Fundamental parameters governing the transmission of conjugative plasmids

Jorge Rodriguez-Grande [(1)] , Maria del Pilar Garcillan, [(1)] Fernando de la Cruz[(1)] and Raul Fernandez-Lopez[(1) (*)]

(1) Instituto de Biomedicina y Biotecnologia de Cantabria IBBTEC

Spanish National Research Council CSIC – University of Cantabria

Albert Einstein 22, Santander

Spain.

(*) To whom correspondence should be addressed.

ABSTRACT

Plasmid conjugation is a major route for the dissemination of antibiotic resistances and adaptive genes among bacterial populations. Obtaining precise conjugation rates is thus key to understanding how antibiotic resistances spread. Plasmid conjugation is typically modeled as a density-dependent process, where the formation of new transconjugants is proportional to the rate of encounters between donor and recipient bacteria. By analyzing conjugation dynamics at different cell concentrations, here we show that this assumption only holds at low bacterial densities. At higher concentrations, plasmid transmission switches from a density-dependent process to a frequency-dependent one. Mathematical modeling revealed that plasmid conjugation follows a Holling´s Type II functional response, characterized by two fundamental parameters: the searching rate and the handling time. These parameters represent, respectively, the density and frequency dependent limits for plasmid transmission. Our results demonstrate that these parameters are characteristic of the transfer machinery, rather than the entire plasmid genome, and that they are robust to environmental and transcriptional perturbation. Precise parameterization of plasmid conjugation will contribute to a better understanding of the propagation dynamics of antimicrobial resistances.

39 **Introduction**

40 Conjugative plasmids are key mediators of bacterial adaptation shuttling antibiotic
41 resistance genes, virulence determinants and other adaptive genes through
42 horizontal gene transfer (HGT) (Baquero et al., 2009; Bennett, 2008; Li et al., 2019;
43 Lopatkin et al., 2017). Plasmids cluster into discrete taxonomic units, PTUs, which
44 show extensive variation in their host range and transmissibility (Brooks et al., 2019;
45 Jain & Srivastava, 2013; Loftie-Eaton et al., 2016; Redondo-Salvo et al., 2020).
46 Although the molecular mechanisms of plasmid propagation have been extensively
47 analyzed, the reasons why some plasmids are more prevalent and promiscuous than
48 others remain largely unknown. The successful propagation of a given plasmid
49 depends on many factors, including its ability to replicate stably, the burden imposed
50 to the host, and epistatic interactions with other plasmids present in the population
51 (Gama et al., 2017; San Millan et al., 2014; San Millan & MacLean, 2017). However,
52 the first and most important barrier for the invasion of a new population is horizontal
53 transmission, a process that bacterial plasmids mainly achieve by conjugation.
54 Among transmissible plasmids, nearly half of them encode all the genes required for
55 their autonomous transmission, and are called conjugative (Smillie *et al*., 2010,
56 Coluzzi *et al*. 2022). Conjugative plasmids contain the genes required for DNA
57 mobilization (MOB) and the genes required to build the conjugative pilus (TRA).
58 Another set of plasmids, called mobilizable, encode only the MOB genes; and their
59 transfer requires the supplementation of TRA from the chromosome or other co-
60 residing conjugative plasmids.

61

62 The long-term dynamics of plasmid propagation depend on a number of intertwined
63 factors, including their transmissibility, their ability to colonize different species, the
64 adaptive genes they encode, and the burden they impose on the host (San Millan &
65 MacLean, 2017). Plasmid transmission is the first and foremost process for the
66 spread of a conjugative plasmid among bacterial populations, and establishes the
67 overall ability of the plasmid to transfer itself from a population of donors (D) to
68 recipient (R) bacteria. Obtaining quantitative estimates of plasmid transmissibility is
69 key to fully understand their propagation dynamics, and over the years a number of
70 mathematical models of plasmid conjugation have been proposed (Levin et al., 1979;
71 Levin & Stewart, 1980; Simonsen et al., 1990; Smets & Lardon, 2009; Stewart & Levin,
72 1977; Wan et al., 2011; Zwanzig et al., 2019). Most of these models are based on
73 ordinary differential equations (ODEs), and describe plasmid transmission as a mass-
74 action kinetic process, where transconjugants (T) are formed at a pace proportional
75 to the rate of encounters between D and R. Such models are formally and conceptually
76 equivalent to classical density-dependent transmission models (DDT) for infectious
77 agents (Figure 1A). Under this regime, transmission of an infectious agent (in this
78 case, the plasmid) is limited by the probability of donors encountering a susceptible
79 individual (R cells in the case of conjugation). Under this mode of transmission, the
80 higher the density of R cells, the higher the conjugation frequency. In microbial
81 populations, however, there are situations where the rate of encounter with R cells
82 saturates, and conjugation may be limited by factors other than the probability of D-
83 R encounters. This is, for example, the case of biofilms. In such cases, transmission
84 may be limited by a second factor: the time required for the infectious agent to turn a
85 susceptible cell into an infected one (Figure 1B). This mode of transmission is called

86 frequency-dependent (FDT), since it is limited by the maximum pace at which
87 susceptible cells can be turned into infected individuals. This is the transmission
88 dynamics of many sexually transmitted diseases, where the frequency of encounters
89 is not usually constrained by the population density. In strict terms, however, any
90 transmissible agent may shift between DDT and FDT regimes, as the availability of
91 susceptible receptors fluctuates (Anderson & May, 1979; May & Anderson, 1979).
92
93 Ecological models of predation encountered similar dynamics. Predation rates also
94 depend on two factors: the density of prey, and the time invested by the predator to
95 consume its catch. The seminal work of Holling (Holling, 1959a, 1959b) derived the
96 fundamental equations governing the functional response of predation rates
97 depending on prey density. Under Holling's type I functional response, predation
98 increases linearly with prey abundance, as the predator tracks a fixed area per unit
99 time. This response is functionally and formally equivalent to density-dependent
100 models in epidemiology. Holling's type II functional response, however, shows
101 saturation. When the abundance of prey no longer becomes limiting, the rate of
102 predation is bounded by the processing time, the time required for the predator to
103 consume the prey (Figure 1C). Despite their formal similarities, there are important
104 differences between epidemiological models and ecological models of predation.
105 Infectious agents turn susceptible individuals into vectors of infection, thus there is
106 an exponential character in the propagation of infectious agents that is absent in the
107 case of predator-prey relationships. In the case of plasmid transmission, this
108 exponential character is present in conjugative plasmids, which can transmit
109 themselves from the newly formed transconjugants to other receptors. Besides, a key

110 difference between plasmid propagation and classical epidemiological models
111 resides in the timescales involved. In classical epidemiology, most agents spread at
112 rates that are much faster than the vegetative reproduction rate of the susceptible
113 population. The contribution of vegetative growth is thus negligible to the overall
114 transmission dynamics. In the case of plasmids, however, both horizontal and vertical
115 transmission present similar timescales and contribute to the propagation of the
116 plasmid.
117
118 Here we show that plasmid mobilization dynamics follows a Holling's type II
119 functional response, and how this model may be generalized to conjugative
120 transmission dynamics. As in ecological models of predation, two fundamental
121 parameters govern plasmid transmission. The first parameter, the searching rate
122 (hereafter, K_{on}), indicates the rate at which donor cells can encounter suitable
123 recipients. The second parameter, the handling time (τ), represents the average time
124 required for a conjugative donor to turn a recipient cell into a transconjugant. These
125 rates were found to be different among model broad host range conjugative plasmids.
126 Experimental measurements revealed that, in *E. coli,* PTU-N1 prototype plasmid
127 pKM101 exhibited faster searching kinetics (K_{on}) and shorter handling times (τ) than
128 PTU-W R388 and PTU-P1 RP4. This superior conjugation ability resulted in faster
129 invasion of the recipient population. Differences in transmission dynamics were
130 assignable to the conjugation machinery, although, a comparison between members
131 of PTU-W revealed considerable intra-PTU variability. Our results thus demonstrate
132 that plasmid conjugation depends on two key parameters, which vary among natural

133 plasmid isolates. These differences may be behind the distinct prevalence of each PTU
134 in microbial communities.
135

136 Results

137 Conjugation dynamics follows a type II functional response.

138 To obtain an analytic model able to capture the dependence on cell density and the
139 time required to turn a recipient in a suitable donor, we posed the following reaction
140 scheme, where D, R and T correspond, respectively, to Donor, Recipient and
141 Transconjugant cells.
142

143 $$D + R \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} C \overset{kc}{\rightarrow} D + T$$

144

145 Eq.1

146

147 In Eq.1, D and R cells encounter each following mass action kinetics with a K_{on} rate.
148 Formation of the conjugative pair, C, then ensues. C may resolve into the irreversible
149 formation of a new transconjugant T at rate K_c, or, alternatively, in an infructuous
150 mating with a reverse rate K_{off}. The handling time, hereafter τ, is the average time
151 required for a donor to turn a receptor into a transconjugant, and can be expressed
152 as 1/ K_c. The model described in EQ.1 is suited for the transfer of mobilizable
153 plasmids, where transconjugants are not able to become donors themselves.
154 However, minor modifications allow the model to be adapted to self-transmissible
155 conjugative plasmids (Supplementary Materials). Eq.1 reaction scheme is

156 conceptually identical to a Michaelis-Menten dynamics, and the same approximations
157 can be used to obtain an analytic expression for transconjugant formation. As shown
158 by (Andrup & Andersen, 1999), following Haldane´s approximation the number of
159 transconjugants per donor (the conjugation frequency) obtained after time *t* follows:
160
161
162
163 $$\frac{[T]}{[D]} = \frac{[R] \cdot k_{on}}{1 + [R] \cdot k_{on}\tau} t$$
164 Eq.2
165
166 Eq.2 corresponds to Holling´s type II functional response, originally proposed as the
167 relationship between predation rate and prey density (Holling, 1959b). In the context
168 of plasmid conjugation, the density of susceptible recipient cells (R) divides the
169 response into two distinct regimes (Figure 1C). At low R densities, the searching rate
170 (k_{on}) becomes limiting (density-dependent transmission). At high R densities,
171 however, it is the handling time τ which limits plasmid propagation (frequency-
172 dependent transmission). Holling´s type II functional response describes both
173 regimes and the transition between them as the R density changes. Eq.2 is valid for
174 plasmid mobilization, but as shown in Supplementary Materials, plasmid conjugation
175 can be similarly modelled. In this latter case, the autocatalytic nature of transmission
176 (T cells become donors in conjugation, but not in mobilization) results into an
177 exponential relationship of the form:
178

179 $$Ln\left(1+\frac{[T]}{[D]}\right)=\frac{[R]\cdot k_{on}}{1+[R]\cdot k_{on}\tau}\mathrm{t}$$

180 Eq.3

181

182 In order to check whether plasmid transmission dynamics followed a type II
183 functional response, as posed by the model (Figure 1D), we performed a series of
184 conjugation assays with variable recipient densities. As described in Materials and
185 Methods, in all these assays the conjugation time was set to 1h. to minimize the effect
186 of vegetative growth on D, R and T counts, and to reduce the impact of newly formed
187 T cells in the overall conjugation frequency. The donor to recipient ratio was also set
188 to 1D:100R, such that the total cell density may be approximated to [R], and no donor
189 saturation (donors only finding other plasmid carriers) is expected to happen
190 throughout this 1h span. Following the protocol described in Materials and methods
191 we measured the transmission rates of plasmids pKM101 (prototype of IncN/PTU-
192 N1), R388 (IncW/PTU-W) and RP4 (IncP1α/PTU-P1). Transmission was assayed in
193 populations of isogenic donor and recipient *E. coli* strains. Results, shown in Figures
194 1E, F and G, revealed that the three plasmids tested experienced transmission
195 dynamics following a type II functional response. Transition from density-dependent
196 to frequency dependent transmission -labeled as $[N]_{sat}$ for saturation density- was
197 observed at similar cell densities, around 0.01 to 0.1 cells per square micron.

