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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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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 Biolog^{TM} 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.

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

and plasmid free isogenic strains for phenotypic testing and two approaches were used. One was to place the plasmids of interest in a common isogenic background. The other was to generate cured host strains for which a number of methods have been described. Commonly used methods include continuous non-selective passage or growth in the presence of sub-lethal concentrations of DNA intercalating agents, but both methods may accumulate mutations (Ferguson & Denny, 2007). Thus, for this study we opted for the use of incompatibility based curing following the observation that two plasmids bearing the same replicon cannot be stably maintained in a bacterium (Couturier *et al.*, 1988). Plasmid incompatibility based curing has been successfully employed for curing of plasmids (Hale *et al.*, 2010; Tatsuno *et al.*, 2001), but not as yet for IncI1 plasmids. Here we describe the construction of a curing tool for IncI1 CTX-M plasmids and then the phenotypic analyses of plasmid free and plasmid carrying strains.

Materials and methods

Bacterial strains and plasmids

The strains and plasmids described in this study were either wild-type and originating from previous studies (Freire Martín *et al.*, 2014; Horton *et al.*, 2011; Randall *et al.*, 2012), commercially available strains and plasmids, or, in the case of pIFM26 (S1), a modification of previously described plasmid pAKE604 (El-Sayed *et al.*, 2001), which differed from it only in the orientation of the restriction sites within its MCS. The strains were grown in LB medium and, when necessary for plasmid maintenance, in the presence of appropriate antibiotics.

Generation of curing vector pIFM27

Exponentially growing bacteria were made electrocompetent through successive water and glycerol washes. For molecular cloning, amplified PCR products were digested

using commercially available enzymes and ligated to digested vectors using standard techniques, a description of which has been included within the supplementary materials (S1). The final curing vector was named pIFM27 and it harboured negative selection gene *sacB*, resistance markers *kan*^{r}, *amp*^{r} and *aac6*-Ib and IncI1 plasmid replication down-regulator *RNAI* alongside its native promoter (Fig. 1).

Plasmid conjugation

Stationary, antibiotic free LB broth cultures of donor (50µl) and recipient (150µl) were mixed and overlaid on a polycarbonate 0.2µm filter placed on the centre of a LB agar plate and incubated at 37ºC for 24hrs. The bacteria were washed with 2ml of PBS and transconjugants selected by plating on Rambach agar plates supplemented with 10µg/ml of tetracycline and 2µg/ml of cefotaxime. Tetracycline was chosen as the intended recipients had been shown to be resistant during extensive characterisation carried out as part of previous work (Freire Martín *et al.*, 2014). Colonies of the appropriate colour (red for the *S. enterica* strains, purple for the *K. pneumoniae* strains) were re-streaked and checked for the presence of the incoming plasmid by CTX-M universal PCR and plasmid profiling.

Characterization methods used for verification of curing, conjugation and strain identity

Macro-restriction profiles of strains were obtained by *Xba*I Pulsed Field Gel Electrophoresis (PFGE) as described by the PulseNet network (Gerner-Smidt *et al.*, 2006). Electrophoresis conditions were 14°C at 6V/cm for 19.5h with switch times ranging from 2.2 to 54.2s of linear ramping for the *K. pneumoniae* strains and 2.2 to 63.8s for the *S. enterica* strains. Gel images were analysed using of BioNumerics software (v.6.6; Applied Maths, Belgium).

Plasmid profiling was performed following the method of Kado and Liu (1981). As reference supercoiled DNA ladder (Sigma-Aldrich D5292), reference strain 39R861 and reference strain 20R764 were also run.

A number of primers were also used to test for the presence of certain genes within the strains throughout the different stages of curing using PCR, as described in the results. See Table 1 for the primer sequences. The PCR ramping conditions were in every case 25 cycles of 94^{o}C for 30s, 60^{o}C for 30s and 72^{o}C for 1m.

Phenotypic tests of plasmid burden by Biolog Phenotype Microarray

Biolog^{TM} plates PM2 and PM3 were used following manufacturer's instructions. Biolog^{TM} output data files were imported into R (http://www.R-project.org; R Core Team, R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria, 2014) and the kinetic curves for each biological replicate were fitted using the Grofit package (Kahm *et al*., 2010). The features studied were: the area under the curve (AUC), the maximum value achieved (MaxV), and the fastest rate of increase (RateI).

Competitive growth curves

Competition experiments of plasmid-free vs. plasmid-harbouring strains were set up and carried out as previously described (Lenski *et al.*, 1994). A sample of the competition mixture at time 0, and thereafter every 24hrs for 6 days, was serially diluted and plated on LB agar and LB agar supplemented with 2µg/ml of cefotaxime to obtain counts of total bacteria and of resistant bacteria, respectively. To calculate the selection rate the natural log of the ratio of plasmid-bearing/plasmid-free for each day was plotted for each competition. The selection rate was taken as the slope of the equation defining the line of best fit. Each competition was repeated four times and two tailed t-tests were

carried out to determine whether the mean selection rate for each pair of strains competed was significantly different from 0.

