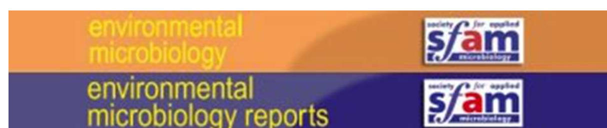

Growth phase-dependent control of R27 conjugation is mediated by the interplay between the plasmid-encoded regulatory circuit TrhR/TrhY-HtdA and the cAMP regulon.

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1 **Growth phase-dependent control of R27 conjugation is mediated by the**
2 **interplay between the plasmid-encoded regulatory circuit TrhR/TrhY-HtdA**
3 **and the cAMP regulon.**

4

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23 Key words: plasmid conjugation, R27, growth phase dependent control, cAMP,
24 transcriptional regulation, TrhR/TrhY-HtdA.

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26 **Originality-Significance Statement**

27 Plasmid conjugation is involved in the spreading of antibiotic resistance among
28 bacteria. Conjugation of IncHI1 plasmids, which are vectors of drug resistance
29 present in many pathogenic Gram-negative bacteria, occurs during bacterial
30 transit outside their warm-blooded hosts. In this report, we explored the
31 mechanisms underlying the regulation of plasmid conjugation by physiological
32 conditions. Our data indicated that the metabolic sensor cAMP plays a key role
33 in the growth phase-dependent control of IncHI1 plasmids transfer in
34 *Escherichia coli* via the TrhR/TrhY-HtdA plasmid conjugation regulators. This
35 work highlights the importance of regulatory crosstalk between conjugative
36 plasmids and the chromosome of recipient bacteria in environmental- and
37 physiological-mediated control of plasmid conjugation.

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39 **Summary**

40 Plasmids of the incompatibility group HI1 (IncHI1) have been isolated from
41 several Gram-negative pathogens and are associated with the spread of
42 multidrug resistance. Their conjugation is tightly regulated and it is inhibited at
43 temperatures higher than 30ºC, indicating that conjugation occurs outside
44 warm-blooded hosts. Using R27, the prototype of IncHI1 plasmids, we report
45 that plasmid transfer efficiency in *E. coli* strongly depends on the physiological
46 state of the donor cells. Conjugation frequency is high when cells are actively
47 growing, dropping sharply when cells enter the stationary phase of growth.
48 Accordingly, our transcriptomic assays show significant downregulation of
49 numerous R27 genes during the stationary phase, including several *tra*
50 (transfer) genes. Growth phase-dependent regulation of *tra* genes transcription
51 is independent of H-NS, a silencer of horizontal gene transfer, and ppGpp and
52 RpoS, regulators of the stationary phase, but highly dependent on the plasmid-
53 encoded regulatory circuit TrhR/TrhY-HtdA. The metabolic sensor cAMP,
54 whose synthesis is chromosomally encoded, is also involved in the growth
55 phase regulation of R27 conjugation by modulating *htdA* expression. Our data
56 suggest that the involvement of regulators encoded by both chromosome and
57 plasmid are required for efficient physiological control of IncHI1 plasmid
58 conjugation.

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60 **Introduction**

61 The exchange of genetic material among bacteria, also known as horizontal
62 gene transfer (HGT), contributes significantly to bacterial evolution. HGT
63 promotes adaptation of the recipient bacteria to new environments through the
64 acquisition of genes encoding metabolic pathways, adhesins, antimicrobial
65 resistance, toxins, etc. Among the mechanisms underlying HGT, plasmid
66 conjugation has become key in the rapid dissemination of antibiotic resistance
67 among bacteria (Bennett, 2008)(Smillie *et al*., 2010). The transfer of plasmids
68 by conjugation is a complex process requiring the expression of a large number
69 of genes and proteins, some of them in large quantities. Therefore, a significant
70 amount of bacterial resources is devoted to conjugation once triggered.
71 Conjugation is very tightly regulated, only promoted under certain environmental
72 and physiological conditions to avoid deleterious effects on the fitness of the cell
73 (Frost and Koraimann, 2010).

74 The plasmids of the incompatibility group HI1 (IncHI1) have been associated
75 with multidrug resistance in several Gram-negative pathogens (Holt *et al*.,
76 2011). Studies on R27, the prototype IncHI1 plasmid, have shown that there are
77 at least 35 genes, called *tra* (transfer) genes, clustered in two separated regions
78 (Tra1 and Tra2) that are required for conjugation (Lawley *et al*., 2002) (Lawley
79 *et al*., 2003). This genetic organisation is shared by all the IncHI1 plasmids. The
80 Tra1 region contains the origin of transfer (oriT) and three polycistronic operons:
81 H, F and R. The H operon encodes relaxosome components and the coupling
82 protein TraG, the F operon encodes proteins needed for H-pilus biosynthesis,
83 and the R operon encodes regulatory proteins involved in the transcriptional
84 activation of many *tra* genes (Lawley *et al*., 2002) (Gibert *et al*., 2014). The Tra2

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85 region contains the operons AC, AN and Z. The AC operon encodes structural
86 proteins of the mating pair formation complex and H-pilus, including the pilin
87 TrhA. The AN operon encodes proteins of the mating pair formation complex
88 and the regulatory protein HtdA, whilst the Z operon encodes an entry exclusion
89 system that stops cells carrying an IncHI1 plasmid from undergoing redundant
90 conjugation (Gunton *et al*., 2008). Two groups of partition genes involved in
91 plasmid segregation are located upstream (*parM* and *parR*) and downstream
92 (*parA* and *parB*) of the Z operon (Rooker *et al*., 1999) (Lawley *et al*., 2003).

93 Regulation of IncHI1 plasmid conjugation has been studied previously using as
94 a model the transfer of the R27 plasmid between *Escherichia coli* cells.
95 Environmental factors such as osmolarity, anaerobiosis, quorum sensing and
96 acidity do not significantly affect the frequency of R27 conjugation (Alonso *et al*.,
97 2005). To date, the only environmental condition described as affecting IncHI1
98 plasmid conjugation is temperature. Remarkably, maximal conjugation
99 frequencies are detected at low temperatures between 22ºC and 30ºC, whereas
100 at 37ºC conjugation is barely detectable. This thermoregulation suggests that
101 IncHI1 plasmids transfer is enhanced in water and soil environments (Taylor
102 and Levine, 1980) (Maher and Taylor, 1993). In this report, we describe that
103 efficiency of R27 plasmid transfer strongly depends on the physiological state of
104 the donor cells. Conjugation frequency is high when cells are actively growing
105 (in the logarithmic phase), but drops sharply when cells enter the stationary
106 phase of growth, correlating with the significant downregulation of the
107 transcriptional expression of several R27 genes, including many *tra* genes. The
108 regulatory circuit formed by TrhR/TrhY and HtdA has been described to
109 modulate the expression of four out of six *tra* operons (Gibert *et al*., 2014).

