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Dissertation

Modelling the spread of plasmid-encoded antibiotic resistance in aquatic environments considering evolutionary modifications, individual heterogeneity and complex biotic interactions

by

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Abstract

Plasmids providing antibiotic resistance to their host bacteria pose a major threat to society, as antibiotics are often the only way to treat infectious diseases. Here the existence conditions of plasmids are investigated in an ecological framework with mathematical methods such as ordinary differential equations and individual-based models. It is shown how (i) the arise of different kinds of compensatory mutation, (ii) intra- and intercellular interactions of plasmids representing opposing plasmid lifestyles as well as (iii) a diverse plasmid community affect plasmid dynamics, community composition and persistence. The results indicate that evolutionary modifications and interactions between plasmids broaden the existence conditions of plasmids in a way that has not been recognized before, but explains their occurrence in nature. This includes that biotic interactions could maintain costly plasmid-encoded antibiotic resistance despite the absence of abiotic selection. These findings open a way to study remaining research questions related to the complexity of natural environments.

Zusammenfassung

Plasmide, die Antibiotikaresistenzen an ihre Wirtsbakterien vermitteln, stellen eine große Bedrohung für die Gesellschaft dar, weil Antibiotika oft die einzige Möglichkeit sind Infektionskrankheiten zu behandeln. In dieser Arbeit werden die Existenzbedingungen von Plasmiden aus einer ökologischen Perspektive mit mathematischen Methoden wie gewöhnlichen Differentialgleichungen und Individuen-basierten Modellen untersucht. Es wird gezeigt, wie (i) das Aufkommen verschiedener Kosten-kompensierender Mutationen, (ii) intra- und interzelluläre Wechselwirkungen von Plasmiden, die gegensätzliche Plasmidlebensstile repräsentieren, sowie (iii) eine vielfältige Plasmidgemeinschaft einen Einfluss auf die Dynamik, Gemeinschaftszusammensetzung und Persistenz von Plasmiden ausüben. Die Ergebnisse deuten darauf hin, dass evolutionäre Modifikationen und Wechselwirkungen zwischen Plasmiden die Existenzbedingungen von Plasmiden in einer Weise erweitern, die bisher nicht erkannt wurde, aber ihr Auftreten in der Natur erklärt. Dazu gehört auch, dass biotische Wechselwirkungen trotz fehlender abiotischer Selektion eine kostspielige Plasmid-vermittelte Antibiotikaresistenz aufrechterhalten könnten. Die Erkentnisse dieser Arbeit können dazu genutzt werden verbleibende Forschungsfragen anzugehen, die im Zusammenhang mit der Komplexität der natürlichen Umwelt stehen.

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Nomenclature

µIBE	microbial individual-based ecology; refered to the combined study of individual microbes by experimentation and individual-based modeling
antibiotics	used as a common synonym for antibiotic drugs in the whole manuscript
EPS	extracellular polymeric substances; secreted by microorganisms to establish and maintain the structural and fucntional integrity of biofilms
HGT	horizontal gene transfer; sometimes also called 'lateral gene transfer'
IBM	inidividual based model(s); equivalent to agent-based model(s) - ABM
MBC	minimum bactericidal concentration; lowest concentration of a bactericidal antibiotic required to kill a bacterium; complementary to MIC
MGE	mobile genetic elements; genetic material able to move within a genome or to be transfered between cells; e.g. transposons, plasmids, bacteriphage
MIC	minimum inhibitory concentration; here referred to the lowest conenctration of an antibiotic, which prevents visible bacterial growth
ODE	ordinary differential equation(s)
PDE	partial differential equation(s)
PLM	population-level model(s)
VGT	vertical gene transfer; transfer of genetic information within a cellular lineage, e.g. by plasmids that are transfered to the daughter cells during bacterial fission
WWTP	wastewater treatment plant

1 Introduction

Antibiotic resistances spread globally throughout bacterial populations and environments (Martínez et al., 2015). They belong to the greatest threats to human health and society (Blair et al., 2015). Since the usage of antibiotics is often the only method to treat infectious diseases, it is crucial for our future ability to combat infections to study the environmental dissemination of resistances against these antibiotics (Allen et al., 2010).

Some bacteria are intrinsically resistant to antibiotics due to structural or functional characteristics (Blair et al., 2015). Other are able to develop or acquire resistance mechanisms in consequence of mutation or horizontal gene transfer (HGT) (Martinez, 2008). The latter refers, for example, to the spread of mobile genetic elements (MGE) such as plasmids through cell-to-cell contact of bacteria. Other MGE and potential sources of antibotic resistance are bacteriophages, genomic islands, insertion sequence elements, transposons and integrons (Dobrindt et al., 2004). Among MGE, conjugative plasmids are the most significant for HGT (Thomas and Nielsen, 2005; Harrison and Brockhurst, 2012) and, at least in the *Gammaproteobacteria* (Garcillán-Barcia et al., 2011), considered to be the most important for the spread of antibiotic resistances.

De novo resistance to antibiotics are induced due to complex interactions of cellular processes involving adaptation of expression levels and mutations (Händel et al., 2014). This includes for example efflux-pump activations enabling the survival of low levels of antibiotics as well as subsequent mechanisms allowing resistance to higher concentrations. The latter are a consequence of a promotion of HGT-stimulating mutations by the SOS response or a result of a stress-induced modulation of genetic instability (Händel et al., 2014).

Blair et al. (2015) recently summarised molecular mechanisms of developed and acquired resistances. These include a prevention of the access of the drug to its target due to a decreased permeability or increased efflux. Furthermore structural changes of the antibiotic target are able to circumvent an efficient binding of the antibiotic. This is induced by point mutations of genes enconding for the antibiotic target, which will be rapidly selected under continuing antibiotic pressure. If the antibiotic target gets protected by a modification, such mutational changes are not necessary. In addition to these preventing mechanisms antibiotics can be directly modified or destroyed by hydrolysis or transfer of a chemical group.

