Group IIC Intron with an Unusual Target of Integration in *Enterobacter cloacae*

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A potential role of group IIC-*attC* introns in integron gene cassette formation, that is, the way in which they could provide the *attC* sequence essential for recombination, has been proposed. Group IIC introns usually target the *attC* site of gene cassettes and more specifically their inverse core. Here we characterized a novel group IIC intron targeting the core site of the *aadA1* gene cassette *attC* site (*aadA1-qacEΔ1* gene cassette junction) from enterobacterial isolates. Intron mobility (retrohomology) was analyzed using a two-plasmid assay performed in *Escherichia coli*. Intron mobility assays confirmed the mobilization-integration of the group II intron into the core site of the *aadA2*, *bla_{VIM-2}*, *bla_{CARB-2}*, *aac(6')*-Ib, *dfrXVb*, *arr2*, *cmlA4*, and *aadB* gene cassettes but not into the *attI* site. This mobility was dependent on maturase activity. Reverse transcriptase PCR showed that this intron was transcriptionally active, and an intermediate circular form was detected by inverse PCR. This element was linked to the *bla_{VEB-1}* extended-spectrum β -lactamase gene in a high number of enterobacterial isolates. A phylogenetic tree showed that the identified element was located in a branch separate from group IIC-*attC* introns, being an IIC intron possessing the ability to integrate using the core site of the *attC* sites as target.

Integrations play an important role in the development of antibiotic resistance in Gram-negative pathogens. Class 1 and class 2 integrons have a worldwide distribution and are described from bacteria colonizing humans, animals, and farmed fish (6, 19, 21, 45). In particular, class 1 integrons are increasingly reported to be a source of diffusion and expression of antibiotic resistance genes in Gram-negative bacteria, but the way in which they are built remains largely unknown (5). Their prevalence is increasing, in particular, in clinical enterobacterial isolates, playing a major role in the overall increased rate of antibiotic resistance worldwide (5, 42). The backbone structure of an integron contains a conserved region encoding an integrase (*intI*) and a variable region with integrated gene cassettes (45). A gene cassette usually contains a single open reading frame (ORF) and a recombination site, the *attC* site. The *attC* sites consist of an inverse core site and a core site separated by an imperfect intervening palindrome of variable length. The inverse core site is defined as RYYAAC, and the core site is defined as GTTRRRY (10, 45, 46). The *attC* sites can vary in length (57 to 141 bp), and their sequence similarities are primarily restricted to the boundaries, which correspond to two pairs of conserved inverted repeats, 1L-2L and 2R-1R (46). It has been proposed that the coding DNA and the *attC* site originally had separate origins and that these elements have been joined through a specific assembly process (34, 38). Considering the unique structural characteristics of most integron cassettes (i.e., the absence of promoters, the paucity of noncoding sequence, and the presence of only one gene), it has been suggested that integron cassette genesis involves reverse transcription of mRNA molecules in an organism that contains a gene that encodes a reverse transcriptase (RT) (10). The idea of reverse transcription became plausible when it was reported that a group IIC intron identified as *Serratia marcescens* I2 (*S.ma*.I2) was inserted into a mobile integron between a structural gene and its associated *attC* site in the multiresistant *Serratia marcescens* SCH909 strain (1). Since then, a specific lineage of several group IIC introns, named the group IIC-*attC* introns, has been identified inside cassettes of mobile or

chromosomal integrons (22, 26, 32, 47, 51) or adjacent to isolated *attC* sites (18, 32). Group II introns are catalytic RNAs and mobile retroelements that self-splice (41). They are separated into several lineages on the basis of their intron-encoded protein (IEP) sequences (44, 53). These mobile elements encode proteins possessing RT, RNA splicing (maturase), and sometimes DNA endonuclease (En) activities. Bacterial group IIC introns are a subgroup of group II introns that differs from the other bacterial introns, and fewer base pairs are required for target site specificity (5 bp compared to 13 bp for groups IIA and IIB) because of a missing intron binding site 2 (IBS2)-exon binding site 2 (EBS2) pairing motif and shorter IBS1-EBS1 as well as IBS3-EBS3 pairing motifs (15). Nevertheless, group IIC introns are site-specific retroelements that are inserted directly after transcriptional terminator motifs or other inverted repeats, such as *attC* sites (22, 26, 32, 33, 35, 47, 51). Another important feature of group IIC introns is the absence of a C-terminal En domain in the IEPs. It has been shown that group II introns that cannot carry out site-specific second-strand cleavage due to the lack of an En domain are still mobile, but the residual mobility shows a pronounced strand bias and dependence on replication (20, 52). Recently, a potential role for group IIC-*attC* introns in gene cassette formation has been suggested (16), in which a group IIC-*attC* intron separately targets a transcriptional terminator adjoining a gene and an isolated *attC*, joins the gene and the *attC* by homologous recombination, and then splices and reverse transcribes a gene-*attC* RNA template, leading to the formation of the cassette.

