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**Real time visualisation of conjugation reveals the molecular strategy evolved by the conjugative F plasmid to ensure the sequential production of plasmid factors during establishment in the new host cell**

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1 **Real time visualisation of conjugation reveals the molecular strategy evolved by the**  
2 **conjugative F plasmid to ensure the sequential production of plasmid factors during**  
3 **establishment in the new host cell**

4  
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14

15 **Abstract**

16 DNA conjugation is a contact-dependent horizontal gene transfer mechanism responsible for  
17 disseminating drug resistance among bacterial species. Conjugation remains poorly characterised at  
18 the cellular scale, particularly regarding the reactions occurring after the plasmid enters the new host  
19 cell. Here, we use live-cell microscopy to visualise the intracellular dynamics of conjugation in real  
20 time. We reveal that the transfer of the plasmid in single-stranded DNA (ssDNA) form followed by  
21 its conversion into double-stranded DNA (dsDNA) are fast and efficient processes that occur with  
22 specific timing and subcellular localisation. Notably, the ss-to-dsDNA conversion is the critical step  
23 that governs the timing of plasmid-encoded protein production. The leading region that first enters  
24 the recipient cell carries single-stranded promoters that allow the early and transient synthesis of  
25 leading proteins immediately upon entry of the ssDNA plasmid. The subsequent ss-to-dsDNA  
26 conversion turns off leading gene expression and licences the expression of the other plasmid genes  
27 under the control of conventional double-stranded promoters. This elegant molecular strategy evolved  
28 by the conjugative plasmid allows for the timely production of factors sequentially involved in  
29 establishing, maintaining and disseminating the plasmid.

30

31

32 **Keywords**

33 Horizontal gene transfer, bacterial DNA conjugation, drug-resistance dissemination, live-cell  
34 microscopy, plasmid transfer

## 35 **Introduction**

36 Bacterial DNA conjugation is a widespread horizontal gene transfer mechanism in which genetic  
37 information is transmitted from a donor to a recipient cell by direct contact (Cruz et al., 2010;  
38 Grohmann et al., 2003; Lederberg and Tatum, 1946; Virolle et al., 2020). Conjugation is responsible  
39 for the intra- and inter-species dissemination of various metabolic properties and accounts for 80%  
40 of acquired resistances in bacteria (Barlow, 2009). The F plasmid was the first conjugative element  
41 discovered (Lederberg and Tatum, 1946; Tatum and Lederberg, 1947) and is now documented as the  
42 paradigmatic representative of a large group of conjugative plasmids widespread in *Escherichia coli*  
43 and other Enterobacteriaceae species, in which they are associated with the dissemination of colicins,  
44 virulence factors, and antibiotic resistance (Fernandez-Lopez et al., 2016; Johnson et al., 2016; Lanza  
45 et al., 2014). Due to their fundamental and clinical importance, F-like plasmids have been the focus  
46 of extensive studies that provided a detailed understanding of the molecular reactions and factors  
47 involved in their transfer by conjugation (*see* (Cruz et al., 2010; Virolle et al., 2020)).

48         Within the donor cell, the relaxosome components, including the integration host factor IHF,  
49 plasmid-encoded accessory proteins TraY, TraM and the multifunctional relaxase TraI (VirD2), are  
50 recruited to the origin of transfer (*oriT*) of the F plasmid (Howard et al., 1995; Nelson et al., 1993;  
51 Schildbach et al., 1998). The relaxosome complex is then recruited to the Type IV secretion system  
52 (T4SS) by the coupling protein TraD (VirD4), resulting in the formation of the pre-initiation complex  
53 (Beranek et al., 2004; Gomis-Rüth et al., 2004; Lang and Zechner, 2012; Llosa et al., 2003; Schröder  
54 and Lanka, 2005). It is proposed that the establishment of the mating pair induces a still  
55 uncharacterised signal that activates the pre-initiation complex. Then, TraI introduces a site- and  
56 strand-specific DNA cut (nick) into the plasmid's *oriT* and remains covalently bound to the 5'  
57 phosphate end. TraI also serves as a helicase that extrudes the ssDNA plasmid to be transferred, called  
58 the T-strand (Clewell and Helinski, 1970; Dostál and Schildbach, 2010; Everett and Willetts, 1980;  
59 Lanka and Wilkins, 1995; Matson and Morton, 1991; Matson and Ragonese, 2005; Reygers et al.,  
60 1991; Traxler and Minkley, 1988; Willetts and Skurray, 1980). It was initially suggested and later

61 confirmed that two relaxases are required to carry out these functions (Dostál et al., 2011; Ilangovan  
62 et al., 2017). At this stage, the 3'OH of the T-strand serves to initiate the rolling-circle replication  
63 (RCR) that converts the intact circular ssDNA plasmid into dsDNA in the donor cell (Cruz et al.,  
64 2010; Llosa et al., 2002; Wawrzyniak et al., 2017), while the 5'phosphate bound to TraI is transferred  
65 into the recipient cell through the T4SS machinery. If the molecular structure of the T4SS has been  
66 well characterised (Christie et al., 2014; Fronzes et al., 2009; Grohmann et al., 2018; Macé et al.,  
67 2022), the way the T-strand-TraI nucleoprotein complex is translocated through the membrane of the  
68 donor and recipient cells' membranes remain unclear.

69         The first transferred segment is the ~13.5 knt leading region, carrying genes which encode the  
70 Ssb<sup>F</sup> protein homolog to the chromosomally encoded essential single-strand-binding protein Ssb, the  
71 PsiB protein (Plasmid SOS Inhibition) (Althorpe et al., 1999a; Bagdasarian et al., 1992; Bailone et  
72 al., 1988; Dutreix et al., 1988) that inhibits SOS induction during conjugation (Baharoglu and Mazel,  
73 2014; Baharoglu et al., 2010), and others proteins of unknown function. Remarkably, the leading  
74 region is conserved in various enterobacterial plasmids belonging to a variety of incompatibility  
75 groups (Cox and Schildbach, 2017; Golub and Low, 1985, 1986a; Golub et al., 1988; Loh et al., 1989,  
76 1990). The adjacent and next transferred ~17 knt maintenance region carries the ParABS-like plasmid  
77 partition system (SopABC) and the origins of vegetative replication (Bouet and Funnell, 2019;  
78 Keasling et al., 1992; Kline, 1985; Thomas, 2000). The last transferred segment of the F plasmid is  
79 the large ~33.3 knt *tra* region that encodes all the protein factors required for plasmid DNA processing  
80 and transfer, including the relaxosome, the T4SS and the exclusion system against self-transfer  
81 (Virolle et al., 2020). Besides, F plasmids often carry cargo genes involved in various metabolic  
82 functions commonly integrated between the maintenance and the *tra* regions (Johnson et al., 2016;  
83 Lanza et al., 2014).

84         Once both 5' and the 3' ends of the T-strand have been internalised into the recipient cell,  
85 now called a transconjugant, the ssDNA plasmid is circularised by TraI and subsequently converted  
86 into dsDNA by the complementary strand synthesis reaction (Chandler et al., 2013; Dostál and

87 Schildbach, 2010; Dostál et al., 2011; Draper et al., 2005; Garcillán-Barcia et al., 2007). The ss-to-  
88 dsDNA conversion reaction is required for plasmid replication and partition and is, therefore, critical  
89 to plasmid stability in the new host cell lineage.

90 The above-described mechanistic model is well-documented; however, the real time dynamics  
91 and intracellular organisation of conjugation remain largely undescribed in the live bacterium. In  
92 particular, we know very little about the subcellular localisation and timing of the reactions in the  
93 recipient cell, including the ssDNA plasmid entry, the ss-to-dsDNA conversion and plasmid gene  
94 expression. Regarding the latter, early works reported that some leading genes (*ssb<sup>F</sup>* and *psiB* in F  
95 plasmid, and *ssb<sup>Collb-P9</sup>*, *psiB* and *ardA* in Collb-P9 plasmid) are expressed rapidly after entry of the  
96 plasmid in the acceptor cell (Althorpe et al., 1999b; Bagdasarian et al., 1992; Cram et al., 1984;  
97 Dutreix et al., 1988; Golub and Low, 1986a; Jones et al., 1992). *In vitro* work by Masai *et al.* (Masai  
98 and Arai, 1997) showed that the single-stranded form of the non-coding *Frpo* sequence, located in  
99 the F plasmid leading region, folds into a stem-loop structure that reconstitutes canonical -10 and -35  
100 boxes. This promoter sequence can recruit the *E. coli* RNA polymerase that initiates RNA synthesis  
101 in *in vitro* assays (Masai and Arai, 1997). Sequences homologous to *Frpo* were also found in the  
102 leading region of Collb-P9 (Bates et al., 1999; Nasim et al., 2004). These observations led to the  
103 proposal that *Frpo*-like sequences could act as ssDNA promoters initiating the early transcription of  
104 leading genes when the plasmid is still in ssDNA form. Whether this regulation mechanism happens  
105 during *in vivo* conjugation remains to be demonstrated.

106 In this study, we use live-cell microscopy imaging to visualise the complete transfer sequence  
107 of the native F plasmid between *E. coli* K12 strains. We inspect the key steps of conjugation using  
108 specifically developed genetic reporters, including a fluorescent fusion of the chromosomally  
109 encoded single-strand-binding protein Ssb (Ssb-Ypet) to monitor the ssDNA transfer, the mCherry-  
110 ParB/*parS* system to reveal the ss-to-dsDNA conversion and subsequent plasmid duplication, and  
111 translational fluorescent fusions to quantify and time plasmid-encoded production in the new host  
112 cell (Goldlust et al., 2022; Nolivos et al., 2019). This approach uncovers the choreography of

113 conjugation reactions in live bacteria and provides new insights into the interplay between plasmid  
114 processing and gene expression.

