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The spread of the plasmid RP4 in a microbial community is dependent on the particular donor strain

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Abstract

The rapid spread of antibiotic resistance challenges modern medicine. So far, mechanistic and quantitative knowledge concerning the spread of resistance genes mainly relies on laboratory experiments with simplified set-ups, e.g., two strain-communities. Thus, the transferability of the obtained process rates is questionable. To investigate the role of a diverse community concerning the dissemination of the multidrug resistance plasmid RP4, an *E. coli* harboring the latter invaded a microbial community consisting of 21 species. Changes in the community composition as well as plasmid uptake by community members were monitored for 22 days. Special focus was laid on the question of whether the observed changes were dependent on the actual invading donor isolate and the ambient antibiotic concentration. In our microcosm experiment, the community composition was primarily influenced by the given environmental variables and only secondarily by the particular invader *E. coli*. The establishment of resistance within the community, however, was directly dependent on the actual donor isolate. The extent to which ambient conditions influence the spread of RP4 depended on the particular isolate.

Keywords: conjugation, RP4 plasmid, bacterial community, epic-PCR

1 Introduction

Nowadays, modern medicine is unthinkable without antibiotics. However, the extensive use of antimicrobials in health care in the last decades fostered the emergence and spread of antibiotic resistance. Worldwide, about 900,000 people per year die from infections with antibiotic resistant bacteria which are no more treatable with the currently available antibiotics (O'Neill, 2016). Although, the problem is most pressing in human and veterinary medicine, the environment constitutes a source and reservoir for antibiotic resistance genes (ARG) and resistant bacteria (ARB). Furthermore, there is a permanent exchange of ARG and ARB between compartments (humans, veterinary medicine, environment). For instance, human-associated ARG and ARB are constantly released in aquatic environments via treated wastewater: A large scale conventional wastewater treatment plant (44,000 population equivalents) discharges about 1.5 x 10^{18} bacteria per day into the receiving water body (Jäger et al., 2018). By eating fresh produce, 'environmental' bacteria get in contact with the intestinal human microbiome. Depending on the season, irrigation mode, and leaf age between 10^5 and 10^7 bacterial cells per g wet weight live on lettuce (Williams et al., 2013). Monitoring studies investigating the stability of the microbiome in the human gut revealed that consequences of such bacterial invasions are, among other variables, dependent on the ambient conditions and especially influenced by a previous antibiotic treatment (e.g., Lazupone et al., 2012; Jeffery et al., 2016; Yassour et al., 2016; Bäumler and Sperandio, 2016; Lange et al., 2016; Vonaesch et al., 2018). Furthermore, changes in microbial community compositions and resistomes of water bodies were attributed to wastewater discharge and water reuse (e.g., Narciso-da Rocha and Manaia, 2017; Dang et al., 2019).

These monitoring studies provide knowledge about the current status of a particular system but do not identify the driving processes creating the seen patterns. To contribute to close this knowledge gap we conducted a microcosm experiment introducing an *Escherichia coli* strain into an assembled community consisting of 21 Gram-positive and -negative species derived from different environments. The invading strain harbored the broad-host range plasmid RP4 which mediates multi-drug resistance. We repeated the experiment with three different *E. coli* strains which revealed different abilities to acquire RP4. By means of 16S amplicon sequencing and epic-PCR, we studied changes in the community composition as well as the uptake of the plasmid by the particular community members. In addition, we tested the ability to express the acquired plasmid by incubating in presence of inhibitory antibiotic concentrations. Specifically, we tested if the observed changes in the community composition and the spread of the plasmid depends on the chosen *E. coli* donor strain (1) and the presence of subinhibitory antibiotic concentrations (2).