198

199 **Differences in conjugation parameters result in faster transmission dynamics.**
200 Data shown in Fig.1 was fitted to Eq.3 as described in materials & methods, to extract
201 the searching rate and handling times of plasmids RP4, pKM101 and R388. Results
202 are shown in Table 1.

203

204 *Table 1. Conjugation parameters obtained by fitting data from Figure 1 to Eq. 3*

Plasmid (PTU)	**K_{on} (Conf. interval at 95%)**	**τ (C.I. at 95%)**
R388 (PTU-W)	35.8 $\mu m^2 \cdot h^{-1}$ (10-62)	0.7 h (0.6- 0.8)
pKM101 (PTU-N1)	275.8 $\mu m^2 \cdot h^{-1}$ (202-350)	0.6 h (0.5 - 0.6)
RP4 (PTU-P1)	88.1 $\mu m^2 \cdot h^{-1}$ (27-149)	0.6 h (0.5 - 0.7)

205

206

207 Results demonstrate that pKM101 had a substantially higher searching rate K_{on} than
208 RP4 and R388. These results indicate that pKM101 is able to find more suitable
209 receptors in the same timespan than the other plasmids tested. The handling times, τ
210 were, however, similar in the three plasmids tested.

211

212 To analyze the impact of differential searching rates on the overall kinetics of plasmid
213 invasion, we monitored the progression of the plasmid in a susceptible population
214 along time. Since plasmids pKM101 and R388 had previously shown the highest
215 differences in searching rates, we performed invasion assays with these two model
216 plasmids in *E. coli* populations. As indicated in Materials and Methods, plasmid
217 progression was measured as the number of transconjugant cells produced per donor
218 cell (T/D). We performed these assays at two different D: R proportions, 1:100 and
219 1:10000, keeping the same total cell concentrations (*i.e.*, using less donors). Results,
220 shown in Figure 2A, demonstrate that both plasmids were able to successfully invade
221 the population after 180 min., reaching T/D>10 in both cases. Plasmid pKM101 was

222 significantly faster at both D:R ratios tested, thus demonstrating that higher searching
223 rates and lower handling times result in faster invasion rates.
224
225 **Differences in the conjugation parameters are intrinsic to the conjugation**
226 **machinery.**
227 Plasmid conjugation is a complex mechanism in which factors other than the transfer
228 machinery often have an impact on the overall rate of plasmid propagation (Dionisio
229 et al., 2005; Guynet et al., 2011). To test whether differences in searching rates (K_{on})
230 and handling times (τ) were intrinsic to the conjugation machinery, or depended on
231 the entire plasmid genomes, we constructed a series of *E. coli* mutants harboring
232 chromosomal constructions of the entire PTU-N1 or the PTU-W transfer systems
233 respectively. For this purpose, the entire TRA operon of each of the plasmids was
234 introduced into the chromosome of *E.coli*. These TRA_N and TRA_W constructions allow
235 the mobilization of plasmids encoding the cognate MOB regions (MOB_N and MOB_W).
236 *Tra* genes from R388 or pKM101 were cloned into *E. coli* MDS42 and MG1655
237 chromosomes respectively under their own, native promoters as indicated in
238 Materials and Methods. We first tested whether these constructions were able to
239 mobilize their cognate MOB regions, and compared the mobilization dynamics of
240 Tra_W to the conjugation dynamics of its cognate R388 plasmid (Figure 2B). As shown
241 in the figure, the strain harboring Tra_W achieved lower T/D ratios than its parental
242 plasmid. This is not surprising, since secondary transfer from the transconjugants is
243 absent in experiments with the Tra_W construction. To test whether the differences
244 observed were solely due to the kinetic difference between conjugation and
245 mobilization, we repeated R388 invasion assays in a population of recipient cells

246 expressing PifC. PifC protein is an inhibitor of R388 conjugation (Getino et al., 2017),
247 thus its presence in the receptors prevents transconjugants to become effective
248 donors. Conjugation of plasmid R388 to a PifC-containing receptor should yield
249 similar dynamics to mobilization from Tra_W if the difference between conjugation and
250 mobilization is solely due to the absence of secondary transfer. As shown in Figure
251 2B, the transfer dynamics in Tra_W are similar to the conjugation of plasmid R388 to a
252 population expressing PifC. This indicates that the differences between R388 and the
253 strain harboring Tra_W can be ascribed to the lack of secondary conjugation in the
254 latter. A similar situation was observed when the conjugation rate from pKM101 was
255 compared to the mobilization rate from a strain containing Tra_N (Figure 2C).
256
257 Once the functionality of the chromosomal constructions had been established, we
258 compared the mobilization rates of Tra_W and Tra_N. Results, shown in Figures 2C and
259 2D demonstrated that the transfer machinery of pKM101 exhibited higher
260 mobilization rates than R388. These results are consistent with the values obtained
261 for wild type plasmids. Results in the searching rates and handling times observed in
262 full plasmids are thus dependent on the differences between the transfer apparatus
263 of both plasmids.
264
265 **Differences in conjugation rates are robust to environmental variables and**
266 **overexpression of the transfer machinery.**
267 In some PTUs, environmental factors such as temperature and nutrient availability
268 have an important impact on plasmid transmission rates (Johnsen & Kroer, 2007).
269 Thus, it was possible that the differences observed between pKM101 and R388 could

270 be explained by a differential sensitivity to environmental conditions. To test this end,
271 we performed conjugations in different temperatures and nutrient compositions.
272 Conjugation assays were performed as detailed in Materials and Methods, and results
273 are shown in Figures 3A and 3B. As shown in Figure 3A, both plasmids exhibited an
274 optimal transmission at 37ºC, yet pKM101 showed higher transfer rates at all
275 temperatures tested. Plasmid conjugation rates were also relatively invariant when
276 different media were used (Figure 3B). LB buffering against acidification produced by
277 cell growth did not change the conjugation rates. Increasing the nutrient availability
278 by performing conjugations in terrific broth did not have an observable effect, either.
279 In all conditions tested, plasmid pKM101 consistently yielded higher conjugation
280 efficiencies than R388.
281
282 Another possible explanation for the differences observed between both plasmids
283 was a differential expression of the conjugation apparatus. The expression of
284 conjugation genes in both plasmids is regulated through two conserved negative
285 feedback loops. TRA operon is auto repressed by KorA, its first ORF, while MOB
286 functions are controlled by second negative feedback under control of the accessory
287 protein ($TrwA_{R388}$/ $TraK_{pKM101}$). Changes in these repressors or their cognate
288 operators could result in different expression levels, thus we tested whether
289 increasing the expression of conjugation genes altered plasmid transmissibility.
290 Specifically, we analyzed whether increasing the expression of transfer genes
291 increased the conjugation frequency of R388. For this purpose, MOB and TRA
292 operons, as well as each individual conjugation gene were cloned under the control
293 of pBAD promoter and introduced into donor cells. Overexpression of these genes

294 was achieved as indicated in Materials and Methods, and the conjugation rates
295 determined as previously described (Rodriguez-Grande & Fernandez-Lopez, 2020).
296 Results, shown in Figure 3C revealed that the overexpression of transfer genes did
297 not increase the conjugation frequency. Minor, yet statistically significative changes
298 were achieved when overexpressing TrwJ. TrwJ is the pilus adhesin, the protein
299 responsible for contacting the recipient cell (Backert et al., 2008; Yeo et al., 2003).
300 Thus, it is possible that overexpression of this protein results in better donor to
301 recipient adhesion (Lacroix & Citovsky, 2011; Schmidt-Eisenlohr, Domke, & Baron,
302 1999; Schmidt-Eisenlohr, Domke, Angerer, et al., 1999).
303
304 **Variation in the fundamental parameters within the PTU-W**
305 Results demonstrated that pKM101 and R388 exhibited different conjugation
306 parameters, which resulted in different invasion dynamics. These differences could
307 be ascribed to their transfer machineries and were considerably orthogonal to
308 environmental and transcriptional perturbations. We thus wondered whether the
309 searching rate and the handling time were characteristic of a given plasmid PTU, or
310 were variable, with each plasmid showing different values. To test this end, we
311 compared plasmid R388 with three other PTU-W plasmids: pMBUI4, pIE321 and R7K.
312 PTU-W plasmids have a highly conserved genomic backbone (Fernández-López et al.,
313 2006), pIE321 being 97% identical at the DNA sequence level to R388, while R7K
314 being 97.5% (Revilla et al., 2008). Despite this high level of sequence conservation,
315 these plasmids were isolated from different bacterial species. Plasmid pIE321 was
316 isolated from *Samonella* Dublin (Götz et al., 1996), while R7K was found in
317 *Providencia rettgeri* (Coetzee et al., 1972). Searching rates and handling times were

318 obtained, as previously described, by analyzing the T/D obtained after 1h conjugation
319 against a population of recipients with variable cell density (Figure 4). While in
320 pIE321 the handling time was slightly higher than that of R388, we observed a 10x
321 increase in the case of plasmid R7K. To check whether these differences had an impact
322 on the speed of propagation of R7K, we performed a conjugation kinetics assay, as
323 described before (Figure 4B). As shown in the figure, plasmid R7K invasion of the
324 recipient population was much slower than that of R388, taking >150 minutes to
325 reach a conjugation frequency of 1 T per D, while R388 reached these levels in <30
326 min. Results thus demonstrated that within a given plasmid PTU, the conjugation
327 parameters may exhibit significant variation.
328

329 **Discussion**

330 Understanding the factors that facilitate or curtail plasmid conjugation is essential to
331 devise strategies against the propagation of antibiotic resistances. Plasmid
332 propagation is a complicated process, in which transmission, vegetative growth and
333 plasmid-host co-adaptation play important roles (San Millan, A. & MacLean, R. C.,
334 2017). Plasmid transmission is classically modelled as a mass-action kinetics process,
335 where the rate of propagation is proportional to the density of donor and recipient
336 bacteria. Here we showed that this model does not faithfully capture the dynamics of
337 transfer. Our results demonstrated that plasmid conjugation followed a Holling's
338 Type II Functional Response (Holling, 1959b). This dynamical response is
339 characteristic of processes for which there exist density and frequency dependent
340 constraints. Density dependent transmission occurs at low cellular concentrations,
341 when the rate of encounters becomes a limiting step. In situations where density is

342 high enough, the time required for a donor cell to "turn" a recipient into a newly
343 formed transconjugant becomes limiting. The hallmark of a Holling´s Type II response
344 is a transition between a regime where the rate of reaction is proportional to density
345 to a regime where it becomes independent (Figure 1). This double dependence can
346 be captured by two characteristic parameters: the searching rate (k_{on}, with units
347 corresponding to area per time, $\mu m^2 \cdot h^{-1}$) and the handling time, τ (with units of time,
348 h). The interplay between these two parameters (Eqs.2 and 3) determines the cell
349 density at which density or frequency become limiting. By fitting conjugation
350 frequencies obtained at different total cell densities, we were able to obtain estimates
351 of both parameters for plasmids from the PTU-P1, PTU-N1 and PTU-W. Estimates of
352 the searching rate, k_{on}, were in the range of hundreds of square microns per hour,
353 which roughly correspond to a searching radius of 3-10 μm per hour. These results
354 are in accordance with earlier estimates for the effective distance for plasmid
355 transmission, obtained for PTU-F_E and PTU-P1 plasmids (Harrington & Rogerson,
356 1990; Lagido et al., 2003; Babić et al., 2008; Seoane et al., 2011).
357
358 Interestingly, although both the searching rate and the handling time showed
359 significant variation between plasmids, the cell density at which plasmids
360 transitioned from one regime to the other was similar in all cases. Transition from a
361 density-dependent to a frequency-dependent regime occurred at cell densities of
362 approximately 0.02-0.05 cells per μm^2 (that is, a 1 μm-long cell every 20-50 μm^2).
363 This figure roughly corresponds to a cellular density where each transconjugant
364 would be, on average, contacting 1 recipient. This is a surprisingly low cellular
365 concentration, indicating that in denser environments such as biofilms (Fux et al.,

