGAAGCCCAGAAAGGAT	19	
ICE*Sp1116* mapping				
orf2	ORF2-for	TTTTCCGAGTGCCGAAGGTGC	This study	11,230
orf11	ORF11-rev	GCGTTACCCCTTTTTCTTTTCG	This study	
orf12	ORF12-for	GACGGTTCCACCATCATTTATC	This study	11,490
orf23	ORF23-rev	CTTTTTCTTCACTCGTAACCGT	This study	
orf24	ORF24-for	AGACAATCAAGTTACTAATCAT	This study	9,372
orf31	ORF31-rev	GTTTCCCATTTAGCCGTCA	This study	
orf31	ORF31-for	GAAAAGAAACCCTGGTAAAAG	This study	12,394
Downstream of *orf45*	1116end-rev	GAGAATAATCATCAAGCCTAT	This study	
ICE*Sp1116* junctions				
SPYALAB49_000242[b]	JUG-for	GCGTCTTGGAATCTGGTCA	This study	621
orf1	ORF1-rev	CCTCTCTCAGCAAGAACTCGCCG	This study	
orf45	ORF45-for	GTATGACAGGTATGAGATTTG	This study	2,448
SPYALAB49_000245[b]	EPI-rev	AGGCTCTTGCGGCGGGTAA	This study	
ICE*Sp1116* circular form				
orf1	ORF1-rev	CCTCTCTCAGCAAGAACTCGCCG	This study	1,937
orf45	ORF45-for	GTATGACAGGTATGAGATTTG	This study	
Chromosomal empty target site				
SPYALAB49_000242[b]	JUG-for	GCGTCTTGGAATCTGGTCA	This study	1,134
SPYALAB49_000245[b]	EPI-rev	AGGCTCTTGCGGCGGGTAA	This study	
Inverse PCR assays				
orf2	REPA-inv	GCACCTTCGGCACTCGGAAAA	This study	
	REPA-for	CTAATGATACTGATATTAGT	This study	
orf30	BETA-inv	AGAAACGCCCTGTAACGCTT	This study	
	BETA-for	GGAATCAGAACGCAAACG	This study	
orf36 [*erm*(B)]	ERMB-inv	GTAAACAATTTAAGTACCGTTACT	This study	
	ERMB-for	TATTTGGTTGAGTACTTTTTC	This study	
orf44 (*tndX*)	TNDX-inv	CTTTGCTCGATAGGCTCTA	This study	
	TNDX-for	CGGATTGATGAAATAGACAGT	This study	

[a] Except for resistance genes and unless otherwise specified, designations are according to the ORF numbering of ICE*Sp1116*.
[b] From the *S. pyogenes* Alab49 genome.

cial functions appear to be associated with *orf6* and *orf7*. The former encodes a putative SspB protein, a large multidomain protein of the AgI/II family, i.e., cell surface proteins that may be key factors in the formation of streptococcal biofilms and that play multiple roles in streptococcal adherence, colonization, and microbial community development (6). *orf7* encodes a bacteriocin-processing endopeptidase belonging to the peptidase family C39 (14).

(ii) *orf29-orf37* (pSM19035 fragment) (bp 32,828 to 39,611; 34% G+C). This region, initially explored by inverse PCR, was formed by an ~6.8-kb *erm*(B)-containing fragment whose ORF encoded proteins with 99% amino acid identity to those encoded by a corresponding portion of pSM19035. This plasmid has extraordinarily long inverted repeats (~80% of the plasmid genome) separated by two nonrepeated sequences (NR1 and NR2) (5). The pSM19035 fragment comprises seven ORFs (*orf29-orf35*) from one of the inverted repeats (from alpha [copy 2] to zeta [copy 2]) plus NR2 [containing *erm*(B) (*orf36*) and its leader peptide (*orf37*)]. Interestingly, *orf33* (omega [copy 2]), *orf34* (epsilon [copy 2]), and *orf35* (zeta [copy 2]) correspond to a pSM19035 omega-epsilon-zeta operon constituting a plasmid addiction system where the epsilon and zeta genes encode antitoxin and toxin, respectively, while omega plays an autoregulatory function (35). Accordingly, the zeta-epsilon toxin-antitoxin cassette might con-