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110 Here, we show that this circuit plays a crucial role in the growth phase-
111 dependent control of R27 conjugation. Moreover, the metabolic sensor cAMP
112 participates in the growth phase regulation of R27 conjugation by modulating
113 *htdA* expression. Our results suggest that an interplay between chromosomal
114 and plasmid-encoded factors is required for efficient physiological control of
115 IncHI1 plasmids conjugation.

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117 **Results**

118 **R27 transfer is promoted in actively growing cells.**

119 The effect of the physiological state of the bacterial cells on IncHI1 plasmid
120 conjugation was studied using the R27 plasmid. Conjugation frequencies at the
121 permissive temperature (25ºC) were monitored using donor and recipient cells
122 cultures grown up to an OD_{600nm} of 0.4 and 2.0, from now denoted respectively
123 as cultures in logarithmic (log) and early stationary (e-stat) phase of growth (Fig.
124 1A) . Conjugation frequency was affected by the physiological state of the donor
125 cells, since R27 plasmid was more efficiently transferred from actively growing
126 cells (log) than e-stat cells (Fig. 1B). The physiological state of the recipient
127 cells did not notably affect conjugation efficiency.

128 **Most of the genes involved in R27 plasmid transfer are upregulated at the**
129 **transcriptional level in log cells.**

130 To elucidate the mechanism behind the observed differences in conjugation
131 frequency, transcriptomic analyses were performed to compare the expression
132 pattern of the R27-encoded genes in log and e-stat cells. RNA samples from
133 AAG1(R27) cultures grown at 25ºC to either the log or e-stat phase were
134 isolated, retrotranscribed to cDNA and used to hybridise against a microarray
135 containing probes for the 205 genes of the R27 plasmid. At least a two-fold
136 difference between the signals for log and e-stat samples was required to
137 qualify a gene as being significantly affected by the growth phase. Moreover, to
138 ensure that the accepted ratio was not based on very low signal strengths, we
139 only considered genes with a signal value higher than the arbitrary value of 100
140 units of fluorescence intensity in at least one of the conditions. The fold change

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141 in the transcriptional expression between log and e-stat phase cells is shown in
142 Figure 2A and Table S1. Of the 205 R27 genes, 68 showed growth phase-
143 dependent expression, representing 33% of the genes. A total of 42 genes had
144 higher expression in the log phase, whereas 26 genes were expressed more in
145 the e-stat phase. Among the genes with altered expression, 21 belonged to the
146 *tra* operons, representing over 60% of the *tra* genes. Expression of *tra* genes is
147 indicated in both relative and absolute values in Figure 2A (black bars) and 2B,
148 respectively. As a control, the mRNA level of 7 selected genes, representing all
149 six *tra* operons, was monitored by RT-PCR (Fig. 2C). The results obtained were
150 in agreement with the transcriptomic data.

151 Consistent with the increased conjugation frequency detected in log cells, 18 of
152 the 21 *tra* genes were induced in actively growing cells, including those
153 encoding crucial proteins for conjugation such as the pilin TrhA and the
154 relaxase TraI (see Table S1 for a functional description of the affected genes).
155 By contrast, only three genes (*htdA*, *htdF* and *htdK*) were induced in e-stat cells.
156 Interestingly, *htdA* encodes a repressor of the transcriptional expression of the
157 H, F, Z and AC operons (Gibert *et al*., 2013).

158 When considering genes located outside the *tra* operons, the expression of 47
159 genes was significantly affected by the growth phase, with 24 being highly
160 expressed in log cells and 23 in e-stat cells. Although very few non-*tra* R27
161 genes have been fully characterised, protein homology studies allowed us to
162 predict the putative function of some of them (Table S1). Among the genes
163 induced during the log phase, some encode proteins that might contribute to
164 efficient conjugation, such as partition proteins (R0013, R0014, R0042 and
165 R0153),, DNA helicase (R0003), muramidase (R0130) and proteins involved in

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166 the turnover of disulphide bonds (R0131 and R0135) (Guynet *et al*., 2011)
167 (Gruber *et al*., 2016)(Zahrl *et al*., 2005)(Elton *et al*., 2005). Among the 23 genes
168 more highly expressed in e-stat than log cells, we were only able to assign a
169 putative function to two of them, a transposase (R0148) and a citrate
170 transporter (R0144), which are not involved in plasmid conjugation.

171 **The master regulators of the stationary phase, ppGpp and RpoS, and the**
172 **HGT-silencer protein H-NS are not essential for the growth phase-**
173 **dependent modulation of R27 conjugation.**

174 The significant repression of R27 conjugation when cells enter the stationary
175 phase of growth is similar to that observed for cellular processes regulated by
176 ppGpp. The concentration of this secondary messenger increases rapidly in the
177 interphase between the log and e-stat phases, mediating the changes in gene
178 expression required for bacterial adaptation. ppGpp can directly regulate gene
179 expression, as well as affect a large set of genes by promoting, to different
180 degrees, the activity of RpoS, the stationary-phase sigma subunit (Magnusson
181 *et al*., 2005)(Battesti *et al*., 2011). To determine whether the growth phase
182 control of R27 conjugation is mediated by ppGpp and/or RpoS, conjugation
183 experiments were performed using donor cells lacking either ppGpp or RpoS
184 (Fig. 3). In ppGpp^0 and *rpoS* derivative strains, although a slight increase was
185 observed in the conjugation frequency in e-stat cells, there was still a
186 pronounced difference in the conjugation frequency between log and e-stat
187 cells. Therefore, ppGpp and RpoS did not seem to be essential for the growth
188 phase dependency of R27 conjugation. However, they could still be involved in
189 the fine tuning of R27-transfer control.

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190 It has been postulated that H-NS silences HGT by repressing the expression of
191 suddenly acquired DNA to diminish its potential deleterious effect on the fitness
192 of the cell (Doyle *et al*., 2007). Remarkably, a gene encoding an H-NS-like
193 protein has been identified in several conjugative plasmids, including R27
194 (reviewed by Dorman, 2014). Previous studies have described the role of H-NS
195 in repressing R27 *tra* gene expression (Forns *et al*., 2005) (Gibert *et al*., 2014).
196 We performed conjugation experiments using donor cells deficient in both
197 chromosomal- and plasmid-encoded H-NS orthologues (Fig. 3). As expected, a
198 higher conjugation frequency was detected in the H-NS deficient strain
199 compared to WT in both the log and e-stat cells. However, there was still a
200 significant difference in conjugation frequency being more than 1,500-fold
201 higher in the log cells than in e-stat cells. These results indicated that H-NS is
202 not involved in the growth phase control of R27 conjugation.