Bacteria which do not harbour any or simply not the appropriate resistance have to suffer in the presence of antibiotics due to the antibiotic action, which comprises bactericidal and bacteriostatic effects (Finberg et al., 2004). The effect of an antibacterial agent can differ between species or strains of the same species. Bacteriostatic drugs only inhibit bacterial growth and treated bacteria may resume their growth if the antibacterial agent gets diluted. Bactericidal drugs are able to kill the bacteria and cause cell lysis, but they may only inhibit bacterial growth as well, if they not reach the minimum bactericidal concentration (MBC). The superiority of bactericidal drugs compared to bacteriostatic drugs appears obvious, since they are able to eliminate bacteria rapidly and decrease the potential development of resistances or infection recurrences, but their usage can be undesirable in certain clinical settings (Finberg et al., 2004). Thus the antibiotic impact depends on the general effect of a drug to a target bacterium and on the antibiotic concentration or its dynamics.

Environmental factors associated with stress for a bacterial population are able to promote HGT (Beaber et al., 2004). Antibiotics used for therapeutical treatment of human infections, in agriculture and animal or fish farming pollute the environment and act as a selective pressure for resistances, potentially increasing the risk of transfer of resistance genes to human pathogens (Martinez, 2008; Allen et al., 2010). For example it has been observed that high background concentrations of quinolones in a river enriches the population of waterborne bacteria carrying plasmid-encoded quinolone resistances (Cattoir et al., 2008). Additionally, heavy metal pollution is considered to be a selective force for antibiotic resistances (Seiler and Berendonk, 2012). It is also suggested that natural environments in general serve as a reactor for the evolution and propagation of antibiotic resistances (Martinez, 2008; Allen et al., 2010; Berendonk et al., 2015). Especially the mixture of environmental species carrying intrinsic resistance mechanisms with allochthonous species in the freshwater may play a major role (Lupo et al., 2012). It should be further noted that some antibiotics serve as signaling molecules for bacteria and thus are kept at a low background concentration, which could favor the selection of antibiotic resistances (Martinez, 2008).

Martínez et al. (2015) suggest that the transfer of resistances from their original (natural) hosts to human pathogens is restricted by stringent bottlenecks. Ecological connectivity is considered to be important, since transfer events require a contact between recipients and donors. If recipients are under positive selection in one habitat, only the connection to another habitat with different conditions is able to maintain genetic exchange. Experimental evolution highlighted the importance of migration between such environments with heterogeneous positive selection for the maintenance of a plasmid carrying a mercury-resistence (Harrison et al., 2018). Martínez et al. (2015) further assumed that the chance that a new gene is established in a population is reduced, when other genes with a similar substrate profile are already stably spread within this population (founder effect). Additionally the physiological-epigenetic background of a resistance determinant, that means its fitness cost and ability to be balanced by compensatory adaptations regulate if it is likely to be spread in a bacterial community. Hall et al. (2015) observed that the fitness effect of plasmids was highly variable and depended, among others, on the plasmid status (of competing hosts) and the degree of abiotic selection of the plasmid-encoded resistance. They also observed a high diversity within the plasmid community and suggested that this is strongly influenced by environmental heterogeneity.

Martínez et al. (2007) proposed some guidelines that were drawn up to predict the risk of emergence of resistances to antibiotics which will become clinically introduced. A prediction of the dissemination of antibiotic resistances should take into account evolutionary constraints, selection pressures and environmental variations. To assess the probability that resistant bacteria become established these authors recommend to develop mathematical models which investigate the population dynamics of resistant and sensitive bacterial populations in the absence and presence of antibiotics. Furthermore they propose to measure the propagation of resistances under different growth phases, anitbiotic-inducing conditions and in a biofilm context.

In the following subsections the characteristics of aquatic environments and processes influencing the propagation of plasmid-encoded antibiotic resistances will be reviewed. The last subsection will provide a short introduction into different modelling techniques and give some examples of their usage in the context of the spread of antibiotic resistances. Finally, the objectives and content of the dissertation will be summarised.

1.1 Current knowledge and methodological approaches

1.1.1 Aquatic environments

Biofilm and plankton

Microbial processes and all kind of ecological interactions greatly depend on the features of the respective environment. In general, bacteria living in aquatic environments are either dispersed in a planktonic environment or embedded in a biofilm context, which can be attached to a kind of ground surface or to another kind of substrate that itself might be dispersed in the free water column, e.g. an activated sludge floc of a water treatment reactor (Ofiţeru et al., 2014). Both systems are schematically illustrated and compared in Fig. 1.1. Whereas bacteria are mixed in plankton and all cells could theoretically encounter each other, the overall cell density is usually reduced due to a lower nutrient availability or a high impact of dilution or washout that is likely given, for example, in a river stream. Opposed to this, bacteria in a biofilm predominantly interact with their local neighbours, which can enhance the possibilities and the efficiency of horizontal gene transfer. At the interface of both, bacteria might either attach from the plankton to the biofilm or otherwise be dispersed from the biofilm to the plankton, which can also be modulated by signal factors (Papenfort and Bassler, 2016). Colonising planktonic cells likely remain in the same location until they either die or re-enter the planktonic phase (Cook et al., 2011).

