

Identification of the Novel Narrow-Spectrum β -Lactamase SCO-1 in *Acinetobacter* spp. from Argentina[∇]

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By studying the β -lactamase content of several *Acinetobacter* spp. isolates from Argentina, producing the expanded-spectrum β -lactamases (ESBL) VEB-1a or PER-2, a novel Ambler class A β -lactamase gene was identified. It encoded the narrow-spectrum β -lactamase SCO-1, whose activity was inhibited by clavulanic acid. SCO-1 hydrolyzes penicillins at a high level and cephalosporins and carbapenems at a very low level. β -Lactamase SCO-1 was identified from unrelated VEB-1a-positive or PER-2-positive *Acinetobacter* spp. isolates recovered from three hospitals. The *bla*_{SCO-1} gene was apparently located on a plasmid of ca. 150 kb from all cases but was not associated with any ESBL-encoding gene. The G+C content of the *bla*_{SCO-1} gene was 52%, a value that does not correspond to that of the *A. baumannii* genome (39%). β -Lactamase SCO-1 shares 47% amino acid identity with CARB-5 and ca. 40% with the enzymes TEM, SHV, and CTX-M. A gene encoding a putative resolvase was identified downstream of the *bla*_{SCO-1} gene, but its precise way of acquisition remains to be determined.

Whereas many β -lactamases are being increasingly reported worldwide, the repertoire of acquired narrow-spectrum penicillinases remains limited among gram-negative bacteria. They are mostly of the Ambler class A and of the TEM, SHV, and CARB types (1). The most recently identified plasmid-encoded class A narrow-spectrum β -lactamase, LAP-1, has been identified together with the quinolone resistance determinant QnrS1 in *Enterobacter cloacae* isolates (17). Production of narrow-spectrum Ambler class A β -lactamases is common in members of the family *Enterobacteriaceae* but has been very rarely reported for *Acinetobacter* spp., including β -lactamases TEM-1 (7), TEM-2 (4), and CARB-5 (15), conferring additional resistance to carboxy- and ureidopenicillins.

By analyzing the β -lactamase content of expanded-spectrum- β -lactamase (ESBL)-producing *Acinetobacter* isolates collected from several hospitals in Argentina from October 2000 to July 2005 (of which several coproduce the carbapenem-hydrolyzing oxacillinase OXA-58 [20]), we have identified a novel gene encoding a narrow-spectrum β -lactamase (14). Thus, the aim of the present study was to characterize the biochemical properties of this novel β -lactamase and the genetic context of the gene.

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MATERIALS AND METHODS

Bacterial strains. Thirteen *Acinetobacter* spp. isolates were first identified with an API20NE system (bioMérieux, Marcy l'Etoile, France). Those isolates produced ESBL PER-2 ($n = 6$) or VEB-1a ($n = 6$) (16, 21). Four isolates also produced the carbapenem-hydrolyzing oxacillinase OXA-58 (13, 22). In addition, a single isolate that produced OXA-58 but which was ESBL negative was included in the study. Further analyses were performed to confirm identification of the isolates at the species level by sequencing the *rrs* gene, followed by a phylogenetic analysis, as described previously (5) (Table 1). Genomic DNA of *Acinetobacter johnsonii* producing the ESBL VEB-1a was used for cloning experiments (Table 1). *Escherichia coli* TOP10 was the host for cloning experiments, and azide-resistant *E. coli* J53 and *A. baumannii* CIP7010T or *A. baumannii* BM4547 were used as recipient strains for conjugation and transformation experiments (9, 19).

Susceptibility testing. Antibiotic-containing disks were used for routine antibiograms performed by disk diffusion testing (Sanofi-Diagnostic Pasteur, Marne-la-Coquette, France), as previously described (16). MICs were determined by an agar dilution technique as described previously (3). MICs of β -lactams were then determined alone or in combination with a fixed concentration of clavulanic acid (4 μ g/ml) or tazobactam (4 μ g/ml) and interpreted according to the guidelines of the CLSI (3).

