Integrase-Mediated Recombination of the veb1 Gene Cassette Encoding an Extended-Spectrum β-Lactamase

Daniel Aubert, Thierry Naas*, Patrice Nordmann

Service de Bactériologie-Virologie, INSERM U914 “Emerging Resistance to Antibiotics,” LabEx LERMIT, Hôpital de Bicêtre, Assistance Publique/Hôpitaux de Paris, Faculté de Médecine Université Paris-Sud, Paris, France

Abstract

The veb1 gene cassette encodes the extended spectrum β-lactamase, VEB-1 that is increasingly isolated from worldwide Gram-negative rods. Veb1 is commonly inserted into the variable region of different class 1 integrons in which it is always associated with a downstream-located aadB gene cassette encoding an aminoglycoside adenylyltransferase. In Pseudomonas aeruginosa, the majority of veb1-containing integrons also carry an insertion sequence, IS1999 that is inserted upstream of the veb1 gene cassette and disrupts the integron specific recombination site, attI1. Investigation of the recombination properties of the sites surrounding veb1 revealed that insertion of IS1999 reduces significantly the recombination frequency of attI1 and that veb1 attC is not efficient for recombination in contrast to aadB attC. Subsequent sequence optimisation of veb1 attC by mutagenesis, into a more consensual attC site resembling aadB attC, successfully improved recombination efficiency. Overall, this work gives some insights into the organisation of veb1-containing integrons. We propose that IS1999 and the nature of veb1 attC stabilize the veb1 gene cassette environment likely by impairing recombination events upstream or downstream of veb1, respectively.

Introduction

Class 1 integrons are increasingly reported as a reservoir for antibiotic resistance genes in Gram-negative rods [1,2]. These structures possess two conserved regions located on each side of a variable region consisting of integrated gene cassettes [1–3] (Figure 1A). The 5′ conserved segment (5′-CS) classically includes a gene encoding a site-specific recombinase of the DNA integrase family, intI1, the cassette integration site, attI1, and the promoter Pc, which is oriented toward the integration point of the gene cassettes and is responsible for gene cassette expression [2–6]. Class 1 integrons may not always contain the entire 3′ conserved segment (3′-CS), which typically includes along with an open reading frame of unknown function (orf3), the truncated disinfectant (quinEA) and the sulfonamide (sulI) resistance determinants [1–3]. Gene cassettes from the variable region are composed of a gene, usually an antibiotic resistance gene, and a downstream recombination site known as gene, usually an antibiotic resistance gene, and a downstream [1–3]. Gene cassettes from the variable region are composed of a

Gene cassettes are non-replicative mobile elements that exist under a free circular or integrated linear form [3,7]. Site-specific recombination leading to gene cassette excision or integration is catalyzed by the integrase IntI1, which recognizes two structurally distinct sites, attI1 and attC [7,8]. The attI1 site is particularly conserved and includes four integrase binding domains (Figure 1B). A pair of inversely oriented binding sites is located at the core site and two other integrase binding sites in direct repeat (DR1, DR2) are located further upstream [2,9,10]. A full attI1 site containing four integrase binding domains is required for optimal recombination with an attC site [11,12]. The attC sites that are associated with the gene cassettes are more complex and weakly related to each other [2]. They differ greatly in sequence and length but contain two pairs of inversely oriented integrase-binding domains (1L-2L and 2R-1R) [13,14] (Figure 1C and 1D). Recombination mediated by IntI1 involves recognition of the bottom strand of the attC site [15]. Upon folding into a hairpin structure, single-stranded attC sites present an almost canonical core site consisting of 2L-2R and 1L-1R duplexes separated by a bulged area [2,16–20]. However attC site recognition and proper interaction with the integrase are not dependent on canonical DNA but on the position of two extrahelical bases that interact with the integrase and originate from symmetrical folding of the bottom strand of attC [19].

Integrase-mediated recombinations between two attC sites, between two attI1 sites, and between an attI1 site and an attC site have been documented, the latter being the most efficient [8,21]. During recombination the crossover point is located between the G and TT of the 7-bp core site motif, GTRRRRY, found at the 3′ end of the recombination sites [2,14].

Among the antibiotic resistance genes that are integron-located, the extended spectrum β-lactamase (ESBL) blaVEB-1 gene has been identified in a series of Gram-negative rods that are scattered...
worldwide [22–26]. At least seven different types of veb1-containing integrons were identified based on cassette content [22,23,26]. It is more than likely that the different veb1-containing integrons evolved from a common ancestor, however they have maintained interesting characteristics. In all cases, veb1 is associated with a downstream-located aadB cassette encoding an aminoglycoside acetyltransferase (Figure 1A) [26]. An insertion sequence (IS1999) is inserted in the majority of the veb1-containing integrons characterized in P. aeruginosa [26,27]. Upon insertion, IS1999 disrupts the integron-specific recombination site, attI1, but provides an outward-directed promoter P out, which increases blaVEB-1 expression in P. aeruginosa (Figure 2A) [28]. Moreover, veb1 has always been reported as the first cassette within the variable region of IS1999-containing integrons carrying different cassette arrays [26,27].

The stability of the veb1 environment was puzzling given the fact that the variable region of an integron is normally in constant evolution since it is subject to gene cassette rearrangement, loss and acquisition [3]. Cointegration assays were performed to determine the relative recombination efficiency of the different recombination sites present in the veb1 vicinity (i.e. attI1, disrupted attI1, veb1 attC and aadB attC). This work revealed that veb1 attC and the disrupted attI1 site of IS1999-containing integrons are not efficient for recombination and consequently might preserve the associations veb1-aadB and IS1999-veb1 from being disrupted, respectively.

**Results**

The veb1 attC site is not efficient for recombination

Integrase-mediated recombination involving integrons located on multicopy plasmids can generate different recombination products including: (i) free circular DNA molecules comprising one or more gene cassettes, resulting from recombination between the gene cassette attC and either attI1 or another attC site from another gene cassette [7]; (ii) cointegrates, resulting from recombination between two copies of the same plasmid. Different cointegrates can be formed depending on the sites that are available for recombination; (iii) gene duplications, which can arise by either insertion of a second gene copy encoded on a previously excised gene cassette or by formation and resolution of cointegrates (Figure S2). While the abundance of circular intermediates is very low, plasmid cointegrates and gene duplications are predominantly formed during recombination when intI1 is overexpressed [29].

