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Plasmids persist in a microbial community by providing fitness benefit to multiple phylotypes

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Abstract

The current epidemic of antibiotic resistance has been facilitated by the wide and rapid horizontal dissemination of antibiotic resistance genes (ARGs) in microbial communities. Indeed, ARGs are often located on plasmids, which can efficiently shuttle genes across diverse taxa. While the existence conditions of plasmids have been extensively studied in a few model bacterial populations, their fate in complex bacterial communities is poorly understood. Here, we coupled plasmid transfer assays with serial growth experiments to investigate the persistence of the broad-host-range IncP-1 plasmid pKJK5 in microbial communities derived from a sewage treatment plant. The cultivation conditions combined different nutrient and oxygen levels, and were non-selective and non-conducive for liquid-phase conjugal transfer. Following initial transfer, the plasmid persisted in almost all conditions during a 10-day serial growth experiment (equivalent to 60 generations), with a transient transconjugant incidence up to 30%. By combining cell enumeration and sorting with amplicon sequencing, we mapped plasmid fitness effects across taxa of the microbial community. Unexpected plasmid fitness benefits were observed in multiple phylotypes of *Aeromonas*, *Enterobacteriaceae*, and *Pseudomonas*, which resulted in community-level plasmid persistence. We demonstrate, for the first time, that plasmid fitness effects across community members can be estimated in high-throughput without prior isolation. By gaining a fitness benefit when carrying plasmids, members within complex microbial communities might have a hitherto unrecognised potential to maintain plasmids for long-term community-wide access.

Introduction

Plasmids—extra-chromosomal replicons—can support rapid bacterial adaptation by moving genes between phylogenetically diverse bacteria, a process known as horizontal gene transfer [1]. This is particularly important in the case of antibiotic resistance, where acquisition of plasmid-borne antibiotic resistance genes (ARGs) can instantly render a strain impervious to antibiotic treatment [2]. The current global antibiotic resistance crisis has been largely attributed to plasmid-mediated ARG dissemination [3].

Strategies to combat the antibiotic resistance epidemic, therefore, require an understanding of the mechanisms that underlie plasmid fate in microbial communities. Upon entering a microbial community, a plasmid will only persist if its original or secondary hosts (transconjugants) survive. If it persists, it will constitute a long-term reservoir of auxiliary genes for that community. In fact, persistence of IncP-1 plasmids and their derivatives has recently been observed in ex situ communities like anaerobic sludge microcosms [4] and an artificial multi-species system [5] over periods longer than 10 days, as well as in murine gut microbiota experiments [6]. However, by simply interpreting plasmid persistence as the frequency of plasmid occurrence, these observational studies did not explicitly examine the mechanisms that underlie plasmid persistence. Therefore, we are currently far from able to predict plasmid fate in a microbial community.

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In contrast to our limited understanding of plasmid persistence at the community level, the fate of plasmids at the population level has received much more attention. There, theoretical and empirical studies have demonstrated that the conditions for plasmid persistence are governed by the interplay of segregational loss, conjugal transfer, and the effect of the plasmid on host fitness [7–9]. The rates of these processes depend on the plasmid, the host, and the environmental conditions, in a complex manner. First, although partitioning mechanisms exist to ensure ordered plasmid segregation prior to cell division [10], plasmid loss during vegetative growth can typically be detected [7]. Second, plasmid carriage often imposes a reduction in fitness to the host [11]. Therefore, in the absence of mechanisms countering segregational loss and fitness cost, plasmids would eventually be removed from bacterial populations. To persist, plasmids lacking stringent post-segregational killing systems thus depend on one or both of two mechanisms: fitness cost amelioration and horizontal transfer [9].

In the majority of long-term cultivation experiments, plasmid persistence is associated with a reduction in the burden of plasmid carriage through adaptation (mainly by compensatory mutations) [9]. This lessens selection against plasmid carriage, and therefore reduces the rate at which plasmids are removed from the population. For example, by experimentally evolving *Pseudomonas fluorescens* carrying a high-fitness-cost plasmid, Harrison et al. observed amelioration of the cost-of-carriage across a wide gradient of environmental conditions, even without positive selection for plasmid carriage [12]. In an evolutionary experiment conducted by Dionisio et al. [13], strong fitness-increase effects were noted of conjugative plasmid R1 both in the original host *Escherichia coli* and other bacterial lineages including *Salmonella enterica*. Recent studies on the genetic basis underlying such adaptations among different plasmid-host pairs suggest that poor plasmid persistence can be caused by a high cost involving helicase-plasmid interactions, which can be rapidly ameliorated [14]. Another explanation for plasmid persistence within a population is conjugal transfer. In contrast to conjugation-deficient pKJK5 derivatives, the ability of the original pKJK5 plasmid to conjugate played an essential role in its stable maintenance in populations of *E. coli* and *Kluyvera sp* [15, 16]. More recently, Lopatkin et al. [17] experimentally demonstrated that transfer rate of common conjugative plasmids was necessary and sufficient to maintain plasmids in an *E. coli* population.

Detailed investigations of plasmid fate have been limited to pure or defined cultures of isogenic or closely related strains of a few model species. Such research on species like *E. coli* and *P. putida* have confirmed the notion that plasmid persistence is strain dependent [18–20]. Strains of *P. putida* can differ largely in their ability to maintain the IncP-1 plasmid pB10 [21, 22]. The cause of this inter-strain variability is currently unknown and our ability to identify it is likely limited by the fact that testing single strains is labour intensive.

Since most bacteria exist within complex communities of hundreds to thousands of species, plasmid persistence is ideally examined within this context. However, tracking the fate of a plasmid and its hosts within a complex community poses a great challenge. Population-level experiments ignore the complex interactions within communities, which further prevents inferring plasmid fate in communities. For example, interspecies transfer enabled persistence of plasmid pQBR57, which would have been impossible within a single population [23]. Besides, multi-level ecological dynamics play a role in a community, since fitness differences do not only exist between plasmid-free and plasmid-bearing cells within populations of a singular strain, but also between strains. Therefore, a plasmid can be lost because of low intrinsic fitness of its hosts or even merely because of ecological drift.

Previous work on community permissiveness (i.e., the ability to take up a plasmid from an exogenous donor strain) has revealed that IncP-1 plasmids can readily be transferred to diverse members of complex environmental communities [24–26]. However, it is not clear to what extent the communities can maintain the plasmid and in which way the individual members can contribute to community-level plasmid persistence.

