Novel Ambler Class A β -Lactamase LAP-1 and Its Association with the Plasmid-Mediated Quinolone Resistance Determinant QnrS1[∇]

Laurent Poirel,¹ Vincent Cattoir,^{1,2} Ana Soares,¹ Claude-James Soussy,² and Patrice Nordmann^{1*}

Service de Bactériologie-Virologie, Hôpital de Bicêtre, Assistance Publique/Hôpitaux de Paris, Faculté de Médecine Paris-Sud, Université Paris-Sud, K-Bicêtre,¹ and Service de Bactériologie-Virologie, Hôpital Henri Mondor, Créteil,² France

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The plasmid-mediated quinolone resistance determinant QnrS1 was identified in non-clonally related *Enterobacter cloacae* isolates in association with a transferable narrow-spectrum β -lactam resistance marker. Cloning experiments allowed the identification of a novel Ambler class A β -lactamase, named LAP-1. It shares 62 and 61% amino acid identity with the most closely related β -lactamases, TEM-1 and SHV-1, respectively. It has a narrow-spectrum hydrolysis of β -lactams and is strongly inhibited by clavulanic acid and sulbactam and, to a lesser extent, by tazobactam. Association of the *bla*_{LAP-1} gene with the *qnrS1* gene was identified in *E. cloacae* isolates from France and Vietnam. These genes were plasmid located and associated with similar insertion sequences but were not associated with *sulI*-type class 1 integrons, as opposed to the *qnrA* genes.

Whereas numerous β -lactamases are increasingly reported worldwide, the diversity of acquired narrow-spectrum penicillinases remains quite limited in gram-negative organisms. They are mostly Ambler class A β -lactamases of the TEM, SHV, or CARB types (1). Most of the TEM- and SHV-type enzymes are inhibited by clavulanic acid, and many variants have a hydrolysis spectrum expanded to cephalosporins. Their genes are mostly plasmid located.

Plasmid-mediated determinants conferring resistance to quinolones (QnrA, QnrB, and QnrS) are increasingly reported in *Enterobacteriaceae* (15). Those pentapeptide repeat proteins usually confer resistance to nalidixic acid and reduced susceptibility to fluoroquinolones (14, 23). QnrA1 and QnrS1 share 59% amino acid identity, whereas both determinants share 40% amino acid identity with QnrB1. Association of the QnrA determinant with clavulanic acid-inhibited extended-spectrum β -lactamases (ESBL) (VEB-1, SHV-7, CTX-M-9, and CTX-M-14 [15, 22]) has been reported repeatedly. The *qnrA*-like genes have been identified in a large variety of enterobacterial species systematically associated with *sulI*-type integrons, involving the insertion sequence ISCR1 (formerly CR1 element) (27), likely at the origin of their acquisition and source of their expression (13, 24, 25, 28). The *qnrB1* gene was first identified on a plasmid that carried the ESBL gene *bla*_{CTX-M-15} in a *Klebsiella pneumoniae* isolate from India and then in several isolates of *Enterobacter cloacae*, *Citrobacter koseri*, and *Escherichia coli* from the United States (8). Other QnrB variants have been subsequently identified, with QnrB2 and QnrB5 being identified in non-Typhi *Salmonella enterica* isolates from the United States (4). The *qnrS1* gene was associated with the gene encoding the narrow-spectrum penicillinase TEM-1 in *Shigella flexneri* and *E. cloacae* from Asia (6, 21). The *qnrS1* gene was also identified in *Salmonella enterica* serotype Bovis-

morbificans from the United States (4) and *Salmonella enterica* serotype Infantis from Germany (10), whereas the *qnrS2* gene was found in *S. enterica* serotype Anatum from the United States (4).

Recently, by analyzing the prevalence of QnrA and QnrS resistance determinants in a collection of ESBL⁺ and ESBL[−] enterobacterial isolates recovered at the Bicêtre Hospital (France) from 2002 to 2005, the very first *qnrS* gene in Europe was identified (19). This *qnrS1* gene was identified in six *E. cloacae* isolates corresponding to four distinct genotypes, a single *E. coli* isolate, and a single *Serratia marcescens* isolate. When transferring the plasmid harboring the QnrS1 determinant in an *E. coli* recipient strain, we had observed that an ampicillin resistance marker had been cotransferred that was not related to a known β -lactamase (preliminary PCR analysis). Thus, the aim of the present study was to characterize this novel β -lactamase and to identify the genetic structures linking β -lactam and quinolone resistance determinants in these *qnrS1*-positive isolates.

