Curing vector for IncI1 plasmids and its use to provide evidence for a metabolic burden of IncI1 CTX-M-1 plasmid pIFM3791 on Klebsiella pneumoniae

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Abstract:	Using a sequence based approach we previously identified an IncI1 CTX-M-1 plasmid, pIFM3791, on a single pig farm in the UK that was harboured by K. pneumoniae, Escherichia coli and Salmonella enterica serotype 4,5,12,i:-. To test the hypothesis that the plasmid had spread rapidly into these differing host bacteria we wished to assess whether the plasmid conferred a fitness advantage. To do this an IncI1 curing vector was constructed and used to displace the IncI1 CTX-M-1 plasmids from K. pneumoniae strain B3791 and several other unrelated IncI1 harbouring strains indicating the potential wider application of the curing plasmid. The IncI1 CTX-M-1 plasmid was re-introduced by conjugation into the cured K. pneumoniae strain and also a naturally IncI1 plasmid free S. enterica serotype 4,5,12,i:-, S348/1. Original, cured and complemented strains were tested for metabolic competence using BiologTM technology and in competitive growth, association to mammalian cells and biofilm formation experiments. The plasmid-cured K. pneumoniae strain grew more rapidly than either the original plasmid-carrying strain or plasmid-complemented strains in competition experiments. Additionally, the plasmid-cured strain was significantly better at respiring with L-sorbose as a carbon source and putrescine, γ-amino-n-butyric acid, L-alanine, L-proline as a nitrogen sources. By contrast, no differences in phenotype were found when comparing plasmid harbouring and plasmid free S. enterica S348/11. In conclusion, the IncI1 curing vector successfully displaced multiple IncI plasmids. The IncI1 CTX-M1 plasmid conferred a growth disadvantage upon K. pneumoniae, possibly by imposing a metabolic burden the mechanism of which remains to be determined.

1   **Curing vector for IncI1 plasmids and its use to provide evidence for a**

2   **metabolic burden of IncI1 CTX-M-1 plasmid pIFM3791 on *Klebsiella***

3   ***pneumoniae***

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25   **Abstract**

26   Using a sequence based approach we previously identified an IncI1 CTX-M-1 plasmid,

27   pIFM3791, on a single pig farm in the UK that was harboured by *K. pneumoniae*,

28   *Escherichia coli* and *Salmonella enterica* serotype 4,5,12,i:-. To test the hypothesis that

29   the plasmid had spread rapidly into these differing host bacteria we wished to assess

30   whether the plasmid conferred a fitness advantage. To do this an IncI1 curing vector

31   was constructed and used to displace the IncI1 CTX-M-1 plasmids from *K. pneumoniae*

32   strain B3791 and several other unrelated IncI1 harbouring strains indicating the

33   potential wider application of the curing plasmid. The IncI1 CTX-M-1 plasmid was re-

34   introduced by conjugation into the cured *K. pneumoniae* strain and also a naturally

35   IncI1 plasmid free *S. enterica* serotype 4,5,12,i:-, S348/1.   Original, cured and

36   complemented strains were tested for metabolic competence using Biolog[TM] technology

37   and in competitive growth, association to mammalian cells and biofilm formation

38   experiments. The plasmid-cured *K. pneumoniae* strain grew more rapidly than either the

39   original plasmid-carrying strain or plasmid-complemented strains in competition

40   experiments. Additionally, the plasmid-cured strain was significantly better at respiring

41   with L-sorbose as a carbon source and putrescine, γ-amino-n-butyric acid, L-alanine, L-

42   proline as a nitrogen sources. By contrast, no differences in phenotype were found when

43   comparing plasmid harbouring and plasmid free *S. enterica* S348/11. In conclusion, the

44   IncI1 curing vector successfully displaced multiple IncI plasmids. The IncI1 CTX-M1

45   plasmid conferred a growth disadvantage upon *K. pneumoniae*, possibly by imposing a

46   metabolic burden the mechanism of which remains to be determined.

Introduction

Extended Spectrum β-Lactamases (ESBL) confer resistance to most penicillins and monobactams and, more significantly, the clinically important cephalosporins (Gniadkowski, 2001). The CTX-M class of ESBLs has become the most prevalent type isolated from clinical settings (Livermore *et al.*, 2007), and is increasingly common in community and veterinary isolates (Batchelor *et al.*, 2005; Pitout *et al.*, 2005). Awareness of the potential spread of this resistance in livestock has increased (Horton *et al.*, 2011). Furthermore, recent genome sequencing data of human and food animal *Escherichia coli* isolates from the Netherlands indicate that the dissemination of resistance across reservoirs is caused by the expansion of successful plasmid lineages (de Been *et al.*, 2014).

During a diagnostic investigation in 2009 into pig mortality related to diarrhoea on a single pig farm in the UK, CTX-M-1 harbouring *Klebsiella pneumoniae, Escherichia coli* and *Salmonella* of serotypes 4,5,12,i:- and Bovismorbificans were recovered. Genotyping methods suggested the dissemination of a common IncI1-CTX-M-1 plasmid, which was demonstrated by sequencing a plasmid isolated from each of the bacterial species present in the farm. These plasmids, pIFM3791, pIFM3804 and pIFM3844 had been found to be identical, excepting a small number (n=7) of mutations (see supplementary data for Table S1) (Freire Martín *et al.*, 2014). These findings prompted the question whether or not this plasmid conferred a fitness advantage to the host bacterium, possibly beyond the resistance phenotype. Plasmids may confer a selective advantage to their host even in the absence of antibiotic selective pressure as suggested by Enne *et al.* (2004). The plasmids present in this farm may themselves confer a selective advantage, especially as there was no recent evidence of use of lactam based antibiotics on this farm. To assess this, our aim was to prepare plasmid bearing

72   and plasmid free isogenic strains for phenotypic testing and two approaches were used.

73   One was to place the plasmids of interest in a common isogenic background. The other

74   was to generate cured host strains for which a number of methods have been described.

75   Commonly used methods include continuous non-selective passage or growth in the

76   presence of sub-lethal concentrations of DNA intercalating agents, but both methods

77   may accumulate mutations (Ferguson & Denny, 2007). Thus, for this study we opted for

78   the use of incompatibility based curing following the observation that two plasmids

79   bearing the same replicon cannot be stably maintained in a bacterium (Couturier *et al.*,

80   1988). Plasmid incompatibility based curing has been successfully employed for curing

81   of plasmids (Hale *et al.*, 2010; Tatsuno *et al.*, 2001), but not as yet for IncI1 plasmids.

82   Here we describe the construction of a curing tool for IncI1 CTX-M plasmids and then

83   the phenotypic analyses of plasmid free and plasmid carrying strains.

84

85   ## Materials and methods

86   ### Bacterial strains and plasmids

87   The strains and plasmids described in this study were either wild-type and originating

88   from previous studies (Freire Martín *et al.*, 2014; Horton *et al.*, 2011; Randall *et al.*,

89   2012), commercially available strains and plasmids, or, in the case of pIFM26 (S1), a

90   modification of previously described plasmid pAKE604 (El-Sayed *et al.*, 2001), which

91   differed from it only in the orientation of the restriction sites within its MCS. The

92   strains were grown in LB medium and, when necessary for plasmid maintenance, in the

93   presence of appropriate antibiotics.

94   ### Generation of curing vector pIFM27

95   Exponentially growing bacteria were made electrocompetent through successive water

96   and glycerol washes. For molecular cloning, amplified PCR products were digested

97   using commercially available enzymes and ligated to digested vectors using standard

98   techniques, a description of which has been included within the supplementary

99   materials (S1). The final curing vector was named pIFM27 and it harboured negative

100   selection gene *sacB*, resistance markers *kan^r*, *amp^r* and *aac6*-Ib and IncI1 plasmid

101   replication down-regulator *RNAI* alongside its native promoter (Fig. 1).

