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Two Distinct Genetic Elements Are Responsible for *erm*(TR)-Mediated Erythromycin Resistance in Tetracycline-Susceptible and Tetracycline-Resistant Strains of *Streptococcus pyogenes*▽†

Andrea Brenciani,[1] Erika Tiberi,[1] Alessandro Bacciaglia,[1] Dezemona Petrelli,[2] Pietro E. Varaldo,[1] and Eleonora Giovanetti[1]*

Department of Biomedical Sciences, Section of Microbiology, Marche Polytechnic University, Ancona, Italy,[1] and School of Pharmacy, Microbiology Unit, University of Camerino, Camerino, Italy[2]

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In *Streptococcus pyogenes*, inducible erythromycin (ERY) resistance is due to posttranscriptional methylation of an adenine residue in 23S rRNA that can be encoded either by the *erm*(B) gene or by the more recently described *erm*(TR) gene. Two *erm*(TR)-carrying genetic elements, showing extensive DNA identities, have thus far been sequenced: ICE10750-RD.2 (~49 kb) and Tn*1806* (~54 kb), from tetracycline (TET)-susceptible strains of *S. pyogenes* and *Streptococcus pneumoniae*, respectively. However, TET resistance, commonly mediated by the *tet*(O) gene, is widespread in *erm*(TR)-positive *S. pyogenes*. In this study, 23 *S. pyogenes* clinical strains with *erm*(TR)-mediated ERY resistance—3 TET susceptible and 20 TET resistant—were investigated. Two *erm*(TR)-carrying elements sharing only a short, high-identity *erm*(TR)-containing core sequence were comprehensively characterized: ICE*Sp*1108 (45,456 bp) from the TET-susceptible strain C1 and ICE*Sp*2905 (65,575 bp) from the TET-resistant strain iB21. While ICE*Sp*1108 exhibited extensive identities to ICE10750-RD.2 and Tn*1806*, ICE*Sp*2905 showed a previously unreported genetic organization resulting from the insertion of separate *erm*(TR)- and *tet*(O)-containing fragments in a scaffold of clostridial origin. Transferability by conjugation of the *erm*(TR) elements from the same strains used in this study had been demonstrated in earlier investigations. Unlike ICE10750-RD.2 and Tn*1806*, which are integrated into an *hsdM* chromosomal gene, both ICE*Sp*1108 and ICE*Sp*2905 shared the chromosomal integration site at the 3′ end of the conserved *rum* gene, which is an integration hot spot for several mobile streptococcal elements. By using PCR-mapping assays, *erm*(TR)-carrying elements closely resembling ICE*Sp*1108 and ICE*Sp*2905 were shown in the other TET-susceptible and TET-resistant test strains, respectively.

In *Streptococcus pyogenes*, erythromycin (ERY) resistance is due to two principal mechanisms: target site modification or active efflux (13, 29). The latter is normally mediated by the *mef*(A) gene, adjacent to an *msr* gene usually designated *msr*(D), and is associated with low-level resistance to 14- and 15-membered macrolides only (M phenotype). Conversely, target site modification generally consists in posttranscriptional methylation of an adenine residue in 23S rRNA caused by *erm* gene-encoded methylases and is associated with either constitutive (cMLS phenotype) or inducible (iMLS phenotype) coresistance to macrolide, lincosamide, and streptogramin B (MLS) antibiotics. While cMLS isolates are rather homogeneous in susceptibility patterns and their methylase gene is normally *erm*(B), iMLS isolates are more heterogeneous, and their methylase gene is either *erm*(B) or an *erm*(A) subclass commonly referred to as *erm*(TR) (26).

Until the present study, only two *erm*(TR)-carrying genetic elements had been completely sequenced: an integrative and conjugative element (ICE), designated ICE10750-RD.2 (~49 kb), from the sequenced genome of a *S. pyogenes* strain (accession no. CP000262) (2), and a genetic element from *Streptococcus pneumoniae*, a species where *erm*(TR) is very uncommon, which was designated Tn*1806* (~54 kb, accession no. EF469826) (6). There are substantial identities between ICE10750-RD.2 and Tn*1806*, despite the occurrence of open reading frames (ORFs) unique to either element. Furthermore, both elements are integrated into an *hsdM* chromosomal gene.

Before ICE10750-RD.2 and Tn*1806* were detected, we had demonstrated that the *erm*(TR) gene from inducibly resistant *S. pyogenes* donors could be transferred by conjugation to susceptible recipients of *S. pyogenes* and other Gram-positive species (9). Intraspecific transfer was associated with the insertion of a new DNA fragment whose size was dependent on the donor, suggesting that *erm*(TR) could be carried by different genetic elements. Partial sequencing of the transposable element from one of these donors (accession no. FM162351) enabled us to compare its *erm*(TR)-flanking region with those of ICE10750-RD.2 and Tn*1806* and to document extensive similarities to both elements (29). Remarkably, other antibiotic (tetronasin and spectinomycin) resistance genes were found in the *erm*(TR)-flanking regions of the three elements.