By coupling permissiveness assays using a *gfp*-tagged plasmid with serial growth experiments, we monitored the persistence of the IncP-1 plasmid pKJK5 not only at the community level but also at the level of individual phylotypes to identify the relative importance of the processes acting on plasmid persistence and how they vary across phylotypes. To reduce complexity, we chose conditions where conjugal transfer is limited, and thus, where segregational loss, plasmid fitness effects, and competition between phylotypes drive plasmid fate. We identified that plasmid fitness benefits in multiple phylotypes played an essential role in community-level plasmid persistence.

Materials and methods

Donor strain and recipient microbial community

E. coli MG1655 carrying pKJK5, was used as donor (further denoted *E. coli* (pKJK5)). pKJK5, originally recovered from the barley rhizosphere by exogenous isolation [27], is a 54 Kb low-copy-number conjugative plasmid. It encodes the maintenance/stability systems that are typically associated with IncP-1 plasmids (i.e., *kle*, *klc*, *kor*, and *par*). Despite an identified putative post-segregational killing

system, we have frequently observed plasmid loss from transconjugant cells (data not included here), indicating its loose control on plasmid-free segregants. An accessory region carries genes encoding tetracycline and trimethoprim resistance, which is associated with transposons [28]. Neither these nor the other plasmid-encoded genes are known to provide a selective advantage to the host in the absence of antibiotics. pKJK5 transfer host range has been measured in different microbial communities; a broad transfer host range was observed in soil and wastewater communities [24–26]. In this experiment, pKJK5 is marked with *gfpmut3b* under the control of a LacI^q repressible promoter P_{A1O4O3} [24]. The donor strain is chromosomally tagged with a constitutively expressed *lacI^q* and *mCherry* using the mini-Tn7 tagging system [24]. Therefore, plasmid-encoded *gfp* expression is repressed in the donor strain, but de-repressed following successful transfer to a recipient strain. As a result, recipients that acquire the plasmid can be quantified and retrieved as green fluorescent cells by fluorescence-activated cell sorting (FACS). The donor strain was cultured overnight in LB medium supplemented with tetracycline, harvested by centrifugation and washed in 0.9% (w/v) NaCl solution. Cell density was adjusted to 3.0×10^7 cells per ml by direct measurement in a Thomas counting chamber, and used in filter mating assays as described previously [29]. Activated sludge (microbial biomass) grab samples were collected from a municipal wastewater treatment plant (WWTP) (Mølleåværket, Lyngby-Taarbæk, Denmark) in March 2018, then transported refrigerated to the laboratory and processed immediately. Bacteria were extracted from 50 ml activated sludge by washing (centrifuging at approx. $5000 \times g$ for 10 min, carefully removing supernatant and resuspending the pellet in 0.9% NaCl) and sonication (500 J for 4 min with alternating pulses and pauses of equal duration every second, tip diameter 3.0 mm, Branson 250). After 10 min settling, 5 ml supernatant was used for counting and adjusting to 3.0×10^7 cells per ml before filter mating assays.

Filter mating assay and serial growth experiment

As the activated sludge microbial community is a mixture of enteric and non-enteric bacteria with distinct growth condition preferences, we used two media in both filter mating and serial growth experiments to selectively enrich the two community fractions (Fig. S1). MacConkey (MC) medium and synthetic wastewater (SW) medium [30] were used to favour enteric and non-enteric bacteria, respectively. In addition, we set low (100 RPM) and high (500 RPM) stirring rates to vary the degree of oxygen transfer during the serial growth experiment. The high stirring rate was sufficient to ensure fully oxic conditions while the low stirring rate would result in oxygen-limited to anoxic

growth in the substrate rich medium (hereafter 500 and 100 RPM are referred to as oxic and anoxic conditions). Initial plasmid transfer in the activated sludge community was facilitated via filter mating assays. Donor and recipient suspensions of 3.0×10^7 cells per ml were mixed at 1:1 ratio (for matings on SW medium) or 1:5 ratio (for matings on MC medium, to compensate for the selectivity of MC media for enterobacteria), and immediately filtered onto a track-etched membrane (pore size: 0.2 μm, Whatman Cyclopore^{TM}, UK). Filters were placed on agar-solidified (15 g/l) SW or MC medium. After 48 h incubation at 25 °C, cells from triplicate filters were combined in 2 ml 0.9% NaCl solution and detached by vortexing at 3000 RPM for 3 min. Cell number was adjusted to 3.0×10^7 cells to initiate serial growth in 50 ml Erlenmeyer flasks containing 15 ml of appropriate media. The serial growth experiments were run in triplicate for 10 days under one out of four combinations of media type and stirring rate (i.e., SW100, SW500, MC100, and MC500), without antibiotic selection. Daily, 1% (v/v) of each culture was transferred to a new flask carrying fresh medium, ensuring average community-level growth of about six generations per day. Control experiments were run in parallel to assess the potential for plasmid transfer during the serial growth experiment. To this effect, donor and recipient cells were separately grown on filters (on either SW or MC medium) for 48 h at 25 °C. Then donor and recipient cells were mixed directly in flasks with liquid growth media; triplicate flasks were subjected to serial growth and treated exactly as the experimental groups under the four conditions.

Cell sorting and 16S rRNA gene amplicon sequencing

Throughout the serial growth experiment, the relative abundance of donor, recipient, and transconjugant cells were quantified daily by FACS (BD FACSAria III, USA) with the same gate settings as for sorting (see below). At selected time points during the serial growth experiment (day 2, 4, 6, 8, and 10), cell fractions were also subject to sorting and 16S rRNA gene amplicon sequencing. Transconjugant and recipient cells were sorted using FACS settings as described earlier [24]. In all sortings, we targeted a minimum of 10,000 cells. Samples with low transconjugant cell fractions (<0.1%) were not sorted, as those conditions would require excessive time to ensure purity. Sorted cells were concentrated by centrifugation, and subject to cell lysis and DNA extraction using GenePurgeDirect^{TM} DNA releasing agent (NimaGen, NL). The hypervariable regions V3–V4 of the 16S rRNA gene were PCR amplified using primer set 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) originally published by Yu et al. [31] and modified as described in

Sundberg et al. [32]. Tagging using loci-specific index primers for individual samples was done in a second PCR step. After purification using HighPrep PCR magnetic beads (MagBio, USA), sample-wise indexed PCR products were pooled in equimolar proportions and concentrated. Sequencing of pooled libraries was performed on a MiSeq platform using the v3 600 cycles sequencing kit in 300 bp paired-end mode (Illumina, USA). Based on index sequences, samples were directly demultiplexed by Casava 1.8 (Illumina, USA) on the MiSeq instrument to produce the paired-end FASTQ files for sequencing analysis.