It has been reported that bacteria carrying conjugative plasmids promote the formation of loose cell aggregates rather than biofilms on a solid surface (Røder et al., 2013). Biofilms were also sometimes described as non-growing, or slowly growing (Cook et al., 2011), which seems to be likely considering the high cell density and potential huge distance to the nutrient supplying interface, which is either the surface the biofilm is attached to or the fluid flowing over and through the biofilm matrix. In general, the differences in growth, structure, behaviour, and physiology are reported to impact the susceptibility to antimicrobials (Sedgley and Dunny, 2015). This is especially addressed to the role of the EPS matrix that can represent a physical barrier to antimicrobial agents as well as to horizontal gene transfer (HGT), which is facilitated in such a matrix and can provide resistance to the population, e.g. by persister cells that survived a previous antibiotic treatment (Sedgley and Dunny, 2015). Additionally, a higher concentration of antibiotics might be required to achieve bactericidal action on biofilm-bacteria compared to planktonic bacteria, because of biofilm impermeability features (Rodríguez-Martínez and Pascual, 2006).

Cook and Dunny (2014) suggested that plasmid carriage is highly heterogeneous in biofilms and their role much more complex than in homogeneous cultures. It has also been hypothesised that HGT in a biofilm context may play a key role in microbial sociality, because many accessory traits of the plasmid are expressed outside of the cell (Harrison and Brockhurst, 2012). Many clinical pathogens possessing an intrinsically low susceptibility to antibiotics have a large genome, which enables them not only to colonise diverse environmental habitats, but also to modify, utilise and resist to antibiotics (Martinez, 2008).

Artificial and natural conditions

Despite differences between these ecological settings, there are also differences between artificial and natural aquatic environments regarding their pollution with antibiotics, which might be either increased due to the direct input into the sewage system and waste water treatment plant or rest at a low natural background concentration in river systems.

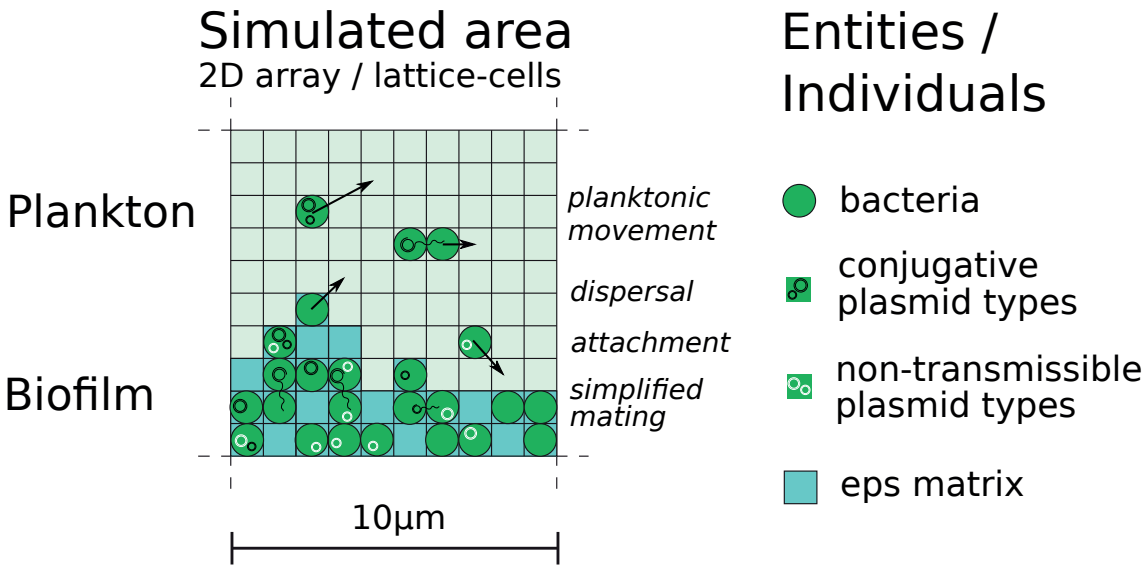

Figure 1.1: Exemplary model abstraction of an aquatic environment. Bacteria may be dispersed to plankton or attach to the biofilm, which embeds bacteria in a self-produced matrix of extracellular polymeric substances (EPS). Bacteria may carry different plasmid types that spread only with them (through movement and cell fission), referred to non-transmissible plasmid types, or even autonomously (through bacterial conjugation), referred to conjugative plasmid types (see Fig. 1.3 and 1.2 for more details). Plasmids may vary in their size and cost for the host cell, whereas a single cell can also harbour multiple plasmid types simultaneously.

Nonetheless, antibiotic resistance genes have been found in any type of aquatic environment, even in drinking water (Zhang et al., 2009). In the danube river, one resistant isolate could be detected for each tested antibiotic, which also opens the possibility that these can spread to related human pathogens (Kittinger et al., 2016).

Of course, there are also differences between laboratory and natural conditions. Conventional laboratory cultivation techniques as batch and continuous-culture fail to appropriately reproduce the natural conditions of organisms (Kovárová-Kovar and Egli, 1998). Whereas closed batch cultures impose a drastic change of the environmental conditions during the experiment, open continuous-cultures fix these conditions, but may neglect a number of microbial kinetic phenomena (Kovárová-Kovar and Egli, 1998). Studies that focus on the dissemination of antibiotic resistance in environmental matrices remain relatively scarce if not inexistent (Rizzo et al., 2013). If environmental matrices are maintained in a laboratory context, the microbial communities may behave and evolve differently than in their natural equivalent (Rizzo et al., 2013). Nonetheless, recent methodological advances (Cairns et al., 2018a; Lambrecht et al., 2018) focus on such issues, whereas, for example, flow cytometry has been demonstrated to be applicable to characterise even single cells of a bacterial community (Liu et al., 2018).

1.1.2 Plasmid propagation

The conditions and processes affecting plasmid persistence and occurrence are manifold, including many properties inherent to the plasmid types themselves. This section aims to provide an overview and ecological framework for the study of plasmid dynamics with mathematical methods, which will be summarised in the next section 1.1.3.

