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Carbapenem-Hydrolyzing GES-5-Encoding Gene on Different Plasmid Types Recovered from a Bacterial Community in a Sewage Treatment Plant

Delphine Girlich,[a] Laurent Poirel,[a] Rafael Szczepanowski,[b] Andreas Schlüter,[c] and Patrice Nordmann[a]

Service de Bactériologie-Virologie, INSERM U914, Emerging Resistance to Antibiotics, Hôpital de Bicêtre, Assistance Publique/Hôpitaux de Paris, Faculté de Médecine Paris Sud, Le Kremlin-Bicêtre, France[a]; Department of Periodontology, University Hospital Münster, Münster, Germany[b]; and Center for Biotechnology (CeBiTec), Institute for Genome Research and Systems Biology, Bielefeld University, Bielefeld, Germany[c]

Plasmids pRSB113 and pRSB115 were recovered from an activated sludge bacterial community of a municipal wastewater treatment plant in Germany. Both plasmids carry the same *bla*$_{GES-5}$ carbapenemase gene, located within two distinct class 1 integrons. These plasmids have different backbones, belong to different incompatibility groups, and could replicate in both *Pseudomonas aeruginosa* and *Escherichia coli*.

The aquatic environment may be considered a reservoir for dissemination of antibiotic resistance determinants, since resistant bacteria, resistance genes, and mobile genetic elements carrying resistance determinants have been isolated frequently from bacterial communities residing in lakes, rivers, or wastewater treatment plants (WWTP) (10, 11, 12, 13, 22, 29, 31). Genes encoding carbapenem-hydrolyzing β-lactamases have been identified in several water samples, such as those encoding the Ambler class A β-lactamases IMI-2 from *Enterobacter asburiae* (1, 2) or BIC-1 from *Pseudomonas fluorescens* (11), the class B β-lactamase VIM-2 from *Pseudomonas pseudoalcaligenes* (27), or the class D β-lactamase OXA-23 from *Acinetobacter baumannii* (10). Ambler class A GES-type carbapenemases (GES-2, GES-4, GES-5, and GES-8) are inhibited by clavulanic acid, and the corresponding genes, located on plasmids, have been identified in the *Enterobacteriaceae* and in *Pseudomonas aeruginosa* (23, 24, 26). GES-5 was originally found in a clinical isolate of *Escherichia coli* collected in Athens, Greece, in 2004 (33). GES-5 has also been identified in *Klebsiella pneumoniae* from Korea and Brazil (3, 17, 24), in *Enterobacter cloacae* from Canada (19), and in *P. aeruginosa* from different countries, such as China (34, 35), Spain (32), Republic of South Africa (20), and Brazil (23). The *bla*$_{GES-5}$ gene was always plasmid located and usually is part of class 1 integrons. Therefore, the carbapenemase *bla*$_{GES-5}$ gene seems to be quite widespread worldwide.

The mobilization of antibiotic resistance genes in Gram-negative bacteria occurs through several mechanisms. The transfer of plasmids (through natural transformation or conjugation) is one of the most frequent factors for the horizontal transfer of resistance genes. Additionally, their acquisition is related mainly to different mechanisms, including recombination, integron-mediated mobilization of gene cassettes, transposition, or mobilization via integron-mobilization units, as recently described for the *bla*$_{GES-5}$ gene (IMU) (25).