115

116

## 117 **Results**

### 118 *Dynamics of the ssDNA plasmid during transfer*

119 We monitored the dynamic localisation of a fluorescent fusion of the chromosomally encoded single-  
120 strand-binding protein Ssb (Ssb-Ypet) in donor and recipient cells, during vegetative growth and  
121 conjugation (Figure 1A-B and Figure S1). During vegetative growth, Ssb-Ypet forms discrete foci at  
122 midcell and quarter positions within the inner region of donors and recipient cells (Figure 1C and  
123 Figure S2A-B). These Ssb foci, termed Ssb replicative foci hereafter, are associated with the ssDNA  
124 that follows the replication forks onto the nucleoid DNA (Reyes-Lamothe et al., 2008, 2010). During  
125 conjugation, the intracellular localisation of Ssb changes dramatically. As previously reported  
126 (Goldlust et al., 2022; Nolivos et al., 2019), the entry of the ssDNA plasmid in the recipient cell, now  
127 called a transconjugant, triggers the recruitment of Ssb molecules and the formation of bright  
128 membrane-proximal foci, we termed Ssb conjugative foci (Figure 1B, Figure S1). Here, we also  
129 observe the formation of Ssb conjugative foci in the donor cells, thus revealing the presence of ssDNA  
130 plasmid on each side of the conjugation pore during transfer (Figure 1B, Figure S1). Foci localisation  
131 analysis reveals that plasmid exit and entry occur at specific membrane positions within the mating  
132 pair cells. Ssb conjugative foci are mainly distributed along the donor cells' side with a noticeable  
133 enrichment at the cell quarter positions (Figure 1C, Figure S2A-B), reflecting the preferred position  
134 for the exit of the ssDNA plasmid through active conjugation pores. By contrast, ssDNA plasmid  
135 entry predominantly occurs within the polar regions of the transconjugant cells (Figure 1C, Figure  
136 S2A-B). Our data also allow us to address whether conjugation occurs at a specific cell cycle stage.  
137 Analysis of cell length as a proxy of cell age reveals that donor and recipient cells engaged in plasmid  
138 transfer exhibit similar length distribution than during vegetative growth (Figure 1D). This shows

139 that conjugation is cell-cycle independent as the donors can give, and recipients can acquire the  
140 plasmid at any stage of their cell cycle, from birth to cell division.

141 In  $77.8 \pm 7\%$  ( $n = 131$ ) of individual plasmid transfer events visualised by time-lapse imaging  
142 (1 min/frame), Ssb conjugative foci appear in the donor and transconjugant cells on the same frame  
143 (Figure 1E). In these cases, Ssb conjugative foci are, on average brighter in the transconjugant than  
144 in the donor cells, reflecting the relative amount of ssDNA plasmid on each side of the conjugation  
145 pore (Figure 1F). In the remaining 22.2 % of transfer events, Ssb conjugative foci first appear in the  
146 transconjugant and then in the donor one or two minutes later (Figure 1E). The delayed accumulation  
147 of ssDNA in the donor relative to the recipient is corroborated by the quantification of a  $2.9 \pm 1.1$   
148 min ( $n = 294$ ) average lifespan of Ssb-Ypet conjugative foci in the transconjugants, compared to  $2.5$   
149  $\pm 1.1$  min ( $n = 197$ ) in the donor cells (Figure 1G). These data indicate that the appearance of  
150 conjugative foci is asynchronous in the mating pair cells and suggest a specific sequence of ssDNA  
151 transfer. The first segment of the T-strand generated by the helicase activity of TraI in the donor cell  
152 does not dwell long enough to recruit Ssb molecules and is immediately transferred to the recipient.  
153 Only after this brief transfer stage does the ssDNA accumulates on the donor's side as well, where it  
154 can correspond to either or both the non-transferred plasmid strand or to the T-strand. This implies  
155 that the rate of ssDNA formation by TraI helicase activity is faster than that of ssDNA removal by  
156 the RCR and transfer through the T4SS (See discussion).

157 The internalisation of a large amount of ssDNA plasmid provokes the massive recruitment of  
158 the intracellular pool of Ssb molecules at the periphery of the donor and transconjugant cells. This  
159 change in Ssb-Ypet subcellular distribution is revealed by skewness analysis, which provides a non-  
160 biased measure of the asymmetry of fluorescence distribution within the cells without a requirement  
161 for threshold-based foci detection (Figure 1H). Cells producing a free mCherry (mCh) exhibit a low  
162 skewness corresponding to the homogeneous pixel fluorescence distribution inside the cell's  
163 cytoplasm. During vegetative growth, Ssb-Ypet fluorescence is partly diffuse in the cytoplasm and  
164 partly locally concentrated within replicative foci, resulting in a skewness of  $\sim 1.2$ . By comparison,



165 Ssb-Ypet exhibits a strong skewness of  $\sim 4.1$  in donors and transconjugants during plasmid transfer,  
166 reflecting the increased proportion of Ssb molecules clustered within foci. Hence, we wondered what  
167 part of Ssb molecules are contained within conjugative foci and if their formation was associated with  
168 a depletion of Ssb within replicative foci in the transconjugant cell. To address this question, we  
169 performed Ssb-Ypet foci automatic detection and brightness quantification during plasmid transfer  
170 (Figure 1I). We observe that one minute after the beginning of plasmid entry Ssb-Ypet replicative  
171 foci are still present but exhibit half their initial intensity, while conjugative foci are 35 times brighter.  
172 Since the total Ssb-Ypet intracellular fluorescence is unchanged during the transfer (Figure S2C),  
173 these variations can be attributable to the displacement of Ssb-Ypet molecules onto the incoming  
174 ssDNA plasmid rather than Ssb-Ypet *de novo* synthesis. This dynamic reflects that the incoming  
175 ssDNA plasmid recruits most Ssb-Ypet molecules in the acceptor cell during transfer.

176 It has been estimated that Ssb is present at about  $\sim 1320 \pm 420$  monomers per *E. coli* cell and  
177 that a dimer of tetramers covers about 170 nt *in vivo* (Reyes-Lamothe et al., 2010). Consequently,  
178 there are not enough Ssb copies per cell to accommodate the 108 000 nucleotides ssDNA F plasmid,  
179 plus the few hundreds of nucleotides of ssDNA associated with replication forks ( $\sim 650$  nt at 22°C  
180 (Lohman and Ferrari, 1994)). This raises the possibility that the reduced availability of Ssb molecules  
181 during plasmid entry could provoke a transitory disturbance of the host chromosome DNA  
182 replication. One way to address this question *in vivo* is to monitor a fluorescent fusion of the  $\beta_2$ -clamp  
183 replisome component (mCh-DnaN), which is diffuse in the cytoplasm of non-replicating cells and  
184 forms discrete replisome-associated foci during DNA replication progression (Moolman et al., 2014;  
185 Reyes-Lamothe et al., 2008, 2010). Microscopy imaging and skewness analysis showed no change  
186 in DnaN localisation pattern before, during or after Ssb conjugative foci formation (Figure S2D). This  
187 indicates that Ssb recruitment onto the incoming ssDNA plasmid does not result in the collapse of  
188 the replication fork. Whether the rate of DNA replication is affected during this transient and short  
189 process remains a possibility.

190

191 ***ss-to-dsDNA conversion and subsequent plasmid replication in the transconjugant cells***

192 The conversion of the newly acquired ssDNA plasmid into dsDNA by the complementary strand  
193 synthesis reaction and the subsequent plasmid duplication events were analysed using the *parS*/ParB  
194 DNA labelling system (Goldlust et al., 2022; Nolivos et al., 2019). The *parS* binding site is inserted  
195 in the F plasmid, while the ParB binding protein fluorescently labelled with the mCherry (mCh-ParB)  
196 is produced from a plasmid in recipient cells only. Under the microscope, the ss-to-dsDNA  
197 conversion is reported by the disappearance of the Ssb-Ypet conjugative focus and the formation of  
198 a mCh-ParB focus in the transconjugant cells (Figure 2A). We first performed time-lapse imaging (1  
199 min/frame) to visualise the success rate and timing of ss-to-dsDNA conversion after ssDNA entry  
200 (Figure 2B). Analysis shows that the appearance of the Ssb-Ypet conjugative focus is followed by  
201 the formation of the mCh-ParB focus in  $83.3 \pm 2.3$  % ( $n = 311$ ) individual transconjugant cells  
202 analysed, indicating that the vast majority of internalised ssDNA plasmids are successfully converted  
203 into dsDNA plasmids (Figure 2C). Notably, we observe that  $40 \pm 3.2$  % ( $n = 286$ ) of transconjugant  
204 cells where the newly acquired ssDNA plasmid has already been converted into dsDNA subsequently  
205 receive additional ssDNA (Figure 2D, Figure S3A). We quantify that  $92 \pm 3.1$  % of these multiple  
206 ssDNA acquisition events originate from the same donor, among which  $79 \pm 5.3$  % appear to take  
207 place at the same membrane position, suggesting that they occur through the same conjugation pore  
208 (Figure S3A). The evidence for multiple transfers within an established mating pair demonstrates that  
209 a single donor can successively give several copies of the T-strand and that transconjugants in which  
210 the ss-to-dsDNA conversion has already been achieved do not become instantly refractory to *de novo*  
211 plasmid acquisition. Accordingly, establishing immunity to conjugation by transconjugant cells is  
212 expected to require the production of the plasmid-encoded exclusion proteins TraS and TraT.

213         Considering successful ss-to-dsDNA events only, we calculate an average  $4 \pm 1.6$  min ( $n =$   
214 475) time lag between the appearance of the Ssb-Ypet conjugative focus and the formation of the  
215 mCh-ParB focus (Figure 2E). This period reflects the time required for the completion of a reaction  
216 cascade that comprises the complete internalisation of the ssDNA plasmid, the circularisation of the

217 ssDNA plasmid by TraI, the initiation and completion of the complementary strand synthesis  
218 replication, and the recruitment of ParB molecules on the *parS* site in dsDNA form. Though our  
219 system does not allow evaluating each step's contribution, results show that the complete sequence  
220 of reactions is achieved within a relatively short and consistent period.

221 Next, we first performed time-lapse imaging (5 min/frame) to examine the timing of plasmid  
222 duplication in transconjugant cells (*i.e.*, replication and visual separation of the plasmid copies)  
223 (Figure 2B). We estimate an average of  $10.4 \pm 4.7$  min ( $n = 158$ ) period between the ssDNA-to-  
224 dsDNA conversion and the first plasmid duplication event (from one to two mCh-ParB foci) and  
225 similar  $10.1 \pm 5.1$  min ( $n = 124$ ) between the first and the second duplication event (from two to three  
226 or four mCh-ParB foci) (Figure 2F). We then decided to compare the rate of plasmid duplication in  
227 transconjugants to the rate of plasmid duplication in a vegetatively growing F-carrying donor strain.  
228 To do so, we plotted the number of plasmid foci per cell from the ss-to-dsDNA conversion (mCh  
229 focus appearance) to cell division in transconjugants and from cell birth to cell division in F-carrying  
230 donor cells (Figure 2G). Results show that the number of F per cell increases significantly faster in  
231 transconjugant cells than in vegetatively growing F-carrying cells (75 % increase of the fit curve  
232 slope), yet to reach a similar final number of  $\sim 4 \pm 1$  copies per cell before division (Figure 2G). F  
233 copy number, like chromosome replication, is known to be controlled by the cell cycle progression,  
234 where initiation occurs when a constant mass per origin is achieved (Keasling et al., 1991). Therefore,  
235 our observations are consistent with the interpretation that when a single plasmid copy arrives in a  
236 recipient cell that can be at any cell cycle stage, plasmid replication initiation is unrepressed until the  
237 specific number of plasmid copies per cell mass is restored. This accelerated plasmid replication  
238 allows for the rapid increase in F copy number before the division of the transconjugant cells, thus  
239 facilitating the segregation of plasmid copies to daughter cells.

