1 **pKBuS13, a KPC-2 encoding plasmid from *Klebsiella pneumoniae* Sequence Type 833,**

2 **carrying Tn*4401b* inserted into a Xer site-specific recombination locus.**

3

4

5 Luigi Garbari[a], Marina Busetti[b], Lucilla Dolzani[a], Vincenzo Petix[b], Anna Knezevich[c],

6 Raffaela Bressan[a], Fabrizia Gionechetti[a], Enrico A. Tonin[a] and Cristina Lagatolla[a]#

7

8 Department of Life Sciences, University of Trieste, Italy[a]; Institute for Maternal and Child

9 Health IRCCS "Burlo Garofolo" Trieste Italy[b]; Department of Laboratory Medicine,

10 Microbiology CS, Azienda Ospedaliero-Universitaria "Ospedali Riuniti" Trieste, Italy[c]

11

12

13 Running head: KPC-2 encoding plasmid carrying Tn*4401b* in a Xer locus

14

15

16 #Address correspondence to Cristina Lagatolla, clagatolla@units.it

17

18

19 **ABSTRACT**

20 Here we report the first detection of a KPC-2 producing *Klebsiella pneumoniae* strain
21 belonging to ST833, collected in an Italian hospital from a patient coming from South
22 America. Its *bla*$_{KPC}$ determinant was carried by a ColE1 plasmid, named pKBuS13, that
23 showed the Tn*4401b*::*bla*$_{KPC-2}$ transposon inserted into the regulatory region of a Xer site-
24 specific recombination locus. This interfered with the correct resolution of plasmid multimers
25 into monomers, lowering plasmid stability and leading to overestimation of the number of
26 plasmids harboured by a single host cell. Sequencing of the fragments adjacent to Tn*4401b*
27 detected a region that did not have significant matches in databases other than the genome of
28 a carbapenem-resistant *E. coli* collected during the same year in a hospital of Boston. This is
29 interesting in an epidemiologic context, as it suggests that despite the absence of *tra* genes
30 and the instability under nonselective conditions the circulation of pKBuS13 or of analogous
31 plasmids might be wider than reported.

32

33 **INTRODUCTION**

34 During the last decade, *Klebsiella pneumoniae* (KP) strains producing KPC (*K. pneumoniae*
35 carbapenemase) enzymes have become a matter of great concern, as they are often susceptible
36 to only a few antibiotics, cause high mortality among patients with bloodstream infections and
37 are increasingly being reported worldwide (1).

38 KPC-type beta-lactamases include 22 variants (http://www.lahey.org/Studies/other.asp) that
39 have been detected in a large number of KP lineages. Among them, KPC-2 and KPC-3 are
40 predominant and are largely disseminated worldwide by strains belonging to the clonal
41 complex 258 (CC258), including the sequence type (ST) 258 lineage defined by multilocus
42 sequence typing (MLST) and its single-locus variants (e.g.: ST11, ST437, ST512) (2-6).

43 Dissemination of bla_{KPC} genes is fuelled by their association with Tn*4401*, a 10 kb Tn3-like
44 element, that has been detected on plasmids belonging to different incompatibility groups
45 (FII, N, L/M) and of different sizes (10 to 170 kb) (7).

46 In Italy, KPC producing *K. pneumoniae* (KPC-KP) have increasingly been reported since
47 2009 (8). Most of them belong to the globally spread ST258 and ST512 clones, but some
48 isolates of different STs (ST101, ST307) have been detected too (9).

49 In the present work we report the isolation in the Trieste area (northeast Italy) of a KPC-KP
50 belonging to ST833, from the blood culture of a patient coming from a Venezuelan hospital.
51 ST833 is a single locus variant of ST11, which has recently been described as one of the
52 lineages responsible for dissemination of bla_{KPC} determinants carried on different plasmids in
53 Latin America (2, 10, 11). To our knowledge, this is the first finding of a KPC-KP belonging
54 to ST833. In addition we describe its $bla_{\mathrm{KPC-2}}$ carrying plasmid, which displays interesting
55 features in an epidemiologic context.

56

57 **MATERIALS AND METHODS**

58 **Bacterial strains and growth conditions**

59 The carbapenem-resistant strain *K. pneumoniae* KBu-1 was recovered from the blood culture
60 of a three year old patient, coming from Venezuela, admitted to the Trieste Pediatric Hospital
61 to undergo marrow transplantation. Both identification and antimicrobial susceptibility were
62 determined by VITEK2 (bioMérieux, Marcy L'Etoile, France). ESBL production was further
63 investigated by the Etest method (AB Biodisk, Solna, Sweden). Detection of carbapenemase
64 production was performed by disc diffusion synergy test (Rosco Diagnostica, Taastrup,
65 Denmark).

66 *Escherichia coli* J53 (*met-63, pro-22*, Rif^r) and J62 (*lac-28, proC23, his-51, trp-30*, Rif^r) were
67 used as recipients for conjugation experiments.

68 *E. coli* JM101 [*supE thi* Δ(*lac-pro*AB) F'(*lacI*^{q} *lac*ZΔM15 *traD*36 *pro*AB$^+$)] was used as
69 recipient for electroporation of plasmid DNA isolated from *K. pneumoniae* KBu-1 and for
70 plasmid DNA preparation for further studies (DNA sequencing, restriction analysis).

71 Bacteria were grown in Luria-Bertani (LB) medium, supplemented with rifampin 100 μg/ml,
72 ampicillin 100 μg/ml or imipenem 10 μg/ml when required.

73 Antibiotic susceptibility profile of all strains was evaluated according to the guidelines of the
74 CLSI, using Sensititre plates produced by Trek diagnostics (Westlake, OH, USA) and, in the
75 case of imipenem, meropenem and ceftazidime, by standard microdilution method. (12).
76 Antimicrobial agent powders were obtained from Sigma Chemical Co. (St Louis, Mo, USA).

