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Diversity of Class 1 Integron Gene Cassette Rearrangements Selected under Antibiotic Pressure

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ABSTRACT

Integrons are bacterial genetic elements able to capture and express genes contained within mobile gene cassettes. Gene cassettes are expressed via a Pc promoter and can be excised from or integrated into the integron by integrase IntI. Although the mechanisms of gene cassette integration and excision are well known, the kinetics and modes of gene cassette shuffling leading to new gene cassette arrays remain puzzling. It has been proposed that under antibiotic selective pressure, IntI-mediated rearrangements can generate integron variants in which a weakly expressed gene cassette moves closer to Pc, thus leading to higher-level resistance. To test this hypothesis, we used an integron with four gene cassettes, *intI1-aac*(6′)*-Ib-dfrA15-aadA1-catB9*, and applied selective pressure with chloramphenicol, resistance to which is encoded by *catB9*. Experiments were performed with three different Pc variants corresponding to three IntI1 variants. All three integrases, even when not overexpressed, were able to bring *catB9* closer to Pc via excision of the *dfrA15* and *aadA1* gene cassettes, allowing their host bacteria to adapt to antibiotic pressure and to grow at high chloramphenicol concentrations. Integrase $IntI1_{R32\_H39}$, reported to have the highest recombination activity, was able, when overexpressed, to trigger multiple gene cassette rearrangements. Although we observed a wide variety of rearrangements with *catB9* moving closer to Pc and leading to higher chloramphenicol resistance, "cut-and-paste" relocalization of *catB9* to the first position was not detected. Our results suggest that gene cassette rearrangements via excision are probably less cost-effective than excision and integration of a distal gene cassette closer to Pc.

IMPORTANCE

Integrons are bacterial genetic elements able to capture and express gene cassettes. Gene cassettes are expressed via a Pc promoter; the closer they are to Pc, the more strongly they are expressed. Gene cassettes can be excised from or integrated into the integron by integrase IntI. The kinetics and modes of gene cassette shuffling, leading to new gene cassette arrays remain puzzling. We used an integron with 4 antibiotic resistance gene cassettes and applied selective pressure with the antibiotic for which resistance was encoded by cassette 4. All IntI variants were able to bring cassette 4 closer to Pc. Rearrangements occur via excision of the previous gene cassettes instead of cut-and-paste relocalization of the fourth gene cassette.

Integrons are bacterial genetic elements able to capture and express genes contained within mobile gene cassettes (1). Integrons are composed of three key elements: (i) an *intI* gene encoding an integrase that mediates gene cassette integration and excision through site-specific RecA-independent recombination, (ii) recombination site *attI*, and (iii) a promoter, Pc (2). Integrons containing antibiotic resistance gene cassettes are located on transposons or plasmids and are widely involved in the spread of antibiotic resistance among Gram-negative bacteria. More than 130 gene cassettes, encoding resistance to nearly all antibiotic families, have been described (3). Several classes of integron have been described on the basis of the amino acid sequence of the IntI integrase (4), class 1 being the most frequently described for multidrug-resistant Gram-negative bacteria (5–7).

Gene cassettes are mobilizable units composed of an open reading frame followed by a recombination site, *attC*. Gene cassettes are usually promoterless and are transcribed under the control of Pc (8). Expression of gene cassettes is also influenced by their position within the cassette array: the closer they are to Pc, the more strongly they are expressed (9). Among the 13 Pc variants that have been described for class 1 integrons, 4 predominate, namely, PcS, PcW_{TGN-10}, PcH1, and PcW (listed from strongest to weakest, with a 25-fold difference in strength between PcS and PcW) (8). In class 1 integrons, Pc is located within the *intI1* gene and Pc polymorphism affects the IntI1 amino acid sequence at positions 32 and 39; three IntI1 variants, $IntI1_{R32\_N39}$, $IntI1_{P32\_H39}$, and $IntI1_{R32\_H39}$, correspond to the four main Pc variants (8). We have previously shown that the weaker the Pc variant, the more active the encoded IntI1: the $IntI1_{R32\_H39}$ variant (corresponding to the weak promoter sequences PcW and PcH1) has the most efficient recombination activity (8). Moreover, *intI1* transcription is regulated via the SOS response (10). This global regulatory network is controlled by the transcriptional repressor LexA (11) and is induced by stresses such as exposure to some antibiotics (quinolones, β-lactams, and trimethoprim). PintI1 lies

Received 31 October 2014 **Accepted** 15 April 2015

Accepted manuscript posted online 20 April 2015

Citation Barraud O, Ploy M-C. 2015. Diversity of class 1 integron gene cassette rearrangements selected under antibiotic pressure. J Bacteriol 197:2171–2178. doi:10.1128/JB.02455-14.

Editor: V. J. DiRita

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/JB.02455-14.

doi:10.1128/JB.02455-14

face to face with Pc. Pc interferes with the level of *intI1* transcription, but this effect depends on the Pc variant: the strong Pc variant PcS prevents *intI1* expression, contrary to the other variants (12). Drug resistance driven by class 1 integrons thus results from (i) the level of gene cassette expression, (ii) the level of integrase expression, (iii) the recombinogenic efficiency of the integrase variant, and (iv) the shuffling capacity of the gene cassettes, driven by their *attC* site folding (13).

