Evolution of a Plasmid Regulatory Circuit Ameliorates Plasmid Fitness Cost

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ABSTRACT

Plasmids play a major role in rapid adaptation of bacteria by facilitating horizontal transfer of diverse genes, most notably those conferring antibiotic resistance. While most plasmids that replicate in a broad range of bacteria also persist well in diverse hosts, there are exceptions that are poorly understood. We investigated why a broad-host range plasmid, pBP136, originally found in clinical *Bordetella pertussis* isolates, quickly became extinct in laboratory *Escherichia coli* populations. Through experimental evolution we found that inactivation of a previously uncharacterized plasmid gene, *upf31*, drastically improved plasmid maintenance in *E. coli*. This gene inactivation resulted in decreased transcription of the global plasmid regulators (*korA*, *korB,* and *korC)* and numerous genes in their regulons. It also caused transcriptional changes in many chromosomal genes primarily related to metabolism. *In silico* analyses suggested that the change in plasmid transcriptome may be initiated by Upf31 interacting with the plasmid regulator KorB. Expression of *upf31 in trans* negatively affected persistence of pBP136Δ*upf31* as well as the closely related archetypal IncP-1β plasmid R751, which is stable in *E. coli* and natively encodes a truncated *upf31* allele. Our results demonstrate that while the *upf31* allele in pBP136 might advantageously modulate gene expression in its original host, *B. pertussis*, it has harmful effects in *E. coli*. Thus, evolution of a single plasmid gene can change the range of hosts in which that plasmid persists, due to effects on the regulation of plasmid gene transcription.

41 **INTRODUCTION**

42 Bacterial evolution and rapid adaptation are frequently shaped by the horizontal transfer
43 of genes, including those transferred by extra-chromosomal DNA elements known as plasmids
44 (Norman et al. 2009; Soucy et al. 2015). One example is the plasmid-encoded spread of
45 antibiotic resistance, which enables bacterial pathogens to resist traditional therapeutic antibiotic
46 treatments, leading to the rise of highly resistant "superbugs" that increasingly threaten human
47 health (Murray et al., 2022; San Millan, 2018). Understanding how plasmids evolve to
48 successfully transfer and maintain themselves in bacterial populations and communities is thus a
49 critical step towards limiting the spread of antibiotic resistance.

50 A central metric for assessing the degree to which a plasmid remains in a bacterial
51 population over time is termed 'plasmid persistence'. Plasmid-host pairs often differ in plasmid
52 persistence, and even the same plasmid has been shown to persist differently in closely related
53 species (De Gelder et al. 2007; Kottara et al. 2018). Instances of poor plasmid persistence can
54 often be attributed to decreased fitness of the plasmid-containing bacterium relative to its
55 plasmid-free counterpart. This fitness cost of plasmid carriage imposed on the bacterial host is
56 due to several factors, including metabolic costs and molecular conflicts between plasmid and
57 host machinery (Modi and Adams, 1991). Several studies have shown compensatory mutations
58 that arise during serial batch cultivation of plasmid-host pairs; these mutations ameliorate
59 plasmid fitness cost and thereby restore plasmid persistence. Some of the important early studies
60 showed that such cost amelioration could occur through genetic changes on the plasmid but were
61 unable to explore the underlying molecular mechanisms (Bouma and Lenski, 1988; Dahlberg
62 and Chao, 2003). With the advent of next generation sequencing, compensatory mutations have
63 been identified in specific plasmid genes, including those encoding accessory functions (Bottery

64 et al. 2017; Stalder et al. 2017), replication initiation (Sota et al. 2010; Hughes et al. 2012; Yano
65 et al. 2016), and conjugation machinery (De Gelder et al. 2008; Jordt et al. 2020; Porse et al.
66 2016; Yang et al. 2023). Additionally, chromosomal evolution can stabilize host-plasmid pairs
67 through amelioration of plasmid cost via mutations targeting global regulators (Harrison et al.
68 2015; Stalder et al. 2017) and putative helicases (San Millan et al. 2014; Loftie-Eaton et al.
69 2017). Thus, compensatory mutations that increase plasmid persistence can occur on the
70 plasmid, chromosome, or both (Hall et al. 2021).

71 Some plasmid types, such as those of the incompatibility group IncP-1 used in this study,
72 are known to be highly persistent across many Proteobacteria (Schmidhauser and Helinski, 1985:
73 Shintani et al. 2010; Yano et al. 2012, 2013; Jain and Srivastava, 2013; Klümper et al. 2015;),
74 with few exceptions (De Gelder et al. 2007; Kottara et al. 2018). This remarkable persistence is
75 aided by several conserved regions (Thorsted et al. 1998; Thomas, 2000; Sen et al. 2013) of
76 backbone genes, which ensure the fidelity of critical functions like replication, stable inheritance
77 and control, mating pair formation and conjugative DNA transfer. These backbone genes are
78 controlled by a complex genetic regulatory circuit with four repressors located chiefly within the
79 stable inheritance and control region (Bingle et al. 2005; Rajasekar et al. 2016). The regulators
80 are part of a negative feedback loop referred to as autogenous control (Bingle and Thomas, 2001)
81 that limits their own expression and that of the operons they control. This in turn allows fine-
82 tuned and temporal plasmid gene expression to minimize cost of plasmid carriage to the host and
83 prevent potential conflicts with host cellular machinery. Collectively, this regulatory network
84 enables IncP-1 plasmids to replicate, transfer, and be stably maintained in multiple classes of the
85 Proteobacteria (Schmidhauser and Helinski, 1985; Shintani et al. 2010; Yano et al. 2012, 2013);
86 Jain and Srivastava, 2013; Klümper et al. 2015).

87 Given the known broad host range of IncP-1 plasmids and their role in the spread of
88 antibiotic resistance (Popowska and Krawczyk-Balska, 2013) we sought to examine the
89 previously reported but unexplained poor persistence of subgroup IncP-1β plasmid pBP136Km
90 in several *Escherichia coli* strains (Sota and Top, 2008). This plasmid was previously obtained
91 by inserting a kanamycin resistance gene in pBP136 (Sota et al. 2007), which was found in a
92 *Bordetella pertussis* strain isolated from a lethal infection in an infant in Japan (Kamachi et al.
93 2006).

94 We report that a single genetic change in pBP136Km that occurred within 50 generations
95 of evolution in *E. coli* in the absence of antibiotic selection drastically increased the plasmid's
96 persistence. This improved persistence was explained by a decrease in the plasmid fitness cost
97 due to partial deletion and presumed inactivation of a plasmid-encoded gene with unknown
98 protein function, *upf31*. This gene was previously thought to encode a putative adenine
99 methylase that is associated with an intriguing set of clustered repeats specific to the IncP-1β
100 plasmids (Thorsted et al. 1998). The high cost of plasmid carriage caused by this *upf31* allele
101 was shown to extend to the archetypal IncP-1β plasmid R751 (Thorsted et al. 1998), which is
102 otherwise stable in *E. coli*. In pBP136Km the presence of Upf31 was associated with changes in
103 expression of plasmid backbone regulatory genes and chromosomal genes, which we propose
104 may be due to its physical interaction with plasmid regulator KorB. Our study highlights that
105 evolution of the intricate gene regulatory systems of self-transmissible plasmids can improve
106 plasmid-bacteria pairings by amelioration of plasmid fitness costs.

107

108 **RESULTS**

109 **Plasmid-encoded *upf31* results in poor plasmid persistence in *E. coli* hosts.**

110 We first sought to confirm a previous finding (Sota and Top, 2008) that plasmid
111 pBP136Km shows poor persistence in various *E. coli* hosts. Using *E. coli* K-12 MG1655
112 (hereafter K-12), we performed a plasmid persistence assay entailing daily passage of triplicate
113 cultures in non-selective conditions (*i.e.* without antibiotics). This initial assay with the ancestral
114 host-plasmid pair showed poor plasmid persistence (Figure 1a in blue). In line with the previous
115 study, we randomly isolated one plasmid-bearing clone from each of the triplicate populations on
116 Day 5 and tested plasmid persistence in these clones. The persistence of the plasmid in these
117 clones was markedly improved (clones A, B, and C in Figure 1a in red, green, magenta,
118 respectively).

