Conjugation dynamics of self-transmissible and mobilisable plasmids into *E. coli* O157:H7 on *Arabidopsis thaliana* rosettes

Mitja N.P. Remus-Emsermann[a,b,c,#], Cosima Pelludat[d], Pascal Gisler[a], David Drissner[a,e]

Running title: Conjugation dynamics on *Arabidopsis thaliana* rosettes

[a]Agroscope, Microbiology of Plant Foods, Wädenswil, Switzerland; [b]School of Biological Sciences, University of Canterbury, Christchurch, New Zealand; [c]Biomolecular Interaction Centre, University of Canterbury, Christchurch, New Zealand; [d]Agroscope, Research Division Plant Protection, Switzerland; [e]Faculty of Life Sciences, Albstadt-Sigmaringen University, Sigmaringen, Germany.

[#]correspondence to

Mitja Remus-Emsermann

mitja.remus-emsermann@canterbury.ac.nz, Tel.: +64 (3) 36 95351, School of Biological Sciences, University of Canterbury, Private Bag 4800, Christchurch, 8140, New Zealand

Abstract

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

Importance

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

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

Introduction

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

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

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

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

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

Materials and methods

Bacterial strains and growth conditions

Strains and plasmids used in this study and their abbreviations are listed in Table 1. *Escherichia coli* strains and *Pe*299R were routinely grown on lysogeny broth agar (LB). To determine total colony forming units (CFU) of *E. coli* after conjugation experiments, M9 minimal medium agar containing lactose as sole carbon source (15 g L^{-1} agar, 100 mL 10 × M9 salts (85.1 g L^{-1} $Na_2HPO_4 \times 2H_2O$, 30 g L^{-1} KH_2PO_4, 5 g L^{-1} NaCl, and 10 g L^{-1} NH_4Cl, pH7), 2 ml 1 M $MgSO_4$, 1 mL 0.1 M CaCl, 40 mL 10% w/v lactose solution) or LB supplemented with rifampicin were employed. *Escherichia coli* colonies were assessed after 7 days of incubation at room temperature, *Pe*299R colonies on the same agar plates after additional 7 days of incubation. To select for *Ec*O157:H7red transconjugants, M9 minimal 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 µg mL^{-1}, gentamicin 15 µg mL^{-1}, streptomycin 100 µg mL^{-1},
123 rifampicin 100 µg 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-1α 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 ×
139 PBS (8 g L^{-1} NaCl, 0.24 g L^{-1} KCl, 1.42 g L^{-1} Na_2HPO_4, 0.24 g L^{-1} KH_2PO_4) by vortexing
140 and pipetting, washed twice by centrifugation at 3,500 × *g*, and resuspended in 10 mL 1 ×
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 µL 1 × PBS, pipetted onto a nitrocellulose filter (0.22 µm pore diameter,
144 Millipore, USA), placed on top of LB agar plates, and were incubated at 30 °C. Bacteria were
145 harvested after 24 hours by placing the filter in an Eppendorf vial containing 1 ml 1 × 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 µL 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 × *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 ddH_2O, 20 µL
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 × *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 °C for four days.
162 For plant cultivation, all wells of 24-well microtiter plates were filled with 1 mL ½ 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

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

Plant inoculation with conjugation partners and harvest

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

scalpel. Plants were transferred to pre-weighed 2 mL tubes and their weight was determined. To dislodge bacteria from plants, 1 mL 1 × PBS was added to a tube, vortexed for 15 seconds, and after 7 minutes of sonication vortexed again for 15 seconds. 100 µL of the wash were spread on $M9_{lactose\ +\ appropriate\ antibiotic}$ to select for transconjugants when *Ec*S17-1 or *Pe*299R were used as donors. When *Ec*O157:H7 was used as a donor, transconjugants were selected on $LB_{rif\ +\ appropriate\ antibiotic}$. To extend the range of transconjugants detection, a 10-times dilution series was performed from the leaf wash and 3 µL droplets were placed on appropriate agar selective for transconjugants.

Statistical analysis

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

Results

Transconjugant frequencies after filter mating

Classical matings on nitrocellulose filters were performed to determine transconjugation frequencies to the recipient *Ec*O157:H7red (Fig. 1). Besides *Pe*299R (pKJK5), all donors were able to transfer plasmids to *Ec*O157:H7red. In case of *Ec*S17 being the donor, all plasmids were transferred at high rates and the transconjugant frequency was between 10^{-1} and 10^{-4} per recipient cell depending on the transmitted plasmid (transconjugant frequencies pUC18<pKJK5<RP4). When *Pe*299R was donor of RP4, transconjugants were on average detected at frequencies of 1.63×10^{-6} per recipient cell. *Ec*O157:H7 donors transferred plasmids pKJK5 and RP4 with the highest efficiency to *Ec*O157:H7red with transconjugants being detected at frequencies of 2.8×10^{-1} and 2.4×10^{1} (transconjugant frequencies RP4<pKJK5).