Sequence data analysis

The DADA2 pipeline based on amplicon sequence variants (ASVs) was used for 16S rRNA gene amplicon sequence analysis [33]. Briefly, filtration and trimming ($maxN = 0$, $maxEE = 2$, $truncQ = 2$) were performed to clean raw sequences, which then went through error learning, de-replication, and merging. By discriminating biological sequences from sequencing errors, ASVs were then inferred. Taxonomy classification of chimera-free ASVs was done by searching against the SILVA non-redundant database (release 132) [34]. The generated sample-wise ASV abundance table was used for further analysis. Each ASV is hereafter referred to as a phylotype. Only ASVs with a relative abundance threshold above 0.1% were considered for downstream analysis to limit noise attributable to rare taxa. To better place the phylogeny of *Aeromonas* phylotypes, *Aeromonas* reference 16S rRNA gene sequences were retrieved from NCBI RefSeq database, and the V3–V4 region sequence was extracted using the same primer set (341F and 806R). The phangorn R-package [35] was used to construct a maximum-likelihood phylogenetic tree, using a neighbour-joining tree as initial tree. Results were visualised using the R-package ggplot2 [36]. All sequences obtained in this study were deposited in NCBI under accession number PRJNA515836.

Statistical analysis

Non-metric multidimensional scaling (NMDS) was carried out using Bray-Curtis distance matrices that were based on relative abundance of ASVs. Diversity indexes (Shannon, richness, and evenness) were calculated by the R-package Vegan [37]. Permutational multivariate analysis of variance (PERMANOVA) tests were conducted to evaluate the effect of the experimental treatments (e.g., oxygen level and medium type), since it is robust to deviation from parametric assumptions. Pearson correlation analysis was performed on individual phylotypes to investigate relationships between their absolute abundance and the total transconjugant density as estimated from FACS signals. Linear regression models were applied to estimate the temporal trends in the relative richness, relative fitness, and loss of unique phylotypes. Linear models were fitted because of the approximate linearity of most of data and because the limited number of data points made fitting more complex models irrelevant.

Identification of the fate of plasmid at the phylotype level

Flow cytometry allows one to quantify the density of three pools of community members and sort them: the transconjugants (cells with green fluorescence), the recipients (which we define here as all community members derived from the wastewater community, irrespective of whether or not they carry, or are permissive to, the pKJK5, i.e., cells with green fluorescence or without fluorescence), and the initial donors (cells with red fluorescence). The absolute abundance of each phylotype in the community was estimated by multiplying the cell densities of each pool by the relative abundance of the phylotype in the transconjugant or recipient pool based on the 16S rRNA gene amplicon sequencing. Phylotypes detected at least once in the transconjugant pool during any serial growth experiment were defined as permissive; phylotypes never detected in the transconjugant pools were defined as non-permissive.

The overall fitness of all permissive phylotypes relative to all recipient phylotypes was calculated by examining the temporal dynamics of the ratio of their absolute abundance (absolute abundance of all permissive phylotypes/absolute abundance of all recipient phylotypes). A positive or negative slope of this time-series ratio indicates an overall growth advantage or disadvantage of permissive phylotypes vs. recipient phylotypes during the experiment, respectively.

For each permissive phylotype, the temporal dynamics of the fraction of plasmid-bearing cells (transconjugants) is impacted by two processes (in the absence of its de novo formation by horizontal gene transfer): first, the positive or negative effect of the plasmid on the phylotype's fitness relative to plasmid-free cells of the same phylotype; second, the segregational loss of the plasmid. These two processes cannot be distinguished in our experiments. To quantify the combination of these two processes, the temporal dynamics of the ratio of the abundance of each transconjugant phylotype relative to its the total abundance as measured in the recipient pool (absolute abundance of phylotype i in transconjugant pool/absolute abundance of phylotype i in recipient pool) was examined. The slope of this ratio as function of time (hereafter referred to as $S_{\alpha+\delta}$) is the combined result of plasmid segregational loss and plasmid fitness effects. In order to minimise noise and identify true positive/negative $S_{\alpha+\delta}$, we only consider slopes biologically significant when they exceed an arbitrary cutoff of 0.01 in absolute value.
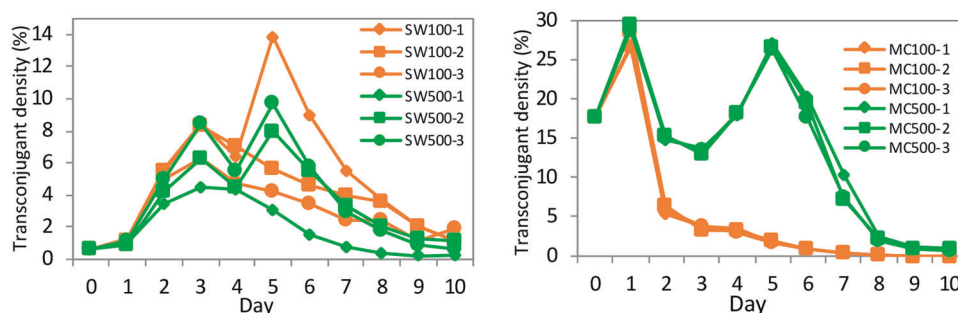
Fig. 1 Transconjugant density (%) detected by FACS in the initial filter mating samples (0 day) and in the daily samples through 10-day serial growth experiments (1–10 days). Transconjugant density in serial growth in SW and MC media are shown on the left and right panels, respectively. Transconjugants are cells only with green fluorescence. Three replicates are shown for each experimental condition.

Results

Plasmids persisted in microbial communities for more than 60 generations despite negligible horizontal transfer

We created a range of environmental conditions to study plasmid persistence in complex microbial communities. The composition of the recipient pools derived from activated sludge and propagated under different conditions became rapidly and significantly different (SW vs. MC media, oxic vs. anoxic) (PERMANOVA test on media and RPM effect, P value < 0.001) (Fig. S2). The effect of mixing on community composition was the strongest in the MC medium, probably due to the oxygen-sensitivity of enterobacteria, which was also evident in the enrichment of lactose fermenting *Enterobacteriaceae* under anoxic compared with oxic conditions.

The plasmid persisted across all examined environmental conditions, except in anoxic conditions in MC media (Fig. 1). During the first 3 days in the SW medium, the transconjugant density increased from <1% in the initial filter mating to 4–8% on the 3rd cultivation day. Subsequently, transconjugant densities decreased except for treatments SW100-1, SW500-2, and SW500-3, where a transient increase was observed (8–14%) before densities gradually decreased to low but stable levels (<1%) on 10th day. In the MC medium, transconjugant densities increased from 18% of initial filter mating up to 30% on the 1st day. The degree of oxygen transfer had a significant impact on transconjugant density in the MC medium, but not in the SW medium. The transconjugant densities decreased monotonically to undetectable in the anoxic (MC100) condition, while they increased transiently to 25% under oxic conditions (MC500). Because of the significant variability among replicates, under all experimental conditions, data were presented for all three replicate groups rather than the means.