The integrase is able to catalyze gene cassette excision by *attC* × *attC* recombination events and gene cassette integration by *attC* × *attI* recombination events. The mechanisms of gene cassette integration and excision are well known (14, 15), notably the *attC* sites, for which recombination occurs in single-stranded form, only the folded bottom strand being active (16, 17). However, there are very few data on the dynamics of gene cassette shuffling leading to new gene cassette arrays. It has been proposed that under antibiotic selective pressure, integrase-mediated gene cassette rearrangements could create integron variants in which a weakly expressed gene cassette moves closer to Pc, leading to higher-level resistance (9). For this to occur, the integrase has to recombine two *attC* sites, leading to excision of a circular gene cassette that can subsequently be integrated at the *attI* site. To verify this hypothesis experimentally, we constructed a class 1 integron [*aac*(6′)*-Ib-dfrA15-aadA1-catB9*] and applied selective pressure with chloramphenicol, the antibiotic to which resistance is encoded by the last gene cassette (*catB9*). Experiments were performed with three different Pc variants and the three different IntI1 variants, under different conditions of *intI1* expression. Although we observed a wide variety of rearrangements with *catB9* closer to Pc and leading to higher chloramphenicol resistance, "cut-and-paste" relocalization of *catB9* to the first position was not detected. The most frequent rearrangement was obtained by excision of the two gene cassettes upstream of *catB9*.

MATERIALS AND METHODS

Bacteria and growth conditions. Bacteria (Table 1) were grown at 37°C in brain heart infusion broth (BHI; AES Chemunex, Bruz, France) supplemented when necessary with kanamycin (Km; 25 mg/liter), ampicillin (Amp; 100 mg/liter), chloramphenicol (Cm; at concentrations depending on exerted selection pressure), and arabinose (0.2%). Antibiotics and arabinose were obtained from Sigma-Aldrich, Lyon, France.

Construction of synthetic class 1 integrons (Fig. 1). PCR assembly was used to generate two synthetic class 1 integrons: integron 1S, *intI1-aac*(6′)*-Ib-dfrA15-aadA1-catB9* (see Text S1 in the supplemental material) (3.9 kb) and integron 2S, *intI1-catB9* (1.9 kb). Integron 1S contains four gene cassettes, encoding resistance to amikacin, tobramycin, and netilmicin [*aac*(6′)*-Ib*], trimethoprim (*dfrA15*), spectinomycin and streptomycin (*aadA1*), and chloramphenicol (*catB9*). Fragments A (1.8 kb) and D (1.2 kb) were amplified from *Enterobacter aerogenes* BM2688 genomic DNA (18) using, respectively, primers 1 and 2 and primers 1 and 7 (Fig. 1; see also Table S1 in the supplemental material). Fragment B (1.5 kb) was obtained from *Shigella dysenteriae* 3Sh genomic DNA (laboratory collection) using primers 3 and 4. Fragments C (0.8 kb) and E (0.7 kb) were amplified from *Escherichia coli* 1314 genomic DNA (19) using, respectively, primers 5bis and 6 and primers 5 and 6. To construct integron 1S, fragment B+C was prepared first and then coupled to fragment A. To construct integron 2S, fragments D and E were fused. The final PCR products (3.9 kb for 1S and 1.9 kb for 2S) were then cloned into the unique restriction sites EcoRI and BamHI of pSU38Δtot*lacZ* to obtain plasmids p1S and p2S (Table 1). The different Pc variants and LexA binding-site mutants were obtained from integrons 1S and 2S by using the GeneEditor Site-directed Mutagenesis system (Promega, Charbonnieres, France) and specific primers (see Table S1).

We also constructed plasmid p1SintI1$_{Y312F}$ containing the inactive integrase IntI1$_{Y312F}$ (20). Using plasmids p1S and p8741 digested with *BsaI* and SpeI enzymes, we replaced the functional integrase of p1S by the inactive integrase IntI1$_{Y312F}$, the catalytic site of which is mutated.

Chloramphenicol selection pressure assays. *E. coli* DH5α containing p1S and its derivatives were cultured in BHI broth and subjected at successive 24-h intervals to increasing chloramphenicol concentrations. Each day, the broth containing the highest Cm concentration permitting visible bacterial growth (turbidity) was diluted 1/100 in fresh BHI broth containing a higher concentration of Cm and was also seeded on antibiotic-free BHI plates. The protocol was continued until no visible growth was observed. Experiments were performed at least three times, with integrons 1S, 1W, and 1W$_{TGN-10}$, with LexA binding-site mutants 1SL, 1WL, and 1W$_{TGN-10}$L (Table 1), and with or without overexpression of *intI1* in *trans*, using the arabinose-inducible plasmid pBad-*intI1* (arabinose was added every day). For *intI1* overexpression, we used the three plasmids corresponding to three different IntI1 variants (pBad-*intI1*$^{\star}_{R32_N39}$, pBad-*intI1*$^{\star}_{P32_H39}$, and pBad-*intI1*$^{\star}_{R32_H39}$) (8).