The three available sequences of *erm*(TR)-carrying elements mentioned above are all from tetracycline (TET)-susceptible strains. However, TET resistance is widespread in *erm*(TR)-positive *S. pyogenes* (10, 11, 16, 19). In previous studies, we demonstrated the association of *erm*(TR) with *tet*(O) in ERY and TET coresistant *S. pyogenes* isolates and the cotransfer of

* Corresponding author. Mailing address: Department of Biomedical Sciences, Section of Microbiology, Marche Polytechnic University, Via Tronto 10/A, 60126 Ancona, Italy. Phone: 39 071 2206297. Fax: 39 071 2206293. E-mail: e.giovanetti@univpm.it.

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the two resistance determinants, but an actual genetic linkage could not be proved (8).

In the present study, in addition to completing the sequencing, characterization, and comparative analysis of the *erm*(TR)-carrying element (designated ICE*Sp*1108) from our TET-susceptible strain, we demonstrate a completely different genetic element (designated ICE*Sp*2905), also carrying the *tet*(O) gene, in TET-resistant *S. pyogenes* isolates with *erm*(TR)-mediated ERY resistance. The two new elements were comprehensively characterized.

MATERIALS AND METHODS

Bacterial strains. Twenty-three strains of *S. pyogenes* were used, all isolated from throat cultures of symptomatic patients and collected from Italian laboratories in the decade from 1998 to 2007. Strain identification was confirmed with bacitracin disks (Oxoid, Basingstoke, England) and by serogroup A agglutination (Streptex; Murex, Chatillon, France). The inclusion criterion was ERY resistance (MIC, $\geq$1 μg ml^{-1}) mediated by the *erm*(TR) gene (determined by PCR using specific primers [12]). All isolates exhibited the iMLS phenotype. Twenty were TET resistant (MIC range, 64 to $>$128 μg ml^{-1}), with resistance mediated by the *tet*(O) gene (determined by PCR using specific primers [17]), and three were TET susceptible (MIC range, $\leq$0.125 to 0.5 μg ml^{-1}). Two test strains, both described in previous studies, were used for sequencing experiments: TET-susceptible C1 (9, 29) and TET-resistant iB21 (8), also called B2 in an earlier report (9).

PCR experiments. The principal oligonucleotide primer pairs used in PCR experiments are listed in Table 1. Inverse PCR (23) was carried out to analyze unknown DNA regions. Genomic DNA digested with endonucleases MunI, HindIII (Roche Applied Science, Basel, Switzerland), BanI, Hpy188I, or AclI (New England Biolabs, Ipswich, MA) was ligated and used as the template in the PCR assays.

DNA sequencing and sequence analysis. Overlapping fragments of the *erm*(TR)-carrying elements were obtained by PCR assays and primer walking techniques using suitable primer pairs. Most oligonucleotides for long PCR experiments were designed from *S. pyogenes* ICE10750-RD.2 and ICE2096-RD.2 (accession numbers CP000262 and CP000261) (2) and from ICE*Cd*630 of *Clostridium difficile* (accession no. AM180355) (25). Amplicons were sequenced by ABI Prism (Perkin-Elmer Applied Biosystems, Foster City, CA) with dye-labeled terminators. ORF analysis was performed by using the online available software ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). The criterion to designate a potential ORF was the existence of a start codon and a minimum coding size of 30 amino acids. Sequence similarity and conserved domain searches were carried out by using tools (BLAST and CDART) available online at the National Center for Biotechnology Information of the National Library of Medicine (Bethesda, MD) (http://www.ncbi.nlm.nih.gov/).

Nucleotide sequence accession numbers. The complete sequences of two new *erm*(TR)-carrying genetic elements, ICE*Sp*1108 and ICESp2905, with their chromosomal junctions, have been submitted to the EMBL database under accession numbers FR691054 and FR691055, respectively.

RESULTS AND DISCUSSION

PCR mapping assays to detect ICE10750-RD.2. The 23 test strains were PCR mapped using primer pairs designed from the sequence of ICE10750-RD.2, thus far the only completely sequenced *S. pyogenes erm*(TR) element. While the 3 TET-susceptible strains yielded positive PCR results with most primer pairs, none of the 20 TET-resistant isolates yielded PCR evidence of ICE10750-RD.2, except for a short region including *erm*(TR).