This work was initiated by the selection of carbapenem-resistant *Pseudomonas knackmussii* B13 (30), formerly *Pseudomonas* sp. B13 (7), which had been transformed with plasmids isolated from bacterial communities residing in the activated sludge compartment of a wastewater treatment plant located in Bielefeld-Heepen, Germany. Transformants were selected on spectinomycin-containing agar (70 μg/ml). Unexpectedly, two resulting recombinants, *Pseudomonas* sp. B13(pRSB113) and *Pseudomonas* sp. B13(pRSB115), exhibited resistance or reduced susceptibility to ceftazidime, aztreonam, and carbapenems. MICs were interpreted according to the CLSI breakpoints (6). MIC values of imipenem were increased from 0.064 μg/ml to 2 and 1.5 μg/ml in *Pseudomonas* sp. B13(pRSB113) and *Pseudomonas* sp. B13(pRSB115), respectively. MIC values of ertapenem were increased from 0.38 μg/ml to >32 μg/ml in both *Pseudomonas* sp. B13 (pRSB113) and *Pseudomonas* sp. B13 (pRSB115). MIC values of meropenem were increased from 0.023 μg/ml to 4 and 6 μg/ml in *Pseudomonas* sp. B13(pRSB113) and *Pseudomonas* sp. B13(pRSB115), respectively. In addition, they showed resistance to chloramphenicol, trimethoprim-sulfamethoxazole, tobramycin, and gentamicin, and *Pseudomonas* sp. B13(pRSB115) was also resistant to netilmicin. Preliminary PCR detection of the most common carbapenemase genes failed (26). However, PCR amplification gave positive results with primers specific for the *bla*$_{GES-like}$ gene (26). Sequencing of the amplicon showed 100% identity with *bla*$_{GES-5}$. Since *bla*$_{GES-1}$-like genes are known to be integron encoded, the consensus primers 5′-CS and 3′-CS for class 1 integrons were used (26), and PCR amplification resulted in a ca. 3.2-kb DNA fragment using both pRSB113 and pRSB115 plasmid DNAs as templates.

The genetic environment of the *bla*$_{GES-5}$ gene was determined by sequencing (Applied Biosystems sequencer ABI 3130) of the class 1 integron and surrounding sequences directly from natural plasmid DNA, using a primer-walking approach (Maxiprep, Qiagen, France). However, for plasmid pRSB113, this primer-walking approach failed. Therefore, a 10,000-bp fragment containing the *bla*$_{GES-5}$ gene was subcloned into plasmid pBKCMV by using EcoRI-restricted DNA. The resulting recombinant plasmid, p113Eco, was introduced into *E. coli* DH10B by electroporation as

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Address correspondence to Patrice Nordmann, nordmann.patrice@bct.aphp.fr.

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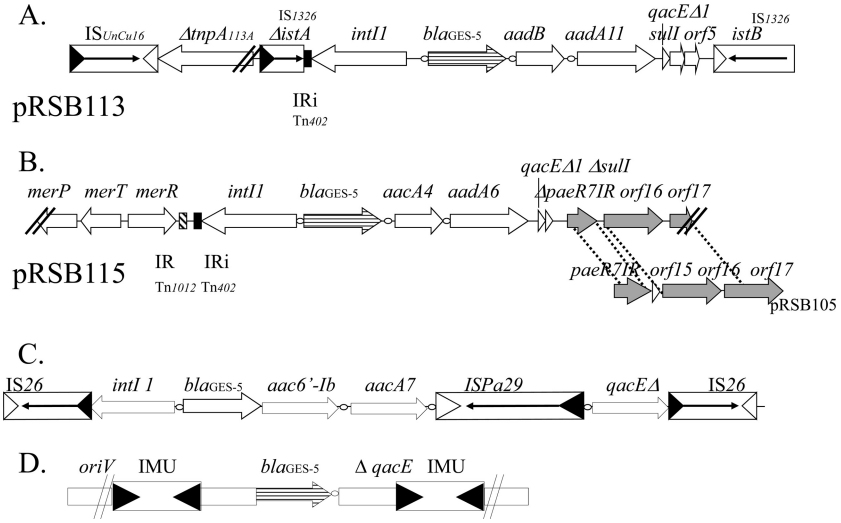

