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MECHANISMS OF RESISTANCE

Integrase-Mediated Recombination of the *bel-1* Gene Cassette Encoding the Extended-Spectrum β-Lactamase BEL-1

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ABSTRACT Integrons are genetic elements that can acquire and rearrange gene cassettes. The bla_{BEL-1} gene encodes an extended-spectrum β-lactamase, BEL-1, that is present at the second position of the variable region of class 1 integrons identified in *Pseudomonas aeruginosa*. The mobility of the *bel-1* gene cassette was analyzed under physiological conditions and with the integrase gene being overexpressed. Cassette mobility in *Escherichia coli* was detected by excision/integration into the recipient integron In*3* on the conjugative plasmid R388 with the overproduced integrase. Despite several antibiotic pressures, the *bel-1* cassette remained at the second position in the integron, highlighting its stability in *P. aeruginosa*. Overexpression of the integrase gene in *E. coli* induced *bel-1* cassette recombination. However, cassettes containing two genes (bla_{BEL-1} and *smr2* or bla_{BEL-1} and *aacA4*) were excised, suggesting that the *bel-1* cassette *attC* site was defective. We show that *bel-1* is a stable gene cassette under physiological growth conditions, irrespective of the selective antibiotic pressure, that may be mobilized upon overexpression of the integrase gene.

KEYWORDS integron, ESBL, *Pseudomonas aeruginosa*, site-specific recombination, BEL-1, integron

Class 1 integrons are genetic elements that can acquire and rearrange gene cassettes, including genes carrying antibiotic/disinfectant resistance genes, therefore participating in the evolution toward multidrug resistance. Integrons are bracketed by two segments at their 5′ (5′ coding sequence [5′-CS]) and 3′ (3′-CS) ends. The 5′-CS includes *intI1*, a gene encoding a site-specific recombinase of the DNA integrase family, with *attI* being the cassette integration site and the promoter P_c (sometimes associated with a second promoter, P_2) driving the expression of the cassettes (1–5). The 3′-CS includes disinfectant (*qacEΔ1*) and sulfonamide (*sul1*) resistance determinants and an open reading frame (ORF) of unknown function (ORF5) (1, 2, 6). Between those two conserved regions occurs the variable region, which is basically a gene cassette array.

The gene cassettes are independent units each consisting of a gene bracketed by

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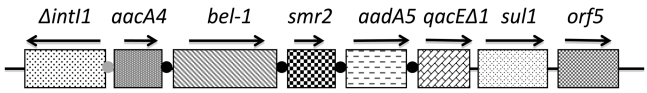

FIG 1 Schematic representation of integrons containing the bla_{BEL-1} gene found in clinical strain *P. aeruginosa* 51170 (22). Arrows, orientation of gene transcription; black and gray circles, *attC* and *attI1* sites, respectively.

copies of a recombination site named *attC* (also named 59-be) (1, 2, 6). The *attI* and *attC* sites do not share the same architecture. The *attI* site is conserved among class 1 integrons and possesses four integrase-binding sites. Two of those integrase-binding sites (L and R) are located at the core recombination sites, and two are 5′ to the core site and are organized as direct repeats (DR1 and DR2) (1, 7, 8). Even though *attC* sites are poorly conserved among gene cassettes, they share an imperfect inverted repeated structure, comprising two pairs of inversely oriented integrase-binding domains separated by a spacer of 7 to 8 bp (9, 10). The boundaries of the *attC* site are defined by degenerate core sites separated by a central region that is highly variable in sequence and size (20 to 104 bp) (11). The only sequences that are fully conserved within *attC* sites are two triplets, 5′-AAC-3′ and 5′-GTT-3′ (11). Nevertheless, *attC* sites present a conserved palindromic organization, allowing the formation of a cruciform structure through the extrusion and self-pairing of both DNA strands (1, 10). When folded, single-stranded *attC* sites present an almost canonical core site consisting of L″-L′ and R″-R′ duplexes separated by a bulged region (12–14). Both *attC* and *attI1* sites are involved in site-specific recombination catalyzed by the integrase IntI1 (15–18). During recombination leading to cassette integration or excision, recombination crossover occurs on the bottom strand only, between the G and TT of the 7-base core site, GTTRRRY (1, 10). Recombination between two *attI* sites and between two *attC* sites can also occur, but the most efficient is between an *attI* site and an *attC* site (18, 19).

