Complete Sequence of Broad-Host-Range Plasmid pRIO-5 Harboring the Extended-Spectrum- β -Lactamase Gene *bla*_{BES-1}

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Broad-host-range plasmid pRIO-5, harboring the extended-spectrum β -lactamase *bla*_{BES-1} gene in *Serratia marcescens*, was fully sequenced. Analysis of the 12,957-bp sequence of this IncP6-type plasmid revealed that the *bla*_{BES-1} gene was associated with two copies of the insertion sequence IS26. The promoter responsible for the *bla*_{BES-1} expression was hybrid, made of a –35 box located inside the inverted repeat of IS26 and a –10 box inside a remnant of an insertion sequence.

TABLE 1 MICs of β -lactams for *S. marcescens* RIO-5, *E. coli* TOP10 pRIO-5, *A. baumannii* CIP 70.10 pRIO-5, and *E. coli* TOP10 and *A. baumannii* CIP 70.10 reference strains

β -Lactam(s)	MIC (μ g/ml) ^a				
	<i>S. marcescens</i> RIO-5	<i>E. coli</i> TOP10 pRIO-5	<i>A. baumannii</i> CIP 70.10 pRIO-5	<i>E. coli</i> TOP10	<i>A. baumannii</i> CIP 70.10
Amoxicillin	>256	>256	>256	2	32
Amoxicillin + CLA	256	16	64	2	8
Ticarcillin	>256	>256	>256	2	8
Ticarcillin + CLA	256	8	64	2	8
Piperacillin	>256	>256	>256	1	16
Piperacillin + TZB	256	4	32	1	4
Cephalothin	>256	>256	>256	4	>256
Cefuroxime	>256	256	>256	2	64
Cefoxitin	>256	2	>256	2	>256
Cefotaxime	64	64	>256	0.06	8
Ceftazidime	4	16	64	0.12	2
Ceftazidime + CLA	1	0.12	4	ND	ND
Cefepime	4	4	64	0.06	1
Cefpirome	4	4	64	0.06	2
Aztreonam	>256	>256	>256	0.03	32
Aztreonam + CLA	16	1	64	ND	ND
Imipenem	0.5	0.12	0.25	0.12	0.25

^a CLA, clavulanic acid (4 μ g/ml); TZB, tazobactam (4 μ g/ml); ND, not determined.

Serratia marcescens is an enterobacterial species expressing a chromosomally encoded and inducible class C β -lactamase. Consequently, *S. marcescens* is naturally resistant to aminopenicillins and many cephalosporins. Clavulanic acid-inhibited extended-spectrum β -lactamase (ESBL) and metallo- β -lactamases have been reported in that species (19). Thus, *S. marcescens* may become resistant to all β -lactams (9). The ESBL BES-1 has been identified only once, from *S. marcescens* isolate RIO-5 in Rio de Janeiro, Brazil (2). BES-1 is an Ambler class A β -lactamase (1) that confers resistance to penicillins, aztreonam, and cefotaxime and to a lesser extent to ceftazidime, mirroring the resistance conferred by many ESBLs of the CTX-M type (2). The activity of BES-1 is inhibited by clavulanic acid and sulbactam.

Whereas the biochemical properties of BES-1 have been previously determined (2), the genetic context of the *bla*_{BES-1} gene remained unknown. The aim of this study was to characterize the genetic structure and support that explain *bla*_{BES-1} acquisition. The complete DNA sequence of pRIO-5 from *S. marcescens* RIO-5 harboring the *bla*_{BES-1} gene was determined.

The transfer of a ceftazidime resistance marker by liquid mating-out assays into *Escherichia coli* J53 (16) and by electrotransformation as described previously (14) into *E. coli* TOP10 and *Acinetobacter baumannii* CIP 70.10 showed that the *bla*_{BES-1} gene was located on a nonconjugative plasmid as previously shown (2) and that this plasmid had a broad host range. Expression of BES-1 conferred resistance to aztreonam and cefotaxime in *E. coli* and *A. baumannii* together with a decreased susceptibility to ceftazidime (Table 1).

A primer walking approach was used to sequence the natural

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TABLE 2 Open reading frames identified in pRIO-5