PCR and hybridization experiments. Total DNA of *A. baumannii* isolates was extracted as described previously (16). Southern hybridizations were performed as described by Sambrook et al. (22), using an ECL nonradioactive labeling and detection kit (Amersham Pharmacia Biotech, Orsay, France). Screening of the *bla*_{SCO-1} gene among our strains was performed by PCR using primers SCO-1A (5'-GGCGGCTATCGCGCTAAAGC-3') and SCO-1B (5'-TGGCAGCGTCC TTTCTCC-3'), and this PCR product was used as the specific probe for detection of the *bla*_{SCO-1} gene, as described previously (19).

Cloning experiments, recombinant plasmid analysis, and DNA sequencing. Total DNA of VEB-1a-positive *Acinetobacter johnsonii* isolate 7037 was BamHI or EcoRI restricted, ligated into the corresponding sites of plasmid pBK-CMV, and then used to transform the *E. coli* TOP10 reference strain, as described

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TABLE 1. Features of the *Acinetobacter* isolates included in this study

Strain ^a	<i>bla</i> _{SCO-1}	ESBL	<i>bla</i> _{OXA-58}	Date of isolation (month/yr)	Hospital ^b	Reference	Number ^c
<i>A. johnsonii</i> 7037	+	VEB-1a	–	12/2004	HHH	This study	9
<i>A. baumannii</i> 5179	+	VEB-1a	–	03/2003	GUT	16	1
<i>A. baumannii</i> 5311	+	VEB-1a	–	10/2003	GUT	This study	4
<i>A. baumannii</i> 101	+	VEB-1a	–	07/2002	GUT	This study	3
<i>A. baumannii</i> 102	+	VEB-1a	–	12/2002	GUT	This study	2
<i>Acinetobacter</i> spp. strains 10 and 5597	–	VEB-1a	+	05/2004	HVV	This study	12
<i>A. baylyi</i> 5400	+	PER-2	–	11/2003	GUT	This study	7
<i>A. baumannii</i> FAV-1	–	PER-2	+	10/2000	FAV	16	10
<i>Acinetobacter</i> spp. strains 15TU and 7368	+	PER-2	+	04/2005	GUT	This study	6
<i>Acinetobacter</i> spp. strains 13BJ and 7415	–	PER-2	–	05/2005	HAP	This study	13
<i>A. junii</i> 7579	+	PER-2	–	07/2005	GUT	This study	8
<i>A. junii</i> 7446	+	PER-2	+	06/2005	HAC	This study	5
<i>A. baumannii</i> 5277	–	None	+	09/2003	GUT	This study	11

^a Isolates indicated as *A. baumannii* actually belong to the *A. calcoaceticus*-*A. baumannii* (ACB) complex.

^b Argentinian hospital (hosp.) sources, as follows: HHH, Hosp. Heller, Neuquen, province of Neuquen; GUT, Hosp. de Ninos Ricardo Gutierrez, Buenos Aires City; HVV, Hosp. Vilela, Rosario, province of Santa Fe; FAV, Fundacion Favalaro, Buenos Aires City; HAP, Hosp. Pinero, Buenos Aires City; HAC, Hosp. Area Cipoletti, Cipoletti, province of Rio Negro.

^c Numbers 1 to 13 correspond to lane numbers shown in Fig. 3.

previously (16). Recombinant plasmids were selected onto Trypticase soy (TS) agar plates containing amoxicillin (30 µg/ml) and kanamycin (30 µg/ml). The cloned DNA fragments of the p7037-B2 and p7007-E1 recombinant plasmids were sequenced on both strands with an ABI 3100 sequencer (Applied Biosystems, Foster City, CA). 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>).

Genetic support. Plasmid DNAs of the *bla*_{SCO-1}-positive isolates were extracted using the Kieser method (10). *E. coli* NCTC50192, harboring four plasmids of 154, 66, 38, and 7 kb, was used as the size marker for plasmids. Transformation assays were performed by electroporation with plasmid extracts from the *bla*_{SCO-1}-positive isolates identified in that study as donors and either *E. coli* J53, *A. baumannii* CIP7010T, or *A. baumannii* BM4547 as recipient strains, as described previously (8). Selection was performed on agar plates supplemented with amoxicillin (30 µg/ml) for *E. coli* or supplemented with ticarcillin (30 µg/ml) for *A. baumannii*.

