

## Nucleotide Sequence of the *bla*<sub>RTG-2</sub> (CARB-5) Gene and Phylogeny of a New Group of Carbenicillinases

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**We determined the nucleotide sequence of the *bla* gene for the *Acinetobacter calcoaceticus*  $\beta$ -lactamase previously described as CARB-5. Alignment of the deduced amino acid sequence with those of known  $\beta$ -lactamases revealed that CARB-5 possesses an RTG triad in box VII, as described for the *Proteus mirabilis* GN79 enzyme, instead of the RSG consensus characteristic of the other carbenicillinases. Phylogenetic studies showed that these RTG enzymes constitute a new, separate group, possibly ancestors of the carbenicillinase family.**

The assumption that carbenicillin-inactivating  $\beta$ -lactamases were a homogeneous cluster of enzymes confined to *Pseudomonas aeruginosa* strains (9) did not last long. First, some *Pseudomonas*-specific enzymes may have spread, on rare occasions, to various enterobacteria (19). Second, evidence is accumulating that, in addition to *Proteus mirabilis* in Japan (27) and other enterobacteria, strains of *Vibrio cholerae*, *Alcaligenes xylosoxidans*, *Aeromonas hydrophila*, and *Acinetobacter calcoaceticus* also produce such  $\beta$ -lactamases (SAR-1 and CARB-6 for *V. cholerae* and PSE-1, AER-1, and CARB-5 for *A. xylosoxidans*, *A. hydrophila*, and *A. calcoaceticus*, respectively). Major progress in the comprehension and classification of carbenicillinases is expected from structural data. Four main structures account for the diversity of carbenicillinases. The major structure is that of the CARB group, which includes CARB-1 (PSE-4), CARB-2 (PSE-1), CARB-3 (17, 18), CARB-4 (22, 25), CARB-6 (6), and *P. mirabilis* N29  $\beta$ -lactamase (12). The three other enzyme structures do not belong, at this time, to any particular group: PSE-3  $\beta$ -lactamase (5), *P. mirabilis* GN79 (24), and AER-1  $\beta$ -lactamases (25). Sequence information is currently unavailable for two  $\beta$ -lactamases, CARB-5 and SAR-1, which have been defined as carbenicillinases due to their hydrolytic properties. Our aim was to determine the nucleotide sequence of the *bla* gene (CARB-5) from *A. calcoaceticus* strain A85-145, the resistance properties and enzyme characteristics of which have been previously published (21).

**Clinical strains producing CARB-5.** Several clinical isolates of *Acinetobacter calcoaceticus* subsp. *anitratus*, resistant to ticarcillin alone but susceptible to ticarcillin in combination with clavulanic acid, have been found to produce a new  $\beta$ -lactamase, with a pI of 6.35 (14). Its molecular weight of 28,000 and substrate profile are typical of a carbenicillin-hydrolyzing enzyme. The enzyme was inhibited by anti-CARB-3 serum, *p*-chloromercuribenzoate, cloxacillin, clavulanic acid, and sulbactam (21).

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**Nucleotide sequence of *bla*<sub>CARB-5</sub>.** Several sets of oligonucleotide primer pairs specific for most CARB sequences were unsuccessfully used for amplification. Unexpected positive results were obtained with primers based on the sequence of the *P. mirabilis* GN79 *bla* gene (24). The nucleotide sequence of the structural gene was then determined by direct sequencing of PCR fragments amplified from chromosomal DNA extracted by the X-Trax procedure (6) with two pairs of synthetic primers (Table 1) comprising the whole reference sequence of the *P. mirabilis* GN79 gene. Amplification by PCR was performed at 44°C as described elsewhere (6).

The nucleotide sequence of the PCR product (912 bp) contains a 905-bp open reading frame. The coding region is 897 bp (positions 12 to 908) and encodes a protein of 298 amino acids (Fig. 1).