During the serial growth experiments, the observed transconjugant cells must derive either from new plasmid transfer events from donors to recipient cells or from persistent transconjugant cells that were formed during the initial filter mating. We included a control experiment to measure the extent of plasmid transfer during the serial growth experiment. Donor cells decreased rapidly in both control and experimental groups (e.g., from >10% on 1st day to <0.1% on 10th day) (Figs. S3 and S4). Transconjugant cells were absent in almost all control groups, except for 0.1% transconjugant density in MC media on the 1st day (Fig. S5). Hence, horizontal transfer of pKJK5 during the serial growth experiment was negligible.

Persistent transconjugants spanned multiple phylotypes

While the recipient community diversity decreased through the experiment (e.g., Shannon index decreased from 4.0 to 2.0 in SW; from 3.0 to 1.7 in MC), substantial diversity remained by the end of the experiment (12–16 genera of 8–10 orders in SW; 7–14 genera of 5–6 orders in MC (Fig. 2)). Although the loss of recipient diversity restricted potential plasmid persistence to the remaining phylotypes, we did not observe significant correlation between recipient and transconjugant community structures by Procrustes analysis of NMDS ordination plots (P value > 0.1).

During the filter mating on SW medium, pKJK5 transferred to 29 genera (four phyla) (Fig. 2a). This diversity of transconjugants eroded gradually during serial growth in SW liquid medium (Shannon index decreased from 3.8 to 2.0). The overall composition of transconjugant pools under oxic and anoxic conditions became distinguishable (PERMANOVA, P value = 0.027). The dynamics of transconjugant density in the different serial growth experiments may mainly be driven by a few specific (abundant) phylotypes. We indeed identified multiple phylotypes with absolute abundance highly correlated with the transconjugant density profile during serial growth experiment (r > 0.9, Pearson correlation), e.g., phylotypes in *Citrobacter*
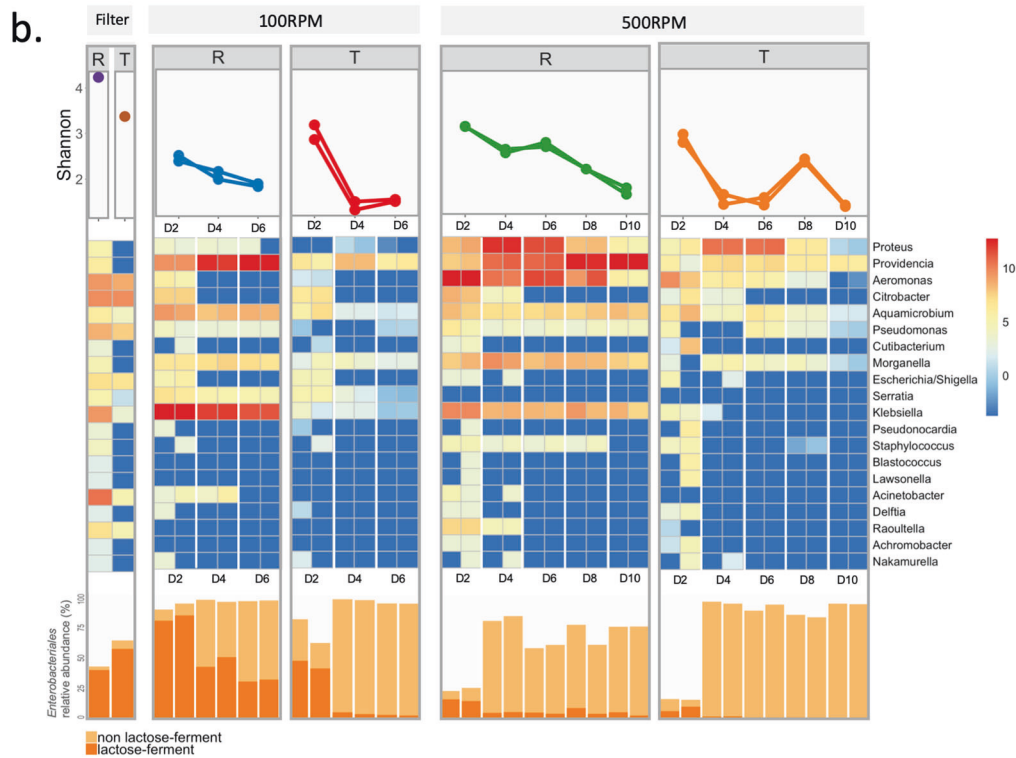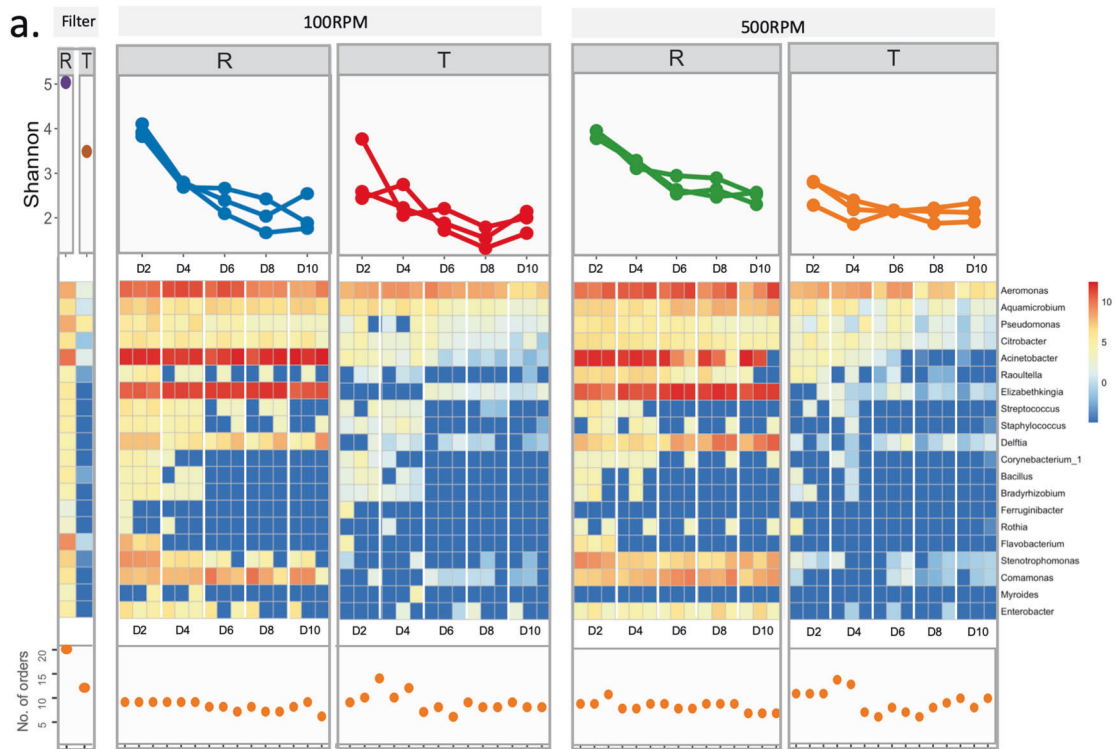
and *Enterobacter* genera in SW100-1, and in *Aeromonas* and *Stenotrophomonas* in SW500-2 (Fig. S6). After 10 days, 62 phylotypes in 9–14 genera were detected across the transconjugant pools. A core of 12 persistent phylotypes in five genera (*Aeromonas*, *Aquamicrobium*, *Citrobacter*, *Elizabethkingia*, and *Pseudomonas*) were shared among all

