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Survival and Evolution of a Large Multidrug Resistance Plasmid in New Clinical Bacterial Hosts

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Abstract

Large conjugative plasmids are important drivers of bacterial evolution and contribute significantly to the dissemination of antibiotic resistance. Although plasmid borne multidrug resistance is recognized as one of the main challenges in modern medicine, the adaptive forces shaping the evolution of these plasmids within pathogenic hosts are poorly understood. Here we study plasmid–host adaptations following transfer of a 73 kb conjugative multidrug resistance plasmid to naïve clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli*. We use experimental evolution, mathematical modelling and population sequencing to show that the long-term persistence and molecular integrity of the plasmid is highly influenced by multiple factors within a 25 kb plasmid region constituting a host-dependent burden. In the *E. coli* hosts investigated here, improved plasmid stability readily evolves via IS26 mediated deletions of costly regions from the plasmid backbone, effectively expanding the host-range of the plasmid. Although these adaptations were also beneficial to plasmid persistence in a naïve *K. pneumoniae* host, they were never observed in this species, indicating that differential evolvability can limit opportunities of plasmid adaptation. While insertion sequences are well known to supply plasmids with adaptive traits, our findings suggest that they also play an important role in plasmid evolution by maintaining the plasticity necessary to alleviate plasmid–host constrains. Further, the observed evolutionary strategy consistently followed by all evolved *E. coli* lineages exposes a trade-off between horizontal and vertical transmission that may ultimately limit the dissemination potential of clinical multidrug resistance plasmids in these hosts.

Key words: **clinical isolates, antibiotic resistance, horizontal gene transfer, ESBL plasmid evolution, IS26 restructuring, experimental evolution.**

Article

Introduction

Conjugative plasmids are key contributors to horizontal gene transfer and carry a wide variety of accessory genetic elements important for the ecology and adaptation of bacterial species (Frost et al. 2005; Norman et al. 2009; Soucy et al. 2015). The role of plasmids in the dissemination of antibiotic resistance is increasingly worrisome for human health; allowing pathogenic bacteria to obtain multiple resistance genes in a single transfer event (Carattoli 2013). Indeed, strains of *Klebsiella pneumoniae* and *Escherichia coli* carrying multidrug resistance plasmids are currently recognized as one of the most urgent antibiotic resistance problems (WHO 2014). In these strains, plasmids encoding carbapenemases and extended spectrum β-lactamases (ESBLs) are of particular concern because they greatly limit effective treatment options (Davies and Davies 2010; Dhillon and Clark 2012).

Currently, the abundance and persistence of large plasmids in competitive environments with little or no selection pressure remains an evolutionary puzzle (Simonsen 1991; Bergstrom et al. 2000). While the range of hosts in which plasmids can successfully replicate is fairly well understood (Mazodier and Davies 1991; Carattoli 2009; Jain and Srivastava 2013), our knowledge of the influence of different host backgrounds on the long-term stability and evolution of natural plasmids remains limited. A number of studies have demonstrated that plasmids confer a cost upon entering a naïve host and that this can be compensated through adaptive evolution (Bouma and Lenski 1988; Dahlberg and Chao 2003; Dionisio et al. 2005; Harrison and Brockhurst 2012). Furthermore, recent studies have used next-generation sequencing to investigate the genetic basis underlying such plasmid–host adaptations (Sota et al. 2010; Harrison and Brockhurst 2012; San Millan et al. 2014; Harrison et al. 2015; Loftie-Eaton et al. 2015; San Millan et al. 2015). In the case of small nonconjugative plasmids, plasmid–host adaptations have been shown to occur via mutations in the plasmid replication machinery (Sota et al. 2010), in chromosomal genes interacting with replication proteins (San Millan et al. 2015) or by acquisition of stabilizing traits via interplasmid transposition (Loftie-Eaton et al. 2015). For plasmid–host evolution of a conjugative mercury-resistance plasmid to *Pseudomonas fluorescens*, adaptation occurred via translational down regulation caused by inactivation of a host-encoded two-component system (Harrison et al. 2015). However, less effort has been made to study the dynamics

and adaptation of large multidrug resistance plasmids in the bacterial hosts they are likely to encounter in a clinical environment.

Here, we investigate the molecular basis for adaptations of a large plasmid, isolated from a clinical *K. pneumoniae* strain, to naïve clinical isolates of *E. coli* and *K. pneumoniae*; two species highly implicated in plasmid mediated dissemination of multidrug resistance. To determine the factors implicated in the long-term plasmid survival, we characterize the influence of the plasmid on maximum growth rate, overall stability, and evolutionary potential as well as genetic adaptations in three novel plasmid–host combinations (fig. 1). We observe a consistent and rapid adaptation pattern in *E. coli* that is dependent on plasmid-borne insertion sequences (ISs) and driven by the cost a plasmid region encompassing the main conjugational machinery. We are the first to describe the role of IS mediated intramolecular restructuring in plasmid host-expansion and how these reactions, although broadly beneficial, were only observed in certain host backgrounds.

Results

The pKP33 plasmid (fig. 2) was obtained from a *K. pneumoniae* clinical host (Kp33) and sequenced using *Pacific Biosciences RS II* single molecule real time sequencing as well as *Illumina MiSeq* technology. While the short reads (~150 bp) obtained from the paired-end *Illumina MiSeq* run were not sufficient to capture the complex repetitive nature of the pKP33 plasmid, such as the localization and orientation of identical mobile genetic elements, the longer reads offered by single molecule real time sequencing enabled complete assembly of the plasmid.

The sequence of pKP33 revealed that it belongs to the IncN incompatibility group. Although IncN plasmids are able to replicate in a variety of enterobacterial pathogens, they are most frequently observed in *E. coli* and *K. pneumoniae* isolates where backbones similar to pKP33 contribute to the global dissemination of cephalosporin and carbapenem resistance (Miriagou et al. 2010; Eikmeyer et al. 2012; Carattoli 2013; Conlan et al. 2014). pKP33 carries eleven antibiotic resistance genes including the endemic CTX-M-15 ESBL variant (Bush and Fisher 2011). These genes confer resistance to multiple major drug classes including β-lactams (CTX-M-15, TEM-1, OXA-1), aminoglycosides (*aac-(6′)-III*, *aac(6′)-Ib and strA*, *strB*), quinolones (*qnrB*), sulfonamides (*sul2*) and dihydrofolate reductase inhibitors (*folA*). These were functionally confirmed by antibiotic susceptibility testing (supplementary table S1, Supplementary Material online). To support stable maintenance, pKP33 contains the *stb* operon known to encode factors involved in active segregation and regulation of conjugative transfer (Guynet et al. 2011). In addition, an *ecoRII-dcm* restriction–antirestriction pair may function as a toxin–antitoxin stability system by inhibiting growth of plasmid-free segregants (Mruk and Kobayashi 2014). The plasmid backbone shows a typical mosaic structure where functionally similar gene clusters are flanked by mobile genetic elements. In pKP33, the IS*6* and Tn*3* family of ISs were highly abundant; exemplified by several occurrences of identical IS*26* copies throughout the backbone.

Initial stability of pKP33 in the original *K. pneumoniae* host (Kp33) was investigated by serial passaging in the laboratory for 35 days in the absence of antibiotic selection (supplementary fig. S1, Supplementary Material online). We estimate, based on the transfer volumes and maximum culture density that this period corresponds to approximately 280 bacterial generations. Remarkably, plasmid loss was never detected for pKP33 in its native host, emphasizing that large plasmids carrying multiple antibiotic resistance genes can persist for extended time periods without selection given the right host environment.

Three clinical strains that did not carry multidrug resistance plasmids (naïve) were obtained to investigate their response to acquisition of pKP33. The *E. coli* isolates Ec37 (ST127) and Ec38 (ST1170) originate from clinical UTIs, belong to the B2 *E. coli* phylogroup, and share 80% of their protein families. The naïve Kp08 (ST36) *K. pneumonia* strain shares 54% of its protein families with both *E. coli* strains and 83% with the original pKP33 plasmid ancestor Kp33 (ST301).