102   **Plasmid conjugation**

103   Stationary, antibiotic free LB broth cultures of donor (50μl) and recipient (150μl) were

104   mixed and overlaid on a polycarbonate 0.2μm filter placed on the centre of a LB agar

105   plate and incubated at 37°C for 24hrs. The bacteria were washed with 2ml of PBS and

106   transconjugants selected by plating on Rambach agar plates supplemented with 10μg/ml

107   of tetracycline and 2μg/ml of cefotaxime. Tetracycline was chosen as the intended

108   recipients had been shown to be resistant during extensive characterisation carried out

109   as part of previous work (Freire Martín *et al.*, 2014). Colonies of the appropriate colour

110   (red for the *S. enterica* strains, purple for the *K. pneumoniae* strains) were re-streaked

111   and checked for the presence of the incoming plasmid by CTX-M universal PCR and

112   plasmid profiling.

113   **Characterization methods used for verification of curing, conjugation and strain**

114   **identity**

115   Macro-restriction profiles of strains were obtained by *Xba*I Pulsed Field Gel

116   Electrophoresis (PFGE) as described by the PulseNet network (Gerner-Smidt *et al.*,

117   2006). Electrophoresis conditions were 14°C at 6V/cm for 19.5h with switch times

118   ranging from 2.2 to 54.2s of linear ramping for the *K. pneumoniae* strains and 2.2 to

119   63.8s for the *S. enterica* strains. Gel images were analysed using of BioNumerics

120   software (v.6.6; Applied Maths, Belgium).

121   Plasmid profiling was performed following the method of Kado and Liu (1981). As

122   reference supercoiled DNA ladder (Sigma-Aldrich D5292), reference strain 39R861 and

123   reference strain 20R764 were also run.

124   A number of primers were also used to test for the presence of certain genes within the

125   strains throughout the different stages of curing using PCR, as described in the results.

126   See Table 1 for the primer sequences. The PCR ramping conditions were in every case

127   25 cycles of 94°C for 30s, 60°C for 30s and 72°C for 1m.

128   **Phenotypic tests of plasmid burden by Biolog Phenotype Microarray**

129   Biolog^{TM} plates PM2 and PM3 were used following manufacturer's instructions.

130   Biolog^{TM} output data files were imported into R (http://www.R-project.org; R Core

131   Team, R: A Language and Environment for Statistical Computing, R Foundation for

132   Statistical Computing, Vienna, Austria, 2014) and the kinetic curves for each biological

133   replicate were fitted using the Grofit package (Kahm *et al.*, 2010). The features studied

134   were: the area under the curve (AUC), the maximum value achieved (MaxV), and the

135   fastest rate of increase (RateI).

136   **Competitive growth curves**

137   Competition experiments of plasmid-free vs. plasmid-harbouring strains were set up and

138   carried out as previously described (Lenski *et al.*, 1994). A sample of the competition

139   mixture at time 0, and thereafter every 24hrs for 6 days, was serially diluted and plated

140   on LB agar and LB agar supplemented with 2μg/ml of cefotaxime to obtain counts of

141   total bacteria and of resistant bacteria, respectively. To calculate the selection rate the

142   natural log of the ratio of plasmid-bearing/plasmid-free for each day was plotted for

143   each competition. The selection rate was taken as the slope of the equation defining the

144   line of best fit. Each competition was repeated four times and two tailed t-tests were

145 carried out to determine whether the mean selection rate for each pair of strains

146 competed was significantly different from 0.

Association of *K. pneumoniae* to cultured monolayers of mammalian cells

148 The porcine intestinal epithelial cell line IPEC-J2 was used. Specifically, 2 day old cells

149 cultured to confluency in 24 well plates were used. On the day of the assay, the culture

150 medium was removed from the wells and the cells were washed twice with Hank's

151 balanced salts solution to remove residual antibiotics. The bacterial inocula were

152 resuspended in Ham's F12/DMEM supplemented with 2% mannose and delivered in

153 volumes of 1ml at a density of ~10^7 CFU/ml per well in triplicate. The plates were then

154 incubated for 1hr in an atmosphere of 5% CO2 at 37°C with 100% humidity. After

155 incubation, the bacterial inocula were removed and the wells washed three times with

156 HBSS. After washing, the cells were disrupted by addition of 1% Triton-X 100 (in PBS)

157 with mechanical stirring followed by serial dilution and plating on LB agar (Favre-

158 Bonte *et al.*, 1999).

Association and invasion of *S. enterica* to cultured monolayers of mammalian cells

160 Association of *S. enterica* was evaluated in the same manner as above with minor

161 differences. Specifically, the bacterial inocula were delivered as 1ml of ~3.5×10^7

162 CFU/ml suspended in Ham's F12/DMEM with a co-incubation step of 2hr. A second

163 plate was set up identically and used for the enumeration of intracellular bacteria

164 (invasion). After co-incubation and washing, the monolayers were overlayed with

165 Ham's F12/DMEM supplemented with 150mg/L colistin and incubated for 90min. The

166 antibiotic containing medium was removed and the wells were washed three times with

167 HBSS. After washing, the cells were disrupted and the bacteria were enumerated as

168 described above (Searle *et al.*, 2009).

Biofilm formation

170 Stationary bacteria were seeded at 0.05_{OD570} and cultured statically in LB (*K.*
171 *pneumoniae)* or LB without salt (*S. enterica*) within triplicate wells in microplates for
172 24h at 25° C and 37°C. The culture was discarded, the wells were washed 3 times with
173 PBS and 130μl of 1% crystal violet was added to the wells and incubated at room
174 temperature for 30 min. The crystal violet solution was then discarded and the wells
175 were washed 4 times with distilled water. To solubilise the bound crystal violet, 130μl
176 of ethanol/acetone (70/30 v/v) was added to the wells and after 10min the $_{OD570}$
177 determined (Woodward *et al.*, 2000). The experiments were carried out three times for
178 each strain and temperature.

Statistical analysis of phenotypic assays

180 For each of the measured phenotypes statistical analysis to compare strains was
181 performed using the triplicate measurements for each strain as input to a one way
182 ANOVA test. Any ANOVA test having a P-value <0.05, indicating that there is a
183 difference in feature values between at least two strains, was followed by a Tukey's
184 HSD post-hoc test, where a P-value < 0.05 for a specific strain comparison denoted a
185 significant difference. For the Biolog experiment this analysis was carried out in R
186 whereas for the remaining experiments statistical analysis was carried out using
187 GraphPad Prism 5.

188

Results

Construction of the IncI1 curing plasmid: selection of targets and vector

191 The DNA sequences of IncI1-CTX-M-1 plasmids from *K. pneumoniae, E. coli* and *S.*
192 *enterica* 4,5,12;i;- identified during a pig farm investigation (Freire Martín *et al.*, 2014)
193 were analysed. The aim of this analysis was to identify genes involved in replication
194 and maintenance in order to target them with the curing vector. A single replicon was

195   identified as well as addiction system pndA/C. In the first instance, the RNA gene,

196   *RNAI*, which controls the copy number in IncI1 plasmids by translational regulation of

197   replication initiator *repZ* (Praszkier & Pittard, 2005), was amplified by PCR and cloned

198   into suicide vector pIFM26 (see supplementary material). This vector is not stable in the

199   absence of selection which, together with the presence of negative selection marker

200   *sacB* (confers sucrose sensitivity to Gram negative organisms), would enable recovery

201   of vector free clones by growth on antibiotic-free sucrose-containing media (Hale *et al.*,

202   2010).

203   *K. pneumoniae* strain B3791 is naturally resistant to both kanamycin and

204   ampicillin, the markers present in pIFM26. An alternative selectable marker was

205   required and so an amikacin resistance determinant *aac6*-Ib was cloned from plasmid

206   CB01 and inserted to create pIFM27 (pIFM26::*RNAI*::*aac6*-Ib) (Fig. 1). CB01 was a

207   wild type plasmid isolated by C. Boinet (unpublished thesis).