To locate the position of the *catB9* gene cassette within the integron, 100 colonies were analyzed each day by PCR with primers 36854 located in the *attI1* site, *catB9*R located in the *catB9* gene, and Fwd located in the vector downstream of the integron (Fig. 1; see also Table S1 in the supplemental material). When PCR products of unexpected sizes were obtained, plasmid DNA was extracted and digested with BamHI and EcoRI and/or sequenced. When two or three fragments were obtained, each fragment was cloned into the unique restriction sites EcoRI and BamHI of plasmid pSU38Δtot*lacZ*, and the sequence of each fragment was analyzed.

Chloramphenicol susceptibility testing and quantification of *catB9* transcripts. Chloramphenicol MICs were determined at least three times for each integron-containing *E. coli* strain according to CLSI recommendations (http://www.clsi.org).

Total RNA was extracted with the FastRNA Pro Blue kit (Q-BIOgene, Illkirch, France) by following the manufacturer's recommendations. Contaminating DNA was removed from RNA samples by using the Turbo DNA-free kit (Ambion, Courtaboeuf, France). cDNAs were synthesized from 1 μg of DNase-treated total RNA by using degenerate primers and Superscript III reverse transcriptase (Invitrogen, Cergy-Pontoise, France). cDNA was quantified in an Mx3005P qPCR system (Agilent Technologies) using the LightCycler FastStart DNA Master Hybridization Probes mix (Roche) according to the supplier's instructions, with appropriate oligonucleotides *catB9*LC1, *catB9*LC2, *dxs*LC1, and *dxs*LC2 and *dxs and catB9* probes (see Table S1 in the supplemental material). Three independent experiments were performed, each in triplicate. Expression of the *catB9* gene was estimated by normalizing absolute transcript values to those of the housekeeping gene *dxs* and by comparing *catB9* expression to that in the native integron (DH5α plus p1S), used as calibrator.

RESULTS

Construction of the integron and chloramphenicol susceptibility. Up to 10 gene cassettes have been described for class 1 integrons (GenBank accession number DQ112222). Each gene cassette can move independently from the others through site-specific recombination catalyzed by the integrase. To investigate the different modes of gene cassette rearrangement under antibiotic selective pressure, we used a chimeric integron in order to obtain an array of at least four gene cassettes. The gene cassettes encoded different mechanisms of resistance to different antibiotic families, thereby avoiding bias during antibiotic selective pressure. We chose four gene cassettes amplified from clinical strains harboring a class 1 integron. This integron possesses all the features of wild-type integrons; the *attC* sites required for recombi-

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
Strains		
Enterobacter aerogenes BM2688	Contains class 1 RI In40 with gene cassette array *aac(6′)-Ib*, *qacF*, *cmlA2*, and *oxa-9* and the strong Pc variant (PcS). Resistant to tobramycin.	13
Shigella dysenteriae 3Sh	Contains class 1 RI with gene cassette array *dfrA15-aadA1*. Resistant to streptomycin, spectinomycin, and trimethoprim.	Laboratory collection
Escherichia coli 1314	pSU38::*catB9*	14
Escherichia coli DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ- *thi-1 gyrA96 relA1*. Resistant to nalidixic acid. Used as recipient strain	Laboratory collection
Plasmids		
pSU38Δtot*lacZ*	Vector carrying the *lacZ* coding sequence with no translation initiation region or promoter	8
p1S	Integron 1S *intI1-aac(6′)-Ib-dfrA15-aadA1-catB9* cloned into pSU38Δtot*lacZ* with PcS promoter	This study
p1W	p1S mutated with primer PcWmut to create p1W with PcW promoter	This study
$p1W_{TGN-10}$	p1S mutated with primer PcW_{TGN-10}mut to create $p1W_{TGN-10}$ with PcW_{TGN-10} promoter	This study
p1SL	p1S mutated with primer LexAmut2L to create p1SL (constitutive expression of $intI1_{R32\_N39}$)	This study
p1WL	p1W mutated with primer LexAmut2L to create p1WL (constitutive expression of $intI1_{R32\_H39}$)	This study
$p1W_{TGN-10}L$	$p1W_{TGN-10}$ mutated with primer LexAmut2L to create $p1W_{TGN-10}L$ (constitutive expression of $intI1_{P32\_H39}$)	This study
pBad-$intI1^*_{R32\_H39}$	*intI1* containing the PcW variant cloned into pBAD18 (arabinose-inducible expression vector) in which PcW is inactivated	8
pBad-$intI1^*_{R32\_N39}$	*intI1* containing the PcS variant cloned into pBAD18 in which PcS is inactivated	8
pBad-$intI1^*_{P32\_H39}$	*intI1* containing the PcW_{TGN-10} variant cloned into pBAD18 in which PcW_{TGN-10} is inactivated	8
p2S	Integron 2S *intI1-catB9* cloned into pSU38Δtot*lacZ* with PcS promoter	This study
p2W	p2S mutated with primer PcWmut to create p2W with PcW promoter	This study
$p2W_{TGN-10}$	p2S mutated with primer PcW_{TGN-10}mut to create $p2W_{TGN-10}$ with PcW_{TGN-10} promoter	This study
p2SL	p2S mutated with primer LexAmut2L to create p2SL (constitutive expression of $intI1_{R32\_N39}$)	This study
p2WL	p2W mutated with primer LexAmut2L to create p2WL (constitutive expression of $intI1_{R32\_H39}$)	This study
$p2W_{TGN-10}L$	$p2W_{TGN-10}$ mutated with primer LexAmut2L to create $p2W_{TGN-10}L$ (constitutive expression of $intI1_{P32\_H39}$)	This study
p3S[a]	*intI1-aac(6′)-Ib-catB9* cloned into pSU38Δtot*lacZ*	This study
p4S[a]	*intI1-aac(6′)-Ib-aac(6′)-Ib-catB9* cloned into pSU38Δtot*lacZ*	This study
p5S[a]	*intI1-dfrA15-aadA1-catB9* cloned into pSU38Δtot*lacZ*	This study
p6S[a]	*intI1-aac(6′)-Ib* cloned into pSU38Δtot*lacZ*	This study
p7S[a]	*intI1-aac(6′)-Ib-dfrA15-catB9* cloned into pSU38Δtot*lacZ*	This study
p8S[a]	*intI1-aadA1-catB9* cloned into pSU38Δtot*lacZ*	This study
p9S[a]	*intI1-aac(6′)-Ib-dfrA15-aadA1- dfrA15-aadA1-catB9* cloned into pSU38Δtot*lacZ*	This study
p10S[a]	*intI1* cloned into pSU38Δtot*lacZ*	This study
p11S[a]	*intI1-aac(6′)-Ib-dfrA15-aac(6′)-Ib-dfrA15-aadA1-catB9* cloned into pSU38Δtot*lacZ*	This study
p12S[a]	*intI1-aac(6′)-Ib-aac(6′)-Ib-dfrA15-aadA1-catB9* cloned into pSU38Δtot*lacZ*	This study
p13S[a]	*intI1-aac(6′)-Ib-catB9-aac(6′)-Ib-catB9* cloned into pSU38Δtot*lacZ*	This study
p14S[a]	*intI1-aac(6′)-Ib-dfrA15-aadA1-aac(6′)-Ib-dfrA15-aadA1-dfrA15-aadA1-catB9* cloned into pSU38Δtot*lacZ*	This study
p15S[a]	*intI1-aac(6′)-Ib-catB9-catB9* cloned into pSU38Δtot*lacZ*	This study
p16S[a]	*intI1- aac(6′)-Ib-dfrA15-aadA1-catB9- aac(6′)-Ib-dfrA15-aadA1-catB9* cloned into pSU38Δtot*lacZ*	This study
p8741	pBad::$intI1_{Y312F}$	20
p1SintI1$_{Y312F}$	p1S with the inactive integrase $IntI1_{Y312F}$	This study