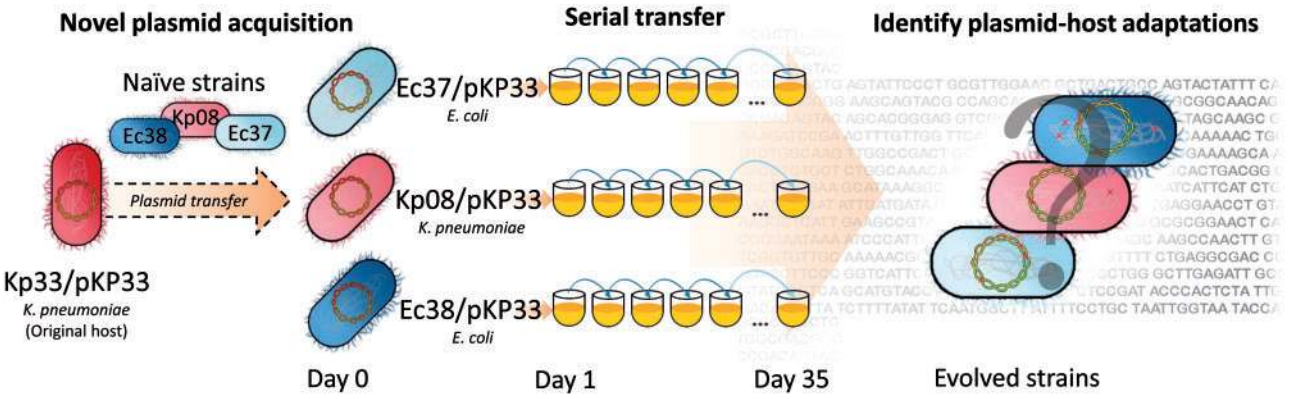

Fig. 1. Experimental overview. A large conjugative plasmid (pKP33) originating from a clinical *Klebsiella pneumoniae* isolate (Kp33) was transferred by conjugation into two different *Escherichia coli* (Ec37 and Ec38) strains and one *K. pneumoniae* (Kp08) strain isolated from urinary tract infections (UTIs) and blood infections, respectively. Quantification of the growth rate and plasmid stability was done immediately after plasmid acquisition (Day 0) as well as following 35 days of serial transfer in plasmid-selective conditions (Day 35—evolved strains). The genome sequences of the evolved strains were analyzed to determine the genetic adaptations involved in plasmid–host adaptations.

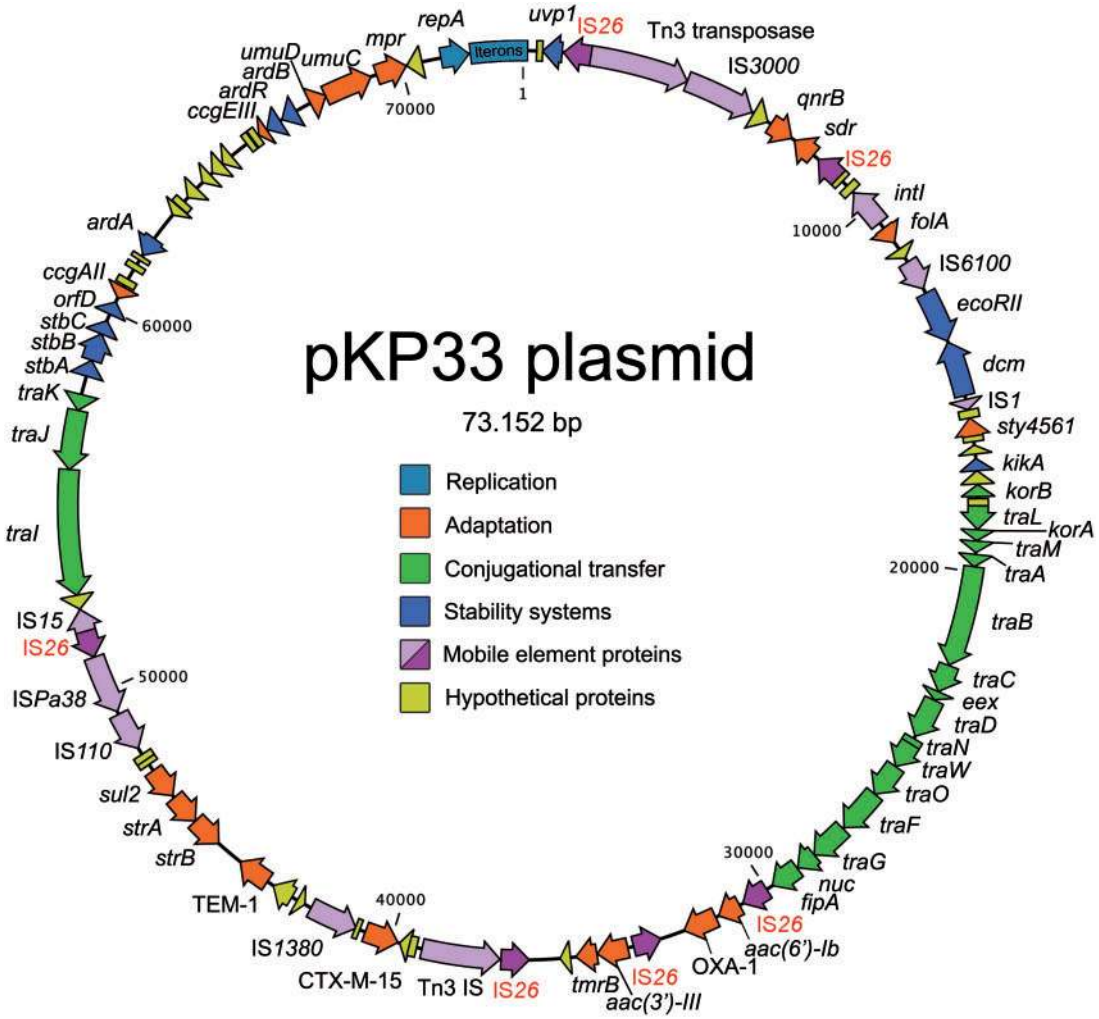

FIG. 2. Genetic map of the pKP33 ESBL plasmid originating from the clinical Kp33 strain. The plasmid belongs to the IncN incompatibility group and contains accessory genes (orange) involved in metabolism and antibiotic resistance along with several stability mechanisms (blue) and conjugational transfer machinery (green). Three β-lactamases, including the endemic CTX-M-15 ESBL, are encoded by the plasmid along with several other antibiotic resistance genes. Furthermore, the plasmid is sectioned by a number of mobile genetic elements (purple) dominated by the IS*26* insertion sequence (dark purple) as well as predicted genes that encode proteins of unknown function (yellow).

Additional information on these strains can be found in table 1 and the full proteome comparison is summarized in supplementary table S2, Supplementary Material online.

Plasmid Cost and Stability in pKP33-Naïve Clinical Isolates

While pKP33 was stably maintained in its native host strain in the absence of selection, this is likely a result of long-term co-evolution. To study the processes that contribute to plasmid success in new hosts, we set out to investigate the stability of pKP33 in naïve clinical *E. coli* and *K. pneumoniae* strains. We transferred pKP33 by conjugation to one *K. pneumoniae* (Kp08) and two *E. coli* (Ec37 and Ec38) clinical isolates (see table 1). To characterize the influence of the host strain on the cost of pKP33 carriage we measured the maximum growth rate as a fitness proxy for UTI pathogens (Gordon and Riley 1992; Nilsson et al. 2003). The plasmid imposed a significant burden on all three strains quantified as a reduction in growth rate of 6%, 11%, and 12.5% for Kp08, Ec37, and Ec38, respectively, compared to the plasmid-free ancestor (one-sample *t*-test. Ec38: $P < 0.001$; Ec37: $P < 0.001$; Kp08: $P < 0.001$) (fig. 3). While there was no significant difference in relative growth rate between Ec37/pKP33 and Ec38/pKP33 (two-sample *t*-test $P = 0.3$), the burden imposed by pKP33 on the Kp08 *K. pneumoniae* strain was significantly lower than for the *E. coli* isolates (two-sample *t*-test Ec38: $P = 0.0034$; Ec37: $P = 0.01$), implying that host specific factors influence the fitness cost of the newly acquired plasmid.

Due to the presence of various plasmid borne stability systems, pKP33 might persist in spite of the burden imposed on naïve hosts. To investigate the stability of pKP33 in the naïve strains, serial passaging of five lineages of each strain in nonselective medium was carried out for 35 days, corresponding to 280 generations of growth (fig. 4A, triangles). The stability of pKP33 differed between the isolates with the naïve Kp08 *K. pneumoniae* strain being the most stable (50% plasmid-free cells after 12.5 days) and *E. coli* the least stable (50% plasmid-free cells after 2.5 and 6 days for Ec37 and Ec38, respectively) (fig. 4B). Notably, while still detectable in all Ec37 and Kp08 populations, pKP33 was present in less than

Table 1. Strains and Plasmids. Clinical isolates were obtained from the Department of Clinical Microbiology at Hvidovre Hospital (Hvidovre, Denmark).

