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New Small Plasmid Harboring bla_{KPC-2} in *Pseudomonas aeruginosa*

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The aim of this study was to characterize the genetic context of bla_{KPC-2} in *Pseudomonas aeruginosa* sequence type 244 from Brazil. The bla_{KPC-2} gene was detected in a new small plasmid, pBH6. Complete sequencing revealed that pBH6 was 3,652 bp long and included the Tn*3* resolvase and Tn*3* inverted repeat (IR), a partial copy of IS*Kpn6*, and a putative *ori* region but no *rep* genes. pBH6 replicated stably into *Escherichia coli* strain DH10B and *P. aeruginosa* strain PAO.

Carbapenem resistance mediated by the production of *Klebsiella pneumoniae* carbapenemase (KPC) enzymes has been reported worldwide in *Enterobacteriaceae*, and the bla_{KPC-2} gene was found to be associated with different transposons and plasmids (1–3). However, bla_{KPC} has also emerged in clinical isolates of *Pseudomonas aeruginosa*. First reported in Colombia in 2006 (4), subsequent worldwide reports described plasmid and chromosomal genetic contexts for this gene. In Brazil, KPC-producing *P. aeruginosa* was first described in 2012 (5), followed by a second report (6) that highlighted a worrying increase in KPC-producing *P. aeruginosa*. However, the genetic context of bla_{KPC} in the Brazilian strains remained unknown. At present, two complete sequences of *P. aeruginosa* bla_{KPC-2}-carrying plasmids, pCol-1 (GenBank accession number KC609323) from *P. aeruginosa* sequence type 308 (ST308) and pPA-2 (GenBank accession number KC609322) from *P. aeruginosa* ST1006 (7), are available in public databases. The aim of this study was to characterize the genetic context of bla_{KPC-2} in a *P. aeruginosa* isolate from Brazil. Carbapenem-resistant *P. aeruginosa* (referred to here as BH6) was isolated in Belo Horizonte, Brazil, in 2011. BH6 was recovered from tracheal secretions of a female patient in the intensive care unit of a tertiary medical care center. This isolate was characterized as multidrug resistant, KPC-2 producing, and of ST244 (8). MIC values were ≥32 μg/ml for imipenem, ≥32 μg/ml for meropenem, ≥256 μg/ml for ceftazidime, ≥256 μg/ml for aztreonam, ≥256 μg/ml for cefepime, and 1.0 μg/ml for polymyxin B (9, 10). The bla_{KPC-2} gene was isolated by PCR amplification, as previously described (11), followed by Sanger sequencing. The ST was characterized according to the *P. aeruginosa* MLST database guideline (see http://pubmlst.org/paeruginosa/).

The genomic location of bla_{KPC-2} was determined, as previously described, by hybridization on I-CeuI pulsed-field gel electrophoresis (PFGE) using bla_{KPC-2} and 16S rRNA genes as probes (to determine whether it was located on the chromosome) and S1 PFGE using bla_{KPC-2} as a probe (to determine whether it was plasmid associated) (12). We observed three bands in the gel after S1 PFGE, and all three of them hybridized with the bla_{KPC-2} probe. Since it seemed possible that they represented the linear, open-circular, and closed-circular forms of a single plasmid, plasmid DNA was extracted and purified using a PureLink HiPure plasmid filter midiprep kit (Invitrogen) and digested with BamHI (Thermo Fisher Scientific). This treatment generated a single plasmid band that indicated the presence of a single plasmid species. To determine whether this plasmid, pBH6, belonged to a recognized incompatibility (Inc) group, we used a PCR-based replicon typing (PBRT) scheme (13); however, we were unable to identify a particular replicon by using this procedure. Finally, plasmid DNA was sequenced using the Ion PGM system (Life Technologies). *De novo* assembly was carried out using the CLC Genomics work-

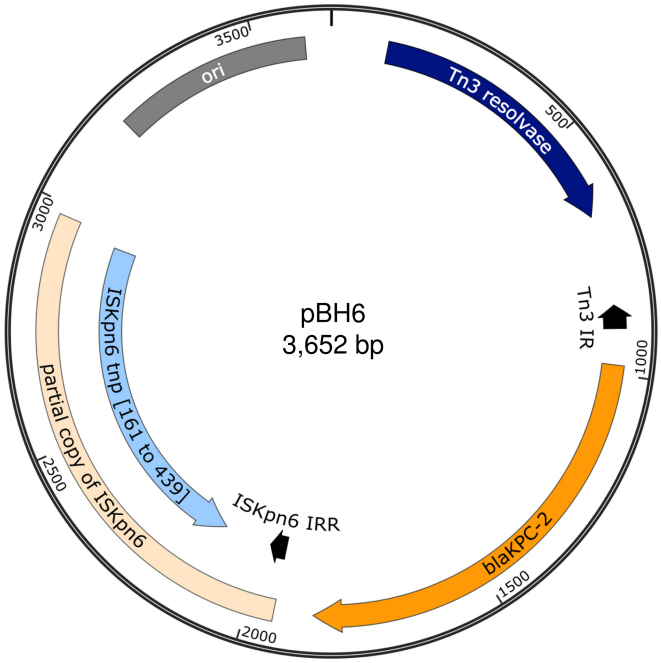

FIG 1 Circular plasmid carrying the bla_{KPC-2} and origin of replication (*ori*) genes. The partial copy of IS*Kpn6* carrying the IRR (black arrow) is shown. A Tn*3* IR is located between the bla_{KPC-2} and the Tn*3* resolvase (longer black arrow).