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description or relevant characteristic	Source or reference
Strains		
<i>E. coli</i> DH10B	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>dlacZ</i> Δ M15 Δ <i>lacX74</i> <i>endA1</i> <i>recA1</i> <i>deoR</i> Δ (<i>ara leu</i>)7697	Invitrogen
<i>E. cloacae</i> GOC	Strains carrying group IIC intron characterized in this study and also containing <i>attC</i> sites of <i>aadB</i> and <i>arr2</i> gene cassettes	This study
<i>E. coli</i> TC GOC	<i>E. coli</i> J53 transconjugant strain harboring the natural plasmid coding for group IIC intron	This study
<i>Salmonella enterica</i> DT104	Strain containing <i>attC</i> site of <i>aadA2</i> and <i>bla</i> _{CARB-2} gene cassette	29
<i>P. aeruginosa</i> COL-1	Strain containing <i>attC</i> site of <i>bla</i> _{VIM-2} gene cassette	31
<i>K. pneumoniae</i> ORI-1	Strain containing <i>attI</i> site and <i>attC</i> sites of <i>aac</i> (6')-Ib, <i>dfrXVb</i> , and <i>cmlA4</i> gene cassettes	28
Plasmids		
pBK-CMV	pUC-derived cloning vector	Stratagene
pTOPO-BluntII	pUC-derived cloning vector used for cloning of PCR fragments	Invitrogen
pACYC184	Low-copy-number vector for cloning that is compatible with pUC vectors	NEB
pGOCXN1	9,996-bp XbaI fragment cloned in pBK-CMV vector containing the partial integron coding for group IIC intron	This study
pGOCXA11	7,347-bp XbaI fragment cloned in pBK-CMV vector containing the partial integron coding for group IIC intron	This study
pIntronIIC	2,062-bp PCR fragment amplified with <i>aadA1</i> -3ext and 3'CS primers containing full group IIC intron from <i>E. cloacae</i> GOC and cloned in pTOPO-BluntII	This study
pIntronIIC-Mut	Clone derived from pIntronIIC by site-directed mutagenesis to inactivate the IEP in the start of the retrotranscriptase region	This study
pattC-aadA2	394-bp PCR fragment of <i>attC-aadA2</i> genes amplified from <i>Salmonella enterica</i> DT104, cloned in pTOPO-BluntII, digested by EcoRI, and subcloned into pACYC184	This study
pattC-blavim2	300-bp PCR fragment of <i>attC-bla</i> _{VIM-2} gene amplified from <i>P. aeruginosa</i> COL-1, cloned in pTOPO-BluntII, digested by EcoRI, and subcloned into pACYC184	This study
pattC-blacarb2	360-bp PCR fragment of <i>attC-bla</i> _{CARB-2} gene amplified from <i>Salmonella enterica</i> DT104, cloned in pTOPO-BluntII, digested by EcoRI, and subcloned into pACYC184	This study
pattC-aac(6')-Ib	330-bp PCR fragment of <i>attC-aac</i> (6')-Ib amplified from <i>K. pneumoniae</i> ORI-1, cloned in pTOPO-BluntII, digested by EcoRI, and subcloned into pACYC184	This study
pattC-dfrXVb	373-bp PCR fragment of <i>attC-dfrXVb</i> amplified from <i>K. pneumoniae</i> ORI-1, cloned in pTOPO-BluntII, digested by EcoRI, and subcloned into pACYC184	This study
pattC-cmlA4	327-bp PCR fragment of <i>attC-cmlA4</i> amplified from <i>K. pneumoniae</i> ORI-1, cloned in pTOPO-BluntII, digested by EcoRI, and subcloned into pACYC184	This study
pattC-aadB	336-bp PCR fragment of <i>attC-aadB</i> amplified from <i>E. cloacae</i> GOC, cloned in pTOPO-BluntII, digested by EcoRI, and subcloned into pACYC184	This study
pattC-arr2	378-bp PCR fragment of <i>attC-arr2</i> amplified from <i>E. cloacae</i> GOC, cloned in pTOPO-BluntII, digested by EcoRI, and subcloned into pACYC184	This study
pattI	434-bp PCR fragment of <i>attI</i> amplified from <i>K. pneumoniae</i> ORI-1, cloned in pTOPO-BluntII, digested by EcoRI, and subcloned into pACYC184	This study

So far, all introns associated with an *attC* site (group IIC-*attC* introns) have been found inserted into the inverse core sites at the position RYYYA_AC and with an inverse orientation with respect to the gene cassettes. In this study, we characterized a new group IIC intron with atypical integration, being inserted into the core site of the *aadA1* gene cassette (GT_TRRRY) and with a leading orientation on the top strand. The mobilization and specific integration of this new element were evaluated.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and nucleic acid extraction. Bacterial strains and plasmids are described in Table 1. Bacterial strains were grown in Luria-Bertani (LB) broth at 37°C. When necessary, antibiotics were used at the following concentrations: ampicillin (Ap), 100 µg/ml; chloramphenicol (Cm), 25 µg/ml; tetracycline (Tc), 20 µg/ml; and kanamycin (Km), 30 µg/ml. Total DNA was isolated using a phenol-chloroform purification method. Total RNA extraction was done using an RNeasy mini-kit from Qiagen following the manufacturer's procedures.

PCR assays. Phusion DNA polymerase (Finnzymes) was used for PCR experiments according to the manufacturer's instructions. PCR primers are indicated in Table 2.

Intron mobility (retrohomology) assay. Intron mobility was evaluated by PCR amplification of the target sites using an *Escherichia coli* two-plasmid assay in which the studied intron sequences had previously been cloned into a pUC18-derived vector and which is aimed to target a specific site cloned into pACYC184 (Table 1). For each mobility assay, the intron donor plasmid (Km^r) and the recipient plasmid (Tc^r and Cm^s) were simultaneously transformed into *E. coli* TOP10 competent cells and subjected to Km and Tc selection. One colony of each double transformant was grown in 5 ml of LB medium in the presence of Km and Tc at 35°C overnight. The overnight preculture (100 µl) was pelleted, inoculated into 5 ml of LB medium with both antibiotics, and incubated at 37°C until the optical density at 600 nm reached a value of 0.5. Expression of the intron was then induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), followed by incubation at 37°C for 3 h. Plasmid DNAs were extracted using a QIAprep kit (Qiagen, Courtaboeuf,

