

Conjugation dynamics on *Arabidopsis thaliana* rosettes

1 **Conjugation dynamics of self-transmissible and mobilisable plasmids into *E. coli***

2 **O157:H7 on *Arabidopsis thaliana* rosettes**

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5 **Running title:** Conjugation dynamics on *Arabidopsis thaliana* rosettes

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19

20 **Abstract**

21 Many antibiotic resistance genes present in human pathogenic bacteria are believed to
22 originate from environmental bacteria and conjugation of antibiotic resistance conferring
23 plasmids is considered to be one of the major reasons for the increasing prevalence of
24 antibiotic resistances. A hotspot for plasmid-based horizontal gene transfer is the
25 phyllosphere, *i.e.* the surfaces of aboveground plant parts. Bacteria in the phyllosphere might
26 serve as intermediate hosts with transfer capability to human pathogenic bacteria. In this
27 study, the exchange of mobilisable and self-transmissible plasmids via conjugation was
28 evaluated. The conjugation from the laboratory strain *E. coli* S17-1, the model phyllosphere
29 colonizer *Pantoea eucalypti* 299R, and the model pathogen *E. coli* O157:H7 Δ *stx* to the
30 recipient strain *E. coli* O157:H7::MRE103 Δ *stx* in the phyllosphere of *Arabidopsis thaliana*
31 was determined. The results suggest that short-term occurrence of a competent donor is
32 sufficient to fix plasmids in a recipient population of *E. coli* O157:H7::MRE103 Δ *stx*. The
33 spread of self-transmissible plasmids was limited after initial steep increases of
34 transconjugants that contributed up to 10% of the total recipient population. The here-
35 presented data of plasmid transfer will be important for future modelling approaches to
36 estimate environmental spread of antibiotic resistance in agricultural production
37 environments.

38

39 **Importance**

40 This study investigated the transfer of antibiotic resistance conferring plasmids to
41 enteropathogenic *E. coli* on plant leaf surfaces. The results indicate that plasmid transfer may
42 be high within the first 24 hours after inoculation. Transconjugant populations are maintained

43 and stable for a considerable time frame on plant leaves, but invasion of the plasmid to the
44 recipient population is limited.

45

46 **Introduction**

47 With the introduction of penicillin in the 1940s, mankind entered the era of antibiotics (AB)
48 which revolutionized therapeutic medicine (Kardos and Demain 2011; Aminov 2010). For
49 the first time, physicians were able to cure their patients of deadly bacterial diseases and
50 saved millions of lives (Neu 1992). Less than a century later, bacterial diseases have yet
51 again become a major threat to human welfare as infectious bacteria acquired antibiotic
52 resistances (ABR) that are able to overcome every antibiotic currently available (Neu 1992;
53 Kumarasamy et al. 2010). ABR *per se* is a natural phenomenon in bacteria (D’Costa et al.
54 2011) and its main function is likely a countermeasure against antibiotic-producing
55 microorganisms that compete for the same resources (Martínez, Coque, and Baquero 2015).
56 It is the use of AB in anthropogenic applications such as medical treatment, animal
57 husbandry and agricultural practice that spreads this natural phenomenon in infectious
58 bacteria whilst pushing the selection pressure on a level beyond the natural evolutionary
59 clock (Palumbi 2001).

60 Many ABR genes present in human pathogenic bacteria are believed to originate from
61 environmental bacteria (Cantas et al. 2013; O’Brien et al. 1985; Davies and Davies 2010;
62 Allen et al. 2010). This implies that, for an ABR gene to reach a human pathogenic
63 bacterium, there needs to be an exchange of genetic material from environmental bacteria
64 towards pathogens. Transfer of genetic material can be achieved by: uptake of environmental
65 DNA due to natural competence, phage-mediated transduction, integrative and conjugative
66 elements or conjugation of plasmids (Thomas and Nielsen 2005; Burrus et al. 2002). The
67 latter is considered to be one of the major reasons for the increased prevalence of ABR

68 (Cantas et al. 2013; O'Brien et al. 1985). The ability of conjugative plasmids to move genes
69 from one bacterium to another, not necessarily related to each other, is responsible for the
70 rapid spread and accumulation of resistances (Baquero, Tedim, and Coque 2013; Klümper et
71 al. 2015; O'Brien et al. 1985; Colombi et al. 2017). A hotspot for plasmid-based horizontal
72 gene transfer is the phyllosphere (Powell et al. 1993; Normander et al. 1998a; Björklöf et al.
73 2000a; van Elsas, Turner, and Bailey 2003; Blau et al. 2018), representing the surface of all
74 above-ground organs of land plants (Ruinen 1961) thereby including the fresh plant products
75 that are considered an important part of a healthy diet.

76 In today's intensive agricultural production, fertilizers are needed to replenish soil nutrients,
77 such as nitrogen and phosphorus. They are essential for crop growth and increased crop yield.
78 Animal manure is an excellent source for such nutrients but it often originates from intensive
79 animal husbandry farms, where the widespread use of AB to preventively treat animals is the
80 rule rather than the exception (Landers et al. 2012). This leads not only to a relative increase
81 of ABR bacteria in fecal waste, but also to an accumulation of ABR-conferring genetic
82 elements, such as plasmids (Heuer, Schmitt, and Smalla 2011; Landers et al. 2012; Wolters et
83 al. 2014). Bacteria that constitute the normal phyllosphere microbiota are generally not
84 considered harmful (Vorholt 2012; Rastogi, Coaker, and Leveau 2013), but for ABR-
85 conferring plasmids they might serve as intermediate hosts with transfer capability to human
86 pathogenic bacteria and most ABR genes present in human pathogenic bacteria are believed
87 to originate from environmental bacteria (Allen et al. 2010; Davies and Davies 2010; Cantas
88 et al. 2013). Little is known about the number of transfer steps involved in the propagation of
89 resistance genes and the efficacy of the mechanism participating in the exchange of genetic
90 material in the environment. However, information about plasmid transfer and plasmid
91 persistence will be important for future modelling and risk assessment approaches to estimate
92 environmental spread of antibiotic resistance in agricultural production environments.