Most of the gene cassettes are promoterless, and thus, their expression depends on promoters in the *intI* gene or in the *attI* site that are oriented toward the integration point, namely, the P_c/P_2 promoters (1, 5, 20). The excision activity of an integrase is inversely proportional to the promoter strength (5), and this inverse correlation is due to a phenomenon of transcriptional interference. P_{int} and P_c are embedded and oriented in outward orientations, and strong transcription from P_c hinders transcription from P_{int}, inhibiting expression of the integrase gene (21).

Numerous antibiotic resistance genes are found on integrons. The bla_{BEL-1} and bla_{BEL-2} genes, encoding extended-spectrum β-lactamases (ESBLs), have been identified in numerous *Pseudomonas aeruginosa* isolates (22–24). The *bel*-1 and *bel-2* cassettes are associated with *aacA4* and *aadA5* gene cassettes, coding for an aminoglycoside-modifying enzyme, and also with the *smr* cassette, encoding resistance to antiseptics (Fig. 1) (22, 23).

We investigated here the putative mobility of the *bel-1* gene cassette and the role of several nonrelated antibiotic molecules in the putative induction of its mobility.

RESULTS

Role of antibiotic exposure in *bel-1* mobility within In*120*. After 10 days of *P. aeruginosa* culture with subinhibitory concentrations of antibiotics, only 2.1-kb PCR products could be obtained, consistent with the permanent presence of the *bel-1* cassette at the second position of the class 1 integron (Fig. 2). That gene cassette therefore remained at the second position of the integron over time under all conditions and upon exposure to nonrelated antibiotic molecules. The changes of MIC values for aztreonam and piperacillin between day 4 and day 10 were probably due to changes in cell permeability or in the upregulation of efflux pumps that is likely to occur in *P. aeruginosa* (see Table S1 in the supplemental material) (25).

Influence of the environment of the *bel-1* gene cassette on its mobility. As described above, the *bel-1* gene cassette associated with native integrase expression in

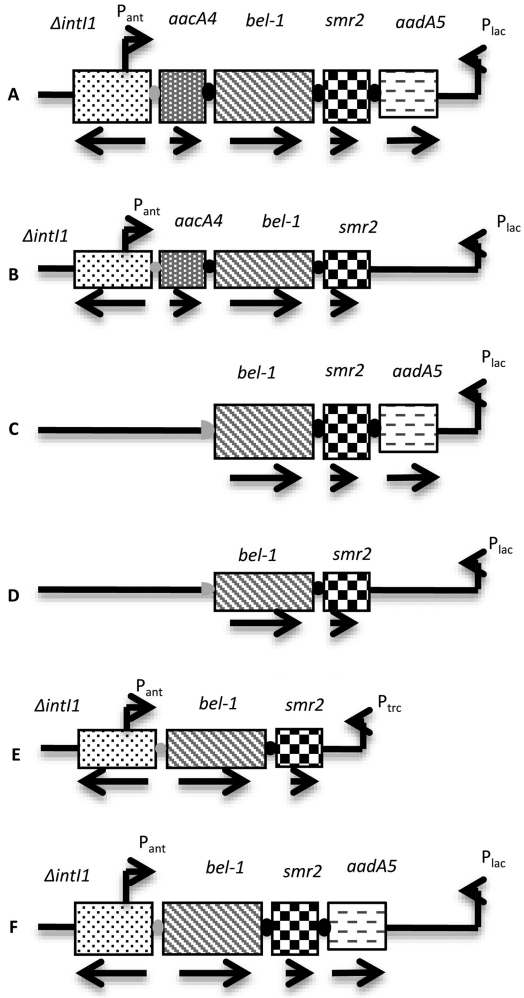

FIG 2 Schematic map of the plasmid constructs used in this study. Fragments were inserted into the multiple-cloning site of the shuttle vector pBBR1MCS.3, represented as a solid line. The coding regions are shown as boxes, with the arrows indicating the orientation of their transcription. Black and gray circles, *attC* and *attI1* sites, respectively; truncated gray circles, incomplete *attI1* site; broken arrows, the P_c and P_{lac} promoters. (A) pAac.Bel.Smr.Aad; (B) p.Aac.Bel.Smr; (C) p.Bel.Smr.Aad (incomplete *attI1* site); (D) p.Bel.Smr (incomplete *attI1* site); (E) p.IntI1.Bel.Smr; (F) p.IntI1.Bel.Smr.Aad.