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

To determine the ability of the different strains to colonize Arabidopsis, *Ec*S17-1, *Ec*O157:H7red and *Pe*299R were inoculated onto gnotobiotic plants. When grown individually, all bacterial strains including the auxotrophic laboratory strain *Ec*S17-1 were able to grow to high densities on Arabidopsis, reaching CFU counts of 10^{8}-10^{10} bacteria per gram plant material (Fig. 2). When *Ec*S17-1 or *Pe*299R carrying either self-transmissible plasmids RP4 or pKJK5 were co-inoculated with *Ec*O157:H7red, population development of individual strains behaved differently (Fig. 3). When co-cultured with *Ec*S17-1, the *Ec*O157:H7red population reached similar densities as grown on Arabidopsis alone, *i.e.* *Ec*O157:H7red multiplied to densities of $>10^{8}$ CFU g^{-1} and maintained those densities till the end of the experiment. The *Ec*S17-1 population reached or maintained densities of approximately 5×10^{6} CFU g^{-1} initially, but after seven days dropped below 10^{6} CFU g^{-1}

(Fig. 3 A, B). When in competition with *Pe*299R (pKJK5), the *Ec*O157:H7 population never reached densities of above 10^8 CFU g^{-1}, while *Pe*299R (pKJK5) reached densities above 10^9 CFU g^{-1} (Fig. 3 C). In competition with *Pe*299R (RP4), *Ec*O157:H7red reached densities of approximately 10^8 CFU g^{-1}, and *Pe*299R (RP4) reached similar densities (Fig. 3 D). When *Ec*O157:H7 represented donor and recipient, the combined *Ec*O157:H7 population reached cell numbers above 10^8 CFU g^{-1} (Fig. 4).

Conjugation dynamics *in planta*

*Ec*S17-1 was able to transfer pKJK5 and RP4 to *Ec*O157:H7red on Arabidopsis (Fig. 3 A, B). When co-inoculated for 24 h with *Ec*S17-1 (pKJK5), on average more than 10^3 *Ec*O157:H7red (pKJK5) transconjugants g^{-1} plant were detected. After an initial increase of *Ec*S17-1 to a maximum of $>10^6$ CFU g^{-1}, the population steadily declined. The *Ec*O157:H7red population increased by three magnitudes to 10^8 CFU g^{-1} and remained stable. The average relative proportion of *Ec*O157:H7red (pKJK5) transconjugants in the recipient population slowly increased over time, but not significantly (Fig. 3 E). When co-inoculated with *Ec*S17-1 (RP4), $\sim 10^2$ *Ec*O157:H7red transconjugants g^{-1} plant carrying RP4 were detected after 24 hours. The initial population size of *Ec*S17-1 was 10^7 CFU g^{-1} and the population did not further increase and steadily declined during the experiment. The *Ec*O157:H7red population increased by two magnitudes to 5×10^8 CFU g^{-1} and remained stable. The average relative proportion of *Ec*O157:H7red (RP4) transconjugants in the recipient population slowly increased over time, however not significantly (Fig. 3 E). No transconjugants could be detected after co-inoculation of *Pe*299R (pKJK5) and *Ec*O157:H7red (Fig. 3 C). The initial population size of *Pe*299R increase from 10^6 CFU g^{-1} to 10^9 and the population did not further increase and steadily declined during the experiment. The *Ec*O157:H7red population increased by two magnitudes to 5×10^8 CFU g^{-1} and

259 remained stable. After co-inoculation of *Pe*299R (RP4) and *Ec*O157:H7red, 5×10^2
260 *Ec*O157: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 *Ec*S17-1 carrying the
268 mobilisable, but non-self-transmissible, plasmid pUC18 and *Ec*O157: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 *Ec*O157: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 *Ec*O157: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 *Ec*O157: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 *Ec*O157: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 *Ec*O157:H7 (RP4) donors and *Ec*O157: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).

Discussion

To study the probability of horizontal gene transfer towards enteropathogenic bacteria on plant leaf surfaces, a model system for the exchange of self-transmissible- and non-self-transmissible but mobilisable plasmids was established. The model system provided insights into the conjugation between Enterobacteriaceae in the phyllosphere of Arabidopsis. Besides the phyllosphere colonizing strain *Pe*299R (pKJK5), all donor strains tested were able to transfer plasmids in measurable rates to the model human pathogenic *E. coli* O157:H7red after being co-inoculated onto nitrocellulose filters, though *Pe*299R did so at a much lower frequency. The reason for this transfer barrier (Heinemann 1991; Thomas and Nielsen 2005) is currently unclear, given that *Pe*299R was a competent recipient of the mobilisable plasmid, that *Ec*O157:H7red had no issue with receiving the same plasmids from *Ec*S17-1 and that both donor and recipient are members of the family Enterobacteriaceae.

In planta, *Ec*O157:H7 outcompeted *Ec*S17-1. This is not unexpected, since both *E. coli* should have a close to identical resource demand and *Ec*S17-1 is an auxotroph, lab-adapted strain (Simon, Priefer, and Pühler 1983) thereby being prone to be less competitive. When co-inoculated with the phyllosphere-competent strain *Pe*299R carrying plasmid pKJK5, *Ec*O157:H7red did not reach the same high densities as in a monoculture and the cell density was decreased to less than 10^7 CFU on average. Potentially, *Pe*299R is outcompeting *Ec*O157:H7red due to nutritional competition (Wilson and Lindow 1994). There is no indication that *Pe*299R produces antibiotics which inhibit the growth of *Ec*O157:H7red (Smits et al. 2011) as no antibiotic production genes are annotated in the *Pe*299R genome (Remus-Emsermann et al. 2013) and no growth halos were formed around *Pe*299R colonies on agar plates indicative for growth inhibition of *Ec*O157:H7red (data not shown). When co-inoculated with *Pe*299R (RP4), the population of *Ec*O157:H7red is less affected than in combination with *Pe*299R (pKJK5). The reason for this reduced fitness is currently unknown

but a likely explanation is a seemingly reduced fitness of *Pe*299R *in planta* when carrying plasmid RP4.

