Erythromycin Esterase Gene *ere*(A) Is Located in a Functional Gene Cassette in an Unusual Class 2 Integron

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The gene *ere*(A) of the plasmid pIP1100 is larger than originally reported and is organized as an integron gene cassette. The *ere*(A) gene cassette carries its own promoter and is propagated by a class 2 integron with an insertion sequence element, IS*1*, inserted upstream of the *intI2* gene. The mobility of the *ere*(A) cassette has been demonstrated.

Integrons are natural cloning and expression systems (21). The integron platform codes for an integrase (IntI) that mediates recombination between a proximal primary recombination site (*attI*) and a target recombination sequence called an *attC* site (or 59-base element). The *attC* site is usually found associated with a single open reading frame in a structure termed a gene cassette (10, 25, 26). Insertion of the gene cassette at the *attI* site, which is located downstream of a resident promoter, Pc, internal to the *intI* gene, drives expression of the encoded proteins (12). More than 70 different antibiotic resistance genes have been characterized within integrons thus far (22). Multiresistance integrons (MRIs) evolved through successive integrations of antibiotic resistance loci within the integron platform. Five classes of MRIs have been reported based on the divergence of their integrase genes (22). Class 1 integrons are mostly found associated with the Tn*21* transposon family, while the class 2 integrons are associated with the Tn*7* transposon family (10).

Most of the *attC* sites of integron resistance gene cassettes identified to date share little homology. Their lengths and sequences vary considerably (lengths from 57 to 141 bp) and their sequence similarities are primarily restricted to their boundaries, which correspond to the inverse core site (ICS; RYYYAAC) and the core site (CS; G ↓ TTRRRY, where R is a purine, Y is a pyrimidine, and the arrow shows the recombination point) (7, 25).

Enterobacteria are intrinsically resistant to high levels of erythromycin (EM; MIC > 250 μg/ml) (2). However, oral EM is still prescribed to overcome intestinal infections due to antibiotic-resistant bacteria such as *Vibrio cholerae* (http://www.who.int/inf-fs/en/fact107.html), due in part to the high local concentrations of EM in the intestinal lumen (0.5 to 6 mg/g of feces). Several clinical isolates of *Escherichia coli* that are highly resistant to EM have been described, and their resistance mechanisms have been characterized to various extents. Interestingly, two types of EM-inactivating esterases have been identified among these *E. coli* strains. Further characterization has shown that these EM esterases were encoded by two genes,

ere(A) (*ereA* from pIP1100 [14]) and *ere*(B) (*ereB* [3]), which share little similarity. Recently, a cassette carrying a gene showing about 90% identity with *ere*(A) was identified in a class 1 MRI carried on plasmid pLQ1723 in *Providencia stuartii* (16) and in other class 1 MRIs from different clinical bacterial isolates (6, 15, 28).

Determining the genetic context of the *ere*(A) gene in plasmid pIP1100. The peculiar *attC* site carried by the *ere*(A) of the pLQ1723 cassette is related to the nucleotide sequence located immediately downstream of the *ere*(A) gene of pIP1100 (accession no. M11277) (14), suggesting that *ere*(A) of pIP1100 was also carried in an integron cassette. However, the region of similarity ended before an identifiable CS sequence could be found. Two features suggest that the deposited sequence was inaccurate: (i) the sequenced fragment was originally cloned from a *Sau*3A library, with the end of the *attC* site corresponding to a *Sau*3A site, and likely corresponding to a cloning artifact, and (ii) the annotated *ere*(A) gene of pIP1100 was shorter (345 codons) than the *ere*(A) gene of pLQ1723 (409 codons), but the homology between them extended a further 246 nucleotides (nt) upstream. Furthermore, the original investigators noted that even though the putative *ere*(A) gene product was a 38-kDa protein, when the protein was expressed in minicells its apparent M_r was about 43,000 (14). These observations led us to characterize the original *ere*(A) locus in pIP1100 in the original strain BM2195 (1). Since we suspected that the *ere*(A) gene was inside an integron, we screened the strain for the presence of the class 1, 2, and 3 integron-specific *intI* genes through PCR amplification using specific primers (Int1.F and Int1.R; I2UP and I2DW; and I3UP and I3DW, respectively; PCRs were performed with PCR Reddy mix (Abgene, Epsom, United Kingdom) following the manufacturer's instructions. Primer sequences can be found at http://www.pasteur.fr/recherche/unites/pmtg/integ/ere_primers). Primer sets specific for *intI1* and for *intI2* gave rise to amplified products, showing that BM2195 contained both class 1 and class 2 integrons. The association of *ere*(A) with the class 2 integron was established by PCR amplification using an *ere*(A)-specific primer (ereQ.L2) and the *intI2* primer, I2DW. Finally, the entire class2 integron was obtained by PCR amplification with primers I2DW and orfX-Tn7, a primer that hybridizes just upstream of the Tn7 *tnsE* gene. The sole amplified fragment,