the presence or absence of various antibiotics remained stable (Fig. 2). Therefore, the effect of IntI1 integrase overproduction was investigated. The ceftazidime- and rifampin-resistant (Caz^r-Rif^r) cointegrates were obtained only in strains containing p112.Kan (IntI1 overexpression), indicating that they are the result of integrase-mediated recombination (Table 1). The only site available for recombination with In*3* was the *bel-1 attC* site. The cointegrates that resulted from R388::pBel conjugation were selected as Caz^r-Rif^r colonies and were found at only a very low frequency (10^{-5}) (Table 1), suggesting that the *bel-1 attC* site was likely inefficient for recombination with the *attI1* site or an *attC* site in In*3*. The

TABLE 1 Cointegration and cassette excision-integration frequencies

Plasmid	Sites	Mean ± SD cointegration frequency
pAac.Bel.Smr.Aad	*attI1*, *aacA4–bel-1–smr2*	$6.8\ 0.10^{-3} \pm 1.8.10^{-3}$
p.Aac.Bel.Smr	*attI1*, *aacA4–bel-1*	$1.2.\ 10^{-3} \pm 4.35.10^{-4}$
p.IntI1.Bel.Smr.Aad	*attI1*, *bel-1–smr2*	$5.33.\ 10^{-4} \pm 1.75.10^{-4}$
p.IntI1.Bel.Smr.Aad	*attI1*, *bel-1*	$1.\ 10^{-4} \pm 9.10^{-5}$
p.Bel.Smr.Aad	*attI1*, *bel-1–smr2*	$8.3.10^{-5} \pm 6.3.10^{-6}$
p.Bel.Smr	*attI1*, *bel-1*	$1.\ 10^{-5} \pm 5.56.10^{-6}$

cointegration frequency was 5- to 10-fold higher for pAac.Bel.Smr.Aad, p.Bel.Smr.Aad, and p.IntI1.Bel.Smr.Aad, all of which carried the *smr2 attC* site, than for p.Bel.Smr and p.IntI1.Bel.Smr, which carried a *bel-1 attC* site. The *smr2 attC* site was thus more efficient for recombination than the *bel-1 attC* site itself. All these constructs contained a highly efficient recombination site (*smr2 attC*) that was likely mainly involved in the cointegration process. Overall, the cointegration frequencies seemed to depend on the recombination efficiency of the *attC* site available for recombination in the donor integron.

DISCUSSION

Recombination assays were carried out to investigate the recombination activities of the sites surrounding the *bel-1* gene cassette encoding the ESBL BEL-1 in *P. aeruginosa*. *Escherichia coli* DH10B containing an inactivated form of the recombinase RecA (*recA1*) limiting recombination between homologous sequences was used for that purpose. This feature ensures that the recombination events involved IntI1-mediated site-specific recombination and not RecA-mediated homologous recombination. The excision/mobilization experiments showed that the *attC* site of the *bel-1* gene cassette was inefficient and that this gene cassette was not mobilizable independently. This might likely be explained by the sequence of the *bel-1 attC* site itself, which does not correspond to the ones better recognized by the Int1 integrase (*attC* containing the T-N_6-G or T-N_6-C sequence), as demonstrated previously (26). Here the extrahelical bases constituting the *bel-1 attC* bottom strand (Fig. 3) are distantly related to those well recognized by Int1. We also showed that the *smr2 attC* site is enhanced over the *bel-1 attC* site, as it is involved in almost all recombination events when both gene cassettes are present on a plasmid. *smr2* always remained associated with the *bel-1* gene cassette. Noticeably, most of the gene cassettes were unnecessary for bacterial growth and may have been lost when the integrase was overexpressed in the absence of the corresponding selection. The excision of gene cassettes, considered independent units, depends on the flanking recombination sites; in this case, the flanking recombination site is *attC*.