2 Material and methods

2.1 Microcosm experiment

To investigate the aforementioned questions, microcosm experiments lasting for 22 days were conducted. Every second day, 1% of the culture (total volume: 6 mL) was transferred into fresh HT-media. The latter consists of different not easily degradable carbon sources and was developed to allow for coexistence of a multitude of bacterial species (reference). The assembled microbial community comprised 21 species (Table 1) which were negatively tested for plasmids being incompatible with RP4 (Cairns et al., 2018). In parallel to the bi-daily transfers, 1 x 10^6 cells of the donor and approx. 5 x 10^5 cells of each community member were added from cryoculture to prevent the extinction of less competitive species. The microcosms were incubated at 28 °C and shaken at 50 rpm. We used three different *E. coli* donors (one at a time) to test the dependence of the spread of the RP4 plasmid on particular isolates belonging to the same species. RP4 is a naturally occurring broad-host range plasmid mediating resistance to ampicillin, kanamycin, and tetracyclin. One donor (donor a) originated from the HAMBI culture collection (University of Helsinki, Finland) and the other two (donors c and e) derived from a previous experiment (Heß et al., 2020). The latter were cultured first with and later without antibiotics to ensure plasmid stability in the donor strain. Additionally, we tested the effect of three different kanamycin concentrations (0 μg/mL, 0.0025 μg/mL and 0.25 μg/mL) on the spread of RP4 within the community. To ensure reproducibility of the obtained results, each treatment was repeated four times resulting in a total of 36 microcosm experiments (3 donors x 3 antibiotic levels x 4 replicates each).

Before each transfer, a subsample of 1 mL was taken, supplemented with glycerol and stored at -80 °C for further analysis (analysis of the community composition via 16S amplicon sequencing and the spread of the RP4 plasmid via epic-PCR).

At the end of the experiment, 60 μL were transferred into 5.4 mL HT-broth containing 25 μg/mL kanamycin to test the expression of the uptaken RP4 by the particular species.

2.2 16S amplicon sequencing and epic-PCR

16S amplicon sequencing: For DNA isolation, 0.5 mL of the preserved samples were centrifuged and the supernatant was discarded. The DNA of the pelleted cells was subsequently isolated using the DNeasy 96 Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Subsequently, the V3/V4 region of the 16S gene was amplified by PCR as previously described (Cairns et al., 2018). The 16S amplicons were

sequenced at the Institute of Biotechnology of the University of Helsinki (Finland) using a MiSeq device.

epic-PCR: By means of emulsion, paired isolation and concatenation (epic) PCR, it was tested which species possessed the plasmid at the end of the experiment (after the 10th transfer). The detailed protocol was already published by Cairns et al. (2018) where primer sequences as well as PCR protocols are listed. Briefly, depending on cell density, up to 10 μL of the preserved culture were used to trap single cells in polyacrylamide beads. Subsequently, successful bead formation was checked and single occupancy of the beads was confirmed using fluorescence microscopy. By fusion PCR, fragments of the *aph*A gene (mediates resistance to kanamycin) located on the RP4 plasmid and the 16S rDNA gene were linked to form one combined amplicon. A smaller fragment of this amplicon was later on replicated in a nested PCR to obtain enough product for subsequent next-generation sequencing. The latter was done at the Institute of Biotechnology of the University of Helsinki (Finland).

2.3 Processing of the sequencing data

The obtained reads (16S amplicons and fused 16S-*aph*A-sequences) were quality checked and trimmed using TrimGalore (q=28, length=100) (https://gitub.com/FelixKrueger/TrimGalore). The retained reads were subsequently merged using PANDAseq (Masella et al., 2012). As an additional trimming step for the epic reads, the *aph*A fragment was cut off. The so trimmed reads were subsequently compared to a self-assembled database consisting of the 16S rDNA sequences of the community members (see Table 1) which are deposit in the NCBI database (www.ncbi.nlm.nih.gov). Only matches with an alignment length of at least 100 bp and an e-value smaller than 1e-50 were counted. In case of one read hit to several database entries the one with the highest (alignment length)/(e-value) ratio was taken. The described pipeline was automated using R (www.r-project.org).

2.4 Statistical analysis

Tabelle 3: Punkt bedeutet, dass Spezies in mindestens 3 der 4 biologischen Replikate Träger des Plasmids war

3 Results and dicussion

3.1 Dynamics of the community composition

The community composition was stable after the third transfer and essentially dominated by the same 8 species independent on the particular *E. coli*

Table 1: Community composition.