240 Localisation analysis reveals that the ss-to-dsDNA conversion and the first duplication event  
241 occur at distinct subcellular positions. The initial mCh-ParB focus preferentially appears in the polar  
242 region of the transconjugant cell, comparable to the ssDNA's entry location (compare Figure 2H to

243 Figure 1C and Figure S3B to Figure S2A). A noticeable difference is that mCh-ParB foci appear less  
244 peripheral, indicating that they are not as close to the cell membrane as Ssb-Ypet conjugation foci  
245 (compare Figure 2H to Figure 1C, and Figure S3C to Figure S2B). We observe that the mCh-ParB  
246 focus subsequently migrates to the midcell position before duplication (Figure 2H, Figure S3B-C).  
247 These data show that the two DNA synthesis reactions involved in plasmid processing (*i.e.*, ss-to-  
248 dsDNA conversion and plasmid replication) are separated in time and space in the new host cell. The  
249 recruitment of the complementary strand synthesis machinery and the ss-to-dsDNA replication  
250 reaction occur in the vicinity of the polar position of entry of the ssDNA plasmid, while plasmid  
251 replication occurs in the midcell region. Altogether, these analyses reveal that plasmid processing  
252 steps (ssDNA entry, ss-to-dsDNA conversion and plasmid replication) occur at specific intracellular  
253 positions within the new host cell and follow a precise chronology.

#### 254

#### 255 ***Program of plasmid-encoded protein production in transconjugant cells***

256 We constructed *superfolder gfp* (*sfgfp*) C-terminal translational fusions to several genes located in  
257 the different functional regions of the F plasmid to examine the production timing of plasmid-encoded  
258 proteins in transconjugant cells, which we use to get insights into the timing of plasmid gene  
259 expression (Figure 3A, Figure S4A). *YgfA*, *ygeA*, *psiB*, *yfjB*, *yfjA* and *ssb<sup>F</sup>* are located in the leading  
260 region and are transferred in order after the origin of transfer *oriT*. The *sopB* gene is part of the  
261 SopABC partition system and is located in the maintenance region. The *traM*, *traC*, *traS* and *traT*  
262 genes are located in the *tra* region that encodes factors involved in plasmid transfer. TraM is the  
263 accessory protein of the relaxosome complex that is recruited to the *oriT* (Di Laurenzio et al., 1992);  
264 TraC is the traffic ATPase organised as a hexamer of dimers docked to the cytoplasmic faces of the  
265 T4SS (Hu et al., 2019); TraS and TraT correspond to the F plasmid exclusion (immunity) system that  
266 protects against self-transfer (Achtman et al., 1977; Jalajakumari et al., 1987; Manning et al., 1980).

267 We first performed time-course experiments where microscopy snapshot images of the  
268 conjugating population were acquired 1, 2, 4 and 6 hours after mixing donors and recipient cells. For

269 each time point, the frequency of transconjugants (T/R+T) was directly measured at the single-cell  
270 level from the proportion of recipient cells exhibiting diffuse mCh-ParB fluorescence (R) or  
271 transconjugant cells harbouring mCh-ParB foci (T), and the intracellular green fluorescence Signal  
272 to Noise Ratio (SNR) was automatically measured (Figure S4B-D). This snapshot analysis shows  
273 that all F plasmid derivatives carrying sfGFP fusions retained their transfer ability and raised  
274 frequencies of transconjugants between 57 and 93 % after 6 hours of mating. Also, fusion-carrying  
275 plasmid acquisition is systematically followed by an increase in sfGFP signal in transconjugant cells,  
276 with highly variable timing and levels (Figure S4B-D).

277 Better resolution of the production level and timing of sfGFP fusions with respect to the ss-  
278 to-dsDNA conversion (appearance of the mCh-ParB focus) in individual transconjugant cells was  
279 obtained using time-lapse imaging of conjugation performed in the microfluidic chamber (Movie S1  
280 and S2). We performed transconjugant cell detection and quantification of the intracellular sfGFP  
281 SNR cells over time (Figure S5A-D). When the transconjugant cell divided, we continued  
282 fluorescence quantification in the resulting daughter cells to monitor sfGFP production over a longer  
283 period. From this raw data, we calculated the fold-increase in SNR per ten-minute interval, where a  
284 fold-increase superior to one reveals that the fusions are being produced in the transconjugants  
285 (Figure S5A-D). These data were finally translated into a comprehensive diagram presenting the  
286 production time windows for each fusion in transconjugant cells relative to the ss-to-dsDNA  
287 conversion event (Figure 3B). This analysis reveals that fusions belonging to the different plasmid  
288 regions exhibit specific production timings with respect to plasmid processing steps.

289 Remarkably, we detect the synchronous production of the leading YgeA, PsiB, YfjB, YfjA  
290 and Ssb<sup>F</sup> fusion proteins even before the appearance of the mCh-ParB focus (Figure 3B and Figure  
291 S5A). Furthermore, the production of these fusions is only transient as it peaks at ~5 minutes and  
292 stops 25-35 minutes after the ss-to-dsDNA conversion event. This unexpected observation indicates  
293 that leading fusions start being produced when the plasmid is still in ssDNA form and stops rapidly  
294 after the plasmid is converted into dsDNA form. An interesting exception is YgfA-sfGFP, for which

295 production is only detected in the 10-20 minutes interval after mCh-ParB focus appearance. The *ygfA*  
296 gene is the closest to the *oriT* and is, therefore, the first gene to be transferred into the recipient (Figure  
297 3A, Figure S4A). However, *ygfA* gene orientation is opposite to other tested leading genes, meaning  
298 that the T-strand does not correspond to the template strand for *ygfA* transcription. Consequently, and  
299 consistent with our observations, *ygfA* expression can only occur after synthesising the  
300 complementary template strand by the ss-to-dsDNA conversion.

301 The ss-to-dsDNA conversion is followed by the production of maintenance and Tra proteins,  
302 starting with SopB and TraM, then TraC, and eventually TraS and TraT fusions (Figure 3B, Figure  
303 S5B-C). The production of these fusions is expected to require the presence of the plasmid in dsDNA  
304 form since the corresponding genes are known to be controlled by dsDNA promoters ( $P_{sopAB}$  for *sopB*,  
305  $P_M$  for *traM* and  $P_Y$  for *traC* and *traST*). However, what could explain the observed differences in the  
306 production timings? We addressed whether timing discrepancies could simply account for the  
307 fusions' position on the genetic map of the F plasmid. This possibility was excluded by the  
308 observation that insertion of the constitutive fluorescent reporter  $P_{lacIQ1}$ sfGFP (*sfgfp* gene under the  
309 control of the  $P_{lacIQ1}$  constitutive promoter) in the *repE-sopA*, *tnpA-ybaA* and *traM-traJ* intergenic  
310 regions resulted in similar sfGFP production timings, within the 0-10 minutes interval after the  
311 appearance of the mCh-ParB focus (Figure 3B, Figure S5D). Instead, we propose that the differential  
312 production timings of maintenance and *tra* genes reflect the activity and regulation of the promoters  
313 of the corresponding genes. The *sopAB* operon is under the control of the  $P_{sopAB}$  promoter, which is  
314 repressed by SopA binding. Therefore, the  $P_{sopAB}$  promoter is expected to be fully unrepressed and  
315 active in transconjugant cells devoid of SopA, thus allowing the rapid production of the SopAB  
316 partition complex required for plasmid stability and inheritance over cell divisions. The *traM* gene is  
317 controlled by the  $P_M$  promoter, which is weakly but constitutively active, even before its full  
318 activation by binding the TraY protein (Penfold et al., 1996). By contrast, the  $P_Y$  promoter that  
319 controls the expression of *traC*, *traS* and *traT* genes needs to be activated by the TraJ protein, encoded  
320 by the *traJ* gene under the control of its own promoter  $P_J$  and located upstream of  $P_Y$  (Virolle et al.,

321 2020). The requirement for this activation cascade probably explains the delayed production of TraC,  
322 TraS and TraT. The additional delay between TraC and TraS/TraT fusions production could  
323 potentially reflect the relative distance of these genes to the *P<sub>Y</sub>* promoter (5.9 kb for *traC* and 20.4 kb  
324 for *traST*).

325 Notably, the intracellular levels of Tra proteins within transconjugant cells reach a plateau  
326 between 60 to 90 minutes after the ss-to-dsDNA conversion and remain stable throughout our  
327 observations (Figure 3B, Figure S5C). This involves that at that point, transconjugant cells have  
328 produced the transfer machinery and the exclusion system and have most likely been converted into  
329 proficient plasmid donors. In support of this interpretation, TraM, TraC, TraS, TraT and SopB are  
330 detected at similar levels in vegetatively growing F-carrying donor cells (Figure 3C, Figure S4C-D  
331 and S5B-C). This is not the case for YgeA, PsiB, YfjB, YfjA, and Ssb<sup>F</sup> leading proteins, which  
332 intracellular levels start decreasing 25-35 minutes after the ss-to-dsDNA conversion in the  
333 transconjugants, and which are not detected in vegetatively growing donor cells (Figure 3C, Figure  
334 S4B and S5A). These results are consistent with the interpretation that leading proteins are produced  
335 rapidly and only transiently upon entry of the ssDNA plasmid in the recipient cells and not when the  
336 plasmid is maintained in dsDNA form during vegetative replication.

337

338 ***Single-stranded promoters allow the early expression of the leading genes in the transconjugant***  
339 ***cell***

340 Together with previous works (Althorpe et al., 1999b; Bagdasarian et al., 1992; Bates et al., 1999;  
341 Jones et al., 1992), the early and transiently expression of leading genes in transconjugant cells  
342 support the existence of specific sequences that would act as single-stranded promoters to initiate the  
343 transcription of leading genes from the internalised ssDNA plasmid. Using bioinformatics analysis,  
344 we identified a region upstream of the *ssb<sup>F</sup>*, *yfjA*, *yfjB*, *psiA* and *psiB* genes, which we named *Frpo2*,  
345 that shares 92% identity with the previously reported *Frpo* region (renamed *Frpo1*) located upstream  
346 *ygeA* and *ygeB* and previously characterised *in vitro* (Masai and Arai, 1997) (Figure 4A). DNA



347 folding prediction using mFold (<http://www.unafold.org>) indicates that the single-stranded form of  
348 *Frpo2* can fold into a highly stable stem-loop structure that also carries canonical -10 and -35 boxes,  
349 similar to the *Frpo1* region (Figure S6A) (Masai and Arai, 1997). We addressed the effect of *Frpo1*  
350 or *Frpo2* deletions on the expression of the downstream genes in transconjugant cells using live-cell  
351 microscopy. Microscopy analysis of transconjugant cells receiving the F  $\Delta$ *Frpo1* *ygeA-sfgfp*, the F  
352  $\Delta$ *Frpo2* *ssb<sup>F</sup>-sfgfp*, or the F  $\Delta$ *Frpo2* *yjfa-sfgfp* revealed no significant fold-increase in sfGFP  
353 fluorescence before or after the ss-to-dsDNA conversion in the transconjugant cells (Figure 4B).