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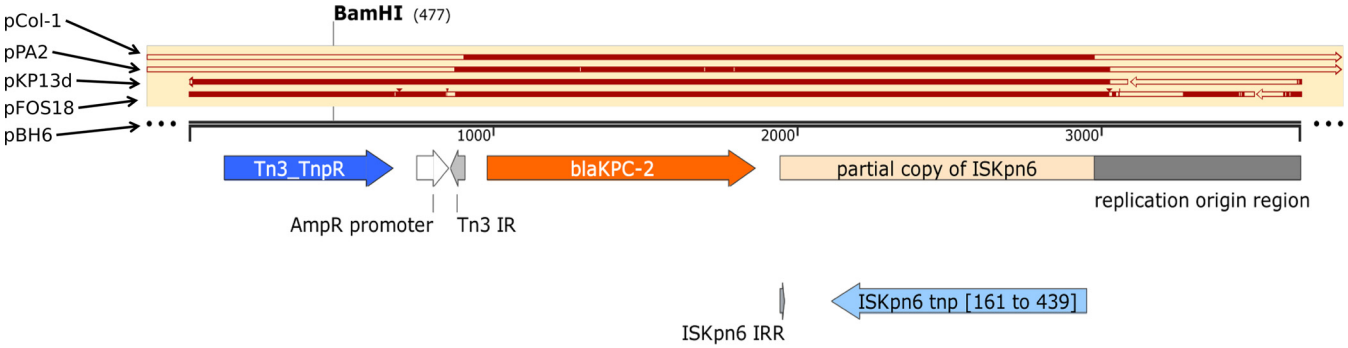

FIG 2 Similarities among pBH6 (3,642 bp), pCol-1 (31,529 bp, coordinates 17066 to 19135), pPA2 (7,995 bp, coordinates 1133 to 3283), pFOS18 (23,939 bp, coordinates 4910 to 11956), and pKP13d (45,574 bp, coordinates 4910 to 11956). Red lines indicate the region with 100% similarity among the 5 plasmids. Uncolored lines indicate different regions among the plasmids.

bench 8.5.0 (CLCbio, Aarhus, Denmark), which generated a single contig that represented the entire circular pBH6 plasmid. This sequence includes a single BamHI site. Gene prediction was carried out with the Prokka pipeline (14). Data files were compiled using Sequin (see http://www.ncbi.nlm.nih.gov/Sequin/), and the SnapGene viewer was used for visualization and analysis. Pairwise alignment was performed by BLASTN and BLASTP homology searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (pBH6 GenBank accession number LGVH01000782.1).

There were no chromosomal bla_{KPC-2} copies. Sequencing revealed a circular genetic element of 3,652 bp and included the Tn*3* transposon resolvase (15), a Tn*3* inverted repeat (IR), a carbapenemase-encoding gene (bla_{KPC-2}), a partial transposase of IS*Kpn6*, and the right end of IS*Kpn6*, IRR (Fig. 1). Positions 3 to 3,027 displayed homology with plasmids pKP13d (82% query coverage, 99% identity [GenBank accession number CP003997]) from a KPC-2-producing *Klebsiella pneumoniae* Kp13 isolate from Brazil (16) and pFOS18 (89% query coverage, 99% identity [KJ653815]) from a KPC-2-producing *K. pneumoniae* isolate from China (17) (Fig. 2). Furthermore, *P. aeruginosa* ST1006 plasmids pCol-1 (56% query coverage, 100% identity [KC609323]) from *P. aeruginosa* ST308 and pPA-2 (58% query coverage, 99% identity [KC609322]) also show homology covering the bla_{KPC-2} gene and the partial IS*Kpn6* copy.

Further analysis found that the region between ~3200 and 3600 (400 bp) of pBH6 (which includes the DNA outside the known genes and transposon features and may contain the replication origin) is similar to that of several KPC-2-producing plasmids deposited in GenBank. Interestingly, almost all related plasmids bear a *rep* gene upstream of this region and, as observed in

TABLE 1 Plasmids related to pBH6 that show similar *ori* regions (more than 97% identity)

