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CARB-9, a Carbenicillinase Encoded in the VCR Region of *Vibrio cholerae* Non-O1, Non-O139 Belongs to a Family of Cassette-Encoded β-Lactamases†

Alejandro Petroni,[1]‡ Roberto G. Melano,[1,2]‡ Héctor A. Saka,[1] Alicia Garutti,[1] Laura Mange,[1] Fernando Pasterán,[1] Melina Rapoport,[1] Mariana Miranda,[1] Diego Faccone,[1] Alicia Rossi,[1]§ Paul S. Hoffman,[2] and Marcelo F. Galas[1]*

Servicio Antimicrobianos, Dpto. Bacteriología, Instituto Nacional de Enfermedades Infecciosas-ANLIS "Dr. Carlos G. Malbrán," Buenos Aires, Argentina,[1] and Department of Microbiology and Immunology, Dalhousie University, Halifax, Nova Scotia, Canada[2]

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The gene bla_{CARB-9} was located in the *Vibrio cholerae* super-integron, but in a different location relative to bla_{CARB-7}. CARB-9 (pI 5.2) conferred β-lactam MICs four to eight times lower than those conferred by CARB-7, differing at Ambler's positions V97I, L124F, and T228K. Comparison of the genetic environments of all reported bla_{CARB} genes indicated that the CARB enzymes constitute a family of cassette-encoded β-lactamases.

The clinical significance of *Vibrio cholerae* non-O1, non-O139 strains is being increasingly recognized since several outbreaks of diarrhea occurred in the early 1990s (3, 8, 29). A high prevalence of *V. cholerae* non=O1, non-O139 ampicillin (AMP)-resistant (Amp^r) isolates from both clinical and environmental origins was reported (3, 7, 10, 19, 29). To date, only three β-lactamases have been characterized in *V. cholerae* non-O1, non-O139 strains: CARB-2 (PSE-1), CARB-6, and CARB-7, which belong to the CARB family (Table 1). This major group of carbenicillinases is characterized by an RSG amino acid triad in positions 234 to 236 instead of the K-T/S-G motif of other class A β-lactamases (15). The proposed ancestors of carbenicillinases are the recently designated RTG enzymes (Table 1), which have an RTG triad instead of the CARB family RSG motif and share low identity with the CARB enzymes (5).

The bla_{CARB} genes have been broadly dispersed among distantly related bacteria, probably through mobile genetic elements (14, 18). Several bla_{CARB} genes were found as cassettes of class 1 integrons (Table 1), which have already been identified in *V. cholerae* (8, 29). In addition, a 126-kb-long integron (named the super-integron [SI]), comprising 216 open reading frames (ORFs) mainly of unknown functions, was found in the *V. cholerae* chromosome 2 (9, 17). Approximately 179 of these ORFs were found as cassettes, flanked by highly conserved sequences of 123 to 126 bp showing imperfect symmetry, named *V. cholerae* repeats (VCRs) (6, 9, 24).

In Argentina, we had previously observed a similar prevalence of AMP-nonsusceptible phenotype (Amp^{NS}; resistant plus intermediate categories) in clinical (29%) and environmental (32%) isolates of a sample of 669 *V. cholerae* non-O1, non-O139 strains. The analysis of a subset of 131 Amp^{NS} isolates detected two carbenicillinases, with pIs at 5.4 (CARB-7; 77 isolates) and 5.2 (54 isolates). The bla_{CARB-7} gene was located in the VCR island (19). Here, in order to characterize the β-lactamase-encoding gene of the second pool (pI 5.2), we selected the representative strain BA5, recovered from water samples in Buenos Aires province (Argentina, December 1993). The β-lactam MICs for BA5, determined as reported previously (19), were four to eight times lower than the corresponding figures for the CARB-7-producing isolate ME11762 (19). The MICs for BA5 and ME11762, respectively, were as follows: AMP, 64 and 256 μg/ml; AMP-sulbactam, 4 and 16 μg/ml; ticarcillin, 128 and 512 μg/ml; ticarcillin-clavulanic acid, 1 and 8 μg/ml; piperacillin, 8 and 32 μg/ml; cephalothin, 0.25 and 2 μg/ml; and cefoxitin, 1 and 8 μg/ml. By biparental conjugation (19), this resistant phenotype could not be transferred to *Escherichia coli*.