Species	Strain ID	Natural habitat of the species
Acinetobacer lwoffii	HAMBI 97	ubiquitous in the environment
Aeromonas caviae	HAMBI 1972	water
Agrobacterium tumefaciens	HAMBI 105	soil
Brevundimonas bullata	HAMBI 262	ubiquitous in the environment
Chitinophaga sancti	HAMBI 1988	soil
Citrobacter koseri	HAMBI 1287	normal flora of human and animal guts
Comamonas testosteroni	HAMBI 403	soil, member of human microbiome
Cupriavidus necator	HAMBI 2164	soil
Hafnia alvei	HAMBI 1279	normal human gut flora
Kluyvera intermedia	HAMBI 1299	water, soil, sewage
Microvirga lotononidis	HAMBI 3237	Nitrogen-fixing nodule of *Lotononsis angolensis*
Moraxella canis	HAMBI 2792	water, soil, mucosal membranes of humans and animals
Niabella yanshanensis	HAMBI 3031	soil
Paraburkholderia caryophylli	HAMBI 2159	soil
Paraburkholderia kururiensis	HAMBI 2494	groundwater, soil
Paracoccus denitrificans	HAMBI 2443	soil, wastewater, sludge
Pseudomonas chlororaphis	HAMBI 1977	soil
Pseudomonas putida	HAMBI 6	soil, water
Sphingobacterium spiritivorum	HAMBI 1896	soil, compost
Sphingobium yanoikuyae	HAMBI 1842	often isolated from human specimen
Stenotrophomonas maltophilia	HAMBI 2659	water, plant rhizospheres, animals

invader: *A. tumefaciens*, *K. intermedia*, *S. spiritivorum*, *A. caviae*, *P. chlororaphis*, *S. maltophilia*, *P. putida*, and *E. coli* (exemplary shown for donor e in absence of antibiotics in the supplementary Fig. S.1). The remaining 14 species made up less than 5% (Fig. S.1). Overall, the microbial communities in the biological replicates with the same treatment were very similar at all time steps analyzed (data not shown). The relative abundances of the respective species differed depending on the invader strain. For 4 out of the 6 chosen indicators describing the diversity and eveness of the community composition, the donor had a significant effect on the final abundance of the members (Tab. 2).

In comparison to the impact of a particular *E. coli* on the community composition, the effect of the ambient kanamycin concentration was stronger

and statistically highly significant independent of which indicator was chosen (Tab. 2). For instance, after the 10th transfer, *P. putida* made up to 20% of the community in absence of any antibiotic and in presence of 0.0025 μg mL^{-1} kanamycin but the strain went extinct at an ambient concentration of 0.25 μg mL^{-1} kanamycin (Fig. 1 top left). The same phenomenon was observed for *P. chlororphis* but with a lower relative abundance in complete absence or presence of the low kanamycin concentration (Fig. 1). On the contrary, *Comamonas testosteroni*, was only able to conquer a niche within the community in presence of the highest tested kanamycin concentration. After the last transfer, *C. testosteroni* cells made up to 10% of the community (Fig. 1).

Based on the ANOVA-test, interaction between the two parameters 'donor strain' and 'antibiotic concentration' was unlikely (Tab. 2).

Table 2: Result of a two-way ANOVA testing for effects of kanamycin exposure and the chosen donor strain on the final composition of the bacterial community. Numbers represent p-values (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). Antibiotic levels were treated as factors (none: 0, low: 0.0025, high: 0.25 μg mL^{-1}).