**CARB-5 is a class A  $\beta$ -lactamase.** Analysis of the deduced amino acid sequence showed that specific motifs were very similar to those of class A  $\beta$ -lactamases: the *bla* active-site (STFK) tetrad at positions 72 to 75 (70 to 73 according to the standard numbering scheme of Ambler [2]) and the consensual boxes I to VI described by Joris et al. (15) were conserved (Fig. 2). In contrast, box VII consisted of an RTG triad instead of the conserved RSG motif specific to the carbenicillinases (3). All the residues specific to class A  $\beta$ -lactamases were conserved except that the Leu at position 177 (164 according to Ambler numbering) was replaced with a Pro, as reported for *P. mirabilis* GN79 (24).

Nucleic and amino acid sequence analyses with the BLAST and FASTA (1) programs showed substantial homology (more than 99%) to the *P. mirabilis* GN79 sequence but only 43, 44, and 45% identity to the SHV (8), CARB (17, 18), and TEM (11)  $\beta$ -lactamases, respectively. Pairwise alignment between

TABLE 1. Oligonucleotide primers used in PCR amplification

Primer	Location (5'-3') <sup>a</sup>	Sequence (5'-3')
A1	301–320	GTAACTCATTATGAACGTA
A1'	866–852	TGTTGTCGTGTCTCG
A2	667–680	GGATGTCGCTCGCA
A2'	1212–1193	TAAATCAGTTACGGCTATTC

<sup>a</sup> Oligonucleotide positions are given according their location on the GN79 *bla* gene sequence (24).

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gttaaccaccatt M N V R K H K A S F F S V V I 15
ATG AAC GTA CGT AAA CAC AAG GCT AGT TTT TTT AGC GTA GTA ATT 54
T F L C L T L S L N A N A T D S V L 33
ACT TTT TTA TGT CTC ACG CTA TCA TTA AAT GCT AAT GCA ACA GAC TCA GTA CTT 108
E A V T N A E T E L G A R I G L A V 51
GAA GCG GTT ACC AAT GCT GAA ACT GAA TTA GGC GCT AGA ATT GGT CTA GCT GTG 162
H D L E T G K R W E H K S N E R F P 69
CAT GAT TTG GAA ACG GGA AAA CGT TGG GAA CAT AAA TCT AAT GAA CGT TTT CCT 216
L S S T F K T L A C A N V L Q R V D 87
CTA AGT AGT ACC TTT AAA ACA CTT GCC TGT GCA AAC GTT CTT CAA AGA GTT GAT 270
L G K E R I D R V V R F S E S N L V 105
CTA GGT AAA GAA AGA ATT GAT AGA GTT GTG AGA TTC TCT GAA AGC AAT CTC GTT 324
T Y S P V T E K H V G K K G M S L A 123
ACA TAC TCA CCT GTA ACA GAA AAA CAT GTG GGT AAA AAA GGG ATG TCG CTC GCA 378
E L C Q A T L S T S D N S A A N F I 141
GAG CTG TGT CAG GCC ACA TTA TCA ACC AGT GAT AAT TCA GCT GCC AAT TTT ATT 432
L Q A I G G P K A L T K F L R S I G 159
CTA CAA GCG ATT GGT GGA CCT AAG GCT CTA ACG AAA TTT TTG CGT TCC ATT GGC 486
D D T T R L D R W E T E L N E A V P 177
GAC GAT ACT ACG CGC CTT GAT CGC TGG GAA ACA GAA CTT AAC GAA GCG GTG CCT 540
G D K R D T T T P I A M V T T L E K 195
GGA GAT AAG CGA GAC ACG ACA ACA CCA ATT GCA ATG GTA ACG ACA CTT GAA AAG 594
L L I D E T L S I K S R Q Q L E S W 213
TTA CTA ATT GAC GAA ACA CTA TCT ATC AAA TCT CGT CAA CAA CTA GAA TCT TGG 648
L K G N E V G D A L F R K G V P S D 231
CTT AAA GGT AAT GAG GTT GGC GAT GCA TTG TTT CGT AAA GGC GTT CCA AGT GAC 702
W I V A D R T G A G G Y G S R A I T 249
TGG ATA GTA GCA GAT AGA ACA GGC GCT GGT GGT TAT GGG TCG CGT GCT ATT ACT 756
A V M W P P N R K P I V A A L Y I T 267
GCG GTG ATG TGG CCT CCA AAT CGC AAG CCT ATC GTA GCC GCT CTA TAC ATT ACA 810
E T D A S F E E R N A V I A K I G E 285
GAG ACA GAC GCC TCG TTT GAA GAA AGA AAT GCT GTC ATT GCA AAA ATT GGT GAG 864
Q I A K T V L M E N S R N * 299
CAA ATA GCG AAG ACA GTA TPA ATG GAG AAT AGC CGT AAC TGA tttt 910
    