Characterization of bla_{CARB-9} and phylogenetic analysis. A Sau3AI-based genomic library of *V. cholerae* BA5 was prepared as described previously (19). An ~4-kb HindIII fragment, conferring Amp^r, was subcloned from a 9-kb Sau3AI insert and sequenced, resulting in a 3,985-bp-long sequence. Putative Sau3AI fragment rearrangements produced in the construction of the library were discarded by PCR-restriction fragment length polymorphism of two amplimers from genomic DNA of BA5 (primers P1/P2 [forward, 5′-CAGGTTGTCAGTTCTCTG; reverse, 5′-GCTAGCTAAAGGTTACTCG] and CARB-F/CARB-R [forward, 5′-CCATCTGTAGTTTTTGCAAGCAG; reverse, 5′-CAACGCGACTGTGATGTATAAAC]), which comprised all of the Sau3AI sites found in the sequenced region. The amplification conditions used were described previously (19), with annealing temperatures of 49°C (P1/P2) or 60°C (CARB-F/CARB-R).

Sequence analysis revealed a major ORF of 867 bp, named

* Corresponding author. Mailing address: Servicio Antimicrobianos, INEI-ANLIS "Dr. Carlos G. Malbrán," Av. Velez Sarsfield 563 (1281), Buenos Aires, Argentina. Phone/Fax: 54-11-4303-2812. E-mail: mgalas@anlis.gov.ar.

‡ A.P. and R.G.M. contributed equally to this work.

† This work is dedicated to the memory of our dear Ali.

§ Deceased.

TABLE 1. Sequences used in the alignments of the *bla* genes and the flanking regions of the bla_{CARB} genes

bla gene	Source, location (reference)	GenBank (accession no.)	FR-CARB (nucleotide position)[a]
bla_{CARB}			
bla_{CARB-1}[b]	*Pseudomonas aeruginosa*, CHR,[k] Tn2521, *ln*33 (22)	AF313471	*CARB-1* (1239–2450)
bla_{CARB-2}[c]	*P. aeruginosa*, pRPL11, Tn*1403*, *ln*28 (23)	AF313472	A.*CARB-2* (1324–2535)
(bla_{CARB-2})[d]	*Salmonella typhimurium*, pMG217 (unpublished)	Z18955	B.*CARB-2* (1–1199)
(bla_{CARB-2})[d]	*S. typhimurium*, CHR, class 1 integron (2)	AF071555	C.*CARB-2* (11109–12320)
(bla_{CARB-2})[d]	*V. cholerae*[e] plasmid, class 1 integron (8)	AF221899	D.*CARB-2* (1–1195)
bla_{CARB-3}	*P. aeruginosa*, CHR, Tn*1408* (13)	S46063	*CARB-3* (1–1060)
bla_{CARB-4}	*P. aeruginosa*, pUD12, Tn*1408*, class 1 integron (27)	U14749	*CARB-4* (501–1734)
bla_{N29}	*Proteus mirabilis*, unknown location (11)	D86225	*N29* (50–1260)
bla_{CARB-6}	*V. cholerae* non-O1, non-O139, CHR (4)	AF030945	*CARB-6* (1–967)
bla_{CARB-7}	*V. cholerae* non-O1, non-O139, SI (19)	AF409092	*CARB-7* (697–1933)
*bla*P2[f]	*S. typhimurium*, pST2301, class 1 integron (21)	AY123251	*blaP2* (3432–4651)
bla_{CARB-9}	*V. cholerae* non-O1, non-O139, SI (this work)	AY248038	*CARB-9* (1881–3119)
bla_{RTG}			
bla_{RTG-1}[g]	*P. mirabilis*, CHR (26)	D13209	NC[h]
bla_{RTG-2}[i]	*Acinetobacter calcoaceticus*, unknown location (5)	AF135373	NC
bla_{RTG-3}[j]	*Oligella urethralis*, CHR (16)	AY178993	NC