Curing *K. pneumoniae* strain B3791 of IncI1 CTX-M plasmid by transformation

with pIFM27

210   To validate the use of curing vector pIFM27 curing tests were performed with

211   wild type strains carrying IncI1-CTX-M-1 plasmids from sources other than the farm

212   pertaining to this study. LREC215, a commensal *E. coli* isolated from a broiler chick

213   (Horton *et al.*, 2011) and LREC447, an avian pathogenic *E. coli* isolated from a broiler

214   (Randall *et al.*, 2012), were confirmed by PCR to carry a CTX-M group 1 gene on an

215   IncI1-γ plasmid and to be sensitive to kanamycin, a strong selectable marker present in

216   pIFM27. Electrocompetent cells were transformed with curing vector pIFM27 with

217   selection on kanamycin (50mg/L). Four colonies from each transformation were tested

218   for the acquisition of pIFM27 by vector PCR. 100% of the colonies tested were shown

219   to be sensitive to cefotaxime and no longer carried their respective CTX-M plasmids as

220   shown by both CTX-M PCR and plasmid profiling. pIFM27 was excluded from the

221   transformants by culture in LB followed by recovery on LB agar supplemented with 5%

222   sucrose. Eight colonies recovered on sucrose supplemented agar for each strain were

223   tested by vector PCR and plasmid profiling which showed that the vector had been

224   successfully excluded. As the presence of *RNAI* in this vector was sufficient for the

225   exclusion of the target plasmid there was no need to pursue the making of a pndA/C

226   harbouring construct.

227   In the case of *K. pneumoniae* B3791 curing vector pIFM27 was electroporated

228   with selection on amikacin 2mg/L. This resulted in forty putative transformant colonies

229   of which three had acquired pIFM27, as confirmed by vector PCR. The remainder were

230   most likely spontaneous resistant mutants which is possible given the recipient strain

231   harboured another aminoglycoside resistance gene, a family of compounds against

232   which cross-resistance is known to occur (Shaw *et al.*, 1993). The three genuine

233   transformants were sensitive to cefotaxime (2mg/L) and had lost pIFM3791, as verified

234   by CTX-M PCR and plasmid profiling. pIFM27 was excluded from these three by

235   culture on LB agar supplemented with 5% sucrose. Six randomly selected sucrose

236   resistance derivatives from each transformant were negative for pIFM27 by PCR, their

237   *Xba*I PFGE banding patterns were all highly similar if not identical to that of B3791 and

238   plasmid profiles showed loss of a ~8kb plasmid, namely pIFM27. The remaining native

239   plasmids present in B3791 remained unaffected throughout the curing process (Fig. 2).

240   Collectively, these data indicated that curing vector pIFM27 excluded the IncI1 plasmid

241   from strain B3791. One cured derivative, designated CL32, was taken forward for

242   further analysis. The curing of an *E. coli* and a *Salmonella* 4,5,12,i:- originally isolated

243   from the same farm as *K. pneumoniae* B3791 (Freire Martín *et al.*, 2014) was also

244   attempted. This was, however, unsuccessful due to high levels of resistance to multiple

245   antibiotic and we did not investigate other selectable markers in this proof of concept

246   study.

247

248   **Re-introduction of the IncI1 CTX-M1 plasmids into cured *K. pneumoniae*, strain**

249   **CL32.**

250   Having prepared CL32, the plasmid cured derivative of *K. pneumoniae* B3791,

251   the last step in constructing strains for plasmid phenotype comparisons was to re-

252   introduce the outbreak plasmids into CL32. Solid surface mating conjugations were set

253   up with strains CL32 as recipient and laboratory strains of *E. coli* (NEB10iβ)

254   harbouring pIFM3791 (isolated from *K. pneumoniae*), pIFM3904 (isolated from *E. coli*)

255   and pIFM3844 (isolated from *Salmonella* 4,5,12:i:-), respectively, as donors. DNA

256   sequence analysis had previously shown these to plasmids to be very similar to each

257   other except for a small number of SNPs. As part of this work e wished to test whether

258   these differences represented adaptations to the harbouring species (Freire Martín *et al.*,

259   2014).

260   Transconjugants were selected by plating on Rambach agar supplemented with

261   tetracycline at 10μg/ml, which selected for CL32, and cefotaxime at 2μg/ml, which

262   selected for the incoming plasmid. Transconjugant colonies were sub-cultured and

263   tested by PCR using the CTX-M universal primers (Table 1) and by plasmid profiling

264   (Fig. 3) and the presence of a plasmid of ~100kb was confirmed in transconjugants of

265   CL32 (Table 2).

266

267   **Phenotypic impact of plasmid carriage upon *K. pneumoniae* B3791**

268   In growth competition studies, strain CL32 outcompeted *K. pneumoniae* strain B3791

269   (P=0.08), CL32-pIFM3791 (P<0.05), CL32-pIFM3804 (P<0.05) and CL32-pIFM3844

270   (P<0.05). A representative example of the data is shown in Fig. 4 and the summary of

271   the data in Table 3. For *K. pneumoniae* B3791 and its cured and complemented

272   derivatives no differences in association to IPEC-J2 monolayers were found. Crystal

273   violet biofilm assays performed at 25°C and 37°C found no differences when comparing

274   plasmid-free to plasmid-carrying strains.

275   In order to investigate subtler impacts in the metabolism of the strain caused by

276   plasmid carriage, Phenotype Microarrays (Biolog™) were performed on wild type *K.*

277   *pneumoniae* strain B3791, its IncI1 plasmid cured derivative CL32 and complemented

278   strain CL32-pIFM3791. There were four compounds for which one measurable

279   parameter of respiration for the plasmid free strain CL32 was significantly higher (P-

280   value <0.05) than B3791 and CL32-pIFM3791 (Table 4). These were rate of increase

281   (RateI) on L-sorbose as a carbon source and maximum value achieved (MaxV) on

282   putrescine, γ-Amino-N-Butyric and Ala-Gly dipeptide as nitrogen sources.

283   Using caproic acid, 4-hydroxy benzoic acid and putrescine as carbon sources

284   there were significant differences between CL32 and B3791 or between CL32 and

285   CL32-pIFM3791. For L-alanine, L-proline and L-threonine although CL32 was better

286   able to respire on these compounds than the other two strains the difference was only

287   significant when compared to plasmid-complemented strain CL32-pIFM3791. Finally,

288   for dipeptide Ala-Thr significant differences were found for two different measures of

289   growth, MaxV and AUC, although, the comparisons differed for each measure. Whilst

290   the AUC for CL32 was significantly different only from CL32-pIFM3791, for MaxV all

291   three comparisons revealed significant differences (Table 4).

292

293   **Effect of plasmid carriage on *Salmonella* 4,5,12:i:- strain S348/11**

294   Having demonstrated the phenotypic impact of pIFM3791 upon *K. pneumoniae*, the

295   question arose as to whether similar phenotypic impacts might occur in other Gram

296   negative bacteria. We had previously noted that *E. coli* and *Salmonella*

297   Bovismorbificans and 4,5,12:i:- from the same farm carried pIFM3791-like plasmids,

298   which however could not be cured due to problems with cross-resistance. For this

299   reason cefotaxime-sensitive *Salmonella* 4,5,12:i:- originating from the original farm

300   were screened by PFGE, plasmid profiling, susceptibility testing and CTX-M PCR. An

301   isolate designated S348/11 was identified which was identical to the *Salmonella*

302   4,5,12:i:- by these measures except for the lack of carriage of a pIFM3791-like plasmid.

303   Plasmid pIFM3791was introduced by conjugation into S348/11 and the phenotype of

304   transconjugants was tested as described above. The presence of the plasmid did not

305   affect competitive growth of the strains, their association and invasion of monolayers of

306   mammalian cell line IPEC-J2, or their ability to form biofilm.

307

Discussion

309   PFGE and PCR tests confirmed curing plasmid pIFM27 successfully excluded IncI1

310   CTX-M plasmids from *K. pneumoniae* B3791, and other unrelated strains with 100%

311   efficiency. We showed also that the use of the curing plasmid could be extended by

312   incorporation of alternative resistance markers demonstrating that this approach has

313   wide applications dependent upon the incompatibility and selectable marker genes

314   cloned. Here, limited selectable markers precluded use in other multiple antibiotic

315   resistant strains. However, this should no longer be a major obstacle due to the presence

316   of restriction sites flanking the resistance cassette allowing for easy exchange of

317   markers.