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FIG. 1. Nucleotide and deduced amino acid sequence of *A. calcoaeticus*  $\beta$ -lactamase (CARB-5). The active site is boxed, and the differences from the *P. mirabilis* GN79  $\beta$ -lactamase sequence are underlined.

the CARB-5 and GN79 nucleic acid sequences revealed four mutations in the coding region (C to A at position 24, C to T at 163, G to T at 450, and C to A at 520) giving rise to three amino acid substitutions (Fig. 1 and 2): Gln to Lys at position 5 (2 according to Ambler), Ala to Val at position 51 (48 according to Ambler numbering), and Pro to Thr at position 170 (167 according to Ambler numbering). Multiple sequence alignment (28) of the CARB-5 amino acid sequence with the previously described sequences of the CARB-1, CARB-2 and CARB-3 (17, 18), CARB-4 (22, 25), CARB-6 (6), *P. mirabilis* N29 (12), and *P. mirabilis* GN79 (24)  $\beta$ -lactamases showed that the first amino acid change (Gln to Lys) was located outside the sequence of the other carbenicillinases, the second (Ala to Val) was common to all the carbenicillinase sequences, and the third (Pro to Thr) was unique among all aligned sequences (Fig. 2).

Although the CARB-5 enzyme contains the specific Arg 234 residue characteristic of carbenicillinases, CARB-5 seems to be only distantly related to these enzymes. Moreover, it also contains RTG box VII, reported for the first and only time for *P. mirabilis* GN79 (24), instead of the RSG usually encountered in carbenicillinases (3).

**Phylogenetic analysis.** A phylogenetic tree was constructed from ClustalW multiple sequence alignment of 29 class A  $\beta$ -lactamases (Table 2), using the neighbor-joining method (23). This tree (Fig. 3) exhibited two major branches. One, including

cephalosporinases, carbapenemases, and *Staphylococcus aureus* PC1 enzyme, constitutes the outgroup. The other ramifies into three main subbranches corresponding to three distinct groups. One of these contains the SHV and TEM enzymes and the second consists of the carbenicillinases according to the previously proposed scheme (4). Surprisingly, CARB-5 clustered with *P. mirabilis* GN79 to make up a third group distantly related to the other two groups. These results were confirmed by other methods (data not shown): PROTPARS (protein sequence parsimony method), PRODIST (protein distances) from the PHYLIP package of Joseph Felsenstein (Department of Genetics at the University of Washington), and Web Gene Bee services (Belozersky Institute, Moscow State University) (13). The reliability of the phylogenetic trees was estimated by bootstrapping (26). The node and branching leading to the CARB-5-GN79 cluster were confirmed in 997 and 1,000 of 1,000 bootstrap tests (data not shown). The general configuration of this tree differs slightly from that in Fig. 3 (neighbor-joining method), with PC1, BLAC\_BACSU, and BLA1\_BACMY making up a separate outgroup.

