

IS1999 Increases Expression of the Extended-Spectrum β -Lactamase VEB-1 in *Pseudomonas aeruginosa*

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The integron-borne *bla*_{VEB-1} gene encodes an extended-spectrum β -lactamase. This gene was associated mostly with IS1999 and rarely with an additional IS2000 element in *Pseudomonas aeruginosa* isolates from Thailand, whereas IS1999 was only very rarely associated with *bla*_{VEB-1} in *Enterobacteriaceae*. Expression experiments and promoter study identified promoter sequences in IS1999 that increased the expression of VEB-1 in *P. aeruginosa*.

Antibiotic resistance in many gram-negative clinical isolates is due to resistance genes that are captured and expressed in class 1 integrons. These integrons possess two conserved regions located on each side of integrated gene cassettes (14). The 5' conserved segment (5'-CS) includes a gene encoding an integrase, *intI1*, the cassette integration site, *attI1*, and the promoter P_{ant}, sometimes associated with a second promoter P2, which are responsible for the expression of gene cassettes (6, 14). The 3' conserved segment (3'-CS) includes, along with another open reading frame of unknown function, the disinfectant (*qacE Δ I*) and the sulfonamide (*sulI*) resistance determinants (14). The expression of the inserted gene cassettes depends not only on sequences of promoters P_{ant}/P2 but also on the gene cassette position relative to the 5'-CS (2).

The integron-borne *bla*_{VEB-1} gene encodes the extended-spectrum β -lactamase VEB-1 (Vietnamese extended-spectrum β -lactamase) found initially in an *Escherichia coli* clinical isolate from Vietnam (13). Subsequently, the *veb-1* gene cassette was identified in two *Pseudomonas aeruginosa* clinical isolates from Thailand (9, 18). In those strains, *bla*_{VEB-1} was associated with either one (IS1999) or two (IS1999/IS2000) insertion sequences (IS) that were inserted upstream of *bla*_{VEB-1} in the integron-specific recombination site, *attI1*. Bacterial IS may bring promoters located in or near their inverted-repeat sequences (IR) that are capable of modulating the expression of neighboring antibiotic resistance genes (7).

Previous studies performed with ceftazidime-resistant *Enterobacteriaceae* and *P. aeruginosa* strains isolated in 1999 from the Siriraj Hospital, Bangkok, Thailand (3, 4), resulted in reports that out of 37 enterobacterial isolates, 18 were *bla*_{VEB-1} positive (10 *E. coli*, 4 *Enterobacter cloacae*, 1 *Enterobacter sakazakii*, and 3 *Klebsiella pneumoniae*) and 19 were *bla*_{VEB-1} negative. Out of 33 ceftazidime-resistant *P. aeruginosa* isolates, 31 were *bla*_{VEB-1} positive. *bla*_{VEB-1} was mostly plasmid located in *Enterobacteriaceae*, whereas this gene was mostly chromosome encoded in *P. aeruginosa* (3, 4). Moreover, spreading of *bla*_{VEB-1}-containing *P. aeruginosa* strains was detected in sev-

eral unrelated isolates carrying different integrons of various sizes and structures (3, 4).

Distribution of IS1999 and IS2000 in *bla*_{VEB-1}-positive isolates. The distribution of IS1999, an IS10-like element, and IS2000, which belongs to the IS5 family (7, 9), was investigated using the same *bla*_{VEB-1}-positive isolates (Table 1). Dot blot hybridizations were performed using whole-cell DNAs (12, 15) of the 18 *Enterobacteriaceae*- and the 31 *P. aeruginosa*-positive isolates (Table 1). Using ECL nonradioactive labeling and detection kits (Amersham Biosciences, Orsay, France), hybridizations were performed under high-stringency conditions. The probes consisted of PCR-generated fragments internal to IS1999 and IS2000 (15) (primer sequences are available upon request).

Out of 18 *bla*_{VEB-1}-positive enterobacterial isolates, none possessed IS2000 and only one *K. pneumoniae* isolate carried IS1999. In contrast, IS1999 was found in 28 out of 31 (90%) *bla*_{VEB-1}-containing *P. aeruginosa* isolates and 2 out of these 28 isolates had an additional IS2000 inserted within the IS1999 sequence.