FIG 1 Genetic environment of the *bla*$_{GES-5}$ gene on plasmid pRSB113 (A), plasmid pRSB115 (B), chromosomes of *P. aeruginosa* isolates from Brazil (23) (C), or plasmid pCHE-A from *E. cloacae* (25) (D). Arrows indicate the directions of transcription of the coding regions, the left and right inverted repeats of IS elements are shown by filled and empty triangles, respectively, and core sites are indicated as circles. Tn*1012* and IRi of the Tn*402*-like transposon are shown as small hatched and black rectangles. IS*1326* is a IS*21* family member and encodes two consecutive open reading frames: a long upstream frame, designated *istA*, which is truncated on pRSB113, and a shorter downstream frame, *istB*, which is separated from *istA* in this plasmid. IS*UnCu16* is an IS*66* family member, ISBstXII subgroup, and *tnpA*$_{ISUnCu16}$ encodes a protein sharing similarity with a transposase-like protein from *Alteromonas macleolii* (16). Open reading frame Δ*tnpA*$_{IS113A}$ encodes a truncated transposase-like protein sharing similarity with the product of the IS*408*-like insertion sequence. *paeR7IR*, *orf16*, and *orf17*, previously identified in plasmid pRSB105 (29), are indicated as gray arrows.

described previously (28). *E. coli* DH10B (p113Eco) was selected on ticarcillin (50 μg/ml) and kanamycin (30 μg/ml)-containing Trypticase soy agar (TSA) plates. Plasmid p113Eco was sequenced by using combinations of universal T3 and T7 primers and specific walking primers designed based on the obtained sequences. The sequences of 10,047 bp- and 7,235-bp fragments, respectively, containing the *bla*$_{GES-5}$ gene were determined for plasmids pRSB113 (after subcloning) and pRSB115 (directly from natural plasmid extract). On both plasmids, the *bla*$_{GES-5}$ gene represents a gene cassette, located in the first position of a class 1 integron, as described previously (4). On plasmid pRSB113, the *bla*$_{GES-5}$ gene is followed by the *aadB* and *aadA11* genes, encoding resistance to aminoglycosides, whereas it is followed by the *aacA4* and *aadA6* genes, also encoding aminoglycoside resistance, on plasmid pRSB115. The *bla*$_{GES-5}$-containing class 1 integrons were inserted into two distinct structures: between the two open reading frames (ORFs) *istA* and *istB* of the IS*21* family insertion sequences (IS*1326*) on plasmid pRSB113 and between genes (*paeR7IR*, *orf16*, and *orf17*) of the previously described plasmid pRSB105 (29) and a mercuric resistance operon on pRSB115 (Fig. 1). Notably, the 3′ end of the pRSB115-located class 1 integron was deleted, since the *sul1* gene is partially missing, interrupting the *paeR7IR* gene, encoding a type II restriction enzyme on pRSB115. In contrast to pRSB105, plasmid pRSB115 therefore encodes a nonfunctional restriction/modification system (Δ*paeR7IR*) (Fig. 1). Notably, the 25-bp inverted repeat (IR) element of a Tn*402*-like transposon was identified on the left extremity of the *intI1* integrase-encoding gene on both the pRSB113 and pRSB115 plasmids (Fig. 1). Moreover, the terminal inverted repeat of Tn*1012*, a Tn*21*-related terminal inverted repeat frequently associated with *merR*, was identified on the left-hand extremity of the class 1 integron on pRSB115 (Fig. 1). On pRSB113, two ORFs that encode two transposase-like proteins, termed TnpA$_{113A}$ (truncated) and TnpA$_{ISUncu16}$, were identified on the left-hand extremity of the class 1 integron. Interestingly, TnpA$_{ISUncu16}$ shares 59% amino-acid identity with a TnpA-like protein from *Alteromonas macleodii*, found in the deep water column of the Mediterranean (16). This result strongly suggests genetic exchanges between waterborne bacteria and indicates again that water habitats are important reservoirs of antibiotic resistance genes, as previously shown by the diversity of these genes found in *Aeromonas* spp. in the Seine River, France (13). The capture of the *bla*$_{GES-5}$ gene by integrons containing aminoglycoside resistance gene cassettes, which are overrepresented in environmental plasmids of the *Enterobacteriaceae* or of *Pseudomonas*, may enhance the dissemination of this carbapenemase gene.