Strain or Plasmid	Designation	Characteristics and Comments	Reference or Source
Escherichiacoli TOP10	TOP10	Conjugation donor similar to DH10B (leucine auxotroph)	Invitrogen
Escherichia coli DY329	DY329	Used for recombineering. W3110 derivative with the genotype: Δ*lacU169 nadA*:*Tn10 gal490 λcI857* Δ(*cro-bioA*)	Yu et al. (2000)
Klebsiella pneumoniae CI ESBL 33	Kp33	ST*: *301*; Chromosomal bla: SHV27; Plasmids: pKP33	Clinical isolate (urine)
ESBL plasmid from Kp33	pKP33	Size: 67kb; IncN; Resistance genes: TEM-1, OXA-1, CTX-M 15, *strA*,*strB*, *aac(6′)Ib-cr*, *aac(3)-Iia*, *qnrB*, *sul2*, *folA*	Clinical plasmid
Escherichia coli CI 37	Ec37	ST*: *127*, Phylogroup: *B2**; *Resistance profile*: *None*; Plasmids replicons*: *FII* (61 kb)	Clinical isolate (urine)
Escherichia coli CI 38	Ec38	ST*: *1170*, Phylogroup: *B2**; Resistance profile: *chloramphenicol*; Plasmids replicons*: *FIB* (9.5 kb) & *FIC* (136 kb)	Clinical isolate (urine)
Klebsiella pneumoniae CI 08	Kp08	ST*: *36*; Resistance genes: *fosA*, SHV11, *oqxB*, *oqxA*;Plasmids replicons*: *FIB(K)* (22kb) & *IncR* (90 Kb)	Clinical isolate (blood)

NOTE.—Asterisks indicate computationally derived information obtained through the CGE Servers (MLST, PlasmidFinder and ResFinder available at: https://cge.cbs.dtu.dk/services, last accessed August 8, 2016).

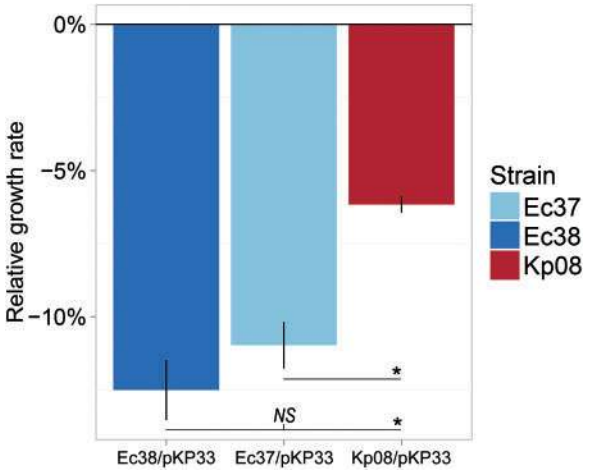

FIG. 3. Growth rate effects of novel plasmid acquisition. Plasmid acquisition leads to a reduction in maximum growth rate relative to the plasmid-free ancestor. Error bars show 95% confidence intervals of 16 pKP33 carrying biological replicates error propagated with the variation of 16 biological replicates of the plasmid-free ancestor. Asterisks indicate significant differences ($P < 0.05$) and *NS* a nondetectable difference ($P > 0.05$).

1% of the population for all three hosts after 35 days of serial passaging, demonstrating that these new plasmid–host combinations are markedly less stable compared to the original pKP33 host (supplementary fig. S1, Supplementary Material online).

In order to gain insight into the origin of the observed instability, we fitted an established mathematical model of plasmid stability to the measured plasmid stability data (Proctor 1994). This model describes the ratio of plasmid bearing to plasmid-free cells as a function of time and assumes the rate of plasmid loss upon cell division and the difference in growth rate between the plasmid bearing and plasmid-free cells as the main drivers of plasmid dynamics (equation 1—Materials and Methods). We did not include a parameter for conjugative transfer as pKP33 displayed negligible rates of conjugation in our liquid setup (see supplementary table S3, Supplementary Material online), which is in agreement with previous studies of IncN plasmid transfer systems (Bradley et al. 1980). The parameter estimates (see supplementary table S4, Supplementary Material online) for segregational loss ranged from 0.0008 to 0.0147 and due to uncertainty of the estimates, these were not significantly different from 0 for Ec37 and Ec38 (one-sample *t*-test, $P = 0.13$ and $P = 0.45$ for Ec37 and Ec38, respectively) but only for Kp08 (one-sample *t*-test, $P = 0.0013$). This indicates that the influence of the segregational loss rate on overall plasmid stability is minor compared to the effect of growth competition from plasmid-free segregants. Consistent with the growth rate reduction imposed by the plasmid (fig. 3), the fitness cost of pKP33 predicted by the model was statistically significant for all three strains (one-sample *t*-test; $P = 0.0054$, $P = 0.00019$, and $P = 0.012$ for Ec37, Ec38, and Kp08, respectively) and determined to be 8.3% (±4.14%), 14% (±2.85%), and 4.6% (±2.94%) for Ec37, Ec38, and Kp08, respectively. These estimates of plasmid fitness costs correspond well with the relative growth rates measured and confirm that the main driver of plasmid loss was competition between plasmid-bearing and plasmid-free segregants.

Adaptive Evolution during Antibiotic Selection Compensates Plasmid Cost in *E. coli* but not *K. pneumoniae*

Based on the plasmid stability experiments without selection, it seems unlikely that pKP33 can be stably maintained even in strains closely related to the original host (fig. 4 and supplementary table S2, Supplementary Material online). However, in clinical settings, plasmid transfer and subsequent host adaptation is likely promoted by selection for plasmid-encoded functions such as antibiotic resistance. In the presence of antibiotic selection pKP33 would confer a strong fitness advantage to an antibiotic sensitive host cell (supplementary table S1, Supplementary Material online). Such periods of selection can provide sufficient time for plasmid–host adaptations to occur, improving plasmid persistence once selection is removed (San Millan et al. 2014).

To investigate plasmid–host adaptation during positive selection, we repeated the serial transfer experiment in liquid medium containing cefuroxime (16 μg/ml) selecting for pKP33 carriage. Five lineages of each host carrying the

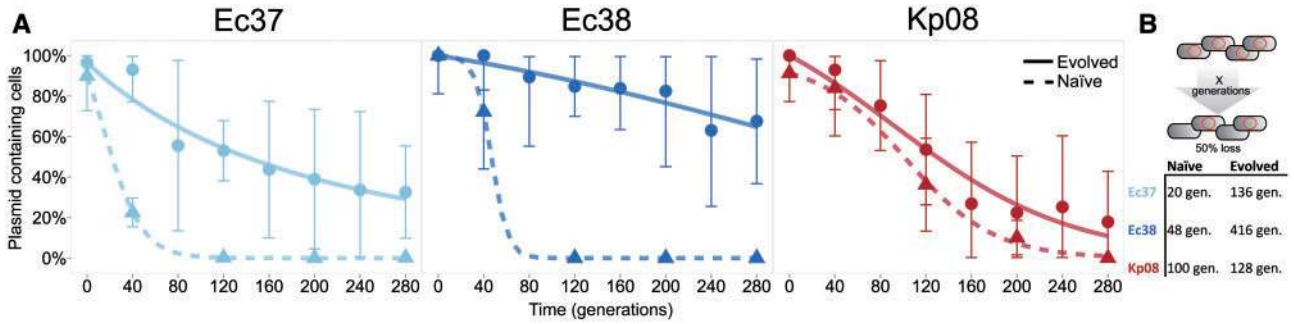

FIG. 4. Adaptive evolution increases stability of pKP33 in *Escherichia coli* hosts. (*A*) The proportion of plasmid-containing cells in initially plasmid bearing populations grown without plasmid selection as a function of time. Immediately after receiving pKP33, the naïve Ec37, Ec38, and Kp08 were propagated in nonselective medium for 35 days to quantify the stability of the newly received plasmid before any adaptations had taken place (triangles). To measure the stabilizing effect of plasmid–host adaptations, the serial passaging procedure was applied to the same plasmid–host combinations evolved for 35 days under plasmid selective conditions (circles). A mathematical model was fitted (lines) to the data points. Error bars illustrate the standard deviation of five independent lineages. (*B*) Plasmid half-life comparison. The average half-life of pKP33 in naïve and evolved lineages was determined from the fitted model and summarized as the number of generations needed for half of the population to be plasmid-free.

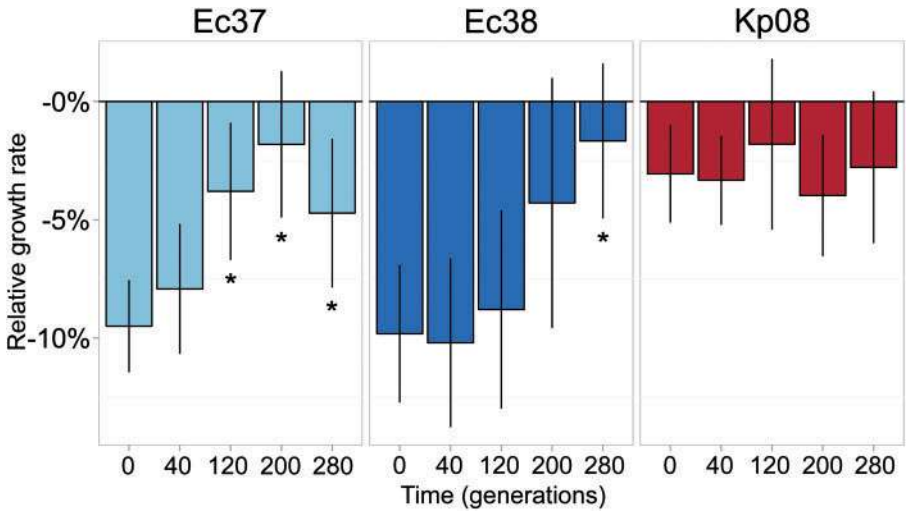

FIG. 5. Adaptive evolution reduces plasmid cost in *Escherichia coli*. The growth rate of each plasmid–host combination was measured throughout the evolution experiment and is shown relative to the plasmid-free host that was evolved in parallel. Means represent 40 randomly picked clones (8 from each evolving lineage) and error bars show the standard deviation of lineage means. Asterisks indicate a significant improvement from Day 0 (*$P < 0.05$, $n = 5$, Dunnett's test).

pKP33 plasmid were evolved in parallel and the growth rates of individual clones from the evolving populations were measured throughout the ~280 generations of the experiment and growth rates were normalized to the plasmid-free host grown in parallel to account for the effect of general medium adaptations (fig. 5. See supplementary fig. S2, Supplementary Material online for absolute values).