![Figure 1. Schematic representations of the veb1-containing integron from P. aeruginosa 14 and of the recombination sites [26].](A) veb1-containing integron from P. aeruginosa 14. The 5’ and the 3’-CSs are underlined. ORFs are shown as boxes with an arrow indicating the orientation of the coding sequence. The promoter P is indicated by a broken arrow. The black diamond represents attI1 and circles represent attC sites. (B–D) Recombination sites. Sequences related to the 7-bp core site are boxed; their relative orientations are indicated with arrows. The crossover points are marked by vertical arrows. The region derived from the downstream-located cassette at the recombination point is in lower case. The extra-helical bases (EHBs) as defined by Bouvier et al. [18] are marked with an asterisk. (B) attI1: the nucleotides belonging to attI1 are indicated in white on a black background. The experimentally determined strong and weak IntI1-binding sites and the pair of direct repeats, DR1 and DR2, as well as the location of the IS1999 insertion in attI1 are indicated. (C) veb1 attC and (D) aadB attC: 7-bp putative core sites (1L, 2L, 2R and 1R) related to the core site consensus as defined by Stokes et al. [14] are shown. The aadB gene cassette is boxed in grey.

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Figure 2. Schematic representation of the vebl-containing integron from *P. aeruginosa* 15 and of the disrupted *attl* recombination site (**A**). vebl-containing integron from *P. aeruginosa* 15. ORFs are shown as boxes with an arrow indicating the orientation of the coding sequence. Promoters *Pc* and *Pout* are indicated by broken arrows. Disruption of site is indicated by boxes. The crossover point is marked by vertical arrow. The region derived from the downstream-located cassette (vebl) at the recombination point is in lower case. The 7-bp from IS1999 replacing the fourth integrase binding site from attl are shown in a dashed box.

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Figure 3. *bla*vebl-specific hybridization and PCR amplifications of vebl-containing fragments. (**A**) *bla*vebl-specific hybridization of small BspE1-digested DNA fragments from *E. coli* DH10B (p112.Kan) harboring pAttI.veb (1), pAttI.veb.aadB (2), pAttI.IS.veb (3), pAttI.IS.veb-1.aadB (4), pAttI.veb* (5), pAttI.veb.aadB* (6), pVeb (7), pVeb.aadB (8) and from *E. coli* DH10B (pTRC99A.Kan) harboring pAttI.veb.aadB (9). Locations of the low (L) 1.1-kb and high (H) 1.6-kb signals are indicated. (**B**) PCR amplifications of vebl-containing fragments using the outward directed primers VEBINV3-VEBINV2 and ca. 1.1-kb (L) and 1.6-kb (H) DNAs that were gel-extracted based on (A), as template. 

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There were excised cassettes that are unnecessary for bacterial growth are often excised and lost from the variable region [21].

The relative recombination efficiency of the sites present within different vebl-containing constructs was tested (Table 1 and Figure S1). Upon induction of integrase expression, circular DNA molecules (including plasmids, cointegrates and excised genes cassettes) were purified, digested with BspE1 and separated according to their size on an agarose gel. The bottom part of the gel containing only small DNA molecules (<2-kb) was analysed and vebl-containing products were detected by hybridization using a *bla*vebl-specific probe (Figure 3A). Our experimental conditions specifically allow for detection of recombination events that involved an *attI* and an *attC* site but do not distinguish whether the product is an excised cassette, a gene cassette duplication or a cointegrate (Figure S2).

Plasmid pAttI.veb contains a full *attI* site, a vebl cassette and a truncated *aadB* cassette, while pAttI.veb.aadB contains, in addition to *attI* and vebl, a full-length *aadB* cassette. While *attI* and vebl *attC* sites are theoretically the only sites available for recombination in pAttI.veb, pAttI.veb.aadB offers an additional recombination site, *aadB* *attC*. Recombination between *attI* and vebl *attC* leading to either vebl cassette excision, duplication or plasmid cointegration should produce, after BspE1 digestion, a vebl-containing DNA fragment of 1.1-kb (Figure S2 and S3). However, no such product was observed with pAttI.veb and pAttI.veb.aadB, suggesting that vebl *attC* is not efficient for recombination (Figure 3A lanes 1 and 2). Instead, a 1.6-kb product was clearly detected using circular DNA extracts from *E. coli* (pAttI.veb.aadB) (Figure 3A lane 2). Additional experiments confirmed that the 1.6-kb product was integrase-mediated since it was only detected when IntI1 was expressed (Figure 3A lanes 2 and 9).

A more sensitive approach using PCR amplification was used. Circular DNA molecules were purified, digested and separated on an agarose gel as before but agarose gel slices were cut at the expected migration of the 1.1-kb and 1.6-kb DNA fragments. Gel-extracted DNAs were then subjected to PCR using outward-directed *bla*vebl-specific primers. These primers are located on each side of the BspE1 restriction site and specifically allow for amplification of vebl-containing DNA fragments that were linearized (excised gene cassettes) or released upon BspE1 restriction from cointegrates and plasmids with vebl duplication (Figure S2 and S3).

PCR amplification using DNA extracted from the 1.1-kb gel slices from *E. coli* (pAttI.veb) and *E. coli* (pAttI.veb.aadB) yielded a 0.7-kb PCR product (Figure 3B lanes 1L and 2L). Sequencing indicated that it corresponded to vebl and further analysis of the recombinant junction confirmed that recombination occurred precisely between *attI* and vebl *attC* (Figure 1B, 1C and 4).
Similarity, PCR amplifications using the 1.6-kb product from E. coli (pAttI.veb.aadB) yielded a 1.3-kb fragment (Figure 3B lane 2H). Sequencing revealed that it contained both veb1 and aadB gene cassettes and that recombination occurred precisely between veb1 and aadB attC (Figure 1B, 1D and 4). The predominant formation of the 1.6-kb product also indicated that aadB remained mostly adjacent to veb1 after recombination.

These results underline the weak activity of veb1 attC for recombination and the stability of the association veb1-aadB. Both veb1 attC and aadB attC can recombine with attI1. However in contrast to veb1 attC, aadB attC is highly efficient for recombination as it is favored over veb1 attC during integrase-mediated recombination.