Thus, the frequent association mostly of IS1999, which is sometimes associated with IS2000, with the *bla*_{VEB-1} gene in *P. aeruginosa* and its absence from *Enterobacteriaceae*, especially from *E. coli*, led us to study the contribution of these IS on β -lactamase expression in both bacterial species.

Influence of IS1999/IS2000 on *bla*_{VEB-1} expression. Using three *bla*_{VEB-1}-positive *P. aeruginosa* isolates as templates (*P. aeruginosa* 14, 1, and JES), recombinant plasmids were constructed containing *bla*_{VEB-1} without any IS (pDA-1), with IS1999 (pDA-2), and with IS1999 and IS2000 (pDA-3) (Fig. 1 and Table 1). Plasmids were constructed by standard recombinant techniques (15). The low-copy-number and broad-host-range cloning vector pBBR1MCS.3 that replicates in *E. coli* and in *P. aeruginosa* (5) was used for subcloning experiments, generating recombinant plasmids pInt-Veb, pInt-1999-Veb, and pInt-1999-2000-Veb (Fig. 1 and Table 1). Recombinant plasmids were introduced by electroporation into *E. coli* DH10B (12) and *P. aeruginosa* KG2505 (11, 17). *P. aeruginosa* KG2505 does not express the naturally chromosome-encoded AmpC β -lactamase and is deficient for the multidrug efflux system MexAB-OprM (11).