77

78 **PCR amplification and DNA sequencing**

79 Molecular confirmation was performed by PCR assays for the ESBL genes (*bla*$_{TEM,}$ *bla*$_{SHV,}$
80 *bla*$_{CTX-M,}$ *bla*$_{OXA-9}$) and for the carbapenemase genes (*bla*$_{IMP,}$ *bla*$_{VIM,}$ *bla*$_{NDM,}$ *bla*$_{OXA-48}$ and

81 *bla*$_{KPC}$). Specific primers, used to amplify the *bla*$_{KPC}$ determinant and other resistant genes,

82 are listed in supplemental material (Table S1). PCR reactions were performed, as previously

83 described (13-17), directly on 2–3 colonies picked from a pure culture. *bla*$_{TEM,}$ *bla*$_{SHV,}$ *bla*$_{CTX-}$

84 $_{M}$ and *bla*$_{KPC}$ amplicons were entirely sequenced to identify the allelic form.

85 Sequencing reactions were carried on at a commercial sequence facility (BMR Genomics,

86 Padua, Italy).

87 The region upstream *bla*$_{KPC}$ was amplified and sequenced with the couple of primers 3098U

88 and KPC-Rev (Table 1) to identify the isoform of Tn*4401*.

89 The region of the plasmid outward transposon Tn*4401* was amplified using the Expand long

90 template PCR system (Roche Molecular Biochemicals, Mannheim, Germany) and two

91 outward-directed primers (EcoRIout and 141R-6). For determination of the sequence of the

92 fragment adjacent to transposon Tn*4401*, primer walking was carried out with primers Bu13-1

93 and Bu13-2, designed from sequences obtained with EcoRIout and 141R-6.

94 Multilocus sequence typing (MLST) was performed according to the protocol described on

95 the *K. pneumoniae* MLST web site

96 (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html).

97

98 **Conjugation experiments**

99 Direct transfer of carbapenem resistance into *E. coli* strains J53 and J62 was attempted by a

100 filter mating procedure (18). Transconjugant selection was performed on LB agar

101 supplemented with rifampin and imipenem.

102

103 **Molecular investigations**

104 Plasmid DNA from *K. pneumoniae* KBu-1 was extracted by the alkaline lysis method (19)

105 and electroporated into *E.coli* JM101 using a Gene-pulser apparatus (Bio-Rad, Hercules, CA,

106 USA) according to the manufacturer's instructions. Transformants were selected on LB agar

107 plus ampicillin and analyzed by PCR for the presence of all the *bla* genes previously detected

108 in the donor strain.

109 Plasmid profile was analysed after S1 nuclease (Roche) digestion (20 U enzyme in each

110 sample), both on crude plasmid extract (30 min at 37°) and on DNA extracted from cells

111 embedded in agarose plugs (20) (1h at 37°C), followed by separation on agarose gel

112 electrophoresis using different running conditions: i) 20V for 20h on 1% agarose gel; ii)

113 pulsed-field gel electrophoresis (PFGE) on 0,8% agarose gel with a CHEF-DR III apparatus

114 (Bio-Rad) at 14°C and 6V/cm for 13h, by using pulse times from 1 to 10 s. Separated DNA

115 was hybridized with a digoxigenin-labeled bla_{KPC}-specific probe, obtained by amplification of

116 an internal fragment of bla_{KPC} with primers KPC-F and KPC-R (21) in the presence of 70 µM

117 dig-11-dUTP (Roche), after capillary blotting onto Hybond-N^{+} membranes (Amersham

118 Biosciences, Piscataway, NJ).

119 Plasmid restriction analysis was carried on with *Bam* HI, *Hind* III, *Pst* I, *Sac* I, restriction

120 enzymes according to the manufacturer's instructions (New England Biolabs, Mississauga,

121 Ontario, Canada), followed by separation on 0,8% agarose gel.

122 The 13 kb band recognized by the bla_{KPC}-specific probe was extracted from low melting

123 agarose by GELase™ digestion (Epicentre, Madison, Wisconsin, USA) and electroporated

124 into *E. coli* JM101.

125

126 **Stability assay**

127 Evaluation of the number of plasmid-free cells among bacteria grown under nonselective

128 conditions was carried out as described by Tolmasky *et al*.(22). Each test was replicated three

129 times.

130

131 **Comparative analysis**

132 The nucleotide and protein sequences were analysed using the *blastn, blastp and bl2seq*

133 algorithms available at the National Center of Biotechnology Information website

134 (http://www.ncbi.nlm.nih.gov).

135 Direct and tandem repeats were detected using the Tandem Repeats Finder software, version

136 4,07b (23).

137

138 **Nucleotide sequences accession number**

139 The regions of pKBuS13 sequenced in this work have been deposited in GenBank under the

140 accession numbers KM076933, KM076934 and KM076935.

141

142 **RESULTS AND DISCUSSION**

143 **Isolation and molecular characterization of KBu-1**

144 In May 2012, a three year old patient coming from Venezuela was admitted to the Trieste

145 Children's Hospital "IRCCS Burlo Garofolo" to undergo bone marrow transplantation.

146 Culture of a surveillance rectal swab detected different multi-drug resistant organisms:

147 extended spectrum beta-lactamase (ESBL) producing *Escherichia coli*, vancomycin resistant

148 *Enterococcus faecium* (VRE) and *K. pneumoniae* resistant to all beta-lactams, with MICs for

149 imipenem and meropenem $\geq$ 16 µg/ml. Unfortunately, at a later stage the patient became

150 neutropenic, developed a severe KP sepsis and died. Further analysis revealed identical

151 features to the previous isolate: i) they showed the same antibiotype (Table 1); ii) both were

152 positive for carbapenemase production. Screening by PCR revealed the presence of the *bla*$_{KPC}$

153 gene and was negative for other carbapenemase determinants; iii) ESBL production was not

154 detected by Vitek2 and resulted non-determinable by Etest, as MIC values were above the test

155 ranges; further analysis by polymerase chain reaction and sequencing of the amplicons

156 revealed the presence of the *bla*$_{CTX\text{-}M\text{-}1}$, *bla*$_{TEM1b}$ and *bla*$_{SHV11}$ genes, while *bla*$_{OXA\text{-}9}$ was not

157 detected.

