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Diversity of Clavulanic Acid-Inhibited Extended-Spectrum β-Lactamases in *Aeromonas* spp. from the Seine River, Paris, France[▽]

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Environmental *Aeromonas* sp. isolates resistant to ceftazidime were recovered during an environmental survey performed with water samples from the Seine River, in Paris, France, in November 2009. Selected isolates were identified by sequencing of the 16S rRNA and *rpoB* genes. PCR and cloning experiments were used to identify broad-spectrum-β-lactamase-encoding genes and their genetic context. Clavulanic acid-inhibited extended-spectrum-β-lactamase (ESBL) genes were identified in 71% of the *Aeromonas* sp. isolates. A variety of ESBL genes were detected, including $bla_{VEB\text{-}1a}$, $bla_{SHV\text{-}12}$, $bla_{PER\text{-}1}$, $bla_{PER\text{-}6}$, $bla_{TLA\text{-}2}$, and $bla_{GES\text{-}7}$, suggesting an aquatic reservoir of those ESBL genes. Moreover, the repeated elements and different insertion sequences were identified in association with the $bla_{PER\text{-}6}$ and the $bla_{VEB\text{-}1a}$ genes, respectively, indicating a wide diversity of mobilization events, making *Aeromonas* spp. a vehicle for ESBL dissemination.

Bacteria with intrinsic or acquired resistance to antibiotics are commonly found in aquatic environments, where *Pseudomonas*, *Serratia*, and *Aeromonas* are commonly identified (2, 17). Recent studies reported that the $bla_{TEM\text{-}1}$ gene was detected in most of the Gram-negative isolates resistant to ampicillin recovered from lakes in Brazil or from wastewater treatment plants in China (24, 40). Furthermore, several studies identified extended-spectrum β-lactamases (ESBLs) from water samples. For instance, the clinically relevant ESBL PER-1 was identified from *Aeromonas media* in a Swiss lake (32), and CTX-M-14 and TEM-52 were detected in *Escherichia coli* recovered from a South Korean river (21). Besides, nonclinically relevant Ambler class A β-lactamases TLA-2 and PER-6 were identified from uncultured bacteria (43, 12) and *Aeromonas allosaccharophila* isolates, respectively (15).

In addition, other studies reported on the identification of carbapenem-hydrolyzing β-lactamases in the environment, such as the Ambler class A β-lactamases IMI-2 and BIC-1 from *Enterobacter asburiae* (1, 5) and *Pseudomonas fluorescens* (14), respectively; the Ambler class B β-lactamase VIM-2 from *Pseudomonas pseudoalcaligenes* (41); and the class D β-lactamase OXA-23 from *Acinetobacter baumannii* (13).

The present study was designed to evaluate the diversity of broad-spectrum β-lactam resistance determinants in *Aeromonas* species in water samples from the Seine River, Paris, France. The working hypothesis was that *Aeromonas* spp. could be an important reservoir for ESBLs.

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MATERIALS AND METHODS

Bacterial strains and plasmids. Three water samples (100 ml) were collected from approximately 0.2 m below the water surface in sterile bottles in November 2009 and filtered through nitrocellulose membranes (pore size, 0.45 μm; Millipore), and the filters were resuspended in 3 ml of sterile water. Aliquots (0.1 ml) were plated on MacConkey agar plates supplemented with ceftazidime (2 μg/ml), and 10-fold dilutions were plated (0.1 ml) in parallel on antibiotic-free MacConkey agar plates for bacterial counts. Samples were processed on the day of collection. *E. coli* DH10B (Invitrogen, Life Technologies, Cergy-Pontoise, France) was used as the host for cloning and transformation experiments as previously described (42). Azide-resistant *E. coli* J53 was used as the recipient in mating-out assays (32). The kanamycin-resistant plasmid pBKCMV (Invitrogen) was used as a cloning vector. Bacterial cultures were grown in Trypticase soy (TS) broth at 37°C for 18 h.