TABLE 2 Primers used in this study

Primer	Sequence (5' to 3')	PCR product size (bp)	Use
PreIntron-A	ACTGTGCGTGATCGTCATGC	1,463	Amplification and cloning
PreIntron-B	GTACACGGCGGTTTCATTTTC		
Intron-5' ext	CAGATGGAAGGCTTCGTGG		Circularization
Intron-5ext-2	CCTCAGATCTGGTTCTTGTAACA		
Intron-3' ext	TTTACATTGCCTTACCTAAC		
aadA1-5ext	GCAAATCAATATCACTGTGTG		Circularization
aadA1-3ext	GCTAGACAGGCTTATCTTGG		
Veb1A	CGACTTCCATTTCGGATGC		Structure integron
Veb1B	GGACTCTGCAACAAATACGC		
Arr2-5ext	GGCTTTGGTTCCATGTAGAACGG		Structure integron
Arr2-3ext	CGCCAGAGATTCCGCGCCTGTTGC		
5'CS	GGCATCCAAGCAGCAAG		Structure integron
aadA2-A-attC	GTTCTGAACAGGATCTATTGCA	394	Cloning <i>attC_{aadA2}</i>
3'CS	AAGCAGACTTGACCTGA		
Vim2-A-attC	CCGTAGCCGATCTGGCTGAATG	300 ^b	Cloning <i>attC_{blavIM-2}</i>
Carb2-A-attC	TGGAACATTGCGGATCGCTCA	360 ^b	Cloning <i>attC_{blaCARB-2}</i>
aac6Ib-attC-A	GATCCCAGAGTACCAAGATCCA	330	Cloning <i>attC_{aac(6')-Ib}</i>
aac6Ib-attC-B	TGGCACTCCATGGAATATCTGG		
dfr-attC-A	GCCTGATCGATAAAGCTGATACT	373	Cloning <i>attC_{dhlfrXVb}</i>
dfr-attC-B	GGTTGCTGCGGCTTACTTGCT		
cmlA4-attC-A	CAACAGTCGTGCTCGGTCTGTCT	327	Cloning <i>attC_{cmlA4}</i>
cmlA4-attC-B	CCATCCACTGCGGAGCCGTACA		
aadB-attC-A	CAGTCCGTTGTAAACAGCTGGGA	336	Cloning <i>attC_{aadB}</i>
aadB-attC-B	TGGTTCCATGATAGAACGGTCC		
arr2-attC-A	CACAGTCCTATAGAACCTGCGA	378	Cloning <i>attC_{arr2}</i>
arr2-attC-B	AGGTTGCTGCGGCTTACTTGTC		
attI-A	CAGTGGACATAAGCCTGTTCCGGT	434	Cloning <i>attC_{attI}</i>
attI-B	CGACGATCGCAACACCGATCTGA		
pACYC-EcoRI-A	GGAAAACGGTGTAACAAGGGTG	183	Structure cloning pACYC
pACYC-EcoRI-B	AGACCGTTCAGCTGGATATTACG		
IEP-GSP1	GTCAAAGAACGCGTCTAAAT	503 ^c	5' RACE
IEP-GSP2	CCTGAATCCATAGCTGTTGTT	404 ^c	
IEP-GSP3	GTATCTCGACCCGTTTACTG	273 ^c	
qnrA-F-Multi	AGAGGATTTCTACGCCAGG	580	<i>qnrA</i> amplification
qnrA-R-Multi	TGCCAGGCACAGATCTTGAC		
intronII-A	CCATGACAGGTTGATGAGTAAGT	465	IntronII internal amplification
intronII-B	CGACGCGTCAGCTCGCGAGTT		
Maturase-STOP-A ^a	CATTGGCCAGCGCTAAAAATAGCAG		IEP inactivation
	<u>T</u> AAGAGACG <u>T</u> GGTAACTACCAA		
Maturase-STOP-B ^a	TTGGTAGTTACCGTCTCTTACT		
	GCTATTTAGCGCTGGCCAATG		

^a Inactivating mutations are underlined.^b Combined with 3'-CS.^c Distance to ATG start codon.

France). Mobility was evaluated using pACYC-EcoRI-A or pACYC-EcoRI-B and intron-specific primers (Table 2).

The homing frequency of the *Enterobacter cloacae* GOC (*E.cl.GOC*) intron was determined by colony replicate hybridization or directly by PCR. Purified plasmids (100 ng) from induced cultures were digested with the PstI or NdeI restriction enzyme. Those enzymes cut only in the intron donor plasmid and outside the intron sequence. *E. coli* TOP10 cells were electroporated with the PstI- or NdeI-digested products and plated on LB medium containing Tc to select for cells containing pACYC184 recipient plasmids that were or were not interrupted by the intron. To calculate the intron mobility efficiency, expressed as the percentage of recipient plasmids that received the intron, 100 Tc^r colonies were replicated onto plates containing LB medium with Tc. The replicated colonies were transferred onto nylon membranes and hybridized with a probe specific for the intron. For each target site tested, randomly chosen posi-

tive colonies were cultured, and the homing site was confirmed by PCR amplification of the target site and sequencing.

Real-time RT-PCR. For real-time RT-PCR experiments, RNA extracts were previously treated with DNase (Qiagen, Courtaboeuf, France) for 30 min at room temperature. The expression of the group IIC *E.cl.GOC* intron was measured using the one-step real-time quantitative RT-PCR described previously (36). Detection of transcripts was performed by using a QuantiFast SYBR green RT-PCR kit on a LightCycler (version 1.0) instrument (Roche Diagnostics, Neuilly-sur-Seine, France). Clinical isolate *E. cloacae* GOC and its respective transconjugants in *E. coli* J53 and clones in *E. coli* TOP10 were studied. A similar procedure was used to amplify the circle form of the *E.cl.GOC* intron.