MATERIALS AND METHODS

Bacterial strains. Enterobacterial isolates were identified with the API20E system (bioMérieux, Marcy l'Etoile, France). The QnrS1-positive isolates included in the study were *E. cloacae* S1 to S6, *E. coli* S7, and *S. marcescens* S8 from our hospital (19) and also *E. cloacae* 287 from Vietnam (21). These isolates produced the same QnrS1 determinant. The QnrA-positive isolates were *E. cloacae* A1, *Enterobacter aerogenes* A2, and *K. pneumoniae* A3 (19). *E. coli* DH10B was the host for cloning experiments, and azide-resistant *E. coli* J53 was used as the recipient strain for conjugation and transformation experiments (13).

Susceptibility testing. Antibiotic-containing disks were used for routine antibiograms performed by disk diffusion testing (Sanofi-Diagnostic Pasteur, Marnes-la-Coquette, France), as previously described (9). MICs were determined by an agar dilution technique, as described previously (20). MICs of β -lactams were determined alone or in combination with a fixed concentration of clavulanic acid (4 μ g/ml) or tazobactam (4 μ g/ml). MIC results were interpreted according to the guidelines of the CLSI (2).

PCR and hybridization experiments. Total DNA of enterobacterial isolates was extracted as described previously (25). This DNA was used as a template under standard PCR conditions (25) with a series of primers designed for the detection of class A β -lactamase genes *bla*_{TEM}, *bla*_{SHV}, and *bla*_{CARB} (5, 13, 18). Southern hybridizations were performed as described by Sambrook et al. (25) using the ECL nonradioactive labeling and detection kit (Amersham Pharmacia

* Corresponding author. Mailing address: Service de Bactériologie-Virologie, Hôpital de Bicêtre, 78 rue du Général Leclerc, 94275 Le Kremlin-Bicêtre cedex, France. Phone: 33-1-45-21-36-32. Fax: 33-1-45-21-63-40. E-mail: nordmann.patrice@bct.aphp.fr.

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FIG. 2. Comparison of the amino acid sequence of β -lactamase LAP-1 to those of β -lactamases TEM-1 and SHV-1. Dashes indicate identical amino acid residues. Critical residues which are involved in the extension of the substrate profile in TEM or SHV enzymes and that are conserved in LAP-1 with respect to TEM-1 or SHV-1 are shaded in gray. Boldfaced amino acids are residues in conserved motifs. Numbering is according to the method of Ambler (1).

Biotech, Orsay, France). Screening of the *bla*_{LAP-1} gene among our strains was performed by PCR using primers LAP-1A (5'-CAATACAAAGCACAGAAGA CC-3') and LAP-1B (5'-CCGATCCCTGCAATATGCTC-3') (Fig. 1), and this PCR product was used as a specific probe for detection of the *bla*_{LAP-1} gene.

Cloning experiments, recombinant plasmid analysis, DNA sequencing, and PCR mapping. Total DNAs of *E. cloacae* S3 and S5 isolates were restricted with EcoRI or with EcoRI and SacI, ligated into the corresponding sites of plasmid pBK-CMV, and then transformed into the *E. coli* TOP10 reference strain, as described elsewhere (16), giving rise to recombinant plasmids pS3-1 (containing an EcoRI fragment) and pS5-1 (containing an EcoRI-SacI fragment), respectively. Recombinant plasmids were selected onto trypticase soy agar plates containing amoxicillin (30 μ g/ml) and kanamycin (30 μ g/ml). The cloned DNA fragments were sequenced on both strands with an Applied Biosystems sequencer (ABI 3100; Foster City, Calif.). The nucleotide and deduced amino acid sequences were analyzed and compared to sequences available over the Internet at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>).

PCR mapping was performed to identify the genetic structures surrounding the *qnrS1* gene in the different isolates tested. Primers specific for the *bla*_{LAP-1} gene were used in combination with primers PBP3B (5'-CGTCCTGAAGTG CACTGG-3'), orfB-B (5'-CAGCAGTCTGCGCAAGG-3'), 213A (5'-GCT TCAGCCTCAGCGTCAAG-3'), 213B (5'-CGATGAATCAGGCTCCAGTC-3'), qnrS-5'ext (5'-GCGAATGAATGTGCAAGCGG-3'), and qnrS-3'ext (5'-GAA CTCGACGGTTTATAGATCC-3') (Fig. 1).