318   Using cured derivative CL32 for phenotypic comparisons carried the risk that

319   differences may be caused by the curing process hence complemented CL32 derivatives

320   were also used. Plasmid cured CL32 out-competed both wild type and plasmid

321   complemented strains, which themselves behaved in essentially the same manner

322   suggesting a fitness burden was imposed by carriage of the test plasmids. In contrast, a

323   recent study of an ST7 IncI1-CTX-M-1 plasmid isolated from broilers in the

324   Netherlands found that its carriage did not impose a burden on the growth of its

325   *Escherichia coli* host (Fischer *et al.*, 2014) although previous findings suggest plasmids

326   can slow down the growth of naïve hosts (Lee & Edlin, 1985; Zünd & Lebek, 1980). In

327   the case of pIFM3791 it may have been recently acquired by *K. pneumoniae* and thus,

328   co-evolution between plasmid and host, which is known to ameliorate growth

329   disadvantage (Bouma & Lenski, 1988), may have not taken place by the time of

330   isolation on the farm. Other reasons for the differences in behaviour could be caused by

331   the actual sequence of the Dutch plasmid, as it displayed a different pMLST sequence

332   type than that of pIFM3791 and its sequence is otherwise unknown.

333   We did not investigate alternative selection markers and this precluded the construction

334   of a *Salmonella* 4,5,12:i:- plasmid cured derivative. As such, we introduced pIFM3791,

335   pIFM3804 and pIFM3844 into strain *Salmonella* 4.5.12:i- S348/11 which was a plasmid

336   free strain from the same farm. Given the available data, S348/11 was highly similar to

337   the original strain harbouring a pIFM3791 like plasmid, but it cannot be ruled out that it

338   may have in the past harboured one such plasmid, and already be somewhat adapted to

339   its carriage. Conversely, highly related versions of the plasmid were originally found in

340   *E. coli* and *Salmonella* 4,5,12:i:- strains as well as *K. pneumoniae*. If the plasmid was

341   introduced by a *Salmonella* 4,5,12:i:- strain into a *K. pneumoniae* strain this could also

342   account for a degree of pre-existing plasmid adaption to S348/11 but not B3791. This is

343 supported by previous description of *in vitro* adaptation of a plasmid genome resulting

344 in the amelioration of the fitness burden imposed upon its host (Modi & Adams, 1991).

345   Another interesting finding was the ability of CL32 to respire more efficiently

346 than the other two strains on ten substrates: *K. pneumoniae* B3791 and CL32-pIFM3791

347 behaved similarly giving confidence that plasmid pIFM3791 did impact metabolism

348 although sequence analysis did not suggest any obvious gene candidates. Nevertheless

349 many genes of unknown function may contribute and future transcriptional and targeted

350 mutagenesis studies may resolve the mechanisms behind the phenotypes. Whether the

351 suppression of respiratory activity using L-sorbose as a carbon source and putrescine, γ-

352 amino-N-butyric acid and dipeptide Ala-Gly as nitrogen sources impacts

353 epidemiologically is worthy of consideration. Of importance is the identification of

354 specific metabolically-based burdens mediated by the plasmid on *K. pneumoniae* which

355 could be explained if expression of plasmid genes diverts resources away from the

356 transcription of metabolic pathways involved in the growth under nutrient limiting

357 conditions. Transcriptional interference mediated by integrated plasmids has been noted

358 before (Zagaglia *et al.*, 1991).

359 Collectively, these data show that the effect that plasmid pIFM3791 has on its host is

360 highly dependent on that host's make up. *Salmonella* 4,5,12:i:- strain 348/11 was better

361 able to carry the outbreak plasmids than the plasmid in *K. pneumoniae* strain B3791.

362 Given its similarity to plasmid bearing isolates it is possible S348/11 is a naturally cured

363 strain and adapted to the burdens imposed by the plasmid. Additionally, the experiments

364 here also show that whether an effect is detectable or not will depend on the exact

365 aspect of fitness that is being measured. The implication of these findings for the

366 reversal of antimicrobial resistance is that even if there is a fitness cost for a certain

367 strain of pathogenic bacteria, as long as there are others in its environment for which

368 such cost does not exist, and given the mobility of conjugative plasmids, once the

369 selection pressure is imposed by means of the antibiotic, the plasmid can spread again.

370 For this reason, it would have been interesting to have carried out the experiments with

371 *E. coli* strains isolated from the farm. *E. coli* are often found to be harmless

372 commensals, and if it was found that plasmid carriage is either beneficial or neutral for

373 them, this could further lend weight to the idea that plasmids are maintained by certain

374 strains and that are then taken up by other strains in times of selective pressure.

375

384 **Transparency declarations**

385 None to declare.

386
387

388   **References**

389   **Batchelor, M., Hopkins, K., Threlfall, E. J., Clifton-Hadley, F. A., Stallwood, A. D., Davies, R. H.**
390   **& Liebana, E. (2005).** bla(CTX-M) genes in clinical Salmonella isolates recovered from
391   humans in England and Wales from 1992 to 2003. *Antimicrob Agents Chemother* **49**,
392   1319–22.

393   **De Been, M., Lanza, V. F., de Toro, M., Scharringa, J., Dohmen, W., Du, Y., Hu, J., Lei, Y., Li, N.**
394   **& other authors**. **(2014).** Dissemination of cephalosporin resistance genes between
395   Escherichia coli strains from farm animals and humans by specific plasmid lineages. *PLoS*
396   *Genet* **10**, e1004776.

397   **Bouma, J. E. & Lenski, R. E. (1988).** Evolution of a bacteria/plasmid association. *Nature* **335**,
398   351–2.

399   **Couturier, M., Bex, F., Bergquist, P. L. & Maas, W. K. (1988).** Identification and classification of
400   bacterial plasmids. *Microbiol Rev* **52**, 375–95.

401   **El-Sayed, A. K., Hothersall, J. & Thomas, C. M. (2001).** Quorum-sensing-dependent regulation
402   of biosynthesis of the polyketide antibiotic mupirocin in Pseudomonas fluorescens
403   NCIMB 10586. *Microbiology* **147**, 2127–2139.

404   **Enne, V. I., Bennett, P. M., Livermore, D. M. & Hall, L. M. C. (2004).** Enhancement of host
405   fitness by the sul2-coding plasmid p9123 in the absence of selective pressure. *J*
406   *Antimicrob Chemother* **53**, 958–63.

407   **Favre-Bonte, S., Joly, B. & Forestier, C. (1999).** Consequences of reduction of Klebsiella
408   pneumoniae capsule expression on interactions of this bacterium with epithelial cells.
409   *Infect Immun* **67**, 554–61.

410   **Ferguson, L. R. & Denny, W. A. (2007).** Genotoxicity of non-covalent interactions: DNA
411   intercalators. *Mutat Res* **623**, 14–23.

412   **Fischer, E. A., Dierikx, C. M., van Essen-Zandbergen, A., van Roermund, H. J., Mevius, D. J.,**
413   **Stegeman, A. & Klinkenberg, D. (2014).** The IncI1 plasmid carrying the blaCTX-M-1 gene
414   persists in in vitro culture of a Escherichia coli strain from broilers. *BMC Microbiol* **14**, 77.

415   **Freire Martín, I., Abuoun, M., Reichel, R., La Ragione, R. M. & Woodward, M. J. (2014).**
416   Sequence analysis of a CTX-M-1 IncI1 plasmid found in Salmonella 4,5,12:i:-, Escherichia
417   coli and Klebsiella pneumoniae on a UK pig farm. *J Antimicrob Chemother* 2098–2101.

418   **Gerner-Smidt, P., Hise, K., Kincaid, J., Hunter, S., Rolando, S., Hyytiä-Trees, E., Ribot, E. M. &**
419   **Swaminathan, B. (2006).** PulseNet USA: a five-year update. *Foodborne Pathog Dis* **3**, 9–
420   19. Mary Ann Liebert, Inc. 2 Madison Avenue Larchmont, NY 10538 USA.