203 **The TrhR/TrhY-HtdA regulatory system is involved in the growth phase-**
204 **dependent regulation of R27 conjugation.**

205 The R27-encoded TrhR, TrhY and HtdA form a regulatory circuit that controls
206 transcription of the *tra* operons and consequently modulates R27 conjugation
207 (Gibert *et al*., 2013)(Gibert *et al*., 2014). TrhR and TrhY have an essential role
208 promoting the transcriptional expression of 4 *tra* operons (H, F, Z and AC),
209 whereas HtdA counteracts their effect, causing an overall repression of the
210 conjugative apparatus expression. Although the exact mechanism has not yet
211 been elucidated, it is known that HtdA does not regulate *trhR* and *trhY*
212 expression and it was proposed that HtdA counteracts TrhR/TrhY activity by
213 establishing protein-protein interactions. Interestingly, *htdA* was one of the few
214 genes induced in the e-stat phase (more than 3-fold compared to the log

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215 phase), while *trhR* was slightly induced in log cells (1.8-fold) (Fig. 2 and Table
216 S1). These fluctuations in expression levels, particularly in *htdA*, may explain
217 the low R27 conjugation frequency observed in e-stat cultures.

218 To dissect the role of TrhR/TrhY and HtdA in growth phase-dependent control,
219 conjugation studies were performed with R27 variants carrying mutations for
220 either *trhR* or *htdA* (Fig. 3). In accordance with the requirement of TrhR/TrhY for
221 the expression of *tra* operons (Gibert *et al*., 2014), conjugation was completely
222 abolished in the *trhR* derivative in both log and e-stat donor cells. By contrast,
223 *htdA* mutation elicited derepression of R27 conjugation in both log and e-stat
224 donor cells. Strikingly, the difference in the averages of the conjugation
225 frequency between log and e-stat cells with WT R27 plasmid (2.78E-3 vs 4.43E-
226 7) was reduced more than 300 times in the absence of *htdA* (3.23E+0 vs 1.59E-
227 1). Consistent with the fact that *htdA* expression was induced in the e-stat
228 phase, a greater derepression of R27 conjugation was observed in the *htdA*
229 mutant strain in the e-stat phase compared to the log phase. These results
230 suggest that HtdA plays an important role in the repression of R27 conjugation
231 observed when cells enter the stationary phase. The transcriptional expression
232 of *trhA*, encoding the H pilus major subunit, was studied by RT-PCR in log and
233 e-stat donor cells carrying R27, drR27 (*htdA*$^{-}$) or R27*trhR*-Tmp (*trhR*$^{-}$) (Fig.3 B).
234 In line with the conjugation frequency data in log cells, a pronounced induction
235 of *trhA* transcription was observed in log cells carrying the R27 plasmid. The
236 growth phase control of *trhA* was abolished in both *htdA* and *trhR* derivative
237 strains since *trhA* expression was only slightly increased in log cells compared
238 to e-stat cells. As expected from the roles of HtdA and TrhR in *tra* gene
239 expression, *trhA* mRNA levels in the *htdA* mutant were higher than in cells

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240 carrying the WT plasmid, and lower in the *trhR* strain (note that the amount of
241 total RNA required for efficient *trhA* detection was 0.1, 1 and 10 ng for *htdA*, WT
242 and *trhR* strains, respectively). Thus, these results were consistent with the
243 observed differences in conjugation frequency (Fig. 3A).

244 Given that HtdA is involved in the growth phase-dependent control of R27
245 conjugation (Fig. 3A) and that *htdA* expression is significantly higher in the e-
246 stat phase (3-fold, see Fig. 2 B), we postulate that fluctuations in the level of the
247 antiactivator (HtdA) and, consequently, in the availability of functional activators
248 (TrhR/TrhY), determine the frequency of R27 conjugation in log and e-stat
249 donor cells. The effect of increasing the amounts of the different regulatory
250 components on conjugation frequency was determined in both log and e-stat
251 donor cells (Fig. 4). Consistent with our previous results, growth phase-
252 dependent regulation vanished when either the antiactivator (HtdA) or the
253 activators (TrhR/TrhY) were overexpressed. HtdA overexpression reduced
254 conjugation frequency in log cells (>100,000-fold) and e-stat cells (10-fold),
255 while TrhR/TrhY overexpression induced conjugation in both e-stat (>10,000-
256 fold) and log (almost 100-fold) cells.

257 **cAMP controls R27 conjugation in an HtdA-dependent manner.**

258 A relevant question to address is how fluctuations in HtdA expression occur in
259 response to the physiological state of the cell. During a random mutagenesis
260 experiment searching for regulators of the R27 *tra* operons, we found that
261 mutation in the *cya* gene, encoding cAMP synthetase, affected expression of
262 the *tra* operons (data not shown). These findings indicated that cAMP may be
263 involved in regulating the synthesis of the conjugative apparatus. In

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264 enterobacteria, the secondary messenger cAMP acts as a physiological sensor.
265 cAMP, in a complex with cAMP receptor protein (CRP), can bind DNA and
266 regulate the expression of a vast number of genes (Shimada *et al*., 2011).
267 Although initially described as playing a role in regulating metabolic gene
268 expression, cAMP-CRP is now known to modulate different cellular processes
269 (Zheng *et al*., 2004). Furthermore, cAMP-CRP has been reported to be involved
270 in the growth phase-dependent expression of colonisation factors in *E. coli*
271 (Müller *et al*., 2009). Here, we studied the effect of *cya* mutation in the
272 expression of the conjugative apparatus by RT-PCR analyses of the *trhA*
273 transcript. cAMP deficiency sharply reduced *trhA* expression (Fig. 5A). To
274 further characterise the involvement of cAMP in regulating R27 transfer,
275 conjugation frequencies were measured for both cAMP-deficient and -proficient
276 cells (Fig. 5B). In log cells, mutation in *cya* significantly decreased conjugation
277 frequency (> 4,000-fold). Moreover, the effect of the *cya* mutation was blocked
278 when the cells lacked HtdA (drR27), indicating that HtdA is crucial for cAMP-
279 mediated control of R27 conjugation. In e-stat cells, no significant differences
280 were detected between the WT and *cya* mutant strains, suggesting that cAMP
281 plays a key role in controlling conjugation in log cells. Since cAMP forms a
282 complex with CRP, R27 conjugation was also monitored in a *crp* mutant strain
283 (Fig. 5C). In log cells the frequency of conjugation drops in the *crp* strain and
284 this reduction was lower in the absence of HtdA. Taken together, our results
285 indicated that cAMP-CRP promotes R27 transfer, possibly by repressing HtdA
286 expression. To explore this hypothesis, *htdA* transcriptional expression was
287 further studied using a transcriptional fusion between the *htdA* promoter and
288 *lacZ* inserted into the chromosomal *attB* locus. Along with the observed