**Fig. 2 Diversity and absolute abundance of the dominant phylo-
types in samples from initial filter mating and serial growth
experiments.** The top and bottom panels present results from the
serial growth experiments in SW (a) and MC (b) media, respec-
tively. (R recipient pools; T transconjugant pools). Within each main
panel, the top subpanel show the Shannon diversity index; the middle
subpanel, the log2-transformed absolute abundance of the 20 most
abundant genera; and the bottom subpanel, the number of unique
orders in SW samples (**a**) and relative abundance of *Enterobacteriales*
(with and without lactose fermenting ability) in MC samples (**b**).
Sampling date is indicated as D2, D4, D6, D8, and D10 on *X*-axis.
Shannon indexes of replicates were plotted along *Y*-axis and grouped
by sampling date, otherwise each replicate data were plotted along *X*-
axis and grouped by sampling date. Scale bar on the right is log2-
transformed absolute abundance.

SW experiments (Fig. 3a). In each transconjugant pool,
Aeromonas phylotypes became enriched (relative abun-
dance > 50%) (Fig. S7). In total, 37 different *Aeromonas*
phylotypes were detected in the recipient pools grown in
SW, 12 of which persisted as transconjugants throughout at
least one serial growth experiment (Fig. S8). These *Aero-
monas* phylotypes spanned multiple species (Fig. S9),
where the dominant ASV6 and ASV1 are closely related to
the human opportunistic pathogens *A. hydrophila* and *A.
veronii* [38], respectively.

In MC medium, both transconjugant and recipient pools
differed significantly from those that developed in SW
medium during the 10-day experiment (Figs. 2b and S2). On
MC medium, pKJK5 was initially transferred to 18 genera
(two phyla) in filter matings, mainly consisting of genera
Citrobacter (48% relative abundance), *Aeromonas* (23%),
and *Pseudomonas* (12%). In the serial growth experiment,
transconjugant diversity continuously decreased, as indi-
cated by Shannon index decreasing from 3.0 to 1.0. Oxygen
conditions significantly affected the composition of trans-
conjugant pools (PERMANOVA, *P* value < 0.001). Several
phylotypes had abundances that were strongly correlated
($r > 0.9$, Pearson correlation) with the observed dynamic
profile of transconjugant density, such as *Proteus* and *Pro-
videncia* under oxic condition (Fig. S6). Transconjugants of
the *Enterobacteriaceae* family were more dominant under
anoxic than oxic condition. At the end of the experiment, 31
transconjugant phylotypes in 13 genera were detected, in
which 8 core phylotypes in *Enterobacteriales* were shared
across all treatments, including 3 *Morganella* phylotypes
and 5 *Providencia* phylotypes (Fig. 3b).

No general fitness deficit of permissive phylotypes relative to the rest of the community

We noted an average 70% decrease in the richness of per-
missive phylotypes during all serial growth experiments
(Fig. S10). In other words, (potential) plasmid hosts dis-
appeared from the community because of insufficient fitness

or ecological drift. For example, several *Acinetobacter* and
Pseudomonas phylotypes, abundant at the start of the serial
growth experiment in SW media, progressively and simul-
taneously became absent from both recipient and trans-
conjugant pools. This process would have limited plasmid
persistence if permissive phylotypes were lost faster than
the rest of the community. The only condition where this
might have been the case was MC100 (Fig. 4), although
three data points are insufficient to test whether that decline
is significant. MC100 was indeed the condition where the
plasmid failed to persist. In this case, strong selection
against some permissive phylotypes contributed to the dis-
appearance of transconjugants, and thus, to plasmid loss.
Elsewhere, the permissive phylotypes appeared to decline
equally or less rapidly than the rest of the community, with
a similar effect of richness loss on recipient and transcon-
jugant pools within a community, and thus a neutral or
positive effect on plasmid persistence.

As removal from the community is an extreme outcome
of low fitness, we also compared average fitness of per-
missive and non-permissive phylotypes to look for an
overall fitness differential. Across all experimental condi-
tions, the average relative fitness of permissive phylotypes
was similar or higher compared with the rest of the com-
munity (no significantly negative slopes in Fig. S11). Thus,
we did not detect selection against the permissive fraction of
the community.

Fitness benefit associated with plasmid carriage improved persistence in multiple phylotypes

The presence of the same phylotype in the plasmid-free and
plasmid-carrying fractions of the community gave us the
opportunity to assess their relative dynamics and explore the
cumulated effect (denoted as $S_{\alpha+\delta}$) of plasmid segregational
loss and plasmid-induced fitness cost/benefit at the phylo-
type level at high throughput (Fig. 5). A positive value of
$S_{\alpha+\delta}$ indicates a net fitness benefit of plasmid carriage
since the contribution of plasmid segregation can only be
neutral or negative. In contrast, a zero to negative value of
$S_{\alpha+\delta}$ makes it difficult to assess the strength and direction of
the plasmid fitness effect because plasmid fitness cost/
benefit and plasmid segregational loss are indistinguishable
in this study. For example, $S_{\alpha+\delta} = 0$ can be caused by
either (segregational loss (negative) + fitness benefit (posi-
tive)) or (segregational stability (neutral) + no fitness effect
(neutral)).