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

The porcine intestinal epithelial cell line IPEC-J2 was used. Specifically, 2 day old cells cultured to confluency in 24 well plates were used. On the day of the assay, the culture medium was removed from the wells and the cells were washed twice with Hank's balanced salts solution to remove residual antibiotics. The bacterial inocula were resuspended in Ham's F12/DMEM supplemented with 2% mannose and delivered in volumes of 1ml at a density of ~10^7 CFU/ml per well in triplicate. The plates were then incubated for 1hr in an atmosphere of 5% CO2 at 37ºC with 100% humidity. After incubation, the bacterial inocula were removed and the wells washed three times with HBSS. After washing, the cells were disrupted by addition of 1% Triton-X 100 (in PBS) with mechanical stirring followed by serial dilution and plating on LB agar (Favre-Bonte *et al.*, 1999).

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

Association of *S. enterica* was evaluated in the same manner as above with minor differences. Specifically, the bacterial inocula were delivered as 1ml of ~$3.5x10^7$ CFU/ml suspended in Ham's F12/DMEM with a co-incubation step of 2hr. A second plate was set up identically and used for the enumeration of intracellular bacteria (invasion). After co-incubation and washing, the monolayers were overlayed with Ham's F12/DMEM supplemented with 150mg/L colistin and incubated for 90min. The antibiotic containing medium was removed and the wells were washed three times with HBSS. After washing, the cells were disrupted and the bacteria were enumerated as described above (Searle *et al.*, 2009).

Biofilm formation

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

Statistical analysis of phenotypic assays

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

Results

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

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

identified as well as addiction system pndA/C. In the first instance, the RNA gene, *RNAI*, which controls the copy number in IncI1 plasmids by translational regulation of replication initiator *repZ* (Praszkier & Pittard, 2005), was amplified by PCR and cloned into suicide vector pIFM26 (see supplementary material). This vector is not stable in the absence of selection which, together with the presence of negative selection marker *sacB* (confers sucrose sensitivity to Gram negative organisms), would enable recovery of vector free clones by growth on antibiotic-free sucrose-containing media (Hale *et al.*, 2010).

K. pneumoniae strain B3791 is naturally resistant to both kanamycin and ampicillin, the markers present in pIFM26. An alternative selectable marker was required and so an amikacin resistance determinant *aac6*-Ib was cloned from plasmid CB01 and inserted to create pIFM27 (pIFM26::*RNAI*::*aac6*-Ib) (Fig. 1). CB01 was a wild type plasmid isolated by C. Boinet (unpublished thesis).

Curing *K. pneumoniae* strain B3791 of IncI1 CTX-M plasmid by transformation with pIFM27

To validate the use of curing vector pIFM27 curing tests were performed with wild type strains carrying IncI1-CTX-M-1 plasmids from sources other than the farm pertaining to this study. LREC215, a commensal *E. coli* isolated from a broiler chick (Horton *et al.*, 2011) and LREC447, an avian pathogenic *E. coli* isolated from a broiler (Randall *et al.*, 2012), were confirmed by PCR to carry a CTX-M group 1 gene on an IncI1-γ plasmid and to be sensitive to kanamycin, a strong selectable marker present in pIFM27. Electrocompetent cells were transformed with curing vector pIFM27 with selection on kanamycin (50mg/L). Four colonies from each transformation were tested for the acquisition of pIFM27 by vector PCR. 100% of the colonies tested were shown to be sensitive to cefotaxime and no longer carried their respective CTX-M plasmids as

shown by both CTX-M PCR and plasmid profiling. pIFM27 was excluded from the transformants by culture in LB followed by recovery on LB agar supplemented with 5% sucrose. Eight colonies recovered on sucrose supplemented agar for each strain were tested by vector PCR and plasmid profiling which showed that the vector had been successfully excluded. As the presence of *RNAI* in this vector was sufficient for the exclusion of the target plasmid there was no need to pursue the making of a pndA/C harbouring construct.

In the case of *K. pneumoniae* B3791 curing vector pIFM27 was electroporated with selection on amikacin 2mg/L. This resulted in forty putative transformant colonies of which three had acquired pIFM27, as confirmed by vector PCR. The remainder were most likely spontaneous resistant mutants which is possible given the recipient strain harboured another aminoglycoside resistance gene, a family of compounds against which cross-resistance is known to occur (Shaw *et al.*, 1993). The three genuine transformants were sensitive to cefotaxime (2mg/L) and had lost pIFM3791, as verified by CTX-M PCR and plasmid profiling. pIFM27 was excluded from these three by culture on LB agar supplemented with 5% sucrose. Six randomly selected sucrose resistance derivatives from each transformant were negative for pIFM27 by PCR, their *Xba*I PFGE banding patterns were all highly similar if not identical to that of B3791 and plasmid profiles showed loss of a ~8kb plasmid, namely pIFM27. The remaining native plasmids present in B3791 remained unaffected throughout the curing process (Fig. 2). Collectively, these data indicated that curing vector pIFM27 excluded the IncI1 plasmid from strain B3791. One cured derivative, designated CL32, was taken forward for further analysis. The curing of an *E. coli* and a *Salmonella* 4,5,12,i:- originally isolated from the same farm as *K. pneumoniae* B3791 (Freire Martín *et al.*, 2014) was also attempted. This was, however, unsuccessful due to high levels of resistance to multiple

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

Re-introduction of the IncI1 CTX-M1 plasmids into cured *K. pneumoniae*, strain CL32.