A study revealed a similar phenomenon for the excision of the *veb-1* gene cassette (encoding another ESBL, namely, VEB-1, also found in *P. aeruginosa*), which occurs at only a low frequency due to an inefficient *attC* site (27). The *aadB* gene cassette includes a highly efficient *attC* site that allows efficient *veb-1–aadB* coexcision (27). This coexcision might also explain the frequent association between the *veb-1* and *aadB* gene cassettes in those class 1 integrons found in clinical isolates. Here we observed the same phenomenon with the *bel-1* donor gene cassette tandem.

Overall, our work provides some insights into the organization of bla_{BEL-1}-containing integrons. It is likely that those later evolved from a common ancestor carrying an early association between the *bel-1* and *smr2* gene cassettes (28). It is also possible that *smr2* was responsible for *bel-1* gene cassette recruitment and for the comobilization of *bel-1–smr2* into class 1 integrons. Although bla_{BEL-1}-containing integrons are subject to gene cassette rearrangements, we propose that the nature of *bel-1 attC* stabilizes its genetic environment, probably by impairing recombination events that could lead to its loss.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture conditions, and MIC determination. The bla_{BEL-1}-positive *P. aeruginosa* clinical isolate 51170 was from a previous study (see Table S2 in the supplemental material) (22). Its class 1 integron contained the *bel-1* gene cassette (Fig. 1). The recombination-deficient strain *Escherichia coli* DH10B (Life Technologies, Eragny, France) was used for bacterial electroporation experiments, and rifampin-resistant *E. coli* DH10B-Rif was used for conjugation experiments. The self-conjugative plasmid R388 (Tmp^r Tra^+) carrying an integron (In*3*) that contains the *dfrB2* gene cassette encoding resistance to trimethoprim (Tmp) was used in our experiments as an integration-recipient plasmid (29). Plasmid p112 (a pTRC99A derivative), conferring resistance to kanamycin and containing the *intI1* gene under the control of an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible synthetic P_{trc} promoter, was used for experiments under conditions of integrase gene overexpression (30). The tetracycline-resistant low-copy-number vector pBBR1MCS.3 was used for cloning experiments (31). Bacterial cells were grown in Trypticase soy (TS) broth or on TS agar plates (Sanofi Diagnostic Pasteur, Marnes-La-Coquette, France) with the following antibiotics, as appropriate: ceftazidime (15 μg/ml),