[a] FR-CARB, flanking region of bla_{CARB} gene. Nucleotide positions of aligned sequences in the corresponding GenBank reports are given in parentheses.
[b] Formerly known as bla_{PSE-4}. A shorter sequence containing bla_{CARB-1} in a Tn*1405* chromosomal background was also reported from *P. aeruginosa* (accession no. J05162) and was identical to a subsequence of AF313471.
[c] Formerly named as bla_{PSE-1}.
[d] Not included in the alignment of *bla* genes (sequences of bla_{CARB-2} in AF313472, Z18955, AF071555, and AF221899 shared 100% identity).
[e] bla_{CARB-2} was detected in both *V. cholerae* O1 and non-O1, non-O139 isolates; the sequence under accession no. AF221899 corresponds to a *V. cholerae* non-O1, non-O139 strain.
[f] Named as bla_{CARB-8} in the GenBank report.
[g] Formerly known as the β-lactamase of *P. mirabilis* GN79.
[h] NC, not considered; the bla_{RTG} genes were not included in the analysis of flanking regions.
[i] Formerly, bla_{CARB-5}.
[j] Also named as bla_{CARB-8}.
[k] CHR, chromosome.

bla_{CARB-9}, as it shares 99 and ~83% identities on both nucleotide and protein levels with bla_{CARB-7} and with other bla_{CARB} genes, respectively. A neighbor-joining-based phylogenetic tree was constructed from multiple alignment of bla_{CARB} and bla_{RTG} genes, using the Clustal X program (ftp://ftp-igbmc.u-strasbg.fr/pub/). Among the bla_{CARB} group, five highly related genes (i.e., $bla_{CARB-1-2-3}$, bla_{N29}, and *bla*P2 [named as the $bla_{CARB-1/2}$ subgroup]) were tightly clustered (Fig. 1). The evolutionary distance between this subgroup and the remainder of the genes suggests a more recent divergence in the evolution of the CARB family. Conversely, bla_{CARB-7} and bla_{CARB-9} appear to have differentiated earlier.

Besides three synonymous changes found between bla_{CARB-7} and bla_{CARB-9}, three nonsynonymous changes differentiated CARB-7 from CARB-9: I97V, F124L, and K228T, in the ABL numbering system (1) (Fig. 2). Although these changes are not located at the conserved positions found in class A β-lactamases, they may have a subtle effect on the activity of CARB-9 relative to CARB-7, which would explain the lower β-lactam MICs conferred by the former. In addition, the change K228T (a basic substituted for a noncharged amino acid) may explain the lower pI of CARB-9 (5.2) in comparison to CARB-7 (5.4) (19).

When comparing the bla_{CARB} genes, we noticed that the sequence of about the first two-thirds of bla_{CARB-6} was identical to that of $bla_{CARB-1-2-3}$, while the last third displayed a high identity with bla_{CARB-7}. This observation was corroborated by constructing two neighbor-joining phylogenetic trees based on separate alignments of partial sequences: (i) nucleotides 1 to 546 (NH_2 two-thirds) and (ii) nucleotides 547 to 867 (COOH third). The first tree showed that the NH_2 two-thirds of bla_{CARB-6} clustered with that of the $bla_{CARB-1/2}$ subgroup, while the COOH third of bla_{CARB-6} grouped with those of bla_{CARB-7} and bla_{CARB-9} in the second tree (data not shown). This uncommon nucleotide structure was reflected not only in the phylogenetic location of bla_{CARB-6}, but also in its deduced amino acid sequence (Fig. 2), suggesting the occurrence of a recombination event (see below).

The flanking regions of bla_{CARB-9}. The analysis of 2,072 nucleotides upstream and 1,046 nucleotides downstream to bla_{CARB-9} revealed six sequences (five of 123 bp and one of 122 bp) which shared an overall 95% identity with the reported VCR consensus sequence (data not shown) (6, 24). These VCRs were in the same orientation relative to one another bordering four predicted ORFs. The upstream region showed high identity mainly with other VCRs when compared with sequences in the GenBank database. Interestingly, almost all of the downstream region (nucleotides 2940 to 3967) shared 96% identity with a fragment of section 35 of *V. cholerae* chromosome 2 (nucleotides 5105 to 6132; GenBank accession no. AE004378). In BA5, this region comprised two VCRs bordering an ORF (nucleotides 3068 to 3664) which showed 99.7% identity with the locus VCA0455 (9) (nucleotides 5232 to 5828; GenBank accession no. AE004378). This analysis, in addition to the fact that bla_{CARB-9} was not transferred to *E. coli* by conjugation, supports the assumption that bla_{CARB-9}, like bla_{CARB-7}, is placed within the *V. cholerae* SI. These results may help to explain the high prevalence of Amp^r observed in