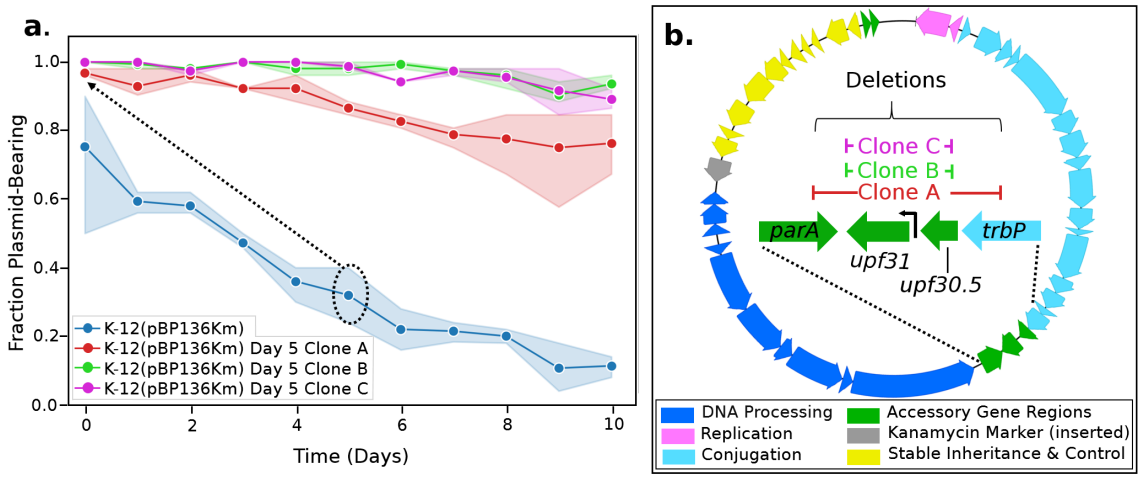

Figure 1: Plasmid evolution rapidly improved pBP136Km persistence. (a) Persistence of the ancestral plasmid pBP136Km (blue) and three populations (red, green, magenta) inoculated with clones that were isolated on Day 5 of the ancestral plasmid assay (see dotted ellipse and arrow). Clones isolated on Day 5 had greatly improved persistence compared to the ancestor. Lighter shades indicate 95% confidence interval. **(b)** Genomic map of pBP136Km in evolved Day 5 clones revealed deletions in the accessory region (green) between the *trb-tra* operons (light and dark blue, respectively).

119 To identify the genetic changes that may explain the observed change in plasmid
120 persistence, we completely sequenced the three isolated clones (Supplemental Table S1). We
121 found deletions within pBP136Km consistently targeting the region often associated with
122 accessory genes between the *tra* and *trb* operons, and clones B and C had identical plasmids
123 (Figure 1b). Additionally, no genetic changes were observed in the chromosomes of these three
124 clones. As the plasmid deletions in all three clones at least included *upf30.5* and *upf31.0*, we
125 suspected the presence of one or both genes to be responsible for the poor plasmid persistence in
126 *E. coli*.

127 We also routinely sequenced clones recovered after transferring the ancestral plasmid
128 between strains of *E. coli* by conjugation (*i.e.* matings) or electroporation. Among these clones
129 were several with deletions either internal to *upf31* or in its putative promoter (Supplemental
130 Table S1). To test if deletion of *upf31* alone could explain improved plasmid persistence, we
131 compared the persistence of one of these recovered variants, named pBP136KmΔ*upf31,* to that
132 of the ancestral plasmid pBP136Km in the same host, *E. coli* K-12 (Figure 2a).

133 This selected plasmid contained a 168-base pair (bp) deletion internal to the open reading
134 frame of *upf31* whereas *upf30.5* was intact (Figure 1b and Supplemental Table S1). Given that
135 the plasmid with inactivated *upf31* was persistent for at least 10 days, this gene of unknown
136 function must be responsible for the very poor persistence of pBP136 (compare red versus blue
137 squares of Figure 2a). The improvement in plasmid persistence after inactivation of *upf31* was
138 also seen in other well-studied strains of *E. coli* including DH5α and JM109 (Figure 2a).
139 Importantly, the pBP136KmΔ*upf31* containing clone was isolated from an overnight mating
140 followed by plating for transconjugants on selective media (Supplemental Table S1). This

141 provides an example of very rapid plasmid evolution that results in remarkably improved

142 plasmid persistence (*i.e.* < 48 hours, see Hall et al. 2020).

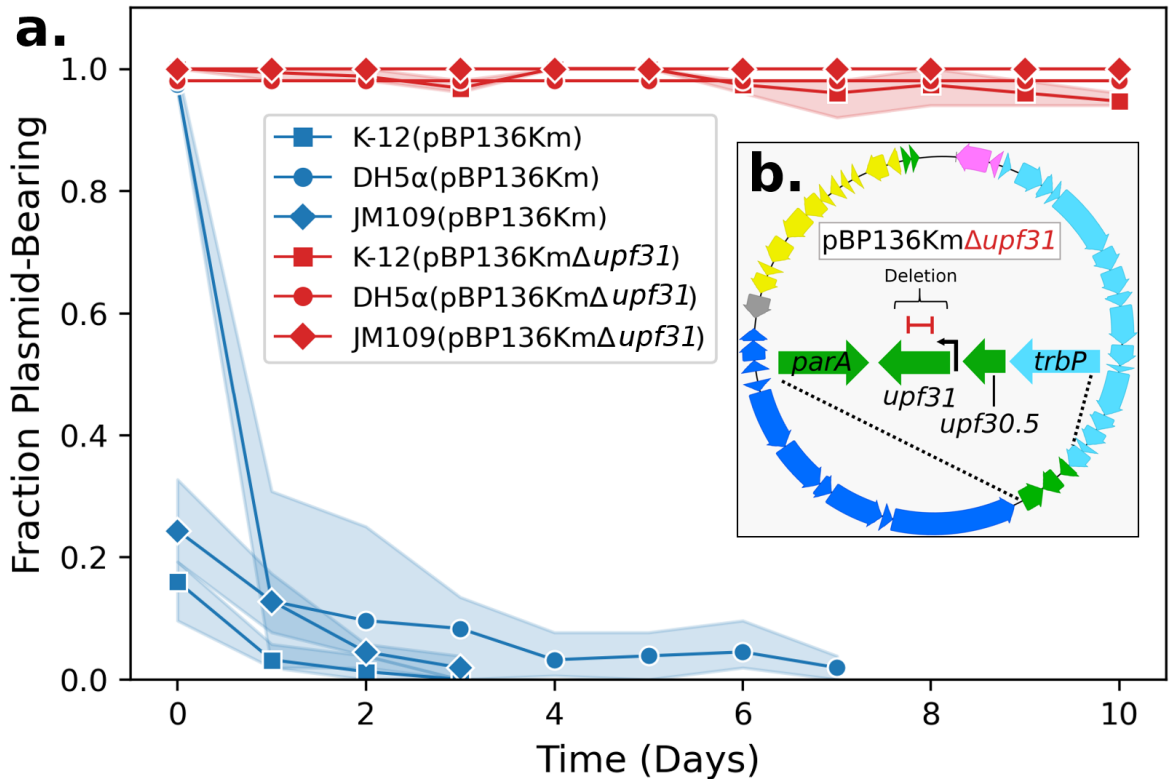

Figure 2: *upf31* inactivation alone explained improved persistence in *E. coli* hosts. (a) The poor persistence of plasmid pBP136Km (blue lines) in *E. coli* strains K-12 MG1655, DH5α, and JM109 (square, circle, and diamond respectively) was markedly improved after an internal 168-bp deletion in plasmid gene *upf31* (red lines). Lighter shades indicate 95% confidence interval. **(b)** A map of pBP136KmΔ*upf31* showing the 168-bp deletion (red) within the coding sequence of *upf31*.

Host fitness was lowered in the presence of upf31 and pBP136Km.

To investigate if the improved plasmid persistence associated with *upf31* inactivation was due to amelioration of plasmid fitness costs, we employed two approaches: *1)* a comparison of the growth dynamics between the two plasmid-host pairs, with a focus on the maximum growth rate and carrying capacity, and *2)* competition assays involving plasmid-bearing and plasmid-free strains. First, we observed that K-12 containing pBP136KmΔ*upf31* had a significantly higher maximum growth rate and reached a higher final density in comparison to K-12 containing ancestral pBP136Km (Figure 3a). These results demonstrate that the presence of *upf31* encoded on pBP136Km imposes a substantial fitness cost on its host.