Genetic support. Mating-out assays were performed with the *bla*_{LAP-1}-positive isolates as donors and azide-resistant *E. coli* J53 as recipient strain, as described previously (13). Plasmid DNAs of the *qnrS1*-positive isolates were extracted using the Kieser method (11).

β -Lactamase purification and isoelectric focusing (IEF) analysis. Cultures of *E. coli* DH10B(pS3-1) were grown overnight at 37°C in 4 liters of trypticase soy broth containing amoxicillin (30 μ g/ml) and kanamycin (30 μ g/ml). β -Lactamase was purified by ion-exchange chromatography. Briefly, the β -lactamase extract was sonicated, cleared by ultracentrifugation, treated with DNase, and dialyzed against 20 mM bis-Tris buffer (pH 6.1). This extract was loaded on a Q-Sepharose column equilibrated with the same buffer, and the β -lactamase-containing fractions were eluted in the flowthrough. After dialysis against 20 mM bis-Tris buffer (pH 8), the β -lactamase was reloaded on the Q-Sepharose column equilibrated with the same buffer, and the β -lactamase activity was retained in the column and then eluted using a NaCl gradient. Finally, the fractions containing the highest β -lactamase activity were pooled and concentrated

using an ultrafiltration filter tip (Sartorius, Goettingen, Germany). The purity of the enzyme was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (25).

IEF analysis was performed with an ampholine polyacrylamide gel (pH 3.5 to 9.5), as described previously (16), using purified β -lactamase extracts from a culture of *E. coli* DH10B(pS3-1). The focused β -lactamases were detected by overlaying the gel with 1 mM nitrocefin (Oxoid, Dardilly, France) in 100 mM phosphate buffer (pH 7.0).

Kinetic measurements. Purified β -lactamase was used for kinetic measurements performed at 30°C with 100 mM sodium phosphate (pH 7.0) with an ULTROSPEC 2000 UV spectrophotometer (Amersham Pharmacia Biotech) (17). The 50% inhibitory concentrations (IC₅₀s) were determined for clavulanic acid, tazobactam, sulbactam, cefoxitin, and imipenem. Various concentrations of inhibitors were preincubated with the purified enzyme for 3 min at 30°C to determine the concentrations that reduced the hydrolysis rate of 100 μ M benzylpenicillin by 50%.

The specific activity of the purified β -lactamase from *E. coli* DH10B(pS3-1) was obtained as described previously (16). One unit of enzyme activity was defined as the activity which hydrolyzed 1 μ mol of benzylpenicillin per min per mg of protein. The total protein content was measured with the DC protein assay kit (Bio-Rad, Ivry-sur-Seine, France).

Nucleotide sequence accession number. The nucleotide sequence data reported in this work have been deposited in the GenBank nucleotide database under accession no. EF026092.

RESULTS

Cloning and sequencing of the β -lactamase gene. Preliminary attempts to detect by PCR several Ambler class A β -lactamase-encoding genes failed (data not shown). Using total DNA of *E. cloacae* S3 as a template in cloning experiments, the pS3-1 recombinant plasmid was obtained. Sequence analysis of the 12-kb cloned EcoRI fragment of pS3-1 revealed an 858-bp-long open reading frame (ORF) encoding a 285-amino-acid preprotein. This protein was a β -lactamase designated LAP-1 and possessed the STFK, SDN, and KTG structural elements characteristic of the active site of Ambler class A β -lacta-

FIG. 3. Dendrogram obtained for representative Ambler class A β -lactamases by neighbor-joining analysis. The alignment used for the tree calculation was performed with ClustalX. Branch lengths are drawn to scale and are proportional to the number of amino acid changes. The distance along the vertical axis has no significance. The scale bar represents a 10% difference in amino acid sequence. Only the bootstrap values superior to 85% are indicated. The amino acid identities of each β -lactamase compared to LAP-1 are indicated in square brackets. Acquired narrow-spectrum β -lactamases in gram-negative organisms are ROB-1, CARB-2, LAP-1, TEM-1, and SHV-1.

mases (Fig. 2) (1). The G+C content of *bla*_{LAP-1} was 43.8%. β -Lactamase LAP-1 was distantly related to other class A β -lactamases. The highest percentages of amino acid identity were 62% and 61% with TEM-1 and SHV-1, respectively, whereas LAP-1 shared only 43% identity with CARB-2 (PSE-1) (Fig. 3).