Plasmid	Size (bp)	Coordinates	% identity	Genomic context		GenBank accession No.
				Upstream to *ori*	Downstream to *ori*	
A. hydrophila pKPC2	44,451	14,647–14,978	100	*repA*	Tn*3 tnpR*	KR014106
E. cloacae WCHECl-14653 pKPC2-EC14653	88,214	10,429–10,612	100	*repA*	Hypothetical	KP868646
K. pneumoniae 565 pKPCAPSS	127,970	62,229–62,565	99	*repA*	Tn*Swi1 tnpR*	KP008371
A. hydrophila AH1 pN6	11,467	437–106	99	*repA*	Tn*Swi1 tnpA*	KC355363
Enterobacter cloacae ECN49 pKPC-ECN49	41,317	40,271–40,469	99	*repB*	Tn*3 tnpR*	KP726894
Citrobacter freundii p112298-KPC	117,288	10,979–11,177	98	*repA*	IS*26 tnpA*	KP987215
K. pneumoniae pFOS18	23,939	5,108–4,910	98	*repA*	Tn*Shes11 tnpR*	KJ653815
Enterobacter aerogenes pHS112625	18,184	7,459–7,261	98	*repA*	Tn*3 tnpR*	KJ210592
E. coli pHS102707	69,453	14,234–14,036	98	*repA*	Tn*3 tnpR*	KF701335
K. pneumoniae pHS092839	15,499	4,142–3,944	98	*repA*	Tn*3 tnpR*	KF724506
K. pneumoniae pCT-KPC LJ04	151,466	81,583–81,385	97	*repA*	Tn*3 tnpR*	KT185451
K. pneumoniae p628-KPC	105,008	15,073–14,875	97	*repA*	Tn*3 tnpR*	KP987218
K. pneumoniae pKP1034	136,848	121,075–120,877	97	*repA*	Tn*3 tnpR*	KP893385
E. coli pHS10842	13,915	4,585–4,387	97	*repA*	Tn*3 tnpR*	KP125892
K. pneumoniae pHS092753	12,966	4,167–3,969	97	*repA*	Tn*3 tnpR*	KF826293
K. pneumoniae pHS10505	15,464	4,716–4,518	97	*repA*	Tn*3 tnpR*	KF826292
E. coli pHS10842	14,757	4,330–4,132	97	*repA*	Tn*3 tnpR*	KF826291
K. pneumoniae pHS082416	12,915	4,116–3,918	97	*repA*	Tn*3 tnpR*	KF724507
K. pneumoniae pHS062105	42,848	32,964–32,766	97	*repA*	Tn*3 tnpR*	KF623109
K. pneumoniae JM45 p1	317,154	120,918–121,116	97	*repA*	Hypothetical	CP006657
K. pneumoniae pKPC-LK30	86,518	61,401–61,203	97	Tn*3 tnpR*	Tn*3 tnpA*	KC405622
K. pneumoniae pKPHS2	111,195	25,304–25,502	97	*repA*	Tn*3 tnpR*	CP003224
K. pneumoniae pK048	151,188	15,712–15,514	97	*repA*	Tn*3 tnpR*	FJ628167

pBH6, a recombinase of the Tn*3* family element downstream. A list of related plasmids is shown in Table 1. Only *Aeromonas hydrophila* pKPC2, *K. pneumoniae* 565 pKPCAPSS, and *Aeromonas hydrophila* AH1-pN6 show high similarity to each other over the entire 331-bp region (82% query coverage, 100% identity). The other plasmids, while maintaining high levels of similarity, only span between 100 and 300 bp, which suggests that this region may be related to plasmid replication and recognized as an origin of replication (*ori*).

It is known that small plasmids, such as pUC19, do not require a plasmid-specific *rep* gene but can replicate from a plasmid origin using bacterial host enzymes. The genetic environment of bla_{KPC-2} here is different from that of those already described. In most *Enterobacteriaceae* and *Pseudomonas* spp., bla_{KPC-2} appears associated with Tn*4401*-like transposons (1) carried by different plasmids (13).

To verify whether pBH6 could be established and replicate in other bacteria, purified plasmid DNA was transformed (18, 19) into *Escherichia coli* DH10B and *P. aeruginosa* PAO recipient strains followed by selection on MacConkey agar supplemented with ceftazidime (8 μg/ml). Transformants were easily obtained in both recipient strains. To evaluate the stability of pBH6, transformants from each strain isolated on LB solid medium were subcultured for 10 consecutive days in Mueller-Hinton (MH) and LB liquid media with or without 8 μg/ml ceftazidime. After the tenth subculture, both transformants still carried bla_{KPC-2} as judged by PCR, suggesting that this small plasmid can replicate and remain stable for several subcultures.

In summary, we have identified and obtained the sequence of a small 3,652-bp plasmid, pBH6, from KPC-producing *P. aeruginosa* ST244. Plasmid pBH6 carries the bla_{KPC-2} gene and remnants of a Tn*3* family transposon and of IS*Kpn6* and may have been derived from the Tn*4401* transposon in which IS*Kpn6* and bla_{KPC} are juxtaposed (20). When known sequences are taken into account, only 675 bp remain for providing replication functions, which implies that this region carries the origin of plasmid replication. Plasmid pBH6 does not include an identifiable *rep* gene and can replicate autonomously in *E. coli* and *P. aeruginosa*.

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