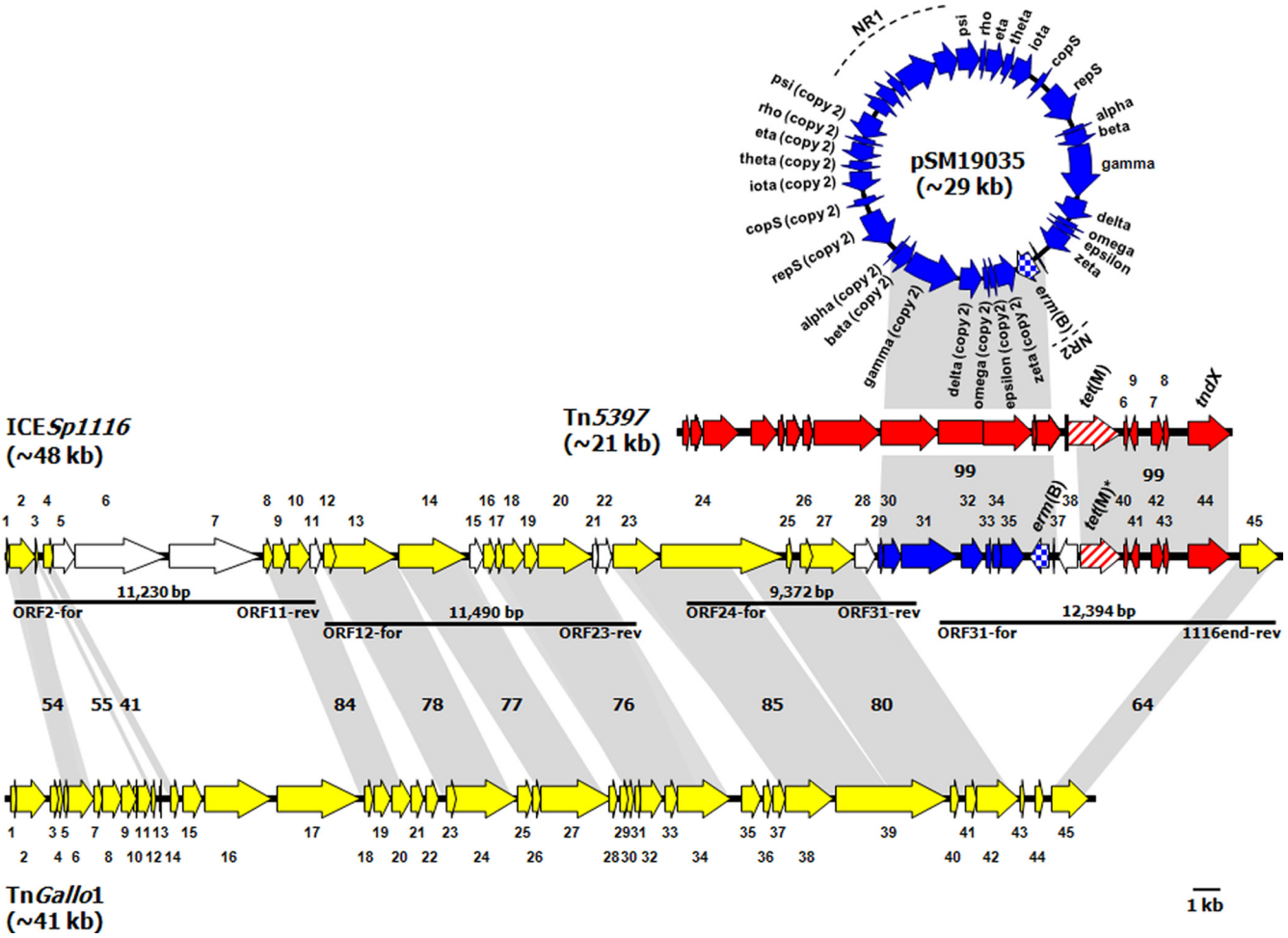

FIG 1 ORF map and genetic organization of ICE*Sp1116* from *S. pyogenes* strain A-3 (accession no. HE802677) and its alignment with the ORF maps of *S. gallolyticus* transposon Tn*Gallo*1 (accession no. FN597254), *S. pyogenes* plasmid pSM19035 (accession no. AY357120), and *C. difficile* transposon Tn*5397* (accession no. AF333235). The ORFs, indicated as arrows pointing in the direction of transcription, are numbered consecutively in ICE*Sp1116* (*orf1* to *orf45*, with predicted functions reported in Table S1 in the supplemental material) and in Tn*Gallo*1 (*orf1* to *orf45*). In pSM19035, where the two nonrepeated sequences NR1 and NR2 are marked by broken lines, the ORFs are numbered according to the specific designations reported in GenBank (the designations of the NR1 ORFs are not indicated). In Tn*5397*, the ORFs are also numbered according to the specific designations reported in GenBank [those upstream of *tet*(M) are not indicated]. Tn*Gallo*1 ORFs and related ORFs of ICE*Sp1116* are depicted as yellow arrows. pSM19035 ORFs and ICE*Sp1116* ORFs of the pSM19035 fragment are depicted as blue arrows [with *erm*(B) checkered]. Tn*5397* ORFs and ICE*Sp1116* ORFs of the Tn*5397* fragment are depicted as red arrows [with *tet*(M) striped]. The other ICE*Sp1116* ORFs are depicted as white arrows. Gray areas between ORF maps denote amino acid identities as reported (%). Horizontal bars indicate the amplicons (each with the relevant primer pair and size) allowing PCR mapping.

tribute to stable maintenance of ICE*Sp1116* in the bacterial population.