Antibiotic susceptibility testing. The antibiotic susceptibility profiles of 80 ceftazidime- or imipenem-resistant isolates were determined by the agar dilution method, and the results were interpreted according to the CLSI guidelines (7). AmpC overproducers were identified by testing susceptibility to ceftazidime on Mueller-Hinton agar plates supplemented with cloxacillin (250 μg/ml). Detection of ESBL production was carried out by the double-disk synergy test (DDST) (19).

Identification of ceftazidime-resistant isolates and genotyping. Isolates were identified by the API 32GN system (bioMérieux, Marcy l'Etoile, France) and/or sequencing of the 16S RNA and/or *rpoB* or *gyrB* genes by using previously described primers (for 16S, primers 27F and 1492R [16]; for *gyrB*, primers *gyrB*3F and *gyrB*14R [17]; for *rpoB*, primers *rpoB*LAPS and LAPS27 [26]). Amplicons were sequenced and their sequences were compared to those in the nucleotide data library using leBIBI software (http://umr5558-sud-str1.univ-lyon1.fr/lebibi/lebibi.cgi) in order to determine their closest phylogenetic relatives. The genetic diversity of the ESBL-producing *Aeromonas* sp. isolates was assessed by pulsed-field gel electrophoresis (PFGE) using XbaI, as previously described (21). The chromosomal fingerprints were compared by eye and assigned to PFGE types and subtypes (44).

PCR amplification for detection of ESBL genes, analysis of genetic environment, and sequencing. Specific primers were used under standard PCR conditions to detect ESBL-encoding genes, namely, bla_{TEM} (9), bla_{SHV} (30), $bla_{CTX\text{-}M}$ (30), bla_{GES} (33), $bla_{PER\text{-}1/\text{-}2}$ (31, 35), $bla_{PER\text{-}6}$ (15), bla_{VEB} (39), and bla_{BEL} (34). The genetic environments of the $bla_{PER\text{-}1}$, $bla_{TLA\text{-}2}$, and bla_{SHV} genes were determined by PCR mapping using previously published specific primers together with primers designed to anneal at the 5′ and the 3′ conserved segments (CSs) of class 1 integrons (35, 12). The genetic environments of $bla_{PER\text{-}6}$, $bla_{VEB\text{-}1a}$, and $bla_{GES\text{-}7}$ were precisely determined by cloning those genes into plasmid pBKCMV. For that purpose, BamHI-, SacI-, ScaI-, or SalI-restricted

TABLE 1. Oligonucleotides used for PCR amplification and sequencing

Primer	Sequence (5′ to 3′)	Reference or source
VEB-inv1	GTCAGTTTGAGCATTTGAATAC	37
VEB-inv2	AAGCGTATTTGTTGCAGAGTC	37
IS26out	CCCACAGAATGATGTCACGC	This study
aadB-B	CGCATATCGCGACCTGAAAGC	This study
Av9_1 A	AAGTGCCTAGAGATTGTTTCC	This study
TnpF-A	AACTACTGCTGGAGTATATGG	This study
IS4B	TAAATCATCAGGCTACAGGTC	This study
IS6100-A	ATTTCTACCTGTCGCCGACC	This study

DNAs were ligated into restricted plasmid pBKCMV and introduced into *E. coli* DH10B by electroporation as described previously (42). Recombinant plasmids were selected on TS agar plates containing ceftazidime (2 μg/ml) and kanamycin (30 μg/ml). All enzymes for DNA manipulations were used according to the recommendations of the supplier (GE Healthcare, Orsay, France). Recombinant plasmids were sequenced by using combinations of universal T3 and T7 primers and specific gene primers (Table 1), designed on the basis of sequences obtained on an Applied Biosystems sequencer (ABI 3130).