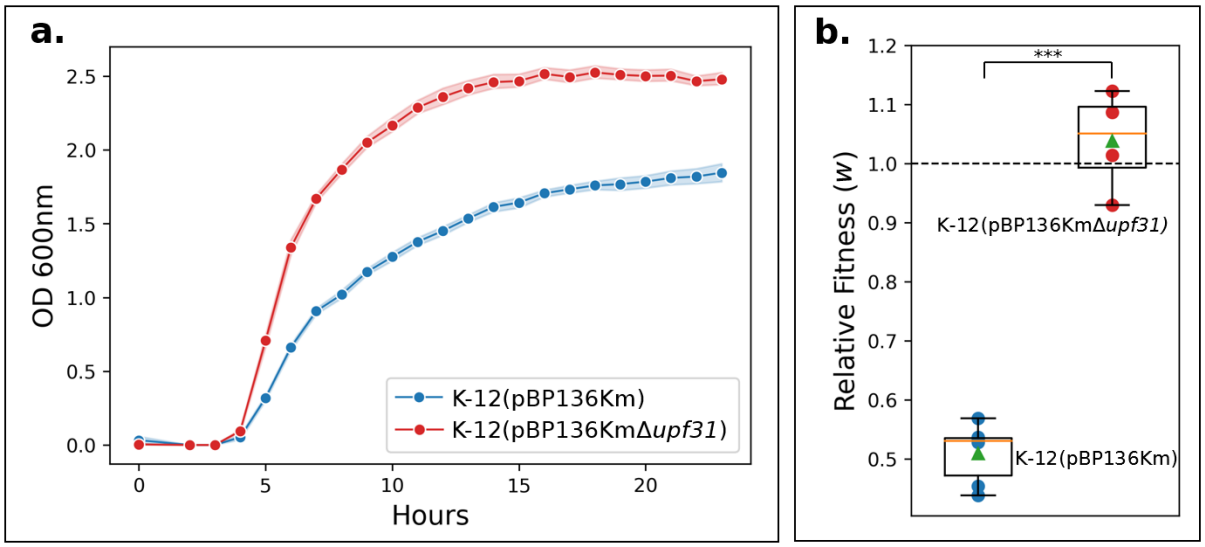

Figure 1: The presence of *upf31* encoded on pBP136Km lowered host fitness. (a) Bacterial growth in batch culture was slower (blue) when carrying pBP136Km with ancestral *upf31* compared to the evolved plasmid pBP136KmΔ*upf31* with inactivated *upf31* (red). Lighter shades indicate 95% confidence interval. (Two sample independent T-test of max growth rates, n=10, p-value= 2.35×10^{-3}) **(b)** With the fitness of plasmid-free K-12 normalized to 1 (dashed line), the relative fitness (w) of *E. coli* with ancestral pBP136Km (in blue) was 0.51, *i.e.,* a ~49% cost (Two sample independent T-test, n=5, p-value=1.70×10^{-6}). The relative fitness of evolved genotype K-12 (pBP136KmΔ*upf31*) to plasmid-free K-12 was statistically indistinguishable (Two sample independent T-test, n=5, p-value=0.40). Box is interquartile range, green triangle is mean, orange line is median, ***=$p \leq 0.001$.

152 Next, we confirmed the results of the growth rate observations by estimating the cost of
153 the ancestral and evolved plasmid in competition assays. This was done by individually
154 competing each plasmid-bearing strain against the plasmid-free K-12. We note that direct
155 competition assays between identical hosts with and without a highly transmissible conjugative
156 plasmid such as pBP136Km can be confounded by plasmid transfer during the assays. While
157 IncP-1 plasmids are less efficient at transferring in liquid than on surfaces, they have been shown
158 to transfer at detectable rates (Zhong et al. 2010). To overcome this limitation, we developed a
159 novel low-density competition assay which allows competing two strains at densities too low for
160 appreciable plasmid transfer to occur (see Methods). Indeed, as conjugation requires cell contact,
161 decreasing initial densities results in decreasing cell collisions and thus plasmid transfer events
162 (Kosterlitz et al. 2022). The results (Fig 3b) show a fitness of K-12 (pBP136Km) relative to K-12
163 of only 0.51, suggesting the plasmid caused a 49% reduction in fitness. By contrast, the fitness of
164 K-12 (pBP136Km∆*upf31*) was statistically indistinguishable from that of K-12. We conclude
165 that the persistence differences in Figure 2 can be attributed to a very large fitness cost conferred
166 by pBP136Km, which was alleviated by the inactivation of one plasmid gene, *upf31.*

167 The fitness cost imposed by *upf31* requires the presence of pBP136Km.

168 We next sought to understand if the expression of *upf31* directly imposed a fitness cost
169 on K-12 in the absence of plasmid pBP136Km. This was done by expressing *upf31* within K-12
170 *in trans* from expression vector pCW-LIC-*upf31.* Production of protein Upf31 was verified via
171 induction of pCW-LIC-*upf31* and observation of the associated 25.4 kDa product (Supplemental
172 Figure S1). As a negative control we used the non-modified vector pCW-LIC-*sacB*. The
173 maximum growth rates of K-12 with or without the Upf31 protein were not significantly
174 different (Fig 4a). This suggests that Upf31 does not impose a significant fitness cost on K-12 in

175 the absence of pBP136Km where it is naturally encoded. In contrast, when *upf31* was expressed
176 *in trans* in the presence of pBP136KmΔ*upf31,* the maximum growth rate was significantly
177 reduced compared to the same strain with the control vector (Fig 4b). Thus, we demonstrated
178 that Upf31 did not directly impose a fitness cost on K-12 but rather the interaction of Upf31 with
179 pBP136Km was responsible for the host fitness cost.

180 Gene *upf31* also effects persistence and fitness cost of archetype Inc-1β
181 plasmid R751 when complemented *in trans*.

182 We examined if the high cost of plasmid carriage observed with *upf31* extends to other
183 IncP-1β plasmids. The archetype IncP-1β plasmid R751(Thorsted et al. 1998) encodes a *upf31*

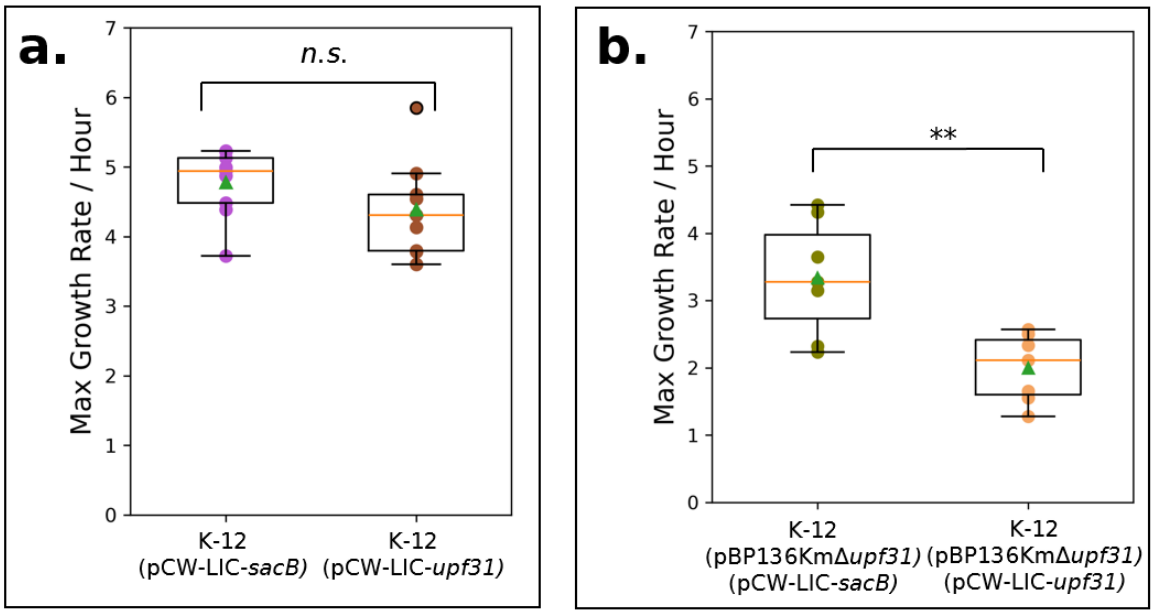

Figure 2: Upf31 requires the presence of pBP136Km to significantly reduce K-12 growth rate. **(a)** In the absence of plasmid pBP136Km there was no significant difference in maximum growth rate between *E. coli* K-12 expressing control *sacB* (purple) and *upf31* (brown) (Two sample independent T-test, *n*=10, p-value=0.46). **(b)** K-12 (pBP136KmΔ*upf31*) with *upf31* expressed *in trans* (orange) showed a lower maximum growth rate than K-12 (pBP136KmΔ*upf31*) with control *sacB* expressed *in trans* (green) (Two sample independent T-test, *n*=10, p-value=4.23×10^{-3}). Box is interquartile range, green triangle is mean, orange line is median, *n.s.* = $p > 0.05$, **=$p \leq 0.01$.

184 homolog with a 66 base pair deletion at the 3' end compared to pBP136Km. The high
185 persistence of R751 in *E. coli* suggests that this allele does not negatively affect *E. coli* fitness
186 (Supplemental Figure S2). To test if *upf31* from pBP136Km affects K-12 (R751) when
187 expressed *in trans*, we compared K-12 (R751) (pCW-LIC-*upf31*) to control K-12 (R751) (pCW-
188 LIC-*sacB*) in terms of plasmid persistence and growth rate. Plasmid R751 showed lower
189 persistence and resulted in a statistically significant lower host growth rate (Supplemental Figure
190 S2) when pBP136Km's *upf31* is present. This suggests that the longer *upf31* allele of pBP136
191 has a negative effect on R751 cost and persistence.