Sequencing was performed using laboratory-designed prim-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or isolate(s) or plasmid	Genotype or description ^a	Source or reference
Strains or isolates		
<i>E. coli</i>		
DH10B	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>dlacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>deoR recA1 endA1 araD139</i> Δ(<i>ara, leu</i>)7697 <i>galU galK</i> λ ⁻ <i>rpsL nupG</i>	Life Technologies, Eragny, France
<i>P. aeruginosa</i>		
KG2505	<i>P. aeruginosa</i> reference strain that does not express the naturally chromosome-encoded AmpC β-lactamase and is deficient for the multidrug efflux system MexAB-OprM <i>mexA::res-ΩSm ampC::res-ΩSm</i> of PAO1; Sm ^r	11
Isolate JES	Clinical isolates from a French patient hospitalized in Thailand; <i>bla</i> _{VEB-1} positive; Caz ^r	9
Isolates 1 to 31	Clinical isolates from the Siriraj Hospital, Bangkok, Thailand; <i>bla</i> _{VEB-1} positive; Caz ^r	3
<i>E. coli</i>		
Isolates 1 to 10	Clinical isolates from the Siriraj Hospital, Bangkok, Thailand; <i>bla</i> _{VEB-1} positive; Caz ^r	4
<i>E. cloacae</i>		
Isolates 11 to 14	Clinical isolates from the Siriraj Hospital, Bangkok, Thailand; <i>bla</i> _{VEB-1} positive; Caz ^r	4
<i>E. sakazakii</i>		
Isolate 15	Clinical isolates from the Siriraj Hospital, Bangkok, Thailand; <i>bla</i> _{VEB-1} positive; Caz ^r	4
<i>K. pneumoniae</i>		
Isolates 16 to 18	Clinical isolates from the Siriraj Hospital, Bangkok, Thailand; <i>bla</i> _{VEB-1} positive; Caz ^r	4
Plasmids		
pBK-CMV phagemid	Neo ^r , Kan ^r	Stratagene, La Jolla, Calif.
pCR-Blunt II-TOPO	Kan ^r	Invitrogen, Cergy Pontoise, France
pBBR1MCS.3	Low-copy-number broad-host-range cloning vector; Tet ^r	5
pDA-1	pBK-CMV recombinant plasmid containing genomic <i>Hind</i> III fragment from <i>P. aeruginosa</i> 14, including <i>bla</i> _{VEB-1}	This work
pDA-2	pBK-CMV recombinant plasmid containing genomic <i>Hind</i> III fragment from <i>P. aeruginosa</i> 1, including <i>bla</i> _{VEB-1} and <i>IS1999</i>	This work
pDA-3	pBK-CMV recombinant plasmid containing genomic <i>Hind</i> III fragment from <i>P. aeruginosa</i> JES, including <i>bla</i> _{VEB-1} and <i>IS1999/IS2000</i>	This work
pInt-Veb	A 2.4-kb <i>SpeI-XhoI</i> fragment from plasmid pDA-1, containing <i>bla</i> _{VEB-1} inserted in the <i>SpeI-XhoI</i> site of pBBR1MCS.3	This work
pInt-1999-Veb	A 3.7-kb <i>SpeI-XhoI</i> fragment from pDA-2, containing <i>bla</i> _{VEB-1} and <i>IS1999</i> inserted in the <i>SpeI-XhoI</i> site of pBBR1MCS.3	This work
pInt-1999-2000-Veb	A 4.9-kb <i>SpeI-XhoI</i> fragment from pDA-3, containing <i>bla</i> _{VEB-1} and <i>IS1999/IS2000</i> inserted in the <i>SpeI-XhoI</i> site of pBBR1MCS.3	This work
pTOP1	A 1.1-kb fragment resulting from PCR amplifications using VEB-CAS-A/VEB-CAS-B primers and genomic DNA of <i>P. aeruginosa</i> 1 as template; this fragment, including the <i>veb-1</i> gene cassette, was inserted in the cloning site of pCR-BluntII-TOPO	This work
pTOP2	A 2.5-kb fragment resulting from PCR amplifications using 5'-CS/VEB-CAS-B primers and genomic DNA of <i>P. aeruginosa</i> 1 as the template; this fragment, including the <i>veb-1</i> gene cassette and <i>IS1999</i> , was inserted in the cloning site of pCR-BluntII-TOPO	This work
pTOP3	A 1.1-kb fragment resulting from PCR amplifications using VEB-CAS-AStu/VEB-CAS-B primers and genomic DNA of <i>P. aeruginosa</i> 1 as the template; this fragment, including a <i>StuI</i> restriction site in the <i>veb-1</i> gene cassette upstream of <i>bla</i> _{VEB-1} , was inserted in the cloning site of pCR-BluntII-TOPO	This work
pTOP4	A 2.2-kb fragment resulting from PCR amplifications using MUTIS1999Stop/VEB-CAS-B primers and genomic DNA of <i>P. aeruginosa</i> 1 as the template; this fragment includes a site-directed mutation generating after transcription a stopcodon within the <i>IS1999</i> transposase sequence; this fragment, inserted in the cloning site of pCR-BluntII-TOPO, includes the two <i>XmnI</i> sites that are present in <i>IS1999</i> and <i>bla</i> _{VEB-1} sequences and that are located on each side of the mutation	This work
pVeb	A 1.1-kb <i>KpnI-ApaI</i> fragment from pTOP1, containing the <i>veb-1</i> gene cassette inserted in the <i>KpnI-ApaI</i> site of pBBR1MCS.3	This work
p1999R-Veb	pInt-1999-Veb from which a 1.9-kb <i>SpeI-StuI</i> fragment has been deleted that includes <i>intII</i> and the left end of <i>IS1999</i>	This work
p1999-Veb	A 2.6-kb <i>KpnI-ApaI</i> fragment from pTOP1, containing the <i>veb-1</i> gene cassette and <i>IS1999</i> , inserted in the <i>KpnI-ApaI</i> site of pBBR1MCS.3	This work
p1999L-Veb	A 2.1-kb <i>KpnI-StuI</i> fragment from pTOP3, containing the <i>veb-1</i> gene cassette, inserted in the <i>KpnI-StuI</i> site of p1999-Veb, containing the left end of <i>IS1999</i>	This work
pInt-1999*-Veb	A 1.5-kb <i>XmnI</i> fragment from pTOP4, containing parts of the mutated <i>IS1999</i> and <i>bla</i> _{VEB-1} sequences inserted in the <i>XmnI</i> site of pInt-1999-Veb	This work
p1999R-2000R-Veb	pInt-1999-2000-Veb from which a 1.8 kb <i>SpeI-PstI</i> fragment has been deleted that includes <i>intII</i> and the right ends of <i>IS1999/IS2000</i>	This work