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TABLE 1. Bacterial strains and relevant plasmids

Strain or plasmid	Description	Source or reference
E. coli		
DH5α	*supE44ΔlacU169* (φ80*lacZ′* Δ*M15*) Δ*argF hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory collection
UB1637	*his lys trp recA56 rpsL*	13
UB5201	*pro met recA56 gyrA*	13
BM2195	Wild-type, pIP1100, pIP1101, pIP1102	1
ω165	UB1637 R388 p2187 p112	This work
ω171	UB1637 R388 p2194 pTRC99A	This work
ω172	UB1637 R388 p2194 p112	This work
ω173	UB1637 R388 p2195 pTRC99A	This work
ω174	UB1637 R388 p2195 p112	This work
P. stuartii	Wild-type, pLQ1723 [*ere*(A)]	P. H. Roy
Plasmids		
R388	Tp^r Su^r Tra$^+$ IncW	27
pSU38	Kan^r oriP15A	4
pTRC99A	Ap^r oriColE1, expression vector	Pharmacia
p112	pTRC99A::*int11*	20
p2247	1,298 bp *Eco*RI-*Bam*HI PCR fragment (ereA1 and ereA2) from pLQ1723 in pSU38, *ere*(A)$_{pLQ1723}$ L101S, Kan^r	This work (AY183454)
p1792	5.4-kb PCR fragment (I2DW and orfX-Tn7) from pIP1100 in pCR2.1, Kan^r Ap^r Em^r	This work (AY183453)
p1818	1,290-bp *Sac*I-*Bam*HI PCR fragment (ereAStQ.L2 and ereA2) from p1792 in pSU38, *ere*(A)$_{pIP1100}$, Kan^r Em^r	This work
p1988	1,298-bp *Eco*RI-*Bam*HI PCR fragment (ereA1 and ereA2) from pLQ1723 in pSU38, *ere*(A)$_{pLQ1723}$, Kan^r Em^r	This work
p2187	1,492-bp *Bam*HI PCR fragment (SatEnd and aadA1) from p1792 in pSU38, *lacZ*α orientation, Kan^r Em^r	This work
p2188	1,492-bp *Bam*HI PCR fragment (SatEnd and aadA1) from p1792 in pSU38, anti-*lacZ*α orientation, Kan^r Em^r	This work
p2194	*Eco*RI deletion in p2187, *ere*(A)(-*aadA1*) *attC* site, Kan^r	This work
p2195	*Pst*I deletion in p2187, *sat*(-*ere*(A)) *attC* site, Kan^r	This work

about 5.4 kb long, was cloned (plasmid p1792) and sequenced. A list of the strains and plasmids used in this study is presented in Table 1.

The *ere*(A) gene cassette and its class 2 integron carrier. Sequence analysis of the p1792 insert revealed a unique class 2 integron, part of a Tn7 derivative, carrying four cassettes in the following order: *sat*, *ere*(A), *aadA1*, and *orfX* (Fig. 1). As for all previously described class 2 integrons, the *intI2** gene was interrupted by a premature stop at codon 179. Compared to the four other class 2 integrons described so far, this integron is the only one showing disruption of the *sat-aadA1-orfX* cassette array (Fig. 1). Indeed, the only difference among the four other class 2 integrons is the presence of distinct cassettes in position 1 (*dfrA1* in Tn7, *orf* in Tn*1825*, and *dfrA14* in Tn*4132*) or the lack of cassettes upstream of *sat* (Tn*1826*) (11, 29, 30). A second exclusive feature of this integron resided in the presence of an IS*1* element inserted 10 nt upstream of *intI2** and 310 bp upstream of the *attI2* recombination point. The rest of the sequence was identical to its Tn7 counterparts.