Distribution of the *bla*_{LAP-1} gene. Since the *bla*_{LAP-1} gene was identified in a *qnrS*-positive isolate, the prevalence of this β -lactamase gene was evaluated in a collection of *qnrS*-positive isolates by PCR amplification. Eight *QnrS*-positive isolates recovered in our hospital during the 2005–2006 period were tested, including six *E. cloacae* isolates (including isolates S1 and S2, producing ESBLs SHV-12 and TEM-52, respectively), one *E. coli* isolate producing the ESBL CTX-M-1, and one TEM-1-positive *S. marcescens* isolate. PCR results indicated that five *E. cloacae* isolates (S1, S3, S4, S5, and S6) were positive for *bla*_{LAP-1}. Pulsed-field gel electrophoresis analysis showed that these five *E. cloacae* isolates corresponded to three distinct genotypes, isolates S1 and S4 on one hand and isolates S5 and S6 on the other hand being clonally related (data not shown). In addition, the *QnrS*-positive *E. cloacae* isolate 287 from Vietnam (21) was also found to harbor the *bla*_{LAP-1} gene.

None of the 10 *QnrA*-positive enterobacterial isolates recovered from our hospital or from other countries such as

Canada, Australia, and Thailand was positive for the *bla*_{LAP-1} gene. In addition, since the five *bla*_{LAP-1}-positive isolates were *E. cloacae*, we searched for the *bla*_{LAP-1} gene among a collection of 30 randomly selected nonreplicate *E. cloacae* isolates recovered in our hospital in 2006. PCR revealed that these isolates were all negative for the *bla*_{LAP-1} gene.

Antibiotic susceptibility. *E. cloacae* S3 was resistant to kanamycin and tobramycin (MICs of >256 μ g/ml), tetracycline, rifampin, and fluoroquinolones (MICs of ofloxacin and ciprofloxacin of >32 μ g/ml) (19). MICs of β -lactams for *E. cloacae* S3 and for *E. coli* DH10B(pS3-1) indicated the expression of a clavulanic acid-inhibited β -lactamase that spared expanded-spectrum cephalosporins and carbapenems (Table 1). MICs of β -lactams for other *bla*_{LAP-1}-positive isolates were distributed in the same ranges (data not shown).

Biochemical properties of LAP-1. IEF analysis showed that *E. coli* DH10B(pS3-1) had a β -lactamase activity with a pI value of 6.7 (data not shown). β -Lactamase activity with a pI value of 8.5 was also detected for *E. cloacae* S3, corresponding to the chromosomal cephalosporinase of *E. cloacae*. The specific activity of the purified β -lactamase LAP-1 was 350 U $\cdot$ mg of protein⁻¹. Its overall recovery was 70% with a 90-fold purification. The purity of the enzyme was estimated to be more than 95% according to sodium dodecyl sulfate-gel electrophoresis analysis (data not shown).

TABLE 1. MICs of β -lactams for *E. cloacae* clinical isolate S3, *E. coli* DH10B harboring recombinant plasmid pS3-1 expressing β -lactamase LAP-1, and the *E. coli* DH10B reference strain

β -Lactam(s) ^a	MIC (μ g/ml)		
	<i>E. cloacae</i> S3	<i>E. coli</i> DH10B(pS3-1)	<i>E. coli</i> DH10B
Amoxicillin	>512	512	4
Amoxicillin + CLA	256	4	4
Ticarcillin	>512	512	4
Ticarcillin + CLA	8	4	4
Piperacillin	16	4	1
Piperacillin + TZB	8	1	1
Cephalothin	>512	16	4
Cefuroxime	256	8	2
Ceftazidime	0.5	0.12	0.06
Cefotaxime	0.25	0.12	0.12
Cefepime	0.5	0.12	0.06
Cefoxitin	256	4	4
Aztreonam	0.12	0.12	0.12
Imipenem	0.12	0.06	0.06

^a CLA, clavulanic acid at a fixed concentration of 4 μ g/ml; TZB, tazobactam at a fixed concentration of 4 μ g/ml.