β-Lactamase purification and isoelectric focusing analysis. Cultures of *E. coli* DH10B(p7037-B2) were grown overnight at 37°C in 4 liters of TS broth containing amoxicillin (30 µg/ml) and kanamycin (30 µg/ml). β-Lactamase was purified by ion-exchange chromatography. Briefly, the bacterial suspension was sonicated, cleared by ultracentrifugation, treated with DNase, and dialyzed against 20 mM Tris-HCl buffer (pH 8.0). This extract was loaded on a Q-Sepharose column, and the β-lactamase-containing fractions were eluted with a gradient of NaCl. The same procedure was repeated using a 20 mM Tris-HCl buffer (pH 7.0). 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 (SDS)-polyacrylamide gel electrophoresis analysis (22).

Isoelectric focusing (IEF) analysis was performed with an ampholine polyacrylamide gel (pH 3.5 to 9.5), as described previously (16), using purified enzyme. 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 model UV spectrophotometer (Amersham Pharmacia Biotech) (18, 19). Fifty percent inhibitory concentrations (IC₅₀s) were determined for clavulanic acid and tazobactam. 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 µM benzylpenicillin by 50%. The specific activity of the purified β-lactamase from *E. coli* DH10B(p7037-B2) was obtained as described previously in 100 mM sodium phosphate (pH 7.0), using 100 µM benzylpenicillin as the substrate (18, 19). One unit of enzyme activity was defined as the activity which hydrolyzed 1 µmol of benzylpenicillin per min per mg of protein. The total protein content was measured with a 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 number EF063111.

RESULTS

Cloning and sequencing of the β-lactamase gene. In the course of cloning the *bla*_{VEB-1a} gene (work in progress) and its surrounding sequences from *A. johnsonii* 7037, recombinant *E. coli* strains were obtained that, surprisingly, gave a narrow-spectrum and clavulanic acid-inhibited β-lactam resistance profile. PCR assays specific for the *bla*_{TEM}, *bla*_{SHV}, *bla*_{LAP}, and *bla*_{CARB} genes did not give positive results with those recombinant strains. Sequence analysis of the 7,193-bp cloned BamHI fragment of recombinant plasmid p7037-B2 obtained from isolate 7037 and exhibiting resistance to penicillins revealed an 867-bp-long open reading frame (ORF) encoding a 288-amino-acid preprotein. This protein had a β-lactamase-like amino acid sequence named SCO-1 and possessed the STFK and SDN structural elements characteristic of the active site of Ambler class A β-lactamases (Fig. 1) (1). In addition, it possessed an RTG motif in box VII of the Ambler class A β-lactamases that has been identified in some CARB-type β-lactamases (CARB-5, CARB-8, and RTG-1), whereas other CARB derivatives usually possess an RSG motif (2, 9, 11). The G+C content of *bla*_{SCO-1} was 52%, whereas it is 39% for the whole genome of *A. baumannii* (6). β-Lactamase SCO-1 was distantly related to all other class A β-lactamases. The highest percentage of amino acid identity was 47% with CARB-5 (and similar percentages with other CARB β-lactamases), whereas it shared only 40% identity with CTX-M enzymes and 40%, 40%, and 37% with TEM-1, LAP-1, and SHV-1, respectively (Fig. 2).

Antibiotic susceptibility. *A. johnsonii* 7037 was resistant to kanamycin, tobramycin, amikacin, gentamicin, rifampin, and sulfonamides. It remained susceptible to chloramphenicol, tetracycline, fosfomycin, nalidixic acid, and fluoroquinolones (data not shown). Its resistance pattern toward β-lactams included most penicillins, expanded-spectrum cephalosporins, cephamycins, and aztreonam. This isolate was fully susceptible to imipenem and meropenem and also to the β-lactam/inhibitor combinations such as amoxicillin/clavulanate, ticarcillin/clavulanate, and piperacillin-tazobactam. MICs of β-lactams for *E. coli* DH10B(p7037-B2) were consistent with the produc-