[a] Obtained after Cm pressure assays and cloned into pSU38Δtot*lacZ*.

nation are conserved, as are the open reading frames encoding antimicrobial resistance.

We determined, as controls, the level of *catB9*-encoded Cm resistance in *E. coli* cells containing p1S or p2S and their derivatives, by determining MICs and by quantifying *catB9* transcripts (see Table S2 in the supplemental material). As expected, higher MICs were obtained when *catB9* was in the first position and the level of resistance differed according to the Pc variant as previously described. No difference with the LexA-binding site mutants was observed, confirming the lack of influence of *intI1* transcription on gene cassette expression (12).

Chloramphenicol selection pressure assays. Three different experiments were performed: one under wild-type conditions where integrase expression was repressed by LexA and two in

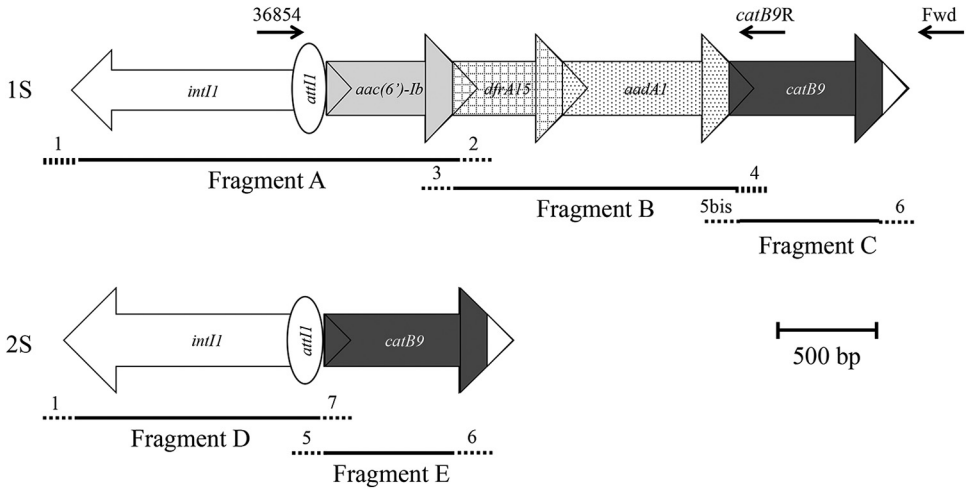

FIG 1 Construction of integrons 1S and 2S. Integrons 1S and 2S were obtained by assembly PCR. To construct integron 1S, fragment B+C (2.2 kb) was first obtained and then coupled with fragment A. To construct integron 2S, fragments D and E were fused. Integron 1S was 3.9 kb, and integron 2S 1.9 kb. The primers used for assembly are represented by dotted lines and indicated by numbers (see Table 1S in the supplemental material), and the primers used for *catB9* detection are represented by black arrows (Fwd is located in pSU38Δtot*lacZ*).

which the integrase was expressed either under the control of its own promoter containing a mutation of the LexA-binding site (derepression) or by overexpression in *trans* by an inducible vector. Durations of the experiments and Cm concentrations reached each day of the assays are shown in Table 2. At the end of the protocol, bacteria were able to grow at Cm concentrations representing 10 times the initial MIC.