Kinetic parameters of LAP-1 showed its narrow-spectrum activity against β -lactams, including penicillins and cephalothin but excluding most expanded-spectrum cephalosporins (except cefepime), cephamycins, and carbapenems (Table 2). IC₅₀ determinations performed with benzylpenicillin as substrate showed that LAP-1 activity was inhibited by clavulanic acid (IC₅₀, 0.08 μ M) and sulbactam (IC₅₀, 0.2 μ M), and to a lesser extent by tazobactam (IC₅₀, 10 μ M). In addition, LAP-1 activity was inhibited by cefoxitin at a lower level (IC₅₀, 100 μ M) but was not inhibited by imipenem.

Genetic environment of *bla*_{LAP-1}. Sequencing of the 12,935-bp insert fragment of plasmid pS3-1 from *E. cloacae* S3 revealed several ORFs. Upstream of *bla*_{LAP-1}, an ORF was present that encoded a protein sharing 53 and 52% amino acid identity with a transpeptidase encoded by the genome of *Salmonella enterica* subsp. *enterica* and of *E. coli*, respectively (GenBank numbers YP151544 and ZP009224346). The ORF ends 64 bp upstream of the start codon of the *bla*_{LAP-1} gene and was truncated in correspondence to the EcoRI site used for cloning. Downstream of the *bla*_{LAP-1} gene, the *qnrS1* gene was identified (Fig. 1). The two resistance genes were separated by 1,597 bp that included the insertion sequence *ISEcl2*, previously identified upstream of the *qnrS1* gene in *E. cloacae* 287 (21) and located 11 bp downstream of *bla*_{LAP-1}. *ISEcl2* is a 1,282-bp insertion sequence belonging to the IS3 family and possessing 24-bp-long inverted repeats. It was made of two ORFs, OrfA being likely truncated by a single base substitution and encoding a 53-amino-acid-long protein and OrfB, encoding a 218-amino-acid-long protein (Fig. 1). No target site duplication was observed at the *ISEcl2* ends. In silico analysis showed that this *ISEcl2* element was also present in the *qnrS1*-positive pINF5 plasmid of *S. enterica* serotype Infantis (with a few nucleotide substitutions that led to a longer OrfA product of 122 amino acids) (10), where it has been truncated at its 3'-end extremity by the insertion of Tn3 (Fig. 1). Downstream of the *qnrS1* gene, the same features identified in *S. enterica* serotype Infantis were found, including a truncated resolvase protein and

TABLE 2. Kinetic parameters of purified β -lactamase LAP-1^a

Substrate	k_{cat} (s ⁻¹)	K_m (μ M)	k_{cat}/K_m (mM ⁻¹ ·s ⁻¹)
Benzylpenicillin	200	20	10,000
Amoxicillin	600	180	3,300
Ticarcillin	30	40	750
Piperacillin	75	50	1,500
Cephalothin	35	400	90
Cefuroxime	>100	>2,000	>50
Ceftazidime	<0.01 ^b	ND ^c	ND
Cefotaxime	<0.01	ND	ND
Cefepime	10	800	12
Cefoxitin	<0.01	ND	ND
Aztreonam	<0.01	ND	ND
Imipenem	<0.01	ND	ND

^a Data are the means of three independent experiments. Standard deviations were within 10% of the means.

^b No detectable hydrolysis (<0.01 s⁻¹).

^c ND, not determinable due to too-low affinity.

a segment showing significant homology with the *E. coli* CS12 fimbrial gene cluster. This region of homology corresponded likely to an inversion system. This element was 2,850-bp long, possessed 14-bp inverted repeats, and contained four open reading frames, one sharing a significant degree of homology with the resolvase of ISXc5, a peculiar transposable element (12). A switch between the sequence from *Salmonella* and that identified in pS3-1 was observed exactly after a TTATT site, which was possibly the original target site of a *bla*_{TEM-1}-borne Tn3 insertion in *S. enterica* serotype Infantis and was also present at the right-hand end of the invertase element (Fig. 1) (10). At the right extremity of that site, no IS26-related features were identified, by contrast to the sequence of *S. enterica* serotype Infantis (10). Sequence analysis revealed an open reading frame sharing 53% amino acid identity with the FipA protein of plasmid pKM101 of *E. coli* (26) or plasmid pGFT1 of *Salmonella enterica* serovar Dublin (3), these proteins being known to be expressed by conjugative broad-host-range plasmids, such as some IncN plasmids, and to represent a fertility inhibition protein that inhibits conjugal transfer of cohabitating IncP plasmids.