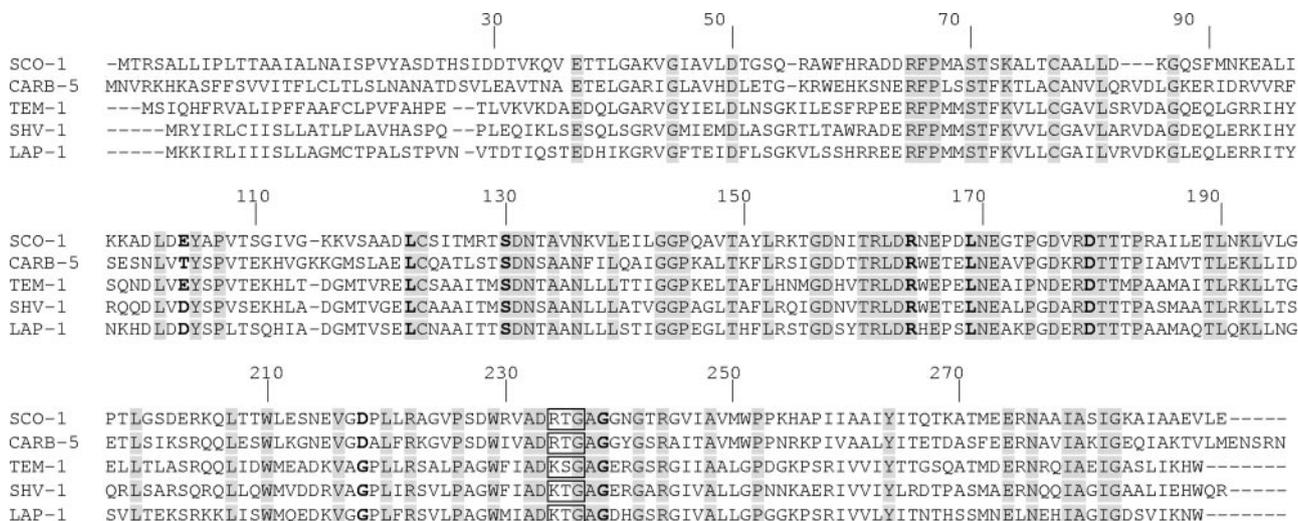


FIG. 1. Sequence alignment of the amino acid sequences of β-lactamases SCO-1, CARB-5, TEM-1, SHV-1, and LAP-1. Dashes indicate amino acids which are lacking in the corresponding sequence. Critical residues that are involved in the extension of the substrate profile in TEM or SHV enzymes are in bold type. Gray-shaded amino acids represent conserved residues. The box VII is bracketed. β-Lactamases are numbered according to the Ambler classification (1).

tion of a clavulanic acid-inhibited and narrow-spectrum β-lactamase that spared expanded-spectrum cephalosporins and carbapenems (Table 2).

Biochemical properties of β-lactamase SCO-1. IEF analysis showed that *E. coli* DH10B(p7037-B2) had a β-lactamase activity with a pI value of 5.5 (data not shown), also detected from a culture extract of *A. johnsonii* isolate 7037. The specific activity of purified β-lactamase SCO-1 for benzylpenicillin was 300 U/mg protein⁻¹. Its overall recovery was 80%, with a 60-fold purification factor. The purity of the enzyme was estimated to be more than 95%, according to SDS gel electro-

phoresis analysis (data not shown). Kinetic parameters of SCO-1 showed its narrow-spectrum activity against β-lactams, including mostly penicillins, and to a lesser extent against cephalothin, ceftazidime, and cefepime. Cephamycins and monobactams were not hydrolyzed, but a very weak hydrolysis of imipenem was noticed (Table 3). IC₅₀ determinations performed with benzylpenicillin as a substrate showed that SCO-1 activity was inhibited by clavulanic acid (IC₅₀, 0.3 μM) and tazobactam (IC₅₀, 1.2 μM).