192 Forty-nine IncP-1β plasmids were identified in NCBI that contain a gene with full-length
193 homology to the ancestral *upf31* and associated promoter in pBP136, although none have an
194 identical protein sequence (Supplemental Data S1). We tested the persistence of two of these
195 plasmids, pAKD1, isolated from forest soil (Drønen et al. 1998), and the wastewater associated
196 pALTS29 (Law et al. 2021). Whereas plasmid pAKD1 showed poor persistence, similar to
197 pBP136Km, pALTS29 demonstrated high persistence (Supplemental Figure S3). The *upf31*
198 gene product of plasmid pAKD1 differs from that of pBP136Km in ten amino acids, with three
199 of these being non-conservative substitutions (D76A, A91V, R124G – see Supplemental Table
200 S2). Upf31 from pALTS29 differed from that of pBP136Km in twelve amino acids, including
201 the same three non-conservative substitutions found in pAKD1. When comparing the Upf31
202 amino acid sequence between all three plasmids, pAKD1 had a unique R60Q change and
203 pALTS29 had three unique differences in L78R, Q82P, S86T. Plasmid pALTS29 may be worth
204 further analysis, especially since the wastewater environment in which it was found might have
205 selected plasmid variants that persist better in a range of *Enterobacteriaceae*.

206 **The computationally predicted methylase function of Upf31 was not**

207 **supported by experimental results.**

208 To provide further insight into the possible function(s) of Upf31 we performed a
209 bioinformatic analysis using Phyre2. The output strongly predicted Upf31 to be a Dam methylase
210 homolog with 70% of the amino acids modeling with 100% confidence (Supplemental Data S2).
211 This is consistent with the pBP136 reference genome including a computationally automated
212 annotation of *upf31* as encoding a DNA methylase based on protein similarity as noted in the
213 original annotation of archetypal IncP-1β plasmid R751 (Thorsted et al. 1998). To test if Upf31
214 had Dam activity, we digested genomic DNA extracted from *dam*$^-$ strain *E. coli* JM110 with and
215 without pBP136Km and *dam*$^+$ K-12, using restriction enzymes that digest only methylated or
216 non-methylated 'GATC' DNA sequences. The results suggest that Upf31 is not a functional Dam
217 homolog as the restriction profiles of DNA from JM110 (pBP136Km) were opposite those of the
218 *dam*+ strain K-12 (Supplemental Figure S4).

219 Next, to determine if Upf31 more broadly encoded a methyltransferase, we used the base
220 pair modification detection available via Single Molecule Real Time (SMRT) sequencing. This
221 method has the benefit of not requiring *a priori* knowledge of the base pair modification
222 chemistry or target DNA sequence of the putative methylase. Briefly, the methylation-free *E.*
223 *coli* ER2796 (Anton et al. 2015) was used with *upf31* expressed from vector pCW-LIC-*upf31* or
224 pCW-LIC-*sacB* as the vector control. Genomic DNA extracts of both strains were analyzed
225 using the base modification analysis on a Pacbio Sequel II, which measures the kinetic
226 incorporation of base pairs to detect patterns distinctive from non-methylated base pairs. The
227 output showed no discernible kinetic patterns associated with methylated base pair incorporation,

228 indicating that Upf31 does not function as a methylase under our experimental conditions, in
229 contrast to the computational predictions.

230 The presence of *upf31* alters the expression of global plasmid regulators and 231 associated operons.

232 To understand how *upf31* and pBP136Km interact to influence *E. coli* fitness, we next
233 performed RNA-seq on K-12 containing pBP136Km and K-12 containing pBP136KmΔ*upf31*.
234 We considered K-12 (pBP136Km) to be the treatment (presence of the intact gene *upf31*), and K-
235 12 (pBP136KmΔ*upf31*) with the internal 168-bp deletion to be the control (absence of a
236 functional *upf31*). The presence of *upf31* led to greater than two-fold expression changes in 654
237 of the ~4,400 chromosomal genes, with 412 of those upregulated and 242 downregulated
238 (adjusted P-value < 0.05, Supplemental Data S3). The gene ontology tool DAVID (Dennis et al.
239 2003) predicted downregulation of cellular pathways related to nitrogen and sugar metabolisms,
240 while biosynthesis of siderophores, ABC transporters, and sulfur metabolism pathways were all
241 upregulated (Supplemental Data S4).

242 Most interestingly, K-12 (pBP136Km) showed greater than two-fold expression increase
243 in 11 of its 46 plasmid genes relative to the *upf31* deletion variant (adjusted P-value < 0.05,
244 Figure 5a and Supplemental Data S3). These genes were primarily part of the stable inheritance
245 and control region made up of regulons controlled by proteins KorA, KorB and KorC (Figure 5a
246 in yellow). Strikingly, K-12 (pBP136Km) showed a relative decrease in expression in only one
247 gene, *upf31* itself. This relative comparison of gene expression was made possible by

248 pBP136KmΔ*upf31* not having a complete *upf31* deletion but rather an internal 168-bp deletion

249 that left an inactivated 507 bp *upf31* product to be expressed (Figure 5b).

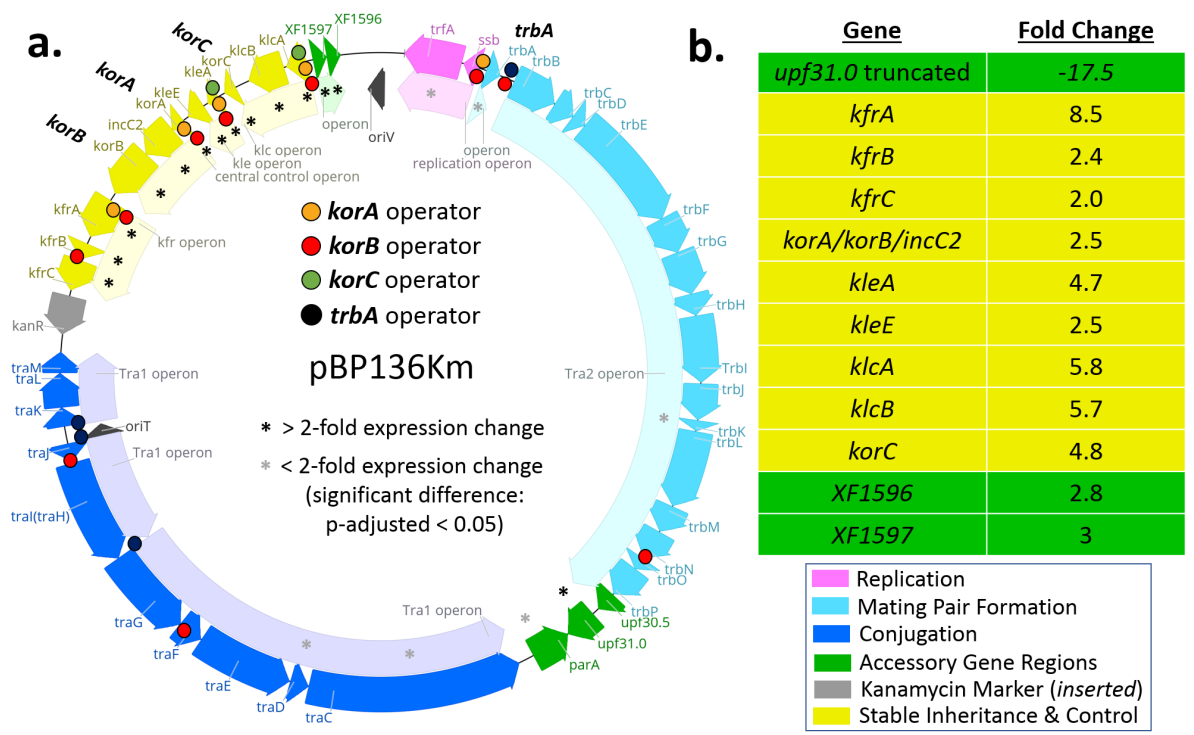

Figure 3: Expression of global plasmid regulators and their associated operons was increased in the presence of ancestral *upf31* compared to the deletion mutant. (a) Genetic map of pBP136Km with operons and operator sites annotated for global regulators KorA, KorB, KorC, and TrbA. Asterisks denote differentially expressed plasmid genes in the presence of *upf31*. **(b)** Table of plasmid-encoded genes with at least 2-fold differential expression (black asterisks in panel a) in the presence of *upf31*. Note expression of the ancestral *upf31* is ~16-fold lower than that of the truncated *upf31* knockout.