Interestingly, detailed analysis of recombinant plasmid pS5-1, obtained from *E. cloacae* isolate S5 and harboring *bla*_{LAP-1} and *qnrS1* genes, showed that its sequence was very similar to that observed in plasmid pS3-1, except that the invertase element, located exactly at the same position, was in the opposite orientation (Fig. 1). However, at the right-hand extremity of that element, no TTATT site was identified and the sequence differed from that of pS3-1, with the presence of an ORF encoding a 134-amino-acid protein of unknown function.

Subsequent PCR mapping revealed that the structures surrounding the *bla*_{LAP-1} and *qnrS1* genes in *E. cloacae* S1 and S6 were identical to those identified in *E. cloacae* S5 (data not shown). However, in isolates S4 and 287, PCR mapping revealed that the invertase element was absent. In these two latter isolates, the sequences present upstream of the *bla*_{LAP-1} gene were identical to those identified in isolates S3 and S5, whereas those present downstream of the *bla*_{LAP-1} gene differed (Fig. 1).

Genetic support of the β -lactamase determinant. Conjugation experiments allowed the transfer of a ca. 100-kb plasmid encoding β -lactamase LAP-1 from *E. cloacae* S3 and S4 isolates to recipient strain *E. coli* J53. These transconjugants exhibited an identical narrow-spectrum resistance profile to β -lactams, including resistance to ticarcillin antagonized by clavulanic acid addition and also a reduced susceptibility to piperacillin (Table 1). In addition, these transconjugants were resistant to nalidixic acid and had reduced susceptibility to fluoroquinolones, whereas no additional antibiotic resistance marker was cotransferred. Using the different *bla*_{LAP-1}-positive isolates as donors, conjugation experiments allowed us to obtain *bla*_{LAP-1}-positive transconjugants from *E. cloacae* S3 and S4 isolates but not with the other isolates as donors. However, plasmid analysis showed that the five *qnrS*-positive isolates possessed a similar-in-size plasmid of ca. 100 kb that cohybridized with the *bla*_{LAP-1}- and the *qnrS*-specific probes (data not shown). The plasmid coharboring the *bla*_{LAP-1} and the *qnrS1* genes in *E. cloacae* 287 was 50 kb in size (19).

DISCUSSION

This study identified a totally novel class A β -lactamase that had weak amino acid identity with known β -lactamases. The β -lactamase LAP-1 is one of the very few acquired narrow-spectrum class A β -lactamases described so far in gram-negative organisms. Most of the acquired β -lactamases reported recently in gram negatives have an extended-spectrum resistance profile, such as CTX-M, PER, VEB, BEL, BES, KPC, and IMI enzymes (7). Detailed analysis of its amino acid sequence showed that LAP-1 shared with the narrow-spectrum β -lactamases TEM-1 or SHV-1 the same amino acids, such as Asp104, Gly156, Arg164, Leu169, Asp179, and Gly238 (Fig. 2). Amino acid substitutions at those positions have been reported to be the source of extension of the resistance profiles (<http://www.lahay.org/studies/>). This observation correlates well with the narrow-spectrum resistance profile of LAP-1.

Analysis of the surrounding sequences of the *bla*_{LAP-1} gene showed that it was located next to the plasmid-encoded quinolone resistance gene *qnrS1*, those two genes being probably part of the same mobilizable unit. Interestingly, the GC content of the *qnrS1* gene (43.8%) is exactly identical to that of the *bla*_{LAP-1} gene. This might indicate that both antibiotic resistance genes come from the same ancestor. The *bla*_{LAP-1} gene is not part of a gene cassette in a class 1 integron structure and is not associated with any ISCR element (27, 28), as opposed to what is known now for recently identified β -lactamase genes, such as the *bla*_{VEB}, *bla*_{GES}, *bla*_{BEL}, and *bla*_{CTX-M} genes.

It is quite surprising that the *bla*_{LAP-1} gene has disseminated in non-clonally related *E. cloacae* isolates from different parts of the world, although its occurrence in the *S. flexneri* isolate from Japan, the *S. enterica* serotype Infantis isolate from Germany, and the *Salmonella* isolates from the United States is yet unknown. This association between the *bla*_{LAP-1} gene and the *qnrS1* gene might provide resistance or reduced susceptibility to both narrow-spectrum β -lactams and quinolones. This association may be quite frequent, and further extended surveys might analyze the distribution of this novel β -lactamase gene in *Enterobacteriaceae* to evaluate whether it represents an emerging threat.

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