366 2005; Hall-Stoodley et al., 2004; Madsen et al., 2012), the soil (Raynaud & Nunan,
367 2014) or the gut microbiota (Contijoch et al., 2019) conjugation is most likely
368 frequency-limited. This indicates that in a significant proportion of microbial
369 environments plasmid conjugation is constrained by the handling time, rather than
370 the probability of encounters, as assumed by classical models of plasmid transmission
371 (Levin et al., 1979; Simonsen et al., 1990; Smets & Lardon, 2009).
372
373 This frequency limitation explains many experimental results obtained for
374 conjugation on solid surfaces and biofilms. In these experiments, a population of
375 donors encountering one of recipients is typically able to generate only a "border" of
376 transconjugants (Christensen et al., 1996; Fox et al., 2008; Reisner et al., 2012 ;
377 Christensen et al., 1998; Hausner & Wuertz, 1999). As handling times of conjugative
378 plasmids are on the same order of magnitude as the doubling times of donors and
379 recipients (Fernandez-Lopez et al., 2014), the layer of contact of recipients is pushed
380 backwards at the same speed that the layer of transconjugants is created. When
381 frequency becomes limiting, the factor constraining plasmid propagation is the time
382 the population enjoys until the exhaustion of resources, compared to the handling
383 time τ. The higher the number of effective transfers achievable by the plasmid before
384 resources run out, the higher the conjugation rate. This explains why most plasmid
385 propagation experiments on highly dense environments produced similar low
386 invasion levels, except those continuously refreshing the growth medium (Turner,
387 2004) (Fox et al., 2008). Thus, to accurately measure the conjugative efficiency,
388 experiments with short mating times (30-90') and an excess of recipients (1:100 D:R
389 ratio) are likely to yield more precise estimations. Results obtained in longer

390 experiments, and with 1:1 D to R ratios will be confounded by vegetative growth and
391 the lower probability of D-R encounters, respectively.
392
393 The handling time, τ, was found the most significant constraint for transfer. The
394 factors that influence this parameter, however, are unclear. The fact that plasmids
395 and their cognate transfer machineries exhibit slightly different handling times
396 (Th=0.5h for pKM101 and Th=0.33h for Tra_N) suggests that factors outside the
397 conjugation region may play a role. The difference in size of the DNA being transferred
398 is the most obvious candidate (pKM101 is 35 kb and the vector mobilized by Tra_N is
399 approximately 5 kb). However classical experiments using HFR strains demonstrated
400 that DNA transfer is fast, and the entire chromosome can be readily conjugated in
401 approximately 100 minutes (Lederberg et al., 1952; Tatum & Lederberg, 1947). Other
402 experiments also demonstrated that plasmid-encoded fluorescent proteins are
403 expressed in transconjugants after 15 minutes of mating, indicating that the whole
404 process of transfer takes shorter times (Gordon, 1992; Lawley et al., 2002). These
405 results suggest that the constraint is not the actual time required to pump the DNA
406 into the recipient, but other factors conditioning the establishment and resolution of
407 an effective conjugative pair. Plasmid copy number may be another possible
408 contributor to τ. R388 and pKM101 plasmids are relatively low copied, compared to
409 the vectors mobilized in assays using Tra_W and Tra_N mediated mobilizations.
410 Moreover, the different IncW plasmids assayed in Figure 4 also present different copy
411 numbers (Fernández-López et al., 2006), which could contribute to the differences
412 observed in transfer rates. Further experiments systematically analyzing the impact
413 of plasmid copy number on the transfer rate are required to clarify this end.

414

415 The searching rate K_{on}, establishes how easily a successful D-R pair is established
416 upon encounter. Physical factors involving cell to cell adhesion are likely to contribute
417 this rate. It is known that conjugation is favored when other, unrelated conjugative
418 plasmids express their pili in the same donor, or even the recipient cell (Gama et al.
419 2017). This suggests that increasing adhesion may facilitate transfer, a phenomenon
420 that was also observed introducing synthetic adhesins in D and R cells (Robledo et al.,
421 2022). Plasmids pKM101 and R388 showed a significant difference in their K_{on} (Table
422 1) a phenomenon which may be related to differences in the ability of the plasmid to
423 facilitate cell to cell adhesion. Plasmid pKM101 contains *pep*, an adhesin-like protein
424 that is absent in R388 and that has been shown to facilitate conjugation (González-
425 Rivera et al., 2019). Also, the only protein that slightly increased the conjugation
426 efficiency in R388 when overexpressed was the VirB5 adhesin TrwJ (Figure3).
427 Similar results have been obtained in pKM101 (Yeo et al., 2003) and Ti plasmids
428 (Lacroix & Citovsky et al., 2011), thus reinforcing the idea that increasing adhesion
429 improves the conjugation rate.

430

431 The fact that plasmids from the same PTU exhibited different searching rates and
432 handling times was surprising. It indicates that conjugation efficiency is not a
433 conserved feature among the members of a PTU, despite the overall sequence
434 similarity. In the case of W plasmids, sequence divergence concentrates in the MOB
435 and replication regions, and are the highest between R388 and R7K plasmids (Revilla
436 et al., 2008; Krol et al., 2013). Interestingly, R7K showed the lowest searching rate
437 and the longest handling time, which resulted in slower invasion dynamics (Figure 4).

438 R7K is the only PTU-W plasmid that was originally isolated from a host outside
439 Enterobacteria, thus its poor performance may represent a maladaptation to *E.coli*,
440 compared to the rest of PTU-W plasmids. Previous analyses have shown that plasmid-
441 host co-evolution has a major impact on key plasmid properties such as host range
442 and burden. (Bouma & Lenski, 1988; de Gelder et al., 2007, 2008; San Millan &
443 MacLean, 2017). It is thus likely that plasmid transmission also depends on co-
444 adaptations between the plasmid and the host. By systematically measuring the
445 searching rates and handling times in plasmids at different stages of co-adaptation,
446 we may be able to correlate transfer efficiencies with genes and mutations. Our model
447 thus provides an analytic framework to unravel the mechanisms and constrains that
448 regulate plasmid propagation in bacterial populations.
449

450 **Materials and Methods**

451 Plasmids and strains employed in this work are detailed in Tables 2 and 3.
452 **Cloning**. Plasmid constructs used for gene dosage experiments were built using the
453 Gibson assembly method (Gibson et al., 2009), using pBAD33 PCR products as vector
454 and genes and operons from *tra* region of R388 as insert (to be inserted downstream
455 the arabinose promoter of pBAD33 (Guzman et al., 1995)). All fragments were PCR
456 amplified with Phusion DNA polymerase (Thermo Fischer), gel-purified and digested
457 with DpnI restriction endonucleases (Promega) before assembly. *E. coli* DH5α strain
458 was used for electroporation and selective culture purposes. Gibson assembly
459 products were introduced in *E.coli* by electroporation. To build the mobilizable
460 plasmid pSEVA121:mobW, the region containing *oriT* of RP4 included in vector
461 pSEVA121 (coordinates 1278 to 1586 of GenBank Acc. No. JX560322.1) was replaced

462 by the *oriT-trwABC* region of R388 (coordinates 11153-16299 of GenBank Acc. no.
463 BR000038) using Gibson assembly.
464
465 **Tra_W and Tra_N constructions**. The MDS42::Tra_W strain was constructed by cloning
466 the *mpf* (also called *tra* operon) region of R388, comprising all genes (*trwD-korA*)
467 required to build up the conjugative pili and inserting this whole region in a MDS42
468 *E.coli* strain (Pósfai et al., 2006) using Wanner-Datsenko protocol (Datsenko &
469 Wanner, 2000). The lacZYA genes of E. coli strain MDS42 (coordinates 287438-
470 292616 of GenBank Acc. No. NC_020518.1) were used as target for the chromosomal
471 insertion. The trwD-korB region of R388 (coordinates 11153-16299 of GenBank Acc.
472 No. BR000038) was PCR-amplified in three overlapping fragments. The kanamycin-
473 resistance gene (KmR) surrounded by FLP recombinase recognition sites (FRT) was
474 amplified from pKD4 (AY048743.1). Finally, 1kb fragments, homologous to the lacI
475 and cynX chromosomal genes, were amplified from MDS42 genomic DNA. The six
476 fragments were assembled (lacI-trwD-korB-KmR-cynX) and introduced by
477 electroporation into MDS42 competent cells expressing the Lambda-Red site-specific
478 recombinase (Datsenko & Wanner, 2000). KmR colonies were checked by PCR to
479 confirm the Tra_W integration in the expected location, and the genome of a selected
480 colony was completely sequenced by Illumina. The phenotype of the MDS42::Tra_W
481 strains was checked by culturing in a chromogenic medium (agar Brilliance™) that
482 differentiates lac- and lac+ phenotypes. The KmR cassette was subsequently deleted
483 following the procedure described by Datsenko and Wanner (Datsenko & Wanner,
484 2000). Resistant derivatives of MDS42::Tra_W were obtained by plating saturated
485 cultures of this strain in LB-agar plates supplemented with Rif10, Nx20 or Sm300.

486 MDS42::Tra_N strain was similarly constructed by amplifying and Gibson-assembling
487 the *mpf* region of pKM101 (genes *korB-traG*) in a pOSIP vector (St-Pierre et al., 2013),
488 then electroporated in a DH5α *pir*$^+$ strain before PCR comprobation, miniprep
489 purification and introduction by electroporation in a *pir*$^-$ MG1655 strain -following
490 St-Pierre et al. protocol- where it recombined at attC position in the *E.coli*
491 chromosome (St-Pierre et al., 2013).
492
493 **Conjugation and mobilization experiments**. Unless otherwise stated, mating
494 assays were performed using BW27783 (Khlebnikov et al., 2000, 2001) Nal^R (donor)
495 and Rif^R (recipient) cells that had been grown to saturation overnight, at 37ºC, in 10
496 ml LB media supplemented with appropriate antibiotics. Antibiotic concentrations
497 used were nalidixic acid 20 µg / ml, rifampicin 10 µg / ml, kanamycin 50 µg / ml,
498 chloramphenicol 25 µg / ml, trimethoprim 20 µg / ml and ampicillin 100 µg / ml. Cells
499 were washed in equivalent volumes of fresh LB without antibiotics and allowed to
500 grow at 37ºC for 3h more. These cultures were collected, centrifuged at 4000g for 10'
501 and concentrated in 1 ml of fresh LB, then mixed at desired D:R ratios. Conjugation
502 on solid surfaces were performed in Corning® Costar® 24 well plates to which 1 ml
503 of LB-Agar had been added at least 3 days before, to solidify and dry. 15 µl of mating
504 mix were placed on top of the LB-Agar and let to conjugate for 1 h. at 37ºC, except
505 when indicated otherwise. Cells were then resuspended using 1 ml of sterile PBS,
506 subject to serial dilution and plated on LB-agar with appropriate antibiotics.
507
508 Conjugation assays using serial dilutions to discern between FDT and DDT were
509 performed as indicated above with the following modifications: For conjugation on