Across different experimental conditions, we identified
diverse $S_{\alpha+\delta}$ profiles with both positive and negative values
among transconjugant phylotypes (hereafter a phylotype
under one specific experimental condition is indicated as
ASV_{expt}, e.g., $ASV1_{SW100-1}$) (Fig. 5a, b). In SW media,
more than half of the ASV_{expt}s were without any negative

Fig. 3 Persistent genera or phylotypes at the end of the serial growth experiment. Given the higher diversity observed in SW compared with MC, genus level results are presented for SW (a) and phylotype-level results for MC (b). Venn diagrams show the shared and unique genera/phylotypes among groups. Phylogenetic trees show all persistent genera/phylotypes. Class names are used as label for SW, while genus names in *Enterobacteriales* order are used for MC.
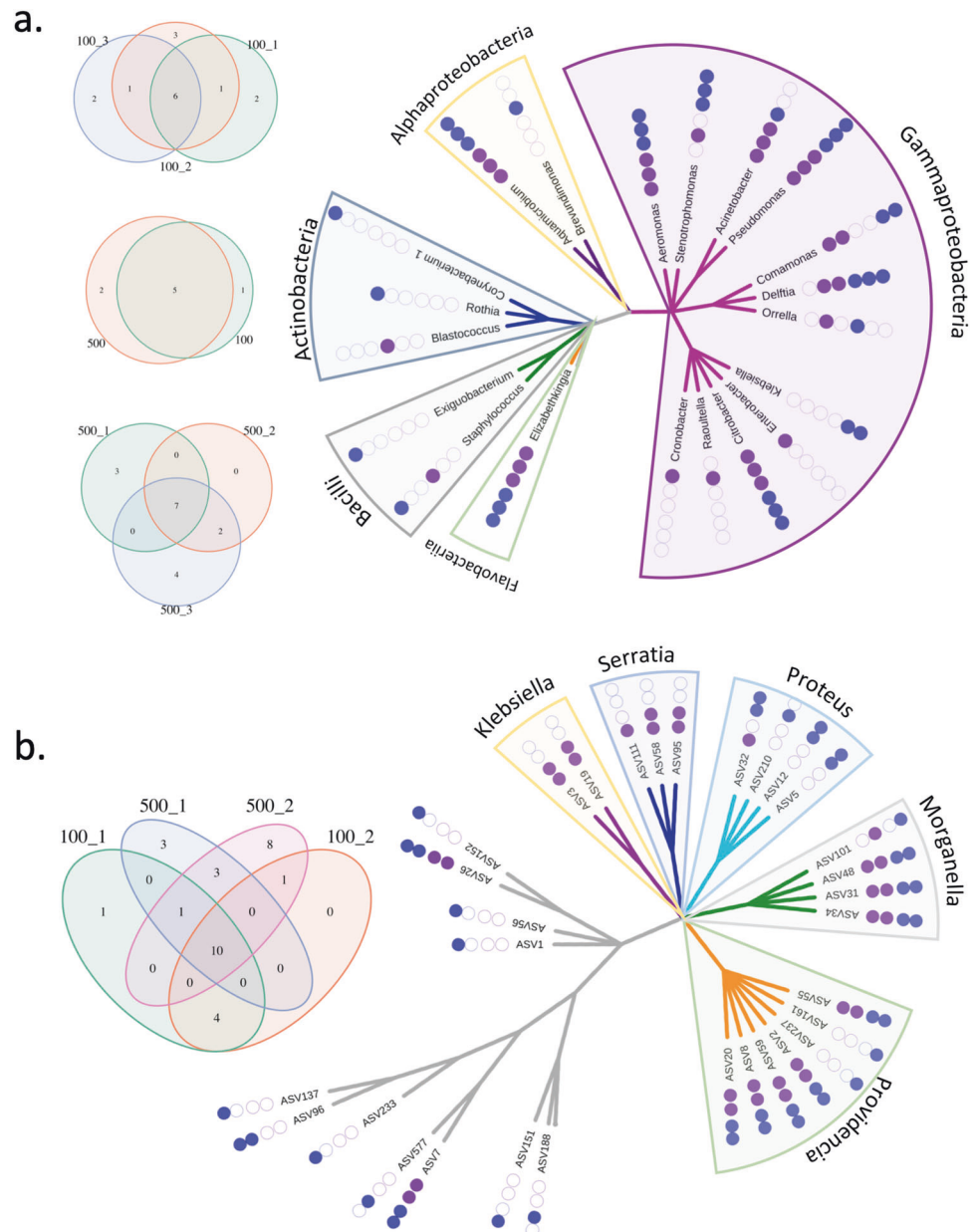
plasmid effect (79 out of 132 ASV_{expt}s with $S_{\alpha+\delta} \geq 0$, of which 25 with $S_{\alpha+\delta} > 0$). Especially, within the genus *Aeromonas*, there was no negative plasmid effect for 40 of 67 ASV_{expt}s ($S_{\alpha+\delta} \geq 0$), with obvious fitness benefit for 20 ASV_{expt}s ($S_{\alpha+\delta} > 0$). In particular, three *Aeromonas* phylotypes (ASV9, ASV13, and ASV42) showed fitness benefits in the majority of the serial growth experiments, whereas some phylotypes of the same genus, such as ASV1 and ASV17, experienced plasmid fitness cost. Variability of plasmid effects within a genus was also observed in *Pseudomonas*, where the incidence of the plasmid in population ASV26 often decreased, in contrast to persistence in ASV70 and ASV81 populations. In other genera like *Delftia* and *Elizabethkingia*, there were a few phylotypes with neutral or