Genetic support of β-lactamase-encoding genes. Plasmid extraction was performed by the Kieser technique (20). *E. coli* NCTC 50192, harboring four plasmids of 154, 66, 38, and 7 kb, was used as a size marker for plasmids. Transformation assays were performed by electroporation with plasmid extracts from the identified positive isolates, and *E. coli* DH10B was used as the recipient strain. Selection was performed on agar plates supplemented with ceftazidime (2 μg/ml). Conjugation experiments were carried out as described previously (32). The I-CeuI restriction enzyme (Ozyme; New England Biolabs, Saint-Quentin-en-Yvelines, France), which digests a 26-bp sequence in the *rrn* genes for the 23S large-subunit rRNA, was used to determine whether the β-lactamase gene had a chromosomal location, and the fragments were separated by pulsed-field gel electrophoresis (36). DNA-DNA hybridization of plasmid extracts was performed after a Southern transfer onto a Hybond N+ nylon membrane (GE Healthcare), as previously described (42). Labeling of the probe and signal detection were carried out using an enhanced chemiluminescence labeling and detection kit, according to the manufacturer's instructions (GE Healthcare). PCR-based replicon-typing (PBRT) analysis was performed as described by Carattoli et al. (6). The 17 primer pairs targeting the FIA, FIB, FIC, HI1, HI2, I1-I, L/M, N, P, W, T, A/C, K, B/O, X, Y, and FII replicons were used in separate PCRs.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper will appear in the GenBank nucleotide database under accession numbers HM453325, HM370390 to HM370393, HM453326, HM453327, and HM626463.

RESULTS

Bacterial counts and susceptibility testing. The total bacterial count on MacConkey agar was 1.5×10^5 CFU/liter of Seine River water, of which 2.6% grew on ceftazidime-agar plates (3.6×10^3 CFU/liter). A total of 73 isolates were selected from ceftazidime-containing plates and subjected to identification. Fifty-six percent ($n = 41$) of the isolates that were resistant or of reduced susceptibility to ceftazidime were identified as *Aeromonas* spp., and the others were mostly *Pseudomonas* spp. and *Stenotrophomonas maltophilia.* Among those 41 ceftazidime-resistant *Aeromonas* spp., 29 (71%) displayed an ESBL phenotype, as evidenced by DDST. These isolates were identified as *A. punctata*, *A. allosaccharophila*, *A. veronii*, and *A. media*. Our study then focused on those 29 ESBL-producing *Aeromonas* isolates. The MICs of β-lactams, the patterns of resistance to other antimicrobial agents, and the molecular typing were used to evaluate that collection, and the findings are presented in Table 2. The 29 ceftazidime-resistant isolates were resistant to ticarcillin and had reduced susceptibility to cefotaxime and cefepime, but none of them were resistant to imipenem (Table 2). Addition of clavulanic acid significantly decreased the MICs of ceftazidime and aztreonam (Table 2). More than 86% of these isolates were resistant to sulfonamides and to nalidixic acid, although *Aeromonas* spp. are naturally fully susceptible to quinolones (Table 2) (22).

Identification of acquired β-lactamase genes. PCRs and sequencing performed on the 29 *Aeromonas* sp. isolates exhibiting an ESBL phenotype, using primers specific for β-lactamase genes, identified the $bla_{\text{VEB-1a}}$ ($n = 11$), $bla_{\text{SHV-12}}$ ($n = 10$), $bla_{\text{PER-1}}$ ($n = 3$), $bla_{\text{PER-6}}$ ($n = 3$), $bla_{\text{TLA-2}}$ ($n = 1$), and $bla_{\text{GES-7}}$ ($n = 1$) genes (Table 2).

Clonal relationship. PFGE analysis performed on the 29 *Aeromonas* sp. isolates showed 19 distinct genotypes, including 4 clonally related *A. allosaccharophila* isolates harboring an identical $bla_{\text{SHV-12}}$ gene (Table 2; data not shown). The ESBL genes were therefore identified in a large diversity of *Aeromonas* species (Table 2).

Genetic support of $bla_{\text{SHV-12}}$ gene. PCR mapping and sequencing revealed that the $bla_{\text{SHV-12}}$ gene, identified in 10 *Aeromonas* sp. isolates, was preceded by insertion sequence (IS) IS*26*, as previously reported (10). Southern blot hybridization of plasmid DNA extracted from the $bla_{\text{SHV-12}}$-positive *Aeromonas* sp. isolates using the corresponding probe did not give any signal, suggesting a chromosomal location of that ESBL gene (data not shown).