158 This KPC-KP isolate remained a unique one, thanks to strict infection control procedures

159 (segregation, barrier nursing) adopted for patient management: culture of rectal swabs of all

160 the patients recovered in the same unit gave negative results.

161 The isolate was named KBu-1 and was further characterized at the molecular level.

162 Sequencing of the *bla*$_{KPC}$ amplicon and of the genes used to determine the MLST group of the

163 isolate revealed that it harboured *bla*$_{KPC\text{-}2}$ gene and belonged to ST833 (allelic profile 3-3-1-1-

164 1-1-12). To our knowledge, this is the first report of a KPC-KP belonging to ST833. It differs

165 for a single point mutation from the ST11 lineage (370 C➔G in the *tonB* allele, leading to the

166 aminoacidic substitution 121 P➔A) and belongs to CC258, which is considered of special

167 concern as it gathers the most common lineages spread worldwide (4-6), including South

168 America (2, 3, 11). The report of the SENTRY antimicrobial surveillance program on strains

169 collected from different South America hospitals during 2010 confirmed the expansion of

170 CC258 in this area and particularly of strains belonging to ST11, mostly detected in Brazil

171 (10).

172 Unfortunately, no data are available about STs circulating in Venezuela, as none of the

173 hospitals were part of the study in 2010, although the circulation of the *bla*$_{KPC}$ determinant in

174 Venezuelan hospitals is documented (24, 25).

175 Plasmid extraction followed by S1 digestion revealed at least 12 bands of various sizes
176 (ranging from 3 to 80 kb), three of which (approximately 13, 25 and 50 kb) were recognized
177 by an internal probe for the *bla*$_{KPC}$ gene (Fig. 1). All attempts to transfer resistance to
178 imipenem by conjugation from KBu-1 to *E. coli* J53Rif^{R} and to *E. coli* J62Rif^{R} were
179 unsuccessful. However, when the plasmid mixture was electroporated into *E. coli* JM101,
180 transformants carrying both the 13 kb and the 50 kb plasmids were obtained (Fig. 1). The
181 same result was achieved when we electroporated the 13 kb band alone, extracted from low
182 melting agarose (Fig. S1). Analysis by polymerase chain reaction on plasmid DNA from *E.*
183 *coli* JM101 transformants revealed the presence of the *bla*$_{KPC}$ determinant, while *bla*$_{CTX}$,
184 *bla*$_{SHV}$ and *bla*$_{TEM}$ were not detected. The 13 kb plasmid was named pKBuS13 and was
185 further investigated.
186

187 **Sequence analysis of plasmid pKBuS13**

188 Besides the spread of few strain lineages, the worldwide dissemination of the *bla*$_{KPC-2}$
189 determinant is favoured by its location on the Tn*4401* transposon, a Tn3-like element that
190 supports replicative transposition and has been found inserted at different loci on a broad
191 variety of plasmids (7, 11).
192 Most of the KPC-KP circulating in South America carry the *bla*$_{KPC-2}$ determinant on the
193 Tn*4401b* variant of Tn*4401*, located on plasmids of variable size (20-300 kb) and belonging
194 to different incompatibility groups (IncFII, IncL/M and IncN) (2, 3, 11).
195 On the assumption that the KBu-1 isolate carried the *bla*$_{KPC}$ determinant inside Tn*4401*, we
196 investigated the variable region of the transposon located upstream *bla*$_{KPC}$. As expected,
197 amplification and sequencing of this region yielded the typical structure of the Tn*4401b*
198 variant, without the deletions of 100 or 200 bp detected in the Tn*4401* or Tn*4401a* isoforms.

199 The region of pKBuS13 adjacent to Tn*4401b* was amplified using outward-directed primers
200 and the 2700 bp amplicon was fully sequenced. The location of genes and genetic structures
201 identified by comparative analysis is shown in figure 2.
202 The 1605 bp region adjacent to the *tnpA*-side of Tn*4401b* contained two genes responsible for
203 replication (*ori p15A*) and control of the copy number (*rop*) of plasmids belonging to the
204 ColE1 family. In addition, an open reading frame (ORF1) containing different direct repeats
205 was found (Fig 2).
206 The insertion site of Tn*4401* looked peculiar, as it was inserted quite inside a Xer site-specific
207 recombination locus. This locus, involved in the resolution of plasmid multimers (26), usually
208 consists of a core region containing the binding sites for two recombinases (XerC and XerD)
209 and an accessory region, which provides the binding sites for specific accessory proteins,
210 needed for the regulation of the entire process. Different core recombination sites have been
211 described (*mwr, psi, cer, dif, dxs, fpr*), which work with different efficiency and are regulated
212 by different accessory proteins (27, 28). Two of them, *mwr* and *fpr*, are osmoregulated, that is
213 at high salt concentrations their recombination efficiency is lower than that required for
214 multimers resolution. These sites have been detected so far only on two natural plasmids,
215 pJHCMW1 (22) and pFPTB1 (29) in a *Salmonella* Typhimurium and in a *K. pneumoniae*
216 isolate respectively (22, 29), both carrying a transposon inserted about 20 bp downstream of
217 Xer. It has been postulated that multimers resolution of these plasmids is provided by the
218 transposon resolvase besides the Xer system, suggesting that they form a group of plasmids
219 whose stability is significantly enhanced by transposon acquisition (28). pKBuS13 is, to our
220 knowledge, the third natural plasmid belonging to this group. However, its Xer recombination
221 system is probably ineffective, because the *fpr* site is the less efficient among those detected
222 in the core region (28) and, most importantly, its accessory region is broken by Tn*4401b*