Primer extension experiments. In order to determine the +1 initiation start site of transcription of the IEP of this group IIC *E.cl.GOC* intron, primer extension experiments were realized using the 5' rapid amplifica-

FIG 1 Original integration of group IIC *E. coli* GOC intron into *attC* site of *aadA1* gene cassette in the In53-like integron in *E. cloacae* GOC clinical strain. (A) Schematic diagram of group IIC *E. coli* GOC intron and surrounding sequences. Gray arrows, open reading frames; gray circles, *attC* sites. (B) *E. coli* GOC homing site for *attC* site of *aadA1* in the original structure. The stop codon (TAA) for the *aadA1* gene cassette is indicated in italics. The inverse core site of *aadA1* gene cassette is indicated in bold. The core site of *aadA1* gene cassette is indicated in bold with a gray background, and its comparison with the conserved sequences for the core site element is shown. The group IIC *E. coli* GOC intron is underlined, and the extremities for the group IIC *E. coli* GOC intron at the integration site are shown.

tion of cDNA ends (RACE) system (version 2.0) according to the instructions of the manufacturer (Invitrogen, Life Technologies, Cergy-Pontoise, France) (37). The design of specific primers (Table 2; see Fig. S2 in the supplemental material) was performed as specified by the manufacturer for first-strand cDNA synthesis (GSP1 primers), PCR of dC-tailed cDNA (GSP2 primers), and nested amplification (GSP3 primers). The 5' RACE PCR products were cloned into pCRBluntII-Topo (Invitrogen). Analysis of the cloned sequence allowed determination of the transcription initiation site and, subsequently, the promoter sequences.

Southern blot analysis and location of the group IIC intron *E. coli* GOC. DNA-DNA hybridizations were performed as described by Sambrook et al. (40) with a Southern transfer of an agarose gel that contained plasmid DNA of 28 enterobacterial clinical isolates positive for the *E. coli* GOC intron. The probe consisted of a 465-bp PCR-generated fragment from *E. cloacae* GOC. Labeling of the probe and signal detection were carried out by use of an enhanced chemiluminescence nonradioactive labeling and detection kit (Amersham Biosciences, Pantin, France) according to the manufacturer's instructions. Southern blot analysis was performed by using plasmid DNA from a Kieser extraction (14), which was separated by electrophoresis on 0.7% agarose gels, transferred onto Hybond N+ membranes, and hybridized with enhanced chemiluminescence-labeled probes overnight at 42°C. The membranes were developed according to the manufacturer's instructions (GE Healthcare, Saclay, France).

Site-directed mutagenesis. The identified group IIC intron was inactivated by modifying the retrotranscriptase domain of the IEP by site-directed mutagenesis, following the manufacturer's protocol (QuikChange site-directed mutagenesis kit; Stratagene). Recombinant plasmid pIntronIIC was used as the template in PCR amplification with primers Maturase-STOP-A and Maturase-STOP-B (Table 2). It gave rise to recombinant plasmid pIntronIIC-Mut, which was subsequently trans-

formed into *E. coli* TOP10. Sequence analysis of the inserts confirmed the presence of the expected mutations, leading to inactivation of the retrotranscriptase.

Epidemiological distribution. A collection of 49 enterobacterial clinical isolates together with three *Pseudomonas aeruginosa* isolates containing *bla*_{VEB-1} was used to analyze the epidemiological distribution of this group IIC intron by PCR with specific primers (Table 2) and also with a primer specific for the *intI1* gene. Additionally, a heterogeneous collection of 50 enterobacterial isolates possessing various extended-spectrum- β -lactamase (ESBL)-encoded genes, including TEM, CTX-M, or SHV, was screened.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to the EMBL/GenBank nucleotide sequence database under the accession number [JF681371](#) for *E. coli* GOC.

RESULTS

Analysis of genomic context of group IIC *E. coli* GOC intron. *E. cloacae* strain GOC was isolated at the Bicêtre Hospital in January 2003. *E. cloacae* GOC was resistant to all β -lactams except carbapenems, fluoroquinolones, aminoglycosides, chloramphenicol, rifampin, and tetracycline. This isolate harbored the plasmid-mediated quinolone resistance gene *qnrA1*, associated with the *bla*_{VEB-1} ESBL gene. Conjugation experiments yielded *qnrA1*- and *bla*_{VEB-1}-positive transconjugants for *E. cloacae* GOC (27, 30), showing that both genes were coharbored on the same plasmid. This *E. coli* transconjugant harbored a single ca. 180-kb plasmid that hybridized with the *qnrA*- and *bla*_{VEB-1}-specific probes. Shotgun cloning followed by sequencing allowed identification of a complex genetic structure in which a group II intron sequence was found (Fig. 1).

FIG 2 Detection of *E. coli* GOC expression by RT-PCR. Lane 1, *E. cloacae* GOC; lane 2, *E. coli* TC GOC; lane 3, pIntronIIC clone; lane 4, negative control; lane M, molecular marker.

FIG 3 Detection of circular form of the *E. coli* GOC intron and effect of mutagenesis by RT-PCR. Lane 1, pGOCXN1 clone; lane 2, pIntronIIC clone; lanes 3 and 4, pIntronIIC-Mut clone; lane 5, negative control; lanes M, molecular marker.