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289 significant drop in R27 conjugation, the absence of either cAMP or CRP
290 increased *htdA* transcription (Fig. 5D). *htdA* expression in *crp* and *cya* mutants
291 was upregulated in both the presence and absence of the R27 plasmid,
292 suggesting that the repression effect was independent of the presence of any
293 R27-encoded factor. Further experiments were performed by adding exogenous
294 cAMP to *cya* mutant cultures (Fig. 5E). The *htdA* upregulation observed in the
295 *cya* mutant strain was suppressed after adding cAMP. Thus, our data indicate a
296 link between the cAMP-CRP regulon and the TrhR/TrhY-HtdA regulatory circuit
297 controlling R27 conjugation.

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299 **Discussion**

300 IncHI1 plasmids - including R27 - are antibiotic resistance-spreading elements
301 prevalent among pathogenic enterobacteria. Their transfer is repressed at
302 temperatures above 30ºC, meaning that dissemination is likely to occur during
303 bacterial transit in natural environments outside warm-blooded hosts (Maher
304 and Taylor, 1993). Our data indicated that R27 conjugation, in addition to low
305 temperature (permissive conditions), is promoted in actively growing cells.
306 Transfer frequency was high when donor cells were in the exponential phase of
307 growth and dropped during entry into stationary phase, after growth rate
308 declined (Fig. 1A). The sensitivity of R27 transfer to the physiological state of
309 the cell was restricted to the donor cell (Fig. 1B). Transcriptional profiling
310 indicated that many R27 genes involved in plasmid transfer are induced in log
311 cells (Fig. 2), suggesting the existence of a regulatory mechanism controlling
312 the transcriptional expression of plasmid transfer genes in a growth phase-
313 dependent manner. Given that plasmid conjugation uses a large amount of
314 energy and resources, our results suggest that when encountering metabolic
315 stress, cells suppress IncHI plasmid conjugation to avoid compromising survival
316 of the donor cell. In harsh conditions, these mechanisms minimise the
317 deleterious effect of conjugative plasmids on bacterial fitness. This plasmid
318 accommodation within the cells lets them act as successful vehicles for DNA
319 spreading among bacteria. An example of coevolution between plasmids and
320 their hosts has been shown with R27 and other IncHI1 plasmids (Paytubi *et al.*,
321 2014). These plasmids have developed a complex interaction with the bacterial
322 chromosome, resulting in an interplay that increases bacterial fitness at low
323 temperatures, thus facilitating the survival of *Salmonella* outside its host.

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324 The impact of the growth phase, nutrients and energy availability on conjugation
325 has been studied with other plasmids. F plasmid transfer in *E. coli* has been
326 reported to be inhibited in the stationary phase by a mechanism involving H-NS
327 (Frost and Manchak, 1998) (Will *et al*., 2004). The expression of the main
328 regulators of the F *tra* operon, *traM* and *traJ*, is significantly derepressed in an
329 *hns* mutant, particularly when the cells enter the stationary phase (Will *et al*.,
330 2004). However, a different observation has been reported for the F-like
331 plasmid, pSLT, in *Salmonella*. pSLT conjugation does not decline in the
332 stationary phase and *traJ* expression is inhibited by H-NS in both the
333 logarithmic and stationary phases of growth (Camacho *et al.*, 2005). Growth
334 rate and nutrient levels also affect IncP and IncI1 plasmids transfer efficiency,
335 although the regulatory mechanism remains unknown (Fox *et al*., 2008)(Händel
336 *et al*., 2015). Moreover, active growth is required for efficient plasmid transfer,
337 which may account for the limited plasmid spread detected in slow-growing cells
338 during biofilm formation (Merkey *et al*., 2011).

339 Our data demonstrated that the stationary phase regulators ppGpp and RpoS
340 were not essential, but could affect the growth phase-dependent transfer of R27
341 to some extent (Fig. 3). Recently, we described that H-NS-mediated
342 thermoregulation of R27 conjugation occurs by repressing the expression of the
343 two plasmid-encoded activators, TrhR and TrhY, at high temperatures (Gibert *et*
344 *al*., 2014). In the present study, H-NS did not seem to be crucial in the growth
345 phase control of R27 conjugation since there was still a significant difference in
346 the conjugation frequency between log and e-stat cells in an H-NS-deficient
347 strain (Fig. 3). Interestingly, growth phase-dependent regulation was strongly
348 governed by the regulatory circuit TrhR/TrhY-HtdA, since it disappeared when

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349 the levels of the regulators were altered either by deletion or ectopic
350 overexpression (Figs. 3 and 4). Moreover, the expression profile of the
351 regulators, particularly HtdA, was consistent with their active role in the growth
352 phase-dependent regulation of R27 conjugation (Fig. 2).

353 In this report we show that factors other than temperature are required to
354 alleviate the HtdA-mediated repression. At the permissive temperature,
355 expression of the conjugative apparatus occurred mostly in actively growing
356 cells. This regulation may be needed to avoid the unnecessary synthesis of the
357 R27 conjugative apparatus when cells are under suboptimal physiological
358 conditions.