It is likely that the class 2 integron also carries a strong promoter, as it allows the phenotypic expression of all the cassettes in the array, as in class 1 integrons (12). Furthermore, the *sat* and *aadA1* cassettes do not contain enough space 5′ of the genes to contain a promoter sequence. The class 2 cassette promoter has still to be precisely identified, but according to Hansen et al. and Levesque et al., it is located upstream of the *intI2** gene (11) and not in the 5′ part of the *intI* gene as in the class 1 integron (12). The structure of this class 2 integron supports a promoter location within the 310-bp region between the IS*1* insertion site and the *attI2* recombination site, as no outward-facing promoter could be identified in the IS*1* sequence itself.

Comparison of the *ere*(A) gene cassettes from pIP1100 and pLQ1723. As indicated above, the *ere*(A) gene of pIP1100 gene was found to be the second cassette in this integron. Sequence analysis showed that this cassette was 1,369 nt long and coded for an *ere*(A) gene product of 406 amino acids, with a molecular mass of 44.5 kDa, similar to the data obtained from previous expression experiments (14). Comparison of the *ere*(A) cassette to the former *ere*(A) locus sequence showed that the differences between the two sequences essentially led to an extension of 62 codons at the 5′ end of the new sequence (Fig. 2). Furthermore, analysis of the *ere*(A) cassette boundaries revealed a 48-nt *attC* site (from the T of the ICS GCATAAC up to the G from the CS located at the junction with the *aadA1* cassette), displaying all the characteristics of such recombination sites. Indeed, a canonical CS consensus could be identified at the recombination point, confirming that, as speculated, the original sequence was a chimera due to ligation between unrelated *Sau*3A fragments.

According to their similarity (94% nt identity), the *ere*(A) gene cassettes from pIP1100 and pLQ1723 should be considered different alleles of the same *ere*(A) cassette. Furthermore, their *attC* sites show only a single nucleotide difference (Fig. 2). Their *ere*(A)-encoded proteins shared 92.5% amino acid identity and are also related to a lesser extent (≈25% identity) to the *ere*(B) protein. The three EM esterases are of similar sizes,
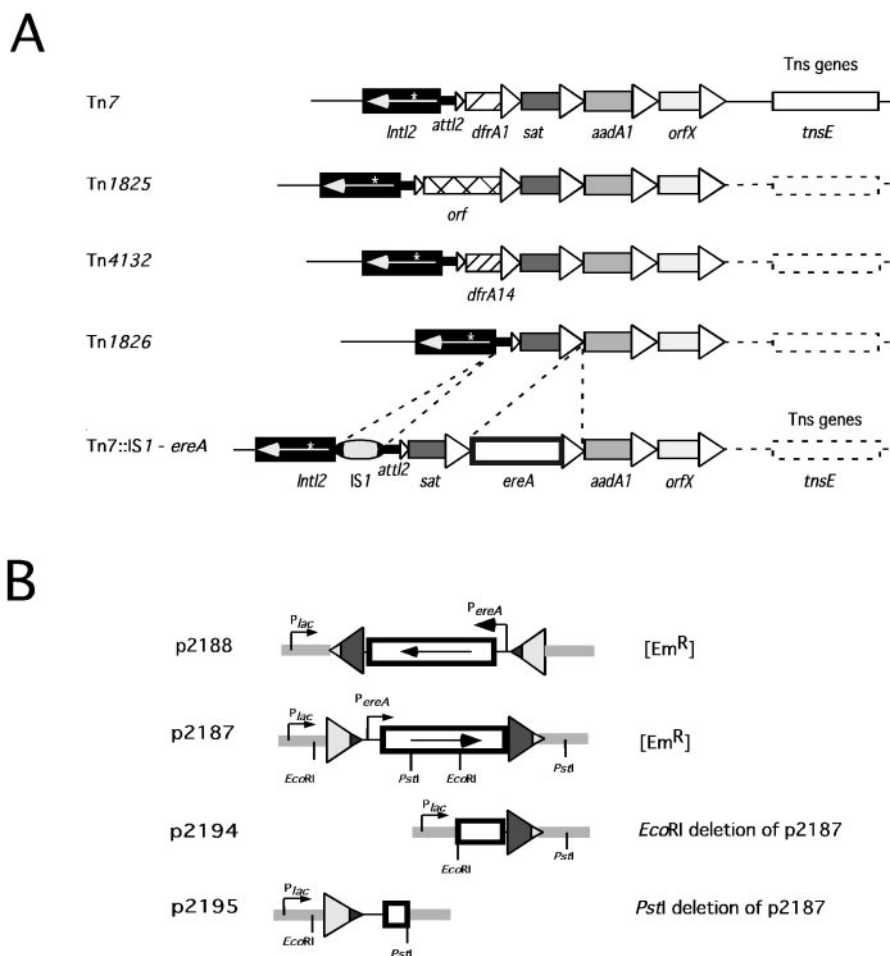
FIG. 1. Physical maps of the different class 2 integrons carried in Tn7-related transposons (A) and of the ere(A)$_{pIP1100}$ cassette derivative constructions used in expression and recombination studies (B). (A) Black boxes symbolize the intI2 integrase genes, and the asterisk indicates the location of the premature stop codon; other boxes correspond to cassette genes. The attI2 recombination site is shown by a small open triangle; attC sites are represented by bigger open triangles. Transposition (Tns) genes are indicated on the right side of the different transposons. The class 2 integron investigated in this study is Tn7::IS1-ere(A) (see the text for details). The IS1 is shown by an oval with black boundaries. (B) The ere(A) gene is represented as an open box; the thin line represents the 5′ untranslated DNA region upstream of the ere(A) start codon. Plasmid constructions are described in Table 1. The sat and ere(A) attC sites are shown as triangles. Relevant EM resistance phenotypes conferred by plasmids are mentioned.