**CARB-5 (RTG-2) and *P. mirabilis* GN79 (RTG-1)  $\beta$ -lactamases are members of a new carbenicillinase group called RTG.** Nucleotide and amino acid sequence features and biochemical properties of the CARB-5 and *P. mirabilis* GN79 enzymes (21, 24) exhibit a great degree of similarity. Although the homology of these sequences with known CARB structures is low, they can be related to the carbenicillinase group in regard to their biochemical properties. However, despite their

TABLE 2. Sequences used in phylogenetic studies

Enzyme designation	Description (SwissProt or GenBank no.)
PC1.....	<i>Staphylococcus aureus</i> PC1 (BLAC_STAAU)
BLAC_BACSU.....	<i>Bacillus subtilis</i> cephalosporinase (BLAC_BACSU)
BLA1_BACMY.....	<i>Bacillus mycoides</i> cephalosporinase (BLA1_BACMY)
OXY-2.....	<i>Klebsiella oxytoca</i> cephalosporinase (BLA4_KLEOX)
BLAC_PROVU.....	<i>Proteus vulgaris</i> cephalosporinase (BLAC_PROVU)
BLAB_PROVU.....	<i>Proteus vulgaris</i> cefuroximase (BLAB_PROVU)
SME-1.....	Carbapenemase (BLAN_SERMA)
ENTCL.....	<i>Enterobacter cloacae</i> carbapenemase (BLAN_ENTCL)
GN79.....	<i>Proteus mirabilis</i> carbenicillinase (BLAC_PROMI)
CARB-1.....	<i>Pseudomonas aeruginosa</i> carbenicillinase (PSE-4) (BLP4_PSEAE)
CARB-2.....	<i>Pseudomonas aeruginosa</i> carbenicillinase (PSE-1) (BLP1_PSEAE)
CARB-3.....	<i>Pseudomonas aeruginosa</i> carbenicillinase (BLC3_PSEAE)
CARB-4.....	<i>Pseudomonas aeruginosa</i> carbenicillinase (PAU14749)
CARB-6.....	<i>Vibrio cholerae</i> carbenicillinase (AF030945)
N29.....	<i>Proteus mirabilis</i> carbenicillinase (D86225)
AER-1.....	<i>Aeromonas hydrophila</i> carbenicillinase (AHU14748)
PSE-3.....	<i>Rhodopseudomonas capsulata</i> sp108 (unpublished data)
TEM-1.....	<i>Escherichia coli</i> pBR322 (BLAT_ECOLI)
TEM-2.....	BLAT_ECOLI
TEM-3.....	<i>Klebsiella pneumoniae</i> penicillinase (KPBLATEM3)
LEN-1.....	<i>Klebsiella pneumoniae</i> bla (BLAC_KLEPN)
OHIO-1.....	<i>Enterobacter cloacae</i> SHV (BLA1_ENTCL)
SHV-1/PIT-2.....	<i>Escherichia coli</i> SHV-1/PIT-2 (BLA1_ECOLI)
SHV-1.....	<i>Klebsiella pneumoniae</i> SHV-1 (BLA1_KLEPN)
SHV-2.....	<i>Escherichia coli</i> and <i>Klebsiella pneumoniae</i> SHV-2 (BLA2_ECOLI)
SHV-3.....	<i>Klebsiella pneumoniae</i> SHV-3 (BLA3_KLEPN)
SHV-4.....	<i>Klebsiella pneumoniae</i> SHV-4/CAZ-5 (BLA4_KLEPN)
SHV-5.....	<i>Klebsiella pneumoniae</i> SHV-5 (BLA5_KLEPN)