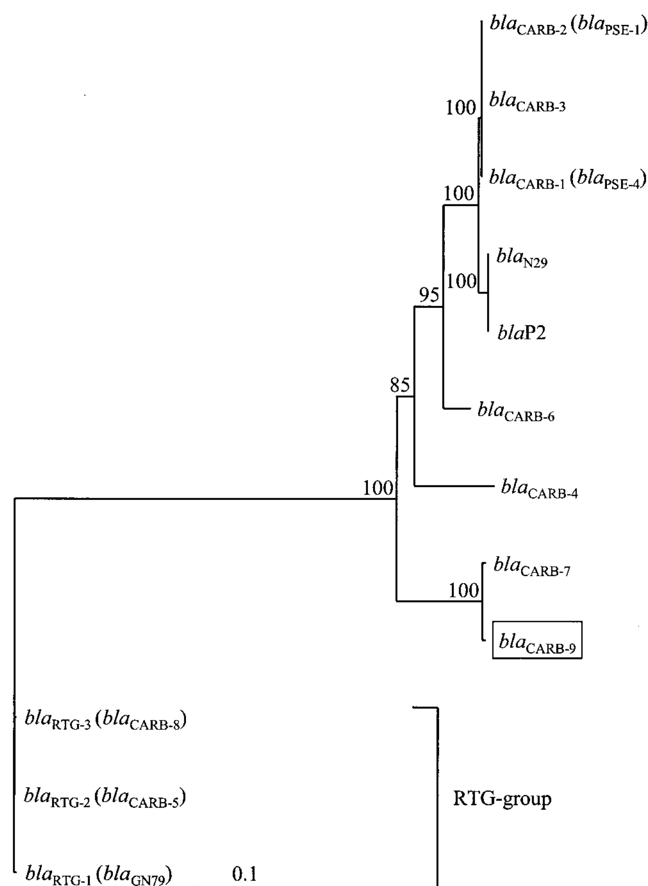

FIG. 1. Phylogenetic neighbor-joining tree of the bla_{CARB} genes. Relevant features of compared sequences are in Table 1. The RTG cluster of presumptive ancestors of the carbenicillinases (5, 16) is indicated. The tree was rooted with $bla_{\mathrm{RTG\text{-}1}}$. Bootstrap percentages (based on 1,000 replicates) of 70% (or higher) of key nodes are shown.