even positive plasmid effects across experiments. In SW media, the plasmid had a similar effect on phylotypes irrespective of oxygen transfer conditions. However, in MC media, a neutral or positive plasmid effect was more often detected under oxic than anoxic conditions, e.g., 72% (22 out of 32) and 28% (6 out of 21) of ASV_{expt}s with $S_{\alpha+\delta} \geq 0$ under oxic and anoxic conditions, respectively. For example, the three phylotypes of *Morganella* were always associated with a positive plasmid effect under oxic condition but were more likely to suffer from fitness cost or plasmid loss under anoxic condition. Within the same genus, we observed phylotype-level variation of plasmid effects in *Aeromonas* (e.g., ASV15 vs ASV6) and *Providencia* (e.g., ASV2 vs ASV20). We identified 28 out of 53
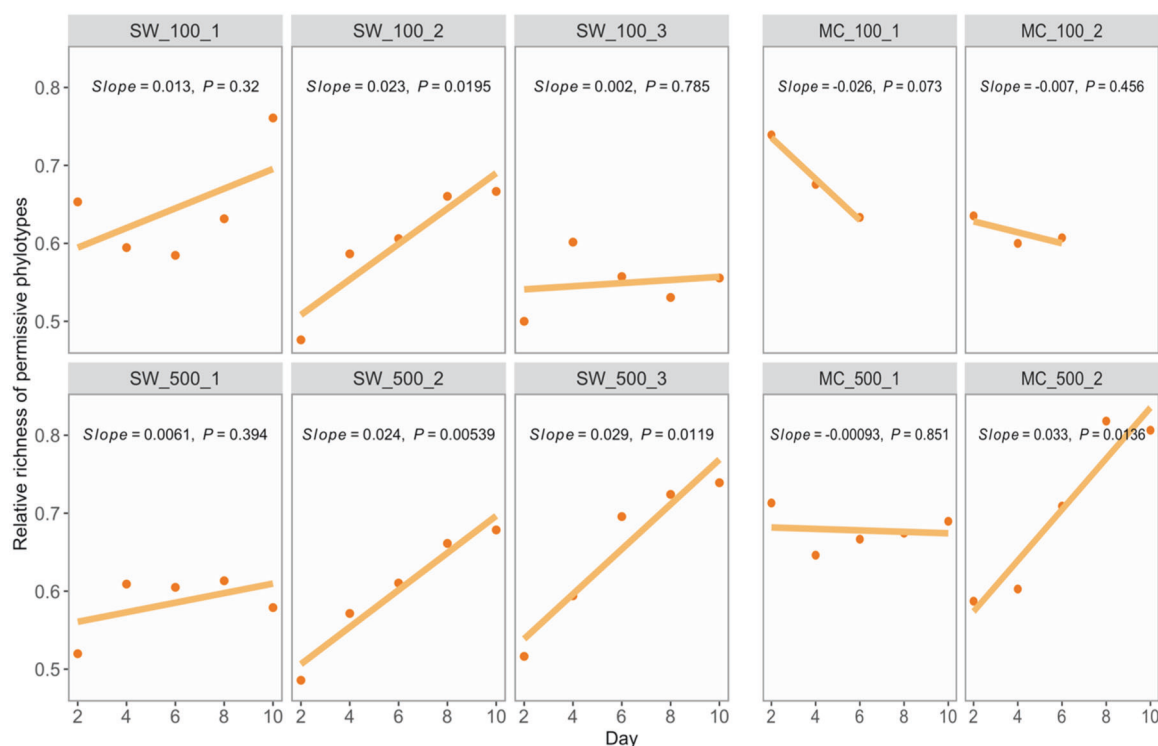
Fig. 4 Richness of permissive phylotypes relative to all recipient phylotypes over time. A linear regression model was applied to estimate the temporal trend of the relative richness. Linear model was fitted because of the approximate linearity of most of data and because the limited number of data points made fitting more complex models irrelevant. Solid lines represent regression lines labelled with slope and P value. Positive or negative slope indicated slower or faster richness loss trends in permissive phylotypes compared with recipient phylotypes.

ASV_{expt}s with neutral (20 with $S_{\alpha+\delta} = 0$) or positive (8 with $S_{\alpha+\delta} > 0$) plasmid effects in MC media. Interestingly, *Pseudomonas* ASV26 and *Aquamicrobium* ASV7 presented a consistent response to plasmid carriage (respectively positive and negative) on the two growth media, suggesting limited impact of the experimental conditions on plasmid maintenance in the two phylotypes. Overall, across experimental conditions in both SW and MC media, more than half (107 out of 185) of the examined ASV_{expt}s maintained the plasmid without any negative plasmid effect, and several (33 out of 185) experienced fitness benefits (Fig. 5c).

Discussion

We examined, for the first time, a complex environmental community to quantitatively monitor persistence of an IncP-1 plasmid pKJK5 among diverse taxa in serial growth experiments. Without any antibiotic pressure to select for transconjugants, we focused on the potential of the community to maintain the plasmid under different nutrient and oxygen conditions. We could discount the contribution of new transfer events to the plasmid fate, as supported by the negligible transfer of pKJK5 in the control experiments and

consistent with the previously reported low transfer of pKJK5 in liquid conditions [16]. Indeed, plasmids (IncP/N/W) with genes encoding short and rigid pili only transfer efficiently on solid surfaces, unlike those with long and flexible pili (IncF/H/T/J), capable of transferring equally well in liquid and on solid surfaces [39, 40].

We experimentally demonstrate community-level plasmid persistence across different environmental conditions in the absence of intentional selection for plasmid carriage and in the absence of significant de novo transfer. In SW and MC media, time-series profiles of transconjugant density and composition became significantly different. The role of the environment in shaping transconjugant composition became obvious, especially under oxic and anoxic conditions in MC media. In fact, earlier observations have consistently identified the importance of the environment in determining plasmid fate, by either setting up microcosms under different oxygen conditions (e.g., aerobic and anaerobic sludge reactors) [4] or manipulating other environmental factors (e.g., spatial structure or presence/absence of antibiotics) [5].