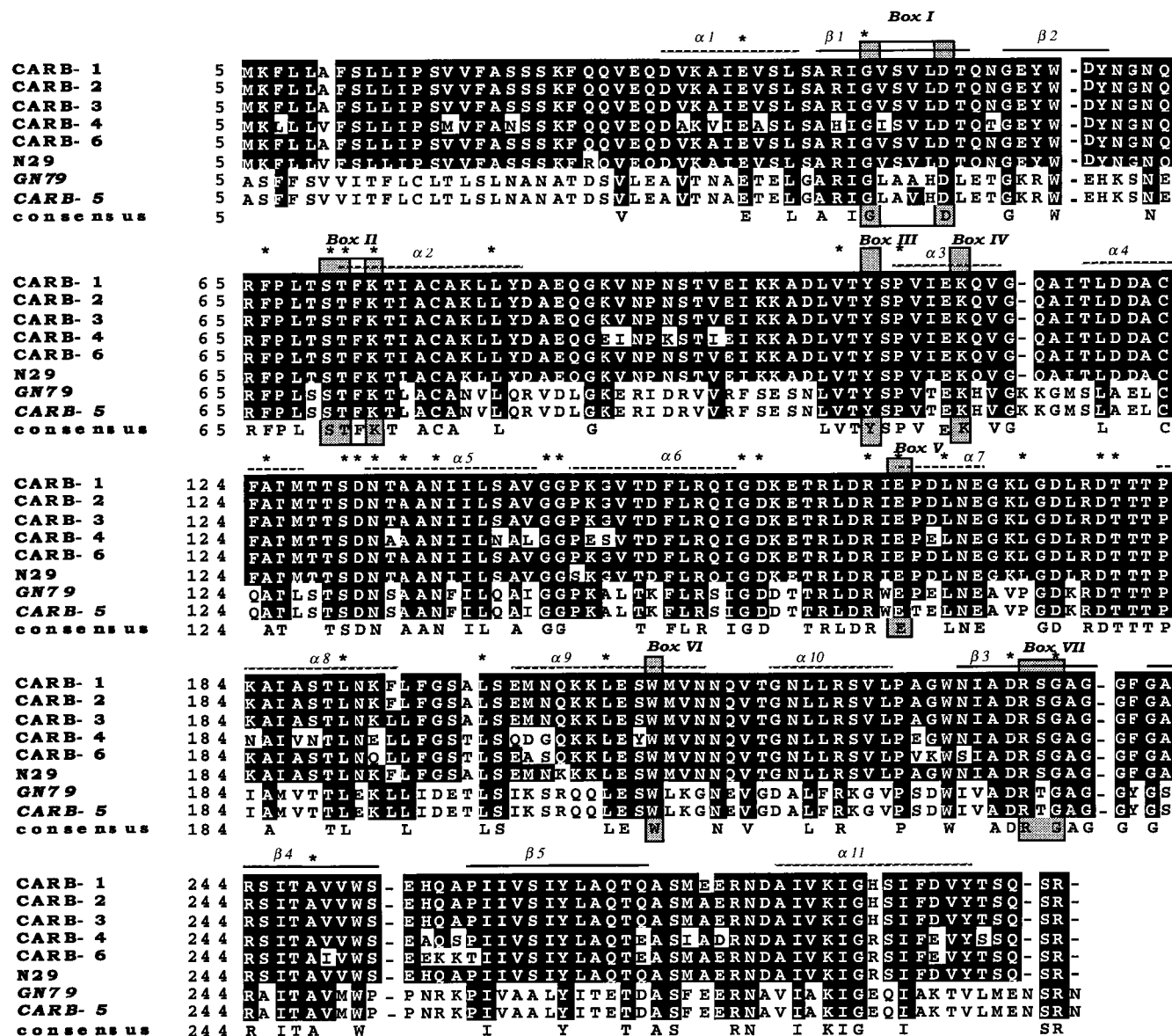


FIG. 2. Multiple sequence alignment of the amino acid sequences of the CARB-1, CARB-2, CARB-3, CARB-4, CARB-6, *P. mirabilis* N29 and GN79, and CARB-5 β-lactamases. The shaded boxes (I to VII) correspond to amino acids conserved in all penicillin-recognizing enzymes, as identified by Joris et al. (15). Alpha-helix and beta-barrel motifs are from the PC1 crystal structure (3, 10). Asterisks indicate the conserved residues specific for class A β-lactamases. Amino acid changes are indicated as black letters on a white background. Sequences are numbered according to the method of Ambler (2).

sequence identities, the β-lactamase neutralization assay for CARB-5 with anti-CARB serum gave results in conflict with those obtained for GN79 in the original study. The *P. mirabilis* GN79 β-lactamase was not neutralized by serum raised against the *P. mirabilis* N29 β-lactamase, which fully neutralized PSE-1 and PSE-4 (27). In contrast, CARB-5 was inhibited by anti-CARB-3 serum that also reacts with other CARB enzymes, including PSE-1 and PSE-4. Moreover, this result accounted for the designation of CARB-5. Such discrepancy in the results may be due to different technical approaches: the neutralization of GN79 was studied in a dilute-liquid-phase assay, whereas CARB-5 was studied undiluted in a specific gel assay. The liquid-phase assay is known to be more specific than the gel assay, which is more sensitive. Indeed, neutralization in gel makes it possible for the three-dimensional structure of the