Plasmids pVeb and pVeb.aadB containing a truncated attI1 site with only the 7-bp core site motif (GTTAGCG) at the junction with the veb1 gene cassette were tested for recombination (Figure 1B and S1). Surprisingly, recombination products were detected in circular DNA extracts from E. coli (pVeb.aadB) albeit at low levels (Figure 3A lane 8 and 3B lane 8H). Sequencing revealed that recombination occurred between aadB attC and a secondary site (1R) instead of the expected GTTAGCG motif of the veb1 gene cassette (Figure 4). This secondary recombination site (GTTAATG) is homologous to a consensus core site GTTRRRY and is located 32-bp downstream of the translation initiation codon of blaVEB-1. Thus, recombination resulted in a truncated veb1 gene cassette associated to aadB (Figure 4). Despite several attempts, we were not able to detect by PCR any product containing the truncated veb1 cassette alone (Figure 3B lanes 7L and 8L). As expected, the 7-bp core site is not sufficient to support precise recombination with attC.

Sequence optimisation of veb1 attC improves recombination

The aadB attC site fits closely to the consensus sequence of an attC site [2,14]. It is 60-bp long and made of two nearly perfect inverted repeats, which are bounded by sequences matching precisely the consensus RYYYYGAC/GTTRRRY (Figure 1D). Moreover, the bottom strand of aadB attC contains the two extrahelical bases (T-N6-G) found in the most easily excisable attC sites [30] (Figure 5). Therefore it is not surprising that aadB attC worked efficiently in our recombination assays. Folding of the bottom strand of veb1 attC revealed a characteristic secondary structure with the three structural elements common to attC sites [2] (Figure 5). However, veb1 attC presents two striking differences as compared to aadB attC: (i) the veb1 attC site (133-bp) has a longer variable terminal structure (VTS) and (ii) the two putative

**Table 1. Strains and plasmids.**

| Strain or plasmid | Relevant characteristics* | Source and/or reference |
|-------------------|---------------------------|-------------------------|
| **Strains**       |                           |                         |
| *P. aeruginosa*   |                           |                         |
| #14               | Clinical isolate containing class 1 integron, veb1 | [26]                     |
| #15               | Clinical isolate containing class 1 integron, IS1999, veb1, aadB | [26]                     |
| **E. coli**       |                           |                         |
| DH10B             | F- mcrA Δ(mrr-hsdRMS-mcrBC) 9800aa2ZAM15 Δloc74 recA1 endA1 araD139 Δ(ara leu) 7697 galU galK rpsL nupG λ- | Life Technologies       |
| DH10B-Rif         | DH10B, RifR                | Laboratory stock        |
| **Plasmids**      |                           |                         |
| R388              | 33kb IncW plasmid containing class 1 integron In3 (dfrB2, orfA), Trα+, TmpR | [38]                     |
| p112              | pTRC99A: intI1, expression vector for integrase, Ptrc, AmpR | [33]                     |
| p112.Kan          | p112 derivative, expression vector for integrase, Ptrc, intI1, KanR | This study               |
| pTRC99A.Kan       | p112.Kan derivative without intI1, KanR | This study               |
| pBBR1MCS.3        | Broad host range cloning vector, TetR | [34]                     |
| pVeb              | class 1 integron containing veb1 in pBBR1MCS.3, CazR, TetR | This study               |
| pVeb.aadB         | class 1 integron containing veb1, aadB in pBBR1MCS.3, CazR, TetR | This study               |
| pAttI.veb         | class 1 integron containing attI1, veb1 in pBBR1MCS.3, CazR, TetR | This study               |
| pAttI.veb.aadB    | class 1 integron containing attI1, veb1, aadB in pBBR1MCS.3, CazR, TetR | This study               |
| pAttI.SS.veb      | class 1 integron containing disrupted attI1, IS1999, veb1 in pBBR1MCS.3, CazR, TetR | This study               |
| pAttI.SS.veb.aadB | class 1 integron containing disrupted attI1, IS1999, veb1, aadB in pBBR1MCS.3, CazR, TetR | This study               |
| pAttI.veb*        | pAttI.veb derivative, sequence modification of veb1 attC to match aadB attC (veb1 attC*) | This study               |
| pAttI.veb*aadB    | pAttI.veb.aadB derivative, sequence modification of veb1 attC to match aadB attC (veb1 attC*) | This study               |
| pAttI.veb*        | pAttI.veb derivative, veb1 with shorter veb1 attC (veb1 attC*) | This study               |
| pAttI.veb†        | pAttI.veb derivative, sequence modification of the integrase binding sites 2L and 2R from veb1 attC (veb1 attC) | This study               |
| pAttI.veb‡        | pAttI.veb derivative, sequence modification of the integrase binding sites 2L and 2R from veb1 attC (veb1 attC) | This study               |

*Antibiotic resistance: AmpR, ampicillin; CazR, ceftazidine; KanR, kanamycin; RifR, rifampin; TetR, tetracycline; TrimR, trimethoprim.

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Figure 4. Recombinant junction analysis. (A) Representation of the pAttI.veb.aadB recombinant plasmid. The location of the different crossover points involved in recombination are indicated by a number: (1) 7-bp core site of attI-veb1; (1a) secondary recombination site found within the bla<sup>vgg</sup> coding sequence; (2), (2*) and (3) 7-bp site found at the veb1 attC-aadB, veb1 attC*-aadB and aadB attC-qacE<sub>AI</sub> junctions, respectively. (B) Sequencing results of the recombinant junctions from experimentally isolated veb1-containing recombination products. The numbers shown on the left indicate the recombination sites that were involved to create the recombinant junctions. Sequences related to the 7-bp core site are boxed. The crossover point is marked by vertical arrow. The nucleotides belonging to the 7-bp core site of veb1 attC are boxed. The crossover point is marked by vertical arrow. The nucleotides belonging to the 7-bp core site of veb1 attC are boxed. The crossover point is marked by vertical arrow. The nucleotides belonging to the 7-bp core site of veb1 attC are boxed. The crossover point is marked by vertical arrow. The nucleotides belonging to the 7-bp core site of veb1 attC are boxed. The crossover point is marked by vertical arrow. The nucleotides belonging to the 7-bp core site of veb1 attC are boxed.