Genetic environment of bla_{SCO-1}. Sequencing of the entire inserts of plasmids p7037-E1 (containing a bla_{SCO-1} and EcoRI

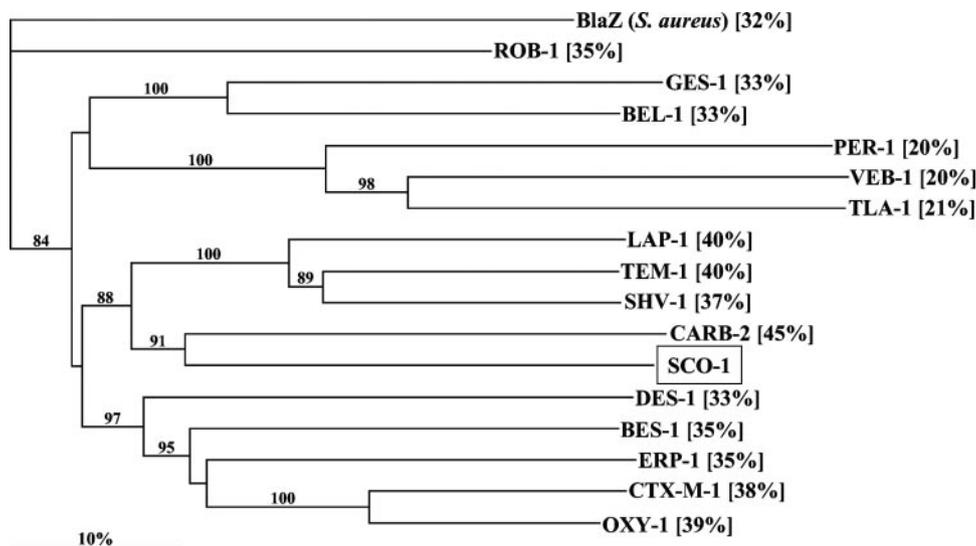


FIG. 2. Dendrogram obtained for representative Ambler class A β-lactamases by neighbor-joining analysis. The alignment used for tree calculation was performed with ClustalX. Branch lengths are drawn to scale and are proportional to the number of amino acid changes. The distance along the vertical axis has no significance. The scale bar represents 10% differences in amino acid sequences. Only the bootstrap values greater than 85% are indicated. The amino acid identities of each β-lactamase to SCO-1 are indicated in square brackets. The acquired narrow-spectrum β-lactamases in gram-negative bacteria are ROB-1, CARB-2, LAP-1, TEM-1, SHV-1, and SCO-1, whereas the others are either naturally occurring ones or extended-spectrum β-lactamases.

TABLE 2. MICs of β -lactams for selected strains^a

β -Lactam(s) ^b	MIC (μ g/ml) for:		
	<i>A. johnsonii</i> 7037	<i>E. coli</i>	
		TOP10(p7037-B2)	TOP10
Amoxicillin	>512	>512	2
Amoxicillin + CLA	1	32	2
Ticarcillin	>512	>512	2
Ticarcillin + CLA	2	128	2
Piperacillin	64	128	1
Piperacillin + TZB	2	1	1
Cephalothin	>512	8	2
Cefuroxime	>512	4	2
Ceftazidime	>512	0.5	0.06
Cefotaxime	>512	0.25	0.12
Cefepime	>512	0.12	0.06
Cefoxitin	>512	4	4
Aztreonam	>512	0.12	0.12
Imipenem	0.12	0.06	0.06

^a MICs (μ g/ml) of β -lactams for the *A. johnsonii* 7037 clinical isolate, *E. coli* DH10B harboring recombinant plasmid p7037-B2 expressing β -lactamase SCO-1, and the *E. coli* DH10B reference strain.

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

fragment from isolate 7037) and p7037-B2, obtained from isolate 7037 and p7037-B2 from *A. johnsonii* 7037, revealed several ORFs. Analysis of the sequences located just upstream of the *bla*_{SCO-1} gene identified, in the opposite orientation, a gene encoding a putative glycosyl hydrolase sharing 51% amino acid identity with that identified in the genome of *Pseudomonas putida* KT2440 (12). The distance separating this gene from the *bla*_{SCO-1} β -lactamase gene was 161 bp. Putative -35 (TGC ATA) and -10 (TAAAAC) promoter sequences separated by 17 bp were identified upstream of the *bla*_{SCO-1} structural gene. Downstream of the *bla*_{SCO-1} gene, a *tnpR*-like gene encoding a putative resolvase was identified that shared 92% amino acid identity with one identified in the antibiotic resistance island of *A. baumannii* strain AYE (6). Downstream of the putative glycosyl hydrolase-encoding gene, a putative *umuDC*-type operon was identified that contained two genes encoding UmuD and UmuC-like proteins that shared 44% amino acid identity with those of *E. coli* and 60% with those of *Roseobacter* sp. (NCBI RefSeq ZP_01058401) (Fig. 2). Those *umuD* and *umuC* genes organized as an operon involved in the SOS response, together with the *recA* gene (23). Downstream of the putative *umuDC* operon, a gene encoding a putative resolvase exhibiting 68% amino acid identity to a similar protein identified on a plasmid in a *Yersinia enterocolitica* strain was identified (24). Then, a gene encoding a protein exhibiting 85% amino acid identity to the OrfA transposase subunit of IS5 was identified.