and the homology between them extends over the entire length of their sequences.

The high degree of similarity between the ere(A) cassettes from pIP1100 and pLQ1723 extended to the 73- and 75-nt sequences found upstream of the respective ere(A) genes. The presence of such a long 5′ untranslated region is in most cases connected to the presence of a promoter within this sequence.

In order to show the effective presence of such a promoter in the ere(A) cassette, we cloned the entire cassette either in the same orientation as or in the opposite orientation to the P$_{lac}$ promoter in the plasmid pSU38 (4). The ere(A) gene was generated by PCR amplification with the primers SatEnd and aadA1, digested with BamHI, and cloned in pSU38. The fragment was designed to include the complete attC site of the upstream sat cassette in order to reduce background expression due to the possible transcriptional read-through originating from plasmid promoters. Two plasmids, representing each of the two possible insert orientations, were selected: p2187,

carrying ere(A) in the same orientation as the P$_{lac}$ promoter, and p2188, carrying ere(A) in the opposite orientation to the promoter. Both were found to confer the Em^r phenotype to DH5α (Fig. 1B). This demonstrated that ere(A) expression was independent of the P$_{lac}$ promoter and relied on a promoter located in the cassette. The only putative promoter sequence we could identify by similarity to the E. coli σ^{70} −35 and −10 consensus sequences is shown in Fig. 2. Interestingly, the homology of this region with ere(A) of pLQ1723 suggests that it too carries its own promoter, as previously speculated by Peters and colleagues (15). This feature is rather unusual for integron cassettes, as only five resistance cassettes, cmlA, cmlA2/4/5, qacE, qacE2, and qacF/H (see Table 1 in reference 22), have been shown or suspected to contain their own promoters (5, 17, 24).

EreA from pIP1100 and pLQ1723 EM resistance phenotypes and characterization of the L101S mutation. The EreA EM esterases from pIP1100 and pLQ1723 show 92.5% iden-

A

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  1 ttatgctctgtgtagccgggttatTGGCGAagcgaacgtatgacgatTACAGCaataaacgcaaaggtaaaaaa ATG ACA TGG AGA ACG ACC  91
  1                                                                            M   T   W   R   T   T     6

 92 AGA ACA CTT TTA CAG CCT CAA AAG CTG GAC TTC AAT GAG TTT GAG ATT CTT ACT TCC GTA ATT GAG GGC GCC CGA  166
  7  R   T   L   L   Q   P   Q   K   L   D   F   N   E   F   E   I   L   T   S   V   I   E   G   A   R    31