Having prepared CL32, the plasmid cured derivative of *K. pneumoniae* B3791, the last step in constructing strains for plasmid phenotype comparisons was to re-introduce the outbreak plasmids into CL32. Solid surface mating conjugations were set up with strains CL32 as recipient and laboratory strains of *E. coli* (NEB10iβ) harbouring pIFM3791 (isolated from *K. pneumoniae*), pIFM3904 (isolated from *E. coli*) and pIFM3844 (isolated from *Salmonella* 4,5,12:i:-), respectively, as donors. DNA sequence analysis had previously shown these to plasmids to be very similar to each other except for a small number of SNPs. As part of this work e wished to test whether these differences represented adaptations to the harbouring species (Freire Martín *et al.*, 2014).

Transconjugants were selected by plating on Rambach agar supplemented with tetracycline at 10µg/ml, which selected for CL32, and cefotaxime at 2µg/ml, which selected for the incoming plasmid. Transconjugant colonies were sub-cultured and tested by PCR using the CTX-M universal primers (Table 1) and by plasmid profiling (Fig. 3) and the presence of a plasmid of ~100kb was confirmed in transconjugants of CL32 (Table 2).

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

In growth competition studies, strain CL32 outcompeted *K. pneumoniae* strain B3791 ($P=0.08$), CL32-pIFM3791 ($P<0.05$), CL32-pIFM3804 ($P<0.05$) and CL32-pIFM3844

(P<0.05). A representative example of the data is shown in Fig. 4 and the summary of the data in Table 3. For *K. pneumoniae* B3791 and its cured and complemented derivatives no differences in association to IPEC-J2 monolayers were found. Crystal violet biofilm assays performed at 25ºC and 37ºC found no differences when comparing plasmid-free to plasmid-carrying strains.

In order to investigate subtler impacts in the metabolism of the strain caused by plasmid carriage, Phenotype Microarrays (Biolog™) were performed on wild type *K. pneumoniae* strain B3791, its IncI1 plasmid cured derivative CL32 and complemented strain CL32-pIFM3791. There were four compounds for which one measurable parameter of respiration for the plasmid free strain CL32 was significantly higher (P-value <0.05) than B3791 and CL32-pIFM3791 (Table 4). These were rate of increase (RateI) on L-sorbose as a carbon source and maximum value achieved (MaxV) on putrescine, γ-Amino-N-Butyric and Ala-Gly dipeptide as nitrogen sources.

Using caproic acid, 4-hydroxy benzoic acid and putrescine as carbon sources there were significant differences between CL32 and B3791 or between CL32 and CL32-pIFM3791. For L-alanine, L-proline and L-threonine although CL32 was better able to respire on these compounds than the other two strains the difference was only significant when compared to plasmid-complemented strain CL32-pIFM3791. Finally, for dipeptide Ala-Thr significant differences were found for two different measures of growth, MaxV and AUC, although, the comparisons differed for each measure. Whilst the AUC for CL32 was significantly different only from CL32-pIFM3791, for MaxV all three comparisons revealed significant differences (Table 4).

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

Having demonstrated the phenotypic impact of pIFM3791 upon *K. pneumoniae*, the question arose as to whether similar phenotypic impacts might occur in other Gram negative bacteria. We had previously noted that *E. coli* and *Salmonella* Bovismorbificans and 4,5,12:i:- from the same farm carried pIFM3791-like plasmids, which however could not be cured due to problems with cross-resistance. For this reason cefotaxime-sensitive *Salmonella* 4,5,12:i:- originating from the original farm were screened by PFGE, plasmid profiling, susceptibility testing and CTX-M PCR. An isolate designated S348/11 was identified which was identical to the *Salmonella* 4,5,12:i:- by these measures except for the lack of carriage of a pIFM3791-like plasmid. Plasmid pIFM3791was introduced by conjugation into S348/11 and the phenotype of transconjugants was tested as described above. The presence of the plasmid did not affect competitive growth of the strains, their association and invasion of monolayers of mammalian cell line IPEC-J2, or their ability to form biofilm.

Discussion

PFGE and PCR tests confirmed curing plasmid pIFM27 successfully excluded IncI1 CTX-M plasmids from *K. pneumoniae* B3791, and other unrelated strains with 100% efficiency. We showed also that the use of the curing plasmid could be extended by incorporation of alternative resistance markers demonstrating that this approach has wide applications dependent upon the incompatibility and selectable marker genes cloned. Here, limited selectable markers precluded use in other multiple antibiotic resistant strains. However, this should no longer be a major obstacle due to the presence of restriction sites flanking the resistance cassette allowing for easy exchange of markers.