223 insertion. Xer system inactivity is supported by two observations: i) under non selective
224 conditions, both *K. pneumoniae* KBu-1 and *E. coli* JM101 lost pKBuS13 at approximately the
225 same rate of pUC19, that lacks a Xer recombination site and is randomly partitioned during
226 cell division (Fig. 3); ii) plasmid stability did not increase in the absence of NaCl (data not
227 shown).
228 The low stability of pKBuS13 proves that the activity of the transposon resolvase alone is not
229 sufficient to stabilize this plasmid, suggesting that the level of dimer resolution needed for
230 stabilization may be achieved by the cooperation between the Xer system and the transposon
231 resolvase, and therefore they are both necessary.
232 These results suggested that the two plasmids detected in the *E. coli* recipient were the
233 monomeric and tetrameric form of pKBuS13; hypothesis that was confirmed by restriction
234 analysis, with four different enzymes (*Bam* HI, *Hind* III, *Sac* I, *Pst* I), of plasmids extracted
235 from *E. coli* JM101, which gave always the pattern expected for pKBuS13 (Fig. 4).
236 The 1118 bp region located downstream of the *tnpR*-side of the transposon carried an
237 unknown ORF2 that retrieved a single match in the database: a fragment of the genome of a
238 carbapenem resistant *E. coli*, named BIDMC43b (GenBank accession number
239 JAPE01000031), detected in a blood culture in a hospital of Boston in December 2012. *E. coli*
240 BIDMC43b is part of the "Carbapenem resistance initiative", an epidemiologic study
241 currently in progress at the Broad Institute of MIT and Harvard (broadinstitute.org). Its entire
242 genome has been sequenced by a shotgun approach and is now at the scaffold assembly level,
243 so little information is yet available (January 2015). The same strain carries a Tn*4401b* too,
244 although in a different region of the genome (GenBank accession number JAPE01000025), so
245 the hypothesis that pKBuS13 might have originated by genomic rearrangements in this strain
246 (or in an analogous one) should be taken into account.

247 In conclusion, pKBuS13 is a small plasmid carrying only one resistance determinant and it is
248 not self-transmissible by conjugation as it does not contain *tra* genes (although its
249 mobilization in presence of a helper plasmid cannot be excluded), so it might be considered
250 unimportant for dissemination of antibiotic resistance. Nevertheless, the finding that part of its
251 sequence did not have significant matches in the database other than the genome of a
252 carbapenem resistant *E. coli* detected very far both from Italy and from South America is
253 interesting for epidemiologic studies, as it might mirror a wider distribution of this kind of
254 plasmids than that reported. Moreover, the finding that it is carried by a strain that hosts many
255 different plasmids (Fig. 1A), along with the ability of Tn*4401* to undergo replicative
256 transposition, agrees with the report that many different bla_{KPC}-carrying genetic platforms are
257 circulating in Latin America (2) and represents a particularly worrisome circumstance.
258 The plasmid instability described for pKBuS13 is a peculiar feature that displays both positive
259 and negative aspects. In the clinic, the detection of unstable plasmids might be considered less
260 alarming compared to that of other plasmids, as their spread might be considered containable
261 providing that appropriate antibiotic control policies were adopted. On the other hand
262 however, researchers that study the epidemiology of resistance determinants should take into
263 account this property as it might lead to overestimation of the number of plasmids harboured
264 by clinical isolates
265
266 **ACKNOWLEDGEMENTS**
267 This work was supported by the University of Trieste, grant FRARizzo 2013.
268 We are grateful to D. Pizzo for the experiments carried out during his thesis.

269 **REFERENCES**

270

271 1. **Munoz-Price LS, Poirel L, Bonomo RA, Schwaber MJ, Daikos GL, Cormican M,**
272 **Cornaglia G, Garau J, Gniadkowski M, Hayden MK, Kumarasamy K, Livermore DM,**
273 **Maya JJ, Nordmann P, Patel JB, Paterson DL, Pitout J, Villegas MV, Wang H,**
274 **Woodford N, Quinn JP.** 2013. Clinical epidemiology of the global expansion of
275 *Klebsiella pneumoniae* carbapenemases. Lancet Infect Dis **13:**785-796.

276 2. **Andrade LN, Curiao T, Ferreira JC, Longo JM, Climaco EC, Martinez R, Bellissimo-**
277 **Rodrigues F, Basile-Filho A, Evaristo MA, Del Peloso PF, Ribeiro VB, Barth AL, Paula**
278 **MC, Baquero F, Canton R, Darini AL, Coque TM.** 2011. Dissemination of bla_{KPC-2} by
279 the spread of *Klebsiella pneumoniae* clonal complex 258 clones (ST258, ST11, ST437)
280 and plasmids (IncFII, IncN, IncL/M) among Enterobacteriaceae species in Brazil.
281 Antimicrob Agents Chemother **55:**3579-3583.

282 3. **Gomez SA, Pasteran FG, Faccone D, Tijet N, Rapoport M, Lucero C, Lastovetska O,**
283 **Albornoz E, Galas M, Melano RG, Corso A, Petroni A.** 2011. Clonal dissemination of
284 *Klebsiella pneumoniae* ST258 harbouring KPC-2 in Argentina. Clin Microbiol Infect
285 **17:**1520-1524.

286 4. **Kitchel B, Rasheed JK, Patel JB, Srinivasan A, Navon-Venezia S, Carmeli Y, Brolund**
287 **A, Giske CG.** 2009. Molecular epidemiology of KPC-producing *Klebsiella pneumoniae*
288 isolates in the United States: clonal expansion of multilocus sequence type 258.
289 Antimicrob Agents Chemother **53:**3365-3370.