167 ATT GTC GGC ATT GGC GAG GCT CAT TTT GTC GCG GAG TTT TCA CTG GCT AGA GCT AGT CTT ATC CGC TAT TTG  241
 32  I   V   G   I   G   E   G   A   H   F   V   A   E   F   S   L   A   R   A   S   L   I   R   Y   L    56

242 GTC GAA AGG CAT GAG TTT AAT GCG ATT GGT TTG GAA TGT GGG GCG ATT CAG GCA TCC CGG TTA TCT GAA TGG CTC  316
 57  V   E   R   H   E   F   N   A   I   G   L   E   C   G   A   I   Q   A   S   R   L   S   E   W   L    81

317 AAC TCA ACA GCC GGT GCT CAT GAA CTT GAG CGA TTT TCG GAT ACC CTG ACC TTT TCT GTG TAT GGC TCA GTG CTG  391
 82  N   S   T   A   G   A   H   E   L   E   R   F   S   D   T   L   T   F   S   V   Y   G   S   V   L   106

392 ATC TGG CTG AAA TCA TAT CTC CGC GAA TCA GGA AGA AAA CTG CAG TTA GTC GGA ATC GAC TTA CCC AAC ACC CTG  466
107  I   W   L   K   S   Y   L   R   E   S   G   R   K   L   Q   L   V   G   I   D   L   P   N   T   L   131

467 AAC CCA AGG GAC GAC CTA GCG CAA TTG GCC GAA ATT ATC CAG CTC ATC GAT CAC CTC ATG AAA CCG CAC GTT GAT  541
132  N   P   R   D   D   L   A   Q   L   A   E   I   I   Q   L   I   D   H   L   M   K   P   H   V   D   156

542 ATG TTG ACT CAC TTG TTG GCG TCC ATT GAT GGC CAG TCG GCG GTT ATT TCA TCG GCA AAA TGG GGG GAG CTA GAA  616
157  M   L   T   H   L   L   A   S   I   D   G   Q   S   A   V   I   S   S   A   K   W   G   E   L   E   181

617 ACG GCT CGG CAG GAG AAA GCT ATC TCA GGG GTA ACC AGA TTG AAG CTC CGC TTG GCG TCG CTT CCC GTC CTG  691
182  T   A   R   Q   E   K   A   I   S   G   V   T   R   L   K   L   R   L   A   S   L   P   V   L   206

692 AAA AAA CAC GTC AAC AGC GAT TTG TTC CGA AAA GCC TCT GAT CGA ATA GAG TCG ATA GAG TAT ACG TTG GAA ACC  766
207  K   K   H   V   N   S   D   L   F   R   K   A   S   D   R   I   E   S   I   E   Y   T   L   E   T   231

767 TTG CGT ATA ATG AAA ACT TTC TTC GAT GGT ACC TCT TTG GAA GGA GAT ACT TCC GTA CGT GAC TCG TAT ATG GCG  841
232  L   R   I   M   K   T   F   F   D   G   T   S   L   E   G   D   T   S   V   R   D   S   Y   M   A   256

842 GGC GTA GTA GAT GGA ATG GTT CGA GCG AAT CCG GAT GTG AAG ATA ATT CTG CTG GCG CAC AAC AAT CAT CTA CAA  916
257  G   V   V   D   G   M   V   R   A   N   P   D   V   K   I   I   L   L   A   H   N   N   H   L   Q   281

917 AAA ACT CCA GTC TCC TTT TCA GGC GAG CTT ACG GCT GTT CCC ATG GGG CAG CAC CTC GCA GAG AGG GTG AAT TAC  991
282  K   T   P   V   S   F   S   G   E   L   T   A   V   P   M   G   Q   H   L   A   E   R   V   N   Y   306

992 CGT GCG ATT GCA TTC ACC CAT CTT GGA CCC ACC GTG CCG GAA ATG CAT TTC CCA TCG CCA AAA AGT CCT CTT GGA 1066
307  R   A   I   A   F   T   H   L   G   P   T   V   P   E   M   H   F   P   S   P   K   S   P   L   G   331

1067 TTC TCT GTT GTG ACC ACG CCT GCC GAT GCA ATC CGT GAG GAT AGT ATG GAA CAG TAT GTC ATC GAC GCC TGT GGT 1141
332  F   S   V   V   T   T   P   A   D   A   I   R   E   D   S   M   E   Q   Y   V   I   D   A   C   G   356