ICE*Sp*1108, the *erm*(TR)-carrying element from TET-susceptible strain C1. The *erm*(TR)-carrying element from *S. pyogenes* C1, one of the 3 TET-susceptible test strains, was sequenced (accession no. FR691054) and characterized. The new element, designated ICE*Sp*1108, was 45,456 bp in size. Its G+C content was 31%. Sequence analysis revealed 40 ORFs. *erm*(TR) was *orf28* (100% identical to the genes of both ICE10750-RD.2 and Tn*1806*). *orf29* encoded a spectinomycin phosphotransferase, 99.8% identical to the gene adjacent to *erm*(TR) in both ICE10750-RD.2 and Tn*1806*. In *orf30* (120 bp, identical to the corresponding gene of Tn*1806*), only the first 85 bp matched (100%) the initial portion of the cytidine deaminase-encoding gene of ICE10750-RD.2 (390 bp). The ICE*Sp*1108 ORF map, aligned with the ORF maps of ICE10750-RD.2 and Tn*1806*, is shown in Fig. 1, while the major characteristics of the ORFs are detailed in the supplemental material (see Table S1 in the supplemental material).

ICE*Sp*1108 displayed close similarities to both ICE10750-RD.2 and Tn*1806*, as demonstrated by extensive DNA identities ($>$90%). However, a significant difference was noted at the right end: *orf40*, the last ORF of ICE*Sp*1108, encoding a recombinase, replaced the last three ORFs of ICE10750-RD.2 and Tn*1806*, which were $>$95% identical in the two elements and encoded recombinases totally unrelated to the one encoded by *orf40*.

ICE*Sp*2905, the *erm*(TR)- and *tet*(O)-carrying composite element from TET-resistant strain iB21. The *erm*(TR)-carrying element from *S. pyogenes* iB21—one of the 20 TET-resistant test strains, previously used as a donor in mating assays yielding cotransfer of *erm*(TR) and *tet*(O) (8)—was sequenced and characterized. High identities to different portions of a segment of the *C. difficile* 630 genome (accession no. AM180355) (25) were detected in both *erm*(TR)- and *tet*(O)-flanking regions, previously sequenced by inverse PCR analysis. We considered this segment (~39 kb, 33 ORFs, G+C content 35% versus 29% of the chromosome), unmentioned in the genome analysis of *C. difficile* 630, as a putative ICE, and arbitrarily designated it ICE*Cd*630. These findings suggested that both *erm*(TR) and *tet*(O), located on separate fragments, were inserted in the same scaffold—ICE*Cd*630—to form a larger structure. The new composite element was designated ICE*Sp*2905 (accession no. FR691055). It was 65,575 bp in size, and its G+C content was 36%. Sequence analysis disclosed 61 ORFs. *erm*(TR) (*orf14*) and *tet*(O) (*orf35*) were far apart (almost 28 kb), explaining previous failures in demonstrating their linkage by PCR (8). The ICE*Sp*2905 ORF map, aligned with that of ICE*Cd*630, is shown in Fig. 2, while the major characteristics of the ORFs are detailed in the supplemental material (see Table S2 in the supplemental material). The organization of ICE*Sp*2905 is summarized below.

(i) Initial region (bp 1 to 5074). This region, spanning from *orf1* to *orf8*, displayed high identity (87%) to a region of ICE*Cd*630 (bp 480415 to 485486 of the *C. difficile* 630 genome). The specific functions associated with some ORFs (*orf2*, *orf3*, and *orf5*) are presumably involved in the ICE conjugative transfer.

(ii) *erm*(TR) fragment (bp 5075 to 17690). This *erm*(TR)-containing fragment (31% G+C), spanning *orf9* to *orf24*, was inserted into *orf8*, close to its 3′ end. *orf8* is 90% identical to the corresponding ORF of ICE*Cd*630. The insertion of the *erm*(TR) fragment did not interrupt the *orf8* coding sequence, which was reconstituted by the first 7 nucleotides of the inserted fragment to produce an ORF shorter than in the wild type (159 versus 215 bp). *erm*(TR) was *orf14* (99.9% identical to the gene of ICE*Sp*1108). *orf15*, encoding spectinomycin phosphotransferase, was 99.8% identical to the gene of