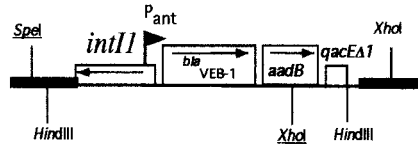
^a Superscript "r," resistance. Abbreviations: Caz, ceftazidime; Kan, kanamycin; Neo, neomycin; Sm, streptomycin; Tet, tetracycline.

ers on an ABI PRISM 3100 automated sequencer (Applied Biosystems, Les Ullis, France). Sequence analysis of inserts of all the recombinant plasmids revealed the presence of the two previously characterized promoters: (i) the weak P_{ant} promoter

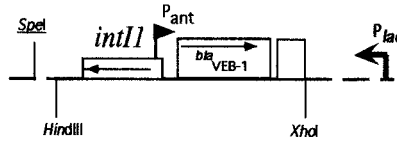
and (ii) the P2 promoter that is likely nonfunctional (as previously shown) due to its reduced spacing between the -10 and -35 promoter sequences (14 bp instead of 17 bp) (2, 6).

Using β-lactamase extracts from cultures of *E. coli* DH10B

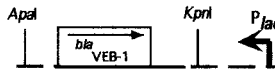
1
pDA-1



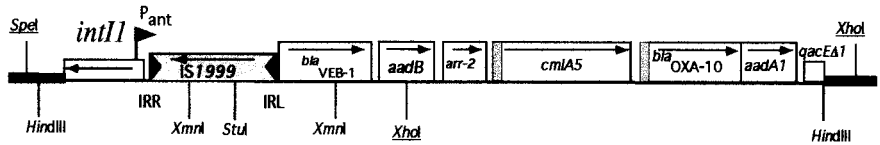
pInt-Veb



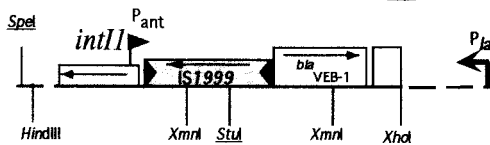
pVeb



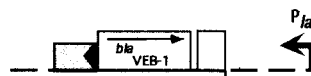
2
pDA-2



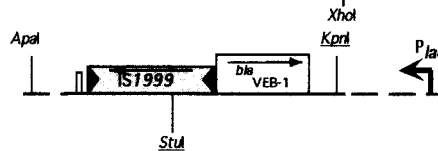
pInt-1999-Veb



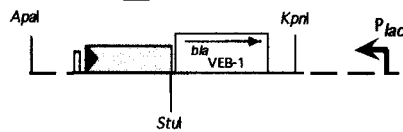
p1999R-Veb



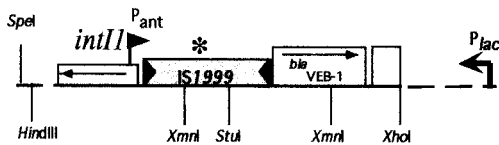
p1999-Veb



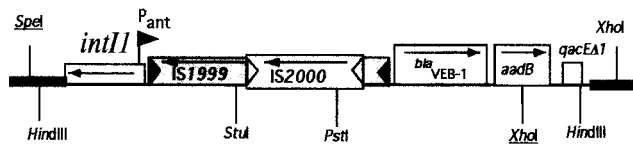
p1999L-Veb



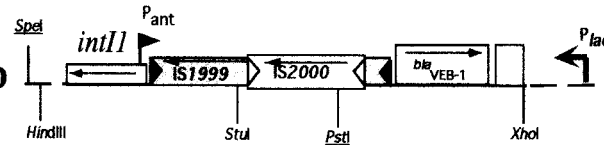
pInt-1999*-Veb