Vebl attC binding sites in thus reducing the size and restoring the 2L and 2R consensus and 1R binding sites was replaced with the one from P. aeruginosa, thus reducing the size and restoring the 2L and 2R consensus binding sites in veb1 attC (veb1 attC*) (Figure 5D). In this configuration, ca. 1.1-kb products were strongly detected with pAttI.veb* or pAttI.veb*aadB (Figure 3A lane 5 and 6), which indicate that recombination between attI and veb1 attC* occurred. Moreover, in cells harboring pAttI.veb*,aadB, veb1 and veb1-aadB-containing recombination products were detected at similar levels (Figure 3A lane 6) suggesting that veb1 attC* and aadB attC have a similar recombination efficiency. Sequencing confirmed that precise recombinant junctions were taken place and involved either the veb1 attC* site or the aadB attC site (Figure 4).

These results indicate that the sequence located between the 1L and 1R sites from veb attC is not optimal for recombination. The veb1 attC site was further modified to determine whether the long intermediate region located between the 2L and 2R integrase binding sites or the 2L and 2R sequences that diverged from an attC consensus sequence was responsible for the low recombination efficiency of veb attC. Two different veb1 attC sites named veb1 attC<sub>A</sub> and veb1 attC<sub>B</sub> were generated. The DNA segment located between the 2L and 2R binding sites from veb1 attC was reduced to 20-bp as found in aadB attC giving rise to veb1 attC<sub>A</sub> (Figure 5E). In veb1 attC<sub>B</sub>, the 2L and 2R sequences were modified to match the 2L and 2R sequences found in aadB attC (Figure 5F).

Plasmids pAttI.veb<sub>A</sub> and pAttI.veb<sub>B</sub> were tested for recombination (Figure 6). In contrast to veb1 attC*, veb1 attC<sub>A</sub> and veb1 attC<sub>B</sub> did not allow for the detection of veb1-containing recombination products by hybridization (Figure 6A lanes 1–2 and 4). However, veb1-containing recombination products were detected after PCR amplification indicating that veb1 attC<sub>A</sub> and veb1 attC<sub>B</sub> were functional (Figure 6B lanes 1–2). Overall, veb1 attC<sub>A</sub>, veb1 attC<sub>B</sub> and wild type veb1 attC had similar activities indicating that modifications made to reduce the VTS or change the 2L and 2R sequences did not improve the recombination efficiency.

**IS1999 insertion decreases attI recombination efficiency**

In *P. aeruginosa*, the attI site of many veb1-containing integrons is disrupted by IS1999 in such a way that only the last 34-bp of the site (containing three integrase binding sites) remain adjacent to veb1 (Figure 2B). Using circular DNA isolated from *E. coli* (pAttI.IS.veb.aadB), veb1-containing recombination products that resulted from recombination between the disrupted attI and aadB attC were detected by hybridization (Figure 3A lane 4). Recombination products involving the disrupted attI and veb1 attC were only detected after PCR amplification (Figure 3B lanes 3L and 4L).

These results suggest that the attI site disrupted by IS1999 insertion is still functional for recombination. Nevertheless, based on signal intensity, the recombination efficiency of the disrupted attI site seemed significantly lowered in comparison to a full-length attI site (Figure 3A lanes 2 and 4, 3B lanes 1L and 3L).

**Recombination frequencies**

Activities of the sites present within different veb1-containing constructs (Table 1 and Figure S1) were assayed in vivo by measuring the frequency of recovery of cointegrates formed between the test plasmids and plasmid R388. The self-conjugative plasmid R388 (TmpR, Tra<sup>+</sup>) contains an integron (In3) that contains the *draB2* cassette conferring resistance to trimethoprim (Tmp) followed by an open reading frame, *orfA*, of unknown function.

*Veb1*-containing constructs (conferring resistance to ceftazidime, Cas<sub>z</sub>) were introduced into a rifampin sensitive *E. coli* containing the integron expressing plasmid and plasmid R388. Upon induction of integrase expression, cointegrates resulting from recombination between the plasmid-located *veb* integrons and the recipient integron In3 located on plasmid R388 were predominantly formed. Plasmid R388 and cointegrates were transferred by conjugation into *E. coli* DH10B-Rif (rifampicin resistant) and cointegration frequencies were measured as ratios of cointegrates (Cas<sub>R-RifR</sub>) to total R388 transconjugants (TmpR-R<sub>RifR</sub>) (Table 2).
Plasmid cointegration was IntI1 mediated since CazR-RifR cointegrates were only recovered from strains over-expressing the integrase. In plasmid pVeb, \textit{veb1 attC} is the only site available for recombination with either \textit{attI1} or an \textit{attC} site from In3. Cointegrates (pVeb::R388) were selected at a very low frequency (4.41×10^{-2}) close to the limit of detection of our assay, thus reflecting the inefficiency of \textit{veb1 attC} for recombination. As compared to pVeb, the presence of \textit{aadB attC} in pVeb.aadB, led to a 50-fold increase in the cointegration frequency, confirming that \textit{aadB attC} is more efficient for recombination than \textit{veb1 attC}. Comparison between (pVeb::R388) and (pAttI.IS.veb::R388) revealed that the disrupted \textit{attI1} site is 3-times more efficient for recombination.

**Figure 5. Bottom strand secondary structures.** (A) consensus \textit{attC} structure as of Cambray et al. [2]; (B) \textit{aadB attC}; (C) \textit{veb1 attC}; (D) \textit{veb1 attC*}; (E) \textit{veb1 attC}'; and (F) \textit{veb1 attC}. The putative IntI1 binding domains are marked with boxes and the inverted repeats 1L-1R and 2L-2R are indicated. The protruding G that determines the recombination strand present in 2L and the protruding T that increases the recombination efficiency, are also boxed. CT, conserved triplet; UCS, unpaired central spacer; EHB, extrahelical base; VTS, variable terminal structure. Folded representations were based on secondary structures obtained thanks to the mfold Web Server. The folded structure of the \textit{veb1 attC*} site was similar to the folded structure of \textit{aadB attC} and possessed a free energy of −30.3 Kcal/mol.