359 We observed that the growth phase-dependent control of R27 conjugation was
360 abolished in a cAMP-CRP-deficient strain. Furthermore, the conjugation
361 frequency in log cells dropped to that seen in e-stat cells in both *cya* and *crp*
362 mutant strains (Fig. 5B, Fig. 5C). The effect of both *cya* and *crp* mutations on
363 conjugation was much more pronounced for the R27 (*htdA*$^+$) than drR27 (*htdA*$^-$)
364 plasmid, suggesting that the pivotal role of cAMP in controlling R27 conjugation
365 requires HtdA. We propose a model (Fig. 6), where cAMP-CRP is involved in
366 maintaining low levels of HtdA during the logarithmic phase of growth, thus
367 promoting R27 conjugation. In the early stages of the stationary phase, HtdA
368 expression increases and, by counteracting the activators TrhR/TrhY,
369 suppresses the expression of most of the *tra* genes, consequently reducing
370 conjugation frequency. Consistent with the proposed model, *htdA* expression
371 was derepressed in both *cya* and *crp* mutant strains and decreased when
372 external cAMP was added (Fig. 5). Although further studies are required to
373 elucidate the molecular mechanism underlying cAMP-CRP-mediated repression

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374 of *htdA* expression, our results clearly indicate that it is crucial for the
375 physiological control of R27 transfer. Indeed, the role of the metabolic sensor
376 cAMP in the growth phase control of transcriptional expression has been
377 previously described. Studies with the F-like plasmid pRK100 showed that,
378 similar to its effect on R27 conjugation, *cya* mutation reduced conjugal transfer
379 and cAMP-CRP stimulated the expression of the central positive regulator TraJ,
380 which is growth phase-dependent (Starcic *et al*., 2003). Moreover, cAMP has
381 been described to be involved in the repression of type 1 fimbriae in *E. coli*
382 during the log phase of growth (Müller *et al*., 2009).

383 Here, we show that R27 plasmid transfer depends on the physiological state of
384 the donor cell and is regulated by HtdA expression under the control of the
385 metabolic sensor cAMP. Plasmid conjugation can be considered an altruistic act
386 from the bacterial perspective or a selfish behaviour from the point of view of
387 the plasmid. In the latter, the main aim of a conjugative plasmid is to spread and
388 safeguard its genes. For some plasmids, such as R27, biological success is
389 achieved by promoting their spread without deleterious effects on the host cells.
390 Therefore, transfer must occur when the physiological conditions of the host
391 cells are optimal to trigger conjugation. Our data demonstrate that cAMP acts
392 as a physiological sensor that modulates the plasmid-encoded regulatory circuit
393 TrhR/TrhY-HtdA, the main controller of R27 conjugation.

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395 **Experimental procedures**

396 **Bacterial strains, plasmids and growth conditions**

397 The *E. coli* strains and plasmids used are listed in Table 1. Oligonucleotides are
398 listed in Table S2. Bacteria were grown in LB (10 g/l NaCl, 10 g/l tryptone and 5
399 g/l yeast extract). For mating experiments, strains were grown in Penassay
400 broth (1.5 g/l meat extract, 1.5 g/l yeast extract, 5 g/l peptone, 1 g/l glucose, 3.5
401 g/l NaCl, 1.32 g/l KH_2PO_4 and 4.82 g/l $K_2HP_4·3H_2O$). For selecting
402 transconjugants, M9 minimal medium plates were used, with the following
403 composition: M9 salts (Sambrook *et al*., 1989), 0.2% (w/v) lactose, 10 μM
404 thiamine and 1.5% (w/v) Bacto agar. When needed, antibiotics were added at
405 the following concentrations: 15 μg/ml tetracycline (Tc), 20 μg/ml
406 chloramphenicol (Cm), 25 μg/ml kanamycin (Km), 50 μg/ml ampicillin (Amp),
407 and 10 μg/ml trimethoprim (Tmp). For monitoring the *lac* phenotype, LB agar
408 plates were supplemented with 40 μg/ml X-gal and 0.5 mM IPTG. When
409 required, cAMP was added at a final concentration of 5 mM. Inducing
410 experiments of pBAD derivative plasmids were performed as described
411 previously (Gibert *et al*., 2014).

412 **Strain construction**

413 The AAG1 *rpoS*, AAG1 *cya*, AAG1-AN *cya*, AAG1 *crp* and AAG1-AN *crp* strains
414 were obtained by P1 transduction using the strains JW5437 (*rpoS::*Km), AAG1
415 *cya* (*cya::*Tmp) from a random mutagenesis experiment and BRE2055 (*crp*::Tc).
416 All genetic constructs were confirmed by DNA sequencing.

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419 **Mating experiments**

420 Mating experiments were performed following the standard protocol for R27
421 conjugation (Taylor and Levine, 1980), slightly modified. In all cases, AAG1 and
422 its derivatives were used as donor cells and MG1655 as recipient cells. Cultures
423 of donor and recipient strains were grown in Penassay broth at 25ºC in shaking
424 conditions to either an OD_{600nm} of 0.4 (log phase) or 2.0 (e-stat phase). When
425 log-phase cultures were used, cell suspensions were concentrated to the
426 OD_{600nm} of the e-stat cultures. Otherwise stated, recipient cells were grown to e-
427 stat phase. Mating frequency was calculated as the number of transconjugants
428 per donor cell.

429 **β-galactosidase assays**

430 β-galactosidase assays were performed as described by Miller (Miller, 1992).
431 Data are given as the means of duplicate determinations in at least three
432 independent experiments, plotted with standard deviations.

433 **Total RNA isolation**

434 Total RNA was isolated from three independent cultures grown at 25ºC under
435 shaking to either log phase (OD_{600nm} of 0.4) or e-stat phase (OD_{600nm} of 2.0).
436 The RNA was purified as previously described (Gibert *et al*., 2014). For
437 microarray experiments, the purified RNA was concentrated using an RNeasy
438 MiniElute Clean-up kit (Qiagen). Purity and quality of the RNA were tested by
439 Bioanalyzer 2100 (Agilent Technologies).

440 **Reverse transcriptase (RT)-PCR assays**

441 Semi-quantitative monitoring of the mRNA levels was performed using the
442 Transcriptor One-Step RT-PCR kit (Roche), as previously described (Gibert *et*

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443 *al*., 2014). Primer pairs used are described in Table S2. The relative amount of
444 cDNA was determined using the ImageJ software (Schneider *et al*., 2012).

445 **Microarray analysis**

446 Transcriptomic analysis was performed on a custom-designed DNA microarray
447 engineered by NimbleGen, containing two replicates of seven selected probes
448 for each of the 205 annotated genes of the R27 plasmid (NC_002305), as
449 previously described (Paytubi *et al*., 2014). The complete data set has been
450 deposited under accession number E-MTAB-4067 at
451 http:/www.ebi.ac.uk/arrayexpress.

452 **Acknowledgements**

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587 **Figure legends.**

588 **Figure 1.** Effect of the physiological state of the cells in the conjugation
589 frequency of R27. A. Growth curve of strains AAG1 (R27) (black lines) and
590 MG1655 (grey lines) in PB medium. Arrows indicate the OD_{600} corresponding to
591 logarithmic (log, OD_{600} of 0.4) and early-stationary (e-stat, OD_{600} of 2.0). A
592 representative experiment is shown. B. Conjugation rates were calculated using
593 cultures of both donor (AAG1(R27)) and recipient (MG1655) strains grown to
594 log or e-stat phase. The median of the conjugation frequency of three
595 experiments is shown.