Using cured derivative CL32 for phenotypic comparisons carried the risk that differences may be caused by the curing process hence complemented CL32 derivatives were also used. Plasmid cured CL32 out-competed both wild type and plasmid complemented strains, which themselves behaved in essentially the same manner suggesting a fitness burden was imposed by carriage of the test plasmids. In contrast, a recent study of an ST7 IncI1-CTX-M-1 plasmid isolated from broilers in the Netherlands found that its carriage did not impose a burden on the growth of its *Escherichia coli* host (Fischer *et al.*, 2014) although previous findings suggest plasmids can slow down the growth of naïve hosts (Lee & Edlin, 1985; Zünd & Lebek, 1980). In the case of pIFM3791 it may have been recently acquired by *K. pneumoniae* and thus, co-evolution between plasmid and host, which is known to ameliorate growth disadvantage (Bouma & Lenski, 1988), may have not taken place by the time of isolation on the farm. Other reasons for the differences in behaviour could be caused by the actual sequence of the Dutch plasmid, as it displayed a different pMLST sequence type than that of pIFM3791 and its sequence is otherwise unknown.

We did not investigate alternative selection markers and this precluded the construction of a *Salmonella* 4,5,12:i:- plasmid cured derivative. As such, we introduced pIFM3791, pIFM3804 and pIFM3844 into strain *Salmonella* 4.5.12:i- S348/11 which was a plasmid free strain from the same farm. Given the available data, S348/11 was highly similar to the original strain harbouring a pIFM3791 like plasmid, but it cannot be ruled out that it may have in the past harboured one such plasmid, and already be somewhat adapted to its carriage. Conversely, highly related versions of the plasmid were originally found in *E. coli* and *Salmonella* 4,5,12:i:- strains as well as *K. pneumoniae*. If the plasmid was introduced by a *Salmonella* 4,5,12:i:- strain into a *K. pneumoniae* strain this could also account for a degree of pre-existing plasmid adaption to S348/11 but not B3791. This is

supported by previous description of *in vitro* adaptation of a plasmid genome resulting in the amelioration of the fitness burden imposed upon its host (Modi & Adams, 1991).

Another interesting finding was the ability of CL32 to respire more efficiently than the other two strains on ten substrates: *K. pneumoniae* B3791 and CL32-pIFM3791 behaved similarly giving confidence that plasmid pIFM3791 did impact metabolism although sequence analysis did not suggest any obvious gene candidates. Nevertheless many genes of unknown function may contribute and future transcriptional and targeted mutagenesis studies may resolve the mechanisms behind the phenotypes. Whether the suppression of respiratory activity using L-sorbose as a carbon source and putrescine, γ-amino-N-butyric acid and dipeptide Ala-Gly as nitrogen sources impacts epidemiologically is worthy of consideration. Of importance is the identification of specific metabolically-based burdens mediated by the plasmid on *K. pneumoniae* which could be explained if expression of plasmid genes diverts resources away from the transcription of metabolic pathways involved in the growth under nutrient limiting conditions. Transcriptional interference mediated by integrated plasmids has been noted before (Zagaglia *et al.*, 1991).

Collectively, these data show that the effect that plasmid pIFM3791 has on its host is highly dependent on that host's make up. *Salmonella* 4,5,12:i:- strain 348/11 was better able to carry the outbreak plasmids than the plasmid in *K. pneumoniae* strain B3791. Given its similarity to plasmid bearing isolates it is possible S348/11 is a naturally cured strain and adapted to the burdens imposed by the plasmid. Additionally, the experiments here also show that whether an effect is detectable or not will depend on the exact aspect of fitness that is being measured. The implication of these findings for the reversal of antimicrobial resistance is that even if there is a fitness cost for a certain strain of pathogenic bacteria, as long as there are others in its environment for which

such cost does not exist, and given the mobility of conjugative plasmids, once the selection pressure is imposed by means of the antibiotic, the plasmid can spread again. For this reason, it would have been interesting to have carried out the experiments with *E. coli* strains isolated from the farm. *E. coli* are often found to be harmless commensals, and if it was found that plasmid carriage is either beneficial or neutral for them, this could further lend weight to the idea that plasmids are maintained by certain strains and that are then taken up by other strains in times of selective pressure.

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Transparency declarations

None to declare.

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Legends

Figure 1: Genetic map of IncI1 curing vector pIFM27. The features coloured in black represent genetic structures already present in vector pIFM26 whilst the features coloured in grey are those added in this study.

Figure 2: Plasmid profiling DNA gel electrophoresis of B3791, its pIFM27 transformant derivatives CL28 and, pIFM27 free derivatives CL31 and CL32. 20R764 is a reference plasmid free *E. coli* for identification of the chromosomal DNA band plasmids of B3791 and its derivatives. The band of approximately 8kb in CL28 corresponds to the introduced pIFM27 curing vector. In lanes CL28 to CL32 the native CTX-M IncI1 p3791 has been lost.