Genetic support of $bla_{\text{TLA-2}}$ gene. PCR mapping to determine the genetic environment of the $bla_{\text{TLA-2}}$ gene in the single *A. allosaccharophila* isolate was performed with combinations of primers located in the 5′ CS, $bla_{\text{TLA-2}}$, and 3′ CS sequences. The $bla_{\text{TLA-2}}$ gene was not part of a gene cassette but was located in a class 1 integron, located on a ca. 50-kb plasmid. Mating-out transfer of the plasmid harboring the $bla_{\text{TLA-2}}$ gene to *E. coli* remained unsuccessful, suggesting that this plasmid has a narrow host range or was nonconjugative (43).

Genetic support of $bla_{\text{PER-1}}$ gene. The $bla_{\text{PER-1}}$ gene identified in three *Aeromonas* sp. isolates was preceded by insertion sequence IS*Pa12* and was followed by IS*Pa13*, thus being part of a composite transposon, Tn*1213*, as previously reported (35). Southern blot hybridization of plasmid DNA extracts recovered from the $bla_{\text{PER-1}}$-positive *Aeromonas* sp. isolates using the corresponding probes did not give any signal, suggesting a chromosomal location of the $bla_{\text{PER-1}}$ gene (data not shown).

Genetic support of $bla_{\text{PER-6}}$ gene. The $bla_{\text{PER-6}}$ gene was identified in an *A. veronii* isolate and in clonally related *A. allosaccharophila* (isolates 26 and 27). Southern blot hybridization of plasmid DNA extracted from these isolates, using a $bla_{\text{PER-6}}$-specific probe, did not give any positive signal, suggesting a chromosomal location of this gene (data not shown). Analysis of the sequences surrounding the $bla_{\text{PER-6}}$ gene by PCR mapping showed that they were different from those initially identified in *A. allosaccharophila* recovered from the Seine River in 2009 (15). Thus, cloning of the $bla_{\text{PER-6}}$ gene from *A. veronii* isolate 25 was performed, and sequencing revealed that the $bla_{\text{PER-6}}$ gene interrupted the Tn*1721* feature, including the *tetA*, *tetR*, and *tnpA*Δ*5* genes, as previously identified in the *A. allosaccharophila* AL-1 isolate (15) (Fig. 1).