250 The effect of Upf31 to cause at least two-fold increased expression of all four of the

251 canonical IncP-1 regulatory genes (Adamczyk and Jagura-Burdzy, 2003)– *korA, korB, korC,* and

252 *trbA* – along with the other genes in the operons they regulate (Figure 5a in bold) is notable.

253 These regulators are known to autogenously repress their own expression (Bingle and Thomas,

254 2001) by binding cognate operators near their respective promoters (orange, red, green, and

255 black dots in Figure 5a). They are also known to repress promoters for the other operons in their

256 regulons (yellow in Figure 5a). The increased repressor gene expression should therefore
257 decrease rather than increase expression of all other regulated operons as the expression of the
258 repressor genes increases. This suggests that *upf31* has a general effect of diminishing the
259 repressive ability of *kor* regulatory genes, both in terms of their autogenous control and
260 repression of their respective regulons.

261 **Upf31 represses its own transcription.**

262 A possible mechanistic explanation for the decrease in *upf31* transcription in the presence
263 of Upf31 is that Upf31 represses its own transcription, like the previously IncP-1 plasmid
264 regulators KorA, KorB, KorC and TrbA studied (Pansegrau et al. 1994). Testing this hypothesis
265 requires first verifying the putative promoter upstream of upf31(*i.e.* upf31p) and then measuring
266 expression from this promoter with and without Upf31 present in the cells. To test the
267 functionality of the putative *upf31* promoter upf31p, a 68-bp region upstream of *upf31* was
268 inserted into *xylE* reporter plasmid pGCMT1 to create pGCMTupf31p (Supplemental Figures S5
269 and S6). The *xylE* assay confirmed that this region possesses significant promoter activity with
270 7.4 XylE units compared to the 0.09 units for the empty vector (Supplemental Table 3).

271 Since the Thomas lab had previously failed to demonstrate strong repressor activity from
272 the 3'-end truncated *upf31* in R751(Akhtar, 2002), we tested if adding the C-terminus of
273 pBP136's Upf31 to R751's Upf31 restored the protein into a functional repressor. To do this, we
274 constructed an R751/pBP136 hybrid *upf31* gene (aa 1 to 172 from R751 and aa 173 to 225 from
275 pBP136) and inserted this hybrid gene into pBR322 to create pBR*upf31*. Expression from the
276 *upf31* promoter on pGCMTupf31p was then measured in the presence and absence of pBR*upf31*.
277 The full-length hybrid Upf31 expressed from pBR*upf31* caused repression of upf31p, resulting in
278 an average of 0.09 XylE units compared to 4.76 XylE units with pBR322 as negative control

279 (Supplemental Table 3). This confirmed that *upf31* expression is likely to be autoregulated via
280 Upf31 binding near upf31p. The most obvious operator-like sequence in the region of this
281 promoter consists of inverted 5'-CAGCATCG-3' repeats, which belongs to the family of
282 inverted repeats (IR) that occur in four groups across the IncP-1β genome (Thorsted et al. 1998).
283 The presence of these strikingly conserved sequences of these IR across IncP-1β plasmids
284 suggests that Upf31 may bind to similar sequences across IncP-1β plasmids. In conclusion, these
285 assays confirmed the transcriptome results that suggested Upf31 represses itself and may have
286 additional regulatory functions.

287 Upf31 is computationally predicted to bind KorB via a C-terminal 288 dimerization domain.

289 A hypothesis to explain our transcriptome results emerges from the fact that the high
290 level of repression associated with the stable inheritance and control region depends on the
291 cooperative interaction of KorA (and TrbA) with KorB (Zatyka et al. 1997; Kostelidou et al.
292 1999; Bingle et al. 2008). If Upf31 were able to disrupt this cooperativity then it might decrease
293 repression while allowing the concentration of the repressors to rise. To explore the hypothesis
294 that Upf31 disrupts the cooperative interaction of KorA (and TrbA) with KorB we used
295 Alphafold2 (Jumper et al. 2021) and ColabFold (Mirdita et al. 2022) to predict the structures of
296 every individual pBP136 coding region in complex with Upf31. We subsequently ranked these
297 interactions using a self-assessment ranking score (ranking_confidence: 0.2pTM + 0.8ipTM, see
298 Supplemental Data S5). The structure prediction for Upf31 is that it possesses a globular DNA
299 binding domain (residues 1 to 174) and a C-terminal dimerization domain (CTD) which is
300 consistent with its proposed autogenous regulation of its own expression. However, the most
301 exciting result of this analysis is the prediction that the CTD mediates interaction with KorB

302 (Figure 6a) in a way that is very similar to KorA. Modeling of Upf31 with KorB gave a robust

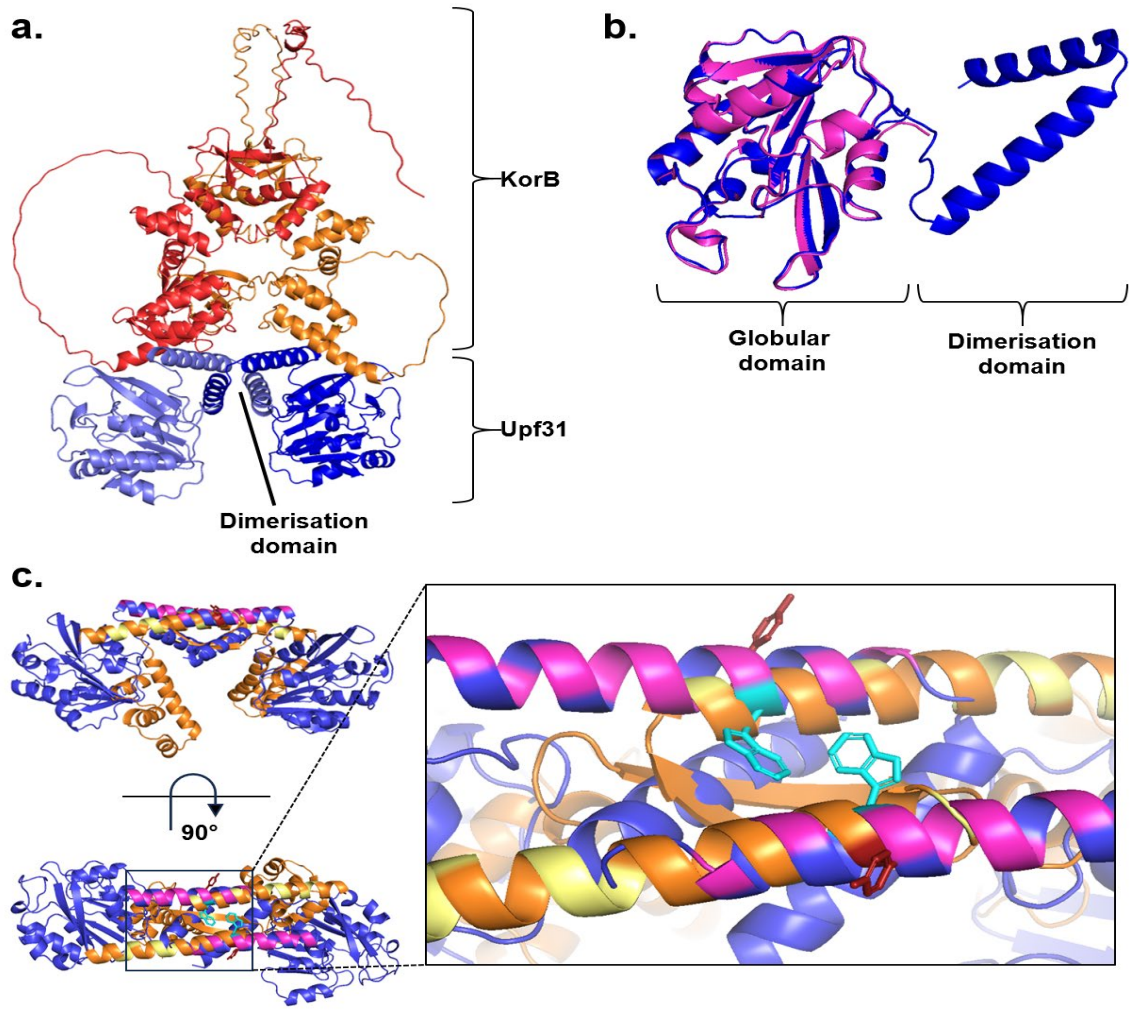

Figure 4: AlphaFold2 modeling reveals the potential interaction between pBP136 Upf31 and KorB is mediated by the C-terminal dimerization domain. (a) AlphaFold2 modelling of the pBP136 Upf31 homodimer (light and dark blue) with the pBP136 KorB homodimer (red and orange). **(b)** Superimposed AlphaFold2 models of pBP136 Upf31 (dark blue) and R751 Upf31 (magenta) highlighting the additional C-terminal dimerization domain of pBP136 Upf31. **(c)** Overlay of the AlphaFold2 model of pBP136 Upf31 homodimer (dark blue) with the crystal structure of the RK2 KorA homodimer (PDB: 5CM3, DNA duplex removed). Basic residues in the dimerization domain are highlighted in magenta or yellow. KorA Y84 is highlighted in deep red, Upf31 W198 is highlighted in cyan.