354 We then addressed the impact of *Frpo1* and *Frpo2* deletions on the efficiency of conjugation  
355 after three hours of mating, as estimated by plating assays (Figure 4C). F  $\Delta$ *Frpo1* exhibits a  
356 dramatically reduced frequency of transconjugants of  $25.2 \pm 2.9$  % compared to  $92.6 \pm 6.6$  % for the  
357 *Fwt*. Comparable results were obtained for F  $\Delta$ *Frpo1*  $\Delta$ *ygeAB* ( $32.7 \pm 7.1$ ) and F  $\Delta$ *Frpo1*  $\Delta$ *ygeA* ( $14.5$   
358  $\pm 0.4$ ). Surprisingly, the single deletion of *ygeA* decreases the conjugation of efficiency even further  
359 ( $3.9 \pm 1.9$  %), and despite our multiple attempts, the deletion of *ygeB* alone could never be  
360 constructed. By contrast, the deletions of *Frpo2* or *ssb<sup>F</sup>* have no significant impact on the conjugation  
361 efficiency. These results show that *Frpo1* and *Frpo2* are required for the early expression of the  
362 downstream genes upon plasmid entry in recipient cells during conjugation *in vivo*. However, genes  
363 under the control of *Frpo1* appear to have a more critical role in conjugation than those under the  
364 control of *Frpo2*.

365

### 366 ***Role of the plasmid-encoded Ssb<sup>F</sup> leading protein in plasmid establishment***

367 The rapid and transient expression of leading genes upon plasmid entry strongly suggests that leading  
368 proteins have an essential role during the early steps of plasmid establishment in the new host cell.  
369 The leading region conserved in various enterobacterial plasmids encodes a homolog of the single-  
370 strand-binding protein Ssb encoded on the *E. coli* chromosome (Golub and Low, 1985, 1986b; Golub  
371 et al., 1988; Howland et al., 1989; Jones et al., 1992; Kolodkin et al., 1983). The chromosomally  
372 encoded *ssb* gene is conserved and essential in all bacterial organisms, raising the question of the



373 *raison d'être* of plasmid-born *ssb* homologues. Early study shows that the Ssb<sup>F</sup> encoded by the F  
374 plasmid can partially complement conditional mutations of the chromosomal *ssb* gene (Golub and  
375 Low, 1986b; Porter and Black, 1991). Consistently, we performed simultaneous visualisation of Ssb<sup>F</sup>-  
376 mCh produced from a pTrc99a-*ssb*<sup>F</sup>-*mch* plasmid and the chromosomally-encoded Ssb-Ypet (Figure  
377 S7A) and observed similar intracellular positioning (Figure S7B) confirmed by colocalisation  
378 analysis (Figure S7C). This indicates that both the plasmid Ssb<sup>F</sup> and the host Ssb are recruited to the  
379 ssDNA that follows the replication forks in vegetatively growing cells. Similarly, Ssb<sup>F</sup>-sfGFP also  
380 forms foci in transconjugant cells that have acquired the F *ssb*<sup>F</sup>-*sfGFP* plasmid, mainly during the first  
381 and second plasmid duplication events (Figure S7D-E). Nonetheless, the role of Ssb<sup>F</sup> during  
382 conjugation is still unclear, and its deletion from the F plasmid has no significant impact on  
383 conjugation efficiency (Figure 4C).

384 To get further insight into the role of Ssb<sup>F</sup> during conjugation, we revisited the dynamics of  
385 ssDNA entry, ss-to-dsDNA conversion and duplication of the F  $\Delta$ *ssb*<sup>F</sup> plasmid. Time-lapse  
386 microscopy image analysis reveals that Ssb<sup>F</sup> deletion has no impact on the dynamics of Ssb-Ypet  
387 conjugative foci (Figure 4D) or the timing of the ss-to-dsDNA conversion (compare Figure 4E to  
388 Figure 2E). However, Ssb<sup>F</sup> deletion dramatically delays the timing of plasmid duplication in  
389 transconjugant cells (compare Figure 4F to Figure 2F). The time lag between mCh-ParB appearance  
390 and the first duplication is increased by ~58 % (from  $10.4 \pm 4.7$  for F<sub>wt</sub> to  $16.4 \pm 9.5$  for F  $\Delta$ *ssb*<sup>F</sup>),  
391 and the time between the first and second plasmid replication event is increased by ~29 % (from  $10.1$   
392  $\pm 4.7$  for F<sub>wt</sub> to  $13 \pm 8$  for F  $\Delta$ *ssb*<sup>F</sup>). This indicates that Ssb<sup>F</sup> has a role in facilitating the first rounds  
393 of plasmid duplication in the new transconjugant cell, possibly by increasing the cellular pool of  
394 single-strand binding protein available for DNA replication. This function appears dispensable since  
395 the absence of Ssb<sup>F</sup> delays plasmid duplication but does not affect the final efficiency of conjugation,  
396 at least when conjugation is performed in optimal conditions between *E. coli* MG1655 strains.

397

398

399 **Discussion**

400 Our current knowledge of conjugation mainly emerges from experimental genetic, biochemical and  
401 structural studies that provided a well-documented understanding of the molecular reactions and  
402 factors involved in DNA transfer, while genomic and computational studies uncovered the diversity  
403 of conjugative plasmids and their importance in the epidemiology of antibiotics resistance  
404 dissemination. It is only recently that the application of optical microscopy has started to provide  
405 insights into the organisation of conjugation at the cellular scale (Aguilar et al., 2011; Babic et al.,  
406 2011; Babić et al., 2008; Carranza et al., 2021; Clarke et al., 2008; Goldlust et al., 2022; Lawley et  
407 al., 2002; Low et al., 2022; Nolivos et al., 2019). In this study, live-cell microscopy combined with  
408 specifically developed fluorescent reporters offers a unique view of the cellular dynamics of  
409 conjugation while providing insights into the timing and localisation of each key step.

410 We report the presence of ssDNA plasmid on both the donor and the recipient's side during  
411 plasmid transfer. Noticeably, the ssDNA plasmid is not randomly positioned but instead allocated to  
412 specific subcellular locations within the mating pair cells. The exit point of the ssDNA is  
413 preferentially located on the side of the donor cell and enriched at quarter positions. This unlikely  
414 reflects a specific positioning of the T4SS machinery, which was reported to be homogeneously  
415 located throughout the periphery of the cells (Aguilar et al., 2011; Carranza et al., 2021). Instead, the  
416 observed lateral localisation of active conjugation pores may reflect the facilitated access to F plasmid  
417 molecules, which are also positioned at quarter positions and excluded from the cell poles (Gordon  
418 et al., 2004; Niki and Hiraga, 1997). By contrast, the ssDNA mainly enters the polar region of the  
419 recipient cells. This could suggest that the pole of the recipients' surface is the preferred location for  
420 the donor's F-pilus attachment or the stabilisation of the mating pair. The latter possibility is  
421 reinforced by the fact that mating pair stabilisation during F conjugation involves interaction between  
422 the plasmid protein TraN exposed at the surface of the donor cells and the host outer membrane  
423 protein OmpA of the recipient cells (Klimke and Frost, 1998; Low et al., 2022). OmpA was shown

424 to be enriched and less mobile in the polar regions of *E. coli* cells (Verhoeven et al., 2013), possibly  
425 favouring the stabilisation of the mating pair and the conjugation pore at this location.

426 The unexpected finding that the ssDNA is present in the donor during conjugation also  
427 provides insights into the activity of TraI and its coordination with the transfer of the T-strand through  
428 the T4SS or the RCR of the non-transferred strand. Before DNA transfer initiation, the relaxosome  
429 bound to the plasmid's *oriT* is docked to the T4SS by the TraD (VirD4) coupling protein, thus forming  
430 the pre-initiation complex (Figure 5A(i)). Contact with the recipient cell is proposed to induce a signal  
431 that activates the pre-initiation complex. We uncover the existence of a brief stage where part of the  
432 T-strand has already been transferred into the recipient cell while no ssDNA is present within the  
433 donor (Figure 5A(ii)). At this stage, the absence of ssDNA in the donor implicates that all the ssDNA  
434 generated by TraI has been removed, both by transfer of the T-strand through the T4SS and by  
435 complementation of the non-transferred ssDNA strand by the RCR. After this transient stage, the  
436 ssDNA also accumulates in the donor, suggesting that the ssDNA is generated by TraI helicase  
437 activity in the donor faster than it is removed by transfer and RCR synthesis (Figure 5A(iii)).

438 Assuming the  $2.9 \pm 1.1$  min lifespan of the Ssb-Ypet foci in transconjugants reflects the time  
439 required to complete the internalisation of the 108 000 nt ssDNA F plasmid, we calculated a  $620 \pm$   
440  $164 \text{ nt}\cdot\text{s}^{-1}$  transfer rate. This is in reasonable agreement with the historical  $770 \text{ nt}\cdot\text{s}^{-1}$  rate estimated  
441 from the 100 minutes required to transfer the whole 4.6 Mb *E. coli* chromosome (Jacob and Wollman,  
442 1958). Besides, the rate of DNA synthesis by the DNA polymerase III holoenzyme during RCR was  
443 estimated at  $650\text{-}750 \text{ nuc}\cdot\text{s}^{-1}$  (Stephens and McMacken, 1997). By comparison, the rate of TraI  
444 helicase activity was measured at  $1120 \pm 160 \text{ bp}\cdot\text{s}^{-1}$  (Sikora et al., 2006). These estimates support the  
445 view that ssDNA accumulation in the donor accounts for the faster rate of TraI helicase activity than  
446 the rate of T-strand plasmid transfer or RCR. Therefore, it is possible that, contrasting with the  
447 previously suggested but never demonstrated proposal, the helicase activity of the relaxase is not  
448 strictly coupled with the activity of DNA translocation through the T4SS.

449 Live-cell microscopy uncovers the global chronology conjugation steps, as summarised in  
450 Figure 5B. The plasmid processing in the transconjugant cell is a relatively rapid process, as the entry  
451 of the ssDNA plasmid and its conversion into dsDNA is completed in about 4 minutes on average.  
452 Most importantly, the ss-to-dsDNA conversion event is the pivotal event that determines the program  
453 of plasmid gene expression. Leading genes are the first to enter the recipient cell and also the first to  
454 be expressed from the F plasmid in ssDNA form. Consistently with previous proposals (Bates et al.,  
455 1999; Masai and Arai, 1997; Nasim et al., 2004), we show that the early expression of leading genes  
456 depends on sequences that act as single-stranded promoters when the plasmid is still in ssDNA form.  
457 As previously described for *Frp1*, we propose that the highly homologous *Frp2* sequences  
458 identified here folds into a stable stem-loop structures that reconstruct -35 and -10 consensus boxes,  
459 resulting in transcription initiation.