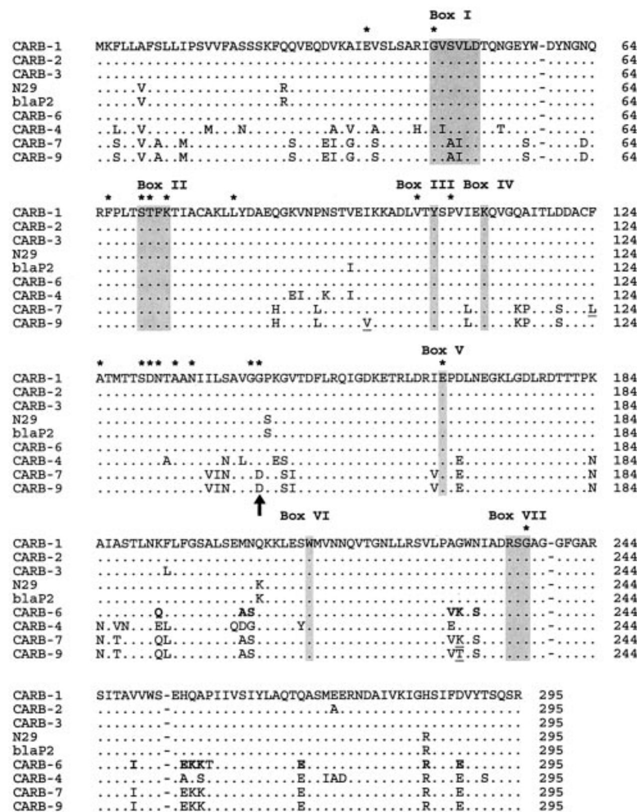

FIG. 2. Alignment of CARB-9 (288 amino acids) with the CARB enzymes. CARB-1 and CARB-2 were formerly named PSE-4 and PSE-1, respectively. Identical residues are indicated by dots. Amino acid motifs conserved in all penicillin-recognizing enzymes (12) are signaled by shaded boxes (I to VII). Residues highly conserved among class A β-lactamases are indicated by asterisks. The unique change, G144D, between CARB-7 and CARB-9 and the remainder of class A β-lactamases reported to date is signaled by the arrow; this change would be located far from the active site, as inferred from the crystallographic structure reported for CARB-1 (15). The three residues that discriminate between CARB-7 and CARB-9 are underlined. Amino acids that differentiate CARB-6 from CARB-1, -2, and -3 and which are identical to residues in the CARB-7 sequence are shown in boldface. Sequences are numbered as described by Ambler et al. Gaps (dashes) at positions 58, 239, and 253 are indicated (1).

clinical and environmental *V. cholerae* non-O1, non-O139 isolates. However, the comparison of the flanking regions of $bla_{\mathrm{CARB\text{-}9}}$ and $bla_{\mathrm{CARB\text{-}7}}$ (19) indicated that these genes share different VCRs (7% of genetic divergence) and that their locations inside the VCR island are clearly different. Moreover, the environments of the ORFs homologous to VCA0455 and VCA0424, in the isolates BA5 and ME11762 (CARB-7 producer) (19), respectively, are different from those found in *V. cholerae* El Tor N16961 (9). These facts constitute new evidence of plasticity in the VCR region (6).

The CARB enzymes constitute a family of cassette-encoded β-lactamases. In order to analyze the genetic background of bla_{CARB} genes, we compared 12 flanking regions reported to date, including four different environments of $bla_{\mathrm{CARB\text{-}2}}$ (Table 1). The upstream bla_{CARB} flanking sequences were identical for *CARB-1*, all of the *CARB-2* variants, *CARB-3*, *N-29*, and *CARB-6* (only 34 bp are available) (Fig. 3). The 3′ terminus of the 5′-conserved segment of class 1 integrons was previously reported in three of these regions (*CARB-1*, A.*CARB-2*, and C.*CARB-2*) (2, 22, 23). By sequence identity, we inferred the presence of the 5′-conserved segment upstream to $bla_{\mathrm{CARB\text{-}2}}$ in plasmid pMG217 (B.*CARB-2*), $bla_{\mathrm{CARB\text{-}3}}$, bla_{N29}, and $bla_{\mathrm{CARB\text{-}6}}$, which had not been previously associated with integrons. Despite the differences in genetic backgrounds, all of the bla_{CARB} genes are contained in cassettes sharing a common array of genetic elements. These elements are located in highly conserved boxes and comprise sequences required for both cassette integration and transcription of bla_{CARB} genes (Fig. 3): a unique core site (CS), putative promoters and ribosome-binding sites (RBS) (boxes 1 to 3); a double translation-stop signal (TGATAA), followed by a unique inverse CS (ICS) (box 4), and the 3′ terminus of *attC* and VCRs (box 5). In addition, sequences identical to the 105-bp-long *attC* previously reported downstream of $bla_{\mathrm{CARB\text{-}1}}$ and $bla_{\mathrm{CARB\text{-}2}}$ (A.*CARB-2*) (22, 23) were found downstream of the remainder of the $bla_{\mathrm{CARB\text{-}1/2}}$ genes (except $bla_{\mathrm{CARB\text{-}3}}$, where the sequence was unavailable). As this *attC* is predicted to form the typical stem-loop structure (DNASIS software v2.5; data not shown) previously reported for both *attC* and VCRs (24), we redefined the 5′ boundary of the *attC* reported for $bla_{\mathrm{CARB\text{-}2}}$ (C.*CARB-2* and D.*CARB-2*) and *bla*P2 cassettes, proposing a