303 ranking_confidence score of 0.64. Removal of the CTD from Upf31 drastically reduced the

304 predicted interaction between KorB and Upf31 to 0.274. When pBP136 KorB was modelled with

305 R751Upf31, which natively lacks a CTD, a similarly low score of 0.252 was predicted. Given
306 the globular domain sequence similarity between the Upf31 of pBP136Km and R751 these
307 results are highly suggestive of pBP136Km Upf31 interacting with KorB via its C-terminus CTD
308 (Figure 6b). Although the Upf31 CTD does not line up with KorA CTD in the way that the CTD
309 of TrbA does, one can detect similarities like the Tyrosine at position 180 and the patch of
310 predominantly basic amino acids. This predicted interaction might allow Upf31 to reduce
311 available KorB either when bound to DNA or free, since we previously observed that KorA and
312 KorB could be copurified in the absence of DNA (McLean et al. 2024). This would in turn
313 explain the increased transcription of the IncP-1 regulatory genes as well as the operons they
314 regulate.

315 Additional experiments were performed to test the computational prediction of Upf31
316 requiring a CTD for functionality. Plasmid R751 encodes a truncated Upf31 allele lacking a
317 CTD and persisted very well in *E.* coli but was rapidly lost in the presence of Upf31/pBP136Km
318 expressed *in trans* (Supplemental Figure S2 and Supplemental Data S6). This suggests the CTD
319 of Upf31 is critical to Upf31's negative effect on plasmid persistence and growth of *E. coli.* To
320 test further whether it is the CTD of pBP136's Upf31 that is critical for poor plasmid persistence
321 in *E. coli*, rather than other amino acid differences between the two alleles, we tested the effect
322 of the hybrid Upf31 (described earlier) on R751. This hybrid Upf31 combines R751's truncated
323 Upf31 with the C-terminus of Upf31/pBP136Km and was expressed *in trans* with vector
324 pBR*upf31*. Strikingly, transformation of pBR*upf31* into competent *E. coli* C600 (R751) gave
325 hundreds of pBR*upf31* transformants on L-agar with ampicillin but less than 10 transformants on
326 L-agar with ampicillin and trimethoprim (selecting for both pBR*upf31* and R751). Together our
327 computational and experimental findings strongly suggest that the presence of the CTD of Upf31

328 from pBP136 is necessary for the poor plasmid persistence phenotype, and likely because of its
329 physical interaction with KorB.

330 **DISCUSSION**

331 This work sheds new light on the biology and evolution of the largest sampled subgroup
332 of the most intensively studied conjugative plasmids. We demonstrate that the poor persistence
333 in *E. coli* of a conjugative broad-host-range IncP-1β plasmid from *Bordetella pertussis* rapidly
334 improved, even in the absence of selection for the plasmid. The associated drastic amelioration
335 of the plasmid fitness cost to *E. coli* was due to the inactivation of a previously uncharacterized
336 plasmid gene*, upf31,* which affected the expression of the plasmid backbone regulatory circuit
337 including all four major regulators as well as hundreds of chromosomal genes. This change in
338 transcriptome may have been initiated by Upf31 interacting with one of these major plasmid
339 regulators, KorB. Even though plasmids like pBP136 of the incompatibility group IncP-1 are
340 notably persistent in many Proteobacteria (Schmidhauser and Helinski, 1985; De Gelder et al.
341 2007; Shintani et al. 2010; Yano et al. 2012, 2013, Jain and Srivastava, 2013; Klümper et al.
342 2015; Kottara et al. 2018;), we show that different alleles of plasmid genes like *upf31* can cause
343 striking differences in plasmid persistence in particular hosts. Understanding how this gene acts
344 and why it may be an advantage in some hosts will expand our understanding of the genetic
345 toolbox plasmids can exploit for their success.

346 The last decade has seen a flood of seminal studies regarding compensatory evolution of
347 plasmid fitness cost, including multiple studies showing that changes in chromosomal regulatory
348 systems can improve plasmid-host relations. One example is mutations of the *fur* transcriptional
349 regulator involved in iron uptake (Hassan and Troxell, 2013; Stalder et al. 2017), while evolution

350 of the chromosomal *gacA/S* regulators (Harrison et al. 2015) was observed to lower the fitness
351 cost of plasmid carriage within 48 hours of plating on agar (Hall et al. 2020). A few studies have
352 found compensatory evolution via putative chromosomally-encoded helicases (Loftie-Eaton et
353 al. 2017), in one case largely restoring gene expression levels of the plasmid-host pair to that of
354 the plasmid-free bacteria (San Millan et al. 2015). Amid these discoveries, the complex and
355 tightly regulated plasmid transcriptional regulatory systems have perhaps been overlooked as a
356 key factor in plasmid-host relations. One exception was a study which found that a plasmid
357 improved persistence via differential expression of a *parAB* operon, with backbone genes and
358 plasmid regulators otherwise remaining unchanged (Hall et al. 2021). Our work shows that the
359 plasmid encoded Upf31 inactivated during evolution in *E. coli* regulates its own transcription as
360 well as that of the primary plasmid regulators associated with the stable inheritance and control
361 region. Thus, the regulatory systems of *both* plasmids and chromosomes should be included in
362 the growing list of evolutionary pathways that can decrease the fitness cost of a plasmid.

363 The exact mechanism of action of Upf31 remains experimentally unvalidated but we
364 hypothesize that its C-terminal dimerization domain interacts with plasmid regulator KorB. This
365 was suggested by our use of AlphaFold2 to predict structures and interactions between Upf31
366 and all other pBP136-encoded proteins, which identified KorB as a highly likely target for Upf31
367 (Supplemental Data S5). Furthermore, the CTD of Upf31 modeled very similar to the CTD of
368 KorA, which was previously shown to interact with KorB (Bingle et al. 2008). KorB is known to
369 interact co-operatively with the other plasmid backbone repressors (Kostelidou et al. 1999;
370 Kostelidou and Thomas, 2000; Zatyka et al. 2001). Therefore, a KorB-Upf31 interaction that
371 reduces KorB's ability to interact with other repressors could explain the observed weakening of
372 KorB autogenous control as well as weaker operon repression. These changes in plasmid

373 transcriptional regulation were associated with greatly reduced *E. coli* host fitness and the
374 associated lowered plasmid persistence. It would also align with our result that plasmid R751
375 with a *upf31* allele that natively lacks a CTD is highly persistent in *E. coli*. In sum, our study
376 suggests that just like KorA, Upf31 may also belong to the family of proteins that can interact
377 with KorB, and that its interaction may override the other interactions with concomitant
378 derepression of gene expression.

379 The Upf31-associated derepression of global plasmid repressors triggers a secondary
380 effect of large-scale differential gene expression. Thus, the next question becomes which of these
381 differentially expressed genes explains poor *E. coli* fitness. As this negative fitness effect of
382 *upf31* was only observed in the presence of IncP-1β plasmids, we focus here on changes in the
383 plasmid transcriptome. First, of the three plasmids in this study that contained full-length *upf31*
384 homologs, only pALTS29 showed high persistence in *E. coli,* which may suggest low fitness
385 cost. Notably, this plasmid lacked a toxin-antitoxin (TA) system found in nearly all IncP-1β
386 plasmids. This suggests that overexpression of TA genes may explain poor *E. coli* fitness.
387 However, the higher persistence of pALTS29 could equally be explained by differences in Upf31
388 amino acid sequences (Supplemental Table S2). Given our hybrid Upf31 results, there are only
389 two amino acid differences between the Upf31/R751/pBP136Km hybrid and Upf31/pALTS29
390 that could explain differences in plasmid persistence, i.e. at positions 82 and 92. Another
391 possible explanation for the negative effect of Upf31 on *E. coli* fitness is a set of four genes in
392 the control region that were upregulated in the presence of Upf31 (*kleA, kleE, klcA, klcB,* or '*kil'*
393 *genes*). These genes have been shown to have harmful effects on the growth of *E.coli* strains
394 when not repressed by the global Kor regulators (Figurski et al. 1982; Kornacki et al. 1993;
395 Larsen and Figurski, 1994), with *klcB* found harmful only in the presence of an IncP-1 plasmid

396 (Bhattacharyya and Figurski, 2001). Both above hypotheses assume that the observed mis-
397 regulation of *non*-regulatory plasmid genes cause physiological change within the bacterial host
398 which would explain the responsive shift in chromosomal gene expression levels. An alternative
399 model is that the observed mis-regulation of the plasmid repressors (KorA/B/C) also directly
400 changes expression of chromosomal genes in an example of plasmid-chromosome 'cross-talk' (
401 Vial and Hommais, 2020; Hall et al. 2021; Marincola et al. 2021; Thompson et al. 2023). Future
402 studies will have to identify the specific overexpressed plasmid genes that have such a negative
403 effect on *E. coli* fitness and by what mechanism, and if the differentially transcribed
404 chromosomal genes are at all responsible. It also remains to be determined whether *upf31*
405 provides an advantage in the original *B. pertussis* host.