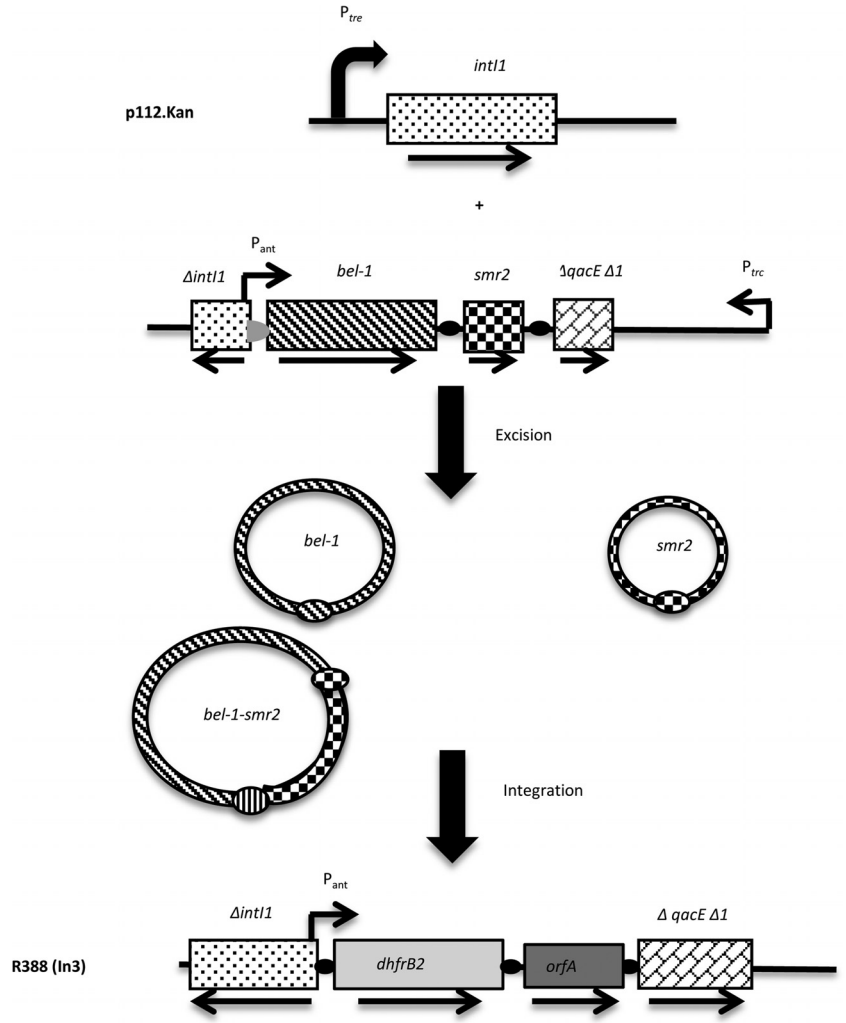

FIG 3 Schematic representation of assay of cassette excision/integration, using p.Bel.Smr.Aad, into the recipient integron In*3* on R388. Black circles, *attC* sites; truncated gray circle, incomplete *attI1* site.

rifampin (200 μg/ml), aztreonam (6 μg/ml), trimethoprim (25 μg/ml), tetracycline (15 μg/ml), kanamycin (30 μg/ml), gentamicin (12 μg/ml), piperacillin (12 μg/ml), and ciprofloxacin (8 μg/ml). MIC determinations were performed using disc diffusion and Etests (AB bioMérieux, La Balme-les-Grottes, France) by standard techniques according to CLSI guidelines (32).

To investigate the stability of the *bel-1* gene cassette under antibiotic exposure, *P. aeruginosa* was grown for 10 days with subinhibitory concentrations of gentamicin (12 μg/ml), to favor the stabilization of the *aacA4* cassette (encoding reduced susceptibility to gentamicin) at the first position, and of aztreonam (6 μg/ml), of piperacillin (12 μg/ml), and of ciprofloxacin (8 μg/ml) (likely to increase integrase expression by inducing the SOS system). *P. aeruginosa* was also grown for 10 days without any antibiotic as a control. Aliquots of the bacteria were plated every day for MIC determinations. Cassette mobility in *P. aeruginosa* was assessed daily for 10 days. DNA was extracted from aliquots collected daily, and primers INTIN and BEL-B were used in the PCR experiments to detect cassette mobility (Table 2). In the absence of *bel-1* cassette mobilization, the expected size of the PCR amplification product was 2.1 kb, although it was 1.5 kb if the cassette was mobilized (Fig. 2).

Nucleic acid extraction, PCR, and sequencing. PCR experiments with FastStart *Taq* polymerase were performed using the primers listed in Table 2 according to the manufacturer's recommendations (Roche Diagnostics, Meylan, France). The PCR products were purified over QIAquick columns (Qiagen, Courtaboeuf, France). An ABI Prism 3100 automated sequencer (Applied Biosystems, Les Ulis, France) and laboratory-designed primers were used for sequencing both strands. Recombinant plasmids were extracted using Plasmid Maxi kits (Qiagen) according to the manufacturer's instructions. Total DNA was extracted as described previously (26).

DNA manipulations and plasmid constructions. T4 DNA ligase and restriction endonucleases were used according to the manufacturer's recommendations (Amersham Biosciences, Orsay, France). An HindIII-digested omega fragment (ΩKm) from plasmid pHP45Ω-Km (18), consisting of a kanamycin resistance gene flanked by transcriptional and translational termination sequences, was inserted into the