TABLE 2. Resistance pattern of ESBL-producing *Aeromonas* sp. isolates

Isolate	*Aeromonas* species	Clone	ESBL identified	MIC (μg/ml)[b]									Non-β-lactam(s) to which isolate was resistant
				CTX	FEP	CAZ	CAZ + CLAV	TIC	TIC + CLAV	ATM	ATM + CLAV	IPM	
1	*A. punctata*	A1[a]	VEB-1a	8	2	32	0.25	128	16	8	<0.06	0.12	KAN, TOB, GEN, AMK, TET, NAL, CIP, FOF, CHL, SXT
2	*A. punctata*	A2	VEB-1a	8	4	32	0.25	128	1	8	<0.06	0.12	KAN, TOB, GEN, AMK, TET, NAL, CIP, FOF, CHL, SXT
3	*A. punctata*	A3	VEB-1a	4	1	32	0.06	128	0.5	2	<0.06	<0.06	KAN, TOB, AMK, TET, NAL, CIP, SXT
4	*A. punctata*	A3	VEB-1a	8	2	32	0.12	64	2	4	<0.06	<0.06	KAN, TOB, TET, NAL, CIP, SXT
5	*A. punctata*	A4	VEB-1a	4	2	16	0.25	128	128	2	<0.06	0.12	SXT
6	*A. punctata*	A4	VEB-1a	8	4	32	0.25	256	128	8	<0.06	0.12	SXT
7	*A. allosaccharophila*	B1	VEB-1a	4	2	16	0.06	32	2	2	<0.06	0.25	KAN, TOB, TET, NAL, SXT
8	*A. allosaccharophila*	B2	VEB-1a	8	2	16	0.06	128	16	4	<0.06	0.12	KAN, TOB, TET, NAL, CIP, SXT
9	*A. veronii*	C1	VEB-1a	0.25	0.12	2	0.06	16	4	0.5	<0.06	0.25	SXT
10	*A. veronii*	C2	VEB-1a	1	0.25	4	0.06	32	4	0.25	<0.06	0.5	
11	*A. media*	D1	VEB-1a	32	4	32	0.5	512	512	16	<0.06	<0.06	NAL
12	*A. allosaccharophila*	B3	SHV-12	0.25	0.25	2	0.06	128	1	1	<0.06	0.25	NAL
13	*A. allosaccharophila*	B3	SHV-12	1	1	4	0.06	512	2	2	<0.06	0.25	NAL
14	*A. allosaccharophila*	B4	SHV-12	4	0.25	4	0.06	256	2	2	<0.06	0.25	NAL
15	*A. allosaccharophila*	B4	SHV-12	1	0.5	4	0.12	512	2	2	<0.06	0.25	NAL
16	*A. allosaccharophila*	B4	SHV-12	1	0.25	4	0.06	256	2	2	<0.06	0.25	NAL
17	*A. allosaccharophila*	B4	SHV-12	1	0.25	4	0.06	256	2	1	<0.06	0.25	NAL
18	*A. veronii*	C3	SHV-12	4	1	4	0.06	512	16	4	<0.06	0.25	NAL
19	*A. veronii*	C3	SHV-12	1	1	4	0.06	512	8	4	<0.06	0.25	NAL
20	*A. veronii*	C3	SHV-12	4	2	8	0.06	128	8	2	<0.06	0.25	NAL
21	*A. media*	D2	SHV-12	8	2	32	0.5	512	256	16	<0.06	<0.06	NAL, RIF, SXT
22	*A. punctata*	A5	PER-1	8	4	32	0.25	256	64	16	<0.06	0.12	KAN, NAL, SXT
23	*A. punctata*	A6	PER-1	8	4	32	0.25	128	128	16	<0.06	0.12	NAL, SXT
24	*A. media*	D3	PER-1	16	4	64	0.25	128	16	64	<0.06	0.12	KAN, NAL
25	*A. veronii*	C4	PER-6	4	2	64	0.25	128	128	32	<0.06	0.12	TET, NAL, SXT
26	*A. allosaccharophila*	B5	PER-6	4	0.5	2	0.06	512	64	4	<0.06	0.5	TET, NAL, SXT
27	*A. allosaccharophila*	B5	PER-6	4	0.5	8	2	512	512	8	0.25	0.5	TET, NAL
28	*A. veronii*	C4	GES-7	2	0.5	16	2	64	32	0.25	<0.06	0.25	KAN, TOB, NAL
29	*A. allosaccharophila*	B6	TLA-2	2	1	8	0.12	128	64	1	<0.06	0.12	NAL, RIF

[a] The letter A was arbitrarily attributed to *A. punctata*, B to *A. allosaccharophila*, C to *A. veronii*, and D to *A. media*. Different numbers correspond to different clones.
[b] Abbreviations: AMK, amikacin; ATM, aztreonam; CHL, chloramphenicol; CAZ, ceftazidime; CIP, ciprofloxacin; CLA, clavulanic acid; CTX, cefotaxime; FEP, cefepime; FOF, fosfomycin; GEN, gentamicin; IPM, imipenem; KAN, kanamycin; NAL; nalidixic acid; RIF, rifampin; SXT, trimethoprim-sulfamethoxazole; SSS, sulfonamide; TET, tetracycline; TIC, ticarcillin; TOB, tobramycin.

Interestingly, the $bla_{\mathrm{PER\text{-}6}}$ gene was bracketed by 135-bp repeated elements (Res; named Re1*Av* and Re2*Av* for *A. veronii*) sharing 84 and 80% nucleotide sequence identities with the reverse complementary sequence of the previously reported Re1 that bracketed the $bla_{\mathrm{VEB\text{-}1a}}$ gene (3). Re1-like sequences have been identified in association with $bla_{\mathrm{VEB\text{-}like}}$ genes in *P. aeruginosa* (3), *Providencia stuartii* (4), and *Proteus mirabilis* (48). *In silico* analysis of previously reported sequences surrounding the $bla_{\mathrm{PER\text{-}6}}$ gene in *A. allosaccharophila* AL-1 showed a downstream-located Re1-like copy (namely, Re1*Aa*, for *A. allosaccharophila*) (Fig. 1). Re1*Aa* shares 80% nucleotide sequence identity with Re1*Av*.