TABLE 1. Principal oligonucleotide primer pairs used

Procedure and gene[a]	Primer	Sequence (5′–3′)	Source or reference	Product size (bp)
ICE*Sp*1108 mapping				
orf1	ETR1	GGGATAGGACTGATTGAA	This study	10,110
orf14	ETR89	GTGTTATACCTGTTGGAATACC	6	
orf14	ETR99	GGTATTCCAACAGGTATAACAC	6	8,093
orf20	ETR94	CTGTTCTTGGATATGTGATTAGC	6	
orf20	ETR94-for	TTGGCTGGTAGGAATGAAT	This study	6,991
orf22	ETR50-rev	TTTGAGTGGTAAGATGGTT	This study	
orf22	ETR50	TATGGTACAAGTAGAGTGAATGCC	6	8,731
erm(TR)	III_8	GCATGACATAAACCTTCA	26	
erm(TR)	TR1	ATAGAAATTGGGTCAGGAAAAGG	12	5,963
orf34	ETR13	GCAAAAAAGCACGCAGAAAG	This study	
orf34	ETR23	GTCTTTCTGCTTGTAGTTCTGCC	This study	5,369
orf40	ETR85	GTTAGCAATAGTGTTTCTGTTTT	This study	
ICE*Sp*2905 mapping				
orf2	CD1-for	AAAACGAACGGGAATATC	This study	8,165
erm(TR)	III_8	GCATGACATAAACCTTCA	26	
erm(TR)	TR1	ATAGAAATTGGGTCAGGAAAAGG	12	14,299
orf29	CD2-rev	TTGATGTAATGTAGGATAAAAG	This study	
orf29	CD3-for	CAAAGCCAAATGTCCTAAATGAAA	This study	13,452
orf33	CD3-rev	TCCGCAAAATCCGTCCTACA	This study	
orf34	TnpV-for	GAATGGACAGGATGCCTCAA	This study	14,319
orf48	CD4-rev	ACTCAAAATAATCGCTGTCCTT	This study	
orf49	CD5-for	ATGTAGTTGAAAGAGAAAATAAGAA	This study	12,981
orf61	CD5-rev	GGAGATTACCATACCGCCAACACC	This study	
ICE*Sp*1108 chromosomal integration site				
Spy1198*	LYT-for	TTACAGGGTCTGCGGCTATT	This study	6,193
orf5	ETR49	TCTTAGCTGGTATATACACTTACC	6	
orf37	ETR45	TCAAAAGCAGCATCTTTATCTTCG	6	5,147
Spy1195*	THIO-rev	TTCAAAGCCGTCTCAGTCAC	This study	
ICE*Sp*2905 chromosomal integration site				
Spy1099†	LYT-for	TTACAGGGTCTGCGGCTATT	This study	2,043
orf1	TR-inv1	CTAACTAAGAATCCAATAATCA	This study	
orf61	CD6-for	TAATGTTGCGGTATTTTTTGA	This study	2,445
Spy1096†	THIO-rev	TTCAAAGCCGTCTCAGTCAC	This study	
Conserved core sequence				
erm(TR)	TR1	ATAGAAATTGGGTCAGGAAAAGG	12	1,497
orf30‡	Cyt-rev	ATGCCCTGAAGTTCCAAAG	This study	
Inverse PCR				
orf1‡	C1LE-inv3	TTCAATCAGTCCTATCCC	This study	
orf1‡	C1LE-inv4	AGTAAAGGGCAAAAGTCT	This study	

[a] *, From the *S. pyogenes* MGAS10750 genome; †, from the *S. pyogenes* MGAS5005 genome; ‡, from ICE*Sp*1108.