1142 ACG GAG AAT TCA TGT CTG ACA TTG ACA GAT GCC CCC ATG GAA GCA AAG CGA ATG CGG TCT CAA AGC GCC TCT GTA 1216
357  T   E   N   S   C   L   T   L   T   D   A   P   M   E   A   K   R   M   R   S   Q   S   A   S   V   381

1217 GAA ACG AAA TTG AGC GAG GCA TTT GAT GCC ATC GTC TGT GTT ACA AGC GCC GGC AAG GAC AGC CTG GTT GCC CTA 1291
382  E   T   K   L   S   E   A   F   D   A   I   V   C   V   T   S   A   G   K   D   S   L   V   A   L   406

1292 TAG gaaaccggaaatgaaatgagggagcaTAACCTGCGAATCCACCGGACGGTTTTCAACCGCCGGTGATCAGCGCG                      1369
407  *                                                                                                 407
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B


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              1L              2L                              2R            1R
         -- >                  <                                 >            <    - -
aadA5.attC    GCCTAACTCGGCGTTCAAGCGGACGG--GCTGCGCC-CGCCGCTCAACT-ATGCGttaggc
aadA1b.attC   GTCTAACAATTCGTTCAAGCCGACGC-CGCTTCGCGGCGCGGCTTACCTTGGCCGttaaac
ORFD.attC     CTCTAACATTTCGGTCAAGCCGACCCGCATTCTGCGGT-CGGCTTACCTCGCCCGttagta
aadA1a.attC   GTCTAACAATTCGTTCAAGCCGACGC-CGCTTCGCGGCGCGGCTTAACTCAAGCGttaaac
aadA3.attC    GTCTAACAATTCGTTCAAGCCGACGC-CGCTTCGCGGCGCGGCTTAACTCAAGCGttagac
aadA6.attC    GTCTAACAATTCGTTCAAGCCGACGC-CGCTTCGCGGCGCGGCTTAATTCAGGCGttagac
qacF.attC     ATCTAACAATTCATTCAAGCCGACGC-CGCTTCGCGGCGCGGCTTAATTCAGGCGttagat
aacAIIa.attC  GCCTAACAACTCATTCAAGCCGACGC-CGCTTCGCGGCGCGGCTTAATTCAGGCGttaggc
catB3.attC    GTCTAACAATTCAATCAAGCCGATGC-CGCTTCGCGGCACGGCTTATTTCAGGCGttagac
catB5.attC    GTCTAACAATTCATTCAAGCCGACGC-CGCTTCGCGGCACGGCTTAATTCTGGCGttagac
aadA7.attC    GTCTAACAATTCATTCAAGCCGACGC-CGCTTCGCGGCGCGGCTTAATTCAAGCGttagac
sat.attC      GCCTAACAATTCATTCAAGCCGACAC-CGCTTCGCGGCGCGGCTTAATTCAGGAGttaggc
aadB.attC     GCCTAACAATTCGTCCAAGCCGACGC-CGCTTCGCGGCGCGGCTTAACTCAGGTGttaggc
aadA2.attC    GTCTAACAATTCATTCAAGCCGACGC-CGCTACGCGGCGCGGCTTAACTCCGGCGttagac
dfrB1.attC    GCCCAACTTGTCGCTCCAGCGGACGG---CTTCGCCGC-CCGCTGAGCTAATTCGttgggc
dfrB3.attC    GCCCAACTGGTCGCTCCAGCGGACGG---CTTCGCCGC-CCGCTGAGCTAGAGCGttgggc
dfrB2.attC    GCCCAACAATTCGCTCCAGCGGACGG---CTTCGCCGC-CCGCTGAGCTTTATCGttaggc
ereA_pLQ.attC GCATAACCTGCCAATCCACCGGACGG---TTTTCAACCGCCGGTGATCA-GCGCGttatgc
ereA_pIP.attC GCATAACCTGCGAATCCACCGGACGG---TTTTCAACCGCCGGTGATCA-GCGCGttatgc
              ***          *  *  **       *        *   *  *        ***
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FIG. 2. *ere*(A)$_{\text{pIP1100}}$ cassette sequence and features (A) and comparison of the *ere*(A) *attC* sites to the short *attC* sites of other resistance cassettes (B). (A) The EreA EM esterase amino acid sequence is shown underneath the coding part of the cassette. The potential ribosome binding site is shown in bold, the proposed start codon in the former characterization of *ere*(A) is shown by a grey box (an additional A upstream of the overlined AA put this ATG in frame with the 3′ end of the gene), and the sequences that are complementary in the CS and ICS of the circularized *ere*(A) cassette are underlined. (B) CLUSTAL X (1.8) multiple sequence alignment of the *attC* sites from the following cassettes: *aadA5* (GenBank no. AF137361), *aadA1b* (GenBank no. M95287), orfD (GenBank no. M95287), *aadA1a* (GenBank no. X12870), *aadA3* (GenBank no. AF047479), *aadA6* (GenBank no. AF140629), *qacF* (GenBank no. AF034958), *aacAIIa* (GenBank no. M29695), *catB3* (GenBank no. U13880), *catB5* (GenBank no. X82455), *aadA7* (GenBank no. AF224733), *sat* (GenBank no. X15995), *aadB* (GenBank no. L06418), *aadA2* (GenBank no. X68227), *dfrB1* (GenBank no. U36276), *dfrB3* (GenBank no. X72585), *dfrB2* (GenBank no. J01773), *ere*(A)$_{\text{pLQ}}$ [*ere*(A) of pLQ1723; GenBank no. AF099140], *ere*(A)$_{\text{pIP}}$ [*ere*(A) of pIP1100; GenBank no. AY183453]. Putative IntI1 binding domains as defined by Stokes et al. (25) are boxed; conserved positions are indicated by stars.