Surprisingly, under wild-type conditions, when the integrase was repressed by LexA, we obtained a rearrangement at day 3 of Cm selection pressure, creating an integron with two gene cassettes, *intI1-aac*(6′)*-Ib-catB9*, regardless of the Pc variant and the IntI1 variant. The only difference between the integrons used was the Cm concentration reached according to the Pc variant, from 15 mg/liter for the weak variant PcW to 100 mg/liter for stronger

TABLE 2 Chloramphenicol selection pressure assays[a]

Integron	Cm concn (mg/liter) on day:					Level of gene cassette rearrangements[d]
	1	2	3	4	5	
Integrons 1S (initial Cm MIC: 32 mg/liter)						
p1S (PcS, IntI1$_{R32_N39}$)	**50**	**75**	**100**	**300**	**400**	**+**
p1S + pBad-*intI1**$_{P32\_H39}$	100	250	400			+
p1S + pBad-*intI1**$_{R32\_H39}$	100	300	400			+++
p1S + pBad-*intI1**$_{R32\_N39}$[b]	100	300	400			+
p1SL[c]	100	150	400			+
p1SintI1$_{Y312F}$	50	75	100	150		−
Integrons 1W (initial Cm MIC: 8 mg/liter)						
p1W (PcW, IntI1$_{R32_H39}$)	**5**	**5**	**15**	**75**		**+**
p1W + pBad-*intI1**$_{P32\_H39}$	ND	ND	ND	ND	ND	ND
p1W + pBad-*intI1**$_{R32\_H39}$[b]	5	10	75			+++
p1W + pBad-*intI1**$_{R32\_N39}$	ND	ND	ND	ND	ND	ND
p1WL[c]	5	15	60			+
Integrons 1W$_{TGN-10}$ (initial Cm MIC: 16 mg/liter)						
p1W$_{TGN-10}$ (PcW$_{TGN-10}$, IntI1$_{P32_H39}$)	**25**	**25**	**100**	**250**	**350**	**+**
p1W$_{TGN-10}$ + pBad-*intI1**$_{P32\_H39}$[b]	25	100	250	350		+
p1W$_{TGN-10}$ + pBad-*intI1**$_{R32\_H39}$	50	150	350			+++
p1W$_{TGN-10}$ + pBad-*intI1**$_{R32\_N39}$	ND	ND	ND	ND	ND	ND
p1W$_{TGN-10}$L[c]	25	50	300			+

[a] Values indicate Cm concentrations reached in BHI broth each day of the protocol using DH5α strains. The native integron organization, without *intI1* overexpression, and its corresponding values are presented in bold. Each experiment was performed at least 3 times. ND, not determined.
[b] *intI1* overexpression was obtained with the same integrase as those present in the synthetic integron.
[c] "L" indicates a mutated LexA-binding site.
[d] To locate the position of the *catB9* gene cassette within the integron, 100 colonies were analyzed each day by PCR and sequencing. Symbols: +, single rearrangement with integron *intI1-aac*(6′)*-Ib-catB9*; +++, multiple rearrangements; −, no rearrangement.

variants (PcS and PcW_{TGN-10}) on day 3 (Table 2). However, the initial integron with the four gene cassettes was still present in all studied colonies, and we failed to separate this integron from the *aac*(6′)-*Ib-catB9*-containing integron, suggesting the presence of cointegrates.

When the integrase was expressed either under the control of its own promoter with a mutation in the LexA-binding site or by overexpression in *trans*, the maximal Cm concentrations reached were identical to those obtained under wild-type conditions but occurred 1 or 2 days earlier, whatever the IntI1 variant (Table 2). When constitutively expressed from their own promoter, the three IntI1 variants gave the same rearrangement as that observed without forced *intI1* expression in *trans*, i.e., *intI1-aac*(6′)-*Ib-catB9*. The dynamics of rearrangement differed when the promoter was constitutively expressed or repressed by LexA: for example, with DH5α plusp1SL, colonies harboring the gene cassette rearrangement represented 1% on day 1, up to 95% on day 2, and 100% on day 3, whereas with p1S the rearrangement occurred only on day 3, representing 66% of colonies on day 3 and 100% on days 4 and 5.

When overexpressed from the strong inducible promoter pBad, the same unique rearrangement was obtained, with $IntI1^*_{R32\_N39}$ and $IntI1^*_{P32\_H39}$ with comparable dynamics (5% of gene cassette rearrangement on day 1 and up to 95% on day 2 in all experiments), whereas $IntI1^*_{R32\_H39}$ overexpression led to multiple gene cassette rearrangements from the first day (95% of colonies), whatever the initial integron (1S, 1W, or $1W_{TGN-10}$) (Table 2). These multiple rearrangements corresponded to gene cassette excisions, always moving *catB9* closer to Pc, and/or to *catB9* duplication (Fig. 2). The observed rearrangement in 60% of colonies on day 1 was the same as that obtained without *intI1* overexpression, i.e., *aac*(6′)-*Ib-catB9*, but we detected up to 15 different gene cassette rearrangements (Fig. 2). However, most of the DH5α strains contained 2 or 3 different integrons, probably due to gene cassette recombination between two plasmid copies, leading to cointegrates, as previously shown (21, 22).