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**Figure 6. vebl-containing recombination products depending on vebl attC variants.** (A) Bl0vebl-specific hybridization of small BspEII-digested DNA fragments from E. coli DH10B (p112.Kan) harboring pAttI.veb (1), pAttI.veb+ (2), pAttI.veb (3), pAttI.veb+ (4), and from E. coli DH10B (pTRC99A.Kan) harboring pAttI.veb (5). (B) PCR amplifications of DH10B (pTRC99A.Kan) harboring pAttI.veb'+ (1), pAttI.veb1 (2), pAttI.veb (3), pAttI.veb* (4), and from E. coli specific hybridization of small BspEII-digested DNA fragments from pAttI.veb (3), pAttI.veb* (4), and from E. coli Sequence optimisation of in the cointegration frequency, as compared to plasmid pAttI.veb. However, recombination than the 7-bp core site from pVebl. However, insertion of IS1999 into attI1 (pAttI.LIS.veb) led to a 6-fold decrease in the cointegration frequency, as compared to plasmid pAttI.veb. Sequence optimisation of vebl attC into vebl attC* led to an almost 20-fold increase in the cointegration frequency of plasmid pAttI.veb+ as compared to pAttI.veb. Similar cointegration frequencies were obtained with plasmids pVebl.vebl.aadB, pAttI.vebl.vebl.aadB, pAttI.LIS.vebl.vebl+aadB and pAttI.vebl+aadB. All these constructs contain the highly efficient recombination site aadB attC that was likely mainly involved in cointegration.

For each plasmid tested, several cointegrates were analyzed by PCR to determine which sites (i.e. attI1, vebl attC, vebl attC* or aadB attC from the vebl-containing plasmids) were involved in the recombination. As expected, all the pVebl:R388 and pVebl.aadB:R388 cointegrates involved vebl attC and aadB attC, respectively. Cointegrates pAttI.vebl:R388 and pAttI.LIS.vebl:R388 were formed by recombination using attI1 or vebl attC. All the pAttI.vebl.aadB:R388 and pAttI.LIS.vebl.aadB:R388 cointegrates involved aadB attC and retained the aadB cassette in association with vebl. All the pAttI.vebl:R388 cointegrates involved vebl attC* and the pAttI.vebl.aadB::R388 cointegrates had systematically lost the aadB cassette. Since aadB cassette excision could occur before or after cointegration, we could not determine whether pAttI.vebl.aadB:R388 arose by cointegration involving vebl attC* or aadB attC.

CazR-RifR transconjugants were further screened for tetracycline susceptibility to discriminate cointegrates from precise vebl insertion into In3. In contrast to cointegration, which results from one recombination event; cassette insertion results from two recombination events (cassette excision-integration or plasmid cointegration-resolution) and occurs at a lower frequency. Despite most colonies (>95%) were resistant to tetracycline, few CazR-RifR-TetS transconjugants were identified. CazR-RifR-TetS transconjugants were recovered only from donor cells containing pAttI.vebl.vebl+aadB, pAttI.vebl* and pAttI.vebl+aadB plasmids with similar frequencies (Table 2). Each of these constructs has the particularity to carry at least two efficient sites that were used during cassette insertion. More than 95% of the CazR-RifR-TetS transconjugants recovered from cells containing pAttI.vebl.vebl+aadB had integrated vebl along with aadB, demonstrating that vebl is co-mobilized with aadB. Using plasmid pAttI.vebl+aadB, both vebl and aadB insertions were found, however vebl inserts were mostly recovered indicating that vebl recombines efficiently and independently of aadB when it contains vebl attC*. 

**Discussion**

Recombination activities of the sites surrounding the vebl gene cassette were investigated by using two independent recombinase-Mediated vebl Mobilization

**Table 2. Cointegration and cassette integration frequencies.**

| Plasmid*                        | Plasmid cointegration frequencyb | Cassette integration frequency | Number of CazR-RifR-TetS colonies* | vebl gene cassette insertion | vebl-aadB gene cassette insertion |
|---------------------------------|----------------------------------|---------------------------------|-------------------------------------|-----------------------------|----------------------------------|
| pAttI.vebl.aadB + pTRC99A.Kan  | 0                                | -                              | -                                   | -                           | -                                |
| pBBR1MCS.3 + p112.Kan           | 0                                | -                              | -                                   | -                           | -                                |
| pVebl + p112.Kan                | 4.41 × 10⁻⁶ (±/-1.96 × 10⁻⁶)      | NDd                            | 0                                   | -                           | -                                |
| pVebl.vebl+aadB + p112.Kan      | 2.15 × 10⁻⁵ (±/-1.80 × 10⁻⁵)      | ND                             | 0                                   | -                           | -                                |
| pAttI.vebl+aadB + p112.Kan      | 7.35 × 10⁻⁶ (±/-3.26 × 10⁻⁶)      | ND                             | 0                                   | -                           | -                                |
| pAttI.LIS.vebl+aadB + p112.Kan  | 3.31 × 10⁻⁶ (±/-1.89 × 10⁻⁶)      | 1.44 × 10⁻⁴                    | 21                                  | 1                           | 20                               |
| pAttI.LIS.vebl+aadB + p112.Kan  | 1.23 × 10⁻⁴ (±/-3.36 × 10⁻⁵)      | ND                             | 0                                   | -                           | -                                |
| pAttI.LIS.vebl+aadB + p112.Kan  | 3.79 × 10⁻⁴ (±/-10.10⁻⁶)          | ND                             | 0                                   | -                           | -                                |
| pAttI.vebl+aadB + p112.Kan      | 1.37 × 10⁻⁴ (±/-2.52 × 10⁻⁶)      | 1.70 × 10⁻⁴                    | 6                                   | 6                           | -                                |
| pAttI.vebl+aadB + p112.Kan      | 6.25 × 10⁻⁷ (±/-7.53 × 10⁻⁸)      | 2.07 × 10⁻⁸ (1.55 × 10⁻⁷)      | 16                                  | 12                          | 4                                |

*Donor strains also contain R388; pTRC99A.Kan did not express IntI1 (negative control); p112.Kan (pTRC99A.Kan derivative) expressed IntI1. The pVebl, pVebl.vebl+aadB, pAttI.vebl, pAttI.vebl.vebl+aadB, pAttI.LIS.vebl.vebl+aadB, pAttI.vebl+aadB and pAttI.vebl+aadB were used for recombination (Figure S1). pBBR1MCS.3, cloning vector (negative control).