After co-inoculation of *Ec*S17-1 containing different self-transmissible and mobilisable plasmids with *Ec*O157:H7red as recipient, transconjugants could be detected after 24 hours (Fig. 3 A, B and E) at high rates underlining the donor's ability to transfer plasmids on plant leaves. Compared to previous studies, the prevalence of transconjugants in the recipient population within similar magnitudes as reported before: Björklöf *et al*. and Lilley *et al*. found transconjugant frequencies of 10^{-3} per recipient, Normander *et al*. much higher transconjugant frequencies of up to 10^{-1} per recipient (Normander et al. 1998b; Lilley et al. 2003; Björklöf et al. 2000b).

The physicochemical nature of plant leaf surfaces presents a spatially segregated, heterogeneous environment that promotes clonal cluster formation and limits movement thereby limiting the potential spread of an invasive plasmid (Remus-Emsermann et al. 2012; Tecon and Leveau 2012). This might explain why self-transmissible plasmids did not further invade the recipient population and the relative contribution of plasmid-bearing transconjugants did not over-proportionally increase in time (Fox et al. 2008). Due to its auxotrophy, the overall number of *Ec*S17-1 is decreasing during the duration of the experiments (Figures 3 A, B and 4).

The extent to which the self-transmissible plasmid RP4 is able to invade the recipient population was tested by using *Ec*O157:H7 as donor and *Ec*O157:H7red as recipient. After an initial steep increase of the emerging transconjugant population, the transconjugant population’s increase exhibited a slope that was slightly higher than the overall recipient’s population increase. This indicates that the plasmid was horizontally propagating to new recipients and not exclusively vertically to daughter cells during growth. Generally, after 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-stransmissible, 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).

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

Conclusions

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

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

Author contributions

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

Acknowledgments

The authors thank Jack A. Heinemann, University of Canterbury, Christchurch, New Zealand, for valuable discussion and insights about plasmid conjugation. Uli Klümper and Barth Smets, University of Copenhagen, Denmark, kindly donated plasmids RP4::Plac::GFP and pKJK5::Plac::GFP. The authors acknowledge André Imboden, ETH Zurich, for providing Arabidopsis seeds and Katharina Schneider for technical assistance. The authors are grateful for financial support of the National Research Programme "Antimicrobial Resistance" (NRP 72, grant number 407240_167068) of the Swiss National Science Foundation and the Agroscope research program Reduction and Dynamics of Antibiotic-resistant and Persistent Microorganisms along Food Chains (REDYMO).

References

Allen, Heather K., Justin Donato, Helena Huimi Wang, Karen A. Cloud-Hansen, Julian Davies, and Jo Handelsman. 2010. "Call of the Wild: Antibiotic Resistance Genes in Natural Environments." *Nature Reviews. Microbiology* 8 (4): 251–59.

Aminov, Rustam I. 2010. "A Brief History of the Antibiotic Era: Lessons Learned and Challenges for the Future." *Frontiers in Microbiology* 1 (December): 134.

Baquero, Fernando, Ana P. Tedim, and Teresa M. Coque. 2013. "Antibiotic Resistance Shaping Multi-Level Population Biology of Bacteria." *Frontiers in Microbiology* 4 (March): 15.

Barth, P. T., and N. J. Grinter. 1977. "Map of Plasmid RP4 Derived by Insertion of Transposon C." *Journal of Molecular Biology* 113 (3): 455–74.

Björklöf, K., E. L. Nurmiaho-Lassila, N. Klinger, K. Haahtela, and M. Romantschuk. 2000a. "Colonization Strategies and Conjugal Gene Transfer of Inoculated Pseudomonas Syringae on the Leaf Surface." *Journal of Applied Microbiology* 89 (3): 423–32.

———. 2000b. “Colonization Strategies and Conjugal Gene Transfer of Inoculated Pseudomonas Syringae on the Leaf Surface.” *Journal of Applied Microbiology* 89 (3): 423–32.

Blau, Khald, Antje Bettermann, Sven Jechalke, Eva Fornefeld, Yann Vanrobaeys, Thibault Stalder, Eva M. Top, and Kornelia Smalla. 2018. “The Transferable Resistome of Produce.” *bioRxiv*. https://doi.org/10.1101/350629.

Brandl, Maria T. 2006. “Fitness of Human Enteric Pathogens on Plants and Implications for Food Safety.” *Annual Review of Phytopathology* 44: 367–92.

Burrus, Vincent, Guillaume Pavlovic, Bernard Decaris, and Gérard Guédon. 2002. “Conjugative Transposons: The Tip of the Iceberg.” *Molecular Microbiology* 46 (3): 601–10.