Plasmids pRSB113 and pRSB115, extracted by using the Kieser method, were of ca. 30 and 40 kb in size, respectively (18). Direct transfer of the *bla*$_{GES-5}$ determinant by electroporation (28) to *E. coli* DH10B (Invitrogen, Life Technologies, Cergy-Pontoise, France) and selection on ticarcillin-containing agar (50 μg/ml)

TABLE 1 Sequences of primers used in this study for replication/mobilization modules characterization[a]

Primer	Sequence (5′–3′)
pRSB105 rep1 F	ATGGCTCGACAATCCACCGTC
pRSB105 rep1 R	TACGAAGAATCGAGAGGCTCC
pRSB105 rep2 F	AAGCACAGCTACGACTTTTCAGCC
pRSB105 rep2 R	TTTGAACACCTCACGATCAGCC
pRSB101MobA-F	ATGATCTCAAAGCACATTCG
pRSB101MobA-R	TATATAGAGCAGAGTCCAGCG
pRSB105MobA-F	GTGATCGTTAAGAAGGTGCC
pRSB105MobA-R	AAGTCTTATCGACTCATGCCGGGTG

[a]Primer sequences were designed *in silico* based on the available sequences of pRSB101 (31) and pRSB105 (29).

resulted in recombinant *E. coli* DH10B (pRSB113) and *E. coli* DH10B (pRSB115) derivatives. Conjugation experiments were attempted between *Pseudomonas* sp. B13 (pRSB113) or *Pseudomonas* sp. B13 (pRSB115) and azide-resistant *E. coli* J53 or between *E. coli* DH10B (pRSB113) or *E. coli* DH10B (pRSB115) and *E. coli* J53, as previously described (21, 22). Mating-out transfer of the plasmids harboring the bla_{GES-5} gene to *E. coli* remained unsuccessful, suggesting that these plasmids were not self-transferable.

Both plasmids replicated in *Pseudomonas* sp. and *E. coli* DH10B, suggesting a possible horizontal transfer between members of the *Enterobacteriaceae* and nonfermenting rods. Incompatibility (Inc)-group typing of resistance plasmids carried out by PCR with primer pairs specific for the Inc groups IncP, IncQ, IncN, IncW, and IncA/C (5) showed that these plasmids did not belong to any of the tested Inc groups. Plasmids pRSB113 and pRSB115 were isolated from an unknown activated sludge bacterium, such as the previously identified erythromycin resistance plasmids pRSB101 and pRSB105 (29, 31). Plasmid pRSB105 possesses two replicase genes: Rep1 clearly derives from plasmids of *Pseudomonas syringae*, while Rep2 is more divergent and might derive from plasmids previously found in *P. aeruginosa* (Rep protein CAI46990). Plasmid pRSB101 possessed a Rep identical to Rep1 from pRSB105. This suggests that they likely derive from the same ancestor; however, the *mobA* gene on those two plasmids was only 61% identical, suggesting a mosaicism or a fusion of the two replicons in a multireplicon status. Although a backbone similarity was observed only between pRSB105 and pRSB115 in the vicinity of the bla_{GES-5} gene (Fig. 1), further comparisons were performed in order to elucidate the typing, replication, and mobilization features. PCR mapping of the *repA* and *mobA* genes was performed using primers listed in Table 1. The two *repA* genes (*rep1* and *rep2*) from pRSB105 were detected by PCR in pRSB113, whereas only the *repA* gene from pRSB101 was detected in pRSB115. Sequencing of the resulting amplicons containing the *repA* genes from pRSB113 showed that the deduced RepA proteins showed 80% and 94% amino acid identity with the corresponding Rep1 and Rep2 proteins from pRSB105 (IncP-6 group) (31). Sequencing of the resulting amplicons containing the *repA* gene from pRSB115 showed that the deduced RepA protein showed 92% amino acid identity with RepA from pRSB101 (unknown Inc group, pRSB101-like) (29). Rep1 is part of a narrow-host-range replicon that is able to replicate in the gammaproteobacteria, and Rep2 extends the host range of the plasmid to the betaproteobacteria. As previously described, the Rep1 replicon may originate with a plant-associated bacterium. pRSB101, pRSB105, and pRSB115 might have evolved from a common ancestor.