FIG 4 Protein alignment of the putative *E.cl.GOC* protein with group II intron-encoded ORFs STH744 (group IIC, GenBank accession no. [AP006840](#)) from *Symbiobacterium thermophilum*, *S.ma.I.2* (group IIC-attC, GenBank accession no. [AY884051](#)) from *Serratia marcescens*, *GBSi1* (group IIC, GenBank accession no. [ZP_04523595.1](#)) from *Streptococcus pneumoniae*, *RmInt1* (group IIB, GenBank accession no. [NP_438012.1](#)) from *Sinorhizobium meliloti*, and *LtrA* (group IIA, GenBank accession no. [AAB06503.1](#)) from *Lactococcus lactis*. Domains conserved among intron-encoded ORFs are denoted by the lines above the alignment according to the *LtrA* sequence. RT1 through RT7 depict RT-like domains (50). The maturase-specific domain is designated x (24), and the zinc finger-like domain is designated Zn (43). Conserved YADD residues essential for the retrotranscriptase activity are indicated in gray. Asterisks are for identical amino acid residues.

The group II intron sequence (named *E.cl.GOC*) was located on a *sulI*-type class 1 In53-like integrator (25) (Fig. 1A). It was bracketed by an *aadA1* gene cassette and the 3' conserved sequence (3'-CS) region of the class 1 integrator and was associated with an *attC* site (Fig. 1B). The overall structure of the 5' part of this class 1 integrator that contained the *veb-1* cassette was identical to that reported in the previously described In53, except for this additional *E.cl.GOC* element (Fig. 1) (8, 25). Additionally, the *ISCR1* element, *qnrA1* resistance gene, and the conserved element 3'-CS were identified, constituting a complex integrator.

This 1,838-bp group II *E.cl.GOC* intron had a 50.4% G+C content, possessed a single open reading frame corresponding to a putative reverse transcriptase, and was identified in the *attC* site of the *aadA1* (*attC_{aadA1}*) gene cassette. This intron had an unusual target of integration since it was identified between the GT and the TRRRY of the core site, as illustrated in Fig. 1B.

***E.cl.GOC* is an active intron capable of self-splicing *in vivo*.** Splicing and transcription of the *E.cl.GOC* intron and of some of the surrounding genes in the original host *E. cloacae* GOC and its transconjugant, *E. coli* TC GOC, were tested by RT-PCR. Transcription of the *bla_{VEB-1}* and *qnrA1* genes was detected using primers *Veb1A/Veb1B* and *qnrA-F-Multi/qnrA-R-Multi*, respectively (data not shown). The *E.cl.GOC* intron precursor mRNA was de-

tected by first-strand cDNA synthesis, followed by PCR with primers IntronIIA/IntronIIB (Table 2; Fig. 2). These results suggest that the *E.cl.GOC* intron RNA is transcribed in the *E. cloacae* GOC clinical strain and its *E. coli* J53 derivative transconjugant. These transcripts were also detected in *E. coli* DH10B(pIntronIIC) containing the cloned intron (Fig. 2).

The self-splicing ability of the *E.cl.GOC* intron was analyzed in order to detect a possible circular form for this element. The study was carried out using four different constructs: (i) the wild-type intron obtained from *E. cloacae* GOC; (ii) its derivative transconjugant, *E. coli* TC GOC; (iii) the recombinant plasmid pGOCXN1, which contains a 9,996-bp *XbaI* fragment cloned in the pBK-CMV vector containing the partial integrator coding for the group IIC intron; and (iv) the clone pIntronIIC, which contains a 2,062-bp PCR fragment amplified with the *aadA1*-3ext and 3'-CS primers containing the entire sequence of *E.cl.GOC* cloned in pTOPO-BluntII. To demonstrate the splicing of the *E.cl.GOC* intron *in vivo*, RNA from the different constructions was reverse transcribed and amplified by inverse PCR with primers Intron-5'ext/Intron-3'ext (Table 2; see Fig. S2 in the supplemental material). A 363-bp amplicon was obtained using recombinant plasmids pGOCXN1 and pIntronIIC as matrix DNAs, and further sequencing confirmed the circular form of the intron (Fig. 3). No product

FIG 5 *attC* target site alignment. Top strands for *attC* sites for *aadA1*, *aadA2*, *bla_{CARB-2}*, *bla_{VIM-2}*, *aac(6')-Ib*, *dfrXVb*, *cmlA4*, *aadB*, and *arr2* gene cassettes are shown (the downstream gene is also indicated). Residue conservation is depicted using sequence logos (weblogo.uberkeley.edu/). *attI* site is also shown. Inverse core sites of the gene cassettes and recombination site for *attI* site are underlined, and core sites of the gene cassette are underlined with a gray background. The integration site for *E. coli* GOC group IIC-*attC* intron is indicated by arrow. nt, nucleotides.

was observed from *E. cloacae* GOC or its derivative transconjugant (data not shown) under our experimental conditions. The 5' (5'-GTGTGTC-3') and 3' (5'-TATCCCCGAT-3') splice site junctions of the intronic circular sequence were consistent with the findings of analysis of the linear sequence (see Fig. S1 in the supplemental material).

Characterization of the IEP encoded by group IIC *E. coli* GOC intron. The *E. coli* GOC intron encodes a putative 434-amino-acid protein showing similarity to other open reading frames identified in prokaryotic group II introns (3, 4, 23). Similar to group II intron-encoded IEPs, this protein contained all seven polymerase-like domains conserved among retroelement reverse transcriptases (RT1 to RT7) (Fig. 4) (50). The IEP of the *E. coli* GOC intron contained both domain Z of undetermined function (48) and domain X, which corresponds to a putative RNA-binding protein associated with RNA splicing or maturase activity (24). However, the C-terminal Zn²⁺ finger-like region associated with DNA endonuclease activity was partially absent in *E. coli* GOC, as described in other group IIC introns (43) (Fig. 4). The highly conserved YADD motif, which is part of conserved sequence RT5 and which forms part of the active site of the retrotranscriptase, was conserved in the *E. coli* GOC protein. Noticeably, the IEP encoded by the *E. coli* GOC intron was more similar to the group IIC intron-encoded ORF (STH744) (50% amino acid identity) and to the group IIC-*attC* intron-encoded ORF (*S. ma*.12) (45% amino acid identity).