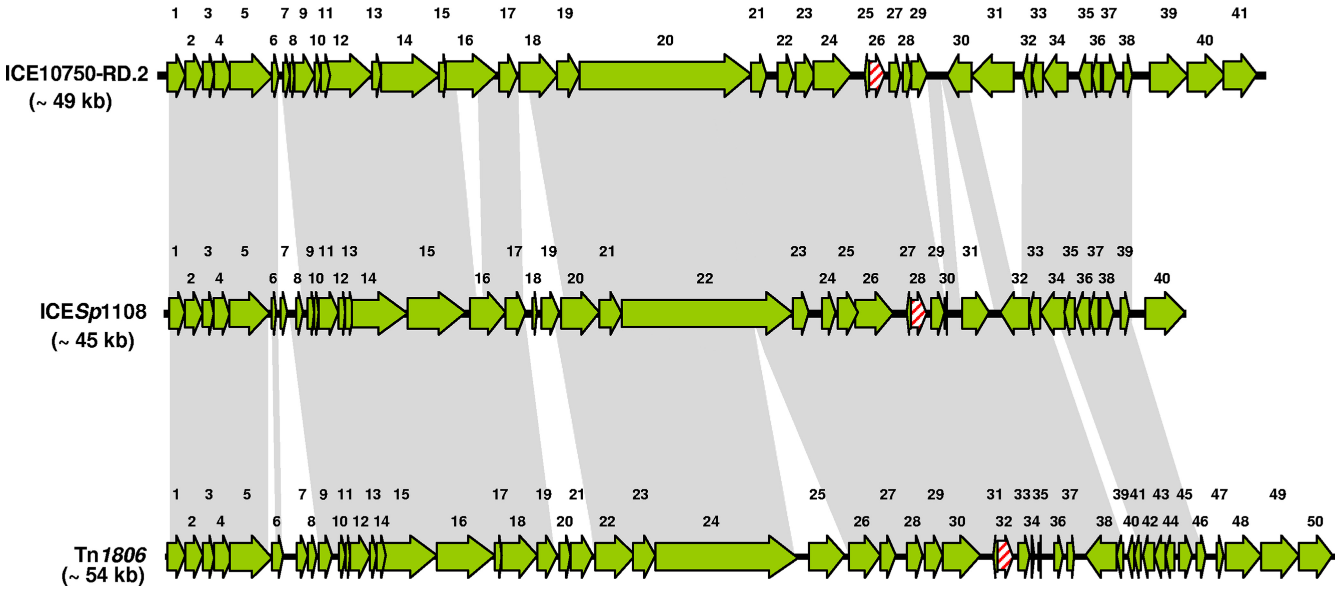

FIG. 1. ORF map of ICE*Sp*1108 from *S. pyogenes* strain C1 (accession no. FR691054) and its alignment with the ORF maps of *S. pyogenes* ICE10750-RD.2 (accession no. CP000262) and *S. pneumoniae* Tn*1806* (accession no. EF469826). The ORFs, indicated as arrows pointing in the direction of transcription, are numbered consecutively (*orf1* to *orf40* in ICE*Sp*1108, with some predicted functions reported in Table S1 in the supplemental material; *orf1* to *orf41* in ICE10750-RD.2; and *orf1* to *orf50* in Tn*1806*). ORFs are depicted as green arrows except for *erm*(TR) (striped red). Gray areas between ORF maps denote >90% DNA identity.

ICE*Sp*1108). *orf16* (141 bp) again displayed the same 85-bp segment mentioned above, matching the initial portion of the cytidine deaminase-encoding gene of ICE10750-RD.2. The region spanning from *orf17* to *orf22* was similar to a region of ICE6180-RD.1—an ~11-kb element of *S. pyogenes* MGAS6180 (accession no. NC_007296)—spanning bases 1083646 to 1087913 of the MGAS6180 genome. *orf24*, the last ORF of the *erm*(TR) fragment, encoded a transposase indicated as *tndX*-like according to CDART analysis, but displayed no significant identity to the *tndX* gene of the Tn*916* family transposon Tn*5397* (14, 21).

(iii) Central region (bp 17691 to 36310). This region is the portion of the ICE*Cd*630-like scaffold of ICE*Sp*2905 encompassed between the two insertions of the composite element,