421   **Gniadkowski, M. (2001).** Evolution and epidemiology of extended-spectrum beta-lactamases
422   (ESBLs) and ESBL-producing microorganisms. *Clin Microbiol Infect* **7**, 597–608.

423   **Hale, L., Lazos, O., Haines, A. & Thomas, C. (2010).** An efficient stress-free strategy to displace
424   stable bacterial plasmids. *Biotechniques* **48**, 223–8.

425 **Horton, R. A., Randall, L. P., Snary, E. L., Cockrem, H., Lotz, S., Wearing, H., Duncan, D., Rabie,**
426 **A., McLaren, I. & other authors**. **(2011).** Fecal carriage and shedding density of CTX-M
427 extended-spectrum {beta}-lactamase-producing escherichia coli in cattle, chickens, and
428 pigs: implications for environmental contamination and food production. *Appl Environ*
429 *Microbiol* **77**, 3715–9.

430 **Kado, C. I. & Liu, S. T. (1981).** Rapid procedure for detection and isolation of large and small
431 plasmids. *J Bacteriol* **145**, 1365–73.

432 **Lee, S. W. & Edlin, G. (1985).** Expression of tetracycline resistance in pBR322 derivatives
433 reduces the reproductive fitness of plasmid-containing Escherichia coli. *Gene* **39**, 173–80.

434 **Lenski, R. E., Simpson, S. C. & Nguyen, T. T. (1994).** Genetic analysis of a plasmid-encoded,
435 host genotype-specific enhancement of bacterial fitness. *J Bacteriol* **176**, 3140–7.

436 **Livermore, D. M., Canton, R., Gniadkowski, M., Nordmann, P., Rossolini, G. M., Arlet, G.,**
437 **Ayala, J., Coque, T. M., Kern-Zdanowicz, I. & other authors**. **(2007).** CTX-M: changing the
438 face of ESBLs in Europe. *J Antimicrob Chemother* **59**, 165–74.

439 **Kahm, M., Hasenbrink, G., Lichtenberg-Fraté, H., Ludwig, J. & Kschischo, M. (2010).** grofit:
440 fitting biological growth curves with R. *J Stat Softw* **33**, 1–21.

441 **Modi, R. I. & Adams, J. (1991).** Coevolution in Bacterial-Plasmid Populations. *Evolution (N Y)*
442 **45**, 656–667. Society for the Study of Evolution.

443 **Pitout, J. D. D., Gregson, D. B., Church, D. L., Elsayed, S. & Laupland, K. B. (2005).** Community-
444 wide outbreaks of clonally related CTX-M-14 beta-lactamase-producing Escherichia coli
445 strains in the Calgary health region. *J Clin Microbiol* **43**, 2844–9.

446 **Praszkier, J. & Pittard, A. J. (2005).** Control of replication in I-complex plasmids. *Plasmid* **53**,
447 97–112.

448 **Randall, L., Wu, G., Phillips, N., Coldham, N., Mevius, D. & Teale, C. (2012).** Virulence genes in
449 bla(CTX-M) Escherichia coli isolates from chickens and humans. *Res Vet Sci* **93**, 23–7.

450 **Searle, L. E. J., Best, a., Nunez, a., Salguero, F. J., Johnson, L., Weyer, U., Dugdale, a. H.,**
451 **Cooley, W. a., Carter, B. & other authors**. **(2009).** A mixture containing
452 galactooligosaccharide, produced by the enzymic activity of Bifidobacterium bifidum,
453 reduces Salmonella enterica serovar Typhimurium infection in mice. *J Med Microbiol* **58**,
454 37–48.

455 **Shaw, K. J., Rather, P. N., Hare, R. S. & Miller, G. H. (1993).** Molecular Genetics of
456 Aminoglycoside Resistance Genes and Familial Relationships of the Aminoglycoside-
457 Modifying Enzymes **57**.

458 **Tatsuno, I., Horie, M., Abe, H., Miki, T., Makino, K., Shinagawa, H., Taguchi, H., Kamiya, S.,**
459 **Hayashi, T. & Sasakawa, C. (2001).** toxB gene on pO157 of enterohemorrhagic
460 Escherichia coli O157:H7 is required for full epithelial cell adherence phenotype. *Infect*
461 *Immun* **69**, 6660–9.

462 **Woodward, M. J., Sojka, M., Sprigings, K. A. & Humphrey, T. J. (2000).** The role of SEF14 and
463   SEF17 fimbriae in the adherence of Salmonella enterica serotype Enteritidis to inanimate
464   surfaces. *J Med Microbiol* **49**, 481–7.

465 **Zagaglia, C., Casalino, M., Colonna, B., Conti, C., Calconi, A. & Nicoletti, M. (1991).** Virulence
466   plasmids of enteroinvasive Escherichia coli and Shigella flexneri integrate into a specific
467   site on the host chromosome: integration greatly reduces expression of plasmid-carried
468   virulence genes. *Infect Immun* **59**, 792–9.

469 **Zünd, P. & Lebek, G. (1980).** Generation time-prolonging R plasmids: correlation between
470   increases in the generation time of Escherichia coli caused by R plasmids and their
471   molecular size. *Plasmid* **3**, 65–9.

472

473 **Legends**

474 **Figure 1:** Genetic map of IncI1 curing vector pIFM27. The features coloured in black

475 represent genetic structures already present in vector pIFM26 whilst the features

476 coloured in grey are those added in this study.

477

478 **Figure 2:** Plasmid profiling DNA gel electrophoresis of B3791, its pIFM27

479 transformant derivatives CL28 and, pIFM27 free derivatives CL31 and CL32. 20R764

480 is a reference plasmid free *E. coli* for identification of the chromosomal DNA band

481 plasmids of B3791 and its derivatives. The band of approximately 8kb in CL28

482 corresponds to the introduced pIFM27 curing vector. In lanes CL28 to CL32 the native

483 CTX-M IncI1 p3791 has been lost.

484

485 **Figure 3: a) Plasmid profiling of S348/11 and its outbreak plasmid harbouring**

486 **transconjugants.** Plasmid profiling DNA gel electrophoresis of strain S348/11 and its

487 outbreak plasmid carrying derivatives. 20R764 is a reference plasmid free *E. coli* for

488 identification of the chromosomal DNA band. 39R861 is a reference strain carrying four

489 plasmids of known sizes as labelled. Strain S348/11 carries no plasmids as shown in this

490 gel, its transconjugants have acquired a plasmid of around 100kb, circled blue. **b)**

491 **Plasmid profiling of CL32 transconjugants complemented with the outbreak**

492 **plasmids.** Plasmid profiling DNA gel electrophoresis of strain CL32 transconjugants.

493 T91-3 plasmid DNA has been included as a reference of the outbreak plasmid size

494 (circled red). 20R764 is a reference plasmid free *E. coli* for identification of the

495 chromosomal DNA band. 39R861 is a reference strain carrying four plasmids of known

496 sizes as labelled. All transconjugants have acquired a plasmid of around 100kb, circled

497 blue.

498

Figure 4: Representative example of a competition experiment of strain CL32 vs.

B3791. The $\log_e$ of the ratio of plasmid bearing over plasmid free organisms on each

day has been plotted. The negative slope of the line of best fit denotes that B3791 is

being outcompeted by CL32.