406 Upf31 might be an important clue to understanding the four groups of conserved inverted
407 repeats (IR) that were previously described (Thorsted et al. 1998) in IncP-1β plasmids but
408 without any explanation of purpose or function. The *upf31* gene itself is embedded in one of the
409 four IR regions and appears to autoregulate its own expression by binding to one of the IR
410 regions. It is therefore likely that Upf31 binds to all the IR sequences although we currently
411 have no indication of purpose. However, it is noteworthy that *klcB* is among the highest
412 overexpressed genes in the presence of Upf31 (Figure 6) and includes several IR within its
413 coding sequence which may correlate with being available to compete with KorB for repression.

414 We next wish to report two important lessons we learned during this study. First, we
415 frequently observed rapid loss of *upf31* during routine laboratory cultivation due to various
416 deletions, prompting us to always sequence the region of *upf31* to confirm its presence. This
417 suggests that rapid evolution of costly plasmid genes may often go unrecognized during
418 cultivation and plasmid transfer between strains by matings and electroporation. Our work aligns

419 with previous findings of rapid (<48 hours) compensatory evolution between plasmid-host pairs,
420 even without known selection (Hall et al. 2020). Second, our work showed that Upf31 is likely
421 not a functional methylase in contrast to automated annotations describing the gene as such
422 (without experimental data) in many plasmid genomes. This highlights the argument that
423 plasmid genes *without* experimental data on their function should be assigned locus tags rather
424 than putative function-related gene names (Thomas et al. 2017).

425 In conclusion, despite decades of great progress in plasmid biology and the pivotal role of
426 conjugative plasmids in antibiotic resistance dissemination, a substantial portion of plasmids
427 harbor multiple genes with unknown functions. Our study highlights the importance of one such
428 gene, which when inactivated led to a lasting host-plasmid pair, effectively rescuing a plasmid
429 from potential extinction (Gomulkiewicz and Holt, 1995) in a bacterial population. The
430 investigation of this specific gene revealed that this evolutionary rescue is closely linked to
431 alterations in the plasmids regulatory circuit. These findings underscore the significance of
432 identifying and experimentally validating uncharacterized plasmid genes to understand how
433 evolution underwrites the spread and persistence of plasmids in bacterial communities.

434

435 # MATERIALS AND METHODS

436 ## Bacterial strains, plasmids, and media

437 IncP-1β plasmid pBP136 was discovered in a strain of *Bordetella pertussis* isolated from
438 a lethal case of infant whooping cough[35]. A kanamycin gene was later inserted to generate
439 pBP136Km (NCBI accession number NZ_OR146256.1), which provided a selectable marker in

440 the originally cryptic plasmid[56]. The expression vector pCW-LIC-*upf31* was derived from pCW-
441 LIC-*sacB* as described below in the cloning section.

442 *Escherichia coli* K-12 MG1655 was derived[57] from an isolate within the stool of a
443 diphtheria patient in 1925, and *E. coli* JM109 and DH5α[58] are later derivatives of this strain with
444 useful attributes relevant to cloning. We generated rifampicin and nalidixic resistant clones of *E.*
445 *coli* K-12 MG1655 by recovering resistant mutants after plating on agar with the respective
446 antibiotic. New constructs and initial, intermediate, and final populations of the plasmid
447 persistence assays were all archived at -70 °C in 30% glycerol.

448 All bacteria in this study were grown at 37°C in lysogeny broth (LB) shaken at 200 RPM
449 or on LB agar (LBA) plates. Strains were grown with 50 μg ml^{-1} kanamycin (km) or 100 μg ml^{-1}
450 ampicillin (amp) when appropriate for plasmid maintenance. Expression vectors were induced
451 with 500 μM isopropyl β- d-1-thiogalactopyranoside (IPTG) when induction is mentioned in the
452 text.

453 **Plasmid Persistence Assay**

454 All plasmid persistence assays were performed in triplicate and first grown overnight
455 (O/N) with kanamycin to select for initial plasmid maintenance (time T0). Daily serial transfers
456 of 4.9 μL of overnight culture into 5 mL of sterile broth *without* antibiotics were then performed
457 for ten more days (times T1-T10). Each day the cultures were diluted 10^{-6} in 1x phosphate
458 buffered saline (PBS) and 100 uL was spread onto dilution plates. The daily plasmid-containing
459 fraction of the population was determined by replica plating 52 randomly chosen colonies from
460 the dilution plates onto LB plates with and without kanamycin.

461 ## Growth Assays

462 We performed growth assays in batch culture to measure the effect of Upf31 on growth
463 rates and final densities. Strains were grown from freezer archives for 24 hours and diluted
464 1:100, then grown another 24 hours and finally diluted 1:1000 in sterile media. From these ten
465 biological replicates each were loaded at a volume of 200uL into the respective wells of a 96-
466 well plate. A SPECTROstar Nano plate reader measured optical density at 600nm every ten
467 minutes for 23 hours with incubation at 37C and 500 RPM orbital shaking. The R package
468 GrowthRates (Hall et al. 2014) v. 0.8.4 was used to calculate maximum growth rate using default
469 parameters.

470 ## Low-Density Competition Assays

471 The low-density competition assay was developed in this study to avoid plasmid transfer
472 during the assay. It contains two distinct steps: (*i*) determining the likely "conjugation-free" time
473 window for specific low donor and recipient densities, and (*ii*) performing a traditional
474 competition assay at low densities within the established time window.

475 To find the likely window of time before density is high enough for conjugation to occur,
476 we used K-12 MG1655 strains[34] with spontaneous mutations conferring resistance to nalidixic
477 acid (Nal) and rifampicin (Rif) (donor K-12N(pBP136Km) and recipient K-12R, respectively).
478 Donor and recipient were mixed1:1 at densities of 10^2/ml into 2.5 mL LB shaken 200 RPM at
479 37°C. To select for possible transconjugants, the cultures were mixed hourly with another 2.5
480 mL of LB with Km and Rif to create a 5mL mixture with 50 μg ml^{-1} Km and 50 μg ml^{-1} Rif.
481 These were grown 24 hours after which the earliest timepoint showing turbidity was associated
482 with the emergence of transconjugants K-12R(pBP136Km). The first turbid culture emerged

483 after six hours, and therefore a five-hour window of likely conjugation-free growth was chosen
484 for the subsequent competition assays.

485 Competition assays were then performed using K-12 vs. K-12(pBP136Km) and K-12 vs.
486 K-12(pBP136KmΔ*upf31*). Each experiment began with 1:1 mixtures at densities of 10^2/ml in 5
487 LB without antibiotics in shaken test tubes. Plating onto LBA occurred at the start time and after
488 five hours (after appropriate dilutions), and the relative increase in cell numbers (cfu/ml) was
489 used as a proxy for relative fitness as follows: (Wiser and Lenski, 2015)

490 $$\boldsymbol{w} = \frac{\boldsymbol{ln}\left(\frac{A_f}{A_i}\right)}{\boldsymbol{ln}\left(\frac{B_f}{B_i}\right)}$$

491 where w is the Malthusian relative fitness term, A_I and A_F are the initial and final plasmid
492 containing K-12 populations, respectively, and B_I and B_F are the initial and final plasmid-free K-
493 12 populations, respectively.