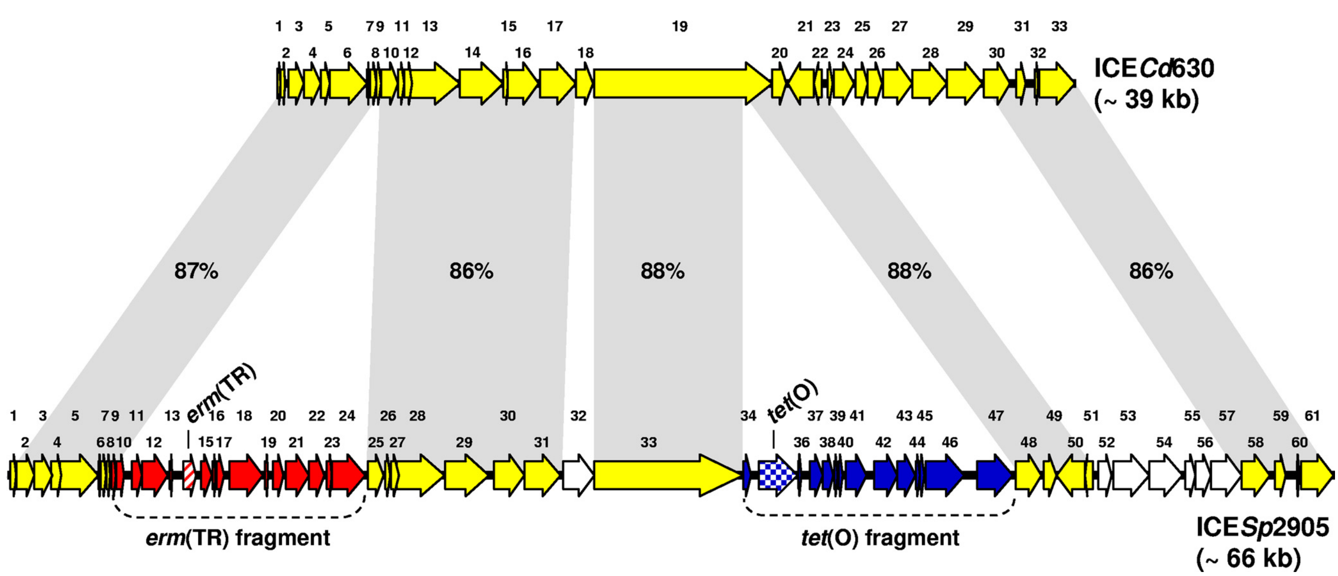

FIG. 2. ORF map and genetic organization of ICE*Sp*2905 from *S. pyogenes* strain iB21 (accession no. FR691055), and its alignment with the ORF map of *C. difficile* ICE*Cd*630 (accession no. AM180355). The ORFs, indicated as arrows pointing in the direction of transcription, are numbered consecutively (*orf1* to *orf61* in ICE*Sp*2905, with some predicted functions reported in Table S2 in the supplemental material; and *orf1* to *orf33* in ICE*Cd*630). ICE*Cd*630 ORFs and related ORFs in ICE*Sp*2905 are depicted as yellow arrows. ICE*Sp*2905 ORFs of the *erm*(TR) fragment and the *tet*(O) fragment are depicted as red and blue arrows, respectively, except for *erm*(TR) (striped red) and *tet*(O) (checkered blue). Other ICE*Sp*2905 ORFs are depicted as white arrows. Gray areas between ORF maps denote significant DNA identities (>70%) as indicated.

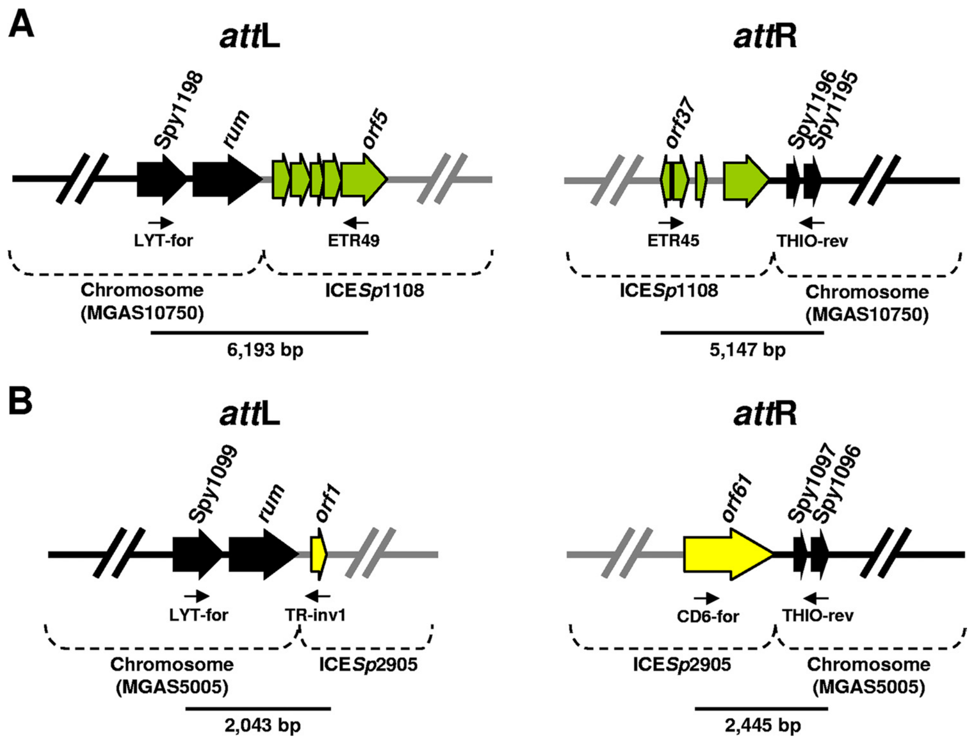

FIG. 3. Chromosomal integration of ICE*Sp*1108 (A) and ICE*Sp*2905 (B). (A) ICE*Sp*1108 was integrated into the chromosome of *S. pyogenes* C1 within the *rum* gene. This gene, detected in all *S. pyogenes* genomes sequenced to date, has the highest DNA identity with the corresponding gene (Spy1197) from *S. pyogenes* MGAS10750. Chromosomal ORF designations are thus from *S. pyogenes* MGAS10750. Chromosomal ORFs at the left (*att*L) and right (*att*R) junctions are indicated as black arrows, and ICE*Sp*1108 ORFs are indicated as green arrows. Amplicons obtained by pairing primers LYT-for/ETR49 (*att*L) and ETR45/THIO-rev (*att*R) are identified by bars. (B) ICE*Sp*2905 was integrated into the chromosome of *S. pyogenes* iB21 within the *rum* gene. This gene has the highest DNA identity with the corresponding gene (Spy1098) from *S. pyogenes* MGAS5005. Chromosomal ORF designations are thus from *S. pyogenes* MGAS5005. Chromosomal ORFs at the left (*att*L) and right (*att*R) junctions are indicated as black arrows, and ICE*Sp*2905 ORFs are indicated as yellow arrows. Amplicons obtained by pairing primers LYT-for/TR-inv1 (*att*L) and CD6-for/THIO-rev (*att*R) are identified by bars.

the one containing *erm*(TR) and the one containing *tet*(O). The region, spanning from *orf25* to *orf33*, largely consisted of two portions (bp 17691 to 27418 and bp 29020 to 36310) displaying high identity (86 and 88%, respectively) to ICE*Cd*630; the only significant difference was *orf32*, which replaced an ORF encoding a different protein in the clostridial ICE.