Expression of the *catB9* gene. We then individually cloned into pSU38Δtot*lacZ* the rearranged integrons obtained under Cm selective pressure from the initial integron 1S with *intI1* overexpression, yielding p3S to p16S (Table 1), and analyzed *catB9* gene expression relative to that observed with the native integron in p1S (Table 3). Cm MICs ranged from 16 to 256 mg/liter and showed no clear relation to the position of *catB9*, whereas transcript numbers gradually increased (from 0.18 to 26.4 copies per cell), and a good correlation was found with the proximity of *catB9* to Pc and/or with the number of *catB9* gene cassettes (Table 3).

DISCUSSION

The aim of this study was to determine if the class 1 integrase is able to catalyze gene cassette rearrangements within an integron under antibiotic selective pressure and under different conditions of gene cassette and *intI1* expression. In our model, whatever the conditions of Cm selection pressure, the *catB9* gene cassette always succeeded in being closer to Pc, even when the integrase was not overexpressed. The latter results were somewhat unexpected, considering that *intI1* expression is repressed by LexA and that, in previous studies, integrase overexpression was systematically required to obtain gene cassette excision or integration at detectable or measurable frequencies (21–23). It might be argued that under our wild-type conditions, *intI1* expression could have resulted from Cm induction of the SOS response, but while Cm indeed induces the SOS response in *Vibrio cholerae*, this is not the case in *E. coli* (24). Moreover, we used *E. coli* strain DH5α, which is D*recA*, thus preventing SOS response induction. We thus showed that the integrase was able to catalyze gene cassette rearrangement at a very low frequency even when the SOS response was not induced. A possible integrase-independent mechanism, such as slippage (20), could not be ruled out. We thus repeated the experiment with the inactive integrase $IntI1_{Y312F}$ ($p1SintI1_{Y312F}$) and found that (i) the Cm concentrations reached on day 4 were lower than with p1S and (ii) no rearrangement occurred (Table 2). Our results thus suggest that the very weak expression which has been observed previously from the promoter of *intI1*, despite LexA repression (12), might be sufficient for gene cassette rearrangement. On the other hand, with strains carrying the LexA binding-site mutation or the pBad-inducing *intI1* overexpression vector, the time required to reach the highest Cm concentrations and gene cassette rearrangements was shortened by 1 or 2 days, whatever the integrase variant (Table 2). Few studies have analyzed the dynamics of gene cassette rearrangement, and these have been carried out mainly under conditions of integrase overexpression (21, 23). We provide comparative data on the ability of the integrase to trigger rearrangements when IntI1 is repressed by LexA and when IntI1 is derepressed or overexpressed. We found that the dynamics of gene cassette rearrangement increased when IntI1 was expressed at a higher level. Even though this might seem logical, it had never previously been shown experimentally.

All our assay conditions, except those in which $IntI1^*_{R32\_H39}$ was overexpressed from pBad, led to the same unique rearrangement, *intI1-aac*(6′)-*Ib-catB9*, resulting from excision of both the *dfrA15* and *aadA1* gene cassettes. This is in keeping with our previous data on recombination efficiency showing that $IntI1_{R32\_N39}$ and$IntI1_{P32_H39}$ were poorly efficient (8, 12). On the other hand, $IntI1_{R32\_H39}$ is known to be the most active integrase (8), and its overexpression led to many gene cassette rearrangements (up to 15 identified) (Fig. 2), whatever the Pc variant present in the initial integron (Table 2).

Most of the rearrangements observed in this study resulted from multiple reordering events, including gene cassette duplication. The main rearrangements likely occurred through the formation of cointegrates. Previous studies have shown that plasmid cointegrates and duplicated gene cassettes are the main IntI1-mediated recombination products (21, 22). These duplications could also been explained by the probability that the *attC* × *attC* recombination process is replicative, leading to replication of the original substrate (15). We never observed that *catB9* excised and reintegrated in first position, in front of the *aac*(6′)-*Ib*, *dfrA15*, and *aadA1* gene cassettes. This could be explained by the fact that gene cassette excision and cointegrate formation, leading to the *intI1-aac*(6′)-*Ib-catB9* rearrangement, require only one recombination event. This recombination event could be either an intramolecular event (excision event on the same plasmid) between the two *attC* sites of the *aac*(6′)-*Ib* and *aadA1* gene cassettes or an intermolecular event (between two copies of the plasmid, generating cointegrates) between the *attC* site of the *aadA1* gene cassette carried by one plasmid copy and the *attC* site of the *aac6′-Ib* gene cassette carried by another plasmid copy. In contrast, the cut-and-paste mechanism leading to *catB9* excision and reintegration in the first position would require two recombination events. Previous ob-