494 Methylation Detection

495 To test if *upf1* encodes a methyltransferase as computationally predicted we utilized the
496 base modification analysis provided by the Pacbio Sequel II DNA sequencer. The methylase-free
497 *E. coli* strain ER2796[43] with expression vector pCW-LIC-*upf31* was tested against ER2796
498 (pCW-LIC-*sacB*) for differential base modification[36] on a Pacbio Sequel II at the Arizona
499 Genomic Institute in Tucson, AZ, USA. Both strains were grown with antibiotic selection and
500 500 μM IPTG induction before genomic extraction using the Sigma GeneElute Bacterial kit.
501 Base Modification Analysis was run on SMRT Link v. 10.2.0.133434 with a reference made by
502 merging the *E. coli* assembly with the respective plasmids and enabling "Find Modified Base

503 Motifs" and "Consolidate Mapped BAMs for IGV" options. All the other options were left as
504 default.

505 RNA-Seq

506 To understand how *upf31* and pBP136Km interact to influence *E. coli* fitness, we
507 performed RNA-seq. Strains K-12WT (pBP136Km) and K-12WT (pBP136KmΔ*upf31)* were
508 grown overnight in 5 ml LB with Km and centrifuged. The cell pellets were sent to Zymo
509 Research for Total RNA-Seq Service. Sequencing libraries were constructed from total RNA
510 samples and were prepared using the Zymo-Seq RiboFree Total RNA Library Prep Kit (Cat #
511 R3000) according to the manufacturer's instructions
512 (https://www.zymoresearch.com/products/zymo-seq-ribofree-total-rna-library-kit). RNA-Seq
513 libraries were sequenced on an Illumina NovaSeq to a sequencing depth of at least 30 million
514 read pairs (150 base paired-end sequencing) per sample.

515 A custom bioinformatics pipeline began with TrimGalore! v. 0.67 to remove adapters and
516 low-quality reads. Bowtie2 (Langmead and Salzberg, 2012) v. 2.4.5 then mapped reads to
517 merged chromosomal and plasmid references. These were counted using featureCounts(Liao et
518 al. 2014) v. 2.0.1, the output of which was input unto DESeq2 (Love et al. 2014) v. 1.38.1, for
519 final calculations of relative expression.

520 DNA Sequencing

521 Whole genome sequencing was performed by SeqCenter, LLC in Pittsburg, PA, USA.
522 Genomic DNA extractions were performed using Sigma GeneElute Bacterial kit. The libraries
523 were prepared using the Illumina DNA Prep kit with IDT 10bp UDI indices and sequenced on an
524 Illumina NextSeq 2000 with 2x151bp reads. Demultiplexing, quality control and adapter

525 trimming was initially performed using Illumina's bcl-convert v. 3.9.3. TrimGalore! v. 0.6 was
526 additionally used to remove remaining adapters and low-quality reads. Breseq (Barrick et al.
527 2014) v. 0.36.0 was used to identify mutations between ancestral and evolved strains.

528 **Cloning**

529 pCW-LIC (hereafter pCW-LIC-*sacB*) was a gift from Cheryl Arrowsmith (Addgene
530 plasmid #26098; http://n2t.net/addgene:26098; RRID:Addgene_26098). Plasmid pCW-LIC-*sacB*
531 was double cut at the *NdeI* and *HindIII* restrictions sites, which created a 4,962 base pair (bp)
532 linearized backbone and a 2,302 bp linear fragment containing *sacB* and its promoter. These
533 were separated and the linearized backbone recovered from an agarose gel using a Thermo
534 Scientific (TS) GeneJET Gel Extraction Kit. Next, gene *upf31* was amplified from pBP136Km
535 miniprep using upstream primer 5'-*GGTGGT*CATATGTCCAGGAAGAAGGCCATGAG-3'
536 and downstream primer 5'- *GGTGG*TAAGCTTCTACTCGGCCGCCTCTAG-3' (flanking
537 sequences for restriction enzymes in italics, *NheI* and *HindIII* sites underlined, respectively). The
538 ends of the *upf31* amplicon were then double digested at the *NdeI* and *HindIII* sites, and the
539 small digested ends removed using a TS GeneJET PCR Purification Kit. The *upf31* segment and
540 linearized pCW-LIC backbone were joined using T4 DNA ligase and the now-circularized pCW-
541 LIC-*upf31* was electroporated into DH5α using standard methods(Sambrook and Russell, 2001).

542 To construct the upf31p-*xylE* reporter gene fusion complementary 68-nt oligomers were
543 designed to create a double stranded fragment with *Xba*I and *Eco*RI sticky ends for insertion into
544 *xylE* reporter plasmid pGCMT1 to create pGCMT-upf31p as shown in Supplementary Figure S6.
545 The 68 bp region upstream of *upf31* was from plasmid R751.

546 To construct the R751-pBP136 hybrid *upf31*, translational start plus codons 1 to 173 were
547 amplified by PCR with primers 5'-

548 TGCAAGCTTTAATGCGGTAGCCAAGTCCCGATTTACTCCAG-3' and 5'-
549 CCGGCTCGGGGTAGTTCATC-3' from R751 template. To create codons 174 to 224 from
550 pBP136 PCR-driven overlapping(Heckman and Pease, 2007) of synthetic oligomers (104 nt and
551 117 nt) were utilized with pBP136 template. The whole segment was amplified with primers 5'-
552 TGCAAGCTTTAATGCGGTAGCCAAG-3' and 5'-TCGGTCGACGCAGGCGTGAC-3' and
553 after cutting with restriction enzymes inserted into both pBR322 (Accession J01749.1) and
554 pACYC184 (Accession X06403.1) between their HindIII and SalI sites so that *upf31* was
555 transcribed from the *tetA* promoter.

556 ## *xylE* reporter assay

557 Overnight LB cultures were inoculated from single colonies of strains with reporter
558 plasmid pGCMT1 or its derivative pGCMTupf31p alone (with just kanamycin selection) or with
559 a second plasmid vector (pBR322) or vector plus the hybrid *upf31* gene (with both kanamycin
560 and ampicillin) and incubated for 16 h. Bacteria from 1 ml aliquots were pelleted, resuspended in
561 0.5 ml sonication buffer, sonicated with three bursts of 5 seconds, the cell debris cleared with 10
562 min centrifugation at maximum speed in a microfuge at 4°C and then measured quantitatively for
563 XylE activity and protein concentration as described previously (Zukowski et al. 1983).

564 ## Upf31 protein expression

565 Induction cultures of pCW-LIC-upf31 in *E. coli* K-12 M1655 were started from an
566 overnight liquid culture of LB (Lennox) with 100 µg/ml ampicillin (LB Amp). Twenty-five
567 milliliter LB Amp cultures were initiated at OD600 0.05 using the liquid overnight culture to
568 serve as the induction cultures. The 25 milliliter cultures were grown at 37° C while shaking at
569 200 rpm. When the 25 milliliter cultures reached OD600 > 1.0, one milliliter of culture was
570 removed, and the cells were harvested by centrifugation for four minutes at 16,000 x g at room

571 temperature in an Eppendorf 5425 centrifuge. This material served as the pre-induction (or 0
572 hour) time point for each culture. ITPG was then added to each induction culture to a final
573 concentration of 0.1 mM, 0.5 mM, or 1 mM and growth was monitored at OD600. At the time
574 points indicated in Figure S2, one milliliter of culture was removed from each culture and cells
575 were isolated as above. Cells were resuspended in 1x Tris-Glycine running buffer (25 mM Tris-
576 Cl, pH 8.3, 192 mM glycine, and 0.1% SDS) at 100 microliters per 1.0 = OD600 cells. Laemmli
577 buffer from Bio-Rad was added to the resuspended cell pellets to a final concentration of 1x and
578 heated 10 minutes at > 98° C prior to gel loading.

579 Upf31 SDS-Page Gel Electrophoresis

580 Heated, denatured cellular extract (see previous section) samples were loaded on a Tris-
581 glycine gel with a 12% acrylamide resolving gel and a 4% acrylamide stacking gel. Each sample
582 lane contained 0.1 OD_{600} of cells. The molecular weight standards used were unstained broad
583 range protein standards (NEB) and then the gel was run at 100 volts until the dye front was 1 cm
584 from the bottom of the gel and then stained one hour in Coomassie blue stain (50% methanol,
585 10% acetic acid, 0.25% Coomassie brilliant blue R-250) followed by destaining (5% methanol,
586 7% acetic acid) overnight.

587 Computational Modeling of Interactions between Upf31 and pBP136-encoded 588 Proteins

589 An '*in silico* pulldown' of Upf31 was carried out using the LazyAF pipeline(McLean,
590 2024). In short, the pBP136 genome (AB237782) was retrieved from NCBI GenBank as a FASTA
591 protein coding sequence. The Upf31 protein coding sequence was used to generate individual
592 concatenated FASTA files with Upf31 and each pBP136 coding sequence. ColabFold v1.5.5:
593 AlphaFold2 w/ MMseqs2 BATCH was then run on Google Colaboratory using the High-RAM

A100 GPU with the following settings: msa_mode: MMseqs2 (UniRef+Environmental), num_models: 5, num_recycles: 3, stop_at_score: 100. Subsequently the JSON files were analyzed to retrieve the pTM and ipTM scores for each top ranked model and calculated the ranking_confidence score (0.2 pTM + 0.8 ipTM).

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