Gene name	Position (sense)	Length of the corresponding protein (amino acids)	Function	Amino acid identity (accession no.)
<i>bla</i> _{TEM-1}	1–858 (+1)	286	Broad-spectrum β -lactamase	100% (AY458016)
IRR-Tn3	971–1008 (+1)		Right inverted repeat sequences of Tn3	
<i>repA</i>	1185–2560 (–1)	458	Replicase	100% with pRSB105 (DQ839391)
<i>parC</i>	2729–3112 (–1)	128	Auxiliary partitioning protein	100% with pRSB105 (DQ839391)
<i>parB</i>	3149–3367 (–1)	73	Auxiliary partitioning protein	100% with pRSB105 (DQ839391)
<i>parA</i>	3392–4027 (–1)	212	ATPase involved in partitioning	100% with pRSB105 (DQ839391)
IRR-IS5075	4375–4386		Right inverted repeat sequences of IS5075	
<i>tnpA</i> IS5075	4633–5634 (–1)	334	Transposase of insertion sequence IS5075	99% (YP_194887.1)
IRL-IS5075	5690–5701		Left inverted repeat sequences of IS5075	
Δ <i>tnpA</i>	5743–6069 (–1)	118	Tn1696 family transposase	96% (ZP_08307045)
IRR-IS26-1	6666–6679		Right inverted repeat sequences of IS26	
IS26-1	6719–7432 (+1)	238	Transposase of IS26	100% (YP_209343)
IRL-IS26-1	7474–7487		Left inverted repeat sequences of IS26	
<i>orf1</i>	8353–9225 (+1)	291	Putative membrane protein	72% with that of <i>Agrobacterium tumefaciens</i> Atu1921 (NP_354897)
<i>bla</i> _{BES-1}	9403–10278 (–1)	292	Extended-spectrum β -lactamase	100% BES-1 (AF234999)
Putative IRR-IS66-like	10349–10368		Deduced right inverted repeat sequences	
Δ IS66-like	10372–11069 (+1)	232	Truncated transposase of an IS66-family transposase	49% with that of an insertion sequence from <i>Cellvibrio japonicus</i> (YP_001980860)
IRR-IS26-2	11070–11083		Right inverted repeat sequences of IS26	
IS26-2	11121–11834 (+1)	238	Transposase of IS26	100% (YP_209343)
IRL-IS26-2	11876–11889		Left inverted repeat sequences of IS26	
Δ <i>tnpR</i>	12129–12772 (+1)	214	Tn3 resolvase	98% (YP_247787)

*bla*_{BES-1} plasmid. Both strands of the plasmid DNA inserts of the plasmids were sequenced by using an Applied Biosystems sequencer (ABI 377) and the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Villebon-sur-Yvette, France). Each predicted protein was compared against the protein database using BlastP (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) with a minimum cutoff of 30% identity over 80% length coverage. Gene sequences were further compared with GenBank data using BLASTN. Assembly of annotated contig was performed with Bioedit software (8), and plasmid mapping was performed using Ape software (<http://biologylabs.utah.edu/jorgensen/wayned/ape/>).

Sequence analysis of the regions flanking the 5' end of the *bla*_{BES-1} gene revealed a partial transposase gene encoding a protein 49% identical to the transposase of IS66 (Table 2). Thirty-one base pairs separated the right inverted repeat (IRR) of this IS66-like element from the start codon of the *bla*_{BES-1} gene (Fig. 1A). This IS66-like sequence had been truncated by a copy of insertion sequence IS26. Analysis of the region downstream of the *bla*_{BES-1} gene revealed that the *bla*_{BES-1} gene was bracketed by a second copy of an IS26 element, therefore forming a hypothetical compound transposon (Fig. 1A). A gene encoding a hypothetical protein sharing 72% amino acid identity with a hypothetical protein

identified in *Agrobacterium tumefaciens* was present downstream of *bla*_{BES-1} and part of the compound transposon (Fig. 1A). By using the 5' RACE (rapid amplification of cDNA ends) PCR technique as previously described (17), the sites of the initiation of transcription of the *bla*_{BES-1} gene were mapped in *S. marcescens* RIO-5. The +1 transcription start was located 769 bp upstream of the start codon of the *bla*_{BES-1} gene in *S. marcescens* RIO-5 (Fig. 1B). Upstream of this transcriptional start site, a –35 sequence (TTGCAA) separated by 17 bp from a –10 sequence (CATACT) constituted a potential promoter (Fig. 1B). The –10 sequence as well as the +1 signal was part of the IS66 family transposase remnant, although the –35 sequence was part of the left inverted repeat (IRL) of IS26.

Cloning of the *bla*_{BES-1} gene from *S. marcescens* RIO-5 using plasmid pBK-CMV and endonuclease BamHI as described previously (14) resulted in *E. coli* TOP10 harboring recombinant plasmid pBamHI-BES-1. Transposition assays were attempted using recombinant plasmid pBamHI-BES-1 containing the entire and putative composite transposon that was transformed into *E. coli* RZ211 harboring the pOX38 conjugative plasmid as described previously (12). Despite repeated attempts, these assays remained unsuccessful. Since the IS26-*bla*_{BES-1}-IS26 structure was not bracketed by any target site duplication, the mobilization process

FIG 1 (A) Overview of the pRIO-5 sequence. Open reading frames are shown by arrows. The origin of plasmid replication is indicated by a circle. Orientations of gene transcription are indicated by arrows. (B) Schematic map representing genetic structure harboring the *bla*_{BES-1} gene. The genes and their corresponding transcriptional orientations are indicated by horizontal arrows. Promoter sequences -35 and -10 are in bold as is the $+1$ position of transcription. The right inverted repeat of IS26-2 is underlined.

of the *bla*_{BES-1} gene could therefore not be the result of a transposition event. The mobilization of that structure might result from a homologous recombination event as hypothesized for the *bla*_{SHV} gene (15).