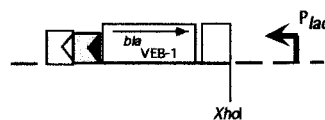
3
pDA-3



pInt-1999-2000-Veb



p1999R-2000R-Veb



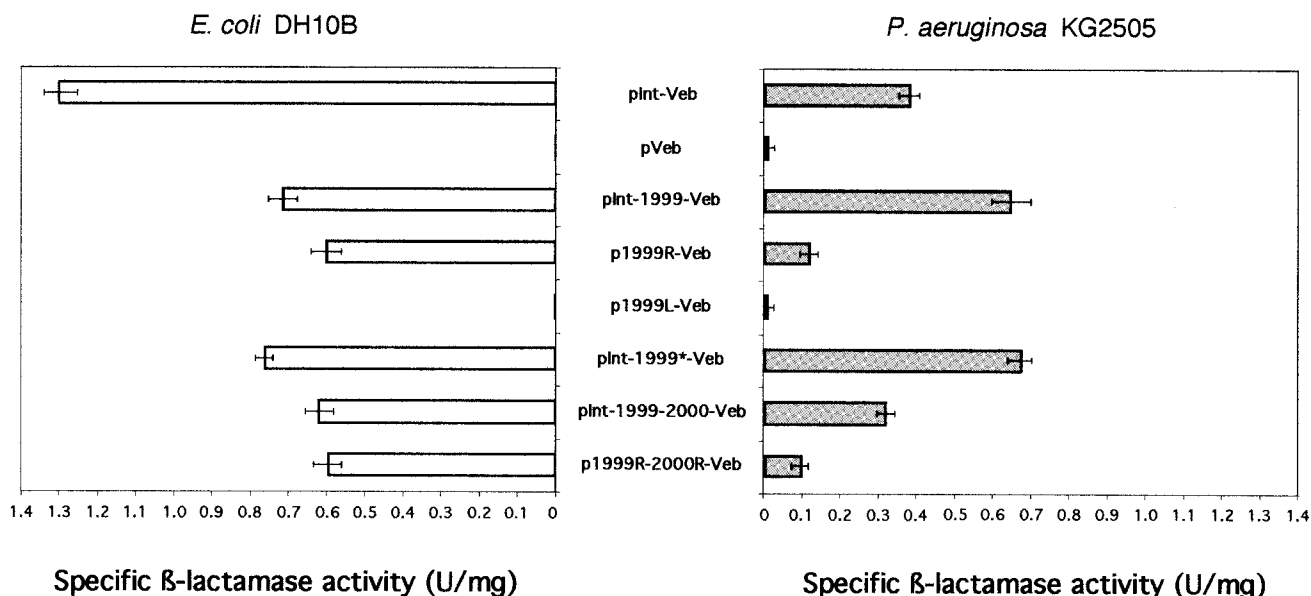


FIG. 2. Comparative study (using cefepime as the substrate) of the specific activity of β-lactamase VEB-1 from cultures of *E. coli* DH10B (left panel) and *P. aeruginosa* KG2505 (right panel) strains harboring recombinant plasmids. Error bars represent standard deviations calculated from five independent cultures. No measurable activity was detected for *E. coli* DH10B(pVeb) and for *E. coli* DH10B(p1999L-Veb).

and *P. aeruginosa* KG2505 harboring recombinant plasmids (Fig. 2) prepared as previously described (10), *bla*_{VEB-1} gene expression was then investigated by measuring the specific β-lactamase activities for cefepime (a β-lactam antibiotic hydrolyzed specifically by VEB-1). Total protein contents and the initial rate of cefepime hydrolysis were determined as previously described (13).

To determine whether IS1999 and IS2000 carry active promoter sequences located in their right ends, P_{ant} was deleted from pInt-Veb, pInt-1999-Veb, and pInt-1999-2000-Veb, generating recombinant plasmids pVeb, p1999R-Veb, and p1999R-2000R-Veb, respectively (Fig. 1 and Table 1).