503

504 **Table 1: Primers used in this study**

Primer	Sequence
RNAFW	TCTAGAATATCTCGAGTGCACAGGGTTGAATCTC
RNARV	GGATCCTGTTCCGGAAGCCATAAA
M13FW	GTAAAACGACGGCCAG
M13RV	CAGGAAACAGCTATGAC
PRE-MCS	GCGATTAAGTTGGGTAACG
aac6'IbFW	GAATTCATGAGCAACGCAAAAACA
aac6'IbRV	GAATTCTTAGGCATCACTGCGTGT

505

506 **Table 2: List of plasmid complemented strains of CL32 and S348/11 as well as**
507 **plasmid donor lab strains T91a, T04a and T44a.**

508

Strain	Parent	Species	CTX-plasmid
T91a	NEB10-beta	*E. coli*	pIFM3791
T04a	NEB10-beta	*E. coli*	pIFM3804
T44a	NEB10-beta	*E. coli*	pIFM3844
CL32-pIFM3791	CL32	*K. pneumoniae*	pIFM3791
CL32-pIFM3804	CL32	*K. pneumoniae*	pIFM3804
CL32-pIFM3844	CL32	*K. pneumoniae*	pIFM3844
S348/11	NA	*S. enterica*	NA
S348/11-pIFM3791	S348/11	*S. enterica*	pIFM3791
S348/11-pIFM3804	S348/11	*S. enterica*	pIFM3804
S348/11-pIFM3844	S348/11	*S. enterica*	pIFM3844

509

510 **Table 3: Effect of plasmid carriage on the competitive fitness of strains B3791 and**
511 **S348/11**

Plasmid-less	Plasmid-carrying	Selection rate	P-value
CL32	B3791	-0.29	0.08
	CL32-pIFM3791	-0.19	0.009
	CL32-pIFM3804	-0.22	0.011
	CL32-pIFM3844	-0.27	0.006
S348/11	S348/11-pIFM3804	-0.01	0.954
	S348/11-pIFM3844	0.01	0.957

512

513 Competition experiment results (Selection Rate) and their statistical significance as
514 assessed by a two tailed T test (P value). Highlighted in grey are those results
515 considered to be significantly different from no selection (P<0.05).

Table 4: Effect of plasmid carriage on the metabolism of *K. pneumoniae* B3791 as assessed by Phenotype Microarray Biolog.

| Compound (Plate, Well)	Feature	Strain order	*P* values of comparisons by Tukey's HSD		
			CL32 vs. B3791	CL32-pIFM3791 vs. B3791	CL32::pIFM3791 vs. CL32
L-Sorbose (PM2, D04)	RateI	CL32 > CL32-pIFM3791 > B3791	0.009	0.974	0.012
Caproic acid (PM2, E02)	MaxV	CL32 > CL32-pIFM3791 > B3791	0.013	0.145	0.194
	AUC	CL32 > CL32-pIFM3791 > B3791	0.031	0.430	0.164
4-Hydroxy Benzoic Acid (PM2, E07)	RateI	CL32 > B3791 > CL32-pIFM3791	0.159	0.174	0.013
Putrescine (PM2, H08)	RateI	CL32 > B3791 > CL32-pIFM3791	0.072	0.904	0.042
	MaxV	CL32 > CL32-pIFM3791 > B3791	0.033	0.736	0.085
	AUC	CL32 > CL32-pIFM3791 > B3791	0.029	0.679	0.082
L-Alanine(PM3, A07)	MaxV	CL32 > B3791 > CL32-pIFM3791	0.165	0.472	0.035
L-Proline (PM3, B09)	MaxV	CL32 > B3791 > CL32-pIFM3791	0.104	0.566	0.028
L-Threonine (PM3, B11)	MaxV	CL32 > B3791 > CL32-pIFM3791	0.054	0.464	0.012
	AUC	CL32 > B3791 > CL32-pIFM3791	0.098	0.526	0.024
Putrescine (PM3, D11)	MaxV	CL32 > B3791 > CL32-pIFM3791	0.029	0.456	0.007
γ-Amino-NButyric Acid (PM03, G08)	MaxV	CL32 > CL32-pIFM3791 > B3791	0.026	0.991	0.030
Ala-Gly (PM3, H04)	MaxV	CL32 > B3791 > CL32-pIFM3791	0.026	0.768	0.012
Ala-Thr (PM3, H07)	MaxV	CL32 > B3791 > CL32-pIFM3791	0.025	0.033	0.001
	AUC	CL32 > B3791 > CL32-pIFM3791	0.236	0.238	0.024

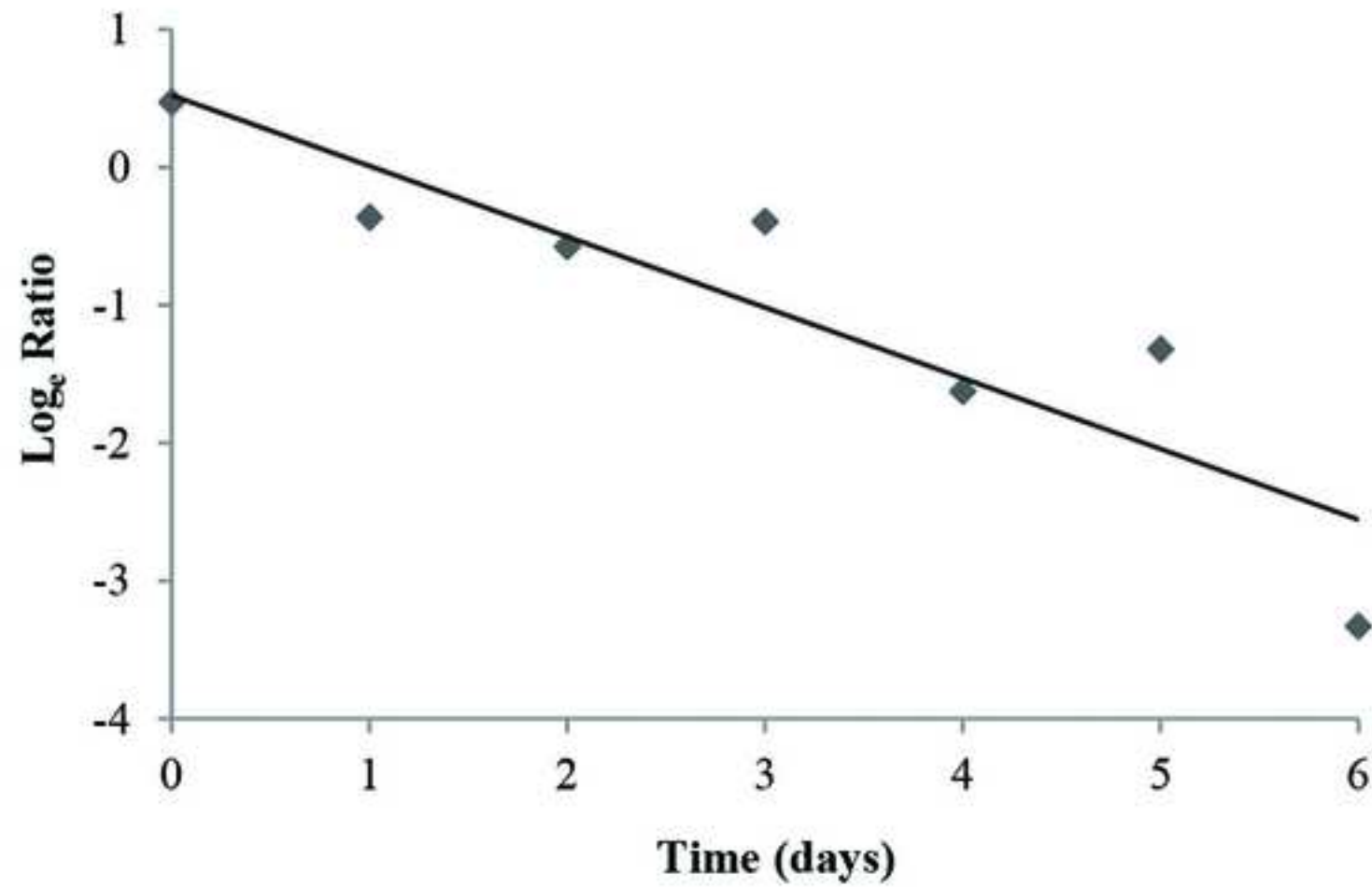Listed are the compounds for which significant differences in their respiration were found for at least one of the features tested. Results that reach statistical significance have been highlighted in grey.

* MaxV = maximum value achieved, RateI = fastest rate of increase, AUC = area under the curve.