TABLE 2 Sequences of primers used in this study

Primer name	Sequence 5′–3′	Target	GenBank accession no.	Positions	Reference or source
BEL-A	CGACAATGCCGCAGCTAACC	bla_{BEL-1}	DQ089809	1374–1393	This work
BEL-B	CAGAAGCAATTAATAACGCCC	bla_{BEL-1}	DQ089809	1822–1802	This work
INTIN	GCCAGGGCAGATCCGTGCAC	*intI1*	AF133699	716–735	30
AttI1-belfor	GCCCTAAAACAAAGTTAGACGTAAGCCTATAATCTC	*bel-1*	DQ089809	306–318, 958–980	This work
AttI1-belrev	TAGGCTTACGTCTAACTTTGTTTTAGGGCGACTGC	*attI1*	DQ089809	958–973, 300–324	This work
Smr-rev	CGCGACCGCAATGCCAACAC	*smr2*	DQ089809	2044–2063	This work
Aad-rev	CGAGCGTGGGACAGCTGCTT	*aadA5*	DQ089809	2369–2388	This work
5′-CS	GGCATCCAAGCAGCAAG	5′-CS class 1 integron	DQ089809	204–220	This work
3′-CS	AAGCAGACTTGACCTGA	3′-CS class 1 integron	AF133699	5381–5397	30

HindIII site in the multiple-cloning site of p112, resulting in recombinant plasmid p112.kan. The 1.2-kb EcoRI-BamHI fragment containing the *intI1* gene was excised from p112.Kan, and then the ends were filled in by using the *Pfu* DNA polymerase and the plasmid was self-ligated, resulting in p112.KanΔ*int*. The primers listed in Table 2 were used to amplify fragments from the genomic DNA of *P. aeruginosa* 51170, and the fragments were inserted into SmaT-restricted pBBR1MCS.3 to give pAac.Bel.Smr.Aad, p.Aac .Bel.Smr, p.Bel.Smr.Aad, p.Bel.Smr, p.IntI1.Bel.Smr, and p.IntI1.Bel.Smr.Aad (Fig. 3). These plasmids were introduced into *E. coli* DH10B by electroporation as previously described (31). TS agar plates containing tetracycline (15 μg/ml) and ceftazidime (15 μg/ml) were used for selection.

Induction of integrase expression. The various plasmids carrying the various amplified fragments were coelectroporated into *E. coli* DH10B(p112.Kan) or into *E. coli* DH10B(p112.KanΔ*int*). The strains were grown to stationary phase in TS broth containing 15 μg/ml tetracycline and 30 μg/ml kanamycin, then diluted 1,000-fold into 200 ml TS broth containing the same antibiotics at the same concentrations, and finally, incubated until growth was exponential (optical density at 600 nm, 0.5). Integrase gene expression was then induced by adding IPTG to a final concentration of 0.6 mM, and cells were harvested 2 h later.