Genetic support of $bla_{\mathrm{GES\text{-}7}}$ gene. Southern blot hybridization of plasmid DNA extracted from *A. veronii* isolate 28, using a probe specific for $bla_{\mathrm{GES\text{-}like}}$ genes, showed that the $bla_{\mathrm{GES\text{-}7}}$ gene was located on a ca. 60-kb plasmid. Partial sequencing of this natural plasmid (named Tf28) showed that the $bla_{\mathrm{GES\text{-}7}}$ gene was located inside an original structure in which the 3′ extremity of the class 1 integron was absent (Fig. 2). The $bla_{\mathrm{GES\text{-}7}}$ gene was in the form of a gene cassette, located at the first position of a class 1 integron and followed by the *aacA4* gene. Downstream of the *aacA4* gene, there was no 3′ CS sequence identified, but instead, the *orf15* and *mobA* gene arrays were identified. The structure containing *orf15*, *mobA*, *orf2*, *orf3*, and *orf4* was previously identified in plasmid pRSB101 (43). Between the *aacA4* and *orf15* genes, the terminal inverted repeat (IRt) of Tn*402* was identified. The terminal inverted repeat of class 1 integrons (IRi) is present upstream of the gene encoding the IntI1 integrase and has the same boundary with the adjacent sequence as in pRSB101 (43). In order to determine whether the backbone of the Tf28 plasmid was the same as that of pRSB101, PCR amplification was performed with primers located in the transporter module (*orf11*, encoding a resistance-nodulation-cell division [RND] efflux mem-

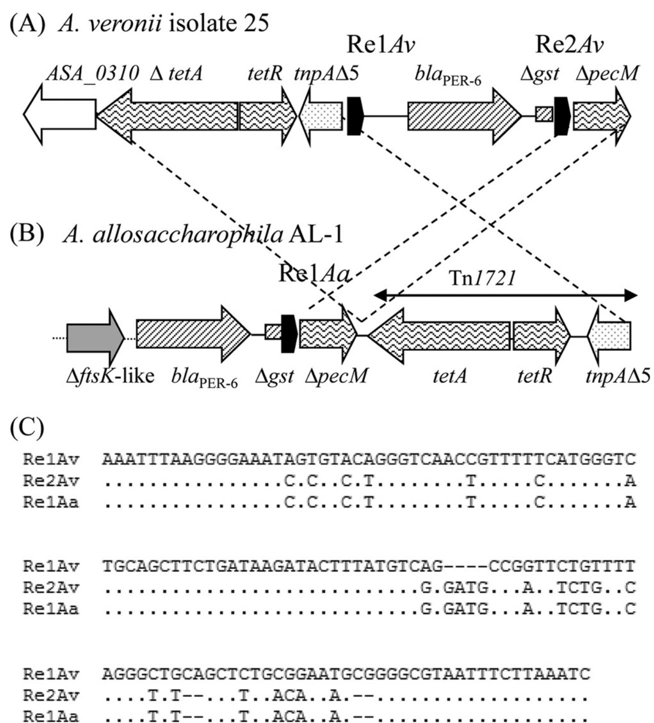

FIG. 1. Schematic representation of the genetic environment of β-lactamase genes. The genetic environment of the bla_{PER-6} gene in *A. veronii* isolate 25 and *A. allosaccharophila* isolates 26 and 27 (A) is compared to the previously identified structure in *A. allosaccharophila* AL-1 (B) (15). Arrows indicate the direction of transcription of the coding regions. Repeated elements Re*Av* and Re*Aa* are indicated as short black arrows. The open reading frame named ASA_0310 is a sugar-phosphate isomerase from *A. salmonicida*; TnpAΔ5 is a truncated relaxase/helicase. (C) Comparison of the sequences of Re1*Av*, Re2*Av*, and Re1*Aa*. Dashes indicate gaps introduced to optimize alignment, and periods indicate nucleotides identical to those in the Re1*Av* sequence.

brane fusion protein) and in the *repA* gene. The absence of PCR amplification suggested a different backbone. Mating-out assays between *A. veronii* isolate 28 and *E. coli* DH10B, performed at 37°C or at 30°C, remained unsuccessful, suggesting that the Tf28 plasmid was not self-transferable.