521

Figure 1

Figure 2

Figure 2

Figure 4
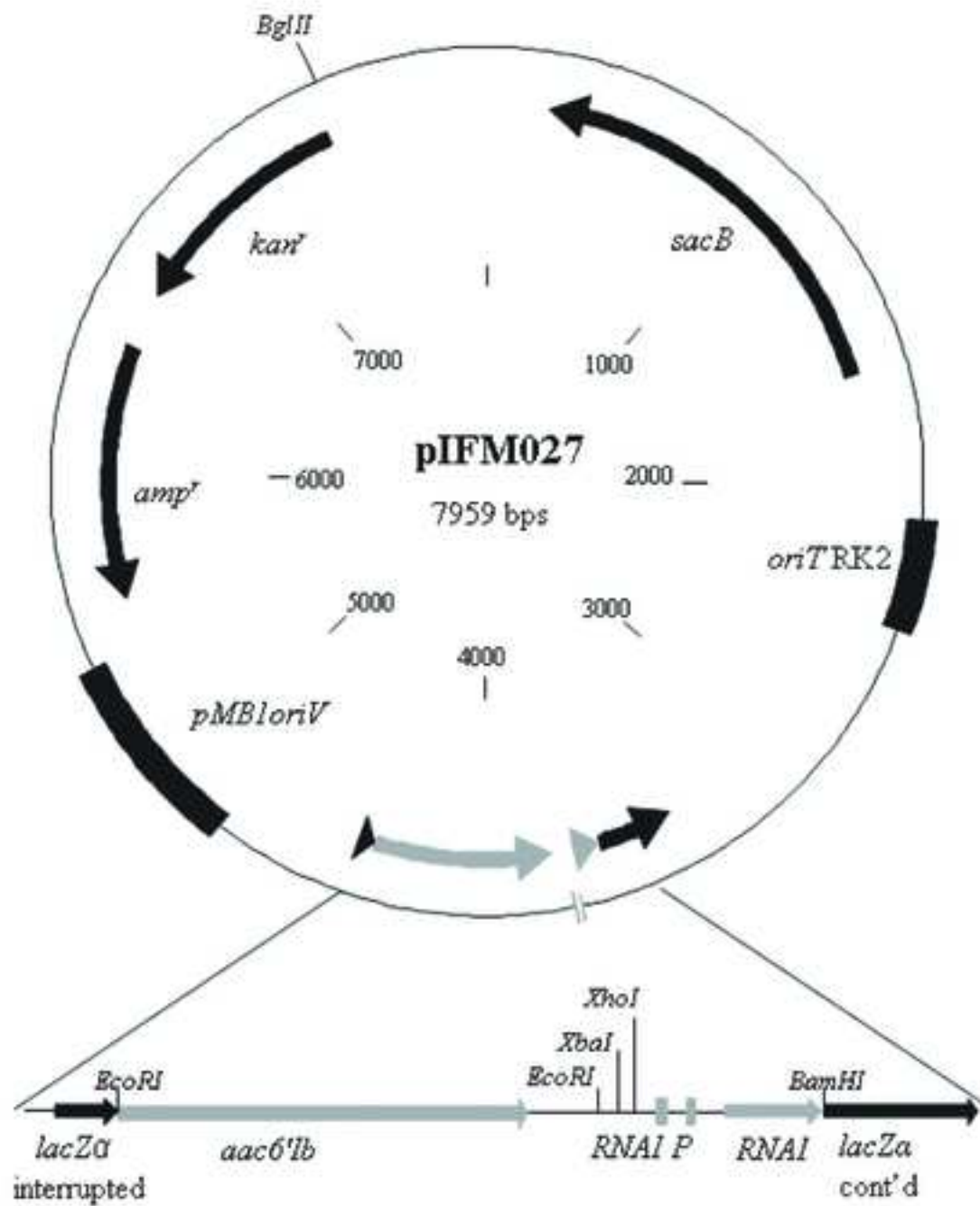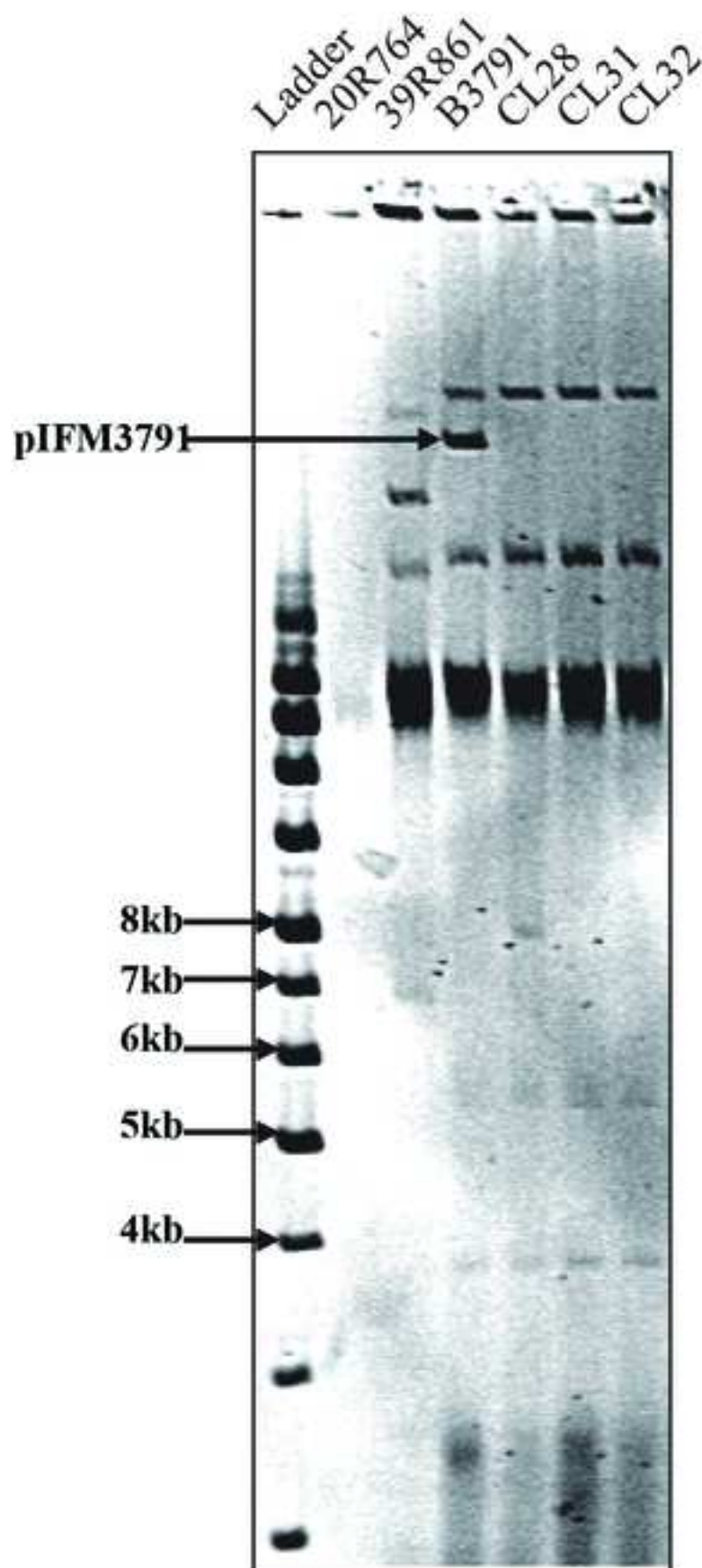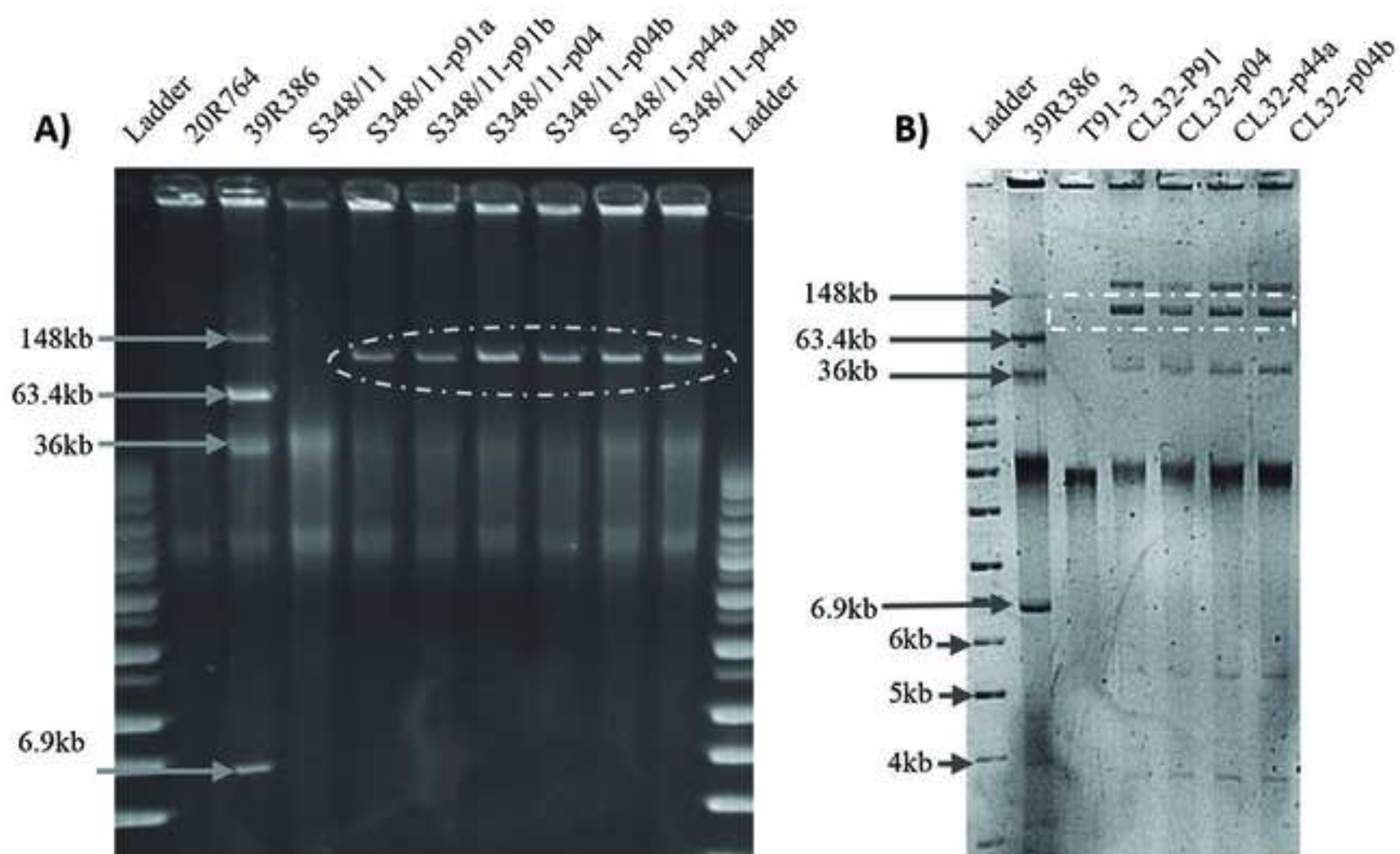
1 **SUPPLEMENTARY MATERIAL**