93 In the presented study, a laboratory-scale model system was established that mimics the
94 shortest possible route for ABR-carrying plasmids into enteropathogen *E. coli* O157:H7 Δ *stx*
95 (*Ec*O157:H7) recipients on *Arabidopsis thaliana* rosettes. The exchange of mobilisable and
96 self-transmissible ABR-carrying plasmids via conjugation in the phyllosphere of *Arabidopsis*
97 *thaliana* was evaluated. Donors are either the model phyllosphere colonizing strain *Pantoea*
98 *eucalypti* 299R (*Pe*299R), the non-pathogenic laboratory strain *E. coli* S17-1 (*Ec*S17-1) or
99 *Ec*O157:H7. The assay takes into account that that plants can carry enteropathogenic
100 contaminations (Brandl 2006; Heaton and Jones 2008; Blau et al. 2018) and that animal
101 manure and digestates from biogas plants used as organic fertilizer are a source for ABR-
102 conferring genetic elements, such as plasmids (Wolters et al. 2014; Heuer, Schmitt, and
103 Smalla 2011). To mimic natural conditions, *in planta* experiments were conducted in absence
104 of antibiotic pressure.

105

106 **Materials and methods**

107 **Bacterial strains and growth conditions**

108 Strains and plasmids used in this study and their abbreviations are listed in Table 1.
109 *Escherichia coli* strains and *Pe*299R were routinely grown on lysogeny broth agar (LB). To
110 determine total colony forming units (CFU) of *E. coli* after conjugation experiments, M9
111 minimal medium agar containing lactose as sole carbon source (15 g L⁻¹ agar, 100 mL 10 ×
112 M9 salts (85.1 g L⁻¹ Na₂HPO₄×2H₂O, 30 g L⁻¹ KH₂PO₄, 5 g L⁻¹ NaCl, and 10 g L⁻¹ NH₄Cl,
113 pH7), 2 ml 1 M MgSO₄, 1 mL 0.1 M CaCl, 40 mL 10% w/v lactose solution) or LB
114 supplemented with rifampicin were employed. *Escherichia coli* colonies were assessed after
115 7 days of incubation at room temperature, *Pe*299R colonies on the same agar plates after
116 additional 7 days of incubation. To select for *Ec*O157:H7^{red} transconjugants, M9 minimal
117 medium agar containing lactose as sole carbon source and appropriate antibiotics was

118 employed. *Ec*S17-1 CFU were determined by plating on LB agar containing streptomycin. To
119 select for *Ec*O157:H7 (RP4) donor cells, LB containing kanamycin was used
120 (transconjugants contributed to less than 10% of the donor population that was also
121 kanamycin resistant). Where appropriate, antibiotics were used in the following
122 concentrations: Kanamycin 50 $\mu\text{g mL}^{-1}$, gentamicin 15 $\mu\text{g mL}^{-1}$, streptomycin 100 $\mu\text{g mL}^{-1}$,
123 rifampicin 100 $\mu\text{g mL}^{-1}$.

124

125 **Plasmids used in the study**

126 The plasmids employed in this study are the two self-transmissible plasmids RP4::Plac::GFP
127 (RP4), pKJK5::Plac::GFP (pKJK5) (Klümper et al. 2015) and the mobilizable plasmid
128 pUC18T-mini-Tn7T-Gm-eYFP (pUC18) (Choi and Schweizer 2006). Both self-transmissible
129 plasmids are promiscuous and have a broad host range, RP4 is a IncP-1 α incompatibility
130 group plasmid (Barth and Grinter 1977) and pKJK5 is an IncP-1 incompatibility group
131 plasmid (Sengeløv et al. 2001). Plasmid pUC18 is a synthetic construct replicating only in
132 Enterobacteriaceae and present in high copy numbers when carried by *E. coli* (Choi and
133 Schweizer 2006).

134

135 **Conjugation on nitrocellulose filters**

136 To determine *in vitro* conjugation rates, donors and recipients were grown as described
137 above. To prepare conjugation mixes, a loop-full of cell material was harvested from freshly
138 grown bacterial lawns on agar plates. Each individual strain was resuspended in 1 mL 1 \times
139 PBS (8 g L⁻¹ NaCl, 0.24 g L⁻¹ KCl, 1.42 g L⁻¹ Na₂HPO₄, 0.24 g L⁻¹ KH₂PO₄) by vortexing
140 and pipetting, washed twice by centrifugation at 3,500 \times g, and resuspended in 10 mL 1 \times
141 PBS. Optical density at 600 nm was determined for the cell suspensions and set to OD 0.2.
142 Donors and recipients were mixed and concentrated by centrifugation. The mixtures were

143 resuspended in 100 μ L 1 \times PBS, pipetted onto a nitrocellulose filter (0.22 μ m pore diameter,
144 Millipore, USA), placed on top of LB agar plates, and were incubated at 30 $^{\circ}$ C. Bacteria were
145 harvested after 24 hours by placing the filter in an Eppendorf vial containing 1 ml 1 \times PBS .
146 The vial was vortexed until the complete bacterial biomass was dislodged and resuspended.
147 From this suspension a serial dilution was prepared up to 10⁻¹¹ and 3 μ L droplets were plated
148 onto M9 lactose agar containing appropriate antibiotics to select for transconjugant *E. coli*
149 O157:H7red. Conjugation data is known to be log-normal distributed, thus, transconjugant
150 and donor CFU numbers were log10 transformed before averages were calculated.

151

152 **Plant growth**

153 *Arabidopsis thaliana* Col0 seeds were surface-sterilized by adding 1 mL 70% EtOH to ~50
154 seeds. The seeds were incubated under constant agitation for 2 minutes, before they were
155 collected by centrifugation at 1,500 \times g for 1 minute. The supernatant was discarded and 1
156 mL sterilization solution was added (1.17 mL bleach (12% NaOCl), 0.83 mL ddH₂O, 20 μ L
157 20% Triton X 100). The seeds were then incubated under constant agitation for five minutes
158 before they were collected by centrifugation at 1,500 \times g for 1 minute. To remove residual
159 sterilization solution, the seeds were washed five times by adding 1 mL sterile water,
160 centrifugation, and dismissing the supernatant, after which 1 mL of sterile water was added.
161 For stratification, seeds were stored at 4 $^{\circ}$ C for four days.

