Characterization of pKP-M1144, a Novel ColE1-Like Plasmid Encoding IMP-8, GES-5, and BEL-1 β -Lactamases, from a *Klebsiella pneumoniae* Sequence Type 252 Isolate

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IMP-8 metallo- β -lactamase was identified in *Klebsiella pneumoniae* sequence type 252 (ST252), isolated in a Portuguese hospital in 2009. *bla*_{IMP-8} was the first gene cassette of a novel class 3 integron, In1144, also carrying the *bla*_{GES-5}, *bla*_{BEL-1}, and *aacA4* cassettes. In1144 was located on a ColE1-like plasmid, pKP-M1144 (12,029 bp), with a replication region of limited nucleotide similarity to those of other RNA-priming plasmids, such as pJHCMW1. In1144 and pKP-M1144 represent an interesting case of evolution of resistance determinants in Gram-negative bacteria.

Acquired carbapenem-hydrolyzing metallo- β -lactamases (MBLs) are resistance determinants of increasing clinical importance in Gram-negative pathogens. Of these, enzymes mainly of the VIM, IMP, and NDM types have been encountered in *Klebsiella pneumoniae* and other *Enterobacteriaceae*. In contrast to *bla*_{NDM} genes, *bla*_{VIM} and *bla*_{IMP} occur as gene cassettes in class 1 integrons or, more rarely, integrons of class 2 or 3 (1–3). Here, we report on a novel class 3 integron, In1144, identified in a *K. pneumoniae* sequence type 252 (ST252) isolate from Portugal. In1144 codes for IMP-8 (4) and two other β -lactamases, GES-5 and BEL-1 (5, 6). It is located on a new ColE1-like plasmid, pKP-M1144, which was entirely sequenced in the study.

In 2009, *K. pneumoniae* strain Kpn-1144 was recovered from a patient in a Portuguese intensive care unit (ICU) of a hospital participating in the European Union (EU)-funded project MOSAR (7). During MOSAR, 17,945 patients in 18 clinical sites in Europe and Israel were screened for rectal carriage of expanded-spectrum cephalosporin-resistant *Enterobacteriaceae* and were tested also for carbapenem susceptibility (7, 8). Kpn-1144 was extended-spectrum β -lactamase (ESBL) positive by the double-disk synergy test (9) and showed reduced susceptibility to carbapenems, according to the EUCAST screening cutoffs (10). The isolate was positive in the MBL EDTA double-disk synergy test (11), and carbapenemase production was confirmed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (12). Table 1 presents the antimicrobial susceptibility data for Kpn-1144 determined by broth microdilution (13) and interpreted using the EUCAST criteria (<http://www.eucast.org/>). Of note, the MICs of carbapenems were relatively low (0.75 to 1 μ g/ml).

PCR screening for various MBL genes (14, 15) followed by sequencing revealed the presence of the *bla*_{IMP-8} gene (4) in Kpn-1144. The multilocus sequence typing (MLST) analysis (16) classified the isolate into ST252. *K. pneumoniae* ST252 was originally identified in the United States in 2007 (<http://bigsd.bweb.pasteur.fr/klebsiella/klebsiella.html>), but recently, it was also found among VIM-1-producing isolates from different health care institutions in Barcelona, Spain (8, 17).

Attempts to transfer β -lactam resistance from Kpn-1144 to rifampin-resistant (Rif^r) *Escherichia coli* strain A15 by conjugation were unsuccessful, in contrast to electroporation of *E. coli* strain DH5 α with purified plasmid DNA of Kpn-1144 (Qiagen maxi kit; Qiagen, Hilden, Germany). Transformants were selected on Luria-Bertani agar plates with ampicillin (50 μ g/ml), confirmed to be IMP producers by PCR (14), and tested for antimicrobial susceptibility (Table 1). The plasmid location of the *bla*_{IMP-8} gene was demonstrated by the S1 nuclease analysis of Kpn-1144 and its transformant (18), followed by hybridization with a digoxigenin-labeled *bla*_{IMP} probe. S1 profiling revealed multiple plasmids in Kpn-1144 comprising molecules of $\sim$ 15 kb, $\sim$ 100 kb, $\sim$ 170 kb, $\sim$ 230 kb, and $\sim$ 340 kb, of which only the $\sim$ 15-kb plasmid was also in the transformant and hybridized with the *bla*_{IMP} probe (results not shown). This plasmid, designated pKP-M1144, was nontypeable by PCR-based replicon typing (PBRT) (19), and its whole nucleotide sequence was determined. Sequencing, assembling of the reads, filling of sequence gaps, and analysis and annotation of the plasmid sequence were performed as described previously (20).