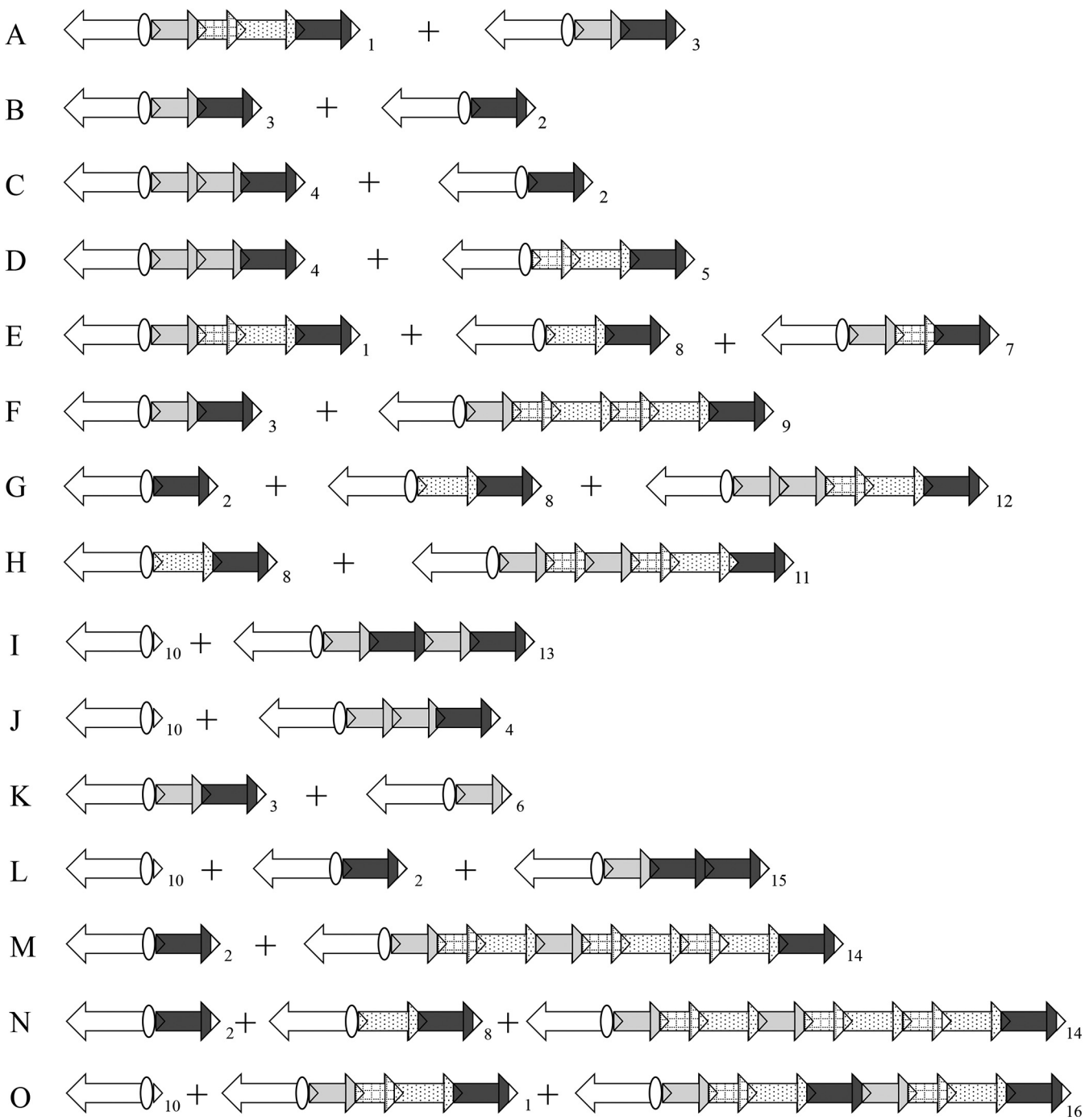

FIG 2 Example of multiple gene cassette rearrangements observed under Cm selection pressure, starting with DH5α plus p1S plus pBad-*intI1**$_{R32\_H39}$. Integron 1 represents the initial integron *intI1-aac*(6′)*-Ib-dfrA15-aadA1-catB9*; the *intI1* gene is represented by a white arrow, the *aac*(6′)*-Ib* gene cassette by a gray arrow, the *dfrA15* gene cassette by a cross-hatched arrow, the *aadA1* gene cassette by a stippled arrow, and *catB9* gene cassette by a black arrow. For example, integron 2 harbors the gene cassette *catB9* (black arrow), and integron 3 harbors the two gene cassettes *aac*(6′)*-Ib* and *catB9* (gray arrow followed by a black arrow). In this assay, after gene cassette rearrangement, all colonies harbored at least 2 different integrons. The different rearrangements are numbered arbitrarily from 3 to 16. The letters (A to O) correspond to integron combinations observed within a given strain.

servations show that single reordering events are more frequent than multiple ones (25).