460 Leading gene expression is also transient as the ss-to-dsDNA conversion turns off leading  
461 protein production by inactivating *Frp1* and *Frp2* promoters while licencing the expression of  
462 maintenance, transfer and other plasmid genes under the control of conventional dsDNA promoters,  
463 often subject to their own regulation specificities. Maintenance and transfer protein levels within  
464 transconjugants reach a steady-state equivalent to that of vegetatively growing F-containing cells in  
465 about 30 to 90 minutes, depending on the protein. Interestingly, our previous work showed that  
466 tetracycline resistance factors encoded by the *Tn10* transposon inserted in the intergenic region *ybdB*-  
467 *ybfA* of the F plasmid are also produced immediately after the ss-to-dsDNA conversion and reach the  
468 resistant cell's level within approximately 90 minutes (Nolivos et al., 2019). These findings  
469 consistently indicate that this time scale corresponds to the period needed for the transconjugant cells  
470 to gain plasmid-encoded functions, including plasmid maintenance, conjugation ability, immunity  
471 against self-transfer and additional resistance potentially carried by the plasmid.

472 The regulation of plasmid gene expression by plasmid processing is an elegant way to ensure  
473 the sequential and timely production of plasmid proteins in the transconjugant cell, and particularly  
474 to restrict the production of leading factors to a narrow time window following the entry of the ssDNA

475 plasmid. However, *de novo* protein synthesis might not be the only way to provide the transconjugant  
476 cell with plasmid-encoded proteins. Recent work by Al Mamun *et al.* reports that the transfer of the  
477 F-like plasmid pED208 (IncFV) is concomitant with the translocation of several plasmid-encoded  
478 proteins, including TraI, ParA, ParB1, Ssb homologue Ssb<sup>ED208</sup>, ParB2, PsiB, and PsiA (Al Mamun  
479 *et al.*, 2021). Protein translocation was detected at low frequency ( $10^{-5}$  recombinants per donor cell  
480 between one and five hours of mating) using a highly sensitive Cre recombinase assay. Protein  
481 translocation might also occur during the transfer of the native F plasmid but could not solely explain  
482 our observations. Indeed, our microscopy analysis shows that YgeA, PsiB, YfjB, YfjA and Ssb<sup>F</sup>  
483 leading fusions are below the microscopy detection threshold in donor cells but are quantified at  
484 significant intracellular levels in all transconjugant cells. This implies that the amounts of leading  
485 proteins observed in the transconjugant cells cannot just originate from donor cells, but result from  
486 *de novo* protein synthesis, which we show depends on *Frpo1* and *Frpo2* sequences.

487 Both the early production and the direct translocation of leading proteins suggest a critical  
488 role of the leading region in conjugation. Several elements support this view. The leading region is  
489 conserved in a variety of conjugative plasmids (Cox and Schildbach, 2017; Golub and Low, 1985,  
490 1986a; Golub *et al.*, 1988; Loh *et al.*, 1989, 1990). In addition, the leading regions of plasmids  
491 belonging to a wide range of incompatibility groups (IncF, IncN, IncP9 and IncW) classified as  
492 MOBF plasmids using the relaxase as a phylogenetic marker were reported to be the preferential  
493 target for CRISPR-Cas systems directed against conjugation (Fernandez-Lopez *et al.*, 2016;  
494 Garcillán-Barcia *et al.*, 2009; Westra *et al.*, 2013). Recently, the leading region was shown to be an  
495 important evolutionary target for the dissemination of the pESLB (IncI) plasmid (Benz and Hall,  
496 2022). Concerning the F plasmid, we can stress that *Frpo1* and *Frpo2* share 92 % similarity at the  
497 nucleotide level and are located only about 5 kb apart. This implies that when in dsDNA form during  
498 vegetative plasmid replication, *Frpo1* and *Frpo2* sequences would be a potential substrate for  
499 homologous recombination, resulting in the deletion of the intervening segment. However, the  
500 intervening segment carries the *flmAB* genes, functional homologues to the *hok/sok* toxin-antitoxin

501 system from the R1 plasmid (Loh et al., 1988), which are likely to safeguard the stability of the  
502 leading region.

503 Despite this body of evidence, it is currently challenging to rationalise the importance of the  
504 leading region since the molecular functions of most leading proteins are still unknown. Our data  
505 indicate that genes downstream of *Frpo1* (*ygeA* et *ygeB*) have a critical function in conjugation. By  
506 contrast, genes located downstream *Frpo2* (*ssb<sup>F</sup>*, *yffA*, *yffB*, *psiB*, *psiA* and *flmC*) appear to be  
507 dispensable since deletions of *Frpo2*, *ssb<sup>F</sup>* or *psiB* (Loh et al., 1989) have no significant impact on  
508 the overall conjugation efficiency addressed by plating assays. However, conjugation efficiency  
509 assays are generally performed between identical or closely related bacterial strains in optimal  
510 medium and temperature conditions. This likely undermines the role of genes that are not strictly  
511 essential but might facilitate or optimise conjugation. Hence, it is possible that the importance of the  
512 leading factors would be best revealed in less favourable conditions, between phylogenetically distant  
513 bacteria, or on the evolutionary scale. Meanwhile, real time microscopy might help uncover the  
514 potentially subtle influence of these genes on the sequence of conjugation in live cells.

515

516

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528 All data to understand and assess the conclusions of this research are available in the main text and  
529 Supplementary Materials.

530

531

## 532 **Materials and Methods**

### 533 **Bacterial strains, plasmids and growth**

534 Bacterial strains are listed in Table S1, plasmids in Table S2, and oligonucleotides in Tables S3.  
535 Fusion of genes with fluorescent tags and gene deletion on the F plasmid used  $\lambda$ Red recombination  
536 (Datsenko and Wanner, 2000; Yu et al., 2000). Modified F plasmids were transferred to the  
537 background strain K12 MG1655 by conjugation. Where multiple genetic modifications on the F  
538 plasmid were required, the *kan* and *cat* genes were removed using site-specific recombination induced  
539 by expression of the Flp recombinase from plasmid pCP20 (Datsenko and Wanner, 2000). Plasmid  
540 cloning were done by Gibson Assembly and verified by Sanger sequencing (Eurofins Genomics  
541 biotech). Strains and plasmids were verified by Sanger sequencing (Eurofins Genomics). Cells were  
542 grown at 37°C in M9 medium supplemented with glucose (0.2 %) and casamino acid (0.4 %) (M9-  
543 CASA) before imaging, and in Luria-Bertani (LB) broth for conjugation efficiency assays. When  
544 appropriate, supplements were used in the following concentrations; Ampicillin (Ap) 100  $\mu$ g/ml,  
545 Chloramphenicol (Cm) 20  $\mu$ g/ml, Kanamycin (Kn) 50  $\mu$ g/ml, Streptomycin (St) 20  $\mu$ g/ml, and  
546 Tetracycline (Tc) 10  $\mu$ g/ml.

547

### 548 **Conjugation assays**

549 Overnight cultures in LB of recipient and donor cells were diluted to an  $A_{600}$  of 0.05 and grown until  
550 an  $A_{600}$  comprised between 0.7 and 0.9 was reached. 25  $\mu$ l of donor and 75  $\mu$ l of recipient cultures  
551 were mixed into an Eppendorf tube and incubated for 90 minutes at 37°C. 1 ml of LB was added  
552 gently and the tubes were incubated again for 90 min at 37°C. Conjugation mix were vortexed, serial



553 diluted, and plated on LB agar X-gal 40  $\mu\text{g/ml}$  IPTG 20  $\mu\text{M}$  supplemented the appropriate antibiotic  
554 to select for recipient or donor populations. Recipient (R) colonies were then streaked on plated on  
555 LB agar containing tetracycline 10  $\mu\text{g/ml}$  to select for transconjugants (T) and the frequency of  
556 transconjugant calculated from the (T/R+T) presented in Figure 4C.

557

### 558 **Live-cell microscopy experiments**

559 Overnight cultures in M9-CASA were diluted to an  $A_{600}$  of 0.05 and grown until  $A_{600} = 0.8$  was  
560 reached. Conjugation samples were obtained by mixing 25  $\mu\text{l}$  of donor and 75  $\mu\text{l}$  of recipient into an  
561 Eppendorf tube. For time-lapse experiments, 50  $\mu\text{l}$  of the pure culture or conjugation mix was loaded  
562 into a B04A microfluidic chamber (ONIX, CellASIC®) (Cayron and Lesterlin, 2019). Nutrient  
563 supply was maintained at 1 psi and the temperature maintained at 37°C throughout the imaging  
564 process. Cells were imaged every 1 or 5 min for 90 to 120 minutes. For snapshot imaging, 10  $\mu\text{l}$   
565 samples of clonal culture or conjugation mix were spotted onto an M9-CASA 1% agarose pad on a  
566 slide (Lesterlin and Duabrry, 2016) and imaged directly.

567

568 *Image acquisition.* Conventional wide-field fluorescence microscopy imaging was carried out on an  
569 Eclipse Ti2-E microscope (Nikon), equipped with x100/1.45 oil Plan Apo Lambda phase objective,  
570 ORCA-Fusion digital CMOS camera (Hamamatsu), and using NIS software for image acquisition.  
571 Acquisitions were performed using 50% power of a Fluo LED Spectra X light source at 488 nm and  
572 560 nm excitation wavelengths. Exposure settings were 100 ms for Ypet, sfGFP and mCherry and 50  
573 ms for phase contrast.

574 *Image analysis.* Quantitative image analysis was done using Fiji software with MicrobeJ plugin  
575 (Ducret et al., 2016). For snapshot analysis, cells' outline detection was performed automatically  
576 using MicrobeJ and verified using the Manual-editing interface. For time-lapse experiments,  
577 detection of cells was done semi-automatedly using the Manual-editing interface, which allows to  
578 select the cells to be monitored and automatically detect the cell outlines. Within conjugation



579 populations, donor (no mCh-ParB signal), recipient (diffuse mCh-ParB signal), or transconjugant  
580 (mCh-ParB foci) category were assigned using the ‘Type’ option of MicrobeJ. Recipient cells were  
581 detected on the basis of the presence of red fluorescence above the cell’s autofluorescence  
582 background level detected in the donors. Among these recipient cells, transconjugants were identified  
583 by running MicrobeJ automated detection of the ParB fluorescence foci (Maxima detection). This  
584 approach was used independently of the presence or the absence of the Ssb-Ypet, or sfGFP fusions  
585 within donor and recipient cells. Within the different cell types, mean intensity fluorescence (a.u.),  
586 skewness, Signal/Noise Ratio (SNR), or cell length ( $\mu\text{m}$ ) parameters were automatically extracted  
587 and plotted using MicrobeJ. SNR corresponds to the ratio (mean intracellular signal / mean noise  
588 signal), where the mean intracellular signal is the fluorescence signal per cell area and the noise is  
589 the signal measured outside the cells (due to the fluorescence emitted by the surrounding medium).  
590 By contrast with the total amount of fluorescence per cell, which is depending on the cell size/age  
591 and accounts for the background, SNR quantitative estimate is more appropriate for unbiased  
592 quantification of intracellular fluorescence over time. Ssb-Ypet, Ssb<sup>F</sup>-mCh and mCh-ParB foci were  
593 detected using MicrobeJ Maxima detection function, and foci localisation and fluorescence intensity  
594 were extracted and plotted automatically. Plots presenting time-lapse data were either aligned to the  
595 first frame where the transconjugant cell exhibits a conjugative Ssb-Ypet focus (ssDNA acquisition)  
596 or a mCh-ParB focus (ss-to-dsDNA conversion) as indicated in the corresponding figure legend.