290 5. **Leavitt A, Carmeli Y, Chmelnitsky I, Goren MG, Ofek I, Navon-Venezia S.** 2010.
291 Molecular epidemiology, sequence types, and plasmid analyses of KPC-producing
292 *Klebsiella pneumoniae* strains in Israel. Antimicrob Agents Chemother **54:**3002-3006.
293 6. **Qi Y, Wei Z, Ji S, Du X, Shen P, Yu Y.** 2011. ST11, the dominant clone of KPC-
294 producing *Klebsiella pneumoniae* in China. J Antimicrob Chemother **66:**307-312.
295 7. **Naas T, Cuzon G, Villegas MV, Lartigue MF, Quinn JP, Nordmann P.** 2008. Genetic
296 structures at the origin of acquisition of the beta-lactamase bla_{KPC} gene. Antimicrob
297 Agents Chemother **52:**1257-1263.
298 8. **Giani T, D'Andrea MM, Pecile P, Borgianni L, Nicoletti P, Tonelli F, Bartoloni A,**
299 **Rossolini GM.** 2009. Emergence in Italy of *Klebsiella pneumoniae* sequence type 258
300 producing KPC-3 Carbapenemase. J Clin Microbiol **47:**3793-3794.
301 9. **Giani T, Pini B, Arena F, Conte V, Bracco S, Migliavacca R, Pantosti A, Pagani L,**
302 **Luzzaro F, Rossolini GM.** 2013. Epidemic diffusion of KPC carbapenemase-producing
303 *Klebsiella pneumoniae* in Italy: results of the first countrywide survey, 15 May to 30
304 June 2011. Euro Surveill **18**.
305 10. **Castanheira M, Costello AJ, Deshpande LM, Jones RN.** 2012. Expansion of clonal
306 complex 258 KPC-2-producing *Klebsiella pneumoniae* in Latin American hospitals:
307 report of the SENTRY Antimicrobial Surveillance Program. Antimicrob Agents
308 Chemother **56:**1668-1669; author reply 1670-1661.
309 11. **Cuzon G, Naas T, Truong H, Villegas MV, Wisell KT, Carmeli Y, Gales AC, Venezia SN,**
310 **Quinn JP, Nordmann P.** 2010. Worldwide diversity of *Klebsiella pneumoniae* that
311 produce beta-lactamase bla_{KPC-2} gene. Emerg Infect Dis **16:**1349-1356.

312 12. **Clinical and Laboratory Standards Institute.** 2012. Methods for dilution antimicrobial
313 susceptibility tests for bacteria that grow aerobically. CLSI M07-A9. Clinical and
314 Laboratory Standards Institute, Wayne, PA.
315 13. **Mugnaioli C, Luzzaro F, De Luca F, Brigante G, Perilli M, Amicosante G, Stefani S,**
316 **Toniolo A, Rossolini GM.** 2006. CTX-M-type extended-spectrum beta-lactamases in
317 Italy: molecular epidemiology of an emerging countrywide problem. Antimicrob
318 Agents Chemother **50:**2700-2706.
319 14. **Hanson ND, Thomson KS, Moland ES, Sanders CC, Berthold G, Penn RG.** 1999.
320 Molecular characterization of a multiply resistant *Klebsiella pneumoniae* encoding
321 ESBLs and a plasmid-mediated AmpC. J Antimicrob Chemother **44:**377-380.
322 15. **Ellington MJ, Kistler J, Livermore DM, Woodford N.** 2007. Multiplex PCR for rapid
323 detection of genes encoding acquired metallo-beta-lactamases. J Antimicrob
324 Chemother **59:**321-322.
325 16. **Poirel L, Walsh TR, Cuvillier V, Nordmann P.** 2011. Multiplex PCR for detection of
326 acquired carbapenemase genes. Diagn Microbiol Infect Dis **70:**119-123.
327 17. **Smith Moland E, Hanson ND, Herrera VL, Black JA, Lockhart TJ, Hossain A, Johnson**
328 **JA, Goering RV, Thomson KS.** 2003. Plasmid-mediated, carbapenem-hydrolysing
329 beta-lactamase, KPC-2, in *Klebsiella pneumoniae* isolates. J Antimicrob Chemother
330 **51:**711-714.
331 18. **Selvaraj G, Iyer VN.** 1983. Suicide plasmid vehicles for insertion mutagenesis in
332 *Rhizobium meliloti* and related bacteria. J Bacteriol **156:**1292-1300.
333 19. **Sambrook J, Fritsch EF, Maniatis T.** 1989. Molecular cloning. A laboratory manual.
334 2nd Ed. Cold Spring Harbor Laboratory Press.

335 20. **Grundmann H, Schneider C, Hartung D, Daschner FD, Pitt TL.** 1995. Discriminatory
336 power of three DNA-based typing techniques for *Pseudomonas aeruginosa*. J Clin
337 Microbiol **33:**528-534.
338 21. **Tenover FC, Kalsi RK, Williams PP, Carey RB, Stocker S, Lonsway D, Rasheed JK,**
339 **Biddle JW, McGowan JE, Jr., Hanna B.** 2006. Carbapenem resistance in *Klebsiella*
340 *pneumoniae* not detected by automated susceptibility testing. Emerg Infect Dis
341 **12:**1209-1213.
342 22. **Tolmasky ME, Colloms S, Blakely G, Sherratt DJ.** 2000. Stability by multimer
343 resolution of pJHCMW1 is due to the Tn1331 resolvase and not to the *Escherichia coli*
344 Xer system. Microbiology **146 (Pt 3):**581-589.
345 23. **Benson G.** 1999. Tandem repeats finder: a program to analyze DNA sequences.
346 Nucleic Acids Res **27:**573-580.
347 24. **Labrador I, Araque M.** 2014. First Description of KPC-2-Producing *Klebsiella oxytoca*
348 Isolated from a Pediatric Patient with Nosocomial Pneumonia in Venezuela. Case
349 reports in infectious diseases **2014:**434987.
350 25. **Marcano D, De Jesus A, Hernandez L, Torres L.** 2011. [Frequency of enzymes
351 associated with reduced sensitivity to beta-lactam antibiotics in enterobacteria
352 isolates, Caracas, Venezuela]. Rev Panam Salud Publica **30:**529-534.
353 26. **Colloms SD.** 2013. The topology of plasmid-monomerizing Xer site-specific
354 recombination. Biochem Soc Trans **41:**589-594.
355 27. **Sarno R, McGillivary G, Sherratt DJ, Actis LA, Tolmasky ME.** 2002. Complete
356 nucleotide sequence of *Klebsiella pneumoniae* multiresistance plasmid pJHCMW1.
357 Antimicrob Agents Chemother **46:**3422-3427.