Cantas, L., Syed Q. A. Shah, L. M. Cavaco, C. M. Manaia, F. Walsh, M. Popowska, H. Garelick, H. Bürgmann, and H. Sørum. 2013. “A Brief Multi-Disciplinary Review on Antimicrobial Resistance in Medicine and Its Linkage to the Global Environmental Microbiota.” *Frontiers in Microbiology* 4 (May): 96.

Choi, Kyoung-Hee, and Herbert P. Schweizer. 2006. “Mini-Tn7 Insertion in Bacteria with Single attTn7 Sites: Example Pseudomonas Aeruginosa.” *Nature Protocols* 1 (1): 153–61.

Colombi, Elena, Christina Straub, Sven Künzel, Matthew D. Templeton, Honour C. McCann, and Paul B. Rainey. 2017. “Evolution of Copper Resistance in the Kiwifruit Pathogen *Pseudomonas syringae* pv. *actinidiae* through Acquisition of Integrative Conjugative Elements and Plasmids.” *Environmental Microbiology* 19 (2): 819–32.

Davies, Julian, and Dorothy Davies. 2010. “Origins and Evolution of Antibiotic Resistance.” *Microbiology and Molecular Biology Reviews: MMBR* 74 (3): 417–33.

D’Costa, Vanessa M., Christine E. King, Lindsay Kalan, Mariya Morar, Wilson W. L. Sung, Carsten Schwarz, Duane Froese, et al. 2011. “Antibiotic Resistance Is Ancient.” *Nature* 477 (7365): 457–61.

Elsas, Jan Dirk van, Sarah Turner, and Mark J. Bailey. 2003. “Horizontal Gene Transfer in the Phytosphere.” *The New Phytologist* 157 (3): 525–37.

Fox, Randal E., Xue Zhong, Stephen M. Krone, and Eva M. Top. 2008. “Spatial Structure and Nutrients Promote Invasion of IncP-1 Plasmids in Bacterial Populations.” *The ISME Journal* 2 (10): 1024–39.

Heaton, J. C., and K. Jones. 2008. “Microbial Contamination of Fruit and Vegetables and the Behaviour of Enteropathogens in the Phyllosphere: A Review.” *Journal of Applied Microbiology* 104 (3): 613–26.

Heinemann, Jack A. 1991. “Genetics of Gene Transfer between Species.” *Trends in Genetics: TIG* 7 (6): 181–85.

Heuer, Holger, Heike Schmitt, and Kornelia Smalla. 2011. “Antibiotic Resistance Gene Spread due to Manure Application on Agricultural Fields.” *Current Opinion in Microbiology* 14 (3): 236–43.

Kardos, Nelson, and Arnold L. Demain. 2011. “Penicillin: The Medicine with the Greatest Impact on Therapeutic Outcomes.” *Applied Microbiology and Biotechnology* 92 (4): 677–87.

Klümper, Uli, Leise Riber, Arnaud Dechesne, Analia Sannazzarro, Lars H. Hansen, Søren J. Sørensen, and Barth F. Smets. 2015. “Broad Host Range Plasmids Can Invade an Unexpectedly Diverse Fraction of a Soil Bacterial Community.” *The ISME Journal* 9 (4): 934–45.

Kumarasamy, Karthikeyan K., Mark A. Toleman, Timothy R. Walsh, Jay Bagaria, Fafhana Butt, Ravikumar Balakrishnan, Uma Chaudhary, et al. 2010. “Emergence of a New Antibiotic Resistance Mechanism in India, Pakistan, and the UK: A Molecular,

Biological, and Epidemiological Study." *The Lancet Infectious Diseases* 10 (9): 597–602.

Landers, Timothy F., Bevin Cohen, Thomas E. Wittum, and Elaine L. Larson. 2012. "A Review of Antibiotic Use in Food Animals: Perspective, Policy, and Potential." *Public Health Reports* 127 (1): 4–22.

Lilley, A. K., M. J. Bailey, M. Barr, K. Kilshaw, T. M. Timms-Wilson, M. J. Day, S. J. Norris, T. H. Jones, and H. C. J. Godfray. 2003. "Population Dynamics and Gene Transfer in Genetically Modified Bacteria in a Model Microcosm: Gene Transfer in Phytosphere Bacteria." *Molecular Ecology* 12 (11): 3097–3107.

Lopatkin, Allison J., Shuqiang Huang, Robert P. Smith, Jaydeep K. Srimani, Tatyana A. Sysoeva, Sharon Bewick, David K. Karig, and Lingchong You. 2016. "Antibiotics as a Selective Driver for Conjugation Dynamics." *Nature Microbiology* 1 (6): 16044.

Martínez, José L., Teresa M. Coque, and Fernando Baquero. 2015. "What Is a Resistance Gene? Ranking Risk in Resistomes." *Nature Reviews. Microbiology* 13 (2): 116–23.

Monier, J-M, and S. E. Lindow. 2005. "Spatial Organization of Dual-Species Bacterial Aggregates on Leaf Surfaces." *Applied and Environmental Microbiology* 71 (9): 5484–93.

Neu, H. C. 1992. "The Crisis in Antibiotic Resistance." *Science* 257 (5073): 1064–73.

Normander, B., B. B. Christensen, S. Molin, and N. Kroer. 1998a. "Effect of Bacterial Distribution and Activity on Conjugal Gene Transfer on the Phylloplane of the Bush Bean (*Phaseolus vulgaris*)." *Applied and Environmental Microbiology* 64 (5): 1902–9.