TABLE 2. Macrolide resistance phenotype conferred by the different *ere*(A) alleles

Strain	MIC (μg/ml)			
	Erythro-mycin	Clarithro-mycin	Azithro-mycin	Telithro-mycin
DB10(pSU38)	1	4	0.6	0.5
DB10(pSU38::*ereAL101S*)	1	4	0.6	0.5
DB10(pSU38::*ere*(A)$_{pLQ1723}$)	25	20	0.6	0.5
DB10(pSU38::*ere*(A)$_{pIP1100}$)	25	20	0.6	0.5

tity. In order to establish whether these differences affected the resistance against different macrolides they conferred, we determined and compared the MICs of several antibiotics for *E. coli* expressing either of the *ere*(A) alleles using the methods of Steers et al. (23) with ca. 10^5 CFU per spot. The EM MIC due to the *ere*(A)-encoded EM esterase in its original context (strain BM2195) had been previously found to be 2,048 μg/ml (1). In order to be able to see subtle differences between *ere*(A)$_{pLQ1723}$ and *ere*(A)$_{pIP1100}$ phenotypes, we cloned the two genes in the same vector and in identical expression contexts and we established the EM MICs for them in *E. coli* DB10, a strain that is sensitive to aminoglycosides and macrolide-linco-samide-streptogramin B molecules and especially to EM (MIC = 1 μg/ml) (9). The resistance phenotypes attributable to EreA from pIP1100 (plasmid p1818) and from pLQ1723 (plasmid p1988) for EM, clarithromycin, azithromycin, and telithromycin were determined in DB10, and the corresponding MICs are shown in Table 2. The two *ere*(A) alleles show identical resistance patterns, conferring resistance to both C_{14} macrolides (EM and clarithromycin), with the strains retaining antibacterial activity for azithromycin, a C_{15} macrolide, or telithromycin.

In these experiments, we isolated an *ere*(A) mutant of pLQ1723 that was sensitive to EM and clarithromycin in DB10 (Table 2). Characterization of this mutant (plasmid p2247) showed a single mutation in codon 101, a TTG-to-TCG transition, which led to the replacement of leucine 101 by a serine (GenBank AY183454). Interestingly, the amino acid position affected by the L101S mutation in this EreA mutant does not correspond to a residue conserved in the different esterases. Indeed, the corresponding amino acid is a valine in EreA of pIP1100 and a glutamate in EreB. Hence, further work will be necessary to understand the biochemical origin of this loss of activity.