Plasmid pKP-M1144 is 12,029 bp in size, with an average G+C content of 51.5%. Analysis of the sequence revealed that *bla*_{IMP-8} is the first gene cassette of a unique class 3 integron structure, In1144 (Fig. 1), composed of 4,679 bp (nucleotides [nt] 1 to 4679). Upstream of *bla*_{IMP-8}, the *intI3* gene encoding the class 3 integrase was

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TABLE 1 Antimicrobial susceptibility of *K. pneumoniae* Kp-1144 and the *E. coli* DH5α transformant harboring the IMP-8-encoding plasmid pKP-M1144

Strain	MIC (μg/ml) of ^a :																
	AMX	AMC	PIP	TZP	CTX	CAZ	FEP	ATM	IPM	MEM	ETP	GEN	AMK	CHL	CST	SXT	CIP
<i>K. pneumoniae</i> Kpn-1144	>256	24	>64	32	>8	>32	16	48	0.75	1	0.5	4	8	>32	0.25	8	0.5
<i>E. coli</i> DH5α (pKP-M1144)	>256	24	64	8	>8	>32	16	2	0.5	0.5	0.25	0.5	1	2	0.25	1	≤0.12
<i>E. coli</i> DH5α	4	2	2	0.5	≤0.12	≤0.25	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12	0.12	0.5	≤1	≤0.12	1	≤0.12

^a AMX, amoxicillin; AMC, amoxicillin-clavulanate (inhibitor fixed at 2 μg/ml); PIP, piperacillin; TZP, piperacillin-tazobactam (inhibitor fixed at 4 μg/ml); CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam; IPM, imipenem; MEM, meropenem; ETP, ertapenem; GEN, gentamicin; AMK, amikacin; CHL, chloramphenicol; CST, colistin; SXT, trimethoprim-sulfamethoxazole; CIP, ciprofloxacin.

identified (21). The pKP-M1144 *intI3* exhibited 100% sequence identity to the *intI3* associated with *bla*_{GES-1} in the IncQ-type plasmid pQ7 from *E. coli* strain TB7 from Switzerland (22) and 99% identity to that with *bla*_{IMP-1} from *Serratia marcescens* strain AK9373 from Japan (23). Downstream of *bla*_{IMP-8}, three other cassettes were found. The second one was *bla*_{GES-5}, encoding the ESBL GES-5 that exhibits weak carbapenemase activity (5). This was followed by *bla*_{BEL-1}, specifying the prototype enzyme of the ESBL family BEL, observed only in *Pseudomonas aeruginosa* so far (6). The last cassette was *aacA4*, encoding the acetyltransferase AAC(6′)-Ib, which confers resistance to tobramycin, netilmicin, and amikacin (24). A putative promoter (TAGACA-N₁₇-TAG GAT) was located within *intI3* and significantly differed from common and relatively strong class 1 integron promoters (25).

In1144 was inserted into a ColE1-like backbone of 2,737 bp (Fig. 1; nt 9293 to 12029). A DNA comparison showed that its 704-bp sequence (nt 9838 to 10541) had limited nucleotide similarity to replication regions of several RNA-priming plasmids (26–29). pKP-M1144 exhibited the highest similarity score with the replicon of pJHCMW1 (100% coverage, 75% identity) from *K.*