510 solid surfaces, cell cultures of donor (10 ml) and recipient (100 ml) cells were grown
511 to saturation on LB broth containing the appropriate antibiotics. Cells were washed,
512 refreshed for 3h in LB without antibiotics, concentrated ~100x in fresh LB and then
513 mixed in 1:100 Donor to Recipient ratio before being serially diluted in LB (dilution
514 ranging from 10^{2}X to 10^{-4}X). From each dilution, 100 µl of cells were deposited on top
515 of a 10cm diameter LB-agar plate (also prepared at least 3 days before to ensure
516 proper LB agar drying) and incubated for 1h at 37ºC. Cells were resuspended in 1000
517 µl of PBS, serially diluted and appropriate dilutions were plated on LB-agar plates
518 with antibiotics to count the number of D, R and T cells present. The same protocol
519 was applied in mating experiments at different times, with only two changes: a
520 1:10.000 ratio was also used (by putting 1/100 less donor volume in the same amount
521 of 10x concentrated recipient cells, as usual) and PBS was used to abort conjugation
522 at different times: 30, 60, 90, 120, 150 and 180 minutes.
523
524 Experiments measuring gene dosage effect on conjugation were conducted as the rest
525 of conjugation experiments explained above, except for the donor and recipients were
526 grown overnight on LB with antibiotics and glucose 0.5% (for pBAD33 arabinose
527 promoter repression) and washed and allowed to grow for 3h more in LB without
528 antibiotics with glucose 0.5% or arabinose 50µM (for arabinose promoter induction).
529 Each experiment was conducted simultaneously with a control donor containing the
530 conjugative plasmid and pBAD33 with no cargo. No transfer of pBAD33-derived
531 plasmids was observed. Conjugation efficiencies were determined as T/D and ratios
532 mean conjugation efficiency (three replicates) for the plasmid + pBAD33::trwX
533 donors divided by mean conjugation efficiency (three replicates) for the plasmid +

534 pBAD33 control were represented in graphs. In this case, if no change in conjugation
535 efficiency is observed, the expected mean ratio clone:control should be 1, while if
536 efficiency is improved by supplementing a gene or operon, the ratio should be greater
537 than 1. In this case, DH5α strain was used as donor to avoid recombination between
538 vector cargo and R388wt. Conjugation experiments in buffered LB were performed
539 at a pH=7.4 using Na_2HPO_4 10mM and NaH_2PO_4 1.8mM and pH adjusted with HCl 35%
540 and NaOH 1M.
541
542 **Statistical tests.** In order to check if conjugation efficiency measurements (as T/D
543 cell counts) did follow a normal distribution we performed a Shapiro-Wilk and an
544 Anderson-Darling test on two independent arrays of 16 measurements of R388
545 conjugation efficiency each. Both groups of T/D counts showed no normality, but
546 when the analysis was performed on their log-transformed values they showed
547 normal distribution (Shapiro-Wilk, W=0.97 both groups, Anderson-Darling test
548 A^{*2}=0.26 and 0.21 respectively), thus indicating log-normal distribution, as suggested
549 before (Gama et al., 2017; Lucas et al., 2010).
550
551 Paired comparisons of log(T/D) values were performed using two-sided t Student's
552 test, while multiple comparisons were performed using ANOVA. Unless otherwise
553 specified, a significance level of α= 0.05 was taken as good enough to reject null
554 hypothesis.
555

556 Nonlinear fitting of conjugation efficiencies at different cell concentrations was
557 performed with nonlinear curve fitting package of GraphPad Prism v8.0.2 using
558 custom equations (namely, Eqs 2 and 3).
559
560

561

562 **Table 2. Strains used in this work**

563

Strain	Genotype	Reference
DH5α	F^- endA1 hsdR17 (rk^-, mk^+) supE44 thi-1 λ^- recA1 gyrA96 relA1 deoRΔ(lacZYA-argF)-U169 φ80d lacZΔM15	(Grant et al., 1990)
BW27783	$lacI^q$ $rrnB_{T14}$ $lacZ_{WJ16}$ hsdR514 Δ($araBAD_{AH33}$) Δ($rhaBAD_{LD78}$) Δ(araFGH) φ(ΔaraEp P_{CP8}-araE)	(Khlebnikov et al., 2000, 2001)
MG1655	Dam $^+$, F $^-$, lambda$^-$, rph-1	(Blattner et al., 1997)
MDS42	MG1655Δ(fhuACDB) $endA^-$, $recA^-$, Δ(699 genes, ISs and prophages)	(Pósfai et al., 2006)
MDS42 Tra_N	MG1655::mpf_R46	This work
MDS42 Tra_W	MDS42::mpf_R388	This work
DH5α *pir*$^+$	endA1 hsdR17 glnV44(=supE44) thi-1 recA1 gyrA96 relA1 φ80dlacΔ(lacZ)M15 Δ(lacZYA-argF)U169 zdg-232::Tn10 uidA::pir^+	(Platt et al., 2000)

564

565

566

567

568

569 **Table 3. Plasmids used in this work**

570

Plasmid	Antibiotic Markers	Description	Reference
pSEVA121	Ap	RP4 replicon, mobilizable (RP4 oriT)	(Martínez-Garćía et al., 2015)
pBAD33	Cm	pACYC replicon, pBAD, araC	(Guzman et al., 1995)
pRL443	Ap, Tc	RP4ΔaphA (Kn^S), IncP1α, conjugative	(Elhai et al., 1997)
R388	Tp	IncW, conjugative	(Datta & Hedges, 1972)
pKM101	Ap	15 kb deletion of R46, IncN, conjugative	(Langer et al., 1981)
R7K	Ap	IncW, conjugative	(Coetzee et al., 1972)
pMBUI4	Tc	IncW, conjugativo	(Brown et al., 2013)
pIE321	Tc	IncW, conjugativo	(Götz et al., 1996)
pSU1027	Tp	R388 with p15a replicon	(Mötsch et al., 1985)
pSU4280	Cm	pSU19::Mob_pKM101	(Llosa et al., 2003)
pSEVA121::mobW	Ap	oriV_RK2, oriT_RP4, oriT-TrwABC_R388, mobilizable by R388 and RP4.	This work

571

572

573

Acknowledgements

This work was supported by the Spanish Ministry of Science and Innovation (Project PCI2021-122067-2A funded by MCIN/AEI/10.13039/501100011033 and the European Union NexGenerationEU/PRTR and by Project PID2019-110216GB-I00 funded by MCIN/ AEI /10.13039/501100011033 to RFL)

References

Anderson, R., & May, R. (1979). Population biology of infectious diseases: Part I. *Nature*. http://www.nature.com/nature/journal/v280/n5721/abs/280361a0.html%5Cnpapers2://publication/uuid/36FFBCB6-E8C2-4E0F-A08E-131E908EDBDF

Andrup, L., & Andersen, K. (1999). A comparison of the kinetics of plasmid transfer in the conjugation systems encoded by the F plasmid from Escherichia coli and plasmid pCF10 from Enterococcus faecalis. *Microbiology*, *145*(8), 2001–2009. https://doi.org/10.1099/13500872-145-8-2001

Babić, A., Lindner, A. B., Vulić, M., Stewart, E. J., & Radman, M. (2008). Direct visualization of horizontal gene transfer. *Science*, *319*(5869), 1533–1536. https://doi.org/10.1126/science.1153498

Backert, S., Fronzes, R., & Waksman, G. (2008). VirB2 and VirB5 proteins: specialized adhesins in bacterial type-IV secretion systems? *Trends in Microbiology*, *16*(9), 409–413. https://doi.org/10.1016/j.tim.2008.07.001

Baquero, F., Alvarez-Ortega, C., & Martinez, J. L. (2009). Ecology and evolution of antibiotic resistance. *Environmental Microbiology Reports*, *1*(6), 469–476. https://doi.org/10.1111/j.1758-2229.2009.00053.x

Bennett, P. M. (2008). Plasmid encoded antibiotic resistance: Acquisition and transfer of antibiotic resistance genes in bacteria. *British Journal of Pharmacology*, *153*(SUPPL. 1). https://doi.org/10.1038/sj.bjp.0707607

Bergstrom, C. T., Lipsitch, M., & Levin, B. R. (2000). Natural selection, infectious transfer and the existence conditions for bacterial plasmids. *Genetics*, *155*(4), 1505–1519.

Blattner, F. R., Plunkett, G., Bloch, C. A., Perna, N. T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J. D., Rode, C. K., Mayhew, G. F., Gregor, J., Davis, N. W., Kirkpatrick, H. A., Goeden, M. A., Rose, D. J., Mau, B., & Shao, Y. (1997). The complete genome sequence of Escherichia coli K-12. *Science (New York, N.Y.)*, *277*(5331), 1453–1462. https://doi.org/10.1126/science.277.5331.1453

Bolland, S., Llosa, M., Avila, P., & de la Cruz, F. (1990). General organization of the conjugal transfer genes of the IncW plasmid R388 and interactions between R388 and IncN and IncP plasmids. *Journal of Bacteriology*, *172*(10), 5795–5802. https://doi.org/10.1128/jb.172.10.5795-5802.1990

615 Bouma, J. E., & Lenski, R. E. (1988). Evolution of a bacteria/plasmid association.
616 *Nature*, *335*(6188), 351–352. https://doi.org/10.1038/335351a0
617 Brooks, L. E., Kaze, M., & Sistrom, M. (2019). Where the plasmids roam: Large-scale
618 sequence analysis reveals plasmids with large host ranges. *Microbial Genomics*, *5*(1).
619 https://doi.org/10.1099/mgen.0.000244
620 Brown, C. J., Sen, D., Yano, H., Bauer, M. L., Rogers, L. M., van der Auwera, G. A., & Top,
621 E. M. (2013). Diverse Broad-Host-Range Plasmids from Freshwater Carry Few
622 Accessory Genes. *Applied and Environmental Microbiology*, *79*(24), 7684–7695.
623 https://doi.org/10.1128/aem.02252-13
624 Cabezón, E., Sastre, J. I., & de la Cruz, F. (1997). Genetic evidence of a coupling role for
625 the TraG protein family in bacterial conjugation. *Molecular & general genetics:*
626 *MGG*, *254*(4), 400–406. https://doi.org/10.1007/s004380050432
627 Chandler, M., & Galas, D. J. (1983). Cointegrate formation mediated by Tn9. II. Activity
628 of IS1 is modulated by external DNA sequences. *Journal of Molecular Biology*.
629 https://doi.org/10.1016/S0022-2836(83)80227-7
630 Chandran Darbari, V., & Waksman, G. (2015). Structural Biology of Bacterial Type IV
631 Secretion Systems. *Annual Review of Biochemistry*, *84*(1), 603–629.
632 https://doi.org/10.1146/annurev-biochem-062911-102821
633 Christensen, B. B., Sternberg, C., Andersen, J. B., Eberl, L., Møller, S., Givskov, M., &
634 Molin, S. (1998). Establishment of new genetic traits in a microbial biofilm
635 community. *Applied and Environmental Microbiology*, *64*(6), 2247–2255.
636 Christensen, B. B., Sternberg, C., & Molin, S. (1996). Bacterial plasmid conjugation on
637 semi-solid surfaces monitored with the green fluorescent protein (GFP) from
638 Aequorea victoria as a marker. *Gene*. https://doi.org/10.1016/0378-
639 1119(95)00707-5
640 Coetzee, J. N., Datta, N., & Hedges, R. W. (1972). R factors from Proteus rettgeri.
641 *Journal of General Microbiology*, *72*(3), 543–552.
642 https://doi.org/10.1099/00221287-72-3-543
643 Charles Coluzzi, Maria Pilar Garcillán-Barcia, Fernando de la Cruz, Eduardo P.C. Rocha,
644 Evolution of Plasmid Mobility: Origin and Fate of Conjugative and Nonconjugative
645 Plasmids, *Molecular Biology and Evolution*, Volume 39, Issue 6, June 2022,
646 msac115, https://doi.org/10.1093/molbev/msac115
647 Contijoch, E. J., Britton, G. J., Yang, C., Mogno, I., Li, Z., Ng, R., Llewellyn, S. R., Hira, S.,
648 Johnson, C., Rabinowitz, K. M., Barkan, R., Dotan, I., Hirten, R. P., Fu, S.-C., Luo, Y.,
649 Yang, N., Luong, T., Labrias, P. R., Lira, S., … Faith, J. J. (2019). Gut microbiota density
650 influences host physiology and is shaped by host and microbial factors. *ELife*, *8*.
651 https://doi.org/10.7554/eLife.40553
652 Datsenko, K. A., & Wanner, B. L. (2000). One-step inactivation of chromosomal genes
653 in Escherichia coli K-12 using PCR products. *Proceedings of the National Academy of*
654 *Sciences of the United States of America*, *97*(12), 6640–6645.
655 https://doi.org/10.1073/pnas.120163297
656 Datta, N., & Hedges, R. W. (1972). Trimethoprim Resistance Conferred by W Plasmids
657 in Enterobacteriaceae. *Journal of General Microbiology*, *72*(2), 349–355.
658 https://doi.org/10.1099/00221287-72-2-349
659 de Gelder, L., Ponciano, J. M., Joyce, P., & Top, E. M. (2007). Stability of a promiscuous
660 plasmid in different hosts: No guarantee for a long-term relationship. *Microbiology*,
661 *153*(2), 452–463. https://doi.org/10.1099/mic.0.2006/001784-0