Indicator	Kanamycin	Donor	Interaction
Shannon diversity index	2e-13***	1e-02*	2e-01
Eveness	1e-06***	4e-01	7e-01
No. species contributing > 1% to community	2e-13***	4e-02*	8e-03**
No. species contributing > 2% to community	6e-05***	3e-03**	6e-01
Share of three dominant species in community	2e-06***	2e-01	1e00
Share of five dominant species in community	7e-13***	1e-04***	4e-01

3.2 Spread of RP4 within the assembled community

Generally, the percentage of community members which harbored the plasmid after the 10th transfer was rather low (0% - 38%). For instance, in the microcosms without antibiotics and *E. coli* a and c as donors, none of them harbored the plasmid after the final transfer (Table 3). Either none of the community members were able to pick up the plasmid or it/they lost RP4 again. For all treatments, a maximum of eight community members possessed the plasmid (0.25 μg mL^{-1}, donor a; Table 3). *S. maltophilia*, *A. caviae*, *C. testosteroni*, *K. intermediae* and *A. lwoffii* formed a core group of plasmid owners which, in case of the spread of RP4 within the community, most likely had picked up the plasmid. Although there was a tendency that the probability of plasmid uptake was higher for more abundant species, RP4 was not detected in all 8 core community species. For instance, *S. spiritivorum* made up to 25% of the community after the 10th transfer (donor a, 0.25 μg mL^{-1}; Fig. 1) but had not picked up the plasmid in any of the