In *E. coli*, the highest β-lactamase activity was measured for *E. coli* DH10B(pInt-Veb), which had *bla*_{VEB-1} located just downstream of the promoter P_{ant} of the class 1 integron (Fig. 1 and 2). Insertion of IS1999 (pInt-1999-Veb), which moved *bla*_{VEB-1} away from P_{ant}, decreased *bla*_{VEB-1} expression (50% decrease). After the removal of the promoter P_{ant} (p1999R-Veb and p1999R-2000R-Veb), significant activity (45%) was still measured, suggesting the presence of an IS1999-located promoter. Similar specific activities were obtained for *E. coli* DH10B(pInt-1999-Veb), (p1999R-Veb), (pInt-1999-2000-Veb), and (p1999R-2000R-Veb), suggesting that P_{ant} made only a minor contribution to the overall β-lactamase expression. Thus, the activity measured in *E. coli* DH10B(pInt-1999-

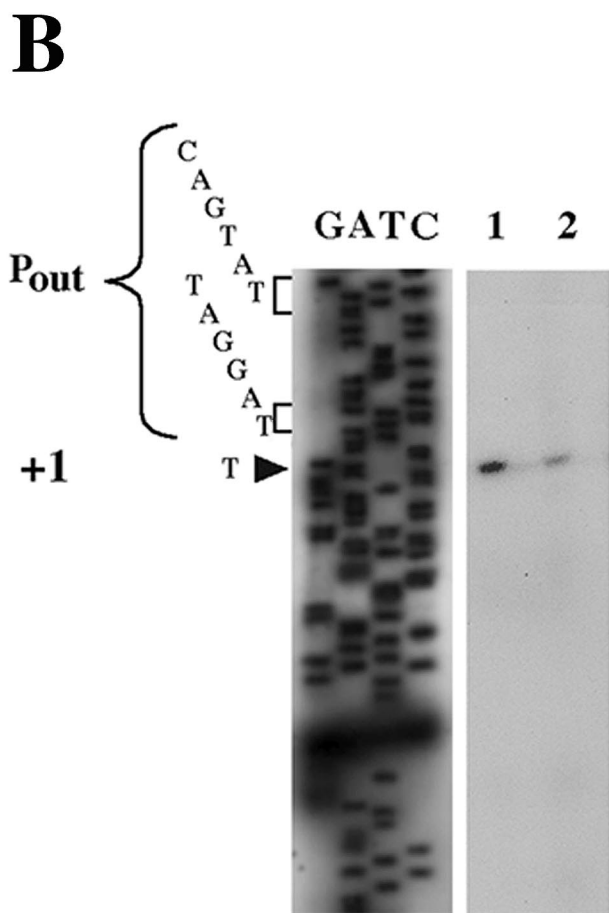
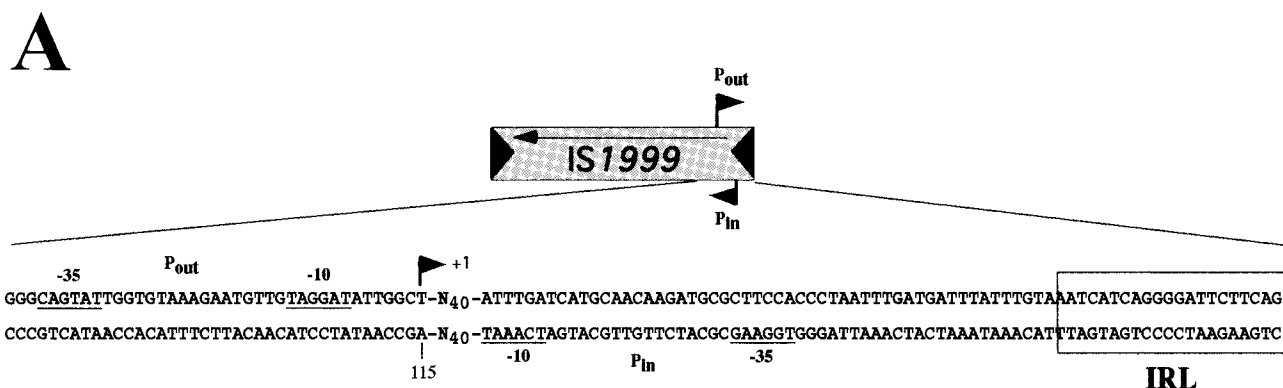
Veb) and (pInt-1999-2000-Veb) is mostly due to the presence of an IS1999-located promoter (and possibly to the presence of IS2000).