———. 1998b. "Effect of Bacterial Distribution and Activity on Conjugal Gene Transfer on the Phylloplane of the Bush Bean (Phaseolus Vulgaris)." *Applied and Environmental Microbiology* 64 (5): 1902–9.

O'Brien, T. F., M. P. Pla, K. H. Mayer, H. Kishi, E. Gilleece, M. Syvanen, and J. D. Hopkins. 1985. "Intercontinental Spread of a New Antibiotic Resistance Gene on an Epidemic Plasmid." *Science* 230 (4721): 87–88.

Palumbi, S. R. 2001. "Humans as the World's Greatest Evolutionary Force." *Science* 293 (5536): 1786–90.

Powell, Bridget J., Kevin J. Purdy, Ian P. Thompson, and Mark J. Bailey. 1993. "Demonstration of Tra Plasmid Activity in Bacteria Indigenous to the Phyllosphere of Sugar Beet; Gene Transfer to a Recombinant Pseudomonad." *FEMS Microbiology Ecology* 12 (3): 195–206.

Rastogi, Gurdeep, Gitta L. Coaker, and Johan H. J. Leveau. 2013. "New Insights into the Structure and Function of Phyllosphere Microbiota through High-Throughput Molecular Approaches." *FEMS Microbiology Letters* 348 (1): 1–10.

Remus-Emsermann, Mitja N. P., Pascal Gisler, and David Drissner. 2016. "MiniTn7-Transposon Delivery Vectors for Inducible or Constitutive Fluorescent Protein Expression in Enterobacteriaceae." *FEMS Microbiology Letters* 363 (16). https://doi.org/10.1093/femsle/fnw178.

Remus-Emsermann, Mitja N. P., Eun Bae Kim, Maria L. Marco, Robin Tecon, and Johan H. J. Leveau. 2013. "Draft Genome Sequence of the Phyllosphere Model Bacterium Pantoea Agglomerans 299R." *Genome Announcements* 1 (1). https://doi.org/10.1128/genomeA.00036-13.

Remus-Emsermann, Mitja N. P., Robin Tecon, George A. Kowalchuk, and Johan H. J. Leveau. 2012. "Variation in Local Carrying Capacity and the Individual Fate of Bacterial Colonizers in the Phyllosphere." *The ISME Journal* 6 (4): 756–65.

Ruinen, Jakoba. 1961. "The Phyllosphere." *Plant and Soil* 15 (2): 81–109.

Sengeløv, G., K. J. Kristensen, A. H. Sørensen, N. Kroer, and S. J. Sørensen. 2001. "Effect of Genomic Location on Horizontal Transfer of a Recombinant Gene Cassette between

Pseudomonas Strains in the Rhizosphere and Spermosphere of Barley Seedlings." *Current Microbiology* 42 (3): 160–67.

Simon, R., U. Priefer, and A. Pühler. 1983. "A Broad Host Range Mobilization System for In Vivo Genetic Engineering: Transposon Mutagenesis in Gram Negative Bacteria." *Nature Biotechnology* 1 (9): nbt1183–1784.

Smits, Theo H. M., Fabio Rezzonico, Tim Kamber, Jochen Blom, Alexander Goesmann, Carol A. Ishimaru, Jürg E. Frey, Virginia O. Stockwell, and Brion Duffy. 2011. "Metabolic Versatility and Antibacterial Metabolite Biosynthesis Are Distinguishing Genomic Features of the Fire Blight Antagonist *Pantoea vagans* C9-1." *PloS One* 6 (7): e22247.

Tecon, Robin, and Johan H. J. Leveau. 2012. "The Mechanics of Bacterial Cluster Formation on Plant Leaf Surfaces as Revealed by Bioreporter Technology." *Environmental Microbiology* 14 (5): 1325–32.

Thomas, Christopher M., and Kaare M. Nielsen. 2005. "Mechanisms Of, and Barriers To, Horizontal Gene Transfer between Bacteria." *Nature Reviews. Microbiology* 3 (9): 711–21.

Vogel, Christine, Gerd Innerebner, Judith Zingg, Jan Guder, and Julia A. Vorholt. 2012. "Forward Genetic in Planta Screen for Identification of Plant-Protective Traits of *Sphingomonas* sp. strain Fr1 against *Pseudomonas syringae* DC3000." *Applied and Environmental Microbiology* 78 (16): 5529–35.

Vorholt, Julia A. 2012. "Microbial Life in the Phyllosphere." *Nature Reviews. Microbiology* 10 (12): 828–40.

Wilson, M., and S. E. Lindow. 1994. "Ecological Similarity and Coexistence of Epiphytic Ice-Nucleating (Ice) *Pseudomonas syringae* Strains and a Non-Ice-Nucleating (Ice) Biological Control Agent." *Applied and Environmental Microbiology* 60 (9): 3128–37.