Distribution of the *bla*_{SCO-1} gene among ESBL-producing *Acinetobacter* spp. isolates. Since the *bla*_{SCO-1} gene was identified from a *bla*_{VEB-1a}-positive isolate, we searched for this gene in a collection made up of other *bla*_{VEB-1a} or *bla*_{PER-2}-positive *Acinetobacter* spp. isolates from Argentina. Six VEB-1a-positive isolates (one of them coproducing β -lactamase OXA-58), six PER-2-positive isolates (three of them coproducing OXA-58), and a single ESBL-negative but OXA-58-positive isolate recovered in different hospitals from different

TABLE 3. Kinetic parameters of purified β -lactamase SCO-1

Substrate	SCO-1 parameter ^a		
	k_{cat} (s ⁻¹)	K_m (μ M)	k_{cat}/K_m (mM ⁻¹ · s ⁻¹)
Benzylpenicillin	150	20	7,500
Ampicillin	270	40	6,700
Ticarcillin	35	80	450
Piperacillin	0.4	10	40
Cephalothin	0.1	120	1
Cefuroxime	0.05	650	0.08
Ceftazidime	0.3	600	0.5
Cefotaxime	0.1	2,000	0.05
Cefepime	>1.5	>2,000	0.8
Cefoxitin	<0.01	ND	
Aztreonam	<0.01	ND	
Imipenem	0.03	3,800	0.008
Meropenem	<0.01	ND	

^a Data are the means of results from three independent experiments. Standard deviations were within 10% of the means. ND, no detectable hydrolysis (<0.01 s⁻¹) was observed using 1 μ M of purified SCO-1 and up to 500 μ M of substrate.

cities of Argentina during a 4-year period were tested (Table 1). PCR results indicated that 9 out of these 13 isolates were positive for the *bla*_{SCO-1} gene (Table 1). The *bla*_{SCO-1}-positive isolates corresponded to different *Acinetobacter* species.

Genetic support of the β -lactamase determinant. Conjugation experiments did not lead to a transfer of any plasmid encoding β -lactamase SCO-1 from different *Acinetobacter* spp. isolates to *E. coli* recipient strains. Attempts to transform a *bla*_{SCO-1}-carrying plasmid into *A. baumannii* recipient strains by electroporation remained unsuccessful. However, plasmid analysis showed that all the *bla*_{SCO-1}-positive *Acinetobacter* isolates (which are either PER-2 or VEB-1a producers) possessed a similarly sized plasmid of ca. 150 kb that hybridized with the *bla*_{SCO-1}-specific probe (Fig. 3) but did not cohybridize with the *bla*_{VEB-1a}- or the *bla*_{PER-2}-specific probe (data not shown). Thus, it is possible that a plasmid with a similar backbone harbored the *bla*_{SCO-1} gene in all these *Acinetobacter* spp. isolates.

DISCUSSION

This study identified a novel class A β -lactamase that had a weak amino acid identity to known β -lactamases. β -Lactamase SCO-1 constitutes one of the few acquired narrow-spectrum β -lactamases described so far in gram-negative bacteria. It possesses a clavulanic acid-inhibited narrow-spectrum hydrolysis toward β -lactams. Detailed analysis of its amino acid sequence showed that SCO-1 shared some structural identities with the CARB-type β -lactamases, possessing in particular an RTG motif which has already been identified in several carbencillinases such as β -lactamases RTG-1, CARB-5, and CARB-8. SCO-1 hydrolyzes mostly penicillins but also weakly ceftazidime and imipenem, which may suggest the possibility that other SCO variants may extend their substrate profile toward these substrates, as already observed for TEM- and SHV-type β -lactamases. Further experiments will be performed to evaluate this ability in vitro.