596 **Figure 2.** Effect of the physiological state of the cells in the transcriptional
597 expression of the R27 genes. **A.** Fold change expression of the 205 ORFs
598 encoded in R27 plasmid between log and e-stat AAG1(R27) cells. The fold
599 change of +2 and -2, indicating significantly higher expressed in log and e-stat
600 cultures respectively, is labelled with dashed lines. Expressed genes were
601 classified as *tra* genes (black bars) and non *tra* genes (grey bars). Genes with
602 signal values lower than 100 fluorescence units in both culture conditions were
603 arbitrarily considered as non-expressed (white bars). **B**. Transcriptional
604 expression of the different *tra* genes in log (grey bars) and e-stat (black bars)
605 cultures is shown as arbitrary units (fluorescence intensity). **C**. Transcription
606 analysis by semi-quantitative RT-PCR of seven selected genes, representative
607 of the six *tra* operons from the AAG1(R27) strain grown as in A up to either log
608 or e-stat phase. 16S rRNA was used as a control.

609 **Figure 3.** Growth phase regulation of R27 conjugation in different genetic
610 backgrounds. **A.** Frequency of conjugation using as donor cells AAG1(R27) and
611 derivative strains grown to either log or e-stat phase. The median of the

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612 conjugation frequency of three experiments is shown. **B.** RT-PCR analyses of
613 *trhA* expression in WT, *htdA* and *trhR* strains. The amount of total RNA used in
614 each case is shown. Relative levels of cDNA are indicated below each panel.

615 **Figure 4.** Ectopic expression of HtdA and TrhR/TrhY vanish the growth phase
616 dependency of R27 conjugation. Conjugation frequencies of the AAG1(R27)
617 strain carrying the indicated plasmids. Donor cultures were grown to either log
618 or e-stat phase in the presence of glucose or arabinose. The median of the
619 conjugation frequency of three experiments is shown.

620 **Figure 5.** cAMP modulates conjugation in an HtdA-dependent manner during
621 logarithmic phase of growth. **A**. RT-PCR analyses of *trhA* in log cultures of WT
622 and *cya* strains. 16S rRNA was used as a control. **B**. Frequency of conjugation
623 using as donor log or e-stat AAG1 cells and its *cya* counterpart carrying either
624 R27 ($htdA^{+}$) or drR27 ($htdA^{-}$). The median of the conjugation frequency of three
625 experiments is shown. **C**. Frequency of conjugation using as donor AAG1 cells
626 and its *crp* counterpart carrying either R27-Cm ($htdA^{+}$) or drR27-Cm ($htdA^{-}$).
627 Cultures of the donor cells were grown until log phase. The median of the
628 conjugation frequency of three experiments is shown. **D**. Transcriptional
629 expression of *htdA* using a chromosomal *lacZ* fusion with the promoter of the
630 AN operon (AAG1-AN strain). β-galactosidase activity (Miller units) was
631 determined in cultures of strain AAG1-AN, and its *cya* and *crp* derivatives in the
632 absence or presence of the R27 plasmid grown as in A. **E**. *htdA* transcriptional
633 expression in cultures of the WT (AAG1-AN) and its *cya* derivative strain in the
634 presence or the absence of cAMP. Bacterial cultures were grown in LB at 25° to
635 an OD_{600nm} of 0.1, cAMP (5 mM) was added and β-galactosidase activity (Miller
636 units) was determined after 2 hours incubation.

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637 **Figure 6**. Proposed model of regulation of R27 plasmid conjugation in
638 logarithmic (A) or stationary (B) phase of growth.
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640 Table 1. Bacterial strains and plasmids used in this work

Strain	**Description**	**Source of reference**
MG1655	F-, *ilvG*, *rph1*	(Guyer *et al*., 1981)
AAG1	MG1655 Δ*lacZ*	(Aberg *et al*., 2008)
JFV2	AAG1 Δ*relA* Δ*spoT*	(Aberg *et al*., 2008)
JW5437	BW25113 *rpoS::*Km^R	(Baba *et al*., 2006)
AAG1 *rpoS*	AAG1 *rpoS::*Km^R	This work
AAG1 *hns*	AAG1 *hns::*Km^R	(Gibert *et al*., 2014)
AAG1 *cya*	AAG1 cya::Tmp^R	(Gibert *et al*., unpublished results)
AAG1-AN	AAG1 P_{AN}::*lacZ*-Km^R in *attB* site	(Gibert *et al*., 2013)
AAG1-AN *cya*	AAG1-AN *cya*::Tmp^R	This work
BRE2055	Δ(*crp*)96 *zhd*- 732::*Tn*10	(Bremer *et al*., 1988)
AAG1 *crp*	AAG1 Δ(*crp*)96 *zhd*- 732::*Tn*10	This work
AAG1-AN *crp*	AAG1-AN Δ(*crp*)96 *zhd*-732::*Tn*10	This work
Plasmid		
R27	IncHI1 Tc^R	(Grindley *et al*., 1972)
drR27	R27 *htdA*::IS10	(Gibert *et al*., 2013)
R27-Cm	insertion of *cat* gene from pAR92 into *tetA* gene of R27, Cm^R	(Gibert *et al*., unpublished results)
drR27-Cm	insertion of *cat* gene from pAR92 into *tetA* gene of drR27, Cm^R	(Gibert *et al*., unpublished results)
R27 *hns*	R27 *hns::*Cm^R	(Gibert *et al*., 2014)
R27*trhR*-Tmp	R27 *trhR::*Tmp^R	(Gibert *et al*., 2014)
pBAD*trhRY*	pBAD18 + *trhRtrhY*	(Gibert *et al*., 2014)
pBAD*htdA*	pBAD/HisB + *htdA*	(Gibert *et al*., 2014)
pAR92	*cat*-$P_{A1/04/03}$-*cfp**-T_0 cassette, Amp^R, Cm^R	(Reisner *et al*., 2002)