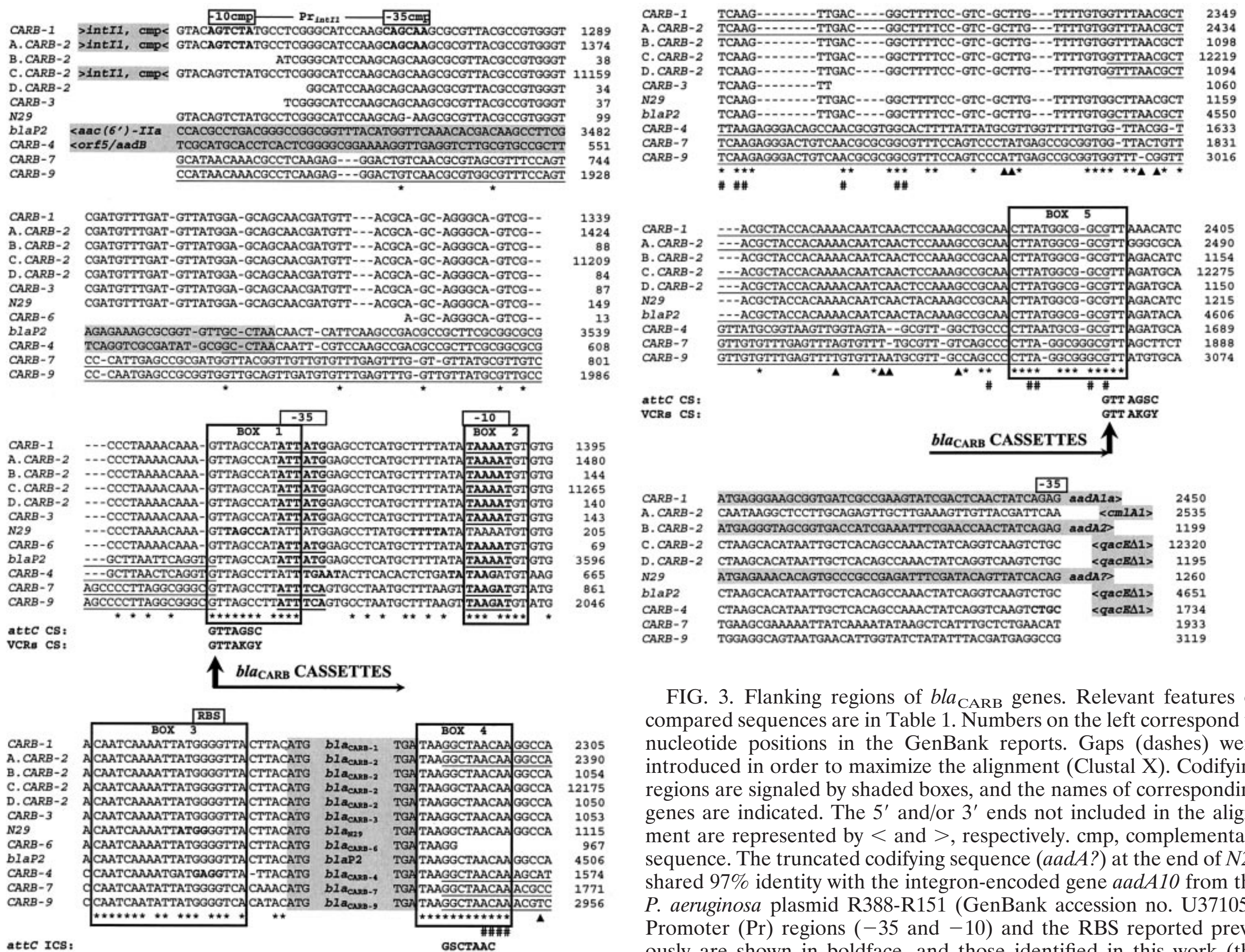

FIG. 3. Flanking regions of bla_{CARB} genes. Relevant features of compared sequences are in Table 1. Numbers on the left correspond to nucleotide positions in the GenBank reports. Gaps (dashes) were introduced in order to maximize the alignment (Clustal X). Codifying regions are signaled by shaded boxes, and the names of corresponding genes are indicated. The 5′ and/or 3′ ends not included in the alignment are represented by < and >, respectively. cmp, complementary sequence. The truncated codifying sequence (*aadA?*) at the end of *N29* shared 97% identity with the integron-encoded gene *aadA10* from the *P. aeruginosa* plasmid R388-R151 (GenBank accession no. U37105). Promoter (Pr) regions (−35 and −10) and the RBS reported previously are shown in boldface, and those identified in this work (the BPROM program, http://www.softberry.com/berry.phtml?topic = promoter) are additionally underlined. For clarity, VCRs and reported *attC* elements (underlined sequences) were defined from the third nucleotide upstream to the motif TAAC in the ICS, up to the G in the GTT of the CS. Consensus sequences for both ICS and CS are shown below the alignment. The identities among all available sequences (*) and positions highly conserved among *Vibrio* repeated sequences (VXRs; #) (24) are indicated. The regions of highest identity are boxed (boxes 1 to 5). Changes between the VCRs associated with bla_{CARB-7} and bla_{CARB-9} are signaled (▲). Horizontal arrows indicate the boundaries of the bla_{CARB} cassettes (5′ to 3′), and vertical arrows indicate the putative recombination points (between G and T in the GTT of the CS).

longer *attC* identical to that of bla_{CARB-1}. Thus, the CS and the ICS of bla_{CARB} cassettes, with the recombination points defined as described previously (24, 28), were perfectly complementary in any circularized cassette (Fig. 3). Interestingly, the occurrence of promoters is very uncommon in the cassettes from class 1 integrons (20, 28), while it would be very unlikely that a single promoter could drive expression of the plethora of cassettes within an SI (25).