Horizontal Gene Transfer

Since the pioneering work of Lederberg and Tatum (1946) it became more and more evident that bacterial cells are able to share genetic information. The acquisition of mobile genetic elements (MGE) by an agent independently of reproduction events in a single evolutionary step is called lateral or horizontal gene transfer (HGT) (Rankin et al., 2011; Romanchuk et al., 2014). This ability to accept and express genetic material from sources external to the cellular lineage broadens the genetic pool to the whole microbial community, instead of a restriction to the parent genome (Skippington and Ragan, 2011). Thus, HGT is considered to be one of the most important evolutionary forces wihtin microbial populations (Baltrus, 2013). A variety of molecular mechanisms for the movement of MGE exist, including conjugation, transformation and transduction (Garcillán-Barcia et al., 2011). Among them only transformation, as the uptake of free DNA, is under the control of bacteria. While conjugation depends on cell-to-cell contact, which is often mediated by conjugative plasmids, transfer of DNA by transduction depends on phage vectors (Harrison and Brockhurst, 2012). For the dissemination of antibiotic resistance genes plasmids are the prefered platform (Garcillán-Barcia et al., 2011).

Plasmid types

The plasmid genome has a modular structure, which means that specific functions are arranged into discrete operons (Harrison and Brockhurst, 2012). Besides some core 'backbone' genes, encoding key functions such as replication, segregation and conjugation, there can be a part of 'accessory' genes, encoding additional functions probably beneficial for the bacterial host (Harrison and Brockhurst, 2012), e.g. antibiotic resistances. In modular descriptions accessory traits are associated with the adaptive or cargo module of the plasmid. In contrast to the backbone module this segment varies more quickly in response to selective pressures (Garcillán-Barcia et al., 2011).

Whereas autonomous replication is the only common function of all plasmid types (Harrison and Brockhurst, 2012), further capabilities provided by core functions can vary remarkably. One of the most important differences is their ability to perform horizontal gene transfer. Plasmid types that encode a set of mobility genes (MOB) as well as a complex enabling mating pair formation (MPF) are called 'conjugative' (Smillie et al., 2010). They can spread from one bacterium to another through a mating channel, provided by a type 4 secretion system (T4SS). The scheme provided in Fig. 1.2 illustrates this process: the bacterium that carries the conjugative plasmid expresses one or more pili. These are extended to capture another bacterium and, if successful, retracted in order to minimize cell distances. This process might take some minutes, as it was shown by live cell imaging by Clarke et al. (2008).

Plasmid types are called 'mobilizable', if they encode the mobility genes, but only hitchhike with through the MPF system of other co-occurring conjugative plasmids performing conjugation (Smillie et al., 2010). This can also occur in the opposite direction, i.e. from the receiving to the donoring cell of the conjugative plasmid, called 'retro-transfer' (Haagensen et al., 2002).

Plasmid types that are neither conjugative nor mobilizable are called 'nonmobilizable' (Smillie et al., 2010). They could also be horizontally transferred by natural transformation or transduction, which refers to the uptake of DNA from the environment or the dissemination by phages (Harrison and Brockhurst, 2012), but the rates of these processes are considered so low that most authors describe this type of plasmid mobility as 'non-transmissible' (San Millan et al., 2014b; Garcillán-Barcia et al., 2011). Therefore,

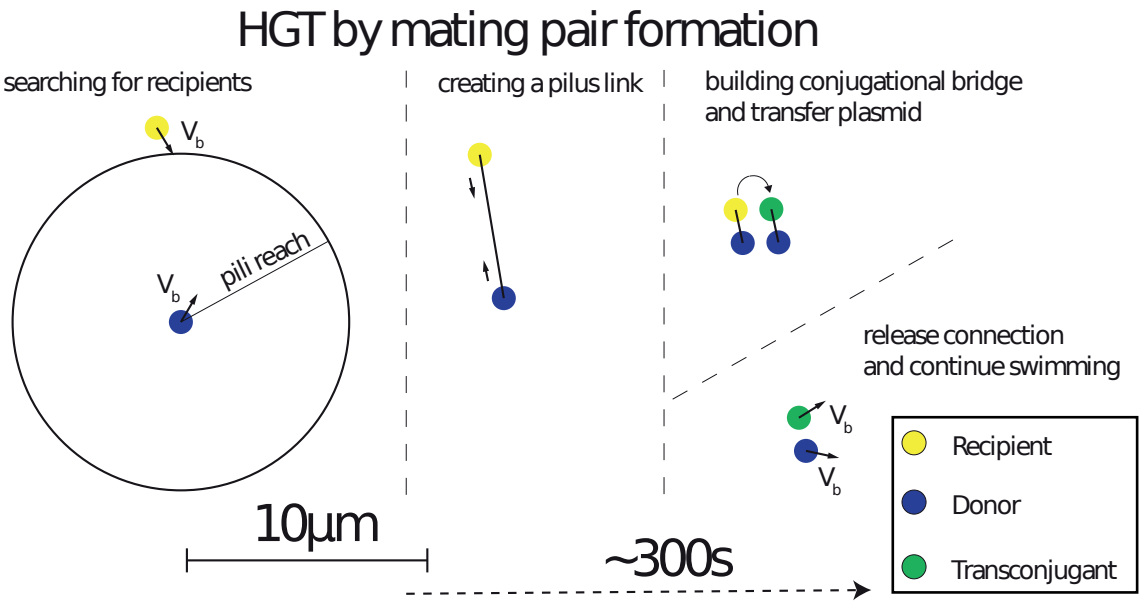

Figure 1.2: Scheme of horizontal gene transfer by mating pair formation of bacteria in a planktonic environment: a bacterium that harbors a conjugative plasmid, called 'donor', couples through it's pilus to another bacterium that will receive the plasmid, called 'recipient'. When a copy of the plasmid was successfully transfered, the recipient finally becomes a new donor, called transconjugant.

non-transmissible plasmids are restricted to spread by cell fission, i.e. vertical gene transfer (VGT). Smillie et al. (2010) and Garcillán-Barcia et al. (2011) found that globally a fourth of the plasmid types are conjugative, one fourth mobilizable and a half non-transmissible.