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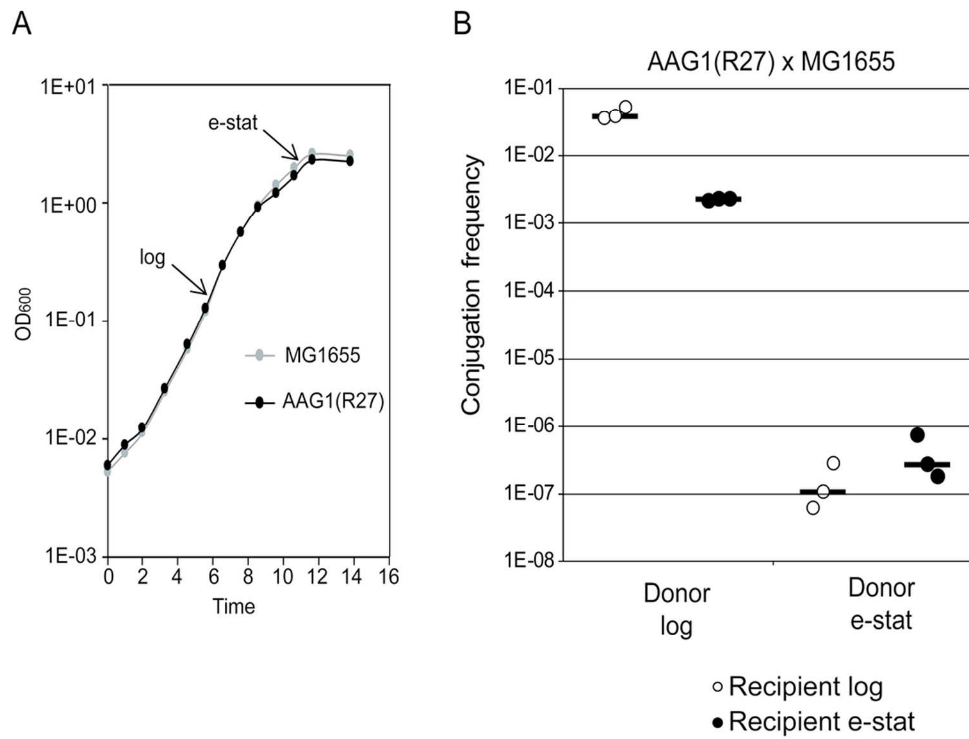

Figure 1
Figure 1
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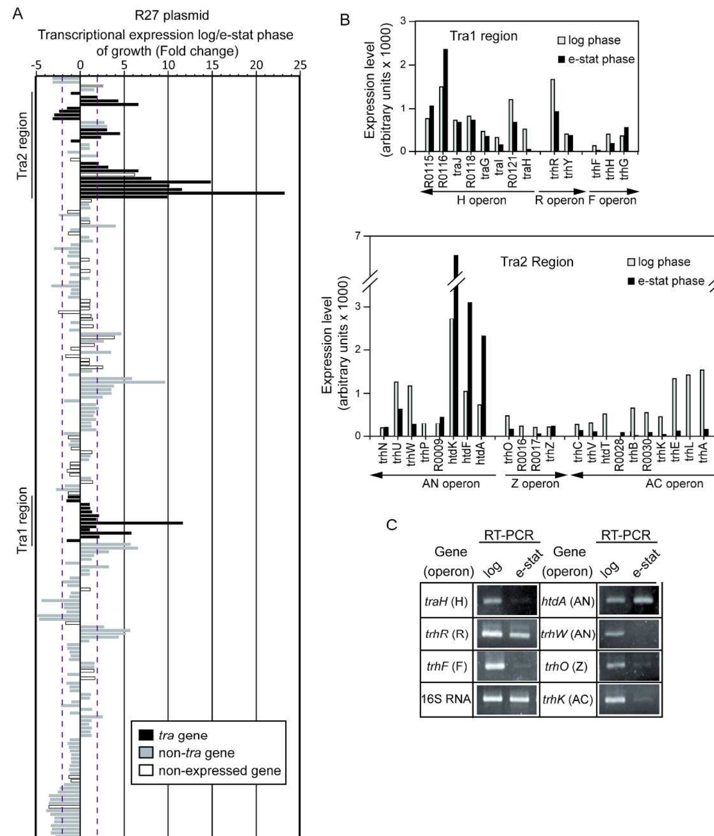

Figure 2
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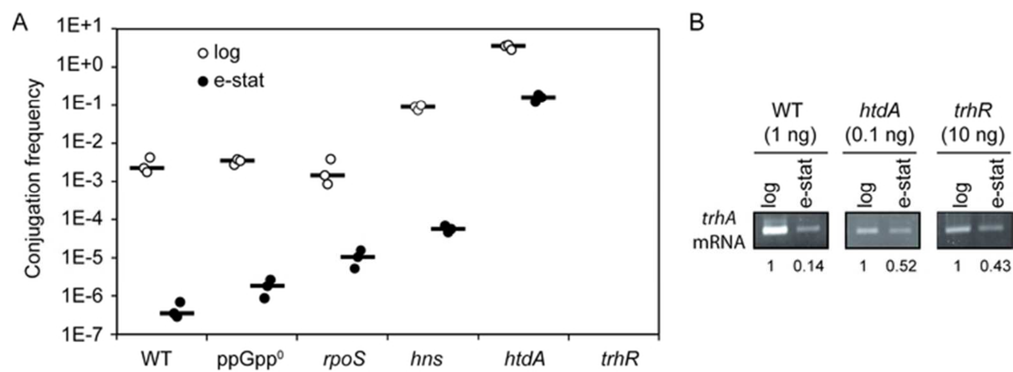

Figure 3
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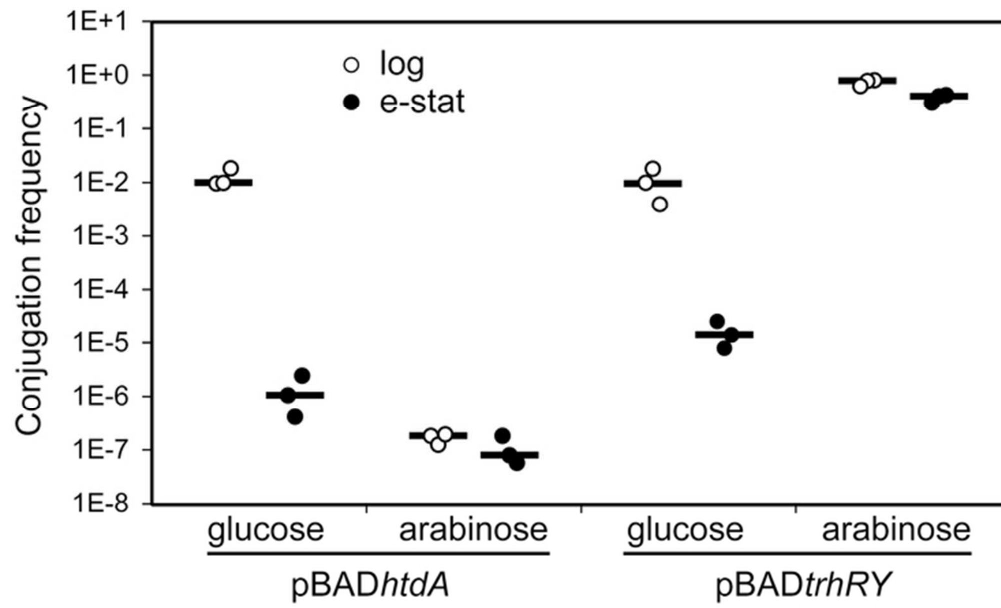