A significant increase in relative growth rate was observed for both *E. coli* carrying pKP33 when comparing the starting point (Day 0) to the evolved endpoint (Day 35) (two-sample *t*-test: Ec38: $P = 0.00215$, $n = 10$; Ec37: $P = 0.0154$, $n = 10$). At the end of the adaptation experiment the average evolved Ec38/pKP33 lineage, now referred to as Ec38/pKP33 D. 35, was indistinguishable from the plasmid-free host (one-sample *t*-test: $P = 0.186$, $n = 5$) . In contrast, the *K. pneumoniae* strain did not experience a significant improvement during the experiment (two-sample *t*-test: $P = 0.380$, $n = 10$).

Our growth rate measurements and stability assays (figs. 4 and 5) revealed that a less costly plasmid–host combination is more stable on the population level. To test whether the stability of the evolved plasmid–host combinations were higher than for the nonevolved ancestors, plasmid stability was measured again by serial passaging in nonselective medium for 35 days or roughly 280 bacterial generations (fig. 4A, circles). A pronounced improvement in the stability of the evolved *E. coli* lineages was evident from the much slower decline in the proportion of plasmid-bearing cells over time compared to the stability of the nonevolved lineages (fig. 4B). The strains evolved under antibiotic selection were able to maintain the plasmid for much longer, with a significant proportion of the *E. coli* populations still carrying

pKP33 after 35 days of serial passaging without antibiotic selection (one-sample *t*-test: Ec37: $P = 0.032$, $n = 5$; Ec38: $P = 0.0075$, $n = 5$). The two *E. coli* strains shows similar improvements in stability with a 6.8- and 8.6-fold increases in pKP33 half-life, respectively compared to the naïve hosts (fig. 4B). In contrast, we could not detect a significant improvement in stability of Kp08/pKP33 D.35 compared to the ancestral Kp08/pKP33 lineage. Comparing the values for ancestral and evolved Kp08/pKP33 lineages at different time points reveal that the plasmid containing fraction of the evolved lineages were not significantly higher than the ancestral plasmid–host combination (two-sample *t*-test: Day 15: $P = 0.31$, $n = 5$; Day 35: $P = 0.18$, $n = 5$) which is in accordance with the overlap of parameter estimates from the mathematical model fitted to data points of the ancestral and evolved Kp08/pKP33 lineages (supplementary table S4, Supplementary Material online).

Population Sequencing Reveals Large Deletions in *E. coli* Evolved Plasmids

To further examine the underlying factors responsible for the dramatic increase in plasmid stability we sequenced all evolved lineages at the population level. Sequencing reads from the evolved strains was mapped to the genome of the nonevolved ancestor representing the starting point before plasmid acquisition and a control population of each strain, evolved without the plasmid, enabling omission of the most frequent general medium adaptations (see supplementary table S5, Supplementary Material online). However, the majority of the chromosomal mutations occurred in virulence genes and none of them could be directly associated with plasmid stability. See supplementary material S1, Supplementary Material online for a discussion of genomic variants.

While no single nucleotide polymorphisms (SNPs) were identified in the *E. coli* evolved plasmids, a consistent deletion of approximately 25 kb (position 8–33 kb, fig. 6A) in pKP33 was observed for all *E. coli* lineages. Similar deletions were never observed in any of the evolved *K. pneumoniae* lineages (fig. 6B). The consistently deleted region encodes three resistance determinants, a restriction-antirestriction system and the main conjugation machinery. See supplementary fig. S3, Supplementary Material online for a detailed view of the region. Interestingly, deletions were always flanked by IS*26* ISs, suggesting that intramolecular transposition or recombination between these elements allowed for the observed loop-out dynamics. IS*26* is over-represented on bacterial plasmids, often associated with antibiotic resistance genes, and believed to catalyse plasmid reorganization through intramolecular replicative transposition events (Cullik et al. 2010; Curiao et al. 2011; Partridge et al. 2011; He et al. 2015).

Deletion Dynamics during Adaptation

Due to the positioning of IS*26* sequences in the pKP33 backbone, partial deletion of the 25 kb region was never observed. Therefore the deletion dynamics of the entire region were assumed to correlate with the presence of individual genes within the region. The trimethoprim (TMP) resistance gene *folA* located inside the region (highlighted in fig. 6A) allowed us to investigate the dynamics of the major deletion by selective plating of the antibiotic evolved populations on TMP containing agar plates (fig. 6B). Here, trimethoprim (TMP) resistance was never lost in neither the original Kp33 *K. pneumoniae* host nor the evolving Kp08/pKP33 lineages, consistent with a high intramolecular stability of pKP33 in these *K. pneumoniae* strains. Conversely, the *E. coli* populations showed a rapid decline in TMP resistance with TMP sensitive mutants dominating the population within 60 generations of evolution.

Plasmid Deletions Are Responsible for the Observed Growth Rate Improvements

The rapid fixation of the 25 kb deletion in all evolved *E. coli* lineages indicated that the main genetic structure involved in adaptation was the plasmid backbone. To verify that the observed improvements were due to the plasmid deletions and not chromosomal mutations, a representative evolved pKP33 (now referred to as pKP33evo) was purified from lineage four of the antibiotic evolved Ec38/pKP33 D.35 strain and transferred back into the ancestral strains. Growth rate measurements showed that the burden of plasmid carriage was indistinguishable between the ancestral Ec37 and Ec38 strains harboring pKP33evo and the evolved plasmid–host combinations (fig. 7). This confirms that the plasmid was the main locus of adaptation and no chromosomal mutations were needed for the observed improvements.

We transferred pKP33evo to the naïve Kp08 to investigate whether the major deletion would confer an advantage beyond the host in which it evolved (fig. 8).

Interestingly, Kp08 cells harboring the *E. coli* evolved plasmid displayed a marginal advantage in terms of a higher growth rate, which was borderline significant ($P = 0.063$, $n = 48$). To gain resolution and test the impact of the presumed fitness benefit of pKP33evo in Kp08, we conducted a 1:1 pairwise competition experiment by mixing Kp08/pKP33 and Kp08/pKP33 and subjecting these cocultures to 110 generations of serial transfer. Here, Kp08 carrying the ancestral pKP33 plasmid was consistently outcompeted in four parallel competitions against the *E. coli* evolved pKP33evo (fig. 8B). These results indicate that the deletion event rather than the adaptive advantage was the bottleneck preventing plasmid adaptation in *K. pneumoniae*.

No Single Locus Is Responsible for the Adaptive Benefit of the Major Plasmid Deletion

The majority of the deleted region encodes the type 4 secretion system (T4SS) comprising the conjugational transfer machinery that allows for autonomous horizontal transfer of the plasmid.