2 All molecular biological methods including DNA extraction, measurement of DNA purity

3 and concentration, PCR, restriction digestion of DNA, agarose gel electrophoresis, ligation,

4 transformation, electroporation and selection followed standard procedures(Sambrook, J., E.

5 F. Fritsch, n.d.) and manufacturer's conditions for use of reagents. Sequencing reactions were

6 carried out by the AHVLA Central Sequencing Unit.

7

8 **Construction of IncI1 curing vector plasmid pIFM27 *RNAI1 aac6-Ib***

9   Primers were designed to amplify the *RNAI* gene along with its native promoter with

10 tails on both forward (RNAIFW), and reverse (RNARV) primers (Table 1) that introduced

11 *Xba*I and *Xho*I sites to the 5' and a *BamH*I site to the 3' of the *RNAI* gene. Using plasmid

12 pIFM3804 DNA as template, the PCR generated a product of the correct size that blunt end

13 cloned into pCR-Blunt. Plasmid DNA extracted from four transformants was digested using

14 *Xba*I and *BamH*I which confirmed the presence of an insert of the predicted size and

15 sequencing with insert flanking primers (M13FW and M13RV) showed complete integrity of

16 *RNAI*.

17   Plasmid DNA from one transformant was digested with *Xba*I and *BamH*I and the

18 *RNAI* insert was gel purified and ligated with vector pIFM26, previously digested with *Xba*I

19 and *BamH*I. pIFM26 was identical to previously described pAKE604,(El-Sayed *et al.*, 2001)

20 with a minor modification to invert its multiple cloning site. The ligated DNA was used to

21 transform *E. coli* DH5α with ampicillin selection (100mg/L). To verify the constructs,

22 plasmid DNA extracted from three well isolated transformants was digested with *Bgl*II and

23 *Xho*I to confirm the predicted total and fragment size of the plasmid and was used as target

24 for PCR and sequencing using RNAI-FW and Pre-MCS primers (Table 1). One transformant

25 designated CL19 was retained for further work.

26   As the outbreak strains were already resistant to kanamycin and ampicillin, the

27   markers present on pIFM26, an alternative resistance marker, amikacin, was chosen for

28   inclusion in the curing vector. The amikacin resistance gene *aac6'-Ib* was amplified using

29   forward and reverse primers, aac6'-IbFW and aac6'-IbFW that included *EcoR*I tails (Table

30   1), using as target the wild type plasmid isolated by C. Boinet as part of her PhD studies

31   (Phenotypic and genotypic analysis of blaCTX-M encoding plasmids isolated from bovine *E.*

32   *coli* samples in the United Kingdom: Royal Holloway, University of London). The *aac6'-Ib*

33   gene product was cloned into pCR-Blunt in *E. coli* NEB5α with selection for kanamycin

34   (50mg/L). Plasmid DNA extracted from five transformants was used in PCR with primers

35   flanking the insertion site (M13FW and M13RV) to confirm presence of the insert (expected

36   ~800bp band) and the product integrity was confirmed by sequence analysis. One

37   transformant designated CL25 was retained for further work.

38   Plasmid DNA extracted from CL25 was digested with *EcoR*I and the *aac6'-Ib*

39   fragment was cloned into the *EcoR1* site of pIFM19 DNA in *E. coli* NEB5α with selection for

40   transformants resistant to ampicillin (100mg/L). Only one colony was found and PCR

41   analysis using primer pairs aac6'-IbFW and Pre-MCS and aac6'-IbRV and Pre-MCS

42   confirmed the correct orientation of the insert with respect to the LacZ promoter to drive its

43   expression. The resulting strain was named CL27 and its plasmid pIFM27 (Figure 1).

44   **Table S1: Sequence differences in outbreak plasmids pIFM3791, pIFM3804 and**

45   **pIFM3844**

Position in pIFM3804	Sequence			Gene	Effect
	pIFM3791	pIFM3804	pIFM3844		
6148	G	G	A	*ISCR2*	serine to phenylalanine in pIFM3844
10994	CG	-G	- -	outside coding region	NA
19884	G	-	G	*ybaA*	frameshift (in pIFM3791 and pIFM3844)
24941	A	-	A	hypothetical protein	frameshift (in pIFM3791 and pIFM3844)
56570	A	-	A	hypothetical protein	frameshift (in pIFM3791 and pIFM3844)
57253	T	-	T	outside coding region	NA
58328	T	-	T	*exc*	frameshift (in pIFM3791 and pIFM3844)

46   **References**

47   **El-Sayed, A. K., Hothersall, J. & Thomas, C. M. (2001).** Quorum-sensing-dependent
48   regulation of biosynthesis of the polyketide antibiotic mupirocin in Pseudomonas
49   fluorescens NCIMB 10586. *Microbiology* **147**, 2127–2139.

50   **Sambrook, J., E. F. Fritsch, and T. M. (n.d.).** *Molecular cloning: a laboratory manual, 2nd*
51   *ed.* Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory Press.