(iv) *tet*(O) fragment (bp 36311 to 49746). This fragment (44% G+C) was formed by an ~11-kb *tet*(O)-containing portion, spanning *orf34* to *orf46*, which was highly identical to a portion of ICE2096-RD.2 (an ~63-kb element of *S. pyogenes* MGAS2096 [accession no. CP000261] [2]), plus *orf47*, the last ORF in the fragment, which was alien to ICE2096-RD.2. The *tet*(O) fragment was inserted into *orf19* of ICE*Cd*630 (encoding a putative helicase), at base 503358 of the *C. difficile* 630 genome. In ICE*Sp*2905, this insertion split the original helicase gene into two ORFs: *orf33* (the last in the central region, encoding a putative helicase) and *orf48* (the first in the terminal region).

(v) Terminal region (bp 49747 to 65575). This region spanned *orf48* to *orf61*. High identities to ICE*Cd*630 were displayed by the two portions spanning from *orf48* to *orf51* (88% identity to the region from bp 503457 to 507442 of the *C. difficile* 630 genome) and from *orf58* to *orf61* (86% identity to the region from bp 515268 to 519796 of the same genome). It is worth noting that *orf61*, the last ORF in ICE*Sp2*905, and the last ORF (*orf40*) in ICE*Sp*1108, both encoding a putative recombinase, displayed 71% identity.

Chromosomal integration of ICE*Sp*1108 and ICE*Sp*2905. DNAs from both strains C1 (harboring ICE*Sp*1108) and iB21 (harboring ICE*Sp*2905) yielded no PCR products using primers, designed from the MGAS10750 sequence, targeting *hsdM*, a chromosomal gene that is the integration site of *erm*(TR)-carrying elements ICE10750-RD.2 (2) and Tn*1806* (6).

The left junction of ICE*Sp*1108 was identified by inverse PCR. In particular, Hpy188I-restricted genomic DNA from strain C1 was ligated and used as the template with primer pair C1LE-inv3/C1LE-inv4 (Table 1). Sequencing of the amplicon revealed significant DNA identity to a conserved RNA uracil methyltransferase (*rum*) gene detected in all *S. pyogenes* genomes sequenced thus far; the highest degree of identity was found to be with the *rum* gene from *S. pyogenes* MGAS10750 (99%). PCR experiments were carried out with two primer pairs, one for the left junction (*att*L) and one for the right junction (*att*R) (Fig. 3A). Sequencing of the resulting amplicons disclosed that ICE*Sp*1108 was integrated at the 3′ end of the *rum* gene at base 1142414 of the *S. pyogenes* MGAS10750 genome. The chromosomal insertion of ICE*Sp*1108 did not interrupt the *orf40* coding sequence, which was reconstituted by the first 39 nucleotides of chromosomal origin.

The junctions of ICE*Sp*2905 in the chromosome were also characterized by inverse PCR and direct sequencing. The highest degree of identity (99%) was with a *rum* gene from *S. pyogenes* MGAS5005 (accession no. NC_007297). Similar to ICE*Sp*1108, the integration site was at the 3′ end of the conserved *rum* gene at base 1070363 of the *S. pyogenes* MGAS5005 genome (Fig. 3B). The chromosomal insertion of ICE*Sp*2905 did not interrupt the *orf61* coding sequence, which was reconstituted by the first 39 nucleotides of chromosomal origin.

Distribution of *erm*(TR)-carrying elements in all test strains. After the complete sequence and the chromosomal integration of ICE*Sp*1108 from strain C1 and ICE*Sp*2905 from strain iB21 were established, the *erm*(TR) elements of all test strains were comparatively examined by PCR mapping using suitable primer pairs (Table 1).

The *erm*(TR) elements of the 2 TET-susceptible strains other than C1 showed an organization comparable to that of ICE*Sp*1108, which was also largely shared by ICE10750-RD.2 and Tn*1806*. Remarkably, the right ends of the elements of both strains reproduced the organization of the right end of ICE*Sp*1108: a single recombinase versus the three unrelated recombinases shared by ICE10750-RD.2 and Tn*1806*. Accordingly, the chromosomal integration site of both elements was at the 3′ end of the *rum* gene as for ICE*Sp*1108 versus the *hsdM* gene which is the integration site of ICE10750-RD.2 and Tn*1806*.