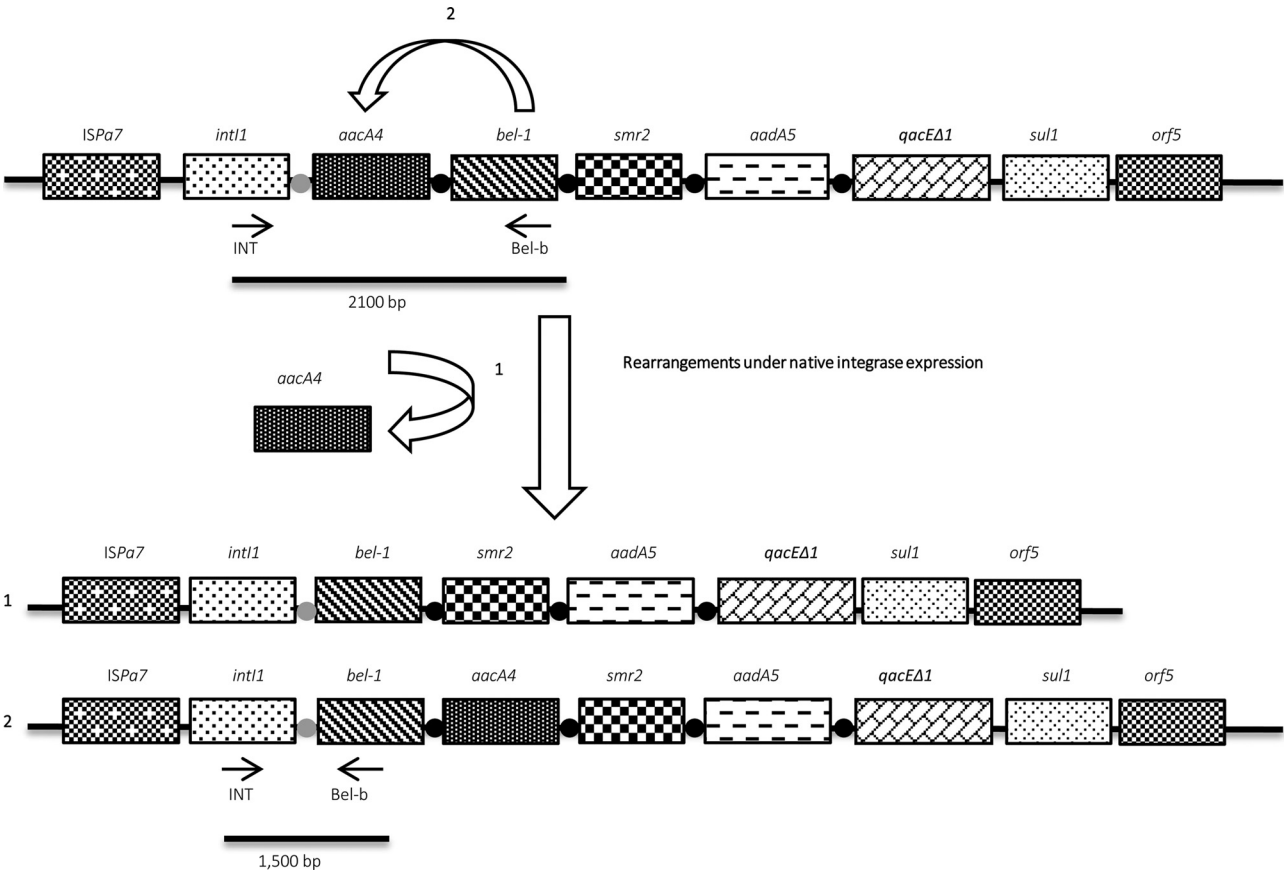

FIG 4 *bel-1* gene cassette mobility assay under conditions of native integrase expression before (day 0) and after 4 and 10 days of antibiotic exposure. In the absence of cassette mobilization, the expected size of the PCR amplification product is 2.1 kb; the expected size is 1.5 kb when the cassette has been mobilized. Black and gray circles, *attC* and *attI1* sites, respectively.

Excision/integration experiments. Integration of cassettes into the recipient integron In*3* on R388 was measured by mating-out assay experiments (Fig. 4). Each of the recombinant plasmids pAac .Bel.Smr.Aad, p.Aac.Bel.Smr, p.Bel.Smr.Aad, p.Bel.Smr, p.IntI1.Bel.Smr, p.IntI1.Bel.Smr.Aad, and pBBRMCS.3 (empty vector) were electroporated into *E. coli* DH10B(p112.kan, R388). Recombinant plasmid pAac .Bel.Smr.Aad was electroporated into *E. coli* DH10B(p112.KanΔ*int*, R388) as a negative control. Three single colonies were picked up for each condition and cultured overnight at 37°C in 10 ml TS broth containing 15 μg/ml tetracycline and 30 μg/ml kanamycin. These overnight cultures were diluted 10-fold in fresh TS medium without antibiotic and cultured under gentle agitation at 37°C for 1 h 30 s. IPTG was then added (induction of *intI1* expression), and culture was further continued for 2 h. Conjugation was performed by incubating 800 μl of recipient *E. coli* DH10B-Rif and 200 μl of the strain to be tested at 37°C for 3 h under gentle agitation. The mating mixture was then vortexed vigorously, placed on ice, and then plated. Aliquots of 100 μl of serial 10-fold dilutions were plated onto plates containing both trimethoprim (25 μg/ml) and rifampin (200 μg/ml) and both ceftazidime (15 μg/ml) and rifampin (200 μg/ml). For *E. coli* DH10B(R388, p112.kan, pBBRMCS.3), aliquots were plated either onto trimethoprim (25 μg/ml) plus rifampin (200 μg/ml)- or onto tetracycline (15 μg/ml) plus rifampin (200 μg/ml)-containing plates. The cointegration frequency was calculated by dividing the number of ceftazidime- and rifampin-resistant (Caz^r-Rif^r) transconjugants by the number of trimethoprim- and rifampin-resistant (Tmp^r-Rif^r) transconjugants.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00030-18.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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