597

## 598 **Statistical analysis**

599 *P*-value significance were analysed running specific statistical tests on the GraphPad Prism software.  
600 Single-cell data from quantitative microscopy analysis were extracted from the MicrobeJ interface  
601 and transferred to GraphPad. *P*-value significance of single-cell quantitative data was performed  
602 using unpaired non-parametric Mann-Whitney statistical test, which allows to compare differences  
603 between independent data groups without normal distribution assumption. *P*-value significance for  
604 the frequency of transconjugants obtained by plating assays were evaluated using One-way analysis

605 of variance (ANOVA) with Dunnetts multiple comparisons test, which allows to determine the  
606 statistical significant of differences observed between the means of three or more independent  
607 experimental groups against a control group mean (corresponding to the *Fwt*). When required, *P*-  
608 value and significance are indicated on the figure panels and within the corresponding legend.

609

610

611 **SUPPLEMENTARY MATERIALS**

612 Figs. S1 to S7

613 Tables S1 to S3

614 Captions for Movies S1 to S3

615 Movies S1 to S3

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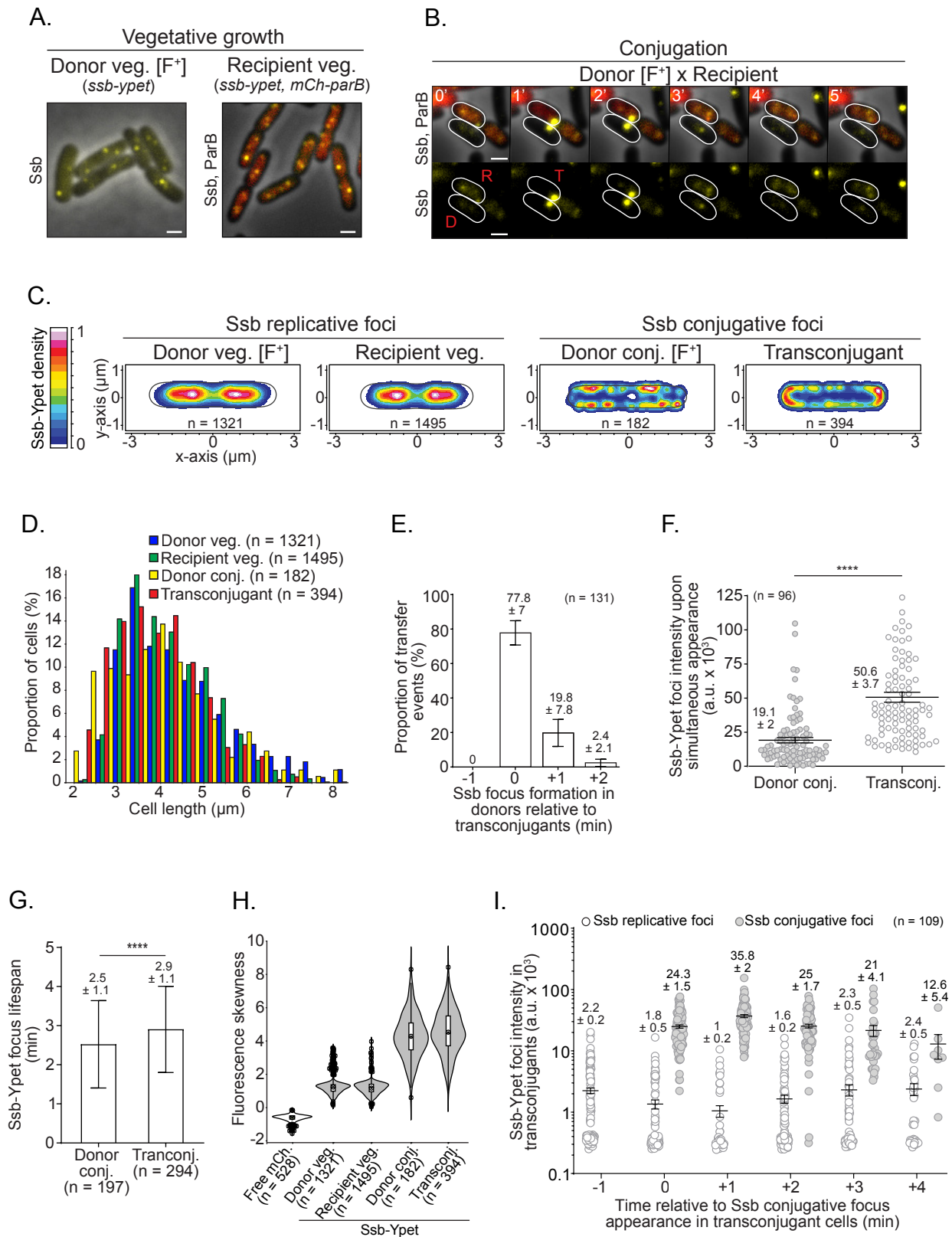


Figure 1

**Figure 1. Real time dynamics of ssDNA plasmid transfer from donor to recipient cells.**

(A) Snapshot microscopy imaging of donor and recipient strains carrying the endogenous *ssb-ypet* fusion gene on the chromosome during vegetative growth. The recipient cells also produce the mCh-ParB fluorescent protein from the pSN70 plasmid that diffuses freely into the cytoplasm in the absence of the F plasmid carrying the *parS*-binding site. Scale bars 1  $\mu$ m. (B) Time-lapse microscopy images of conjugation performed in microfluidic chamber showing a plasmid transfer event between a donor (D) and a recipient cell (R) that is converted into a transconjugant (T). The ssDNA plasmid transfer is reported by the formation of paired bright membrane-associated Ssb-Ypet foci in both donor and transconjugant cells. Scale bars 1  $\mu$ m. Additional transfer events are presented in Figure S1. (C) 2D localisation heatmaps of Ssb-Ypet fluorescent protein in donor, recipient cells in vegetative growth and in donor and transconjugant cells during conjugation. Heatmaps correspond to the merge and normalisation by the cell length of (n) individual cells from at least three biological replicates. The density scale bar is shown on the left. (D) Cell length distribution histogram of donor and recipient cells during vegetative growth, and of donor and transconjugant cells during conjugation (n cells analysed from at least three independent experiments). (E) Apparition timing of the Ssb conjugative focus in donor relative to transconjugant cells. Histograms represent the proportion of individual transfer events in which the Ssb focus appears in the donors before (-1 min), at the same time of (0 min) or after (+1 min; +2 min) the formation of a Ssb focus in the transconjugants. The number (n) of individual transfer events analysed from three independent experiments is indicated. (F) Jitter plot of the fluorescence intensity of Ssb-Ypet conjugative foci upon simultaneous formation in donor and transconjugant cells. The number of foci analysed from three independent experiments (n) is indicated. *P-value* significance from Mann-Whitney statistical test is indicated by \*\*\*\*( $P \leq 0.0001$ ). (G) Histograms of Ssb-Ypet conjugative foci lifespan in donor and transconjugant cells measured at the single-cell level. *P-value* significance from Mann-Whitney statistical test is indicated by \*\*\*\*( $P = 0.0001$ ). The number (n) of cells analysed from at least five independent experiments is indicated. (H) Violin plots showing the fluorescence skewness of a free mCherry produced from a plasmid and of the chromosomally encoded Ssb-Ypet in donor and recipient cells during vegetative growth or donor and transconjugant cells during conjugation. The median, quartile 1 and quartile 3 are indicated by horizontal lines and the mean by a black dot. Black dots above and below the max and min values correspond to outlier cells. The number of cells analysed (n) from one representative experiment is indicated. (I) Jitter plot showing the evolution of the intensity of Ssb-Ypet replicative and conjugative foci in transconjugant cells in the course of the conjugation process. Time 0 minute corresponds to the appearance of the Ssb-Ypet conjugative focus in recipient cells. The number of cells analysed (n) from three independent experiments is indicated. Donor (LY1007), recipient (LY358), transconjugant (LY358 after *Fwt* acquisition from LY1007); free mCherry producing strain (LY318).