662 de Gelder, L., Williams, J. J., Ponciano, J. M., Sota, M., & Top, E. M. (2008). Adaptive
663 plasmid evolution results in host-range expansion of a broad-host-range plasmid.
664 *Genetics*, *178*(4), 2179–2190. https://doi.org/10.1534/genetics.107.084475
665 del Campo, I., Ruiz, R., Cuevas, A., Revilla, C., Vielva, L., & de la Cruz, F. (2012).
666 Determination of conjugation rates on solid surfaces. *Plasmid*, *67*(2), 174–182.
667 https://doi.org/10.1016/j.plasmid.2012.01.008
668 Dimitriu, T., Marchant, L., Buckling, A., & Raymond, B. (2019). Bacteria from natural
669 populations transfer plasmids mostly towards their kin. *Proceedings of the Royal*
670 *Society B: Biological Sciences*, *286*(1905). https://doi.org/10.1098/rspb.2019.1110
671 Dionisio, F., Conceição, I. C., Marques, A. C. R., Fernandes, L., & Gordo, I. (2005). The
672 evolution of a conjugative plasmid and its ability to increase bacterial fitness.
673 *Biology Letters*, *1*(2), 250–252. https://doi.org/10.1098/rsbl.2004.0275
674 Dionisio, F., Matic, I., Radman, M., Rodrigues, O. R., & Taddei, F. (2002). Plasmids
675 spread very fast in heterogeneous bacterial communities. *Genetics*, *162*(4), 1525–
676 1532. http://www.ncbi.nlm.nih.gov/pubmed/12524329
677 Elhai, J., Vepritskiy, A., Muro-Pastor, A. M., Flores, E., & Wolk, C. P. (1997). Reduction
678 of conjugal transfer efficiency by three restriction activities of Anabaena sp. strain
679 PCC 7120. *Journal of Bacteriology*, *179*(6), 1998–2005.
680 https://doi.org/10.1128/jb.179.6.1998-2005.1997
681 Fernandez-Lopez, R., Machón, C., Longshaw, C. M., Martin, S., Molin, S., Zechner, E. L.,
682 Espinosa, M., Lanka, E., & de la Cruz, F. (2005). Unsaturated fatty acids are inhibitors
683 of bacterial conjugation. *Microbiology*, *151*(11), 3517–3526.
684 https://doi.org/10.1099/mic.0.28216-0
685 Fernández-López, R., Pilar Garcillán-Barcia, M., Revilla, C., Lázaro, M., Vielva, L., & de
686 La Cruz, F. (2006). Dynamics of the IncW genetic backbone imply general trends in
687 conjugative plasmid evolution. *FEMS Microbiology Reviews*, *30*(6), 942–966.
688 https://doi.org/10.1111/j.1574-6976.2006.00042.x
689 Fernandez-Lopez, R., Del Campo, I., Revilla, C., Cuevas, A., & de la Cruz, F. (2014).
690 Negative feedback and transcriptional overshooting in a regulatory network for
691 horizontal gene transfer. *PLoS genetics*, *10*(2), e1004171.
692 https://doi.org/10.1371/journal.pgen.1004171
693 Fox, R. E., Zhong, X., Krone, S. M., & Top, E. M. (2008). Spatial structure and nutrients
694 promote invasion of IncP-1 plasmids in bacterial populations. *The ISME Journal*,
695 *2*(10), 1024–1039. https://doi.org/10.1038/ismej.2008.53
696 Fux, C. A., Costerton, J. W., Stewart, P. S., & Stoodley, P. (2005). Survival strategies of
697 infectious biofilms. *Trends in Microbiology*, *13*(1), 34–40.
698 https://doi.org/10.1016/J.TIM.2004.11.010
699 Gama, J. A., Zilhão, R., & Dionisio, F. (2017). Conjugation efficiency depends on intra
700 and intercellular interactions between distinct plasmids: Plasmids promote the
701 immigration of other plasmids but repress co-colonizing plasmids. *Plasmid*,
702 *93*(April), 6–16. https://doi.org/10.1016/j.plasmid.2017.08.003
703 Garcillán-Barcia, M. P., & de la Cruz, F. (2013). Ordering the bestiary of genetic
704 elements transmissible by conjugation. *Mobile Genetic Elements*, *3*(1), e24263.
705 https://doi.org/10.4161/mge.24263
706 Getino, M, Palencia-Gándara, C, Garcillán-Barcia, M.P., & de la Cruz, F (2017). PifC and
707 Osa, plasmid weapons against rival conjugative coupling proteins. *Frontiers in*
708 *Microbiology, 8*.

https://doi.org/10.3389/fmicb.2017.02260
Gibson, D. G., Young, L., Chuang, R. Y., Venter, J. C., Hutchison, C. A., & Smith, H. O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods*, *6*(5), 343–345. https://doi.org/10.1038/nmeth.1318
González-Rivera, C., Khara, P., Awad, D., Patel, R., Li, Y. G., Bogisch, M., & Christie, P. J. (2019). Two pKM101-encoded proteins, the pilus-tip protein TraC and Pep, assemble on the Escherichia coli cell surface as adhesins required for efficient conjugative DNA transfer. *Molecular microbiology*, *111*(1), 96–117. https://doi.org/10.1111/mmi.14141
Gordon, D. M. (1992). Rate of plasmid transfer among Escherichia coli strains isolated from natural populations. *Journal of General Microbiology*, *138*(1), 17–21. https://doi.org/10.1099/00221287-138-1-17
Götz, A., Pukall, R., Smit, E., Tietze, E., Prager, R., Tschäpe, H., van Elsas, J. D., & Smalla, K. (1996). Detection and characterization of broad-host-range plasmids in environmental bacteria by PCR. *Applied and Environmental Microbiology*, *62*(7), 2621–2628. http://www.ncbi.nlm.nih.gov/pubmed/8779598
Grant, S. G. N., Jessee, J., Bloom, F. R., & Hanahan, D. (1990). Differential plasmid rescue from transgenic mouse DNAs into Escherichia coli methylation-restriction mutants. *Proceedings of the National Academy of Sciences of the United States of America*, *87*(12), 4645–4649. https://doi.org/10.1073/pnas.87.12.4645
Guglielmini, J., de La Cruz, F., & Rocha, E. P. C. (2013). Evolution of conjugation and type IV secretion systems. *Molecular Biology and Evolution*, *30*(2), 315–331. https://doi.org/10.1093/molbev/mss221
Guynet, C., Cuevas, A., Moncalián, G., & de la Cruz, F. (2011). The stb operon balances the requirements for vegetative stability and conjugative transfer of plasmid R388. *PLoS Genetics*, *7*(5). https://doi.org/10.1371/journal.pgen.1002073
Guzman, L. M., Belin, D., Carson, M. J., & Beckwith, J. (1995). Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *Journal of Bacteriology*, *177*(14), 4121–4130. http://www.ncbi.nlm.nih.gov/pubmed/7608087%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC177145
Hall-Stoodley, L., Costerton, J. W., & Stoodley, P. (2004). Bacterial biofilms: from the Natural environment to infectious diseases. *Nature Reviews Microbiology*, *2*(2), 95–108. https://doi.org/10.1038/nrmicro821
Harrington, L. C., & Rogerson, A. C. (1990). The F pilus of Escherichia coli appears to support stable DNA transfer in the absence of wall-to-wall contact between cells. *Journal of Bacteriology*, *172*(12), 7263–7264. https://doi.org/10.1128/jb.172.12.7263-7264.1990
Harrison, E., Jamie Wood, A., Dytham, C., Pitchford, J. W., Truman, J., Spiers, A., Paterson, S., & Brockhurst, M. A. (2015). Bacteriophages limit the existence conditions for conjugative plasmids. *MBio*, *6*(3), 1–9. https://doi.org/10.1128/mBio.00586-15
Hausner, M., & Wuertz, S. (1999). High rates of conjugation in bacterial biofilms as determined by quantitative in situ analysis. *Applied and Environmental Microbiology*, *65*(8), 3710–3713. http://www.ncbi.nlm.nih.gov/pubmed/10427070

754 Holling, C. S. (1959a). The Components of Predation as Revealed by a Study of Small-
755 Mammal Predation of the European Pine Sawfly. *The Canadian Entomologist*, *91*(5),
756 293–320. https://doi.org/10.4039/Ent91293-5
757 Holling, C. S. (1959b). Some Characteristics of Simple Types of Predation and
758 Parasitism. *The Canadian Entomologist*, *91*(7), 385–398.
759 https://doi.org/10.4039/Ent91385-7
760 Jain, A., & Srivastava, P. (2013). Broad host range plasmids. *FEMS Microbiology*
761 *Letters*, *348*(2), 87–96. https://doi.org/10.1111/1574-6968.12241
762 Johnsen, A. R., & Kroer, N. (2007). Effects of stress and other environmental factors on
763 horizontal plasmid transfer assessed by direct quantification of discrete transfer
764 events. *FEMS Microbiology Ecology*, *59*(3), 718–728.
765 https://doi.org/10.1111/j.1574-6941.2006.00230.x
766 Khlebnikov, A., Datsenko, K. A., Skaug, T., Wanner, B. L., & Keasling, J. D. (2001).
767 Homogeneous expression of the P(BAD) promoter in Escherichia coli by constitutive
768 expression of the low-affinity high-capacity AraE transporter. *Microbiology*
769 *(Reading, England)*, *147*(Pt 12), 3241–3247. https://doi.org/10.1099/00221287-
770 147-12-3241
771 Khlebnikov, A., Risa, Skaug, T., Carrier, T. A., & Keasling, J. D. (2000). Regulatable
772 arabinose-inducible gene expression system with consistent control in all cells of a
773 culture. *Journal of Bacteriology*, *182*(24), 7029–7034.
774 https://doi.org/10.1128/JB.182.24.7029-7034.2000
775 Kneis, D., Hiltunen, T., & Heß, S. (2019). A high-throughput approach to the culture-
776 based estimation of plasmid transfer rates. *Plasmid*, *101*(October 2018), 28–34.
777 https://doi.org/10.1016/j.plasmid.2018.12.003
778 Król, J. E., Wojtowicz, A. J., Rogers, L. M., Heuer, H., Smalla, K., Krone, S. M., & Top, E. M.
779 (2013). Invasion of E. coli biofilms by antibiotic resistance plasmids. *Plasmid*, *70*(1),
780 110–119. https://doi.org/10.1016/j.plasmid.2013.03.003
781 Lacroix, B., & Citovsky, V. (2011). Extracellular VirB5 enhances T-DNA transfer from
782 agrobacterium to the host plant. *PLoS ONE*, *6*(10).
783 https://doi.org/10.1371/journal.pone.0025578
784 Lagido, C., Wilson, I.J., Glover, L.A., Prosser, J.I. A model for bacterial conjugal gene
785 transfer on solid surfaces, *FEMS Microbiology Ecology*, Volume 44, Issue 1, May
786 2003, Pages 67–78, https://doi.org/10.1016/S0168-6496(02)00453-1
787 Langer, P. J., Shanabruch, W. G., & Walker, G. C. (1981). Functional organization of
788 plasmid pKM101. *Journal of Bacteriology*, *145*(3), 1310–1316.
789 Lawley, T. D., Gordon, G. S., Wright, A., & Taylor, D. E. (2002). Bacterial conjugative
790 transfer: Visualization of successful mating pairs and plasmid establishment in live
791 Escherichia coli. *Molecular Microbiology*, *44*(4), 947–956.
792 https://doi.org/10.1046/j.1365-2958.2002.02938.x
793 Lederberg, J., Cavalli, L. L., & Lederberg, E. M. (1952). Sex Compatibility in Escherichia
794 Coli. *Genetics*, *37*(6), 720–730. https://doi.org/10.1093/genetics/37.6.720
795 Levin, B. R., & Stewart, F. M. (1980). The population biology of bacterial plasmids: a
796 priori conditions for the existence of mobilizable nonconjugative factors. *Genetics*,
797 *94*(2), 425–443. http://www.ncbi.nlm.nih.gov/pubmed/6248416
798 Levin, B. R., Stewart, F. M., & Rice, V. A. (1979). The kinetics of conjugative plasmid
799 transmission: Fit of a simple mass action model. *Plasmid*, *2*(2), 247–260.
800 https://doi.org/10.1016/0147-619X(79)90043-X