In vivo mobility of group IIC *E. coli* GOC intron into various *attC* sites. Since *E. coli* GOC was identified to be integrated into the *attC* site of the *aadA1* gene cassette, inserted between the GT and TRRRY core site features (Fig. 1B), its ability to be mobilized was

evaluated. For that purpose, a two-plasmid mobility assay was used to analyze the ability of intron *E. coli* GOC to integrate into various *attC* sites (33). Eight *attC* sites [*aadA2*, *bla_{VIM-2}*, *bla_{CARB-2}*, *aac(6')-Ib*, *dfrXVb*, *arr2*, *cmlA4*, and *aadB*] were subcloned into pACYC184 in order to remove unnecessary sequences (i.e., resistance gene and other recombination sites) from the corresponding donor plasmids (Fig. 5).

Intron mobility was evaluated by PCR and/or colony patch hybridization methods. Figure 6 shows the results of the PCR assay performed with external primers located in the pACYC184 vector and internal intron-specific primers in order to amplify the 5' or 3' intron integration junction. PCR bands corresponding to specific homing events were detected for all *attC* sites evaluated (Fig. 6). Sequencing of the PCR products confirmed that the intron inserted specifically between the GT and the TRRRY into the core site located in the top-strand sequence of all *attC* sites tested, resembling the original structure identified (Fig. 1B). These results confirmed that this intron was mobilized by retrohoming into the core site of the *aadA2*, *bla_{VIM-2}*, *bla_{CARB-2}*, *aac(6')-Ib*, *dfrXVb*, *arr2*, *cmlA4*, and *aadB* gene cassettes, with a lead orientation (Fig. 1B and 6 and Table 3), in contrast to other group IIC introns, which usually target the *attC* sites of gene cassettes at the inverse core site.

In order to compare the levels of mobility of *E. coli* GOC for the distinct *attC* sites, we used the colony patch hybridization method. Under the experimental conditions used, the *E. coli* GOC mobility frequency (defined as the percentage of homing site plasmids containing introns) varied from 21% for the *attC_{cmlA4}* site to 81% for the *attC_{aadA2}* site (Table 3). To summarize, *E. coli* GOC was specifically mobilized to various *attC* sites into the core site, and

the frequency of mobility varied as a function of the target sequence.

In vivo mobility of cloned group IIC *E. coli* GOC intron into the *attI* site. An assay identical to the one described above was carried out to analyze the possible mobilization of the *E. coli* GOC intron into the *attI* site of class 1 integrons. Under our experimental conditions, *E. coli* GOC mobility into *attI* site was not detected (Fig. 6C and Table 3). The fact that the *attI* sequence could not serve as a homing site could result from an absence of similarity with the so-called ESB1 sequence, as evidenced by Quiroga and colleagues (32, 33). Unfortunately, our analysis did not allow us to define precisely this ESB1 site in the *E. coli* GOC intron. The bottom strand of *attC* sites might therefore not correspond to a suitable homing site for the *E. coli* GOC intron, and we may therefore speculate that the phosphodiester bond that is targeted by subgroup IIC-*attC* introns should be similarly positioned, as evidenced by Leon and Roy (17).

E. coli GOC contains promoter sequences for IEP expression.

In order to determine the +1 initiation start site of transcription of the ORF included in the *E. coli* GOC intron coding for the IEP, primer extension experiments were performed using a 5' RACE system and the primers indicated in Table 2 and Fig. S2 in the supplemental material. Analysis of the cloned sequences allowed determination of the transcription initiation site and, subsequently, the promoter sequences. We found that *E. coli* GOC possessed its own promoter sequences for the expression of the IEP. This promoter was made of a –35 box (CCGAAC) and a –10 box (TACCAA) separated by 17 bp and was responsible for IEP expression. The +1 transcription site was located 26 bp upstream of the IEP start codon of *E. coli* GOC (Fig. 7). Our results showed that the group IIC *E. coli* GOC intron mediates the expression of its IEP.

***E. coli* GOC self-splicing is dependent on IEP activity.** We analyzed the self-splicing ability of the *E. coli* GOC intron under regulated conditions in order to detect any circular form for this element in the pIntronIIC-Mut clone that had presumably lost the activity of its IEP since it corresponded to an inactivated variant of the retrotranscriptase RT1 region induced by site-directed mutagenesis (Fig. 4 and Table 2). The resulting product (363 bp), which was detected for recombinant plasmids pGOCXN1 and pIntronIIC and which represents the circular form of the intron, was not observed from the pIntronIIC-Mut clone under our experimental conditions (Fig. 3), reflecting that the splicing was dependent on this IEP activity. As expected, mobility into different *attC* sites was also not detectable using plasmid pIntronIIC-Mut (data not shown).

Epidemiological distribution of group IIC *E. coli* GOC intron.

Forty-nine nonclonally related enterobacterial clinical isolates (36 *E. coli*, 5 *Proteus mirabilis*, 3 *Klebsiella pneumoniae*, and 2 *E. cloacae* isolates and 1 isolate each of *Enterobacter hormaechei*, *Citrobacter freundii*, and *Providencia stuartii*) plus 3 *P. aeruginosa* isolates recovered from different geographical locations, all of them containing the *bla*_{VEB-1} and *int1* genes, were used to analyze the epi-

FIG 6 In vivo mobility of *E. coli* GOC group IIC-*attC* intron into various *attC* site sequences and *attI* site. Agarose gels contained the purified PCR products obtained using pACYC-EcoRI-B plus intron 5'ext-specific or pACYC-EcoRI-A plus intron 3'ext-specific primers. These primers amplify the 5' and 3' intron-exon integration junctions, respectively, and are specific for homing into the top strand of the cloned targets. +, positive PCR result for integration; –, negative PCR result for integration.