The simplest explanation for to the aforementioned facts is that the bla_{CARB} genes have evolved as cassette-encoded rather than "naked" genes, from a unique ancestral cassette. Additional evidence supports this assumption. First, a neighbor-joining tree based on cassette noncoding sequences showed essentially the same topology as that of the encoding sequences depicted in Fig. 1 (data not shown), suggesting that both regions have evolved in parallel. Second, there are several identities scattered along the fragment from box 4 to box 5, which include all the highly conserved positions previously found among *Vibrio* repeated sequences (VXRs) (Fig. 3) (24). Thus, under this rationale, it is hypothesized that the ancestral cassette could have originated in *V. cholerae*, probably as a unit of its SI, since the earliest differentiated bla_{CARB} genes seem to be bla_{CARB-7} and bla_{CARB-9} (Fig. 1). The bla_{CARB-4} gene, with an *attC* only 3 bp shorter than the $bla_{CARB-7-9}$-associated VCRs (68% identity with them), may have emerged after horizontal exchanges mediated by plasmids and transposons/integrons, as an integron-associated cassette in *Pseudomonas aeruginosa* (27). Finally, the $bla_{CARB-1/2}$ group, showing a unique *attC*, can be considered as more recent cassette variants generated by nonsynonymous point mutations, while bla_{CARB-6} could be generated by recombination between bla_{CARB-7} and bla_{CARB-1}, bla_{CARB-2}, or bla_{CARB-3}. Interestingly, $bla_{CARB-1-2-3}$ are the most broadly dispersed genes among clinical isolates (8, 14, 18), while bla_{CARB-6} was only detected in a unique *V. cholerae* non-O1, non-O139 clinical strain (4). Further se-

quencing of the 3′-flanking region of bla_{CARB-6} will be useful to corroborate the recombination event. In summary, the CARB enzymes constitute the first family of cassette-encoded β-lactamases reported to date.

The data presented here also provide additional evidence to support the assumption of the in vivo capture of VCR cassettes by class 1 integrons, reinforcing the notion that these elements have evolved from SIs (25). Thus, the ORFs of SIs may constitute a big reservoir for horizontal gene transfer, including antibiotic resistance genes, such as bla_{CARB-7} and bla_{CARB-9}.

Nucleotide sequence accession number. The nucleotide sequence determined here will appear in the GenBank database under accession no. AY248038.

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