Figure 4
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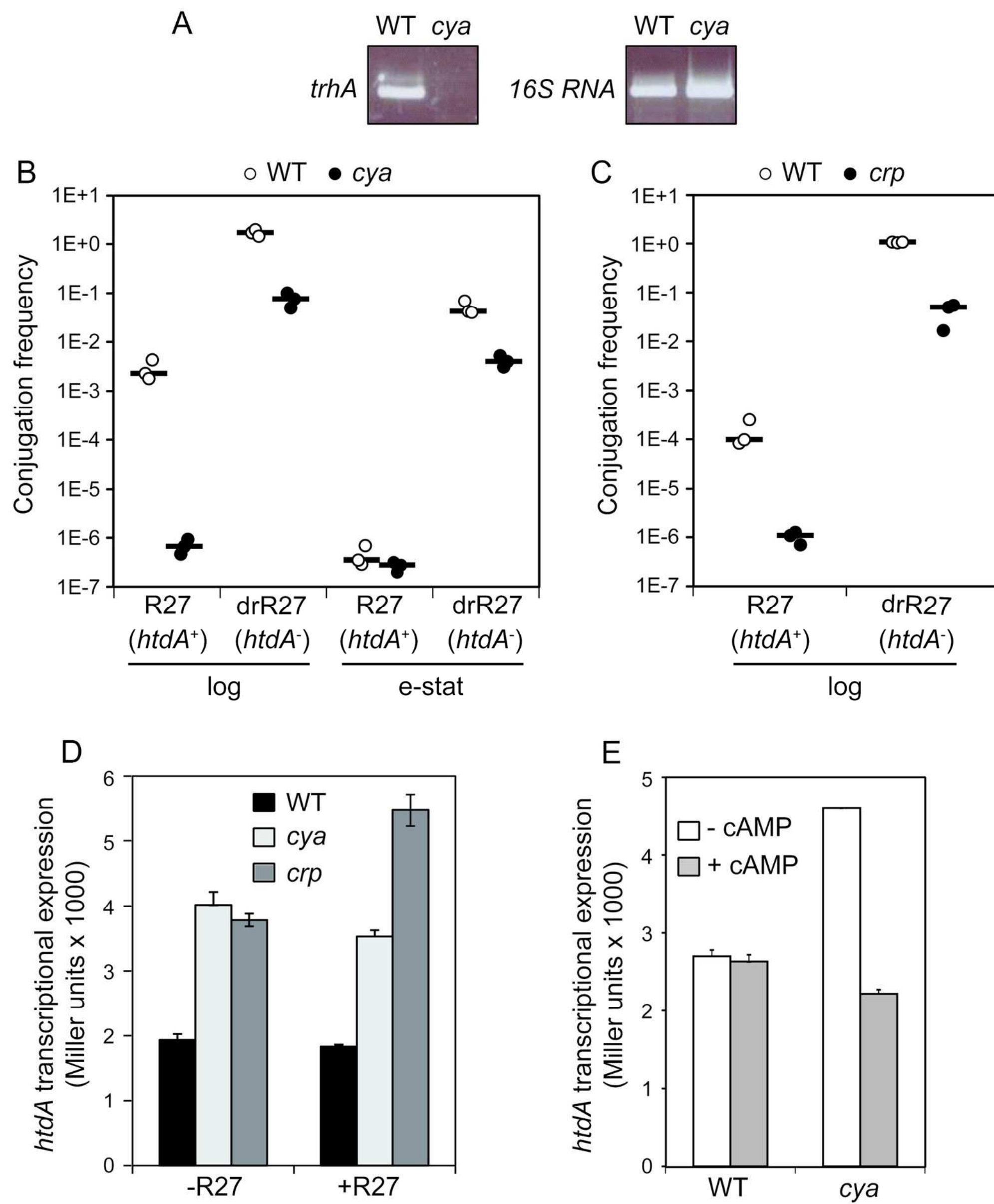

Figure 5
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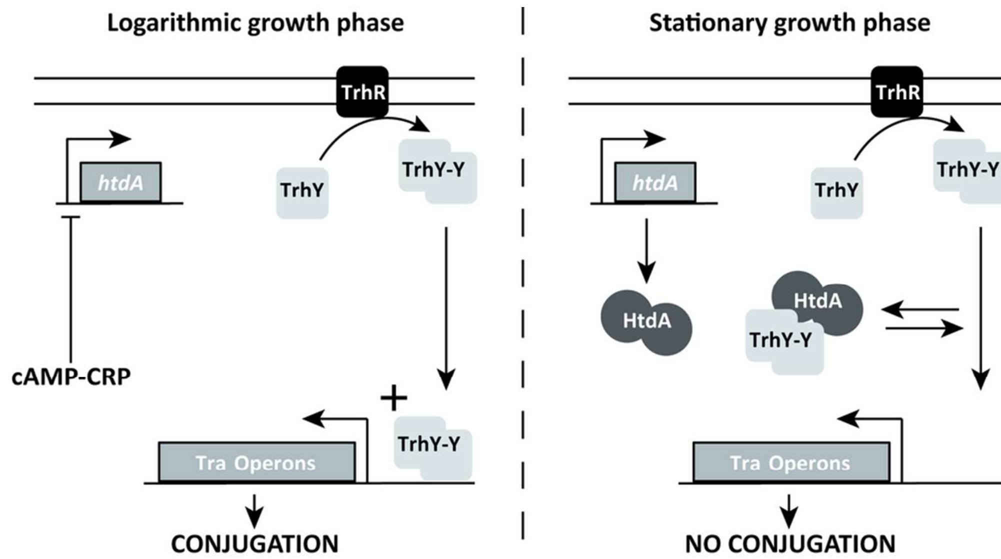

Figure 6
Figure 6
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