Genetic support of bla_{VEB-1a} gene. The bla_{VEB-1a} gene was identified in 11 *Aeromonas* sp. isolates. Plasmid extraction and Southern hybridization showed that the bla_{VEB-1a} gene was chromosomally located in *A. punctata* isolates 1 and 2, whereas it was plasmid located in all the other *Aeromonas* sp. isolates. The sizes of the plasmids harboring the bla_{VEB-1a} gene varied from 30 kb in *A. media* isolate 11 to 50 kb in *A. allosaccharophila* isolates 7 and 8 and up to 170 kb in all other isolates. Investigation of the genetic context with primers designed from the previously identified structures failed. The identification of $bla_{VEB-1-like}$ genes has been reported from the family *Enterobacteriaceae*, *P. aeruginosa*, and *A. baumannii* recovered worldwide (27, 36, 39). The bla_{VEB-1} gene is often part of a gene cassette located in class 1 integrons. However, in some cases, the bla_{VEB-1} and bla_{VEB-1a} genes have been identified in association with Res in *P. aeruginosa* and the *Enterobacteriaceae* (3, 28, 38). More recently, the bla_{VEB-1a} gene has been identified in association with two copies of insertion sequence IS*CR2*

A. veronii isolate 28

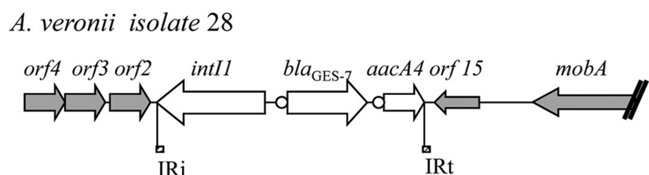

FIG. 2. Genetic environment of the bla_{GES-7} gene in *A. veronii* isolate 28. Arrows indicate the direction of transcription of the coding regions. The *orf2*, *orf3*, *orf4*, *orf15*, and *mobA* genes previously identified in plasmid pRSB101 (43) are indicated as gray arrows. IRt of the Tn*402*-like sequence and IRi are also shown.

(37). PCR assays revealed that none of these previously described genetic structures was bracketing the bla_{VEB-1a} gene in the *Aeromonas* isolates recovered in the present study. Sequence analysis of the obtained recombinant or natural plasmids harboring the bla_{VEB-1a} gene revealed some similarities among all genetic environments, as shown in Fig. 3. In particular, partial sequences of a gene encoding a TnpF-like putative integrase from *Acinetobacter* genomospecies 3 (GenBank accession no. GQ926879) and of insertion sequences IS*6100* and IS*26* were identified (Fig. 3). Mating-out assays performed with *Aeromonas* isolates 3 to 11 as donors and *E. coli* J53 as the recipient, either at 37°C or at 30°C, remained unsuccessful, suggesting that these plasmids were not self-transferable. PBRT analysis showed that the bla_{VEB-1a}-bearing plasmids did not belong to any of the tested Inc groups that correspond to plasmids identified in the *Enterobacteriaceae* (6).

DISCUSSION

Aeromonas spp. were the predominant bacteria recovered in this study, representing 56% of the colonies obtained on ceftazidime-containing plates. The ceftazidime resistance that we have observed was mainly related to the expression of ESBLs (71%). Many ESBLs that are not frequently identified among clinically relevant Gram-negative isolates in Paris (29), namely, VEB-1a, GES-7, TLA-2, and PER-1 and PER-6, have been identified in this screening. It was actually unexpected to identify such an important diversity of class A ESBLs in *Aeromonas* spp., which are species known to already possess intrinsic Ambler class B, C, and D β-lactamase genes. This raises the question of why *Aeromonas* spp. are so often hosts for those antibiotic resistance determinants. This waterborne location may be one part of the explanation. Rivers are subjected to the effects of several human activities. We considered this environment to be a good model for studying the prevalence and molecular diversity of genes that might represent a potential risk for human health. *Aeromonas* spp. living in water were demonstrated to be a potent reservoir of antibiotic resistance genes.