Analysis of the surrounding sequences of the *bla*_{SCO-1} gene showed that it was not part of a gene cassette and was not

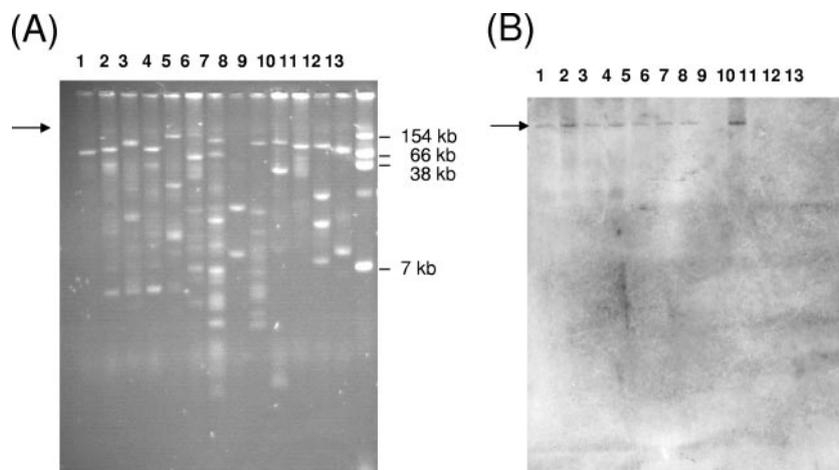


FIG. 3. Plasmid content analysis of the *Acinetobacter* spp. isolates. (A) Ethidium bromide-stained electrophoresis pattern of undigested plasmids and (B) corresponding Southern hybridization assay using a *bla*_{SCO-1}-specific probe. Lane numbers 1 to 13 refer to strains described in Table 1.

associated with integron features. However, a gene encoding a putative resolvase was associated at its 3' extremity that could indicate that the *bla*_{SCO-1} gene could be part of a transposon.

Interestingly, we showed here that the *bla*_{SCO-1} gene has disseminated in different *Acinetobacter* species. It seems very likely that dissemination of this gene could be linked to the spread of a single plasmid among all these isolates. It is noteworthy that the *bla*_{SCO-1} gene has been identified in cases from other Argentinean hospitals which are very distantly localized; Buenos Aires, for example, is distant (1,300 km) from the province of Rio Negro. Overall, these observations suggest the likelihood of a widespread diffusion for a *bla*_{SCO-1}-positive plasmid in Argentina that could also be identified in other South American countries. The impact (although quite moderate) of SCO-1 production under conditions of reduced susceptibility to expanded-spectrum cephalosporins may enhance the selection of *Acinetobacter* isolates exhibiting resistance to these molecules, such as those presented in this study and expressing acquired ESBLs.

Although the *Acinetobacter* isolates studied possessed other β-lactamase genes (*bla*_{VEB-1a}, *bla*_{PER-2}, and *bla*_{OXA-58}), the corresponding β-lactamase genes were not located on the same *bla*_{SCO-1}-positive plasmid. This observation is in accordance with the fact that our isolates were either positive or negative for *bla*_{SCO-1} independently of the presence of genes encoding broad-spectrum β-lactamases OXA-58, VEB-1a, and PER-2. This observation raises the question of the selection agent, if any, at the origin of the presence (or persistence) of the *bla*_{SCO-1} plasmid in *Acinetobacter* spp. isolates that are resistant to most (if not all) β-lactams, whereas SCO-1 possesses a narrow-spectrum of hydrolysis.

Epidemiological studies may be conducted to search for the *bla*_{SCO-1} gene in ticarcillin-resistant *Acinetobacter* isolates and to better estimate its prevalence. In addition, searching for this novel β-lactamase gene in other gram-negative bacteria (*Enterobacteriaceae*, *Pseudomonas* spp., etc.) and in other countries should help to evaluate whether the identification of this novel β-lactamase gene truly corresponds to the emergence of a novel resistance determinant.

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