As shown by Garcillan-Barcia et al. (9), most classical Inc groups of plasmids are located in the relaxase (or MobA) phylogenies. The MobA proteins encoded by plasmids pRSB113 and pRSB115 were therefore investigated as evolutionary markers. The whole *mobA* gene from pRSB113 was successfully amplified with primers specific for the *mobA* gene from pRSB105, and that from pRSB115 was amplified with primers specific for the *mobA* gene from pRSB101 (Table 1). Corresponding proteins showed, respectively, 93 and 90% amino acid identity with the corresponding fragments, and the MobA proteins from pRSB113 and pRSB115 shared only 43% amino acid identity. Dendrograms were derived from the multiple relaxase protein sequence alignment by a parsimony method using the ClustalW2 software program (Fig. 2). MobA from pRSB113 shared the highest amino acid identity with that from plasmids Rms149 from *P. aeruginosa* (15) and pRAS3.2 from *Aeromonas salmonicida* (19), with 98% and 92% identities, respectively. According to classification by Garcillan-Barcia et al., pRSB113 belongs to clade P14 of the MOBp family, a clade composed of a series of mobilizable plasmids (9). MobA from pRSB115 shared low amino acid identity with other relaxase proteins, and the highest amino acid identities were with those from plasmids R64 from *Salmonella enterica* serovar Typhimurium (8) and pCTXM-3 from *Citrobacter freundii* (14) (44% and 39% identities, respectively). The latter could then belong to a clade related to MOB_{P12} and MOB_{P13}. This result suggests that the pRSB113 and pRSB115 plasmids belong to two distinct clades of the MOB_P plasmid family, and it might be speculated that these plasmids are mobilizable (Fig. 2).

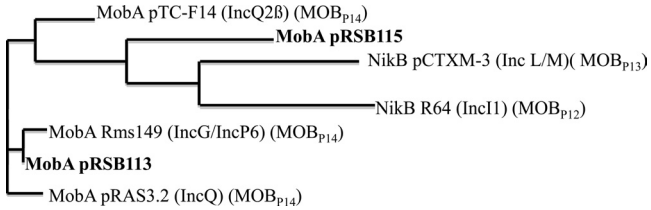

FIG 2 Phylogeny tree of relaxase of plasmids pRSB113 and pRSB115 and MobA of pTC-F14 from *Acidithiobacillus caldus* (GenBank accession no. AAP04747), NikB of pCTXM-3 from *Citrobacter freundii* (GenBank accession no. AAN87675), NikB of R64 from *Salmonella enterica* serovar Typhimurium (GenBank accession no. BAK64476), MobA of Rms146 from *Pseudomonas aeruginosa* (GenBank accession no. CAI46961), and MobA of pRAS3.2 from *Aeromonas salmonicida* (GenBank accession no. AAK97758). Known incompatibility groups and MOB families are indicated.

Conclusions. Comparative genomic analysis of plasmids pRSB113 and pRSB115 in the vicinity of the bla_{GES-5} gene showed that they are related. They may have evolved by integration of distinct modules from different plasmid sources and thus represent mosaic plasmids. No hot spot of integration of the bla_{GES-5}-gene-containing class 1 integron could be evidenced, suggesting a high ability of integration in distinct genetic loci in plasmids found in the environment. This study emphasizes that aquatic environments may be the reservoir of carbapenemase genes of clinical relevance.

Nucleotide sequence accession numbers. The sequences corresponding to the genetic elements described in this work were assigned to GenBank accession numbers JN849689 (pRSB113) and JN849690 (pRSB115).

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