The limit of detection of our assay is <1.5 × 10⁻⁵.

Standard deviations calculated from three independent experiments.

*ND, Not determinable.

up to 484 CazR, RifR transconjugants were tested for tetracycline sensitivity.

Integration frequency of the vebl gene cassette alone.

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tion assays. It is noteworthy that experiments were performed in E. coli DH10B, which contains an inactivated form of RecA (RecA1) excluding recombination between homologous sequences. Thus, despite plasmids share some sequence identity, cointegration is unlikely to have occurred by homologous recombination but is truly the result of site-specific recombination mediated by the integrase.

Dissemination and acquisition of a single gene cassette within the variable region of integrons is the result of integrase-mediated recombination between two different sites, one of which is the gene cassette attC [7]. We demonstrate here that veb1 attC found within the veb1 gene cassette, is not efficient for recombination implying that veb1 alone is not highly mobilizable. Our cointegration experiments also revealed that aadB attC is favored over veb1 attC as it is almost exclusively involved in recombination when both gene cassettes are present on a plasmid. Despite no selection, aadB remained associated to wild type experiments also revealed that veb1 attC recombination sites that are flanking them. Therefore, it was anticipated that aadB would be rarely excised since aadB excision require the involvement of veb1 attC. Stability of the association veb1-aadB within integrons is likely preserved by the nature of veb1 attC, which impairs not only aadB excision but also new insertions of gene cassettes in between them. Our cassette integration assay in plasmid R388 showed that the association veb1-aadB is to the benefit of veb1, which is mobilized along with aadB thanks to the high recombination efficiency of aadB attC.

The aadB attC site is 50 times more efficient for recombination than veb1 attC. Sequence optimisation of veb1 attC into a more consensual attC site resembling aadB attC (veb1 attC*) improved drastically the recombination efficiency and allowed independent excision of aadB from veb1-containing integrons. This experiment revealed also that the features responsible for the weak activity of veb1 attC reside within the DNA sequence located between the 1L and 1R integrase binding sites. Two major differences between veb1 attC and aadB attC were found in this region and concerned: (i) the 2L and 2R integrase binding sites, which sequences in veb1 attC diverged from the consensus and (ii) the length of the variable terminal structure (VTS). However, modification of one or the other in veb1 attC was not sufficient to improve recombination activity. While VTS seem to have a minor role in the attC recombination efficiency, other structural features including the unpaired central spacer (UCS) bulge shape and two extrahelical bases (EHBs) appear to be important for recognition and recombination rate [18]. In contrast to aadB attC, veb1 attC presents slightly larger UCS. Also, while the EHBs in aadB attC are 6 nucleotides apart (T-N-G) as found in the attC sites flanking cassettes that are efficiently excised by IntI1 [30], veb1 attC contains only one EHB (G). It has been recently demonstrated that the identity and spacing of the EHBs in the attC sites has a pronounced effect on the efficiency of cassette excision [30]. The relevance of these structural differences in the activity of veb1 attC remains to be established. It is also possible that the recombination efficiency of veb1 attC is not affected by only one of these features but by several of them.

Several studies have shown that a full attI site containing the four integrase binding sites was required for high efficiency recombination with attC and that progressive 5’ deletions reaching closer to the 7-hp core site of attI leads to decreased recombination rates [11,12,31]. Insertion of IS1999 into attI displaces only the weak integrase binding site located in DR2. Accordingly, the disrupted attI is still sufficient to support recombination albeit at a lower frequency than a full attI site. Analysis of the bases from IS1999 replacing part of attI did not reveal any obvious 7-hp core site sequence, which could have compensated for the loss of the fourth integrase binding site. Also, by reducing the recombination rate of attI, IS1999 likely increases the stability of veb1 and consequently aadB at the first positions within the variable region by impairing integration of new gene cassettes upstream of veb1 and excision of veb1-aadB. However, since the disrupted attI still supports recombination, new gene cassettes could be inserted at the first position and benefit from the additional promoter Pout located in IS1999 for their expression [20].

Several studies have demonstrated that integrase is also able to catalyse recombination between one specific site (attI or attC) and non-specific secondary sites conforming to the consensus GNT at a very low frequency [12,21,31]. Moreover, limitation of attI to the 7-hp core site is insufficient for determining recombination specificity alone and leads to the formation of cointegrates owing to recombination with secondary sites [12]. Using plasmid pVeb.aadB, we demonstrated upon PCR amplification that aadB attC is able to recombine with the secondary site (GTGAAATG) located within the blaVEB-1 gene. This event resulting in a truncated veb1 gene cassette is extremely rare since it has been detected only once in our repeats. The region that is truncated in blaVEB-1 encodes the first eleven amino acids of the pre-enzyme VEB-1 and includes the translation initiation codon and most of the signal peptide. Even upon insertion in an environment providing transcription and translation signals, it is unlikely that the protein encoded by the truncated veb1 cassette would be functional in vivo since it would not be properly targeted to the periplasm.

Overall, this work gives some insights into the organisation of veb1-containing integrons that are widespread among Gram-negative bacteria. It is more than likely that the different veb1-containing integrons evolved from a common ancestor presenting an early association veb1-aadB. It is also possible that aadB is at the origin of the veb1 gene cassette recruitment and of the co-mobilization of veb1-aadB into class 1 integrons. Even though veb1-containing integrons are still subject to gene cassette rearrangements, we propose that IS1999 and the nature of veb1 attC stabilize the veb1 gene cassette environment likely by impairing recombination events upstream or downstream of veb1, respectively.

Materials and Methods

Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. The clinical strains P. aeruginosa 14 and 15 carrying different veb1-containing class 1 integrons were from the Siriraj Hospital, Bangkok, Thailand [26]. The recombination deficient strain E. coli DH10B (Life Technologies, Eragny, France) was used as bacterial host in electroporation experiments. The conjugative plasmid R388 (TnpR, Tra+) includes an integron (In3) that contains the dfrR2 cassette conferring resistance to trimethoprim (Tmp) followed by an open reading frame, qfdA, of unknown function [92]. Plasmid p112 (a PTech99a derivative) was a gift from D. Mazel [33]. This plasmid contains the intI1 gene under the control of an IPTG-inducible synthetic Pout promoter, E. coli DH10B harboring various plasmids and E. coli DH10B-Rif (rifampin resistant) were used for conjugation experiments. The low-copy number cloning vector, pBR1MCS.3 was used for cloning experiments [34]. Bacterial cells were grown in Trypticase.
Soy (TS) broth or onto TS agar plates (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France) with antibiotics when needed.