Plasmid fitness

Within this study, we consider fitness as a measure of ecological success representing the ability to leave offspring relative to others (Kassen et al., 2004), whereas the fitness of plasmids has two dimensions (Summers, 1996): vertical transmission fitness, i.e. spread to the daughter cells of the host by binary fission, and horizontal transmission fitness, i.e. spread to new host cells, for example, by conjugation (Fig. 1.3).

Fox et al. (2008) mentioned that it is not clear how plasmids that provide no apparent benefit persist in a bacterial community, but they summarised some factors that are known to affect plasmid persistence. According to this, the persistence potential of a specific plasmid type might be increased if it is able to replicate in multiple hosts and has a less severe impact on the relative growth rate of the plasmid-bearing hosts in comparison to its plasmid-free counterpart. For example, Seoane et al. (2010) observed that pWW0 plasmid caused a reduced yield and specific growth rate of the host. They reported that plasmids can reduce maximum growth rates by up to 40% compared to the plasmid-free state. In addition, plasmids benefit if they secure their own propagation during cell fission. This can be achieved in different ways: (i) high copy numbers can reduce the rates of mis-segregation, i.e. that a daughter cell receives no plasmid copy, (ii) an active-partitioning system can control successful segregation of the plasmid copies, (iii) post-segregational killing by a toxin-antitoxin system that causes the death of daughter cells that do not receive a plasmid copy (Harrison and Brockhurst, 2012). The latter might prevent that plasmid-bearing cells get outcompeted in the absence of positive selection for plasmid borne traits (Turner et al.,

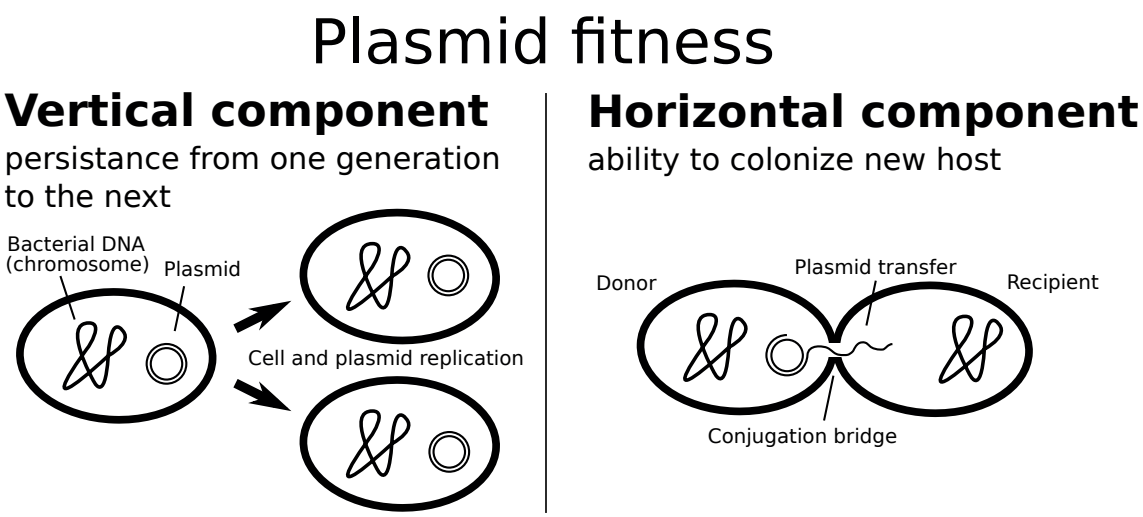

Figure 1.3: The dimensions of plasmid fitness. Vertical transmission fitness (left), mediated by cell fission, and horizontal transmission fitness (right), mediated, for example, by conjugation.

2014). However, this is not the case when one considers that a population consists of a number of bacteria that have never received the plasmid that performs post-segregational killing.

The average number of replicates in a cell is differs between plasmid types. Conjugative plasmids have in general a low copy number and mobilizable plasmids a high copy number (Garcillán-Barcia et al., 2011). This seems to be reasonable, since the bigger size of conjugative plasmids induces higher costs for DNA replication and repair. Moreover the production of plasmid proteins, e.g. for conjugation, uses up raw material and occupies the cellular machinery (Harrison and Brockhurst, 2012). San Millan et al. (2014b) proposed to use a combination of the plasmid copy number and the degree of multimerization, that means the number of division into smaller parts, as a proxy for plasmid stability. Thus, a daughter cell receives one complete set of a plasmid according to a certain probability. If the plasmid does not encode for an active partitioning system and if we assume complete mixing every piece has a chance of 50 percent to belong to one of the resulting daughter cells. In the case of only 3 monomers the chance for segregational loss therefore is $0.5^3 = 0.125$ and is consequently reduced with decreasing plasmid copy numbers and an increasing degree of multimerization. Nonetheless, some studies reported that segregational loss never occurred (Dahlberg and Chao, 2003).

An increased segregation rate of a plasmid can also be the result of incompatibility, which describes the failure of two coresident plasmids to be stably inherited in the absence of external selection, because they share one or more elements of the plasmid replication or partitioning system (Novick, 1987). Thus, related plasmids belong to the same incompatibility group, which means that the introduction of one of both destabilises the first. Incompatibility can also affect horizontal transmission, since cells can actively prevent the transfer of incoming plasmids, if they determine that it belongs to the same incompatibility group as an already present plasmid type (Humbert et al., 2019). This is done by the recognition of proteins involved in conjugation that are specific to the respective incompatibility groups.