Determination of specific activity of *P. aeruginosa* KG2505 cultures showed that surprisingly, the highest activity was measured for *P. aeruginosa* KG2505(pInt-1999-Veb) [a 60% increase compared to the activity of cultures of *P. aeruginosa* KG2505pInt-Veb] (Fig. 2). However, insertion of IS2000 (pInt-1999-2000-Veb) or deletion of the P_{ant} promoter along with the left end(s) of IS1999 and/or IS2000 (p1999R-Veb and p1999R-2000R-Veb) led to a decrease in β-lactamase activity. Thus, the right end of IS1999, which includes the left IR (IRL), most likely carried a functional outward-directed promoter capable of driving *bla*_{VEB-1} transcription in *P. aeruginosa*.

To determine whether the left end of IS1999 carried additional promoter sequences, plasmid p1999L-Veb was constructed. This plasmid contained two-thirds of the IS1999 sequence, including the right IR (IRR) located upstream of *bla*_{VEB-1} (Fig. 1). Strains harboring p1999L-Veb plasmid had very low levels of β-lactamase activity similar to those observed for strains harboring pVeb, suggesting the absence of functional promoter sequences in the left end of IS1999.

To determine whether the functional transposase of IS1999 can influence *bla*_{VEB-1} expression, the plasmid pInt-1999*-Veb, which contains an interrupted open reading frame en-

FIG. 1. Schematic map of the constructs used in this study. Constructs 1 (pDA-1, pInt-Veb, and pVeb), 2 (pDA-2, pInt-1999-Veb, pInt-1999*-Veb, p1999R-Veb, p1999L-Veb, and p1999L-Veb), and 3 (pDA-3, pInt-1999-2000-Veb, and p1999R-2000R-Veb) were cloned from genomic DNAs of *P. aeruginosa* clinical isolates 14, 1, and JES, respectively. The *bla*_{VEB-1} gene was inserted in opposite orientation to P_{lac}, thus removing any contribution of promoter P_{lac} in β-lactamase expression. The stop codon resulting from a site-directed mutagenesis experiment is shown by an asterisk (construct pInt-1999*-Veb). Restriction sites that were used at each cloning step are underlined. The coding regions are shown as boxes, with an arrow indicating the orientation of their transcription. The IR of IS1999 and IS2000 are shown by filled and empty triangles, respectively. IRL and IRR of IS1999 are indicated for pDA-2. The broken arrows indicate the promoter P_{lac}. Thin dashed lines indicate ligation in the multiple-cloning site of the shuttle vector pBBR1MCS.3.



coding the *IS199* transposase, was generated. No significant modification of *bla*_{VEB-1} expression was measured for strains harboring pInt-1999*-Veb compared to those harboring pInt-1999-Veb (Fig. 2). Thus, the transposase had no or little effect on β -lactamase expression in *E. coli* and *P. aeruginosa*.

Mapping of promoter P_{out} of *IS199*. The precise location of the right-end-located promoter of *IS199* was determined with a primer extension system-AMV reverse transcriptase kit (Promega, Charbonnières, France). Total RNAs were extracted with a Qiagen RNeasy Maxi kit (Qiagen, Courtaboeuf, France). cDNAs were synthesized using ³²P end-labeled primers Vebprom and Vebprom 1999, which annealed to the left end of *veb-1* gene cassette and to the right end of *IS199*. Using a