The bla_{VEB-1a} gene was identified in 38% of *Aeromonas* spp. resistant to or with reduced susceptibility to ceftazidime recovered from the Seine River, whereas bla_{VEB-1} has been identified in France only once in *E. coli* (25) and several times in *A. baumannii* (27, 36). The wide dissemination of this gene among so diverse *Aeromonas* species is of interest, since it likely indicates that the natural reservoir of that clinically relevant ESBL gene is waterborne or raises the possibility of Seine River

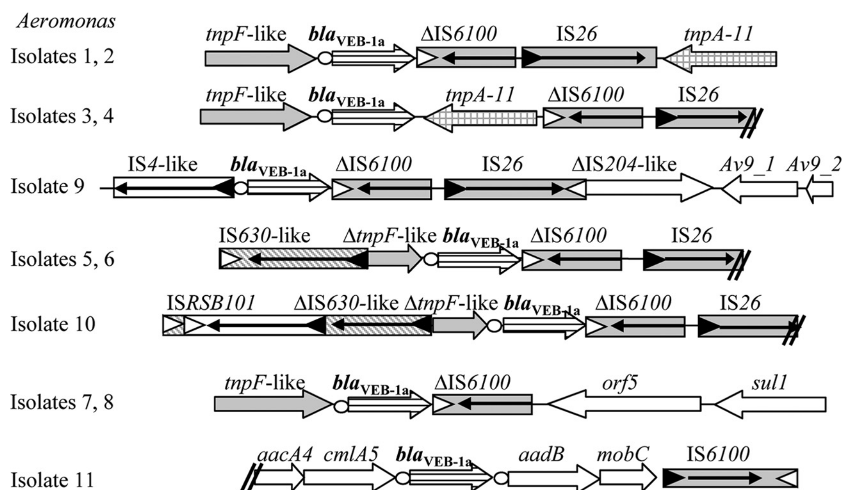

FIG. 3. Genetic environment of the bla_{VEB-1a} gene in *A. punctata* isolates 1 to 6, *A. allosaccharophila* isolates 7 and 8, *A. veronii* isolates 9 and 10, and *A. media* isolate 11. The coding genes are represented by arrows indicating their translation orientation, the left and right inverted repeats of IS elements are shown by filled and empty triangles, respectively, and the core sites are indicated as circles. IS*6100*, IS*26*, and *tnpF*-like genes (gray rectangles) are recovered in most structures. TnpF-like is a putative integrase from *Acinetobacter* genomospecies 3 (GenBank accession no. GQ926879). TnpA-11 (arrow with gray squares) from *A. punctata* 1 to 4 shares 83% amino acid sequence identity with the sequence of transposase_11 from *A. punctata* (GenBank accession no. YP_067863). Av9_1 and Av9_2 from *A. veronii* 9 are proteins of unknown function.

contamination. In addition, our study emphasizes that the dissemination of $bla_{VEB-1-like}$ genes is related to a variety of genetic structures.

In addition, other rare β-lactamase-encoding genes were identified here, in particular, the bla_{TLA-2}, bla_{PER-6}, and bla_{GES-7} genes. The bla_{TLA-2} gene had already been identified on plasmid pRSB101, which had been recovered from wastewater treatment plants in Germany but which has never been reported from clinical samples (43). The bla_{PER-1} gene is mainly reported in Europe and Asia (45, 46, 23) and to a lesser extent in North Africa (18), but it has very rarely been identified in France (35). The bla_{PER-6} gene has been identified once from the Seine River in *A. allosaccharophila* (15). Similarly, the $bla_{GES-7/IBC-1}$ gene has been detected only from clinical *Enterobacteriaceae* in Greece (11, 47) and in Brazil (8) but had never been identified in France.

Analysis of the genetic structures surrounding all these ESBL genes in environmental *Aeromonas* isolates revealed an important diversity of genetic supports and genetic environments. Noticeably, the identification of Re*Av* repeated elements in association with the bla_{PER-6} gene further underlines that those still underinvestigated genetic elements might play a relevant role in antibiotic resistance gene dissemination and mobilization.

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