In addition to this, recent experimental studies also demonstrate that the co-occurrence of plasmids affects the fitness of a single plasmid. On the one hand plasmid survival can

be promoted by positive epistasis (San Millan et al., 2014a), which refers to decreased costs of coresiding plasmids in comparison to a cost that might be expected when their single costs are summed up. On the other hand, epistasis can also be negative or neutral. Besides effects on the vertical transmission fitness, it has also been reported that transfer rates are often decreased, when two (Gama et al., 2017a) or three (Gama et al., 2017b) distinct plasmids interact. Similarly, interference can also be given between plasmids and chromosomal mutations (Silva et al., 2011).

Adaptation

The spectrum of actions bacteria are able to perform under suitable conditions is restricted by the genetic information they have access to. In addition to the stable core genome of around 2000 genes for a single *E. coli* cell (assessed for 20 *E. coli* strains) (Touchon et al., 2009), bacteria gain and loss genes which are highly mobile between bacterial cells (Rankin et al., 2011). These non-core genes increase the diversity of a species pan-genome and can make up 90% of it (Touchon et al., 2009). Hence a bacterial population has a fundamental basis to adapt to various environmental requirements. But these shifts on population level are subjected to adaptations of individual cells. The part of a bacterial genome that encodes for additional traits that are only sometimes beneficial, but do not represent essential cellular functions, is called the 'flexible' gene pool (Dobrindt et al., 2004).

As each action demands energy the genetic information does, since it has to be stored, repaired and replicated (Harrison and Brockhurst, 2012). Therefore, the amount of genetic information is limited for a single bacterium, depending on the respective species strategy and individual needs. Indeed, the genome structure reflects the bacterial lifestyle (Dobrindt et al., 2004). Stoebel et al. (2008) demonstrated that the expression of genes and not only the synthesised products are costly. They argue that this provides an explanation for the evolution of repression systems, which can circumvent that only the environment determines if this function is costly or beneficial. Thus, expression of functions such as antibiotic resistance is costly, even if no antibiotics are present. But if no antibiotics are present, a transcriptional regulatory network might allow bacteria to repress this function in this case.

It has been mentioned that the 'excess baggage' hypothesis, which assumes that the costs of carriage of additional foreign DNA reduces the fitness of their hosts and thus prevents their unintended spread, should not be accepted uncritically (Bouma and Lenski, 1988). This notion is based upon observations of adaptations compensating for the initial costs of additional foreign DNA.

Mechanisms of adaptation

If ecological conditions become harsh bacteria may adapt through alterations of their genome structure. This happens by the selection of point mutations and genetic rearrangements as well as by gene acquisition through HGT or by genome reduction (Dobrindt et al., 2004). It results rather in modifications of the 'flexible' gene pool, which comprises variable chromosomal regions as well as mobile and accessory genetic elements, than in alterations of the conserved 'core' genome, encoding essential cellular functions (Dobrindt et al., 2004). The latter mostly emerge through recombination and mutation (Touchon et al., 2009).

One of the most active platforms capturing antibiotic resistance genes are integrons (Mazel, 2006), which are inserted into the plasmid backbone (Garcillán-Barcia et al., 2011). It was observed that integron integrases were upregulated during conjugative transfer, which

increased rearrengements of the gene cassette (Baharoglu et al., 2010). Moreover, the same authors suggest that conjugation triggers chromosomal rearrangements and transcriptional switches of the recipient cell, even when gene transfer was aborted. Their results indicate an enhanced gene rearrangement of bacteria through conjugation induced SOS, which allows them to evolve and survive in new environments.

Sources of adaptation

Elements of the mobile gene pool can be integrated into the 'flexible' gene pool and may provide advantageous traits to the host, potentially increasing its fitness. Those bacteria possessing highest compatibility and performance in interaction with these mobile genetic elements (MGE) will presumably be most competitive (Martínez et al., 2015). If compatibility is low or the carriage of the MGE in its ancestral form is to expensive compared to its advantages, adaptation has to occur. Otherwise the integrated MGE, the bacterium or both will get lost, at least in the long-term. This becomes evident to a greater extend if the conditions for the selection of the trait are fluctuating or become obsolete and the carriage of these genes represents a competitive burden for the bacteria. In the study of San Millan et al. (2014b) both a reduced cost and periods of positive selection were necessary to maintain the integrated MGE, a non-transmissible plasmid. Environmental conditions, starvation, stress and high copy numbers of a gene increase the probability for genetic mutations, which could be important in the evolution of antibiotic resistances (Martínez et al., 2007). Such mutations may alter the biological fitness of resistant strains to a level similar or even higher than that of the susceptible parental strain.

The mobile gene pool consists of various kinds of elements, including bacteriophages, plasmids, genomic islands, insertion sequence elements, transposons and integrons (Dobrindt et al., 2004). Among these mobile genetic elements (MGE), conjugative plasmids are the most significant for HGT (Thomas and Nielsen, 2005; Harrison and Brockhurst, 2012). For the spread of antibiotic resistances they are considered to be the most important MGE, at least in the *Gammaproteobacteria* (Garcillán-Barcia et al., 2011).

Plasmid-host relationships

Plasmids usally have a circular structure and exist as self-replicating extrachromosomal replicons in cells (Dobrindt et al., 2004). As mobile genetic elements which can become a part of the flexible gene pool of bacteria they directly influence the fitness of their host. Hence, bacteria are able to adapt on their own, but depend under some circumstances on plasmid related impacts on their fitness. This connection is of fundamental relevance for the ecology and evolution of both, bacteria and plasmids. For bacteria it becomes even more important if we consider plasmids as autonomous agents, whose fitness interests do not have to overlap with those of their hosts (Rankin et al., 2011; Harrison and Brockhurst, 2012; Turner et al., 2014). This finding refers to the circumstance that plasmids control their own replication and transmission and are therefore able to adapt too.