162 For plant cultivation, all wells of 24-well microtiter plates were filled with 1 mL $\frac{1}{2}$ strength
163 Murashige and Skoog (MS) agar (2.2 g L⁻¹ MS powder including vitamins (Duchefa, The
164 Netherlands), 10 g L⁻¹ sucrose, 5.5 g L⁻¹ plant agar (Duchefa), pH adjusted to 5.8), after
165 which the plates were exposed to UV-light in a laminar flow for 15 minutes (Vogel et al.
166 2012). Individual stratified seeds were placed into each well of the prepared microtiter plates,
167 the plate was closed using Parafilm[®] and placed in a translucent plastic bag. Plants were then

168 grown in a plant growth chamber (Percival, USA) at long day conditions (16 h day/ 8 h night,
169 22 °C day, 18 °C night, 70% relative humidity). Plants were grown 3 to 3.5 weeks and
170 developed between six to eight leaves before they were inoculated with bacteria.

171

172 **Plant inoculation with conjugation partners and harvest**

173 Bacterial strains were grown overnight on LB-agar plates containing appropriate antibiotics.
174 Freshly grown colonies of each bacterial strain were harvested using an inoculation loop, the
175 bacteria resuspended in 10 mL 1 × PBS, washed twice by centrifugation at 3,500 × g, and
176 resuspended in 1 × PBS. Optical density at 600 nm was determined for the cell suspensions.
177 For single strain growth experiments, the optical density of each strain was set to OD_{600 nm} 0.2
178 before 20 µL of bacterial suspension were pipetted onto the middle of individual plant
179 rosettes. For *in planta* conjugation experiments, donor and recipient were mixed in 1 × PBS
180 and 20 µL of the mixture were pipetted onto individual plants. The inoculation densities were
181 dependent on the experiment and inoculation densities ranged from OD_{600 nm} = 0.05, 0.1,
182 0.25, to 0.5 of donor and recipient. For experiments described in Figure 3, donors and
183 recipients were each co-inoculated at an OD of 0.05. For experiments described in Figure 4,
184 donors and recipients were mixed in ratios 1:2 (OD₆₀₀ 0.05/ 0.1), 1:5 (OD₆₀₀ 0.05/ 0.25), 1:10
185 (OD₆₀₀ 0.05/ 0.5), 2:1 (OD₆₀₀ 0.1/ 0.05), 5:1 (OD₆₀₀ 0.25/ 0.05), or 10:1 (OD₆₀₀ 0.5/ 0.05).
186 For experiments described in Figure 5, donors and recipients were mixed in ratios 1:1 (OD₆₀₀
187 0.05/ 0.05), 2:1 (OD₆₀₀ 0.1/ 0.05), 5:1 (OD₆₀₀ 0.25/ 0.05), or 10:1 (OD₆₀₀ 0.5/ 0.05). The
188 inoculated plants were further incubated at standard growth conditions (16 h day/ 8 h night,
189 22 °C day, 18 °C night, 70% relative humidity). Plants were harvested at different time points
190 and bacteria were washed off to determine the CFU of each strain and transconjugants. To
191 that end, 3 individual plants per treatment were individually processed. Plants were harvested
192 using sterile forceps and the roots cut from the plants on a sterile surface with a sterile

193 scalpel. Plants were transferred to pre-weighed 2 mL tubes and their weight was determined.
194 To dislodge bacteria from plants, 1 mL 1 × PBS was added to a tube, vortexed for 15
195 seconds, and after 7 minutes of sonication vortexed again for 15 seconds. 100 µL of the wash
196 were spread on M9_{lactose} + appropriate antibiotic to select for transconjugants when *EcS17-1* or
197 *Pe299R* were used as donors. When *EcO157:H7* was used as a donor, transconjugants were
198 selected on LB_{rif} + appropriate antibiotic. To extend the range of transconjugants detection, a 10-
199 times dilution series was performed from the leaf wash and 3 µL droplets were placed on
200 appropriate agar selective for transconjugants.

201

202 **Statistical analysis**

203 All experiments were repeated at least three times independently. Data was analyzed using
204 the software Prism 7 (Graphpad Software, USA). All CFU were log-transformed before
205 plotting or statistical tests were performed. To accommodate values below the limit of
206 detection, a 1 was added to all values. To compare the difference of the mean between
207 treatments, a one-way ANOVA using Kruskal-Wallis test with Dunn's correction for multiple
208 comparisons was performed.

209 **Results**

210 **Transconjugant frequencies after filter mating**

211 Classical matings on nitrocellulose filters were performed to determine transconjugation
212 frequencies to the recipient *EcO157:H7red* (Fig. 1). Besides *Pe299R* (pKJK5), all donors
213 were able to transfer plasmids to *EcO157:H7red*. In case of *EcS17* being the donor, all
214 plasmids were transferred at high rates and the transconjugant frequency was between 10^{-1}
215 and 10^{-4} per recipient cell depending on the transmitted plasmid (transconjugant frequencies
216 pUC18<pKJK5<RP4). When *Pe299R* was donor of RP4, transconjugants were on average
217 detected at frequencies of 1.63×10^{-6} per recipient cell. *EcO157:H7* donors transferred
218 plasmids pKJK5 and RP4 with the highest efficiency to *EcO157:H7red* with transconjugants
219 being detected at frequencies of 2.8×10^{-1} and 2.4×10^1 (transconjugant frequencies
220 RP4<pKJK5).

221

222 **Growth dynamics of individual or co-inoculated bacterial strains *in planta***

223 To determine the ability of the different strains to colonize *Arabidopsis*, *EcS17-1*,
224 *EcO157:H7red* and *Pe299R* were inoculated onto gnotobiotic plants. When grown
225 individually, all bacterial strains including the auxotrophic laboratory strain *EcS17-1* were
226 able to grow to high densities on *Arabidopsis*, reaching CFU counts of 10^8 - 10^{10} bacteria per
227 gram plant material (Fig. 2). When *EcS17-1* or *Pe299R* carrying either self-transmissible
228 plasmids RP4 or pKJK5 were co-inoculated with *EcO157:H7red*, population development of
229 individual strains behaved differently (Fig. 3). When co-cultured with *EcS17-1*, the
230 *EcO157:H7red* population reached similar densities as grown on *Arabidopsis* alone, *i.e.*
231 *EcO157:H7red* multiplied to densities of $>10^8$ CFU g⁻¹ and maintained those densities till the
232 end of the experiment. The *EcS17-1* population reached or maintained densities of
233 approximately 5×10^6 CFU g⁻¹ initially, but after seven days dropped below 10^6 CFU g⁻¹

234 (Fig. 3 A, B). When in competition with *Pe299R* (pKJK5), the *EcO157:H7* population never
235 reached densities of above 10^8 CFU g^{-1} , while *Pe299R* (pKJK5) reached densities above
236 10^9 CFU g^{-1} (Fig. 3 C). In competition with *Pe299R* (RP4), *EcO157:H7red* reached densities
237 of approximately 10^8 CFU g^{-1} , and *Pe299R* (RP4) reached similar densities (Fig. 3 D). When
238 *EcO157:H7* represented donor and recipient, the combined *EcO157:H7* population reached
239 cell numbers above 10^8 CFU g^{-1} (Fig. 4).