358 28. **Tran T, Sherratt DJ, Tolmasky ME.** 2010. fpr, a deficient Xer recombination site from
359 a *Salmonella* plasmid, fails to confer stability by dimer resolution: comparative
360 studies with the pJHCMW1 mwr site. Journal of bacteriology **192:**883-887.
361 29. **Pasquali F, Kehrenberg C, Manfreda G, Schwarz S.** 2005. Physical linkage of Tn3 and
362 part of Tn1721 in a tetracycline and ampicillin resistance plasmid from *Salmonella*
363 Typhimurium. J Antimicrob Chemother **55:**562-565.
364
365
366

367 **FIGURE LEGENDS**

368 **Fig. 1: Hybridization with a *bla*$_{\mathbf{KPC}}$ probe of plasmid DNA separated on agarose gel**

369 **electrophoresis.**

370 1A) Plasmid extract from *K. pneumoniae* KBu-1 and from *E.coli* JM101 transformed with

371 KBu-1 plasmid content was run at 20V for 20h before and after S1 nuclease digestion. 1B)

372 Fragments higher than 30 kb obtained by S1 digestion were better separated on PFGE, switch

373 1 s -10 s for 13h.

374 lane 1: KBu-1 plasmid content; lane 2: plasmid extraction from *E.coli* JM101 transformed

375 with KBu-1 plasmid content; lane M: Molecular Weight marker II (Roche); lane M^{dig}:

376 digoxygenin-labeled MWmarker II (Roche); lane λ: λ ladder (New England Biolabs).

377

378 **Fig. 2: Genetic map of relevant region of pKBuS13.**

379 Genes, ORFs and genetic structures in the regions adjacent to Tn*4401b* are shown. The 1605

380 bp region upstream the *tnpA*-side (GeneBank accession n° KM076933) is gray-shaded; the

381 1118 bp region downstream the tnpR-side (GeneBank accession n° KM076935) is cross-

382 hatched.

383 Tn*4401b* is drawn schematically, not to scale, indicating the *tnpA* and *tnpR* genes located at

384 the boundaries. The region evidenced by dots was verified by sequencing (GeneBank

385 accession n° KM076934).

386 The position of some primers used in this work and the sites of the enzymes used for

387 restriction analysis are shown (B=*Bam* HI; H=*Hind* III; P=*Pst* I; S=*Sac* I).

388 Two sequences are enlarged: above, the Xer site, with the *mwr* locus (interrupted by the

389 Tn*4401b* insertion) in the accessory region shown in the grey box, the 5 bp duplication

390 resulting from transposon insertion underlined, the *fpr* locus in the core region boxed, with the

391 binding sites for XerC and XerD shown in bold; below, the sequence containing the direct
392 repeats (DR) identified inside ORF1, with the different DR motifs marked as follows:
393 – ****** 6 bp motif (5 repeats)
394 – underlined 37 bp stretches separated by 25 bp
395 – bold tandem repeat identified by the tandem repeat finder software (23): two
396 61 bp stretches separated by one T; it is an imperfect DR, with three mismatches
397 (lower case) compared with the consensus sequence:
398 CGCGGGTGTACAACAGAATTACATCAAAAGTACA
399
400 **Fig. 3: Stability of pKBuS13** in *K. pneumoniae* KBu-1 (circles) and in *E. coli* JM101
401 (triangles). Plasmid pUC19 carried by *E. coli* JM101 (squares) was used as control, as it lacks
402 a Xer recombination site and is randomly partitioned during cell division.
403 Plasmid content of strains cultured under nonselective conditions for the indicated number of
404 generations was analysed. The graph shows the means of three independent experiments ± the
405 standard deviations.
406
407 **Fig. 4: Restriction analysis of pKBuS13**
408 Separation on 0,8% agarose gel electrophoresis of pKBuS13 extracted from the *E. coli* JM101
409 recipient and digested with *Bam* HI (lane B), *Hind* III (lane H), *Sac* I (lane S), and *Pst* I (lane
410 P). Lane M: GeneRuler^{TM} 1 kb DNA Ladder (ThermoScientific).
411

412 **Table 1. Antimicrobial susceptibility patterns of *K. pneumoniae* KBu-1, the *E. coli***
413 **JM101 recipient and the *E. coli* JM101 transformants.**

Antimicrobial agent(s)	MIC µg/ml[a]		
	K. pneumoniae KBu-1	*E. coli* JM101	*E. coli* JM101 transformants[c]
Imipenem[b]	512	0.25	4
Meropenem[b]	512	0.03	4
Ceftazidime[b]	64	0.12	8
Amoxicillin-clavulanic acid	>8	4	>8
Ampicillin/Sulbactam	>32	≤8	>32
Cefepime	>32	≤1	2
Cefotaxime	>4	≤0.06	4
Piperacillin-Tazobactam	>128	≤2	128
Amikacin	≤4	≤4	≤4
Gentamicin	≤1	≤1	≤1
Colistin	≤0.5	≤0.5	≤0.5
Nitrofurantoin	>64	≤32	≤32
Tigecycline	1	0,25	0,25

Trimethoprim-Sulphametoxazole	>4	≤0.5	≤0.5
Ciprofloxacin	>2	≤0.06	≤0.06
Levofloxacin	>4	≤1	≤1