A plasmids relation to bacteria can range from a mutualistic to a parasitic form (Harrison and Brockhurst, 2012). Carriage of beneficial genes may increase bacterial fitness, whereas some plasmids show parasitic behaviour, e.g. when rapid conjugation slows down growth of infected bacteria and the accompanied vertical plasmid transmission (Turner et al., 2014). According to different strategy needs, the plasmid genome varies in its extend and composition. For example mobilizable plasmids carry and express no transfer genes, are not able to spread horizontally on their own as conjugative plasmids, but are therefore less in size and energy demands.

If a population is confined to conditions without a positive selection for accessory plasmid genes, plasmids might lose this non-beneficial genes. On the other hand consistent positive selection of these accessory genes could lead to their integration into the bacterial chromosome. Instead, plasmid vectors and their accessory elements are maintained. This circumstance, that means the existence of beneficial conjugative plasmids and their persistence under various ecological conditions, has been called the 'plasmid paradox' (Harrison and Brockhurst, 2012).

Mechanisms of compensatory adaptation

Several studies have attempted to examine the extend of evolutionary adaptation of bacteria-plasmid associations (Modi et al., 1991; Levin, 1993; Dahlberg and Chao, 2003; Dionisio et al., 2005; Heuer et al., 2007; Turner et al., 2014). Only in one of the studies summarized by Harrison and Brockhurst (2012) the plasmid was lost after more than 700 generations in co-culture without the selection of plasmid-borne traits. In seven of the mentioned studies the plasmids cost-of-carriage decreased during experimental evolution independent of a selection of plasmid-borne traits. A within-host competition of coinfecting plasmids resulted in evolved plasmids with an increased cost-of-carriage, which were highly virulent in the ancestral background. In four of five host-plasmid co-cultures this was addressed due to adaptations of both, plasmids and bacteria. The reasons for this amelioration of the costs-of-carriage are addressed to three 'key-mechanisms', which could broaden the conditions favoring plasmid persistance (Harrison and Brockhurst, 2012). These include changes in conjugation rate, plasmid gene expression or the loss of plasmid genes.

Changes in conjugation rate can range from a complete loss of the ability to conjugate towards the evolution of lower or higher rates (Dahlberg and Chao, 2003), whereas the imposed costs to the host positively correlate with the extent of the conjugation rate (Turner et al., 2014). Hence, a shift towards a lower rate enables a higher vertical transmission rate, which indicates a closer alignment of plasmid and bacterial fitness interests. In a study comparing plasmids with and without fertility inhibition systems, Haft et al. (2009) concluded that competition favours a decreased horizontal mobility of plasmids.

A downregulation of plasmid genes in general could ameliorate the plasmids costs of carriage, since the expression and not only the carriage of genes causes costs, which potentially decrease growth (Scott et al., 2010). Heuer et al. (2007) reported an improved stability of plasmids evolved after 1000 generations of frequent host switching due to a downregulation of plasmid genes involved in conjugation and stability.

Dahlberg and Chao (2003) reported deletions of plasmid-borne antibiotic resistance genes in plasmid-containing populations during experimental evolution. Also Gelder et al. (2004) observed an increase in the fraction of antibiotic-sensitive, but plasmid-carrying mutants over 500 generations from 0.1 to 7%, caused by a deletion of the *tet* operon. As Dahlberg and Chao (2003) summarized, this leads to an increased fitness of the host, but means that there is still the potential for selection of antibiotic-sensitive populations as their advantage in the presence of antibiotics is lost. Of greater importance is their finding of plasmid-encoded compensatory adaptations which remain the antibiotic resistance genes. As these adaptations are not only confined to one population as adaptations of the bacterial chromosome, their potential to invade new populations by horizontal gene transfer may be enhanced. This was further demonstrated by Dionisio et al. (2005), which found that plasmid adaptations can provide a general fitness improvement, even in alternative hosts. This enhances the potential for plasmid spread and maintenance dramatically, even when it was reported to be very low for a specific plasmid-host-combination.