240

241 **Conjugation dynamics *in planta***

242 *EcS17-1* was able to transfer pKJK5 and RP4 to *EcO157:H7red* on *Arabidopsis* (Fig. 3 A, B).
243 When co-inoculated for 24 h with *EcS17-1* (pKJK5), on average more than 10^3
244 *EcO157:H7red* (pKJK5) transconjugants g^{-1} plant were detected. After an initial increase of
245 *EcS17-1* to a maximum of $>10^6$ CFU g^{-1} , the population steadily declined. The
246 *EcO157:H7red* population increased by three magnitudes to 10^8 CFU g^{-1} and remained stable.
247 The average relative proportion of *EcO157:H7red* (pKJK5) transconjugants in the recipient
248 population slowly increased over time, but not significantly (Fig. 3 E). When co-inoculated
249 with *EcS17-1* (RP4), $\sim 10^2$ *EcO157:H7red* transconjugants g^{-1} plant carrying RP4 were
250 detected after 24 hours. The initial population size of *EcS17-1* was 10^7 CFU g^{-1} and the
251 population did not further increase and steadily declined during the experiment. The
252 *EcO157:H7red* population increased by two magnitudes to 5×10^8 CFU g^{-1} and remained
253 stable. The average relative proportion of *EcO157:H7red* (RP4) transconjugants in the
254 recipient population slowly increased over time, however not significantly (Fig. 3 E). No
255 transconjugants could be detected after co-inoculation of *Pe299R* (pKJK5) and
256 *EcO157:H7red* (Fig. 3 C). The initial population size of *Pe299R* increase from 10^6 CFU g^{-1} to
257 10^9 and the population did not further increase and steadily declined during the experiment.
258 The *EcO157:H7red* population increased by two magnitudes to 5×10^8 CFU g^{-1} and

259 remained stable. After co-inoculation of *Pe299R* (RP4) and *EcO157:H7red*, 5×10^2
260 *EcO157:H7red* transconjugants were detected three days after inoculation. The frequency of
261 transconjugants remained stable after 7 days (Fig. 3 E).

262

263 **Effect of non-self-transmissible but mobilisable plasmids on transconjugant frequencies** 264 *in planta*

265 To separate the effect of secondary horizontal transfer of plasmids from primary
266 conjugations, *i.e.* from a freshly conjugated cell to another recipient *vs.* from an original
267 donor to a recipient cell, four different initial densities of donor *EcS17-1* carrying the
268 mobilisable, but non-self-transmissible, plasmid pUC18 and *EcO157:H7red* as recipient,
269 were tested. Recipient and donor were mixed in ratios 1:1, 1:2, 1:5, and 1:10. Presumably due
270 to its auxotrophy, the donor was outcompeted by the recipient during the experiment and as a
271 consequence the probability over time for recipients to encounter donor cells decreases (Fig.
272 4 A). A strong initial increase of *EcO157:H7red* transconjugants occurred within the first 24
273 hours (Fig. 4 B), which, while not statistically significant, shows a trend of higher
274 conjugation rates in the presence of increased donor densities. While the total *EcO157:H7red*
275 population was increasing by two magnitudes after 7 days post inoculation (d.p.i.), the
276 plasmid-bearing subpopulation increased only by roughly one magnitude, *i.e.* only one tenth
277 of the relative increase of the total *EcO157:H7red* population. The transconjugant frequency
278 reached 10^{-4} per recipient cell after 7 days and did not decrease after 24 days, despite the lack
279 of selective marker and a potential fitness cost of the plasmid (Fig. 4 C).

280 When comparing the transconjugant frequencies in the recipient population after treatment
281 with different donor densities, there is no significant difference between the different donor
282 and recipient ratios. However, similar to self-transmissible plasmids, we found a positive
283 trend between donor density and transconjugant frequencies after 24 hours (Fig. 4 D).

284

285 **Invasion of self-transmissible plasmids into a population of *EcO157:H7* in planta**

286 To test the ability of self-transmissible plasmids to invade a population of *E. coli* O157:H7,
287 we inoculated several different densities of *EcO157:H7* (RP4) donors and *EcO157:H7*
288 recipients onto *Arabidopsis* plants. Donors and recipients were mixed in ratios 1:2, 2:1, 5:1,
289 and 10:1 prior inoculation. All mixtures yielded $>10^4$ transconjugants per gram of plant after
290 24 hours (Fig. 5 A-F), which translates to transconjugant frequencies of 2.5×10^{-2} to 9×10^{-4}
291 per recipient cell (Fig. 5 G). Conjugation efficiency was barely impacted by the number of
292 recipients introduced to the system. If the number of donors was increased, a significant
293 decrease in conjugation efficiency was observed at a ratio of 10:1 donors to recipients. This
294 initial trend in plasmid spread is also impacting the development of the transconjugant
295 population. The transconjugant population was leveling off between 10^6 and 10^7
296 transconjugants per gram of plant (Fig. 5 A-F). In general, this relates to every 10th of the
297 recipient population being conjugated during the invasion population by the plasmid in each
298 treatment after seven days (Fig. 5 G). At that time, the invasion of the plasmid leveled off.
299 The data suggest a low correlation between the donor:recipient ratio and transconjugant
300 frequency after 24 hours (Fig. 5 H).