By examining plasmid effects temporally across taxa in complex microbial communities, we dissected processes contributing to plasmid persistence within a community. We observed that a plasmid fitness benefit in multiple phylotypes contributed strongly to plasmid persistence at
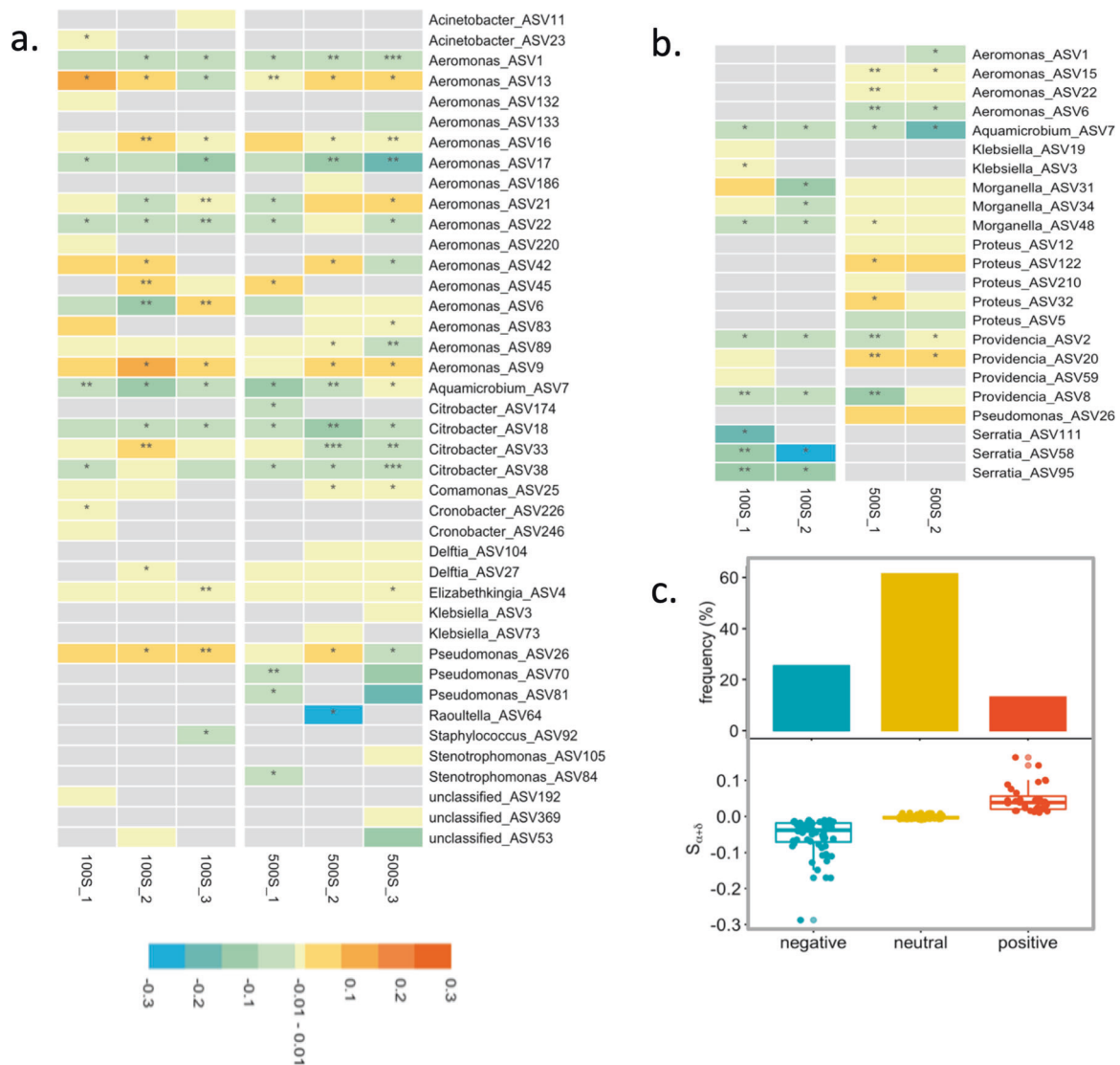
Fig. 5 Plasmid effect of permissive phylotypes during the serial growth experiments. $S_{\alpha+\delta}$ value profile in SW media (**a**), MC media (**b**), and all experimental groups (**c**). $S_{\alpha+\delta}$ is an estimate of the combined plasmid fitness and segregation effects. $-0.01 \le S_{\alpha+\delta} \le 0.01$ or $S_{\alpha+\delta} < -0.01$: neutral or negative plasmid effect; $S_{\alpha+\delta} > 0.01$: positive plasmid effect. P values of slope: *<0.5; **<0.1; ***<0.01. Occurrence frequency of three $S_{\alpha+\delta}$ groups with negative, neutral, and positive values in **a** and **b** are summarised in **c**.

the community level. We expected plasmid pKJK5 to be a neutral or even detrimental extra-chromosomal element for the fitness of its host, as it does not encode apparent beneficial phenotypes under the applied conditions. It is possible that during the experimental period, co-adaptation between plasmid and hosts decreased their negative interactions, thus, improving plasmid maintenance in specific phylogenetic groups. Although such co-adaptation—mainly through plasmid fitness cost amelioration—is frequently observed in population-level studies over evolutionary time scale (>500 generations), it can also appear as early as within 50 generations, such as the rapidly improved stability of IncP-1 plasmid pBP136 in *E. coli* under non-selective conditions [41]. In experiments on plasmid-host compensatory coevolution, genomic changes frequently emerge

within 100 generations [20, 42]. Therefore, early genomic adaptations might have occurred improving persistence within the time scale of the experiment. These adaptions might ameliorate fitness cost of plasmids, and even confer fitness advantages to the host [13]. Indeed, naturally occurring plasmids can confer a fitness advantage upon their hosts, even if the genetic basis of this fitness advantage is not clear [43]. It is also possible that the microbial community we used as potential recipients might already have been subjected to compensatory adaptation for hosting IncP-1 plasmids. Genomic and experimental studies have demonstrated a high prevalence of IncP-1 plasmids spanning all subgroups (IncP-1 α, β, γ, δ, and ε) in WWTP systems [44–46]. If this were the case, community members with prior experience of genetic interaction with IncP-1

plasmids might favour carrying cognate plasmids like pKJK5. While this could explain why pKJK5 might have caused minimal burden to some phylotypes, it does not explain why it would provide a fitness benefit. It is possible that experimental conditions might bias the observed pattern of plasmid effects. However, we observed frequent plasmid fitness benefits with two media and with two mixing conditions. Further experiments with specific knockout mutants of target plasmid genes will likely be necessary to provide a molecular explanation.

The wide range of $S_{\alpha+\delta}$ values across phylotypes and conditions confirmed that the plasmid effect is a phylotype and context dependent feature. In fact, population-level studies have repeatedly revealed that plasmid effects are indeed determined by strain-specific factors [47, 48]. We identify here, for the first time, phylotype-level-specific plasmid effects within a complex microbial community. Although the underlying mechanisms are not yet clear, the heterogenous plasmid effect observed in this study might be the overall result of phylotype-level-specific compensatory adaptations, protective mechanisms, and/or regulatory networks. For example, as revealed in plasmid-host interactions in clinical isolates of *E. coli* and *K. pneumoniae* [20], differential adaptive evolutions among strains and species modify the plasmid effect on hosts in various ways.

Across the experimental conditions, plasmid persistence was especially observed in several phylotypes in the *Aeromonas*, *Proteus*, *Providencia* and *Pseudomonas* genera. In particular, several phylotypes of *Aeromonas* experienced fitness benefits from pKJK5 carriage under different experimental conditions. These genera have often been reported as carriers of antibiotic resistance plasmids in either environmental or clinical settings, such as plasmid-mediated carbapenem- and colistin-resistance in *Proteus* and *Providencia* isolates. Recent studies have demonstrated the high potential of *Aeromonas* for carrying multidrug-resistance plasmids [49–51], and transferring plasmids in various environmental communities [24–26]. Given the ubiquitous presence of *Aeromonas* in aquatic environments [52, 53], *Aeromonas* might play a significant role in facilitating transfer and maintenance of plasmid-mediated ARGs in environmental communities.

We demonstrated that complex microbial communities can maintain plasmids under non-selective conditions, and phylotype-level plasmid fitness benefits significantly facilitate community-level persistence. To further understand the role of the community in modulating plasmid behaviour, the distinct mechanisms of plasmid-host interactions in communities compared with populations remain to be elucidated.

Compliance with ethical standards

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