Figure 3: a) Plasmid profiling of S348/11 and its outbreak plasmid harbouring transconjugants. Plasmid profiling DNA gel electrophoresis of strain S348/11 and its outbreak plasmid carrying derivatives. 20R764 is a reference plasmid free *E. coli* for identification of the chromosomal DNA band. 39R861 is a reference strain carrying four plasmids of known sizes as labelled. Strain S348/11 carries no plasmids as shown in this gel, its transconjugants have acquired a plasmid of around 100kb, circled blue. **b) Plasmid profiling of CL32 transconjugants complemented with the outbreak plasmids.** Plasmid profiling DNA gel electrophoresis of strain CL32 transconjugants. T91-3 plasmid DNA has been included as a reference of the outbreak plasmid size (circled red). 20R764 is a reference plasmid free *E. coli* for identification of the chromosomal DNA band. 39R861 is a reference strain carrying four plasmids of known sizes as labelled. All transconjugants have acquired a plasmid of around 100kb, circled blue.

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.

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

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

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

Table 3: Effect of plasmid carriage on the competitive fitness of strains B3791 and 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

Competition experiment results (Selection Rate) and their statistical significance as assessed by a two tailed T test (P value). Highlighted in grey are those results 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.

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Figure 1

Click here to download Figure Figure 1 [300ppi 8bit CMYK Image].tif

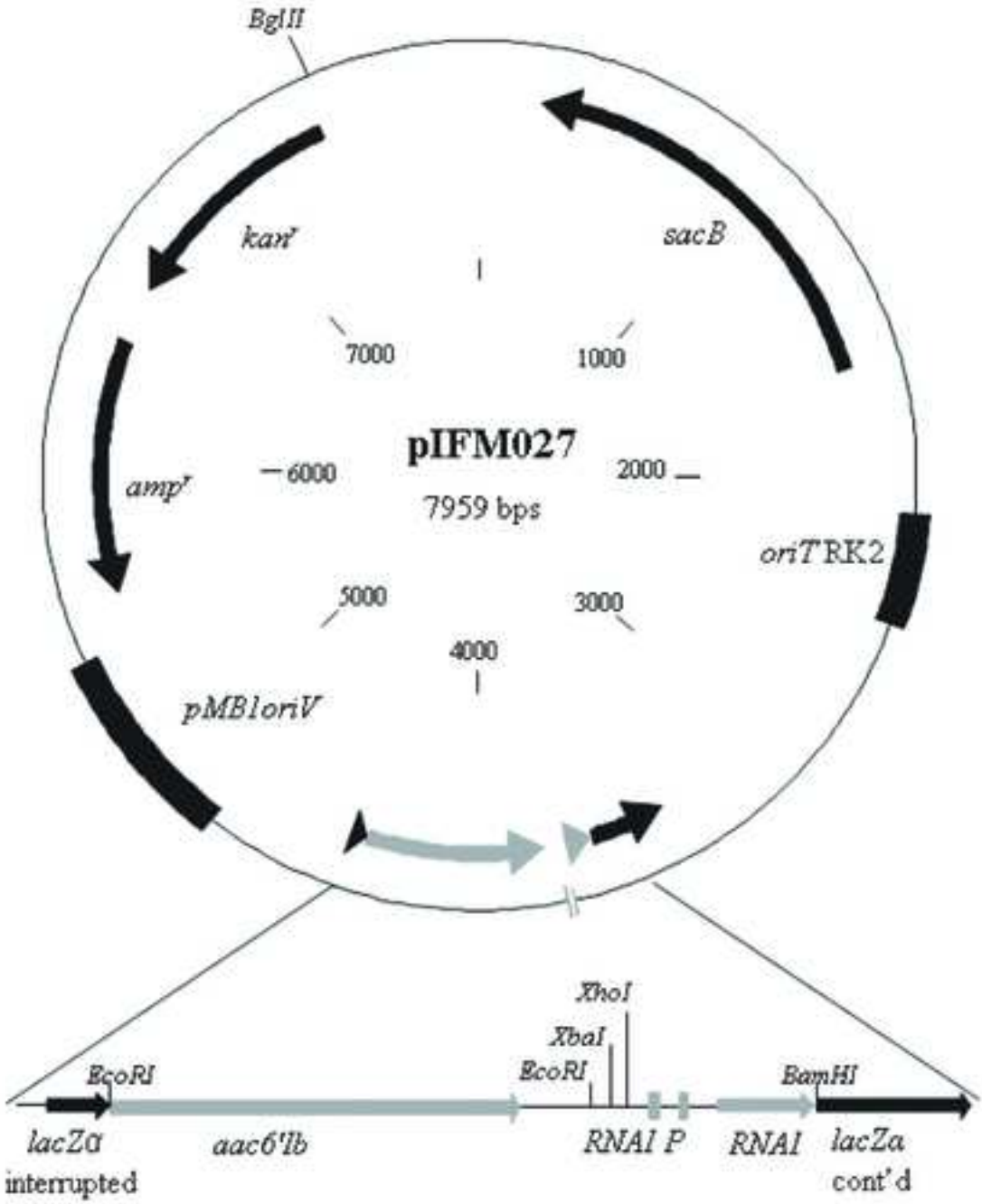

Figure 2

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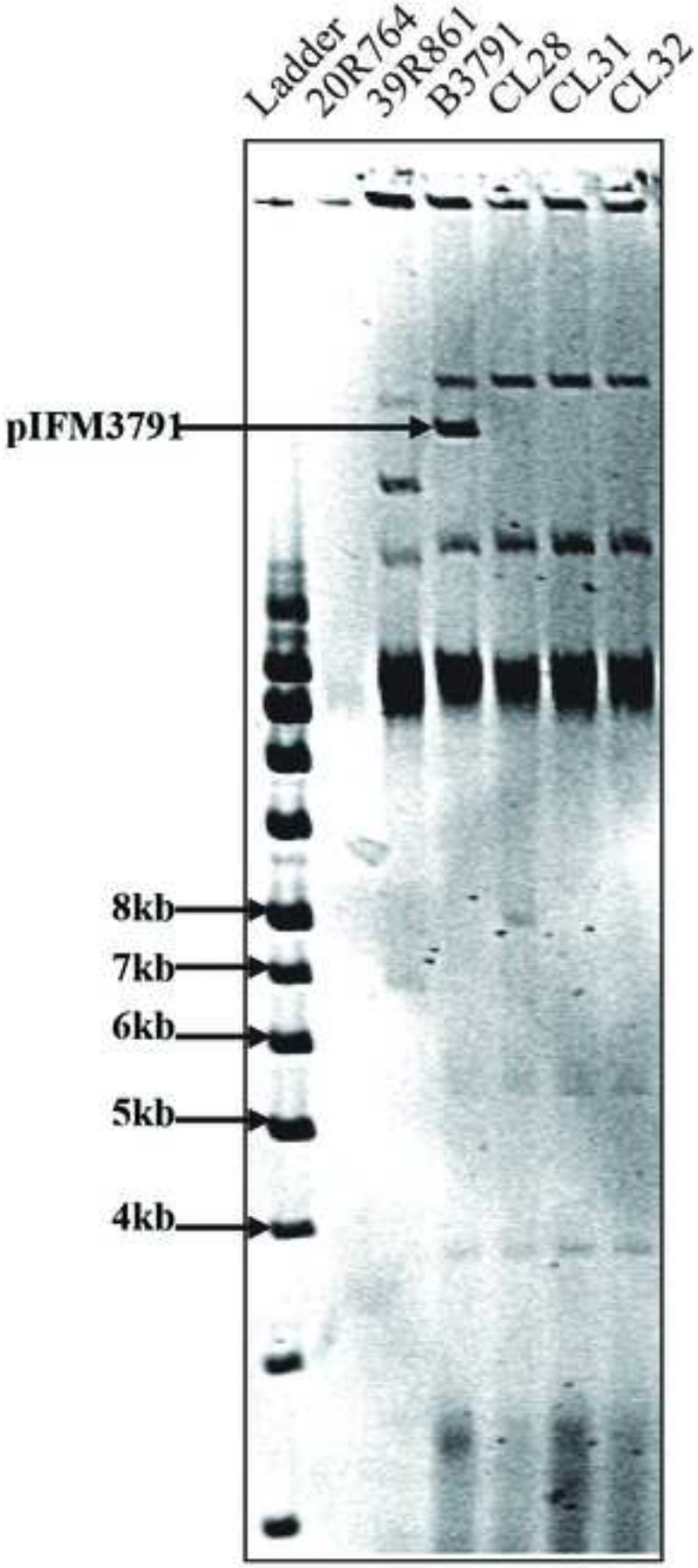