Li, Q., Chang, W., Zhang, H., Hu, D., & Wang, X. (2019). The role of plasmids in the multiple antibiotic resistance transfer in ESBLs-producing Escherichia coli isolated from wastewater treatment plants. *Frontiers in Microbiology*, *10*(APR). https://doi.org/10.3389/fmicb.2019.00633

Llosa, M., Roy, C., & Dehio, C. (2009). Bacterial type IV secretion systems in human disease. *Molecular Microbiology*, *73*(2), 141–151. https://doi.org/10.1111/j.1365-2958.2009.06751.x

Llosa, M., Zunzunegui, S., & de la Cruz, F. (2003). Conjugative coupling proteins interact with cognate and heterologous VirB10-like proteins while exhibiting specificity for cognate relaxosomes. *Proceedings of the National Academy of Sciences*, *100*(18), 10465–10470. https://doi.org/10.1073/pnas.1830264100

Loftie-Eaton, W., Yano, H., Burleigh, S., Simmons, R. S., Hughes, J. M., Rogers, L. M., Hunter, S. S., Settles, M. L., Forney, L. J., Ponciano, J. M., & Top, E. M. (2016). Evolutionary paths that expand plasmid host-range: Implications for spread of antibiotic resistance. *Molecular Biology and Evolution*, *33*(4), 885–897. https://doi.org/10.1093/molbev/msv339

Lopatkin, A. J., Meredith, H. R., Srimani, J. K., Pfeiffer, C., Durrett, R., & You, L. (2017). Persistence and reversal of plasmid-mediated antibiotic resistance. *Nature Communications*, *8*(1). https://doi.org/10.1038/s41467-017-01532-1

Lucas, M., González-Pérez, B., Cabezas, M., Moncalian, G., Rivas, G., & de La Cruz, F. (2010). Relaxase DNA binding and cleavage are two distinguishable steps in conjugative DNA processing that involve different sequence elements of the nic site. *Journal of Biological Chemistry*, *285*(12), 8918–8926. https://doi.org/10.1074/jbc.M109.057539

Madsen, J. S., Burmølle, M., Hansen, L. H., & Sørensen, S. J. (2012). The interconnection between biofilm formation and horizontal gene transfer. *FEMS Immunology and Medical Microbiology*, *65*(2), 183–195. https://doi.org/10.1111/j.1574-695X.2012.00960.x

Martinez, E., & de la Cruz, F. (1988). Transposon Tn 21 encodes a RecA-independent site-specific integration system. *MGG Molecular & General Genetics*. https://doi.org/10.1007/BF00330610

Martínez-Garćía, E., Aparicio, T., Goñi-Moreno, A., Fraile, S., & de Lorenzo, V. (2015). SEVA 2.0: An update of the Standard European Vector Architecture for de-/re-construction of bacterial functionalities. *Nucleic Acids Research*, *43*(D1), D1183–D1189. https://doi.org/10.1093/nar/gku1114

May, R. M., & Anderson, R. M. (1979). Population biology of infectious diseases: Part II. *Nature*, *280*(9), 455–461.

Meyer, R. J., & Shapiro, J. A. (1980). Genetic organization of the broad-host-range incP-1 plasmid R751. *Journal of Bacteriology*, *143*(3), 1362–1373.

Meynell, E., & Datta, N. (1967). Mutant Drug Resistant Factors of High Transmissibility. *Nature*, *214*(5091), 885–887. https://doi.org/10.1038/214885a0

Mötsch, S., Schmitt, R., Avila, P., de la Cruz, F., Ward, E., & Grinsted, J. (1985). Junction sequences generated by "one-ended transposition." *Nucleic Acids Research*, *13*(9), 3335–3342. https://doi.org/10.1093/nar/13.9.3335

Platt, R., Drescher, C., Park, S. K., & Phillips, G. J. (2000). Genetic system for reversible integration of DNA constructs and lacZ gene fusions into the Escherichia coli chromosome. *Plasmid*, *43*(1), 12–23. https://doi.org/10.1006/plas.1999.1433

Pósfai, G., Plunkett, G., Fehér, T., Frisch, D., Keil, G. M., Umenhoffer, K., Kolisnychenko, V., Stahl, B., Sharma, S. S., de Arruda, M., Burland, V., Harcum, S. W., Blattner, F. R., & Posfai, G. (2006). Emergent properties of reduced-genome Escherichia coli. *Science*, *312*(5776), 1044–1046. https://doi.org/10.1126/science.1126439

Raynaud, X., & Nunan, N. (2014). Spatial Ecology of Bacteria at the Microscale in Soil. *PLoS ONE*, *9*(1), e87217. https://doi.org/10.1371/journal.pone.0087217

Redondo-Salvo, S., Fernández-López, R., Ruiz, R., Vielva, L., de Toro, M., Rocha, E. P. C., Garcillán-Barcia, M. P., & de la Cruz, F. (2020). Pathways for horizontal gene transfer in bacteria revealed by a global map of their plasmids. *Nature Communications*, *11*(1). https://doi.org/10.1038/s41467-020-17278-2

Reisner, A., Wolinski, H., & Zechner, E. L. (2012). In situ monitoring of IncF plasmid transfer on semi-solid agar surfaces reveals a limited invasion of plasmids in recipient colonies. *Plasmid*, *67*(2), 155–161. https://doi.org/10.1016/j.plasmid.2012.01.001

Revilla, C., Garcillán-Barcia, M. P., Fernández-López, R., Thomson, N. R., Sanders, M., Cheung, M., Thomas, C. M., & de La Cruz, F. (2008). Different pathways to acquiring resistance genes illustrated by the recent evolution of IncW plasmids. *Antimicrobial Agents and Chemotherapy*, *52*(4), 1472–1480

Robledo, M., Álvarez, B., Cuevas, A., González, S., Ruano-Gallego, D., Fernández, L.A., de la Cruz, F. Targeted bacterial conjugation mediated by synthetic cell-to-cell adhesions, *Nucleic Acids Research*, Volume 50, Issue 22, 9 December 2022, Pages 12938–12950.

Rodriguez-Grande J, Fernandez-Lopez R. Measuring Plasmid Conjugation Using Antibiotic Selection. *Methods Mol Biol*. 2020;2075:93-98. doi:10.1007/978-1-4939-9877-7_6

Sampei, G. ichi, Furuya, N., Tachibana, K., Saitou, Y., Suzuki, T., Mizobuchi, K., & Komano, T. (2010). Complete genome sequence of the incompatibility group I1 plasmid R64. *Plasmid*, *64*(2), 92–103. https://doi.org/10.1016/j.plasmid.2010.05.005

San Millan, A., Heilbron, K., & MacLean, R. C. (2014). Positive epistasis between co-infecting plasmids promotes plasmid survival in bacterial populations. *ISME Journal*, *8*(3), 601–612. https://doi.org/10.1038/ismej.2013.182

San Millan, A., & MacLean, R. C. (2017). Fitness Costs of Plasmids: a Limit to Plasmid Transmission. *Microbiology Spectrum*, *5*(5), 1–12. https://doi.org/10.1128/microbiolspec.mtbp-0016-2017

Schmidt-Eisenlohr, H., Domke, N., Angerer, C., Wanner, G., Zambryski, P. C., & Baron, C. (1999). Vir proteins stabilize VirB5 and mediate its association with the T pilus of Agrobacterium tumefaciens. *Journal of Bacteriology*, *181*(24), 7485–7492. https://doi.org/10.1128/jb.181.24.7485-7492.1999

Schmidt-Eisenlohr, H., Domke, N., & Baron, C. (1999). TraC of IncN plasmid pKM101 associates with membranes and extracellular high-molecular-weight structures in Escherichia coli. *Journal of Bacteriology*, *181*(18), 5563–5571. https://doi.org/10.1128/jb.181.18.5563-5571.1999

Seoane, J., Yankelevich, T., Dechesne, A., Merkey, B., Sternberg, C., & Smets, B. F. (2011). An individual-based approach to explain plasmid invasion in bacterial populations. *FEMS Microbiology Ecology*, *75*(1), 17–27. https://doi.org/10.1111/j.1574-6941.2010.00994.x

Simonsen, L. (1991). The existence conditions for bacterial plasmids: Theory and reality. *Microbial Ecology*, *22*(1), 187–205. https://doi.org/10.1007/BF02540223

Simonsen, L., Gordon, D. M., Stewart, F. M., & Levin, B. R. (1990). Estimating the rate of plasmid transfer: an end-point method. *Journal of General Microbiology*, *136*(11), 2319–2325. https://doi.org/10.1099/00221287-136-11-2319

Smets, B. F., & Lardon, L. (2009). Mass Action Models Describing Extant Horizontal Transfer of Plasmids: Inferences and Parameter Sensitivities (pp. 289–305). https://doi.org/10.1007/978-1-60327-853-9_17

Smillie C, Garcillán-Barcia MP, Francia MV, Rocha EP, de la Cruz F. (2010) Mobility of plasmids. *Microbiol Mol Biol Rev*. 2010 Sep;74(3):434-52. doi: 10.1128/MMBR.00020-10. PMID: 20805406; PMCID: PMC2937521.

Stewart, F. M., & Levin, B. R. (1977). The population biology of bacterial plasmids: a priori conditions for the existence of conjugationally transmitted factors. *Genetics*, *87*(2), 209–228.

St-Pierre, F., Cui, L., Priest, D. G., Endy, D., Dodd, I. B., & Shearwin, K. E. (2013). One-step cloning and chromosomal integration of DNA. *ACS Synthetic Biology*, *2*(9), 537–541. https://doi.org/10.1021/sb400021j

Tatum, E. L., & Lederberg, J. (1947). Gene Recombination in the Bacterium Escherichia coli. *Journal of Bacteriology*, *53*(6), 673–684.