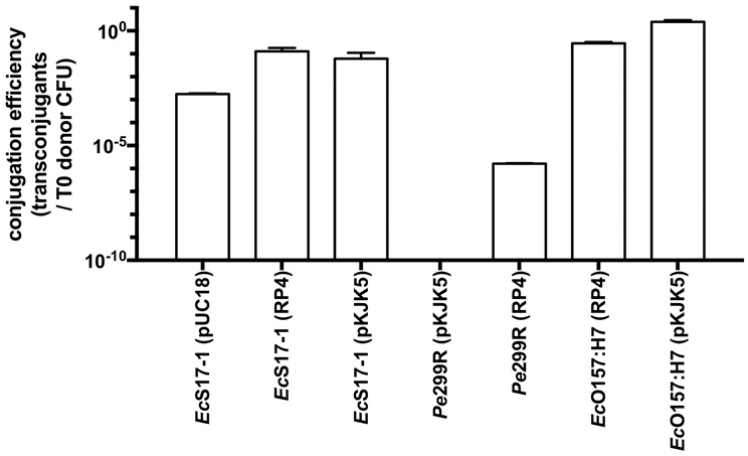
Wolters, Birgit, Martina Kyselková, Ellen Krögerrecklenfort, Robert Kreuzig, and Kornelia Smalla. 2014. "Transferable Antibiotic Resistance Plasmids from Biogas Plant Digestates Often Belong to the IncP-1ε Subgroup." *Frontiers in Microbiology* 5: 765.

Tables

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

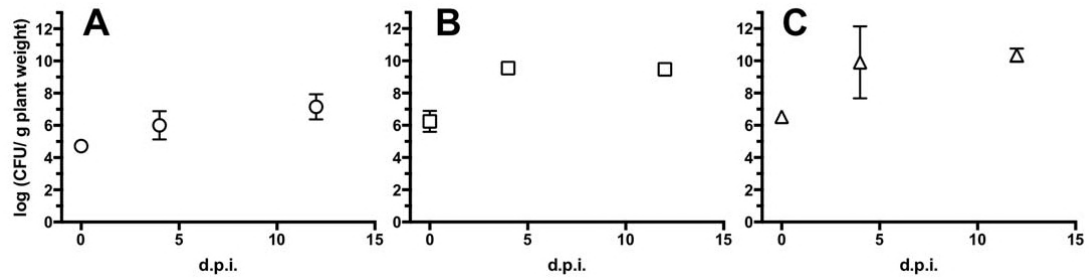
Strain (note of important properties)	**Abbreviation**	**Antibiotic resistance**	**Reference**
E. coli O157:H7::MRE103 Δ*stx* (grows on lactose, red fluorescent, does not produce Shiga toxin)	*Ec*O157:H7red	Rifampicin	(Remus-Emsermann, Gisler, and Drissner 2016)
E. coli O157:H7 Δ*stx* (grows on lactose, does not produce Shiga toxin)	*Ec*O157:H7	n.a.	NCTC 12900
E. coli S17-1 λpir (auxothroph)	*Ec*S17-1	Streptomycin	(Simon, Priefer, and Pühler 1983)
Pantoea eucalypti 299R (grows slowly on lactose)	*Pe*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)