β-lactamase to combine with low-affinity heterologous antibodies. Such unexpected cross-neutralization in the gel assay has also been reported for the TEM and SHV β-lactamases with anti-TEM-1 serum (20).

In the CARB-5 amino acid sequence, all residues specific to class A β-lactamases are conserved, as are six of seven boxes (15). The most striking feature is the presence of an RTG triad (box VII), as for GN79, instead of the RSG characteristic of the CARB family and unique among class A β-lactamases. The CARB-5 and GN79 enzymes seem to constitute a new carbencillinase group.

RTG-1 and RTG-2 are possible ancestors of the carbencillinase family. Phylogenetic trees (Fig. 3 and 4) clearly show the evolution of 29 β-lactamases. *S. aureus* PC1 and the two *Bacillus* cephalosporinases (BLAC\_BACSU and

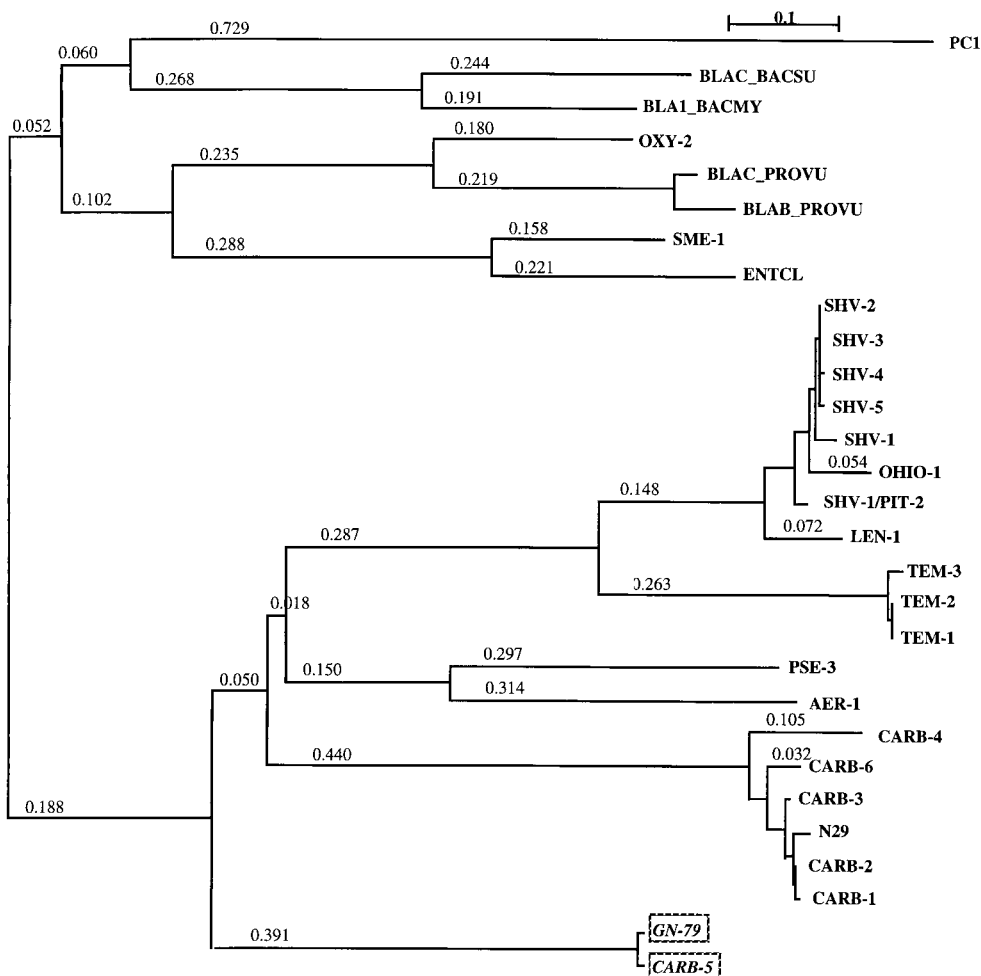


FIG. 3. Dendrograms obtained from multiple alignment of 29 class A  $\beta$ -lactamases according to the neighbor-joining method (23). Branch length values represent relative phylogenetic distances.