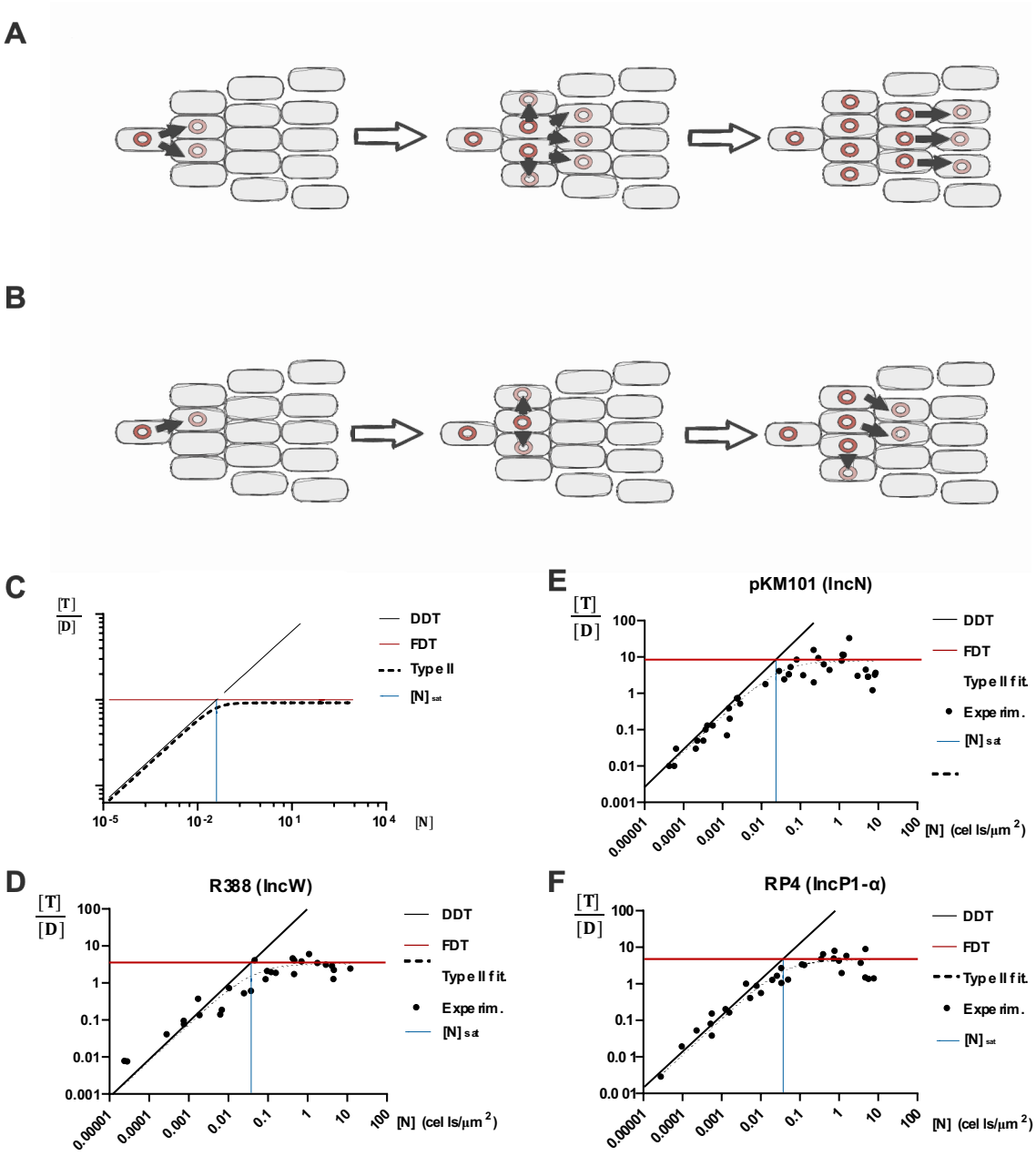
Turner, P. E. (2004). Phenotypic Plasticity in Bacterial Plasmids. *Genetics*, *167*(1), 9–20. https://doi.org/10.1534/genetics.167.1.9

Wan, Z., Varshavsky, J., Teegala, S., McLawrence, J., & Goddard, N. L. (2011). Measuring the rate of conjugal plasmid transfer in a bacterial population using quantitative PCR. *Biophysical Journal*, *101*(1), 237–244. https://doi.org/10.1016/j.bpj.2011.04.054

Watanabe, T., Furuse, C., & Sakaizumi, S. (1968). Transduction of various R factors by phage P1 in Escherichia coli and by phage P22 in Salmonella typhimurium. *Journal of Bacteriology*, *96*(5), 1791–1795.

Yeo, H.-J., Yuan, Q., Beck, M. R., Baron, C., & Waksman, G. (2003). Structural and functional characterization of the VirB5 protein from the type IV secretion system encoded by the conjugative plasmid pKM101. *Proceedings of the National Academy of Sciences*, *100*(26), 15947–15952. https://doi.org/10.1073/pnas.2535211100

Zwanzig, M., Harrison, E., Brockhurst, M. A., Hall, J. P. J., Berendonk, T. U., & Berger, U. (2019). Mobile Compensatory Mutations Promote Plasmid Survival. *MSystems*, *4*(1), 1–11. https://doi.org/10.1128/msystems.00186-18



934
935
936 ***Figure 1. Conjugation follows a type II Functional response. A)*** *In a Density-Dependent Transmission*
937 *(DDT) regime, plasmid conjugation increases linearly with increasingly available recipients; cell-to-cell*
938 *contact is the only limiting factor,* ***B)*** *in a Frequency Dependent-Trasmission (FDT) regime donors are*
939 *limited by the time required to complete the transfer event. Transmission is not limited by encountering*
940 *susceptible receptors.* ***C)*** *Number of transconjugants per donor expected (y axis) as a function of the total*
941 *cell population (N, x axis). In a Holling´s Type II functional response there is a density-dependent region*
942 *where transconjugant formation increases proportionally with N (leftwards from the blue line). When N*
943 *becomes saturating the formation of transconjugants does not scales any further with cell density and*
944 *becomes frequency-limited (rightwards from the blue line).* ***D, E, F)*** *Transconjugants per donor ([T]/[D],*
945 *y axis) measured at different Recipient densities ([R]≈[N], x axis) in E. coli BW27783 mating on solid LB-*
946 *agar surfaces and 1 h. conjugation time for plasmids D) R388, E) pKM101 and F) RP4. The black line*
947 *represents the DDT regime, where different* k_{on} *produce different y-intercepts. The red line represents the*
948 *FDT regime, where the conjugation efficiency is just 1/ τ and the dotted curve corresponds to the fitting*
949 *to Eq.3. Every black dot corresponds to the average of 3 conjugation experiments.*

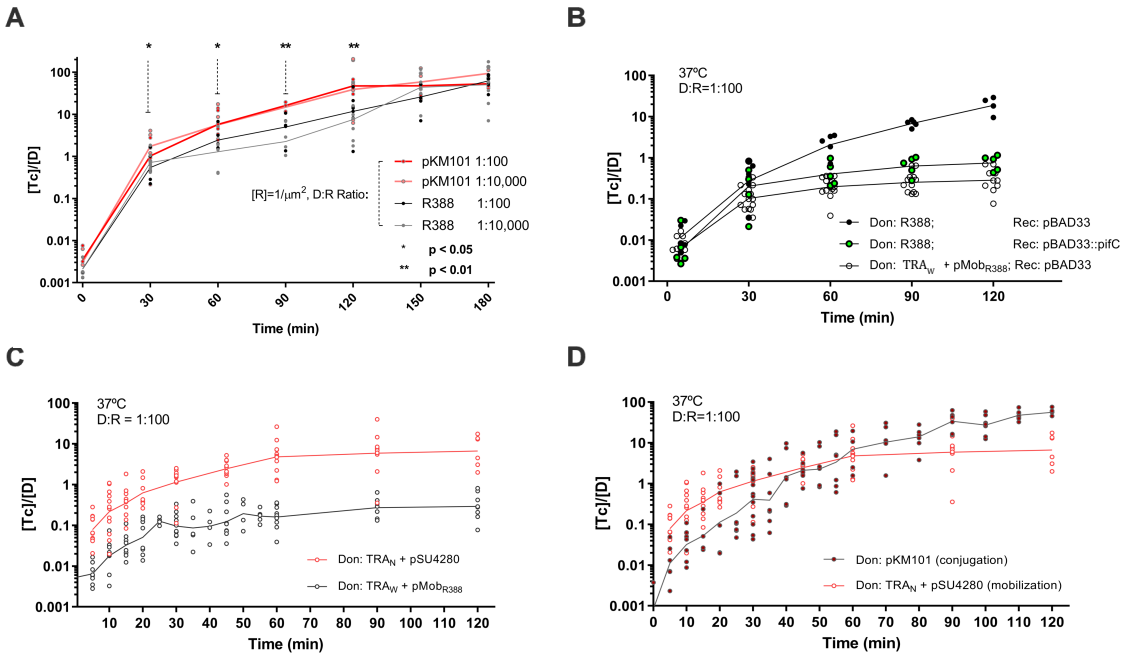


Figure 2.- Plasmid propagation efficiency through time, as transconjugant per donor cell production at different time intervals. A) *Plasmids R388 and pKM101 conjugation rates at 1:100 and 1:10000 D/R ratio.* ***B)*** *Comparison of plasmid R388 conjugation efficiency (black dots) vs R388-derived constructions. Black dots correspond to the wt plasmid transferred to a recipient strain that contains an empty pBAD expression vector. Green dots represent values obtained in conjugations where donors contained wild-type R388, and recipients harboured a pBAD::pifC construction, able to block secondary transmission. White dots correspond to the mobilization of a plasmid containing the MOB region of plasmid R388 from a strain harbouring a chromosomal integration of the* TRA_W *genes.* ***C)*** *Mobilization dynamics obtained using the TRA genes from PKM101 (*TRA_N*, red) and the TRA genes from R388 (*TRA_W*, black) integrated in the chromosome of E.coli . The mobilizable plasmid in each case contained the MOB region of each corresponding plasmid.* ***D)*** *Mobilization (red circunferences) and conjugation (maroon dots) dynamics of PKM101. The mobilization dynamics was obtained using a mobilizable vector containing* MOB_N *harboured by a strain that contained the* TRA_N *genes inserted in the chromosome. The conjugation dynamics was obtained using the wt pKM101 plasmid.*