562 **Figures**



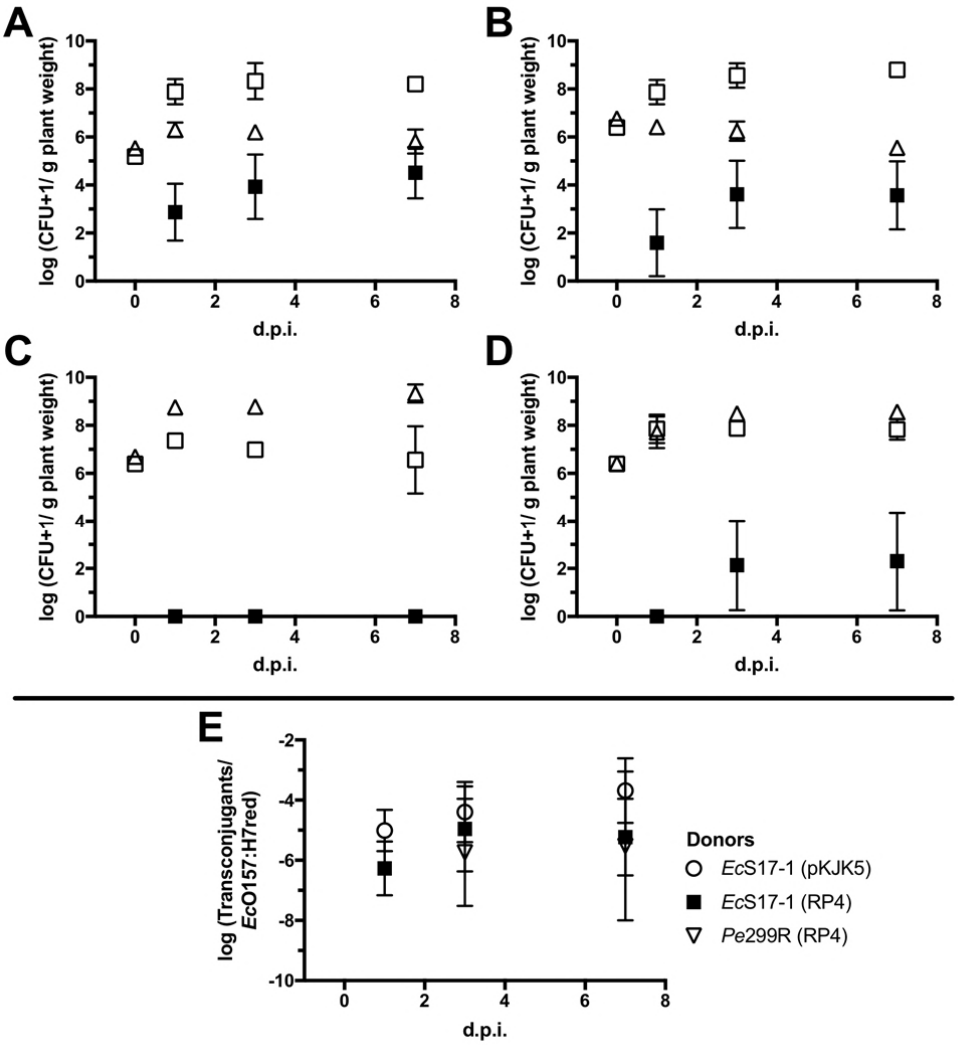
563

564 **Figure 1.** Transconjugant frequencies in the recipient population after mating of the recipient
565 *Ec*O157:H7red with *Ec*S17-1, *Pe*299R and *Ec*O157: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

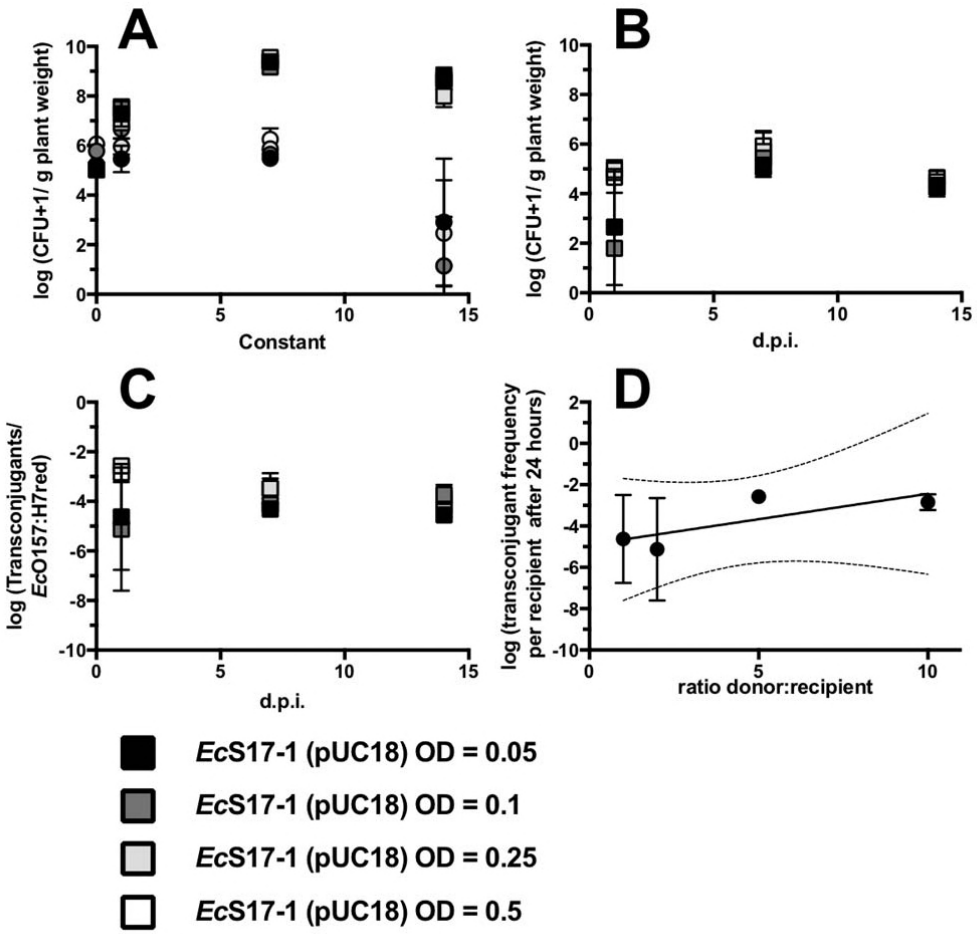
571 **Figure 2.** Bacterial population development after inoculation of individual strains onto
572 gnotobiotic Arabidopsis. A) *Ec*S17-1; B) *Ec*O157:H7; C) *Pe*299R. Error bars represent the
573 standard deviation of the mean.



574

575 **Figure 3.** Population development of bacteria on gnotobiotic Arabidopsis. **A)** Population
576 development of *Ec*O157:H7red (open squares), *Ec*S17-1 (pKJK5) (open circles), and
577 *Ec*O157:H7red (pKJK5) transconjugants (filled squares). **B)** Population development of
578 *Ec*O157:H7red (open squares), *Ec*S17-1 (RP4) (open circles), and *Ec*O157:H7red (RP4)
579 transconjugants (filled squares). **C)** Population development of *Ec*O157:H7red (open
580 squares), *Pe*299R (pKJK5) (triangles), and *Ec*O157:H7red (pKJK5) transconjugants (filled
581 squares). The conjugation with *Pe*299R (pKJK5) did not yield transconjugants above the
582 limit of detection. **D)** Population development of *Ec*O157:H7red (open squares), *Pe*299R
583 (RP4) (triangles), and *Ec*O157:H7red (RP4) transconjugants (filled squares). **E)** Frequencies