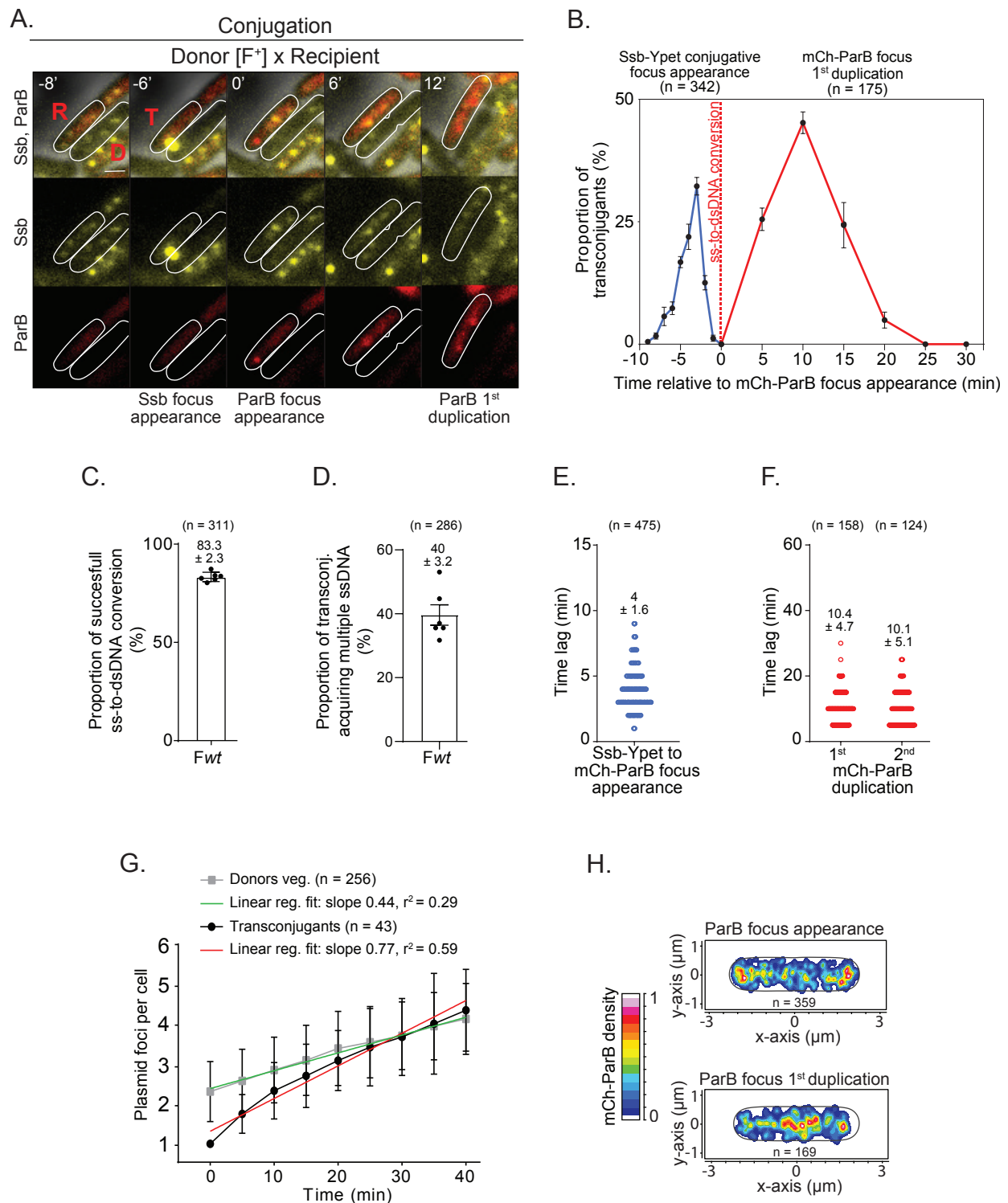


Figure 2

**Figure 2. Timing and spatial localisation of the ss-to-dsDNA conversion and plasmid duplication in transconjugant cells.**

(A) Time-lapse microscopy images performed in microfluidic chamber showing the transfer of the ssDNA plasmid reported by the formation of the Ssb-Ypet conjugative foci in both donor (D) and recipient (R) cells, followed by the ss-to-dsDNA conversion reflected by the appearance of a mCh-ParB focus in transconjugant (T) cells. Scale bar 1  $\mu\text{m}$ . (B) Single-cell time-lapse quantification of Ssb-Ypet focus appearance (blue line) and mCh-ParB focus first duplication (red line) with respect to the ss-to-dsDNA conversion revealed by mCh-ParB focus formation in transconjugant cells (0 min). The number of conjugation events analysed (n) from seven independent experiments is indicated. (C) Histogram showing the frequency of successful ss-to-dsDNA conversion reflected by the conversion of the Ssb-Ypet conjugative foci into a mCh-ParB focus. The mean and SD are calculated from (n) individual transfer events from six biological replicates (black dots). (D) Histogram showing the percentage of transconjugant cells with a mCh-ParB focus that acquire multiple ssDNA plasmids as revealed by the successive appearance of an additional Ssb-Ypet conjugative focus. The mean and SD are calculated from (n) individual transconjugant cells from six biological replicates (black dots). (E) Scatter plot showing the time lag between the appearance of the Ssb-Ypet focus and the mCh-ParB focus in transconjugant cells. The mean and SD calculated from (n) individual ss-to-dsDNA conversion event (blue circles) from seven biological replicates are indicated. (F) Scatter plot showing the time-lag between the apparition of the mCh-ParB focus and its visual duplication in two foci (1<sup>st</sup> duplication), and in three or four foci (2<sup>nd</sup> duplication). The mean and SD calculated from (n) individual duplication events (red circles) from at least six biological replicates are indicated. (G) Single-cell time-lapse quantification of the number of F foci per cell in F-carrying donor strain during vegetative growth and in transconjugants after F plasmid acquisition. For donor, the number of F foci per cell (reflected by the number of SopB-sfGFP foci) with respect to cells birth ( $t = 0$  min) is shown (grey curve). For transconjugants the number of F foci per cell (reflected by the number of mCh-ParB foci) with respect to mCh-ParB focus appearance ( $t = 0$  min) is shown (black curve). Mean and SD calculated from (n) individual cells from four biological replicates are indicated, together with curves' linear fitting lines for donors (green) and transconjugants (red). F-carrying donor strain (LY834), Transconjugant (LY358 after Fwt acquisition). (H) 2D localisation heatmaps of the mCh-ParB focus at the time of its appearance (top) and just before its duplication into two foci (bottom). Heatmaps correspond to the merge and normalisation by the cell length of (n) individual transconjugant cells from seven biological replicates. (A-F and H) Fwt donor (LY1007), recipient (LY358), transconjugant (LY358 after Fwt acquisition).

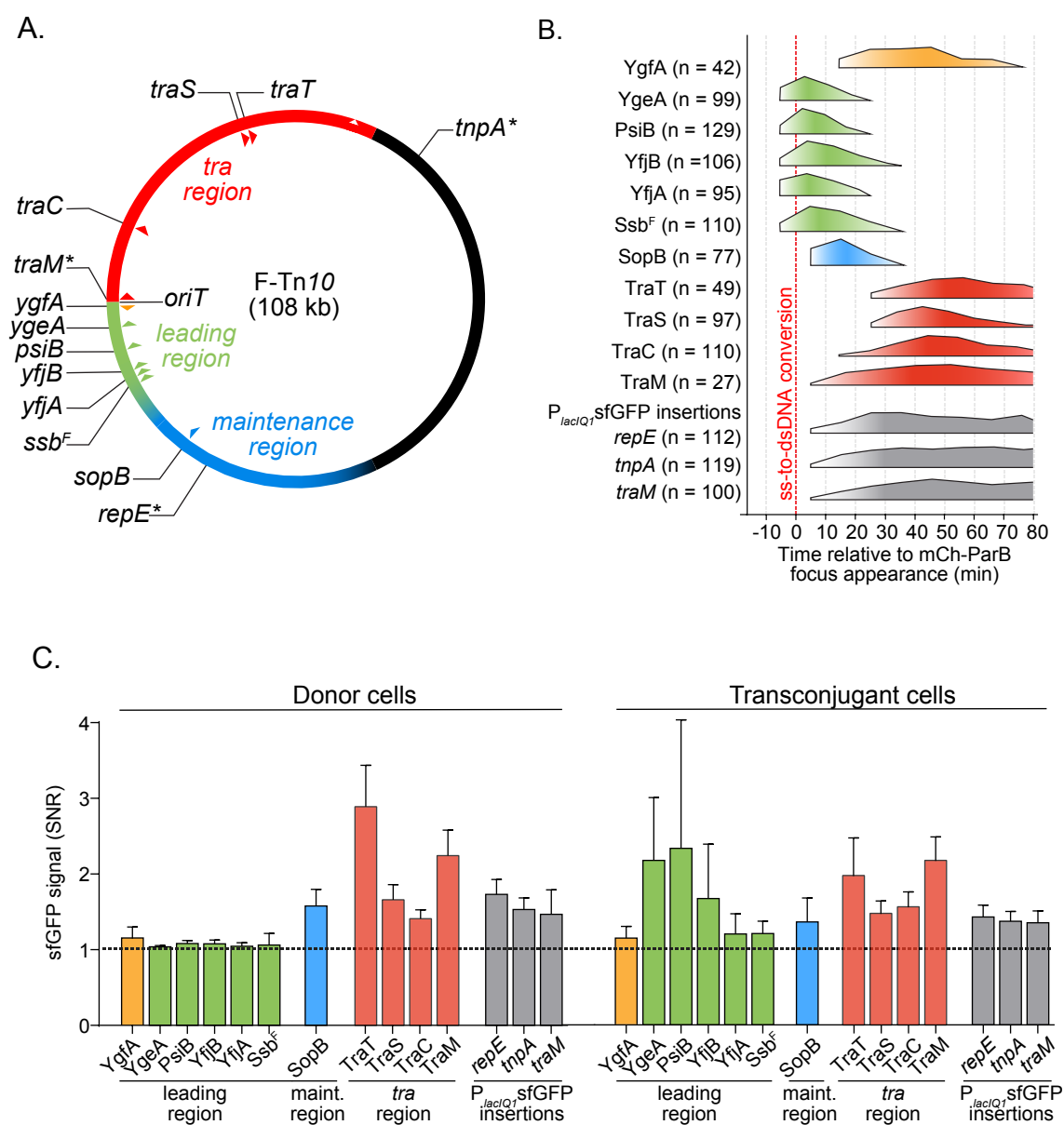


Figure 3



**Figure 3. Timing of plasmid-encoded proteins production in transconjugant cells.**

(A) Genetic map of the 108 kb F plasmid indicating the leading (green), Tra (red) and maintenance (blue) regions, and the positions of the studied genes (triangles). Stars represent the genetic location of the  $P_{lacIQ1}sfGFP$  insertions. (B) Summary diagram of the production timing of each plasmid-encoded protein fusions in transconjugant cells with respect to the timing of ss-to-dsDNA conversion reflected by mCh-ParB focus appearance (0 min). The diagram represent data from the foldchange increase in sfGFP signal from Figure S5. Orange/green, blue and red colours correspond to production of proteins from the leading, maintenance and transfer region respectively. Timings of the cytoplasmic sfGFP production from the  $P_{lacIQ1}$  promoter inserted in the *repE-sopA* (*repE*), *tnpA-ybaA* (*tnpA*) and *traM-traJ* (*traM*) intergenic regions are represented in grey. The number (n) of individual transconjugant cells from at least three biological replicates analysed is indicated. (C) Histograms showing the intracellular green fluorescence (SNR) for each sfGFP fusions and reporters within vegetatively growing donor (left) and transconjugant cells (right) at the maximum SNR value from Figure S5. Means and SD calculated from the same individual transconjugant cells as in (B) are indicated. Donors of F derivatives (see Table S1), Recipient (LY358).