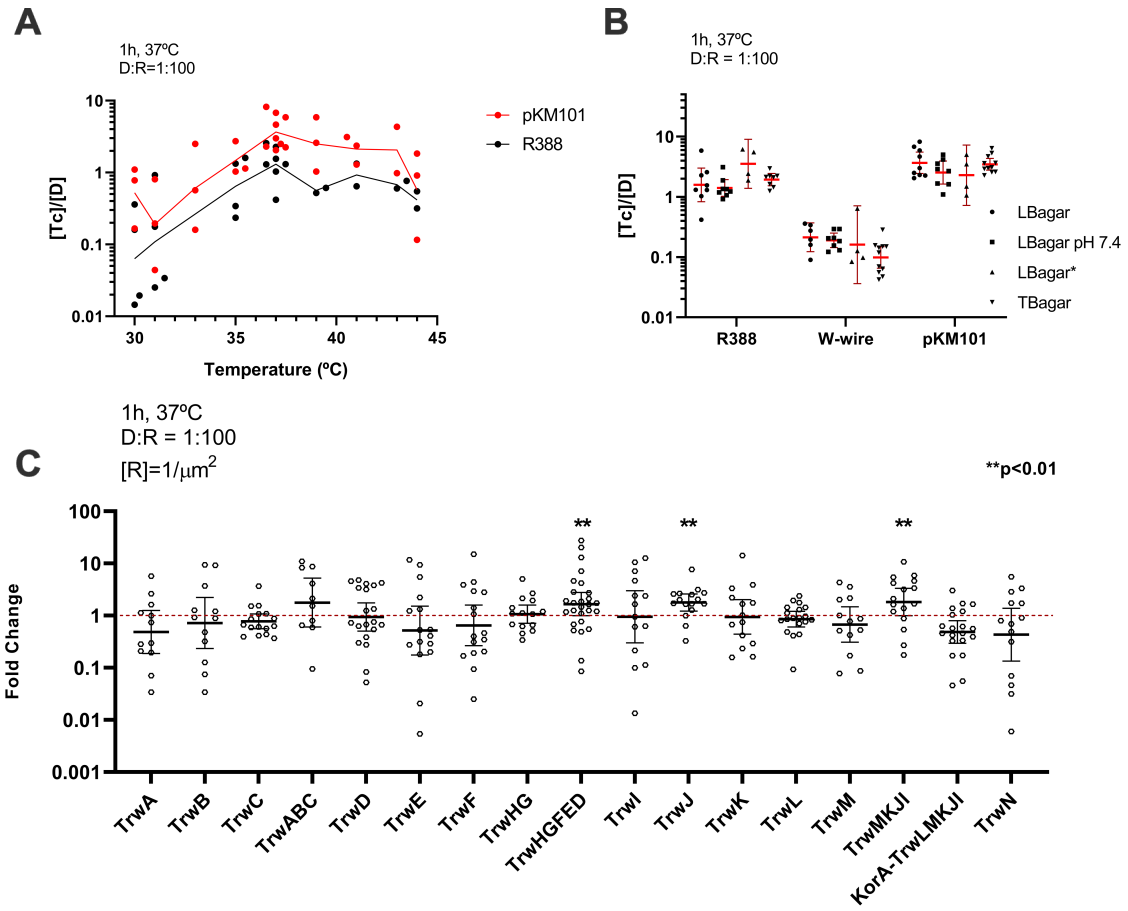


Figure 3. Effect of environmental conditions and protein expression on conjugation rates. ***a)*** *Effect of temperature on the conjugation rate. Conjugation assays were performed as described in Materials and Methods. (1h, 37ºC, 1D:100R). Each point corresponds to an independent experiment* ***b)*** *Effect of media composition on the transfer rate (LBagar: autoclaved LBagar, LBagar pH7.4: PBS-buffered at pH 7.4, LBagar*: non-autoclaved LBagar and TBagar: Terrific Broth agar.* ***c)*** *Overexpression of TRA genes does not increase the transfer rate. On the x axis, genes overexpressed in R388-carrying donors using a pBAD33 vector. On the y axis, the ratio between the conjugation frequency obtained overproducing each of the constructions shown in the figure, and the conjugation frequency obtained with an empty vector.*

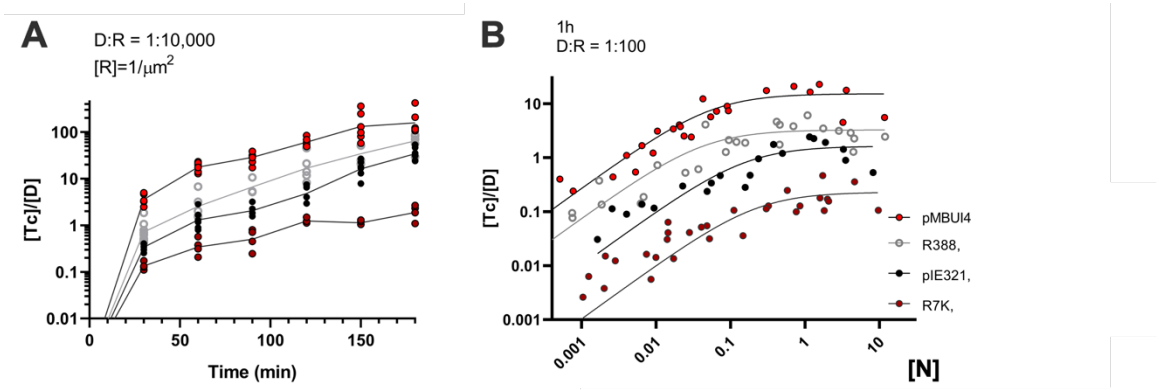


Figure 4. Plasmids from the same PTU display different conjugation dynamics. (A) *Conjugation kinetics for different plasmids of the same PTU. The conjugation frequency, expressed as Tc / D is indicated on the y axis. The conjugation time, in minutes, is indicated on the x axis.* ***(B)*** *Conjugation frequencies (y axis) obtained at different cell densities (x axis) for different plasmids belonging to PTU-W. Estimated handling times (τ) and searching rates (k_{on}) for each plasmid correspond to k_{PMBUI4}= 159 $\mu m^2 \cdot h^{-1}$ τ_{pMBUI4}=0.3 h , k_{R388}= 36 $\mu m^2 \cdot h^{-1}$ τ_{R388}=0.7 h, k_{pIE321}= 8.63 $\mu m^2 \cdot h^{-1}$ τ_{pIE321}=1 h , k_{R7K}= 1.8 $\mu m^2 \cdot h^{-1}$ τ_{R7K}=6.6 h.*

SUPPLEMENTARY CALCULATIONS

Fundamental parameters governing the transmission of conjugative plasmids

Jorge Rodriguez-Grande, Maria del Pilar Garcillan, Fernando de la Cruz and Raul Fernandez-Lopez

1.- Deriving the functional response for plasmid mobilization

To obtain the dynamics of mobilization we pose a model with three different types of cells:

D = donors
R= recipients
T= transconjugants

The model assumes that *D* cells encounter *R* in a density-dependent fashion, producing a complex *C*. The conjugative complex may progress to the formation of a transconjugant *T*, or may result in a non-productive mating (by premature termination of the conjugative pair), such that:

$$D + R \underset{koff}{\overset{kon}{\leftrightarrow}} C \overset{Kc}{\rightarrow} D + T$$

Eq.1

We also will perform our experiments in the regime where $R >> D$, thus the number of recipients may be considered a constant. Under these assumptions, the differential equations governing the progression of the population follow:

$$\frac{dD}{dt} = C\ koff + C\ kc - D\ R\ kon$$

Eq. 2

$$\frac{dC}{dt} = D\ R\ kon - C\ koff - C\ kc$$

Eq. 3

$$\frac{dT}{dt} = C\ kc$$

Eq. 4

The system is thus formally equivalent to a Michaelis-Mentem reaction scheme, following the Haldane approximation, we can pose the following:

1) Pseudo-steady state: Given enough time t , formation of C complexes will be in steady-state, thus:

$$D\ R\ kon = C\ (koff +\ kc)$$

Eq. 5

2) Preservation of the total number of D: the contribution of vegetative growth during the experiment is considered negligible

$$D(0) = D(t) + C(t)$$

Eq. 6

This allows us to pose an equation which is formally equivalent to that of the MM process under the Haldane approximation:

$$C = \frac{D_0\ R}{\frac{koff + kc}{kon} + R}$$

Eq. 7

$$\frac{dT}{dt} = kc\ C = \frac{D_0\ R\ kc}{\frac{koff + kc}{kon} + R} = \frac{D_0\ R\ kc\ kon}{koff + kc + R\ kon}$$

Eq. 8

Now, we can introduce the average lifetime of C, the time required for the C conjugative complex to resolve into the formation of transconjugants or an abortive reversion to D + R. Such average lifetime (τ) is just 1 / ($koff + kc$). Introducing this factor into Eq. 8 yields

$$\frac{dT}{dt} = kc\ C = \frac{D_0\ R\ kc}{\frac{koff + kc}{kon} + R} = \frac{D_0\ R\ kc}{\frac{1}{\tau\ kon} + R} = \frac{D_0\ R\ kon\ \tau\ kc}{1 + R\ kon\ \tau} = \frac{D_0\ R\ kon}{1 + R\ kon\ \tau}\ \frac{1}{1 + koff/kc}$$

Eq. 9

This Eq can be now directly integrated yielding the following expression for the formation of transconjugants per donor :

$$\frac{T}{D_0} = \left(\frac{R\ kon}{1 + R\ kon\ \tau}\right)\left(\frac{1}{1 + \frac{koff}{kc}}\right)t$$

Eq. 10

Now, in conditions where the formation of a transconjugant is much more likely than the abortion of the conjugative pair, such that kc>> koff Eq 10 simplifies to an expression which is formally equivalent to Holling´s type II functional response:

$$\frac{T}{D_0} = \left(\frac{R\ kon}{1 + R\ kon\ \tau}\right)\ t$$

Eq. 11

In this Eq the transconjugants generated per donor cell depend on two constants: a *kon* parameter that indicates the rate at which donor cells find and attach to recipients, and an effective conjugation time, τ, indicating the time required for a donor-recipient complex to resolve into a new transconjugant. In those cases where the abortion rate of the conjugative plasmid is substantial, the rate of transconjugant formation will be affected by a term which is just the probability of the C complex resulting into the successful formation of a new transconjugant:

$$\frac{T}{D_0} = \left(\frac{R\ kon}{1 + R\ kon\ \tau}\right)\left(\frac{kc}{kc + koff}\right)\ t$$

Eq. 12

<u>2.- Deriving the functional response for plasmid conjugation</u>

The dynamics of plasmid conjugation are significantly more complex than that of mobilization. The newly formed transconjugants can act as donors, a process which is often assumed to involve a lag period. Additionally, transconjugants often exhibit higher conjugation rates than the parental donor population, due to the transitory de-repression of the conjugation machinery after plasmid transmission. An autocatalytic process of this nature, involving lags of variable time and non-linear dynamics, frequently exhibits too complicated dynamics to be faithfully parametrized. We can, however, modify the experimental conditions to simplify the dynamics and obtain the searching rate and the effective conjugation time.

The simplest approximation is to assume that, in conjugative plasmids, the rate of formation of new transconjugants is proportional to the number of donors D_0 and already formed transconjugants (T_t). In these conditions:

$$\frac{\mathrm{dT}}{dt} = \frac{R\ kon}{1 + R\ kon\ \tau}\ (T_t + D_0)$$

Eq. 13

This expression can be directly integrated, yielding:

$$Ln(1+\frac{T}{D_0}) = \frac{R\ kon}{1+R\ kon\ \tau}t$$

Eq. 14

For plasmids with low conjugation rates the overall contribution of the newly formed transconjugants is negligible, compared to that of the initial D population. This translates in the convergence of Eq 14 and Eq 11 at low conjugation frequencies. As a rule of thumb, in a plasmid with a conjugation frequency of 0.1 T/D the contribution of secondary conjugation is approximately 0.01 T/D. This means that for most experimental conditions and model plasmids, Eq10 can be directly applied without the introduction of a substantial estimation error. This, however, does not hold for plasmids in which the conjugation frequency observed is higher than 1 T/D (for 1 T/D the error would be 0.3 T/D). This may be the case of highly conjugative plasmids or, more frequently, mating experiments performed for very long times, under a continuous replenishment of nutrients.

<u>3.-Vegetative growth contribution is negligible</u>

In Eq.12 we assumed vegetative growth to yield a negligible contribution to the frequency of conjugation (Fc). Here we will show that this is always the case, as long as the growth rate of T and D is similar.

The FC is defined as the ratio of T to D, such that

$$\frac{dFc}{dt} = \frac{dT_t D_t - dD_t T_t}{dD_t{}^2}$$

Eq. 15

Where

$$\frac{dT}{dt} = \frac{R\ kon}{1+R\ kon\ \tau}\ (T_t + D_t) + \alpha\ T$$

Eq. 16

And the growth of *D* follows:

$$\frac{dD}{dt} = \alpha\, D$$

Eq. 17

$$D_t = D_0 e^{\alpha t}$$

Eq. 18

In these conditions

$$\frac{dFc}{dt} = \frac{R\ kon}{1 + R\ kon\ \tau}\ \frac{D_t + T_t}{D_t} = \frac{R\ kon}{1 + R\ kon\ \tau}\ (1 + Fc)$$

Eq. 19

Which can be directly integrated

$$Ln(1 + Fc) = \frac{R\ kon}{1 + R\ kon\ \tau}\, t$$

Eq. 20