301 Discussion

302 To study the probability of horizontal gene transfer towards enteropathogenic bacteria on
303 plant leaf surfaces, a model system for the exchange of self-transmissible- and non-self-
304 transmissible but mobilisable plasmids was established. The model system provided insights
305 into the conjugation between Enterobacteriaceae in the phyllosphere of *Arabidopsis*. Besides
306 the phyllosphere colonizing strain *Pe299R* (pKJK5), all donor strains tested were able to
307 transfer plasmids in measurable rates to the model human pathogenic *E. coli* O157:H7red
308 after being co-inoculated onto nitrocellulose filters, though *Pe299R* did so at a much lower
309 frequency. The reason for this transfer barrier (Heinemann 1991; Thomas and Nielsen 2005)
310 is currently unclear, given that *Pe299R* was a competent recipient of the mobilisable plasmid,
311 that *EcO157:H7red* had no issue with receiving the same plasmids from *EcS17-1* and that
312 both donor and recipient are members of the family Enterobacteriaceae.

313 *In planta*, *EcO157:H7* outcompeted *EcS17-1*. This is not unexpected, since both *E. coli*
314 should have a close to identical resource demand and *EcS17-1* is an auxotroph, lab-adapted
315 strain (Simon, Priefer, and Pühler 1983) thereby being prone to be less competitive. When
316 co-inoculated with the phyllosphere-competent strain *Pe299R* carrying plasmid pKJK5,
317 *EcO157:H7red* did not reach the same high densities as in a monoculture and the cell density
318 was decreased to less than 10^7 CFU on average. Potentially, *Pe299R* is outcompeting
319 *EcO157:H7red* due to nutritional competition (Wilson and Lindow 1994). There is no
320 indication that *Pe299R* produces antibiotics which inhibit the growth of *EcO157:H7red*
321 (Smits et al. 2011) as no antibiotic production genes are annotated in the *Pe299R* genome
322 (Remus-Emsermann et al. 2013) and no growth halos were formed around *Pe299R* colonies
323 on agar plates indicative for growth inhibition of *EcO157:H7red* (data not shown). When co-
324 inoculated with *Pe299R* (RP4), the population of *EcO157:H7red* is less affected than in
325 combination with *Pe299R* (pKJK5). The reason for this reduced fitness is currently unknown

326 but a likely explanation is a seemingly reduced fitness of *Pe299R in planta* when carrying
327 plasmid RP4.

328 After co-inoculation of *EcS17-1* containing different self-transmissible and mobilisable
329 plasmids with *EcO157:H7red* as recipient, transconjugants could be detected after 24 hours
330 (Fig. 3 A, B and E) at high rates underlining the donor's ability to transfer plasmids on plant
331 leaves. Compared to previous studies, the prevalence of transconjugants in the recipient
332 population within similar magnitudes as reported before: Björklöf *et al.* and Lilley *et al.*
333 found transconjugant frequencies of 10^{-3} per recipient, Normander *et al.* much higher
334 transconjugant frequencies of up to 10^{-1} per recipient (Normander *et al.* 1998b; Lilley *et al.*
335 2003; Björklöf *et al.* 2000b).

336 The physicochemical nature of plant leaf surfaces presents a spatially segregated,
337 heterogeneous environment that promotes clonal cluster formation and limits movement
338 thereby limiting the potential spread of an invasive plasmid (Remus-Emsermann *et al.* 2012;
339 Tecon and Leveau 2012). This might explain why self-transmissible plasmids did not further
340 invade the recipient population and the relative contribution of plasmid-bearing
341 transconjugants did not over-proportionally increase in time (Fox *et al.* 2008). Due to its
342 auxotrophy, the overall number of *EcS17-1* is decreasing during the duration of the
343 experiments (Figures 3 A, B and 4).

344 The extent to which the self-transmissible plasmid RP4 is able to invade the recipient
345 population was tested by using *EcO157:H7* as donor and *EcO157:H7red* as recipient. After
346 an initial steep increase of the emerging transconjugant population, the transconjugant
347 population's increase exhibited a slope that was slightly higher than the overall recipient's
348 population increase. This indicates that the plasmid was horizontally propagating to new
349 recipients and not exclusively vertically to daughter cells during growth. Generally, after
350 three days of growth, the increase in transconjugants leveled off and the contribution of

351 transconjugants to the total *Ec*O157:H7 population did not further increase. This indicates
352 that the ability of plasmids to invade the complete population is limited and directly
353 connected to active growth of the donor and recipient populations. Once the plant is saturated
354 with colonisers, the transmission of the plasmid slows to a hold and can best be explained by
355 vertical transmission rather by horizontal transmission. By using a wide range of donor vs.
356 recipient ratios that were initially inoculated, we could determine the relationship between
357 donor and recipient ratios and transconjugant frequencies. The transconjugation frequency
358 was correlated with the amount of donors inoculated ($r^2 = 0.56$, Fig. 5 H). This is likely a
359 combined effect of the maximal load of local leaf environments (Remus-Emsermann et al.
360 2012) and the probability of members of the two populations to colonise the leaf in the same
361 site (Tecon and Leveau 2012; Monier and Lindow 2005).

362 When a non-self-transmissible, but mobilisable plasmid is conjugated by *Ec*S17-1, the
363 transconjugant population is not over-proportionally increasing in comparison to self-
364 transmissible plasmids. This lack of increase is likely depicting a stable total population of
365 transconjugants that ceased in growth. As pUC18 does not contain the transfer machinery
366 necessary to further conjugate itself, *Ec*O157:H7red transconjugants are incapable of
367 transmitting the acquired plasmid to other cells. As expected, the ability of the pUC18 to
368 invade the recipient population is limited and the transconjugant population is increasing
369 proportionally slower than the total recipient population. As the generation of new
370 transconjugants is limited by the presence of the donor strain and vertical transfer of the
371 plasmid from primary transconjugants to daughter cells, this can be interpreted as a cease of
372 growth or decrease of the donor population and a cease of growth of the primary
373 transconjugant population. Indeed, the donor population stopped growing after 1 day and
374 started to decrease after 7 days (Fig. 4 A).

375 In line with previous findings we observed that conjugation efficiency of plasmids was high
376 in the absence of antibiotic pressure (Lopatkin et al. 2016). Even for mobilisable plasmids,
377 which only propagate vertically after the initial conjugation, we found that transconjugants
378 were not lost from the system, *i.e.* they were not outcompeted by the non-plasmid-bearing
379 population. This finding is concerning as it indicates that even low frequencies of plasmid
380 transfer on plant foodstuffs might fix a plasmid bearing antibiotic resistance in a population
381 of bacteria.