The *erm*(TR) elements of the 19 TET-resistant strains other than iB21 had an organization comparable to that of ICE*Sp*2905, with the *tet*(O) gene detected almost 28 kb downstream of *erm*(TR). Five strains yielded no amplification using the primer pair CD1-for/III_8. All demonstrated a chromosomal integration site at the 3′ end of the conserved *rum* gene.

Twenty-two of the twenty-three test strains (the exception being a TET-resistant isolate) yielded PCR evidence of the conserved region including *erm*(TR), the spectinomycin phosphotransferase-encoding ORF, and the above-mentioned 85-bp sequence.

Conclusions. We show here that two completely distinct categories of *erm*(TR)-carrying elements can be found in *S. pyogenes*: one in TET-susceptible strains, and another, where the *erm*(TR) gene is typically linked with the *tet*(O) gene, in TET-resistant strains. The former category, epitomized here by ICE*Sp*1108 (~45 kb) from strain C1 and detected in the other TET-susceptible test strains, also includes the two previously sequenced *erm*(TR) elements ICE10750-RD.2 (~49 kb) and Tn*1806* (~54 kb). The latter category, epitomized here by ICE*Sp*2905 (~66 kb) from strain iB21 and detected in all of the other TET-resistant strains tested, is a totally new finding. We had documented in a previous study the presence of the *tet*(O) determinant in TET-resistant *S. pyogenes* isolates with *erm*(TR)-mediated ERY resistance (8). However, the genetic basis of the *erm*(TR)-*tet*(O) association was still unknown. Here we demonstrated an *erm*(TR)-*tet*(O) linkage in ICE*Sp*2905 and closely related elements, which turned out to be composite structures where separate *erm*(TR)- and *tet*(O)-containing fragments are inserted in a clostridial scaffold. Intriguingly, in *S. pyogenes*, also *mef*(A)-encoded ERY resistance has been shown to be mediated by different genetic elements in TET-susceptible and TET-resistant strains (4, 29): the closely related Tn*1207.3* (24) and Φ10394.4 (1) in the former strains and Φm46.1 (3), with TET resistance again encoded by *tet*(O), in the latter.

Although the nomenclature of mobile genetic elements is evolving (5, 20), all of the *erm*(TR) genetic elements described in the present study can be considered ICEs (27, 30). In keeping with their exogenous origin, *S. pyogenes* ICEs differ by an average of 5% from endogenous *S. pyogenes* core genomes (2), whose G+C content is ca. 38.5% (2, 7). This is consistent with the detected G+C contents of ICE*Sp*1108 (31%) and ICE*Sp*2905 (36%). In the latter, however, the G+C contents of the two insertions—the *erm*(TR) fragment and the *tet*(O) fragment—are quite far apart (31 and 44%, respectively).

Despite broad differences between the two categories of *erm*(TR) elements, all ICEs from our *S. pyogenes* test strains, both TET-susceptible and TET-resistant, were integrated in the chromosome at the 3′ end of the conserved *rum* gene. This denotes a considerable divergence from the two previously sequenced *erm*(TR) elements—ICE10750-RD.2 and Tn*1806*—which share a different chromosomal integration site, namely, the *hsdM* gene. Interestingly, the 3′ end of the *rum* gene is an integration hot-spot for mobile streptococcal elements that is also shared by other *S. pyogenes* ICEs, such as ICE2096-RD.2 and ICE6180-RD.1 (2), and by prophages such as *S. pyogenes* Φm46.1 (3), *Streptococcus agalactiae* λSa04 (3, 28), and *Streptococcus suis* ΦSsUD.1 (18).

Moreover ICE*Sp*1108 and ICE*Sp*2905, as well as ICE10750-RD.2 and Tn*1806*, shared an almost identical conserved core sequence (2,062 bp, 99.8% identity) that included *erm*(TR), an adjacent ORF encoding a spectinomycin phosphotransferase, and a contiguous segment matching the first 85 bp of the cytidine deaminase-encoding gene of ICE10750-RD.2. PCR evidence of this conserved region was obtained in all but one of the 23 test strains. Since *erm*(TR) is an *erm*(A) subclass (22, 26), it is noteworthy, as underscored previously (6), that a different gene encoding spectinomycin resistance (designated *spc*) is found adjacent to *erm*(A) also in the *Staphylococcus aureus* transposon Tn*554*, where *erm*(A) was first detected and sequenced (15). Compared to the spectinomycin phosphotransferase-encoding gene detected in ICE*Sp*1108 (*orf29*) and ICE*Sp*2905 (*orf15*), the *spc* gene encodes a different enzyme (a spectinomycin adenyltransferase) and is transcribed in the opposite direction.

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