414 [a] Reported MIC values were determined by Sensititre plates (Trek diagnostics), with the
415 exception of those of imipenem, meropenem and ceftazidime.
416 [b] For these antibiotics the CLSI standard microdilution method was used (12), in order to
417 obtain a more precise evaluation.
418 [c] *E. coli* JM101 transformed with the entire *K. pneumoniae* KBu-1 plasmid content and with
419 the 13 kb band alone displayed the same susceptibility profile.
420

Fig. 1

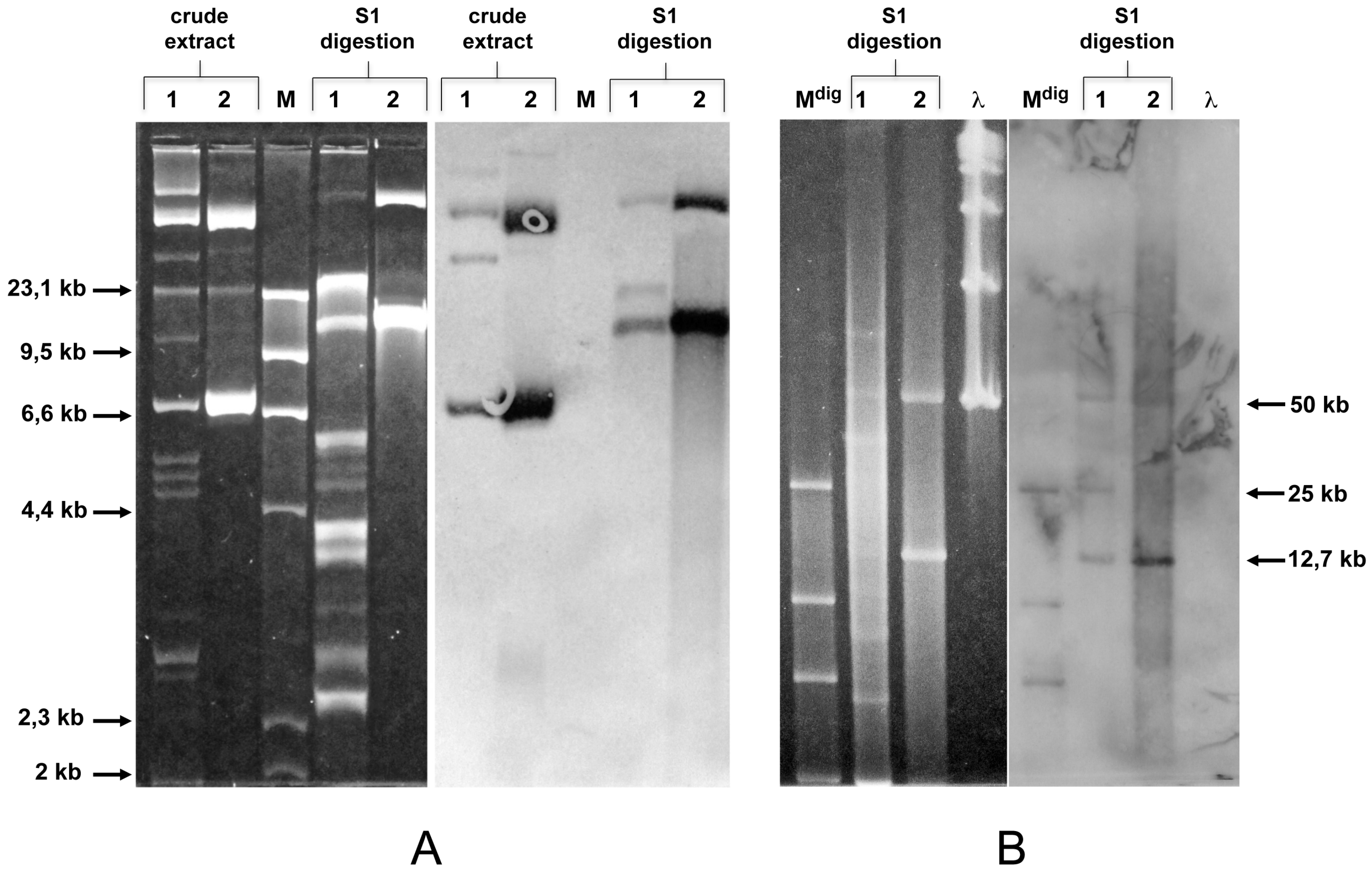
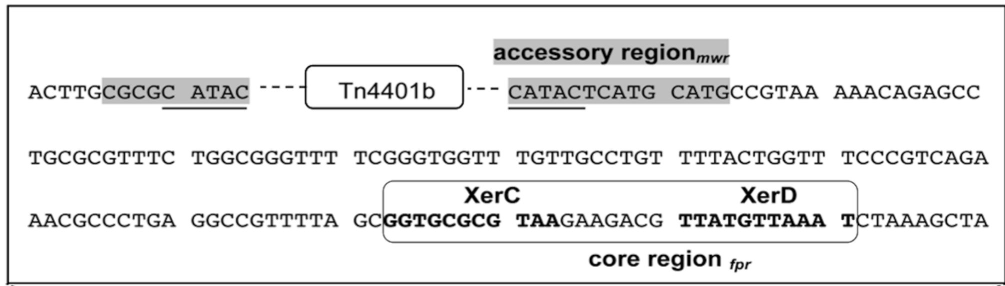
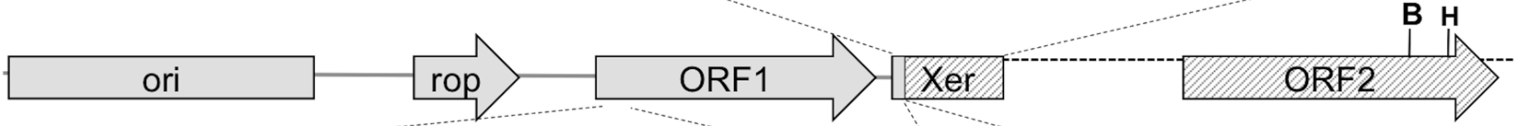
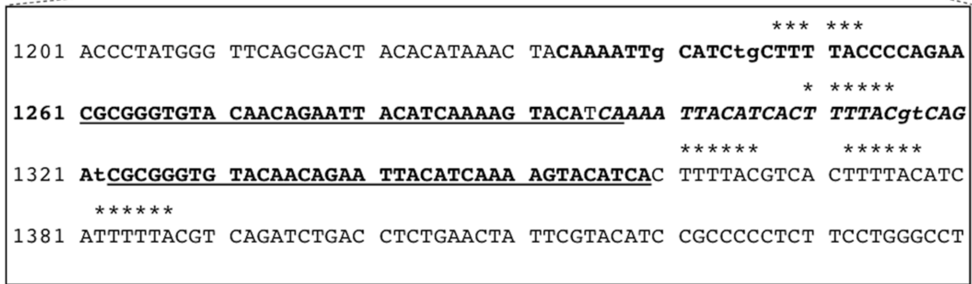
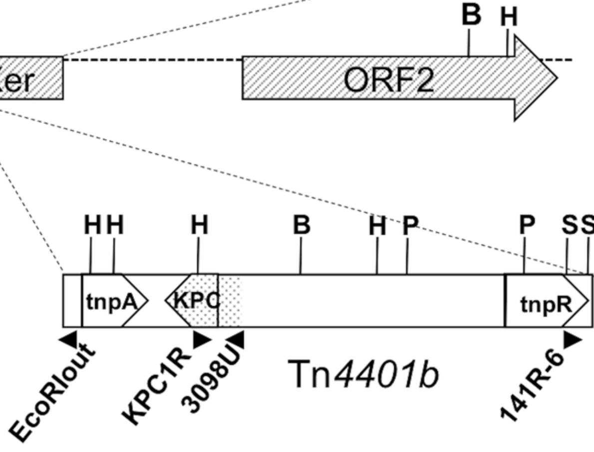