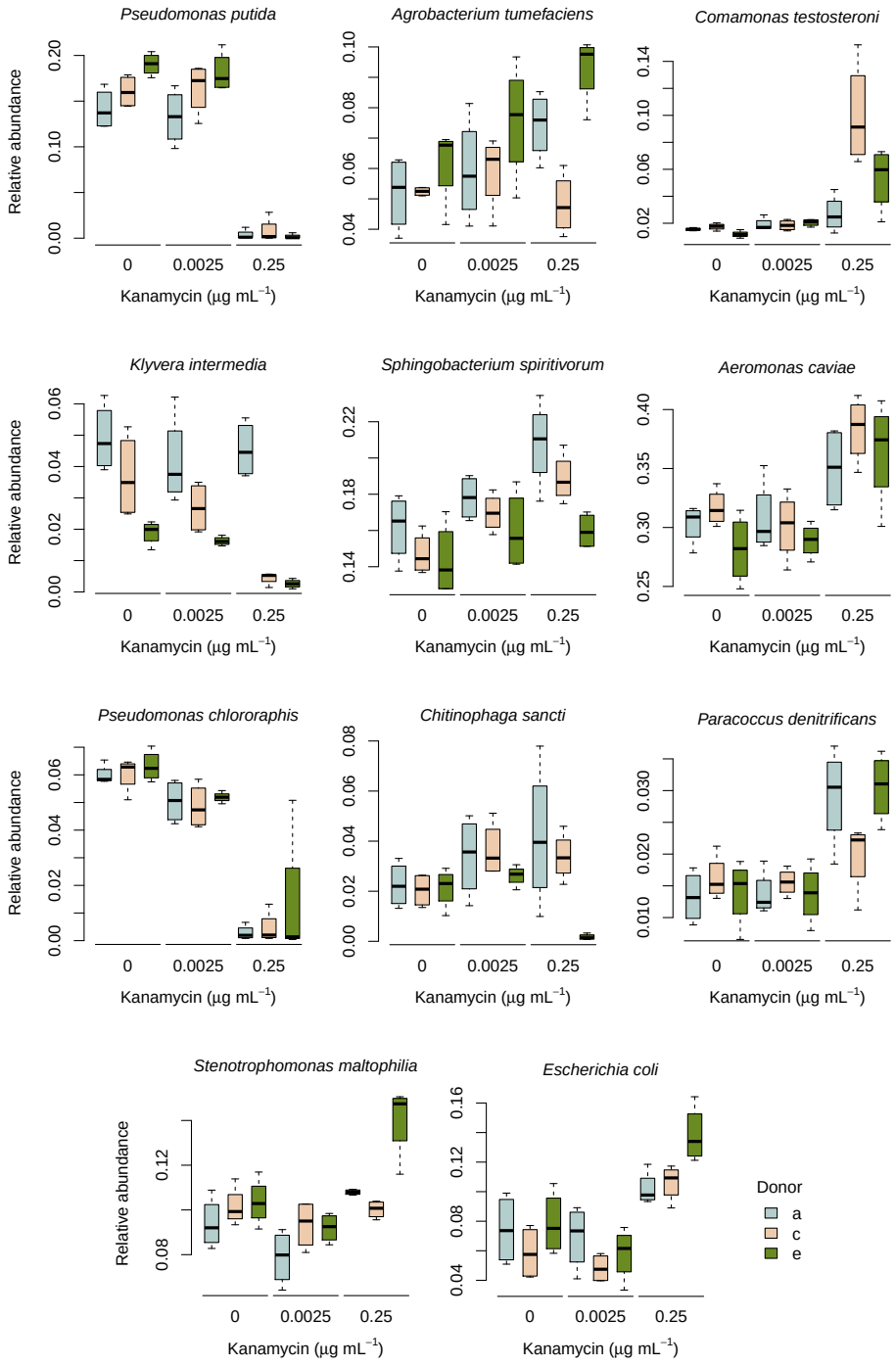

Figure 1: Relative abundance of the predominant species after the 10th transfer.

treatments or lost it again (Table 3). The plasmid was only very rarely detected in species not belonging to the 8 core community members, e.g., *H. alvei*, *S. yanoikuyae* or *C. necator*. In this context, however, it is important to keep in mind that the probability that (a) a less abundant species get in contact with a plasmid harboring cell is less likely as it is for higher abundant species and (b) the probability of the detection of such an event via epic-PCR is lower simply due to their rareness.

The spread of the plasmid within the community differed depending on the particular invading *E. coli* strain. In presence of donor a and 0.25 μg mL^{-1} kanamycin the highest number of species picked up RP4: *A. tumefaciens*, *H. alvei* and *C. necator* possessed the plasmid only in this treatment (Table 3). Unfortunately, the sensitivity of the method did nor allow for the detection of (a) hub-species (starting point for the star-shaped spreading of the plasmid) nor for a detailed traceability of the dissemination path of the plasmid. However, since the abilities of the community members to acquire the plasmid were not donor dependent (Fig. 2), there is an indirect hint that the initial transfer to the possible hub-species was directly from the *E. coli* donor or the *E. coli* strain itself acted as hub-species.

The observation that the probability of plasmid uptake of particular community members seemed not to depend on the invading *E. coli* strain was not surprising. The latter was not necessarily expected because of the possible spread triggered by transconjugants.

Besides the donor, the ambient antibiotic concentration also had an effect on the dissemination of RP4. Interestingly, its impact differed for the used donors: For the donors a and c, the number of species which had taken up the plasmid was higher with increasing kanamycin concentration. The opposite was observed in presence of donor e.

3.3 Expression of RP4 by transconjugants

In presence of an inhibitory antibiotic concentration, antibiotic susceptible cells die independent of whether they do not possess a gene mediating resistance to the respective drug or they do not express the latter. To test whether the transconjugants are able to express the kanamycin resistance gene *aph*A located on the acquired RP4 plasmid, the communities were exposed to 2.5 μg mL^{-1} kanamycin. In addition to *E. coli*, only two other species, namely *S. maltophilia* and *A. caviae*, were able to grow under these conditions - given they picked up the plasmid during the experiment.

4 Discussion

We tested the effect of the invasion of different antibiotic-resistant *E. coli* strains on the composition of an assembled community and the spread of the RP4 plasmid within the latter. Our results revealed that the impact of