382

383 **Conclusions**

384 Thus far, no study existed that determined the rate of plasmid transfer towards potential
385 enteropathogenic bacteria in the phyllosphere. Using a model plant system conjugation rates
386 with high repetition and reproducibility were evaluated and provide estimates for the
387 probability of horizontal plasmid transfer on plants. The here-presented rates of plasmid
388 transmission will be important for future modelling approaches to estimate the spread of
389 antibiotic resistant in the environment and assess the risk for human health through
390 consumption of fresh produce.

391 Future *in planta* studies should also include experiments of donors and recipients that arrive
392 on plant leaves at different times to investigate the importance of growth in conjugation
393 efficiency.

394

395

396 **Author contributions**

397 MRE, CP, and DD conceived the study. MRE planned experiments. MRE, CP, and PG
398 performed the experiments. MRE analyzed the data. DD provided lab infrastructure and
399 project management. MRE and CP wrote the manuscript with input from DD. All authors
400 agreed on the final version of the manuscript.

401

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411 resistant and Persistent Microorganisms along Food Chains (REDYMO).

412

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556

557 **Tables**

558 **Table 1.** Strains and plasmids used in this study and their abbreviations.

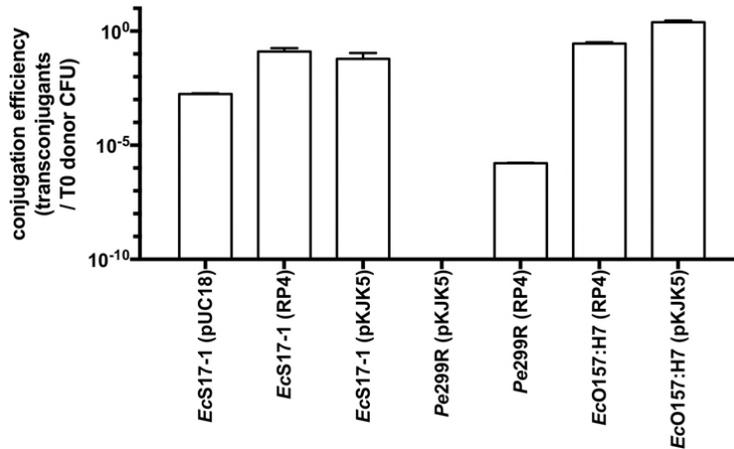
Strain (note of important properties)	Abbreviation	Antibiotic resistance	Reference
<i>E. coli</i> O157:H7::MRE103 Δ <i>stx</i> (grows on lactose, red fluorescent, does not produce Shiga toxin)	<i>Ec</i> O157:H7red	Rifampicin	(Remus- Emsermann, Gisler, and Drissner 2016)
<i>E. coli</i> O157:H7 Δ <i>stx</i> (grows on lactose, does not produce Shiga toxin)	<i>Ec</i> O157:H7	n.a.	NCTC 12900
<i>E. coli</i> S17-1 λ pir (auxothroph)	<i>Ec</i> S17-1	Streptomycin	(Simon, Priefer, and Pühler 1983)
<i>Pantoea eucalypti</i> 299R (grows slowly on lactose)	<i>Pe</i> 299R	Rifampicin	(Remus- Emsermann et al. 2013)
Plasmid (note of important properties; size)		Antibiotic resistance	Reference
pUC18T-mini-Tn7T-Gm-eYFP (mobilisable, confers yellow fluorescence; 5.9 Kbp)	pUC18	Gentamicin	(Choi and Schweizer 2006)
pKJK5::Plac::gfp (self-transmissible, confers green fluorescence; 54 Kbp)	pKJK5	Kanamycin	(Klümper et al. 2015)
RP4::Plac::gfp (self-transmissible, confers green fluorescence; 56 Kbp)	RP4	Kanamycin	(Klümper et al. 2015)