It is generally believed that horizontal transfer comes at the expense of reduced vertical transfer (Turner et al. 1998) and that the conjugational transfer machinery imposes a burden on the bacterial host cell (Zahrl et al. 2006; Fernandez-Lopez et al. 2014). We hypothesized that the T4SS was the main component responsible for the fitness cost of the deleted plasmid region and that the remaining part of the 25 kb

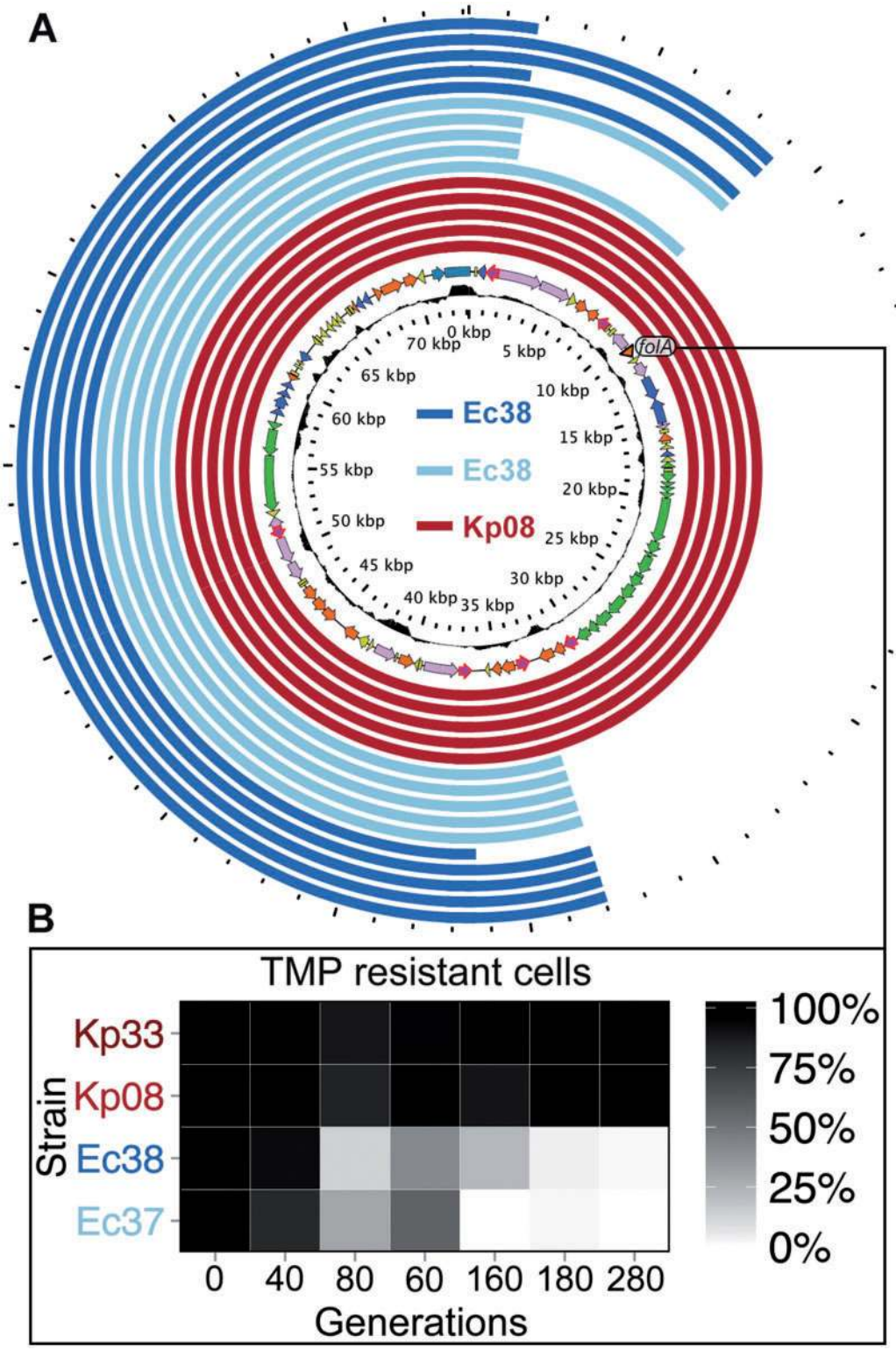

Fig. 6. Large deletions in the pKP33 plasmid backbone occurred in *Escherichia coli* but not *Klebsiella pneumoniae*. (*A*) The ancestral pKP33 plasmid compared to the evolved plasmid genomes displayed as a BLAST atlas. All evolved populations of each strain were sequenced at the 280-generation end point. IS*26* elements are indicated in purple with a red outline and the position of the trimethoprim (TMP) resistance gene *folA* is highlighted. Lineages are depicted in ascending order from the center. The inner-most circle shows the relative GC content. (*B*) Heat-map showing the abundance of TMP resistant cells in the populations grown with plasmid selection as a measure of the major deletion event. Each tile represents the average of five evolving lineages. None of the pKP33 free strains where resistant to TMP.

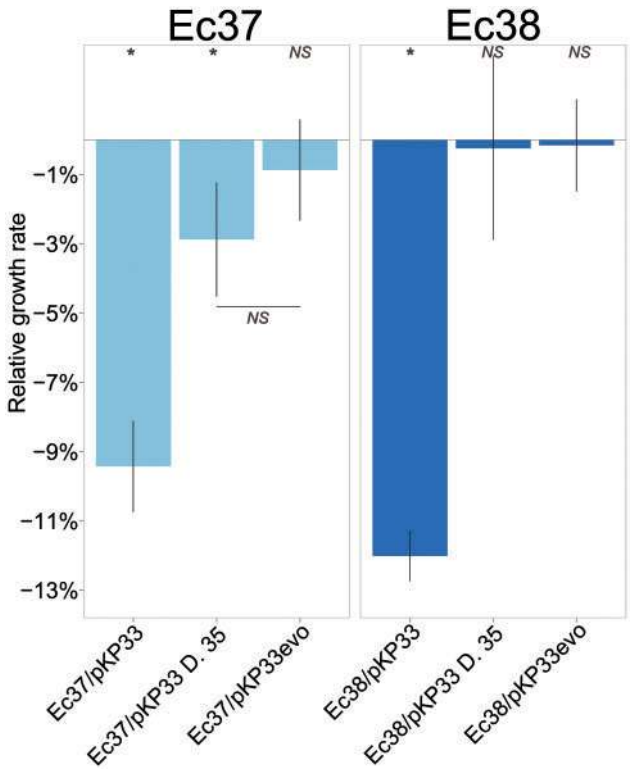

FIG. 7. Plasmid adaptations are responsible for the increased growth rate in the evolved *Escherichia coli* hosts. Growth rates of plasmid–host combinations before evolution (Day 0), after evolution (Day 35), and the evolved plasmid (pKP33evo) transferred back into the ancestral hosts, shown relative to the growth rate of the ancestral plasmid-free ancestors. A significant difference determined by two-sample *t*-test statistics is indicated with asterisks ($P < 0.05$) or NS (nonsignificant, $P > 0.05$). Top indicators are obtained through comparison to the plasmid-free ancestor (no difference). All measurements were done for 24 individually picked colonies and error bars display 95% confidence intervals of the mean.

region was codeleted merely as a result of constraints in IS*26* localization. To investigate this hypothesis, two pKP33 knockout mutants were constructed. In one pKP33 mutant (pKP33ΔT4SS) we deleted the T4SS only; while in the other mutant (pKP33Δ9-17 kb) the majority of the remaining 25 kb region containing the k*ikA*, the *ecoRII* endonucleases as well as the *folA* gene was deleted, leaving the T4SS intact. We tested the ability of the two mutants to transfer autonomously and while the pKP33ΔT4SS did not yield any transconjugants, the pKP33Δ9-17 kb mutant retained the ability to transfer by conjugation on par with the intact pKP33 plasmid (see supplementary table S3, Supplementary Material online). In both mutants, the intermediate part of the 25 kb region, containing the *kor* regulatory genes, were preserved to avoid a growth bias from excessive expression of the *kik*/*kil* or T4SS genes due to the absence of negative regulation by the KorA and KorB regulators (Moré et al. 1996).

The influence of the targeted deletions on the growth rate of the *E. coli* strains was measured along with the ancestral pKP33 and a 9–30 kb deletion mutant encompassing both partial knockouts (fig. 9).

There was no significant difference in terms of growth rate between carrying pKP33 with a targeted knockout of the 9–30 kb region when compared to the evolved plasmid (two-sample *t*-test: Ec38: $P = 0.374$, $n = 32$; Ec37: $P = 0.158$, $n = 32$) emphasizing that this deleted region was responsible for the observed plasmidcosts.

Interestingly, the effect of partial knockouts within the major deleted region differed between the two hosts. Knockout of the transfer machinery alone (pKP33ΔT4SS) resulted in significantly higher growth rates in both strains compared to the ancestral pKP33 plasmid (two-sample *t*-test: Ec37: $P < 0.001$, $n = 32$; Ec38: $P < 0.001$, $n = 32$) that were not distinguishable from the 9–30 kb knockout mutants (two-sample *t*-test: Ec37: $P = 0.115$, $n = 32$; Ec38: $P = 0.20$, $n = 32$). This indicates that the T4SS did indeed impose a cost on the naïve *E. coli* strains (fig. 9). Surprisingly, targeted knockout of the remaining region (pKP33Δ9-17 kb), leaving the T4SS region intact, also increased the growth rate of both *E. coli* strains and completely ameliorated the cost in Ec37 (one-sample *t*-test: $P = 0.388$, $n = 16$) but not in Ec38 (one-sample *t*-test: $P = < 0.001$, $n = 24$) where the T4SS knockout was more beneficial (two-sample *t*-test: $P = 0.0087$, $n = 40$) compared to the 9–17 kb deletion in this strain. See supplementary table S5, Supplementary Material online for an overview of the statistical comparisons made in this section.