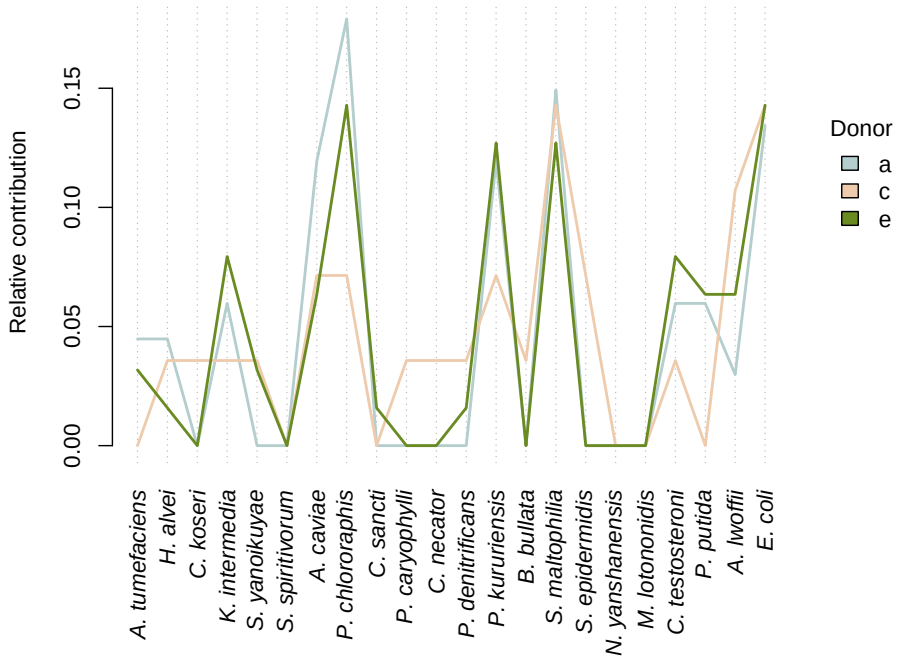

Figure 2: Relative contribution of individual strains to the occurrence of RP4 in the bacterial community (possible range: 0–1).

such an invasion differed between donor isolates even though they all belonged to the same species (*E. coli*). If an invader is successful in conquering an ecological niche depends on its traits which can be encoded in the core genome but can also be acquired. Comparisons on whole genome level depicted that about 2,200 genes form the core genome of the species *E. coli* and thus are common to all *E. coli* cells. The pangenome, however, consists of approx. 13,000 genes comprising six times more genes than the core genome (Rasko et al., 2008). This finding explains the high phenotypical variability within the species and simultaneously provides an indication why it is almost impossible to predict the competitiveness of an invading *E. coli* within a community without a preceding whole genome analyses of all community members.

As expected, strains of the species *E. coli* varied also with regard to their ability to acquire, maintain and donate resistance gene carrying plasmids (Gordon, 1992; Heß et al., 2020). Whereas the donor c was a rather bad recipient (plasmid uptake rate: $10^{-14.2}$) donor e had a higher plasmid uptake rate ($10^{-13.5}$) in absence of any antibiotic (Heß et al., 2020). In contrast to donors c and e, donor a lost the plasmid in complete absence or presence of only traces of kanamycin (0.0025 μg mL^{-1}; Table 3). Cairns et al. (2018) used donor a for their microcosm experiments testing especially the effect of spatial structure and predation on plasmid spread. Their setup only dif-

Table 3: List of community members harboring the plasmid after the 10th transfer.

Kanamycin (μg mL^{-1})	0			0.0025			0.25		
Donor	a	c	e	a	c	e	a	c	e
S. maltophilia		●	●		●	●	●	●	●
E. coli		●	●		●	●	●	●	●
A. caviae			●		●	●	●	●	●
C. testosteroni			●			●	●	●	●
K. intermedia			●			●	●	●	
A. lwoffii		●	●				●	●	
P. putida			●		●	●			
A. tumefaciens							●		●
P. chlororaphis			●			●			
H. alvei							●		
S. yanoikuyae						●			
C. necator							●		
C. koseri									
S. spiritivorum									
C. sancti									
P. caryophylli									
P. denitrificans									
P. kururiensis									
B. bullata									
S. epidermidis									
N. yanshanensis									
M. lotononidis									
Species harboring RP4	0	3	8	0	4	8	9	6	5