559

560

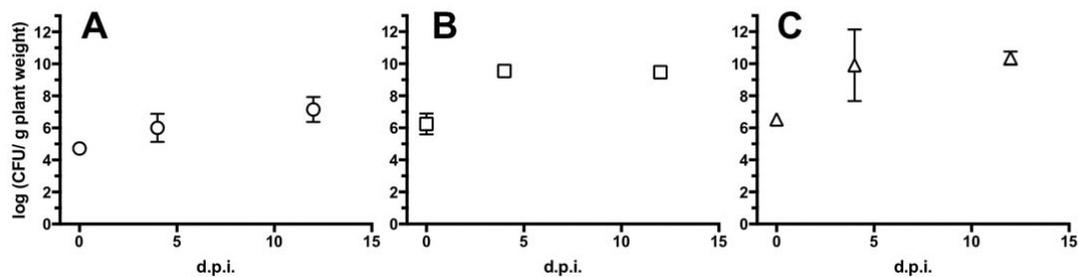
561

562 **Figures**



563

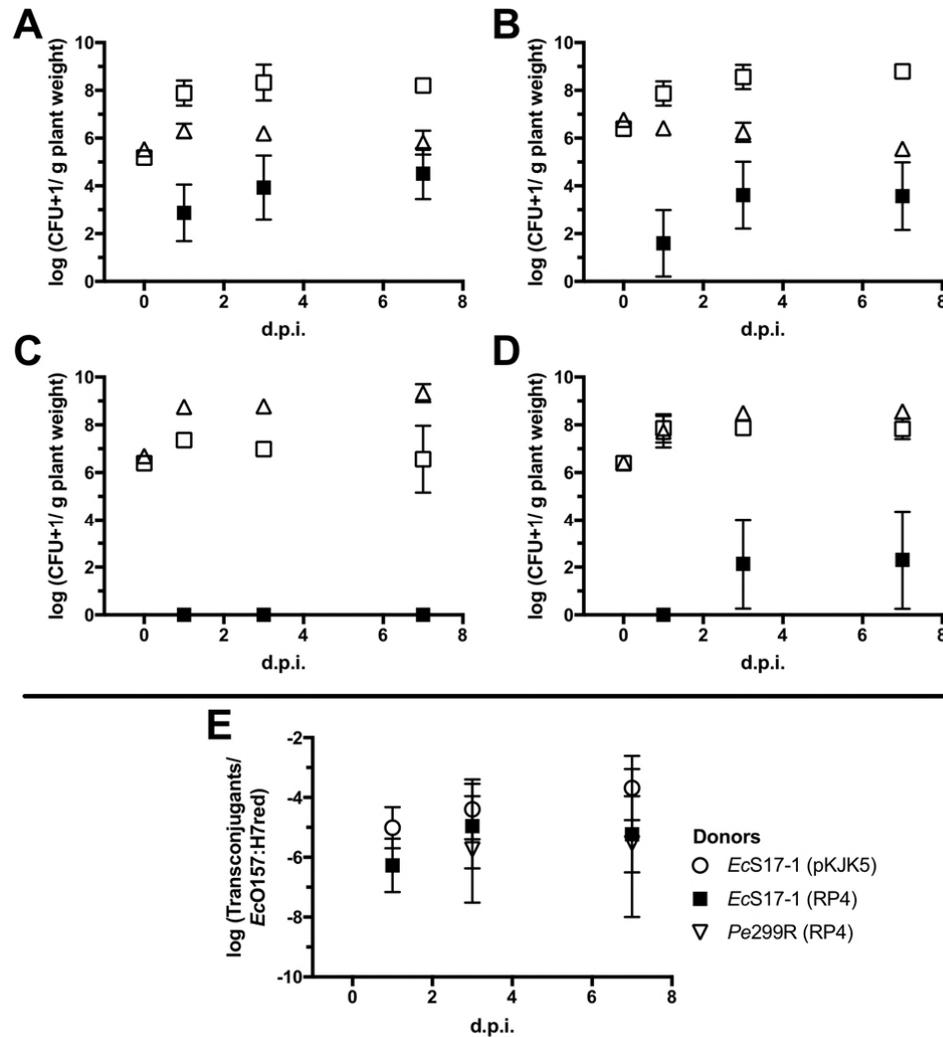
564 **Figure 1.** Transconjugant frequencies in the recipient population after mating of the recipient
565 *EcO157:H7*red with *EcS17-1*, *Pe299R* and *EcO157:H7* donors carrying plasmids pUC18,
566 pKJK5, or RP4 on nitrocellulose filters. "n.a." refers to matings that did not yield any
567 transconjugants. Error bars represent the standard deviation of the mean. Different letters
568 indicate significant differences between treatments (One-way ANOVA, Tukey's multiple
569 comparison test, $p < 0.01$).



570

571 **Figure 2.** Bacterial population development after inoculation of individual strains onto
572 gnotobiotic *Arabidopsis*. A) *EcS17-1*; B) *EcO157:H7*; C) *Pe299R*. Error bars represent the
573 standard deviation of the mean.

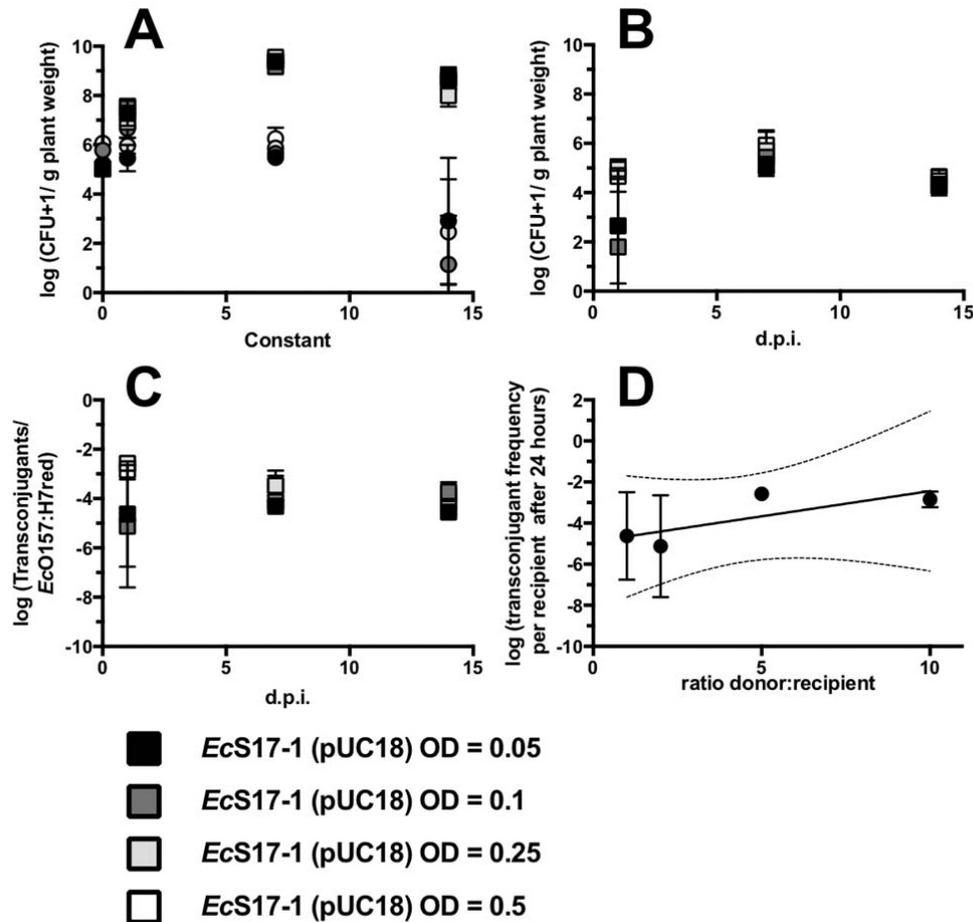
Conjugation dynamics on *Arabidopsis thaliana* rosettes



574

575 **Figure 3.** Population development of bacteria on gnotobiotic *Arabidopsis*. **A)** Population
 576 development of *EcO157:H7red* (open squares), *EcS17-1* (pKJK5) (open circles), and
 577 *EcO157:H7red* (pKJK5) transconjugants (filled squares). **B)** Population development of
 578 *EcO157:H7red* (open squares), *EcS17-1* (RP4) (open circles), and *EcO157:H7red* (RP4)
 579 transconjugants (filled squares). **C)** Population development of *EcO157:H7red* (open
 580 squares), *Pe299R* (pKJK5) (triangles), and *EcO157:H7red* (pKJK5) transconjugants (filled
 581 squares). The conjugation with *Pe299R* (pKJK5) did not yield transconjugants above the
 582 limit of detection. **D)** Population development of *EcO157:H7red* (open squares), *Pe299R*
 583 (RP4) (triangles), and *EcO157:H7red* (RP4) transconjugants (filled squares). **E)** Frequencies

584 of transconjugants in the *EcO157:H7red* population after 1, 3, and 7 days. Error bars
 585 represent the standard deviation of the mean.