Taken together, this implies that the reduction in growth rate imposed by the wild type pKP33 plasmid cannot be attributed to a single factor of the T4SS nor the remaining part of the 25 kb region alone, and that the exact nature of the cost varies between closely related strains.

Discussion

Conjugative plasmids are important mediators of horizontal gene transfer, enabling the direct exchange of multiple genes to allow rapid adaptation of bacteria to dynamic environments (Norman et al. 2009; Tamminen et al. 2012; Soucy et al. 2015). While a plasmid might replicate in, and transfer between, a diverse set of hosts, little is known about the factors determining the long-term persistence of endemic antibiotic resistance plasmids within their compatible range of host species (De Gelder et al. 2007). Here we simulate the scenario of novel host invasion of three pathogenic *E. coli* and *K. pneumoniae* isolates by a large clinically relevant multidrug resistance plasmid. Such transfer events are likely to take place in dense multispecies communities such as the human intestinal microbiota, supplying potential pathogens with a wide array of antibiotic resistance factors (Shoemaker et al. 2001; Sommer et al. 2009; Sommer and Dantas 2011).

We demonstrate that the host genetic background substantially influences initial plasmid cost and stability. The pKP33 plasmid was highly stable in its original Kp33 *K. pneumoniae* host, where the plasmid was maintained throughout 280 generations of culturing in the absence of selection (see supplementary fig. S1, Supplementary Material online). In contrast, pKP33 displayed a high degree of instability in the

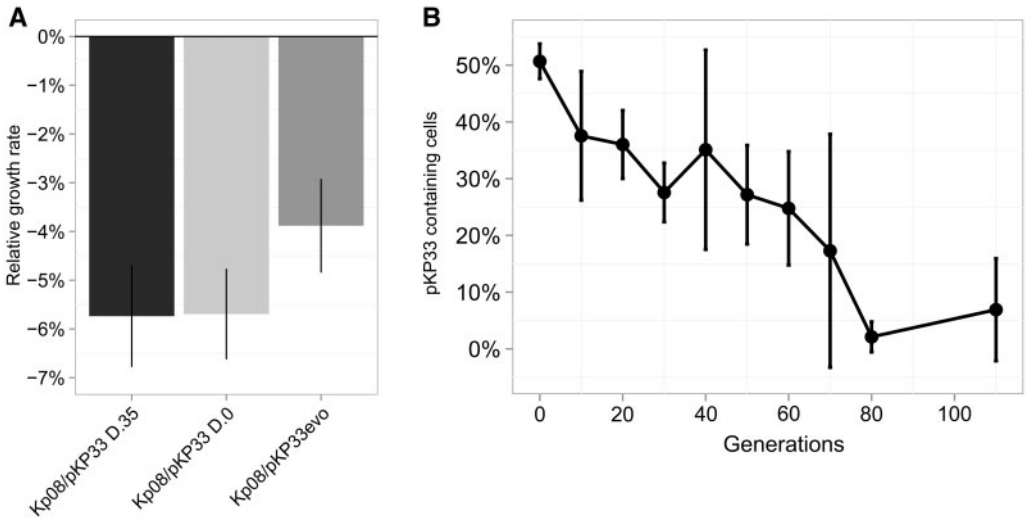

Fig. 8. The ancestral pKP33 as well as pKP33 evolved in *Escherichia coli* (pKP33evo) were transferred to the naïve Kp08 *Klebsiella pneumoniae* strain. (*A*) The exponential growth rate was measured for 24 individual colonies of Kp08 carrying the ancestral plasmid before (Day 0) and after (Day 35) 35 days of serial passaging in plasmid selective medium. These were compared to Kp08 carrying pKP33evo, transferred from the evolved *E. coli*. The growth rate improvement of Kp08/pKP33evo compared to Kp08/pKP33 Day 0 was borderline significant ($P = 0.063$). (*B*) Direct competition of Kp08 carrying the wild-type pKP33 against the Kp08 carrying the *Escherichia coli* evolved pKP33evo. Kp08/pKP33evo and Kp08/pKP33 Day 0 were equally mixed and serially transferred under antibiotic selection to select for both plasmids. The ratio of pKP33 to pKP33evo was quantified throughout the experiment by spotting on TMP and cefotaxime containing agar plates. Error bars depict the standard deviation of four replicate experiments.

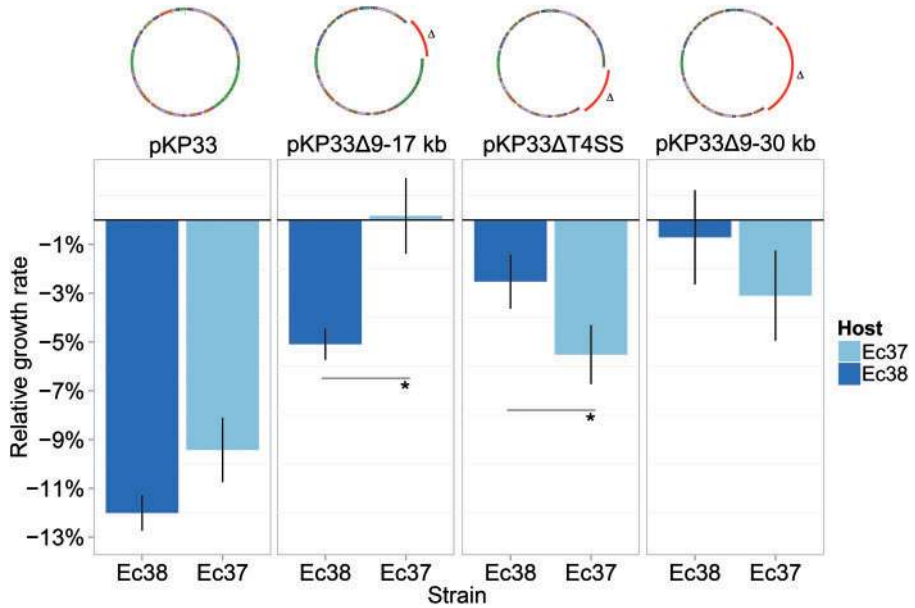

Fig. 9. Growth rate effects of targeted pKP33 knockouts in ancestral hosts. Engineered deletion mutants of pKP33 were generated in the ancestral hosts and the growth rate of the resulting variants was measured and depicted relative to the host without pKP33. The 19–30 kb part of pKP33 was deleted (pKP33ΔT4SS) comprising the conjugational transfer region and compared to deletion of the adjacent 9–17 kb (pKP33Δ9-17 kb) as well as a knockout of both regions (pKP33Δ9-30 kb). The top panel illustrates the position of the deleted parts (red) in pKP33. Error bars depict the 95% confidence interval of the mean measurements performed on at least 16 individual clones. A significant difference is indicated by asterisks (two-sample *t*-test, $P < 0.05$).

novel hosts, where it was lost in 50% of the population after 20, 48, and 100 generations of growth for Ec37, Ec38, and Kp08, respectively (fig. 4B).

Confirming previous observations of plasmid evolution (Heuer et al. 2007; De Gelder et al. 2008; San Millan et al. 2014; Harrison et al. 2015; Loftie-Eaton et al. 2015) we show that adaptive evolution in conditions selecting for plasmid carriage can significantly increase plasmid stability by reducing the fitness cost of an initially costly plasmid–host association. In addition, we show that nature of these

costs and the ability to adapt depends on the host background.

Sequencing of the evolved *E. coli* populations showed that the reduced fitness cost of pKP33evo was achieved by deleting a 25 kb plasmid region containing the T4SS involved in conjugational transfer; an event that was clearly dictated by the positioning of IS*26* elements in the plasmid backbone. Conjugation is considered costly in terms of vertical transfer (Turner et al. 1998), and targeted knockout of the main transfer machinery was indeed beneficial in terms of growth rate for both *E. coli* strains. Although liquid setups are commonly used in experimental evolution to rapidly assess a high number of cell divisions in a controlled environment, we note that such a setup might overemphasize the trade-offs observed here, because the conjugational transfer machinery of IncN plasmids works poorly in liquid environments (see supplementary table S3, Supplementary Material online). By measuring the conjugational transfer rates of pKP33 for all strains in a solid-surface setup we were able assess the potential of conjugative transfer to compensate the observed plasmid loss in a structured environment compared to our liquid setup and evaluate these values against the theoretical minimum requirement for parasitic maintenance of pKP33 in each strain (see supplementary table S3, Supplementary Material online). While pKP33 does show notably higher conjugation rates when surface associated, these rates are still orders of magnitude lower than the theoretical minimum rates required for parasitic plasmid maintenance in all naïve isolates (see supplementary table S3, Supplementary Material online), rendering the conjugational machinery disadvantages in these strains, even in conditions allowing high transfer rates.