TABLE 3 *In vivo* mobility frequency of intron *E.cl.GOC* into *attI* site and various *attC* target sites

Target ^a	Downstream gene ^b	Mobilization into target	Homing strand ^c	Orientation ^d	<i>E.cl.GOC</i> mobility frequency (%) ^e
<i>attC_{aadA2}</i>	<i>qacE</i>	Yes	ts	Lead	81 ± 9
<i>attC_{bla_{VIM-2}}</i>	<i>qacE</i>	Yes	ts	Lead	75 ± 8
<i>attC_{bla_{CARB-2}}</i>	<i>qacE</i>	Yes	ts	Lead	77 ± 7
<i>attC_{aac(6')-Ib}</i>	<i>dfrXVb</i>	Yes	ts	Lead	38 ± 5
<i>attC_{dfrXVb}</i>	<i>cmlA4</i>	Yes	ts	Lead	33 ± 4
<i>attC_{arr2}</i>	<i>cmlA5</i>	Yes	ts	Lead	35 ± 6
<i>attC_{cmlA4}</i>	<i>aadA2</i>	Yes	ts	Lead	21 ± 5
<i>attC_{aadB}</i>	<i>arr2</i>	Yes	ts	Lead	40 ± 6
<i>attI</i>	<i>bla_{GES-1}</i>	No			<1

^a The targets were the gene cassette *attC* site or the *attI* site.^b Downstream gene located upstream of the corresponding target.^c *E.cl.GOC* homing specificity for the bottom-strand (bs) or top-strand (ts) sequences on the basis of the PCR assays.^d Orientation of the homing strand sequence relative to the direction of DNA replication, lagging, or leading strand.^e Percentage of recipient plasmids that received the intron. The mobility frequencies were determined by colony patch hybridization with an intron-specific probe or by PCR and are the means ± standard deviations of positive hybridization signals of at least three independent experiments.

demological distribution of this group IIC *E.cl.GOC* intron. Twenty-eight (53.8%) out of these 52 isolates were positive for the *E.cl.GOC* intron. *E.cl.GOC* was identified in 23 *E. coli*, 2 *K. pneumoniae*, and 2 *E. cloacae* isolates and 1 *C. freundii* isolate. Most of these isolates ($n = 25$) were from Bangkok, Thailand, and three isolates were from Istanbul, Turkey. Southern blot analysis revealed that in most of the isolates ($n = 25$), intron *E.cl.GOC* was always located on an ~160-kb plasmid that carried the *bla_{VEB-1}* gene, thus suggesting the diffusion of a single plasmid (data not shown). Only one *C. freundii* isolate harbored the *E.cl.GOC* intron on a different-size plasmid (~120 kb). In one *E. coli* isolate, the *E.cl.GOC* intron was detected on two different plasmids of ~160 kb and ~70 kb (and thus is present at least in two copies), and in two *E. coli* isolates, the *E.cl.GOC* intron was chromosomally located. The *E.cl.GOC* intron was always integrated into the *attC* site of *aadA1* and upstream of the *qacEΔ1* gene, except for the ~70-kb plasmid, where it was located in the *attC* site of *aadB*, also upstream of the *qacEΔ1* gene. The amino acid sequence of the IEP in all the strains was analyzed and was always identical to that of the original *E.cl.GOC*.

In order to analyze the association of the *E.cl.GOC* intron with class 1 integron features, the co-occurrence of the *intI1* gene and of the *E.cl.GOC* intron was analyzed in a collection of 50 enterobacterial isolates coding for different ESBLs. Thirty-six isolates (72%) were *intI1* positive, while the *E.cl.GOC* intron was not detected in any of those isolates.

DISCUSSION

Integrins and gene cassettes are important structures that play a major role in the horizontal exchange of genes allowing bacteria to adapt to environmental selection pressure, such as that driven by antibiotic usage. However, little information about the origin of those resistance genes and the way in which they are assembled into gene cassettes is available. The large cassette arrays of several chromosomal integrons have been shown to be potential sources of gene cassettes for mobile integrons (2, 11, 39, 49). However, the few antibiotic resistance gene cassettes identified in chromosomal integrons cannot explain the large variety of resistance cassettes found among mobile integrons (5, 6). Interestingly, potential progenitors of some cassette-associated genes (for example, *catB* genes) have been found in the chromosomes of *P. aeruginosa* and *Agrobacterium tumefaciens* (34), but not in the form of cassettes. Despite a >60% nucleotide sequence identity between the gene cassettes and the corresponding chromosomal genes, *attC* sites have never been identified downstream of the latter. This suggests an independent origin for the genes and the *attC* sites. Analysis of known integron cassettes suggested that their genesis involved an intermediate step involving reverse transcription of mRNA molecules in order to explain the absence of promoters, the paucity of noncoding sequence, and the presence of only one gene (9, 10, 34), although this hypothesis has also been discussed (38, 39). Recent studies showed that some group IIC introns target *attC* sequences

FIG 7 Intron group IIC *E.cl.GOC* inserted at core site of *attC* of *aadA1* gene in the original clinical strain *E. cloacae* GOC. The promoter structure for IEP expression of *E.cl.GOC* determined by the 5' RACE experiment is shown. The -35 and -10 motifs of the promoter identified are boxed. The +1 initiation site of transcription is indicated in gray. The IEP from the start ATG codon to the stop codon is indicated by arrows. The full intron group IIC *E.cl.GOC* is underlined. The core site and inverse core site of the *attC* element of *aadA1* gene are shaded in gray.