Recombination properties of the *ere*(A) cassette. The *ere*(A) cassette *attC* site is unique among the *attC* sites identified thus far in integron and superintegron cassettes (18, 25). Its closest structural relatives are the *attC* sites of the *dfrB1*, *-B2*, and *-B3* cassettes, which share about 65% identity with it. Together with the *ere*(A) recombination site, these sites correspond to the shortest *attC* sites characterized so far (57 bp). Their primary sequences, apart from the conserved ICS and CS sequences, are fairly different from the other "short" *attC* sites found in 14 resistance cassettes (Fig. 2). These short *attC* sites are all related and resemble the XCRs and XSRs, the *attC* sites of the *Xanthomonas* superintegron (SI) cassettes (18).

The recombination proficiency of the *ere*(A) cassette was demonstrated from its original context between the *sat* and

aadA1 cassettes and its ability to be integrated in a class 1 integron through IntI1 catalysis using the conduction assay developed by Martinez and de la Cruz (13). Strain ω165 was constructed by successive transformations of strain UB1637 by R388, a natural conjugative IncW plasmid harboring a class 1 integron conferring resistance to trimethoprim through its *dfrB2* cassette (13), by plasmid p2187, and by p112, a plasmid expressing the *intI1* integrase gene under the control of the P$_{trc}$ promoter (Table 1). The plasmid p2187 insert encompassed the complete *attC* site of the *sat* cassette, the entire *ere*(A) cassette, and the beginning of the *aadA1* cassette (see above; Table 1). Induction of *intI1* expression in ω165 was then followed by conjugation using strain UB5201 as a recipient. Recombination and integration of the *ere*(A) cassette alone at the *attI1* site of the R388 integron was found to occur at a frequency of $11.6 \times 10^{-2} \pm 3 \times 10^{-2}$.

In order to precisely determine the contribution of each of the two *attC* sites [the *sat*(-*ere*(A)) and *ere*(A)(-*aadA1*) *attC* sites] in *ere*(A) cassette recombination from p2187, we conducted the same experiments using plasmids p2194 (strain ω172) and p2195 (strain ω174) instead of p2187 as the substrate for IntI1. Plasmids p2194 and p2195 are p2187 derivatives which, respectively, carry either only the *ere*(A)(-*aadA1*) *attC* site or only the *sat*(-*ere*(A)) *attC* site (Table 1). The exconjugants from the different experiments were then plated on different selective media in order to establish the rates of integration through recombination of the different *attC* sites carried by p2194 or p2195 into the *attI1* or *attC* sites of the R388 class 1 integron. Since integration of the entire plasmid at the *attI* site would be expected to generate Kan^r Tp^s transconjugants while integration at the *attC* site would generate Kan^r Tp^r transconjugants, the integration frequency was calculated as the ratio of Kan^r transconjugants to R388 UB5201 transconjugants, where the number of R388 transconjugants was the sum of the number of the Tp^r UB5201 clones and Tp^s clones among the Kan^r UB5201 transconjugants. The precise location of the recombination event was established by PCR using primer attI1.1, which hybridizes to the *attI1* site of R388, and primer M13fd, which hybridizes in pSU38 downstream of the *attC* site recombination point. Twenty random clones per test set were mapped.

The *sat attC* site belongs to the family of related cassette recombination sites mentioned above, and their recombination properties have been extensively studied (see for examples (8, 11, 13). As shown in Table 3, in identical conditions, the *ere*(A) *attC* × R388 *attI1* site recombination frequency was found

TABLE 3. Recombination frequencies

Strain	Relevant characteristics	Integration frequency[a]
ω171	*ere*(A)(-*aadA1*) *attC*; IntI1 not overexpressed	$<7 \times 10^{-9}$
ω172	*ere*(A)(-*aadA1*) *attC*; IntI1 overexpressed	$(2.4 \pm 0.5) \times 10^{-2}$ [56/3]
ω173	*sat*(-*ere*(A)) *attC*; IntI1 not overexpressed	$<2 \times 10^{-9}$
ω174	*sat*(-*ere*(A)) *attC*; IntI1 overexpressed	$(22.6 \pm 6.1) \times 10^{-2}$ [57/3]

[a] Values are averages of three independent trials; values in brackets are *attI1*/*dfrB2 attC* integrations.

repeatedly to be only one-ninth of the *sat attC* × R388 *attI1* frequency. Therefore, as these sites show all the structural features common to the various *attC* sites of MRI and SI cassettes (19, 25), differences in structures should help identify the factors governing the variations in recombination efficiencies.

Nucleotide sequence accession number. The sequence determined in this study has been deposited in GenBank under accession no. AY183453.

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