Although it is unlikely that conjugation alone will maintain pKP33 even in dense, rapidly growing, homogenous populations similar to the ones investigated here, we note that there might be other selective advantages of carrying a conjugational transfer system in natural environments, even if transfer rates are low. We demonstrate that the original Kp33 host can indeed maintain a plasmid for much longer than even closely related naïve hosts. As suggested by Bergstrom et al., the ability to "sample" different host backgrounds for compatibility in diverse bacterial populations increases the opportunity for a stable plasmid–host relation, which along with other benefits of the T4SS such as increased biofilm formation, might improve long-term plasmid survival (Bergstrom et al. 2000; Ghigo 2001).

Surprisingly, the pKP33 knockout mutants lacking the 9–17 kb part whilst retaining the T4SS showed an equal or higher benefit in terms of growth rate compared to the pKP33ΔT4SS mutant, indicating that the cost is not exclusive to the T4SS but stems from multiple loci. While these costs displayed an additive nature, the relative contribution of each plasmid region varied between the two closely related *E. coli* UTI isolates (fig. 9, table 1 and supplementary table S2, Supplementary Material online). Whereas the 25 kb region of pKP33 had a half-life of roughly 60 generations in both *E. coli* strains evolved under plasmid selection, Kp08/pKP33 as well as the original Kp33/pKP33 strain retained the intact plasmid backbone throughout the evolution experiment. These observations emphasize the role of host genetic background in plasmid–host adaptation and highlight the potential role of stable plasmid donors as reservoirs in long-term plasmid survival. This is an important realization because it underlines the irregular fitness landscape navigated by transmissible plasmids in heterogeneous populations; a landscape that is eventually evened out during periods of selection for plasmid-borne traits, thus increasing plasmid frequency and opportunity for dissemination and adaptation towards new hosts. Interestingly, the *E. coli* evolved plasmid was also beneficial in Kp08 and although selection for compensatory mutations is expected to be weaker in Kp08 than for *E. coli* due to the lower cost of plasmid carriage (Qi et al. 2016), the evolved plasmid dominates the population within 50 generations of direct competition against Kp08 carrying the ancestral pKP33 plasmid (fig. 8B). Despite this potential competitive benefit of the large plasmid deletion in Kp08, restructuring was never observed in Kp08 nor in the original Kp33 host (fig. 6B), suggesting that the *E. coli* host were better catalysts of these adaptations than *K. pneumoniae*.

While it is reasonable to assume that the majority of intramolecular restructuring events lead to deleterious outcomes, we show that these events can indeed provide important plasticity for natural selection to work on. There are indications that the frequency at which recombination events occur can vary substantially within bacterial species. For example, Rodríguez-Beltrán et al. show that there is a tendency towards higher frequencies of homologous recombination in uropathogenic *E. coli* strains compared to nonpathogenic *E. coli* strains (Rodríguez-Beltrán and Tourret 2015). This has been suggested to increase adaptability in their native environment and such differences in mutational events, and in particular homologous recombination, could explain the differential evolvability of the *K. pneumoniae* strain compared to *E. coli* UTI isolates. These frequencies of recombination are suggested to be much higher ($>10^{-4}$) than point mutations ($\sim10^{-8}$), which might explain why we did not observe any SNPs able to compensate the plasmid imposed costs. Although we can only speculate on the mechanistic basis for these differences, they seem to correlate with the number of virulence genes and could also be influenced by host global regulators known to target horizontally acquired DNA and influence transposition frequency (Shiga et al. 2001; Doyle et al. 2007; Rodríguez-Beltrán and Tourret 2015). However, the cause is likely multifactorial and further investigations are needed to elucidate the exact nature of these differences.

As demonstrated here, the advancement of long read sequencing technologies aids greatly in the elucidation of the complex repetitive plasmid patterns attributed to abundant IS elements and it has become clear that IS elements, IS*26* in particular, play a major role in the organization of naturally occurring plasmids implicated in health-care associated outbreaks (Conlan et al. 2014; Harmer et al. 2014; He et al. 2015). Whereas it was recently demonstrated that transposition of beneficial genes into the plasmid backbone can improve stability (Loftie-Eaton et al. 2015), we are the first to describe IS mediated deletions as a driving force of plasmid

persistence in novel hosts. Whereas sacrificing important traits such as the conjugational transfer machinery can seem like an evolutionary dead-end strategy, such rapid recombination mediated restructuring might confer an advantage in certain environments (Labat et al. 2005). For a uropathogenic strain residing in the liquid environment of the urine bladder, getting rid of costly transfer machinery to increase growth rate can be a beneficial survival strategy (Bradley et al. 1980; Gordon and Riley 1992; Nilsson et al. 2003).

While domestication of costly plasmid traits seems like a common theme in plasmid–host evolution, the means of cost amelioration are diverse (Modi et al. 1991; Dahlberg and Chao 2003; Doyle et al. 2007; De Gelder et al. 2008; San Millan et al. 2014; Harrison et al. 2015). Recent work shows that the cost associated with carriage of a large conjugative plasmid in *Pseudomonas fluorescens* can be compensated by translational down-regulation attained via chromosomal mutations alone (Harrison et al. 2015). In contrast, we show that IS*26* mediated deletion of costly plasmid genes can provide similar benefits.

It is clear that strain specific factors can be highly influential, and extrapolating observations from single-host experiments to general plasmid behavior in clinical isolates is not a trivial task (De Gelder et al. 2007). The differences in stability observed for pKP33 in the *K. pneumoniae* and *E. coli* strains investigated here, suggests that transfer well within the expected natural host range of a plasmid might be more difficult than generally assumed. Although the initial tolerance, in terms of cost and stability, does seem to correlate with the relatedness of the strains to the original plasmid host (see supplementary table S2, Supplementary Material online), predicting the potential for stability improvements and long-term survival is not straight forward.

While species dependent constrains have been located to replication proteins in a nonconjugative broad host-range plasmid (Sota et al. 2010), our results suggest that dispensable factors of the plasmid backbone can impose similar limitations to the long-term host range of conjugative plasmids. Although chromosomal mutations as well as the presence of native plasmids in the naïve strains might have influenced the cost of pKP33 carriage, we expect these effects to be minimal compared to the major plasmid deletion event. Chromosomal mutations occurred almost exclusively in genes associated with virulence. Such genes are likely acquired by horizontal gene transfer and might be involved in the host specific cost patterns observed; possibly through regulatory interference or antagonistic interactions between for example membrane associated components of the deleted plasmid region (Doyle et al. 2007; San Millan et al. 2015). Although we did not observe any correlation between pKP33 carriage and retention of native plasmids, and we did not find any significant functional overlap in plasmid carried genes when compared to pKP33, we cannot out rule that the native plasmids of the strains have influenced plasmid cost and evolutionary outcomes (see supplementary material S1, Supplementary Material online). We acknowledge that such minor effects on fitness are hard to detect given the relatively low resolution of the growth measurements used here, but might be detectable by more sensitive fitness assays such as direct competition.

Although IS mediated cost amelioration effectively improves plasmid persistence, intramolecular restructuring events might come at the expense of conditionally useful components such as genes involved in antibiotic resistance or horizontal transfer, that might ultimately limit plasmid dissemination. While the evolved plasmid kept most of its resistance determinants, the trimethoprim resistance gene *folA* was consistently deleted along with the T4SS machinery involved in conjugation. These deletion patterns suggest that nonconjugative plasmids can evolve as a result of a strain dependent selection process dictated by transfer associated costs or collateral deletions to improve short term fitness at the expense of future niche expansion potential.

Our data suggests that while the selective forces against plasmid carriage can vary between even closely related isolates, the evolutionary solution might be more general and even broadly beneficial in strains to which genetic resolution is not immediately accessible.

Although the radical restructuring observed here ultimately narrows the plasmid host range by constraining its dissemination, such ongoing dynamics might help explain the dominance of nonconjugative plasmids in sequence databases (Smillie et al. 2010; Shintani et al. 2015). Further investigations of the mechanisms underlying plasmid persistence and the role of transposable elements herein are important to understand and prevent our current epidemic of multidrug resistance. Such studies shall preferentially be carried out on relevant specimen of clinical importance to directly improve our understanding of the barriers and opportunities implicated in successful plasmid dissemination that will provide us with the knowledge necessary to control and predict the rapid emergence of multidrug resistant pathogens.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

The *E. coli* and *K. pneumoniae* strains used in this study were isolated from urinary tract and blood infections by the Department of Clinical Microbiology at Hvidovre Hospital, Denmark. The antibiotic resistance profile of the strains was evaluated by broth dilution antibiotic susceptibility testing (Wiegand et al. 2008) and summarized along with genotypic information in table 1 and supplementary table S1, Supplementary Material online. Culturing was generally done with shaking in lysogeny broth (LB) medium at 37°C. M9 minimal medium lacking leucine was used for counterselection of donors in the conjugation experiments. Cefotaxime (2 μg/ml) was added to ensure stable plasmid maintenance during culturing of strains harboring ESBL plasmids and TMP (16 μg/ml) was added when appropriate to prevent deletions of the 25 kb plasmid region containing the *folA* gene.