FIG 8 Phylogenetic tree for several group IIC introns and group IIC-*attC* intron compared to group IIA and group IIB introns according to the IEP amino acid sequences from various organisms. Evolutionary distances were computed using the neighbor-joining algorithm of the ClustalW and TreeView software using 1,000 bootstrap analyses to estimate the robustness of the phylogenetic inference. The new intron described here is indicated *E.cl.GOC*.

and that their ORFs code for a multidomain protein with RT activity (33, 35).

So far, all introns associated with an *attC* site (group IIC introns) have been found to be inserted into the inverse core sites at the position RYYA_AC and with an inverse orientation in respect to the gene cassettes on the bottom strand (17). Herein we demonstrated an atypical integration of a novel group IIC intron that was found to be inserted into the *attC* site of the *aadA1* gene cassette at the core site (GT_TRRY) and with a leading orientation on the top strand.

Only a minority of bacterial group II introns have been unequivocally shown to be functional or to undergo splicing *in vivo* (33, 48). Our results showed that the *E.cl.GOC* intron RNA was transcribed in the *E. cloacae* GOC clinical strain and its derivative transconjugant and recombinant strains, indicating that it was transcriptionally active (Fig. 2). Additionally, the self-splicing ability of the *E.cl.GOC* intron under regulated conditions was demonstrated by detection of a circular form. No product was, however, observed with *E. cloacae* GOC or its derivative transconjugants as template under our experimental conditions, indicating that this phenomenon likely happened at a low frequency.

The ability of intron *E.cl.GOC* to confer resistance to any antimicrobial agent was also evaluated, because of the frequent association observed with class 1 integrons known to carry mostly antimicrobial resistance determinants. The *E.cl.GOC* intron did

not confer resistance to any antimicrobial agent once cloned in *E. coli* (data not shown).

Noticeably, the IEP encoded by the *E.cl.GOC* intron was more similar to the group IIC intron-encoded ORFs. This protein shared 50% amino acid identity with STH744 from *Symbiobacterium thermophilum* (group IIC introns), 45% amino acid identity with *S.ma.12* from *Serratia marcescens* (group IIC-*attC* introns), 45% amino acid identity with GBSi1 from *Streptococcus pneumoniae* (group IIC introns), 36% amino acid identity with LtrA from *Lactococcus lactis* (group IIA introns), and 30% amino acid identity with RmInt1 from *Sinorhizobium meliloti* (group IIB introns). The most similar protein (GenBank accession number [EDM48718.1](#)) in the NCBI database shared 63% amino acid identity and was identified in *Marinobacter algicola*. Other IEPs with significant amino acid identity found in the NCBI database were present in *Acidobacterium capsulatum* (53%, GenBank accession number [ACO32387.1](#)), *Burkholderia vietnamiensis* (50%, GenBank accession number [ABO59899.1](#)), and *Bacillus clausii* (48%, GenBank accession number [BAD18275.1](#)). A phylogenetic tree was built and showed that *E.cl.GOC* was an intron IIC located in a branch separate from the group IIC-*attC* introns (Fig. 8), being an intron IIC possessing the ability to integrate using the core site of the *attC* sites as the target.

We showed here that this novel group IIC intron was specifically mobilized into the core site between the GT and the TRRY

on the top strand in the different gene cassettes evaluated [*aadA2*, *bla_{VIM-2}*, *bla_{CARB-2}*, *aac(6')-Ib*, *dfrXVb*, *arr2*, *cmlA4*, and *aadB*], resembling the original structure found (Fig. 1B). The hypothesis for the role of group IIC-*attC* introns in gene cassette formation would not include the type of integration found for this novel group IIC intron (16). Analysis of these flanking exon sequences of the *E. coli* GOC intron from the mobility assays showed that this intron recognizes a degenerate consensus homing site, 5'-RRYG T/TRR, which is at the 5' end of the 1R of most *attC* sites (top strand) (Fig. 5) (12). *E. coli* GOC mobility into the *attI* site was not detected, supporting the suggestion that these elements require secondary structure in its process of mobilization, as reported previously (17).

These results confirmed that this intron was mobilized by retrohoming into the core sites of the *aadA2*, *bla_{VIM-2}*, *bla_{CARB-2}*, *aac(6')-Ib*, *dfrXVb*, *arr2*, *cmlA4*, and *aadB* gene cassettes with a lead orientation (Fig. 1B and 6 and Table 3), in contrast to others' group IIC intron, which usually targets the *attC* site of gene cassettes in the inverse core site on the bottom strand (16, 33), although both insertion sites are structurally similar, supporting the specificity of group IIC introns. Variability in terms of mobility frequency that could be a function of the target sequence was observed. Whether the disruption of the 1R region generated by this intron could affect the excision mediated by the integrase remains unclear. Different modifications within *attC* sites have been assayed *in vitro* and *in vivo* (13, 46). Although the *ant(2'')-Ia::S.ma.I2* cassette containing the *S. ma.I2* class C group II intron in 1L has been shown to be recombinationally active (33), further studies will be necessary to determine whether this cassette can also integrate and be considered a fully functional element (46). Additionally, it has recently been demonstrated that integrons can efficiently recombine sequences differing from the canonical core site and inverse core site (GTT/CAA) (7).

It has been suggested that while not all integron-containing strains contain group IIC-*attC* introns, cassettes formed in strains that do contain them would be exchanged by lateral transfer of their integrons in association with plasmids or transposons. The unique structural characteristics of gene cassettes and the rapid appearance of new antibiotic resistance cassettes in mobile integrons (e.g., extended-spectrum β -lactamase and carbapenemase gene cassettes), which do not have homologs among chromosomal integron cassettes, suggest that there is an RT-based cassette formation mechanism that is enhanced by the antibiotic-driven selection (16). Our data provide additional information to understand how these groups of IIC-*attC* introns interact within *attC* sites in gene cassettes.

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