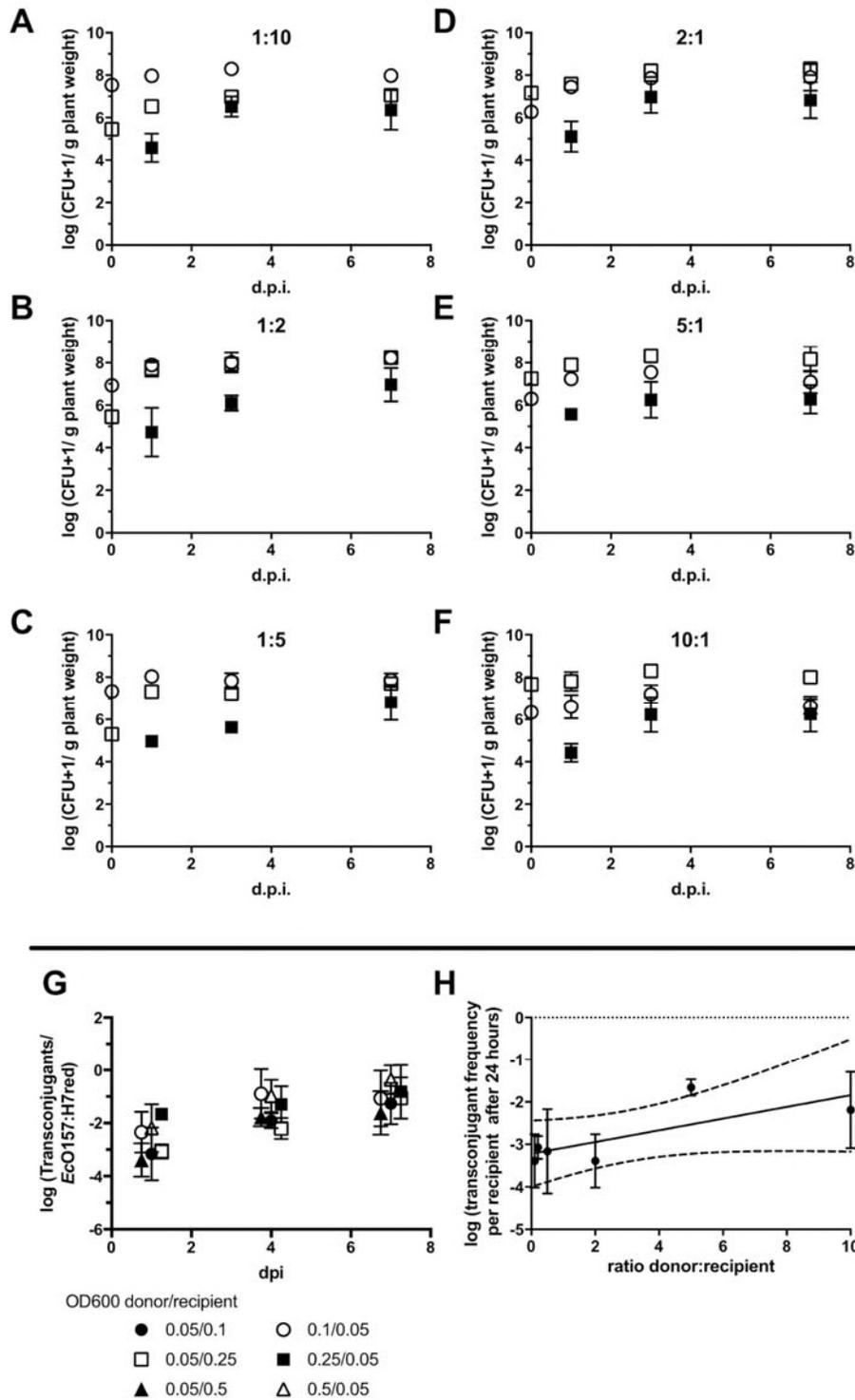


586

587 **Figure 4.** A) Population development after co-inoculation of *EcO157:H7red* (squares) and
 588 *EcS17-1* (circles) on *Arabidopsis*. *EcS17-1* (pUC18) was inoculated in different densities
 589 (treatments indicated by different shadings), while the inoculation density of *EcO157:H7red*
 590 remained constant. B) Population development of the transconjugant *EcO157:H7red*
 591 (pUC18). C) Transconjugant frequencies in the recipient population over time. D)
 592 Transconjugant frequencies after 24 hours. No significant differences in the conjugation
 593 efficiency after treatments with different *EcS17-1* donor concentrations could be detected,
 594 however, the variation within treatments was lower at high donor densities. A linear

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595 regression was fitted ($r^2 = 0.61$, broken lines 95% confidence intervals of the linear
 596 regression). Error bars represent the standard deviation of the mean.



597

598 **Figure 5.** Conjugation dynamics of the self-transmissible plasmid RP4 in a population of
599 *EcO157:H7*. **A-F)** Population development of six different ratios of *EcO157:H7* (RP4)
600 donors (open circles) *EcO157:H7*red recipients (open squares), and *EcO157:H7*red
601 transconjugants (filled squares) after inoculation onto *Arabidopsis*. Inoculation of donors and
602 recipients at a ratio of 1:10 (**A**), 1:5 (**B**), 1:2 (**C**), 2:1 (**D**), 5:1 (**E**), and 10:1 (**F**). **G)**
603 Transconjugant frequency in the recipient population over time. Data points were slightly
604 nudged for better visibility. **H)** A linear regression was fitted and shows the inverse
605 correlation of initial donor density and transconjugant frequency in the recipient population
606 after 24 hours ($r^2 = 0.56$, broken lines 95% confidence intervals of the linear regression).