Plasmid Transfer and Conjugation Assays

Large plasmids were transferred to clinical isolates via either conjugation or direct electroporation when possible. For transfer by conjugation, the *TOP10* strain (Life Technologies, USA) was used as donor strain due to its high competence and leucine auxotrophy. Following incubation with the donor strain, cells were scraped of the agar surface, washed in isotonic salt water and plated on M9 minimal medium agar plates lacking leucine but containing cefotaxime for plasmid selection. Transconjugants were verified as β-galactosidase positive (blue colonies) on *X-gal* containing LB plates. For all conjugation experiments, the donor and recipient strains were grown with appropriate antibiotics to midexponential phase and washed in isotonic salt water before mixing 1:1. Conjugation was performed in either liquid LB medium or on a solid LB agar surface incubated without shaking at 37°C. For transfer rate quantification, conjugation was carried out for 2 h and the cells were plated on selective plates to quantify donors, recipients, and transconjugants.

Plasmid Evolution and Stability Experiments

Five parallel lineages of each plasmid-carrying strain were passaged once daily, in selective medium to ensure plasmid inheritance during the evolution experiment, and subsequently without selection to assess plasmid stability. Serial passaging was performed in 96-well plates containing 150 µl LB medium. Each day of the experiment, 1 µl culture was transferred to the corresponding well of a new plate by pin replication. Plates were incubated at 37°C and subjected to medium shaking at 400 rpm. The plasmid selection (cefuroxime, 16 µg/ml) used for the evolution experiment did not reduce the growth rate of plasmid bearing strains compared to growth in LB without antibiotics.

Growth Rate Measurements

Growth rate was measured as the maximum increase in optical density (OD) over time during exponential growth. OD measurements were conducted in 96-well plates containing 150 µl LB/well by the *ELx808* plate reader (BioTek, USA). Breathe-Easy (Sigma-Aldrich) film was applied to minimize evaporation during measurements. OD at 600 nms was measured with 5 min intervals for maximum 16 h and incubated with medium shaking at 37°C between measurements.

Colonies were picked into a pre-inoculation plate and were grown for 2–3 h with shaking at 37°C before inoculation of the final measurement plate.

Pairwise Competition

Two O/N cultures were diluted to the same OD and mixing equally in LB medium. Cefotaxime (1 µg/ml) was added to prevent plasmid loss. The competition was carried out in 1.5 ml cultures and a volume of 1.5 µl was transferred to a fresh well every 24 h. From OD measurements the number of generations was estimated to ~10 generations/day. The ratio of the competitors was determined as the fraction of colonies on TMP agar plates compared to plates containing cefotaxime (performed as in "Plasmid deletion and loss quantification").

Plasmid Deletion and Loss Quantification

Plasmid loss was measured as sensitivity to cefotaxime; assuming loss of the CTX-M-15 gene. While quantification was done at the population level, periodic verification of plasmid-free colonies was carried out by testing for the presence of the remaining plasmid carried resistance determinants. Deletion of the 9–30 kb plasmid region was measured as sensitivity to TMP, indicating the absence of the *folA* TMP resistance gene. Frozen culturing plates were thawed completely and 10-fold dilutions (10^{-1}–10^{-8}) were made in isotonic salt water. After thorough mixing of each dilution, 5 µl spots were placed on selective and nonselective agar plates (2 µg/ml cefotaxime or 16 µg/ml TMP). After absorption of the liquid, plates were incubated O/N at 30°C to ensure countable colony sizes. CFUs of the lowest countable dilution (<70 CFU) were quantified and the ratio of resistant to total cells was calculated.

Mathematical Modelling of Plasmid Stability

A mathematical model derived in Proctor (1994) was fitted to plasmid loss data using the *nls2* package in *R* (version 3.0.1). The model assumes that the population dynamics of a plasmid bearing population can be described by differential equations modelling the dynamics of plasmid containing $dPc/dt = (\gamma_{Pc} \cdot N_{Pc}) - (\mu \cdot N_{Pc})$, and plasmid-free cells $dPf/dt = (\gamma_{Pf} \cdot N_{Pf}) + (\mu \cdot N_{Pc})$ respectively.

Where *Pc* denotes plasmid containing cells and *Pf* denotes plasmid-free cells. Here, γ is the growth rate and µ is the segregational plasmid-loss rate. An equation describing the plasmid-free fraction as a function of time can be derived:

$$F_{pc} = \frac{(\mu+\rho)\cdot F_{pc,t0}}{(\mu+\rho\cdot(1-F_{pc,t0}))\cdot e^{(\mu+\rho)\cdot t} + \rho\cdot F_{pc,t0}} \quad (1)$$

Here, the starting number of plasmid containing cells is $F_{pc,t0}$ and the differential growth rate $(\gamma_{Pf} - \gamma_{Pc})$ is summarized in the parameter ρ.

Whole Genome and Plasmid Sequencing

Genomes and whole population DNA samples were extracted using the *DNeasy* Blood & Tissue Kit from *QIAGEN* (QIAGEN, Netherlands). Sample libraries for Illumina sequencing were prepared using the *TrueSeq Nano* and *Nextera XT* kits (Illumina, USA). All reference genomes and plasmids were prepared using the *TrueSeq Nano* kit and mechanical shearing. The remaining sequencing libraries were prepared using the *Nextera XT* employing enzymatic fragmentation. Sample concentrations were measured on a *Qubit* fluorometer (Life Technologies, USA) and analysed with the *Agilent 2100 Bioanalyzer* (Agilent, USA) for fragment length distributions. Libraries were sequenced paired-end on a *MiSeq* sequencer (Illumina, USA) with sample amounts ensuring coverage of >30X. In addition, pKP33 was sequences using the *Pacific Biosciences RS II* single molecule real time sequencing technology. PacBio library preparation and sequencing was performed by The Norwegian High-Throughput Sequencing Centre (NSC)

(Oslo, Norway). Sequenced genomes and short read data from the pKP33 evolved populations are deposited within the BioProject: PRJNA325878.

Sequence Analysis

All sequencing data was processed and analysed using the *CLC Genomics Workbench* software from *CLC Bio* (QIAGEN, Netherlands). Further analysis and preparation of final graphics was conducted in *R* (version 3.0.1). Annotation was done using the *RAST* server (Aziz et al. 2008). All annotations were manually inspected and updated via BLAST searches if deemed necessary. Reads from sequenced evolved genomes were mapped to reference genomes via the "Map reads to reference" feature in *CLC*. SNP's and small INDELS were detected automatically using the "Quality based variant detection" tool. Read mappings were manually inspected for each contig to identify larger INDELS. Variants resulting from ambiguous read-mappings were excluded from the variant analysis.

BLAST Analysis and Sequence Comparison Using *GView*

Consensus sequences of the evolved plasmids were extracted from read mappings to pKP33 and the cut-off for inclusion was set to 5× coverage, corresponding to approximately 10% of the average read coverage. These were blasted against the pKP33 plasmid reference using the *GView* web-server available at: https://server.gview.ca/, last accessed August 8, 2016. The blastn algorithm was used to generate a circular BLAST atlas using the following settings: *e* value cut-off was set to 10^{-10}, alignment length cut-off to 100 bp, percent identity cut-off to 85% and the genetic code to "Bacterial and Plant Plastid". The layout of the output was edited in the *GView* Java stand-alone application accessible from the results page.

Generating Plasmid Knock Out Mutants

Recombineering in the *E. coli DY329* strain was performed as previously described (Yu et al. 2000). Targeted deletions were made by introducing a chloramphenicol resistance cassette from the *pKD3* vector (Datsenko and Wanner 2000) into the pKP33 plasmid backbone. The T4SS region was targeted using primers with the following flanking regions: 5′-TTAAA TCTGCAATCAACAGAAGATAGTGAGTAAGGAGAAAGT ATGACCAC-3′ and 5′-AGAAATATAGCCTGCGTCAATCG TTTCTGCCGTGAGGGTACCGCTTTCCC -3′; deleting the approximately 10 kb part of pKP33 containing the main conjugational transfer region. A plasmid mutant with the 9–17.6 kb (pKP33Δ9-17 kb) region deleted was created using the following homologous regions: 5′-CTTCCATTCCGCC CATTTTTAGAAAATTTCGTGTCCATGCGATCAGGTTA-3′ and 5′-GATTTACGTGCATAGCCGATTTTCATTCTTTTCT CGCTAATTAGTTATGG-3′. A combination of both deletions from 9 to 30 kb was made using: 5′-GATTTACGTGCATA GCCGATTTTCATTCTTTTCTCGCTAATTAGTTATGG-3′ and 5′-AGAAATATAGCCTGCGTCAATCGTTTCTGCCGTG AGGGTACCGCTTTCCC-3′ as homology regions. All deletions were confirmed by PCR and subsequent *Sanger* sequencing (Macrogen, Korea).

Supplementary Material

Supplementary material S1, tables S1–S5, and figures S1–S3, are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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