**Antimicrobial agents**

The antimicrobial agents and their sources were as follows: cefazidime, GlaxoSmithKline (Marly-Le-Roi, France); rifampin, Aventis (Paris, France); trimethoprim and tetracycline, Sigma (Saint-Quentin Fallavier, France); kanamycin, Euromedex (Mundolsheim, France).

**Nucleic acid extractions**

Circular DNA molecules were extracted using Plasmid Maxi Kits (Qiagen, Courtaboeuf, France) according to the instructions of the manufacturer. Extractions of whole-cell DNAs were done as described elsewhere [35].

**PCR experiments**

Taq and Pfu DNA polymerases were from Roche Diagnostics (Meylan, France) and Promega Corporation (Madison, Wis.), respectively. PCR experiments [36] were performed using the series of primers listed in Table S1. The PCR products were purified using Qiaquick columns (Qiagen). To determine the insertion sites of the veb1 cassette or derivatives into the In3 integron, plasmids from independent E. coli DH10B-Rif (R388::veb1) transconjugants were extracted and amplifications using combination of primers VEBINV2-TMPB, VEBINV2-ORFAB, VEBINV2-QACEB, and TMPA-VEBINV3 were performed. The veb1 cassette was detected by PCR amplification using the AADBF and AADBB primers. For each construct tested, five cointegrates resulting from cointegration between R388 and veb1-containing plasmids were analyzed. Amplifications using combination of primers T3-5' promoter-3' arentino cassette or derivatives into the In3-3'CS, T7 promoter-3'CS and VEBINV2-3'CS were performed in order to determine which recombination sites were involved in the formation of the cointegrates.

**Cloning experiments and electroporation**

T4 DNA ligase, and restriction endonucleases were used according to the recommendations of the manufacturer (Amersham Biosciences, Orsay, France). The plasmid p112.Kan was constructed by inserting a HindIII-digested omega fragment (ΩKm) from plasmid pHPI5Ω-Km [37], made of a kanamycin resistance gene (aphIII')-HhaI flanked by transcriptional and translational termination sequences, into the HindIII site from the multiple cloning site of p112 plasmid (pTRC99A:aintih) [33]. The pTRC99A.Kan plasmid was constructed by removing the 1.2-kb EcoRI-BamHI fragment containing the intII gene from p112.Kan, filling in its ends with Pfu DNA polymerase and followed by self ligation. The inserts of the recombinant plasmids pVeb and pVeb.aadB, corresponded to fragments of 1.6-kb (containing the veb1 cassette) and 1.7-kb (containing the veb1 and aadB cassettes) that were amplified with the pairs of primers VEBCASF/ADDABB and VEBCASF/3'CS, respectively and genomic DNA of P. aeruginosa 14 as template (Figure S1). The inserts of the recombinant plasmids pAttL.veb and pAttL.veb.aadB corresponded to fragments of 2-kb (containing an entire attI1 site and the veb1 cassette) and 2.1-kb (containing an entire attI site and the veb1 and aadB cassettes) that were amplified with the pairs of primers INTIN/AADDABB and INTIN/3'CS, respectively and genomic DNA of P. aeruginosa 14 as template (Figure S1). The inserts of the recombinant plasmids pAttL.IS.veb and pAttL.IS.veb.aadB corresponded to fragments of 3.4-kb (containing a disrupted attI1 site, IS1999, and the veb1 cassette) and 3.5-kb (containing a disrupted attI1 site, IS1999, and the veb1 and aadB cassettes) that were amplified with the pairs of primers INTIN/AADDABB and INTIN/3'CS, respectively and genomic DNA of P. aeruginosa 15 as template (Figure S1). PCR products were purified prior to cloning into the Smal-restricted pBBR1MCS.3 vector.

Three different modifications of the veb1 attC site (veb1 attC*, veb1 attCA*, and veb1 attC) were generated. Creation of the veb1 attC* site was performed by using the attCVEB1 and attCVEB2 primers that anneal to the beginning of the veb1 attC site up to the inverse core site and to the beginning of the aadB gene cassette (core site), respectively. These primers have floating 5' ends, each corresponding to a half of the aadB attC site, and have in common the portion containing the BsaHI restriction site (Figure S1). The recombinant plasmid pAttL.veb* containing veb1 attC* site was constructed as follows: a 1.5-kb fragment amplified with the pair of primers T7 promoter-attCVEB1 and pAttL.veb as template was digested with SpeI-BsaHI. A 0.7-kb fragment was amplified with the primers attCVEB2-T3 and pAttL.veb as template and was digested with BsaHI-PstI. Then, the digested inserts were purified and mixed in a three-way ligation with the PstI-SpeI-restricted pBBR1MCS.3 vector generating pAttL.veb* (Figure S1). The pAttL.veb.aadB and pAttL.veb* recombinant plasmids were digested with SacI endonucleases. The SacI insert of pAttL.veb* was cloned into the SacI-restricted pAttL.veb.aadB plasmid generating pAttL.veb*aadB (Figure S1).

Similarly, pAttL.vebA (containing veb1 attABC) was constructed as follows: a 1.5-kb fragment amplified with the pair of primers T7 promoter-Shortatt1 and pAttL.veb as template was digested with SacI. A 0.6-kb fragment was amplified with the primers Shortatt2-T3 and pAttL.veb as template and was digested with KpnI. Then, the digested inserts were purified and mixed in a three-way ligation with the SacI-KpnI-restricted pBBR1MCS.3 vector generating pAttL.veb (Figure S1). The recombinant plasmid pAttL.veb' (containing veb1 attC) was constructed as follows: a 1.6-kb fragment amplified with the pair of primers T7 promoter-att2L and pAttL.veb as template and was digested with KpnI-PstI. Then, the digested inserts were purified and mixed before ligation with the SacI-XhoI-restricted pAttL.veb plasmid generating pAttL.veb' (Figure S1).