(iii) *orf38* (bp 39,612 to 40,420; 36% G+C). *orf38* encoded the transposase of IS*1216*, found mainly in enterococcal genomes but also in other Gram-positive bacteria and frequently associated with antibiotic resistance genes in streptococci (2, 15, 29).

(iv) *orf39-orf44* (Tn*5397* fragment) (bp 40,421 to 46,047; 36% G+C). This region was formed by an ~5.6-kb fragment whose ORF encoded proteins with 99% amino acid identity to those encoded by the right-hand portion of Tn*5397*. The fragment starts with a truncated *tet*(M) gene (*orf39*), resulting from the insertion of IS*1216* in the *tet*(M) coding sequence. Clearly, the truncated *tet*(M) gene was silent, in agreement with the tetracycline susceptibility of *S. pyogenes* A-3. The next ORFs of the fragment (*orf40-orf44*) were ~99% identical to the corresponding ORFs of Tn*5397*, the last one (*orf44*) being *tndX*.

(v) *orf45* (bp 46,048 to 48,174; 31% G+C). We expected that *tndX*, the last ORF of Tn*5397*, was also the last ORF of ICE*Sp1116*. However, inverse PCR assays revealed an additional ICE ORF (*orf45*) encoding a protein with 64% amino acid identity to that encoded by the last ORF of Tn*Gallo*1, which encodes a DDE transposase.

Chromosomal integration of ICE*Sp1116*. The left junction of ICE*Sp1116* was identified by inverse PCR. ICE*Sp1116* was found to be integrated at the 3′ end of an ORF, detected in all *S. pyogenes* genomes sequenced so far, whose highest degree of DNA identity (99%) was with the SPYALAB49_000243 gene from *S. pyogenes* Alab49 (accession no. CP003068) (4).

Detection of the circular form and of the core integration site of ICE*Sp1116*. The ICE*Sp1116* circular form was detected using an appropriate outward-directed primer pair (ORF45-for/ORF1-rev) (Fig. 2A). Sequence analysis of the amplicons from the inte-