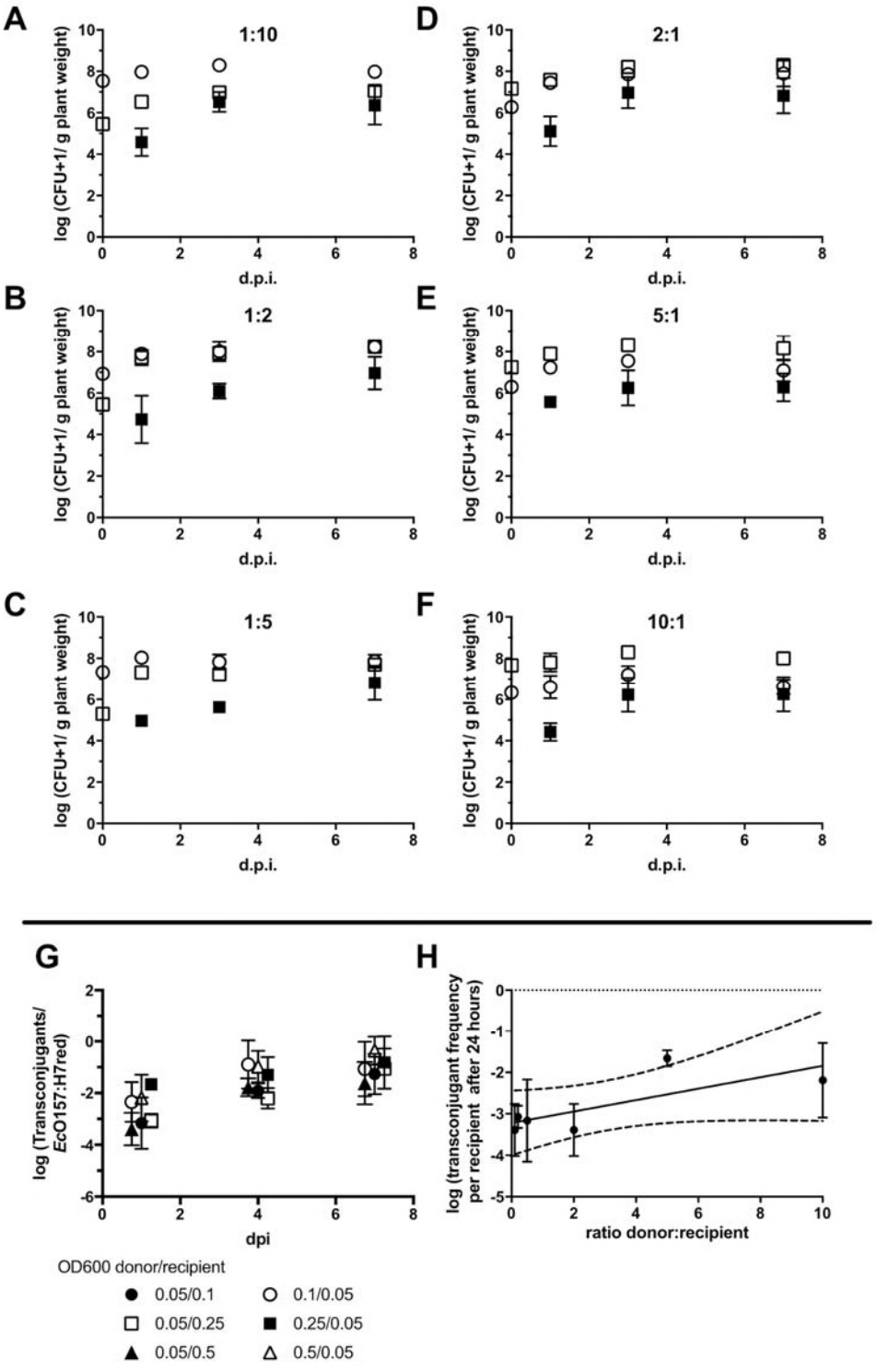
584 of transconjugants in the *Ec*O157:H7red population after 1, 3, and 7 days. Error bars
585 represent the standard deviation of the mean.



586

587 **Figure 4. A)** Population development after co-inoculation of *Ec*O157:H7red (squares) and
588 *Ec*S17-1 (circles) on Arabidopsis. *Ec*S17-1 (pUC18) was inoculated in different densities
589 (treatments indicated by different shadings), while the inoculation density of *Ec*O157:H7red
590 remained constant. **B)** Population development of the transconjugant *Ec*O157: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 *Ec*S17-1 donor concentrations could be detected,
594 however, the variation within treatments was lower at high donor densities. A linear

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

Figure 5. Conjugation dynamics of the self-transmissible plasmid RP4 in a population of *Ec*O157:H7. **A-F)** Population development of six different ratios of *Ec*O157:H7 (RP4) donors (open circles) *Ec*O157:H7red recipients (open squares), and *Ec*O157:H7red transconjugants (filled squares) after inoculation onto Arabidopsis. Inoculation of donors and 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)** Transconjugant frequency in the recipient population over time. Data points were slightly nudged for better visibility. **H)** A linear regression was fitted and shows the inverse correlation of initial donor density and transconjugant frequency in the recipient population after 24 hours ($r^2 = 0.56$, broken lines 95% confidence intervals of the linear regression).