FIG. 3. (A) Structure of *IS199*. The *IS199* IR are shown by filled triangles. The arrows indicate the orientation of transcription. The outward-directed promoter, P_{out} , and the promoter of the transposase gene, P_{in} , are indicated by broken arrows. The -10 and -35 regions for P_{in} and P_{out} are underlined. Nucleotide position 115 (according to the sequence GenBank AF133697) in *IS199* corresponds to the transcription start of P_{out} . The IRL sequence is boxed. (B) Mapping of transcription initiation. Primer Vebprom was extended using RNAs from cultures of *E. coli* DH10B(pInt-1999-Veb) (lane 1) or *P. aeruginosa* KG2505(pInt-1999-Veb) (lane 2) as the templates. Equal volumes (2 μ l) of the extension product obtained from *P. aeruginosa* and *E. coli* were loaded onto the gel. Size markers were from sequencing reactions generated from pInt-1999-Veb DNA primed with Vebprom. G-, A-, T-, and C-specific lanes are indicated. The nucleotide sequences on the left side correspond to that of the complementary strand, which was deduced from the sequencing reaction. The -10 and -35 promoter sequences of P_{out} regions are shown, and the $+1$ transcriptional initiation site is indicated by an arrowhead. Similar results were obtained for RNA extracted from *E. coli* DH10B and *P. aeruginosa* KG2505 harboring pInt-1999-2000-Veb recombinant plasmid (data not shown).

Sequenase version 2 DNA sequencing kit (Amersham Biosciences), manual sequencing was performed with the same primers. Sequencing and extension products were separated on an 8% polyacrylamide gel and were visualized by autoradiography after overnight exposure at -80°C .

Primer extension experiments (performed with Vebprom primer and RNAs from *E. coli* DH10B and *P. aeruginosa* KG2505 strains containing recombinant plasmids pInt-1999-Veb and pInt-1999-2000-Veb) generated a cDNA starting at a thymidine at nucleotide position 115 (Fig. 3). Analysis of the sequence located upstream of bp 115 revealed a putative -35 promoter region (CAGTAT) separated by 17 bp from a -10 region (TAGGAT) (Fig. 3). This promoter was located close to the IRL of *IS199* at a position similar to that of the promoter P_{out} (-35 [CAGAAT] and -10 [TAAAAT]) identified in the related IS element, *IS10* (16). Using the primer Vebprom 1999, which annealed upstream of P_{out} , no extension product was identified, indicating that no promoter sequence was located further inside of *IS199* or in *IS2000*.

Conclusions. This work identified a functional outward-directed promoter, P_{out} , of *IS199* that was capable of driving *bla*_{VEB-1} expression in *E. coli* and in *P. aeruginosa*. The level of expression obtained from P_{ant} or P_{out} was about fourfold higher in *E. coli* than in *P. aeruginosa*. Furthermore, our results suggest that an association between *IS199* and the P_{ant} promoter enhances *bla*_{VEB-1} expression by about 60% in *P. aeruginosa*.

nosa but not in *E. coli*. An increase in β -lactamase expression may change bacteria from being susceptible to being intermediate or even resistant. This is the case for *P. aeruginosa* harboring plasmid pInt-1999-Veb, for which certain β -lactams, such as piperacillin and cefepime, that are extensively prescribed in hospitals display a twofold increase in MICs (data not shown). Thus, in a hospital environment, where bacteria may be under constant antibiotic pressure, IS1999 (by enhancing the bla_{VEB-1} gene expression) might bring a selective advantage to *P. aeruginosa*, at least when it is present on a low-copy-number plasmid (pBBR1MCS). Future experiments will be directed towards determination of bla_{VEB-1} gene expression in its native environment, i.e., the chromosome of *P. aeruginosa* isolates, to see whether our plasmid-mediated system mimics the chromosomal situation.

An increase of bla_{VEB-1} expression in *P. aeruginosa* KG2505 (pInt-1999-Veb) might be the result of a cooperative effect between P_{out} and P_{ant} promoters. Indeed, the maximum amount of expression was obtained only when both promoters were present. In *E. coli*(pInt-1999-Veb), however, both promoters were present and still the expression decreased compared to that seen with *E. coli*(pInt-Veb). These data may reflect major differences in transcriptional properties between *P. aeruginosa* and *E. coli*. The role of IS2000 in the bla_{VEB-1} -containing integron remains unclear, but decrease of bla_{VEB-1} expression after IS2000 insertion into IS1999 argues against its role in β -lactamase expression.

Most of the genes inserted in class 1 integrons are expressed from a common promoter region (P_{ant}/P_2). In a few cases, however, other promoters of the expression of gene cassettes have been reported (1, 8). This work identified for the first time an IS-located promoter capable of driving expression of downstream-located gene cassettes in an integron structure.

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