The *intI1-aac*(6′)*-Ib-catB9* rearrangement yielded high-level resistance to Cm in DH5α as strains carrying an integron with *catB9* in the first position (Table 3). In most of the observed rearrangements, the *aac*(6′)*-Ib* gene cassette remained in first position (Fig. 2). This could be explained by a lower efficiency of the integrase to excise a gene cassette located in first position; indeed, *attI* × *attC* recombinations occur at lower frequencies than *attC* × *attC* recombinations (21). Another explanation lies in the

TABLE 3 Comparison of Cm MICs and *catB9* transcripts in *E. coli* DH5α strains according to the position of *catB9* in the integron[a]

Integron	Relative quantity of *catB9* transcripts	Cm MIC (mg/liter)
p2S (*intI1-catB9*)	26.40 ± 3.54	256
p15S (*intI1-catB9-catB9*)	14.95 ± 1.48	256
p13S [*intI1-aac(6′)-Ib-catB9-aac(6′)-Ib-catB9*]	8.18 ± 0.81	256
p3S [*intI1-aac(6′)-Ib-catB9*]	6.78 ± 0.21	256
p4S [*intI1- aac(6′)-Ib -aac(6′)-Ib-catB9*]	3.78 ± 0.24	256
p5S (*intI1-dfrA15-aadA1-catB9*)	3.54 ± 0.54	128
p8S (*intI1aadA1-catB9*)	3.40 ± 0.46	128
p7S [*intI1-aac(6′)-Ib-dfrA15-catB9*]	2.07 ± 0.28	128
p1S [*intI1-aac(6′)-Ib-dfrA15-aadA1-catB9*]	1	32
p16S [*intI1-aac(6′)-Ib-dfrA15-aadA1-catB9-aac(6′)-Ib-dfrA15-aadA1-catB9*]	0.95 ± 0.08	32
p9S [*intI1-aac(6′)-Ib-dfrA15-aadA1-dfrA15-aadA1-catB9*]	0.49 ± 0.11	32
p11S [*intI1-aac(6′)-Ib-dfrA15-aac(6′)-Ib-dfrA15-aadA1-catB9*]	0.32 ± 0.02	32
p12S [*intI1-aac(6′)-Ib-aac(6′)-Ib-dfrA15-aadA1-catB9*]	0.27 ± 0.01	32
p14S [*intI1-aac(6′)-Ib-dfrA15-aadA1-aac(6′)-Ib-dfrA15-aadA1-dfrA15-aadA1-catB9*]	0.18 ± 0.05	16
p6S [*intI1-aac(6′)-Ib*]	0	4
p10S (*intI1*)	0	4

[a] Strains are classified according to their quantities of *catB9* transcripts relative to DH5α plusp1S (underlined) considered as the calibrator strain.

structure of the folded bottom strand of the *attC* site, with the presence of extrahelical bases (17). IntI1 is more efficient for gene cassette excision using T-N_6-G or T-N_6-C motifs (22, 26), and this was the case in our integron for both *dfrA15 attC* (T-N_6-C) and *aadA1 attC* (T-N_6-G) sites, whereas *aac(6′)-Ib attC* and *catB9 attC* sites contain C-N_6-G motifs for which no excision activity has been observed with IntI1 (22, 26). Furthermore, in our model, the large size of the *catB9 attC* site (125 bp) might also contribute to the relatively low recombination efficiency of *catB9*. Indeed, in their model with bla_{VEB-1} and *aadB* gene cassettes, Aubert et al. demonstrated that both an optimal motif (like T-N_6-G) and a short variable terminal structure (VTS) of the *attC* site (close to 60 bp) are needed to obtain detectable gene cassette rearrangements (22). Moreover, the fact that the *aadA1* and *dfrA15* gene cassettes were rarely excised separately might be due to the imperfect complementarity between the 1R (GTTAAAC) and 1L (GTCTAAC) sites of *aadA1*, as previously shown for other gene cassettes (22). The dynamics of gene cassette shuffling is likely largely dependent on the number of gene cassettes within the gene cassette array and on the *attC* site efficiency of the gene cassettes. Our data were obtained in a model of four gene cassettes with *attC* sites of different recombination efficiencies. Even if these conditions likely exist in natural integrons, the observed dynamics of gene cassette rearrangement cannot be generalized to all integrons, and further experiments with other gene cassettes and gene cassette arrays will be needed.

Analysis of *catB9* expression showed, for all rearrangements, that relocation of *catB9* closer to Pc always enhanced Cm resistance (Table 3). As shown in other studies, the closer the gene cassette was to Pc, the more strongly the gene was expressed (9).

In vivo gene cassette rearrangement was recently described for a class 1 integron in *Pseudomonas aeruginosa* after induction of integrase expression by metronidazole, an SOS-inducing antibiotic, leading to the excision of a gene cassette and allowing the expression of a downstream β-lactamase-encoding gene cassette (27). However, this work did not explore the dynamics of gene cassette shuffling. Our work reinforced this *in vivo* finding, as this was the first study to analyze, under antibiotic selective pressure, the modes and dynamics of the movement of an unexpressed or weakly expressed gene cassette located in a position distal to Pc. In our experimental model, we showed that (i) the class 1 integrase was able to catalyze gene cassette rearrangement even when its promoter was repressed by LexA, (ii) rearrangement of the *catB9* gene cassette closer to Pc was mainly due to movements of the upstream gene cassettes through excision and not to the cut-and-paste movement of the *catB9* gene cassette itself, and (iii) only the IntI1*$_{R32\_H39}$ integrase was able to promote multiple rearrangements.

ACKNOWLEDGMENTS

This work was supported by grants from Ministère de l'Enseignement Supérieur et de la Recherche and Institut National de la Santé et de la Recherche Médicale (INSERM).

We thank Didier Mazel, Thomas Jové, and Sandra Da Re for helpful discussions and Didier Mazel for the kind gift of the *catB9*-containing strain and p8741 plasmid.

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