Figure 3

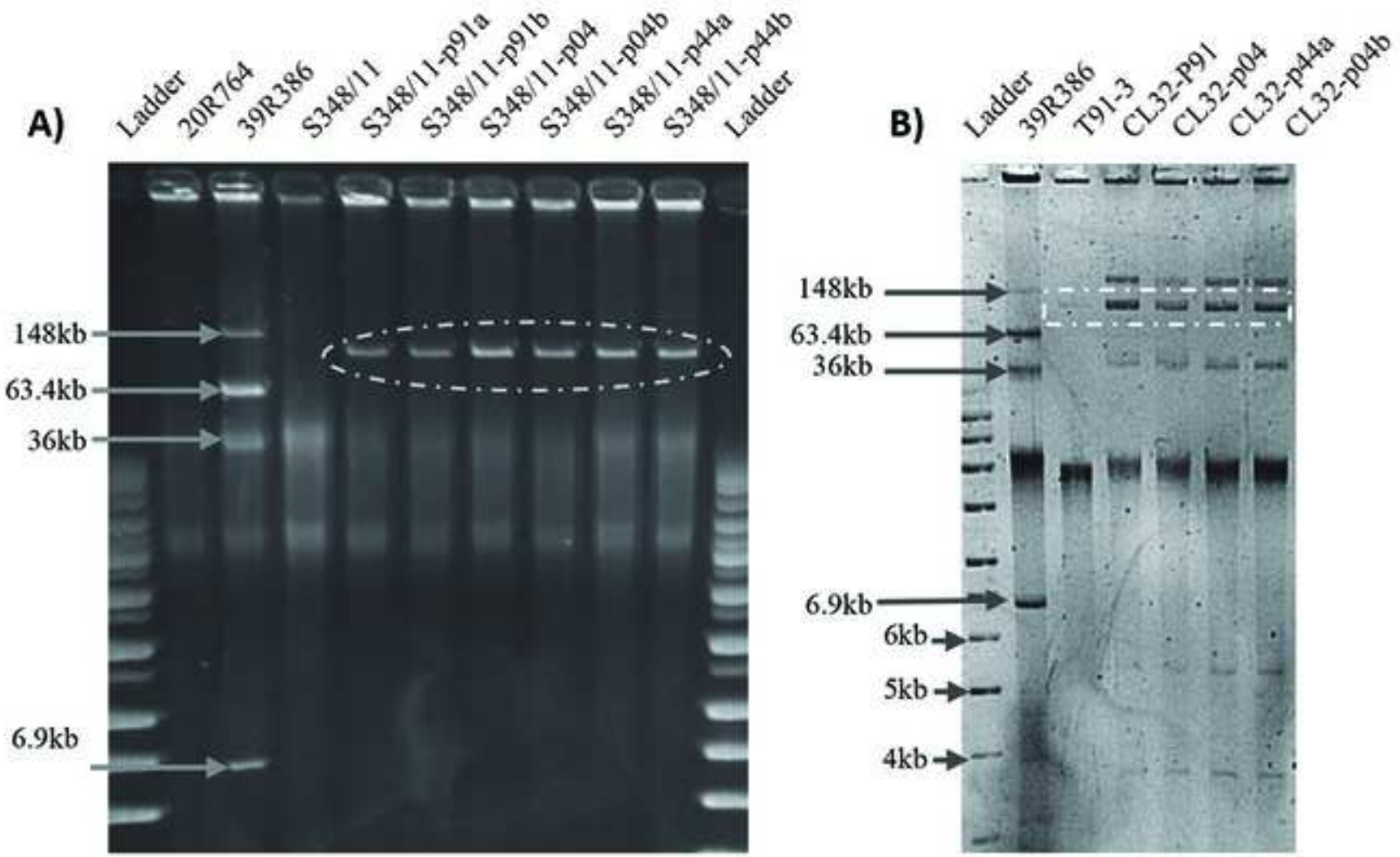
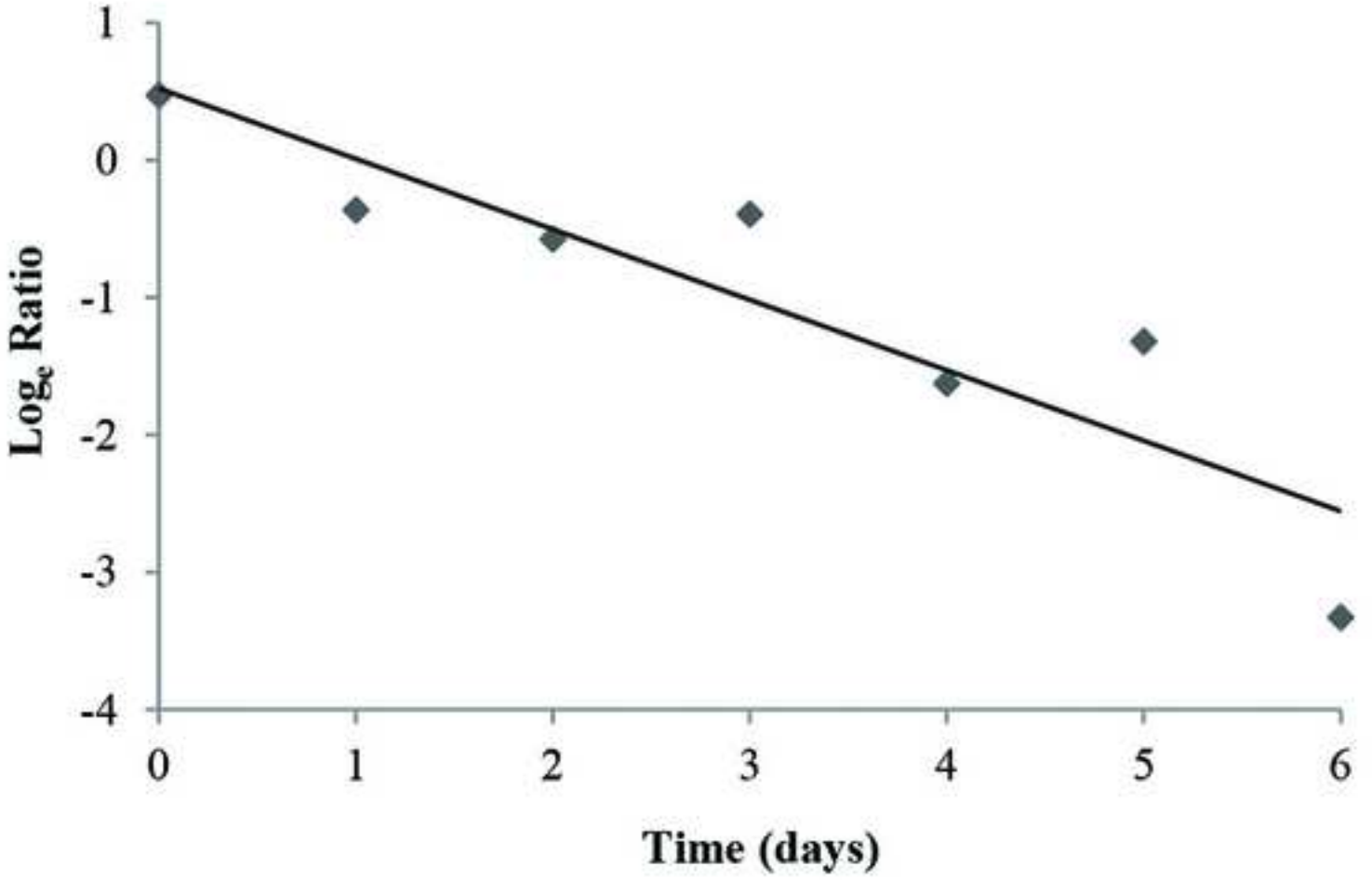