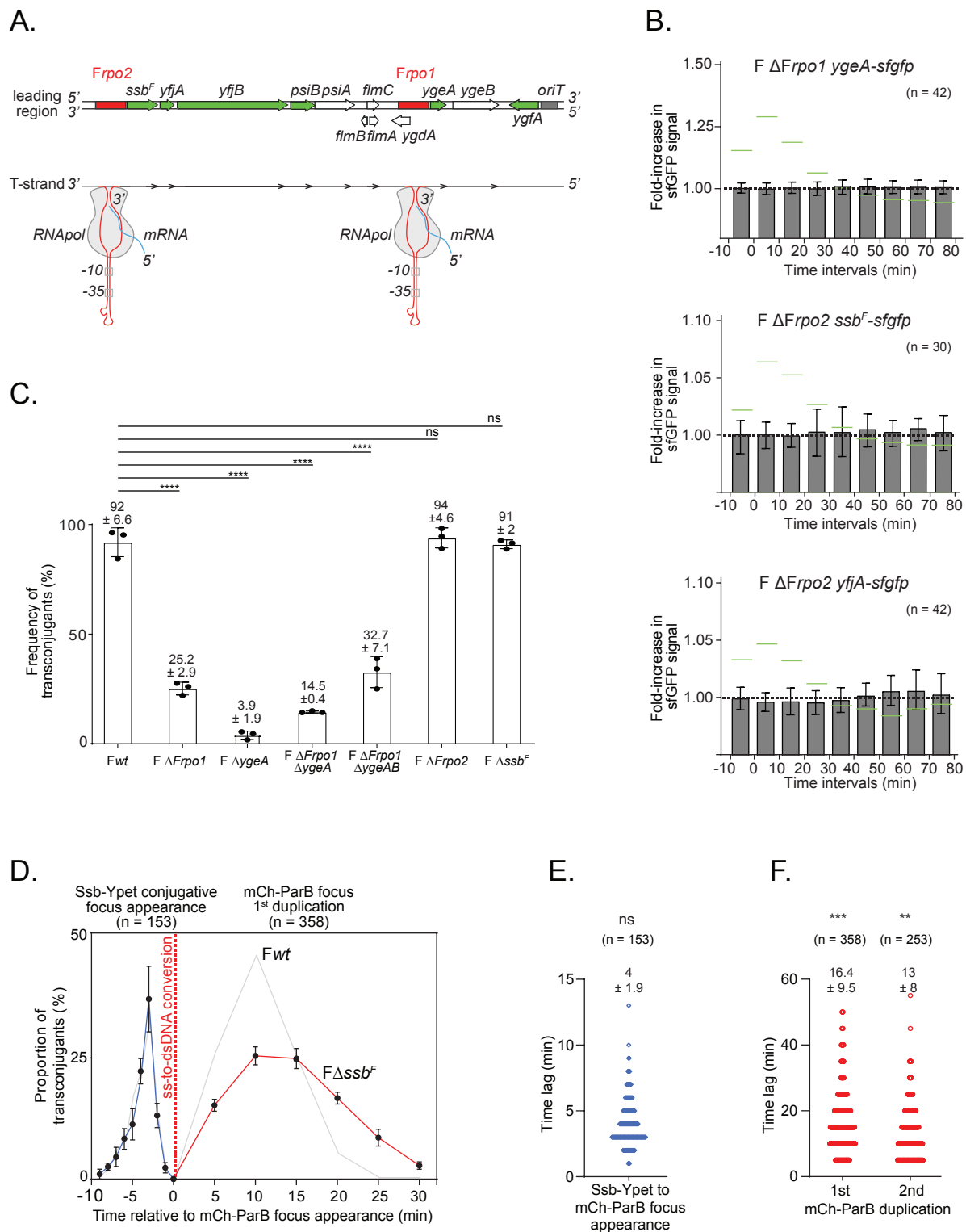
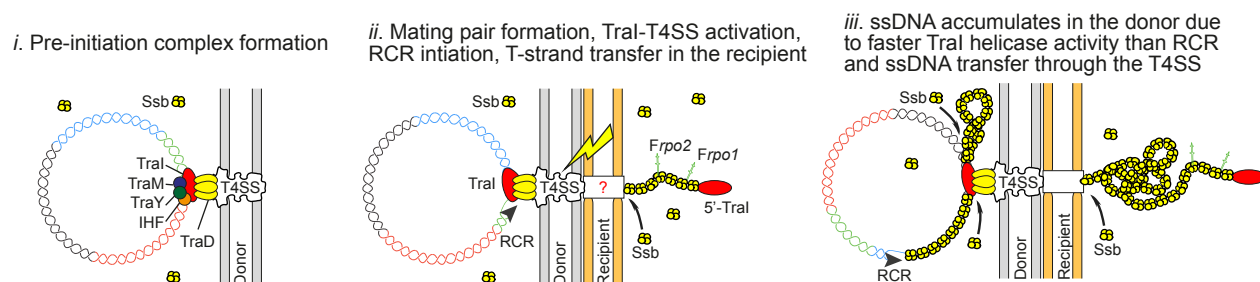


Figure 4

**Figure 4. Role of leading region factors *Frpo1*, *Frpo2* and *ssb<sup>F</sup>* in conjugation.**

(A) Genetic map of the dsDNA leading region showing the position of the genes (green for studied sfGFP fusions and white for the other genes) and *Frpo1* and *Frpo2* promoters (red) (top). The bottom diagram shows the stem-loop structure formed by the ssDNA forms of *Frpo1* and *Frpo2* promoter sequences (detailed in Figure S6). Recognition of the -10 and -35 boxes present in the dsDNA stem region by the RNA polymerase (RNA pol in grey) induces the initiation of transcription and the production of mRNA (blue). (B) Histograms of intracellular sfGFP fold increase in transconjugant after acquisition of F  $\Delta Frpo1$  *ygeA-sfgfp*, F  $\Delta Frpo2$  *ssb-sfgfp* and F  $\Delta Frpo2$  *yjJA-sfgfp*. Mean and SD are calculated from (n) individual transconjugant cells analysed from at least three independent experiments. Levels obtained with the *Fwt* plasmid from Figure S5A are *wt* reported in green as a reference. Donor of F  $\Delta Frpo1$  *ygeA-sfgfp* (LY1368), F  $\Delta Frpo2$  *ssb-sfgfp* (LY1365), F  $\Delta Frpo2$  *yjJA-sfgfp* (LY1364), recipient (LY318). (C) Histograms of *Fwt*, deletion mutants F  $\Delta Frpo1$ , F  $\Delta ygeA$ , F  $\Delta Frpo1$   $\Delta ygeA$ , F  $\Delta Frpo1$   $\Delta ygeAB$ , F  $\Delta Frpo2$  and F  $\Delta ssb^F$  frequency of transconjugant (T/R+T) estimated by plating assays. Mean and SD are calculated from at least three independent experiments. *P*-value significance ns and \*\*\*\**P*  $\leq$  0.0001 were obtained from One-way ANOVA with Dunnetts multiple comparisons test. Donor of *Fwt* (LY875), F  $\Delta Frpo1$  (LY824), F  $\Delta ygeA$  (LY160), F  $\Delta Frpo1$   $\Delta ygeA$  (LY1424), F  $\Delta Frpo1$   $\Delta ygeAB$  (LY1425), F  $\Delta Frpo2$  (LY823), F  $\Delta ssb^F$  (LY755), recipient (MS428). (D) Single-cell time-lapse quantification of Ssb-Ypet focus appearance (blue line) and mCh-ParB focus first duplication (red line) with respect to the ss-to-dsDNA conversion revealed by mCh-ParB focus formation in transconjugant cells (0 min) that receive the F  $\Delta ssb^F$  plasmid. The number of conjugation events analysed (n) from five independent biological replicates is indicated. Results obtained in Figure 2B with *Fwt* plasmid are reported in grey for comparison. (E) Scatter plot showing the time lag between the appearance of the Ssb-Ypet focus and the appearance of the mCh-ParB focus in transconjugant cells after the acquisition of the F  $\Delta ssb^F$  plasmid. The mean and SD calculated from (n) individual ss-to-dsDNA conversion event (blue circles) from five biological replicates are indicated. *P*-value significance ns (>0.05 non-significant) was obtained from Mann-Whitney statistical test against results obtained with the *Fwt* plasmid (Figure 2E). (F) Scatter plot showing the time-lag between the apparition of the mCh-ParB focus and its visual duplication in two foci (1<sup>st</sup> duplication), and in three or four foci (2<sup>nd</sup> duplication) in transconjugant cells after acquisition of the F  $\Delta ssb^F$  plasmid. The mean and SD calculated from (n) individual duplication events (red circles) from eight biological replicates are indicated. *P*-value significance \*\**P* = 0.0023 and \*\*\**P* = 0.0007 were obtained from Mann-Whitney statistical test against results obtained with the *Fwt* plasmid (Figure 2F). Donor F  $\Delta ssb^F$  (LY1068), recipient (LY358).

A.



B.

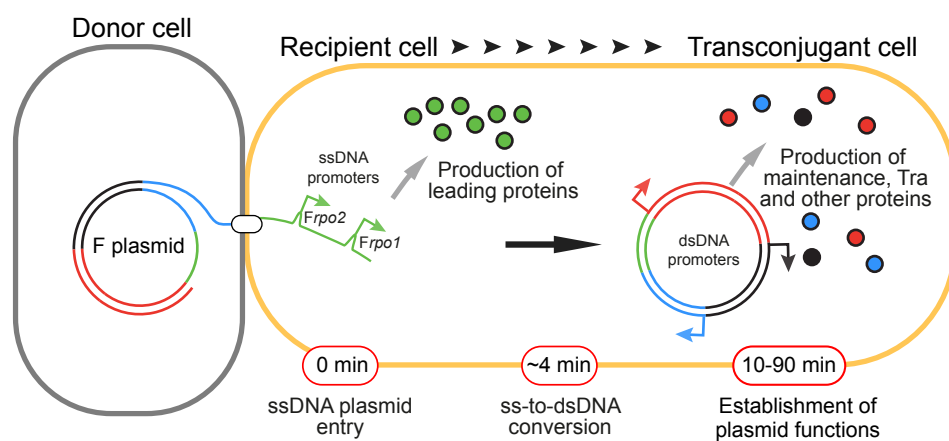


Figure 5

**Figure 5. Model for conjugation initiation and intracellular dynamics.**

**(A)** (i) Before the initiation of conjugation, the pre-initiation complex bound to the plasmid's origin of transfer is docked to the Type IV secretion system (T4SS). (ii) The establishment of the mating pair transduces a signal that activates the pre-initiation complex. Unwinding of the dsDNA plasmid by the helicase activity of TraI produces the first segment of the T-strand, which is immediately transferred into the recipient cell where it recruits Ssb molecules, while the non-transferred strand is being complemented by rolling-circle replication (RCR) in the donor cell. (iii) The helicase activity of TraI generates ssDNA at higher rate than the T-strand is transferred through the T4SS or the non-transferred strand is complemented by RCR, thus resulting in the accumulation of ssDNA plasmid coated by Ssb molecules in the donor cell. **(B)** Upon entry of the ssDNA plasmid in the recipient cell, *Frpo1* and *Frpo2* leading sequences form stem-loop structures that serve as promoters initiating the transcription of the downstream leading genes, rapidly resulting in the production of leading proteins. The subsequent ss-to-dsDNA conversion inactivates *Frpo1* and *Frpo2* and licenses the expression of other plasmid genes under the control of conventional dsDNA promoters. The production of maintenance, transfer and other plasmid-encoded proteins eventually results in the development of new functions by the transconjugant cell.