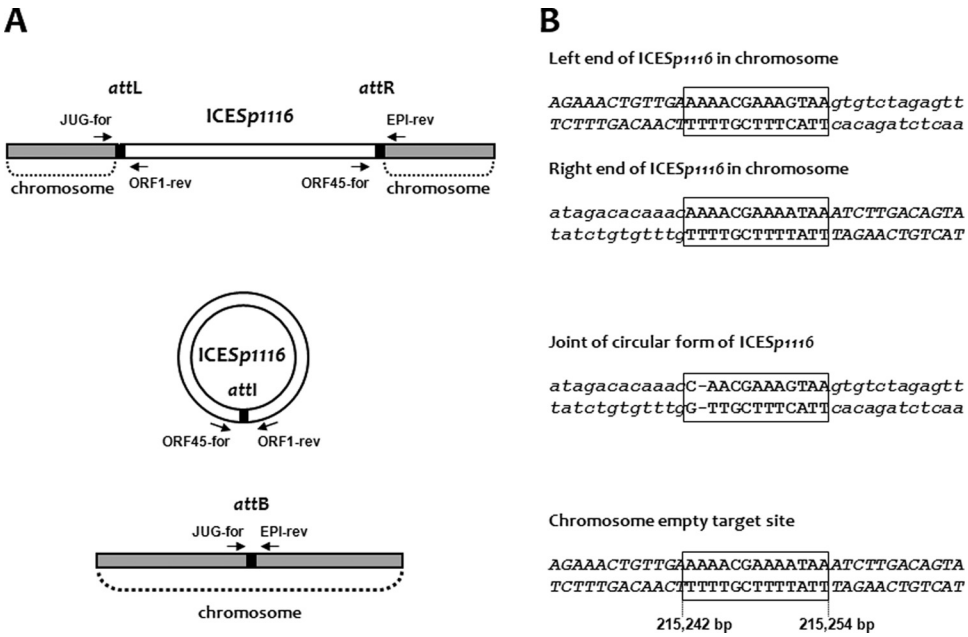

FIG 2 Detection of excision of ICE*Sp1116* from the genome. (A) Scheme of primer binding sites on the element and the genome. The chromosomal region is shown in gray, and ICE*Sp1116* is in white. The integrated (top) and circular (center) forms of the element are shown, as is the regenerated target after excision (bottom). The core integration site is shown as a black box. Oligonucleotide primers and their direction of priming are represented by arrows. The primer pairs JUG-for/ORF1-rev and ORF45-for/EPI-rev detect the junctions between the genome and ICE*Sp1116* (*attL* and *attR*, respectively), ORF45-for/ORF1-rev detects the circular form (containing *attI*) of ICE*Sp1116*, and JUG-for/EPI-rev detects the empty target site (containing *attB*). (B) Partial nucleotide sequences of the ICE*Sp1116* integrated form at the left and right junctions (top), of the circular form (center), and of the chromosomal empty target site (bottom), showing the putative core sites (boxed sequences in uppercase roman letters), corresponding to bases 215,242 to 215,254 of the *S. pyogenes* Alab49 genome (accession no. CP003068). Nucleotides belonging to ICE*Sp1116* are in lowercase italics; nucleotides belonging to the *S. pyogenes* A-3 chromosome are in uppercase italics.

grated (containing *attL* and *attR*) and circular (containing *attI*) forms of ICE*Sp1116* and from the empty chromosome (containing *attB*) of *S. pyogenes* A-3 led to the identification of a 13-bp putative core site (corresponding to bases 215,242 to 215,254 of the *S. pyogenes* Alab49 genome) (Fig. 2B).

Distribution of ICE*Sp1116* in other *S. pyogenes* isolates with *erm*(B)-mediated, inducible erythromycin resistance. Twenty additional *S. pyogenes* test strains—randomly selected among isolates having inducible erythromycin resistance mediated by the *erm*(B) gene, collected in 1998 to 2007—displayed uniform antibiotic susceptibility patterns: all were highly resistant to 14-, 15-, and 16-membered macrolides (MIC, >128 μg/ml); all were susceptible to clindamycin without induction (MIC, 0.03 to 0.5 μg/ml) but resistant after induction with erythromycin (MIC, >128 μg/ml); and all were susceptible to tetracycline (MIC, ≤0.125 to 0.5 μg/ml) in spite of (similar to the case with strain A-3) a *tet*(M) genotype. The 20 strains were subjected to PCR mapping using four primer pairs, designed from the sequence of ICE*Sp1116*, yielding amplicons covering the entire element (Fig. 1). While 19 strains produced amplicons of the expected size with all four primer pairs, the 20th gave no amplification with the fourth primer pair, although it did yield a regular amplicon when ORF31-for was paired with a primer internal to *orf45*. All strains gave positive PCRs with the primer pairs targeting the left and the right junctions, respectively (Fig. 2A). These data confirm that ICE*Sp1116* is typically harbored by *S. pyogenes* isolates with *erm*(B)-mediated, inducible erythromycin resistance.

Conclusions. ICE*Sp1116* shares with other streptococcal elements involved in macrolide resistance a mosaic structure resulting from the insertion of particular DNA fragments into a particular scaffold. These DNA fragments are of the most varied origins and often carry other antibiotic resistance genes—besides erythromycin resistance determinants—and other genes encoding products which may increase bacterial fitness. The scaffolds are also of various origins and natures. In the case of ICE*Sp1116*, the scaffold is related to a streptococcal transposon (Tn*Gallo*1); in other cases, it may consist of a Tn*916* family transposon (10, 22, 30), a clostridial ICE (9), or a bacteriophage (1, 8, 10, 20, 30).

A unique feature of ICE*Sp1116* is its chromosomal integration site. The relevant chromosomal gene (highest identity, the SPYALAB49_000243 gene from *S. pyogenes* Alab49) occurs in all the *S. pyogenes* genomes sequenced so far but has never been shown to represent the chromosomal integration site of a genetic element. It may be hypothesized that *orf45* of ICE*Sp1116* plays a role in such a specific chromosomal integration.

The occurrence of IS*1216* between the pSM19035 fragment and the Tn*5397* fragment suggests that the insertion sequence might have been involved in early mobilization of one of the two fragments to form ICE*Sp1116*. IS*1216* cuts off the coding sequence of *tet*(M), resulting in loss of the 5′ end of the gene (besides, of course, the remaining upstream portion of Tn*5397*) and in a silent *tet*(M). A subpopulation harboring ICE*Sp1116*, sharing a number of typing characters, is highly prevalent in Italy among erythromycin-resistant *S. pyogenes* isolates (7, 13, 21, 24, 27, 34). The success of this subpopulation may have been favored by the acquisition of specific genetic traits, such as *orf6*, whose product, the cell wall-anchored adhesin SspB, may have provided ICE*Sp1116*-harboring isolates with an adaptive advantage and enhanced their

survival through a greater ability to compete with indigenous commensal streptococci in colonizing host surfaces (6).

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