BLA1\_BACMY), all issued from gram-positive bacteria, appeared early in evolution. Analysis of 18 class A  $\beta$ -lactamases has shown the origin of the enzyme to be the actinomycetes, from which it migrated first into nonactinomycete gram-positive lines, such as *Bacillus*, and later into the gram-negative bacteria (16). This is consistent with the results of Huletsky et al. (11) and supports their suggestion that the  $\beta$ -lactamases from gram-negative and gram-positive bacteria constitute two distinct groups. This would imply that the *bla* genes of gram-positive and *Bacillus* species appeared early in evolution, followed by the PSE and CARB enzymes and later by the SHV and TEM enzymes found in enteric bacteria. If we consider the evolutionary representation of the phylogenetic tree (Fig. 4), CARB-5 and GN79 are tightly linked and appeared earlier in evolution than other CARB enzymes, which make a separate cluster.

Interestingly, CARB-5 and GN79 are chromosomally acquired genes. It has been suggested that  $\beta$ -lactamases evolved and spread from the ancestral source; *Streptomyces* chromosomal penicillin-recognizing enzymes would be the oldest known enzymes of this type (7). The LEN-1 chromosomal enzyme was previously thought to be the ancestor of the SHV family (11). A similar hypothesis could be put forward for the GN79 and CARB-5 group, which would then be the ancestors of the carbenicillinase family.

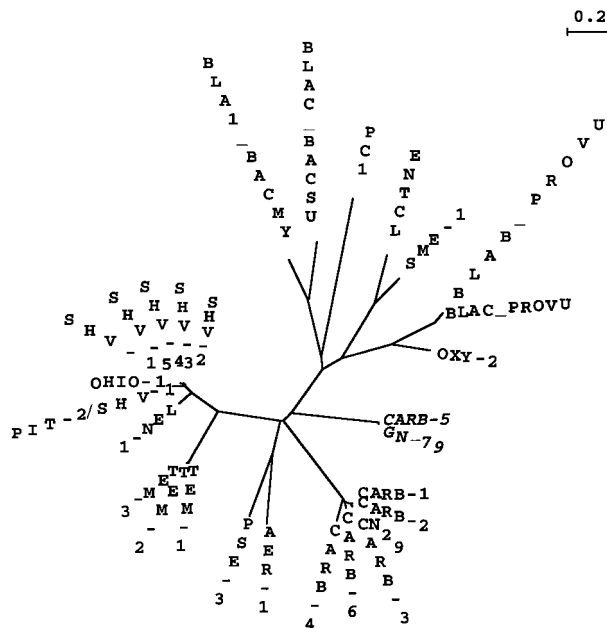


FIG. 4. Evolutionary representation of unrooted phylogenetic tree. The phylogenetic tree was obtained as described in the legend to Fig. 3.



Thus, the carbenicillinase encoded in the *A. calcoaceticus* chromosome is a novel carbenicillinase, structurally related to the *P. mirabilis* GN79 enzyme. These two chromosomal enzymes may be considered to be ancestors of the carbenicillinase family.

All these features lead us to suggest the name of RTG carbenicillinases for this new enzyme group based on the unique RTG triad. The first reference enzymes would be RTG-1 (*P. mirabilis* GN79  $\beta$ -lactamase) and RTG-2 (CARB-5).

**Nucleotide sequence accession number.** The RTG-2/CARB-5  $\beta$ -lactamase sequence has been submitted to GenBank. Its accession number is AF135373.

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