fered from this study by the used culture media (Kings'B- versus HT-broth) and the agitation regime (no shaking versus 50 rpm). However, the two variables do not seem to crucially effect the cost-benefit ratio of the plasmid as the donor also lost the plasmid in absence of antibiotics, predators and glass beads (Cairns et al., 2018). Plasmid loss is an indication of an excessive physiological burden by plasmid carriage under the given ambient conditions. Controlled laboratory experiments suggest that epistasis, co-selection and silencing of transcripts facilitate the maintenance of plasmids that harbor ARG (San Millan et al., 2014; Hughes and Andersson, 2017; Wein et al., 2019; Baker-Austin et al., 2007; Pal et al., 2015). It has been demonstrated that sub-inhibitory antibiotic concentrations promote the persistence of plasmids in populations by altering the cost-benefit ratio resulting in increased fitness and competitiveness (e.g., Gullberg et al., 2011; Andersson and Hughes, 2014). However, for donor a, only the highest tested kanamycin concentration (0.25 μg mL^{-1}) seemed to have an cost altering effect. Another

possibility why the plasmid persisted at the highest concentration might be the emergence of cost-ameliorating mutations (Dahlberg and Chao, 2003). So far, it is unclear why the RP4 plasmid was lost by donor a in absence of antibiotics but maintained by the donors c and e. Further genomic and m-RNA-based analyses comparing donor a with c and e could help to unravel the reason.

Comparing the effect of sub-inhibitory antibiotic concentrations on the spread of resistance in a multi-species community (this study) with their impact on the conjugative plasmid uptake in single or two-strain systems led not necessarily to the same conclusions. Whereas the observed plasmid uptake rate by the *E. coli* from *Serratia marcescens* was only negligibly influenced by traces of antibiotics (Heß et al., 2020), the latter had a significant effect on the spread of RP4 within the assembled community. Already traces of antibiotics, which nowadays can be detected in almost every ecosystem (Felis et al., 2020), thus might be enough to foster the spread of resistance in communities. This needs to be considered when generating and interpreting predictive models concerning the dissemination of ARG (e.g., Blanquart, 2019).

Interestingly, most of the species which picked up the plasmid during the experiment were unable to phenotypically express their resistance and subsequently compete with *E. coli*, *S. maltophilia* and *A. caviae* in the given setting. This might be assigned to too high costs of the plasmid: The plasmid possession might be associated with decelerated growth and thus transconjugants might have been overgrown by the three species in presence of 25 μg/mL kanamycin. In a clinical context where such an inhibitory concentration would be applied, this finding has a rather positive connotation: The treatment would lead to an abolition of cells which took up the plasmid over time since they get extinct. So far, scientists feared that antibiotic treatment kills sensitive cells but enrich resistance gene carrying species/cells. Thus, our results suggest that the relative abundance of donor cells is indeed enriched but the plasmid spread within the members of the microbiome is largely reversed. For the environment, however, where antibiotic concentrations above MIC are usually not reached, our results let assume that ARG carrying plasmids persist in the long-term. So far, it is known which role cells which do not express the resistance genes located on the acquired plasmid play concerning the persistence of AR. To deeper understand their function as passive plasmid possessors or active donors single species studies measuring plasmid costs and persistence would be helpful.

To conclude, if and how an invader species influenced the community composition was primarily dependent on the given environmental parameters and only secondarily by the specific strains. Whether *E. coli* was able to conquer an ecological niche depended both on its fitness and competitiveness within the given environmental conditions and on the microbial community.

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Competing interests

The authors declare no competing interests.

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Supplement

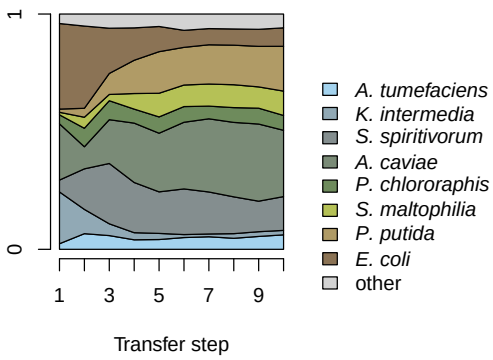

Figure S.1: Community composition in presence of donor e in absence of any antibiotic over time.