Figure 4



SUPPLEMENTARY MATERIAL

All molecular biological methods including DNA extraction, measurement of DNA purity and concentration, PCR, restriction digestion of DNA, agarose gel electrophoresis, ligation, transformation, electroporation and selection followed standard procedures(Sambrook, J., E. F. Fritsch, n.d.) and manufacturer's conditions for use of reagents. Sequencing reactions were carried out by the AHVLA Central Sequencing Unit.

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

Primers were designed to amplify the *RNAI* gene along with its native promoter with tails on both forward (RNAIFW), and reverse (RNARV) primers (Table 1) that introduced *Xba*I and *Xho*I sites to the 5' and a *BamH*I site to the 3' of the *RNAI* gene. Using plasmid pIFM3804 DNA as template, the PCR generated a product of the correct size that blunt end cloned into pCR-Blunt. Plasmid DNA extracted from four transformants was digested using *Xba*I and *BamH*I which confirmed the presence of an insert of the predicted size and sequencing with insert flanking primers (M13FW and M13RV) showed complete integrity of *RNAI*.

Plasmid DNA from one transformant was digested with *Xba*I and *BamH*I and the *RNAI* insert was gel purified and ligated with vector pIFM26, previously digested with *Xba*I and *BamH*I. pIFM26 was identical to previously described pAKE604,(El-Sayed *et al.*, 2001) with a minor modification to invert its multiple cloning site. The ligated DNA was used to transform *E. coli* DH5α with ampicillin selection (100mg/L). To verify the constructs, plasmid DNA extracted from three well isolated transformants was digested with *Bgl*II and *Xho*I to confirm the predicted total and fragment size of the plasmid and was used as target for PCR and sequencing using RNAI-FW and Pre-MCS primers (Table 1). One transformant designated CL19 was retained for further work.

As the outbreak strains were already resistant to kanamycin and ampicillin, the markers present on pIFM26, an alternative resistance marker, amikacin, was chosen for inclusion in the curing vector. The amikacin resistance gene *aac6'-Ib* was amplified using forward and reverse primers, aac6'-IbFW and aac6'-IbFW that included *EcoR*I tails (Table 1), using as target the wild type plasmid isolated by C. Boinet as part of her PhD studies (Phenotypic and genotypic analysis of blaCTX-M encoding plasmids isolated from bovine *E. coli* samples in the United Kingdom: Royal Holloway, University of London). The *aac6'-Ib* gene product was cloned into pCR-Blunt in *E. coli* NEB5α with selection for kanamycin (50mg/L). Plasmid DNA extracted from five transformants was used in PCR with primers flanking the insertion site (M13FW and M13RV) to confirm presence of the insert (expected ~800bp band) and the product integrity was confirmed by sequence analysis. One transformant designated CL25 was retained for further work.

Plasmid DNA extracted from CL25 was digested with *EcoR*I and the *aac6'-Ib* fragment was cloned into the *EcoR1* site of pIFM19 DNA in *E. coli* NEB5α with selection for transformants resistant to ampicillin (100mg/L). Only one colony was found and PCR analysis using primer pairs aac6'-IbFW and Pre-MCS and aac6'-IbRV and Pre-MCS confirmed the correct orientation of the insert with respect to the LacZ promoter to drive its expression. The resulting strain was named CL27 and its plasmid pIFM27 (Figure 1).

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

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