Fig. 2









Direct Repeats

Fig. 3

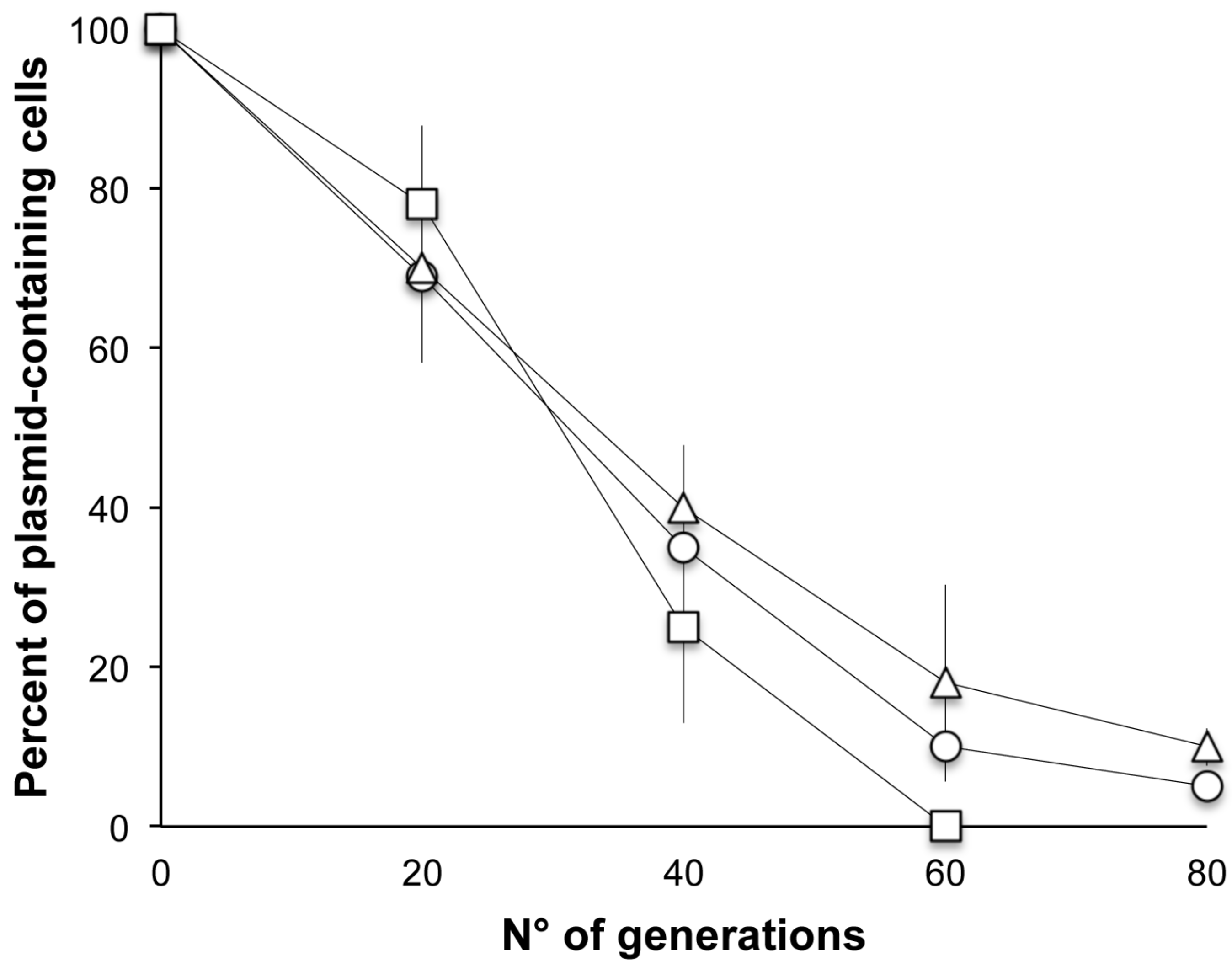


Fig. 4