pneumoniae strain JHCK1 from Argentina (28). A consensus sequence for the ColE1 replication origin (*oriV*) (30) was located at positions 10414 to 10416. Additionally, putative regions containing the RNA transcripts RNA II and RNA I that control the initiation of DNA replication and the plasmid copy number (31) were identified (Fig. 1). RNA II (nt 9882 to 10417) acts as a primer for the initiation of replication, while RNA I (nt 9984 to 9883) is an antisense molecule that controls replication initiation by binding to RNA II and preventing primer formation. The RNA II and RNA I of pKP-M1144 were 78% and 68% identical, respectively, to the corresponding transcripts from pJHCMW1 (28). Downstream of *oriV*, a 192-bp sequence resembling the *rom* gene (96% identity) of pNBL63 from *Klebsiella oxytoca* strain NBL63 (29) was present (nt 10589 to 10780). The Rom (or Rop) protein enhances the interaction of the RNA I inhibitor with its target, thus resulting in a reduction of the replication initiation frequency (32). Just next to *rom*, an *excI*-like sequence of 423 bp, exhibiting 95% identity with that of pNE1280 from *Enterobacter cloacae* strain 1623 (33), was identified (nt 10780 to 11202). The Exc1 protein (entry exclusion protein 1) may reduce the formation of stable mating pairs (26). Of note is that an *excI*-like gene, whose putative product showed low amino acid similarity (41%) with the putative Exc1 of pKP-M1144, has also been found downstream of the *rom* gene in pNBL63 (29). In the remaining part of pKP-M1144 (nt 4680 to 9292), a 37-bp segment similar to the replication region of IncQ plasmids (*ΔrepC*) (22) was found at the boundary of In1144, 258 bp downstream of *aacA4*. The *repC* gene at a similar position was identified in the IncQ plasmid pQ7 with the *bla*_{GES-1}-carrying class 3 integron (22). Upstream of *ΔrepC*, the intact transposon Tn5403 was found (34).

Although no origin-of-transfer (*oriT*) and ColE1 *mob* genes (35) were identified in pKP-M1144, the mobilization capability of the plasmid was tested. pKP-M1144 was introduced into *E. coli* strain XL1-Blue that harbors the fertility factor F′, being an IncFIA-type conjugative plasmid. The resulting transformants were used in mating experiments with *E. coli* A15 Rif^r (8). Transconjugants were selected on MacConkey agar plates with rifampin (150 μg/ml) and ampicillin (50 μg/ml) and confirmed to carry the ~15-kb plasmid pKP-M1144. The transfer of pKP-M1144 was achieved at a relatively high frequency (2×10^{-5} *bla*_{IMP}-positive recombinants per donor cell), indicating its capability to be mobilized by plasmids with apparently different conjugation systems. This finding might be explained by the presence of a sequence with limited identity with the ColE1 *oriT* (28); however, a fur-

FIG 1 Circular genetic map of pKP-M1144. Arrows show directions of transcription of open reading frames and regulatory elements. Sequences characteristic of the ColE1-like plasmid backbone are indicated by black arrows. The *intI3* gene and transposases are shaded gray. White arrows indicate the resistance genes. Position 1 is indicated by a vertical black arrow on strand ruler.

ther study is necessary to prove this hypothesis and characterize the putative *oriT*.

To our knowledge, this is the first description of a *bla*_{IMP}-carrying class 3 integron identified in Europe. In1144 also carries the *bla*_{GES-5}, *bla*_{BEL-1}, and *aacA4* gene cassettes. Of note was that Kpn-1144 showed only reduced susceptibility to carbapenems, even though it produced IMP-8 and GES-5 carbapenemases. It might be due to lower expression driven by a weaker promoter (25), low plasmid copy number, and/or good permeability of the outer membrane of the strain for antibiotics.

In general, IMP-like MBLs have been much more common in the Far East than in Europe (3), and class 3 integrons with MBL genes, exclusively *bla*_{IMP}, have occurred rarely and only in Japan so far (23, 36). In 2010, the *bla*_{IMP-8} cassette was reported in *K. oxytoca* in Spain and *Pseudomonas mendocina* in Portugal, but it was located in class 1 integrons (37, 38); moreover, the cassettes *bla*_{GES-5} and *bla*_{BEL-1} have been found exclusively in such elements so far (5, 6). All these observations suggest that In1144 emerged by the exchange of resistance cassettes between class 1 and class 3 integrons. Moreover, the presence of *bla*_{BEL-1} in Kpn-1144 is the first report on a BEL-type ESBL in *Enterobacteriaceae*, suggesting its acquisition from *P. aeruginosa*. In1144 is carried by a new ColE1-like plasmid, pKP-M1144, but the presence of the IncQ-derived *ΔrepC* next to the integron suggests that it may have originated from an IncQ-like plasmid with a class 3 integron, like pQ7 (22). A plausible hypothesis is that this class 3 integron was acquired by a ColE1-like replicon from an IncQ-type plasmid at a certain step in pKP-M1144 evolution; however, it is not known whether this was In1144 already or any of its possible progenitors. This work confirms the significant role of ColE1-like plasmids in resistance dissemination (29, 39–41) and documents an interesting case of evolution of mobile genetic elements with resistance determinants in Gram-negative bacteria.

The nucleotide sequence of the plasmid pKP-M1144 has been assigned the GenBank accession no. KF745070.

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We declare no conflicts of interest.

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