Plasmid pRIO-5 harbored *bla*_{TEM-1a} and *bla*_{BES-1} but no other antibiotic resistance markers. The *bla*_{TEM-1} gene was found as a part of transposon Tn3. However, the resolvase gene included in Tn3 was truncated, probably as the result of recombination events (Fig. 1A).

Downstream of the *bla*_{TEM-1} β -lactamase gene, the partition and replication modules of pRIO-5 were identified (Table 2). They included the partitioning genes *parA*, *parB*, and *parC*; the replication gene *repA*; and the origin of replication *oriV* (Fig. 1A). The *parA* gene encodes an ATPase involved in plasmid partitioning, whereas the *parB* and *parC* genes encode auxiliary proteins (Table 2) (11). The *parABC* module of pRIO-5 was identical to that of different IncP6-type plasmids, including pRms149 identified from a clinical *Pseudomonas aeruginosa* strain (7) and plasmid pRSB105 (57 kb in size) recovered from an active sludge in Germany (18). This module was also 98.7% identical with that of plasmid pRA2 identified in *Pseudomonas alcaligenes* (10). The *repA* gene of pRIO-5 encoded a protein identical to that of plasmid pRSB105 and 82% identical to that of plasmid pRms149, which differed in the C terminus. This RepA could be a hybrid created by homologous recombination. The RepA of pRms149 was shown to confer replication ability in *E. coli* and *Pseudomonas putida* (7). Interestingly, the pRIO-5 RepA protein differed from that of pRms149 at its C-terminal extremity.

The function of the IncP6-type replicase had been evaluated, and it was shown to replicate in *Ralstonia eutropha* and *Xanthomo-*

nas campestris (18). That of plasmid pRms149 was shown to be functional in *E. coli* and *P. aeruginosa* (7). We further evaluated the broad host range of RepA of pRIO-5 by cloning its complete replication module together with the *bla*_{BES-1} gene into the pBK-CMV vector, which has a restricted host range, resulting in plasmid pRIO-5-RepA. This recombinant plasmid was then transformed by electroporation into electrocompetent *A. baumannii* CIP 70.10 and *P. aeruginosa* PU21 recipient strains prepared as described previously (4, 18), and selection was based on ticarcillin (50 μ g/ml). This showed that pRIO-5-RepA was able to replicate in *A. baumannii* but not in *P. aeruginosa*. Together with data obtained by others (7, 18), our data showed that pRIO-5 might be considered a broad-host-range plasmid.

Conclusion. Here, we determined the entire sequence of plasmid pRIO-5 carrying the extended β -lactamase gene *bla*_{BES-1}. This plasmid is made of truncated genetic structures, including a Tn3 transposon. The *bla*_{BES-1} gene is bracketed by two copies of IS26 as previously found, e.g., for SHV-type β -lactamase genes, ESBL SFO-1, or 16S rRNA methylase ArmA-encoding genes (4–6, 13). However, those structures are not bracketed by target site duplications, which are usually the signature of a transposition event. Therefore, the mobilization of the IS26-*bla*_{BES-1}-IS26 structure likely resulted from a homologous recombination event, even if it could have also resulted from a transposition process followed by a deletion of its adjacent extremities removing the direct repeat sequences.

The IS26 and IS66-like insertion sequences provided promoter sequences for the transcription of the *bla*_{BES-1} gene, constituting a hybrid promoter. The same -35 sequence TTGCAA associated with another -10 sequence had been identified previously upstream of the *bla*_{SHV-2a} ESBL gene in *P. aeruginosa* (13). In that case, the -10 box corresponded to the original one of *bla*_{SHV} originating from *Klebsiella pneumoniae*, whereas it was made here of an IS66-like fragment constituting a hybrid promoter.

Interestingly, we showed here that plasmid pRIO-5 harboring an ESBL-encoding gene and identified in a clinical enterobacterial isolate in Brazil shared at least some parts of the backbone of a broad-host-range plasmid identified in an aquatic environment in Germany. Although having a broad host range, plasmid pRIO-5 harbored only partitioning and replicase genes and lacked most of the accessory genes, including those involved in conjugation and mobilization. That would therefore explain the fact that the *bla*_{BES-1} gene did not spread among Gram-negative bacteria, contrasting with many other ESBL genes located on “successful” plasmids.

Nucleotide sequence accession number. The nucleotide sequence data reported in this work have been deposited in the GenBank nucleotide database under accession no. [JF785550](https://www.ncbi.nlm.nih.gov/nuclotide/JF785550).

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