Ligation products were electroporated first into E. coli DH10B as previously described [35]. Selection was performed onto TS agar plates containing tetracycline (15 µg/ml) and cefazidime (15 µg/ml) except for pVeb and pVeb.aadB plasmids that were selected onto TS-agar plates containing tetracycline (15 µg/ml) only. Clones harboring recombinant plasmids pVeb, pVeb.aadB, pAttL.veb, pAttL.veb.aadB, pAttL.vebA, pAttL.vebB, and pAttL.veb' were retained for further experiments (Figure S1).

**Sequencing**

Sequencing was performed on both strands using laboratory-designed primers on an ABI PRISM 3100 automated sequencer (Applied Biosystems, Les Ullis, France).

**Induction of integrase expression**

The recombinant plasmids pVeb, pVeb.aadB, pAttL.veb, pAttL.veb.aadB, pAttL.IS.veb, pAttL.IS.veb.aadB, pAttL.vebA, pAttL.vebB, and pAttL.veb' were freshly electroporated into E. coli DH10B (p12.Kan); the plasmid pAttL.veb.aadB was also freshly electroporated into E. coli DH10B (pTRC99A.Kan) before each experiment. Strains were grown to stationary
phase in TS-broth containing tetracycline 15 μg/ml and kanamycin 30 μg/ml. The cultures were then diluted 1000-fold into 200 ml TS-broth containing tetracycline 15 μg/ml, kanamycin 30 μg/ml and grown to exponential phase (OD 600 nm: 0.5). Integrase expression was then induced for 2 h by adding IPTG at a final concentration of 0.6 M.

Detection of veb1-containing recombination products by hybridization

This assay allows for the detection of veb1-containing molecules that are the result of a recombination event between attI1 (or disrupted attI1) and an attC site and gives an insight into their recombination efficiency. Total circular DNA content was extracted from E. coli DH10B [p112.Kan] strains harboring recombinant plasmids pVeb, pVeb.aadB, pAttL.veb, pAttL.veb.aadB, pAttL.veb*, pAttL.veb.aadB, pAttL.vebΔ, and pAttL.veb1 and E. coli DH10B (pTRC99A.Kan) harboring pAttL.veb.aadB after IPTG-induction. Five μg of circular DNA extracts were digested in duplicate with 10 units of BspE1, which cleaves at a unique site located in the veb1 cassette (Figure S1). Digested DNA samples were then loaded on two agarose gels (25 cm, 0.7%) and electrophoresed at 45 V for 16 h using Tris-borate-EDTA running buffer [36]. The duplicates that were loaded on the second gel were spaced two wells apart.

The first agarose gel was used for hybridization experiments as follows: The bottom section of the gel that contained small BspEI excision products (<2-kb), was cut and transferred onto a N° Hybond nylon membrane (Amersham Biosciences). Southern blot hybridizations [56] were performed under high-stringency conditions using the ECL nonradioactive labeling and detection kit (Amersham Biosciences). The probe consisted of a PCR-generated fragment internal to blvVEB1 that was amplified using primers VEB1A/VEB1B and whole-cell DNA of P. aeruginosa 14 as template.

The second agarose gel was used to extract BspEI digested veb1-containing fragments. Since DNA amounts were very low and could not be detected visually after ethidium bromide staining, DNA location was spotted by superposing the autoradiography film obtained from the first gel after hybridization. Gel slices were cut with a separate disposable scalpel to avoid sample cross-contamination. DNA was extracted using Qiaquick Gel extraction kit (Qiagen) and subjected to PCR amplification using the VEBINV3-VEBINV2 outward-directed primers. These primers are located on each side of the BspEI restriction site, and thus allowed amplification of the recombinant junction that was created upon recombination (Figure S2 and S3).

Cointegration assay using plasmid R388 and calculation of cointegration frequencies

Precise integration of veb1 gene cassettes into the class 1 integron In3 carried by the conjugative plasmid R388 or cointegration products using the ECL nonradioactive labeling and detection kit [36] were performed under high-stringency conditions using the ECL nonradioactive labeling and detection kit. The probes consisted of a PCR-generated fragment internal to blvVEB1 that was amplified using primers VEB1A/VEB1B and whole-cell DNA of P. aeruginosa 14 as template.

After BspEI digestion when recombination between attI1 attC* or attI1 attC of excised veb1 gene cassette, or by resolution (veb1 attC1 attI1 or attI1 attC1) of cointegrates. In any case, the 1.1-kb fragment is only recovered after BspEI digestion when recombination between attI1 and veb1 attC has occurred.
**Figure S3** Recombination products obtained with pAt-t1.**a** veb-aadB. A) Schematic representation of plasmid pAt-t1.veb-aadB. Construct was made in the pBBR1MCS-3 shuttle-vector represented with a solid line. The coding regions are shown as boxes and the arrow indicates the orientation of their transcription. Dashed lines indicate truncated genes. The black diamond, white and grey circles represent attI1, vebl attC and aadB attC, respectively. The broken arrow indicates the P promoter. Small arrows (1 and 2) located on each side of the BspEI restriction site represent the positions of the VEBINIV3 and VEBINIV2 primers, respectively. B-D) Recombination products. The different vebl-containing recombination products are represented. The sites involved in the recombination and the size of the relevant BspEI digestion products are indicated. B) Vebl-containing excision product. C) Cointegrates. Double lines represent scale breaks. D) Gene duplications. Vebl duplications can arise from integration of an excised vebl-containing gene cassette or by resolution of cointegrates. In any case, the 1.1-kb and 1.6-kb fragments are only recovered after BspEI digestion when recombinations attI1 × vebl attC and attI1 × aadB attC have occurred, respectively.

**Table S1** Sequence of primers used in this study. a Nucleotides that are complementary to the aadB attC site are underlined; b Restriction sites are bolded; c Nucleotides that are mutated are boxed in grey. Supplemental references are as follows:

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**Author Contributions**

Conceived and designed the experiments: DA TN PN. Performed the experiments: DA TN PN. Analyzed the data: DA TN PN. Contributed reagents/materials/analysis tools: DA TN PN. Wrote the paper: DA TN PN.

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