Besides these 'key-mechanims', Modi and Adams (1991) observed an amelioration

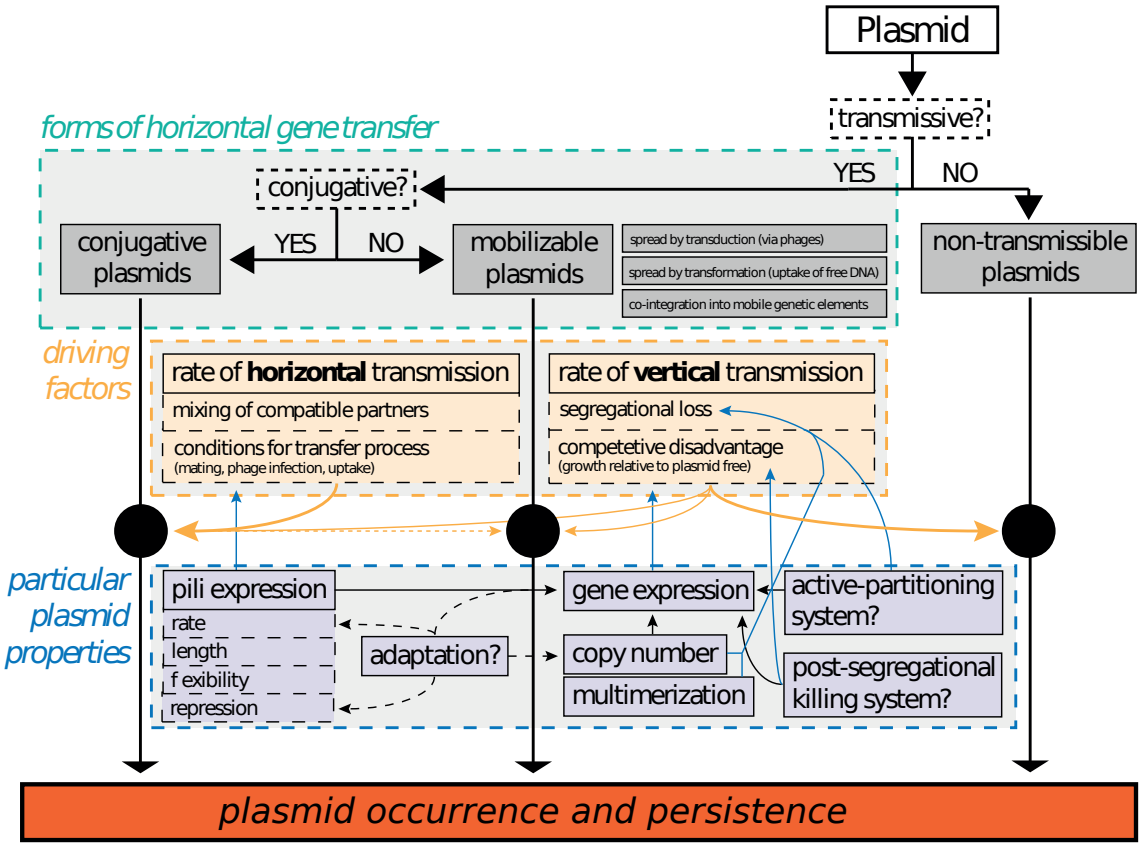

Figure 1.4: Plasmid properties and external factors affecting plasmid occurrence and persistence.

associated with a decrease in plasmid copy number and an increase in segregation rate resulting from a pairwise host-plasmid co-culture. Since plasmid replication is regulated by gene expression, this mechanism is partly similar to the key-mechanism of plasmid gene expression. However, the changes in plasmid copy number represent more profound alterations than a downregulation of the genes associated for plasmid replication itself. Therefore this mechanism can not be classified as one of the 'key-mechanisms' mentioned by Harrison and Brockhurst (2012), although it seems to provide a powerful strategy.

In summary, various factors influence the way how different types of plasmid mobility affect plasmid propagation and persistence (Fig. 1.4).

Ecological setting

It was found that regular switching of the plasmid host is able to increase the amelioration of the plasmids cost-of-carriage compared to plasmid evolution in a single host species (Heuer et al., 2007). Similar findings have also been reported from soil microcosm experiments (Hall et al., 2016), suggesting an increased potential for amelioration of plasmid costs in more complex (natural) environments compared to laboratory conditions, which may raise general concerns about their validity.

Opposed to this, some studies report specific adaptations of the plasmid and its host (Harrison and Brockhurst, 2012), occurring when the evolved plasmid imposes a greater burden on its ancestral host than the ancestral plasmid (Modi et al., 1991). This specificity means that there is a decreased potential of horizontal gene transfer or at least of its speed, which could be of crucial importance in some ecological settings.

Although not specific on plasmid loads, Amos et al. (2015) reported significant geospatial and temporal variation of resistance loads, with a strong correlation to the number, proximity, size and type of the surrounding wastewater treatment plants (WWTP). Nguyen et al. (2014) showed that the level of antibiotic resistant bacteria that are excreted by animals to the environment depends on the applied drug concentration and the duration of the treatment, whereas reductions of the treatment from 5 to 3 days decreased the total amount of excreted resistant enterobacteria by 75% and a ten times lower drug concentration achieved a reduction by 57%. Thus, besides biotic control, the abiotic environment affects resistance loads and may also influence plasmid propagation.

Combining a stochastic model with a statistical analysis, Joyce et al. (2005) predicted that antibiotic resistances will persist for years after an antibiotic treatment, even in the complete absence of antibiotics afterwards. Hall et al. (2018) found that natural communities might even not adapt to abiotic conditions, because such fitness benefits can be negated considering competitive species interactions in natural communities. Similarly, Cairns et al. (2018b) showed that a plasmid-dependend bacteriophage can eliminate a conjugative plasmid providing antibiotic resistance, but this can be prevented by a protozoan predator. It suggests that multitrophic interactions play a significant role, although many experimental setups do not take this into account.

1.1.3 Modelling approaches

Population-level vs. Individual-based models

Among the tools of modern research in environmental and microbial science are simulation models. Existing modelling approaches are differently suited to address certain research questions. A fundamental difference is at what level these models describe interactions. On the one hand there are population-level models (PLM) that can describe the interactions between species. Ordinary differential equations (ODE) are often used to describe how a compartment of the model (e.g. one of the interacting species) changes over time. This is based on the principle of mass action, similar to chemical reactions. Thus, the degree of interaction or outcome of some process depends on the 'concentration', 'proportion' or 'density' of the directly involved compartments. For example, horizontal gene transfer depends on the density of both plasmid-bearing and plasmid-free cells and a conjugation rate that determines how often they successfully encounter each other. If one imagines that the yellow compartment in Fig. 1.5 represents the density of plasmid-free cells and the blue compartment the density of plasmid-bearing cells, such an interaction might increase the area of the green compartment to the detriment of the yellow, considering that recipients become transconjugants as depicted in Fig. 1.2. The composition of the population of bacteria that live in a certain environment therefore changes. Such changes can also be resource-dependend, for example, by an explicit consideration that some nutrients are consumed, which control bacterial growth through some Monod-kinetic. A resource dependency can also be described by a logistic growth function that implicitly considers a decreased resource availability when population density increases. As an illustrative example, the black box surrounding the coloured compartments in Fig. 1.5 might be seen as the maximal cell density. Bacterial growth is the greater the more open (non-colored) space is left in this box. If it is full, as given, the density of bacteria cannot increase, only their proportions can change. Apart from that, some bacteria may die off over time, which means there is always some space left to fill by an increase of the compartments. The cell density of the total population or the single compartments could be theoretically infinitely small or large. Any change that can be observed at the population level arises due to the

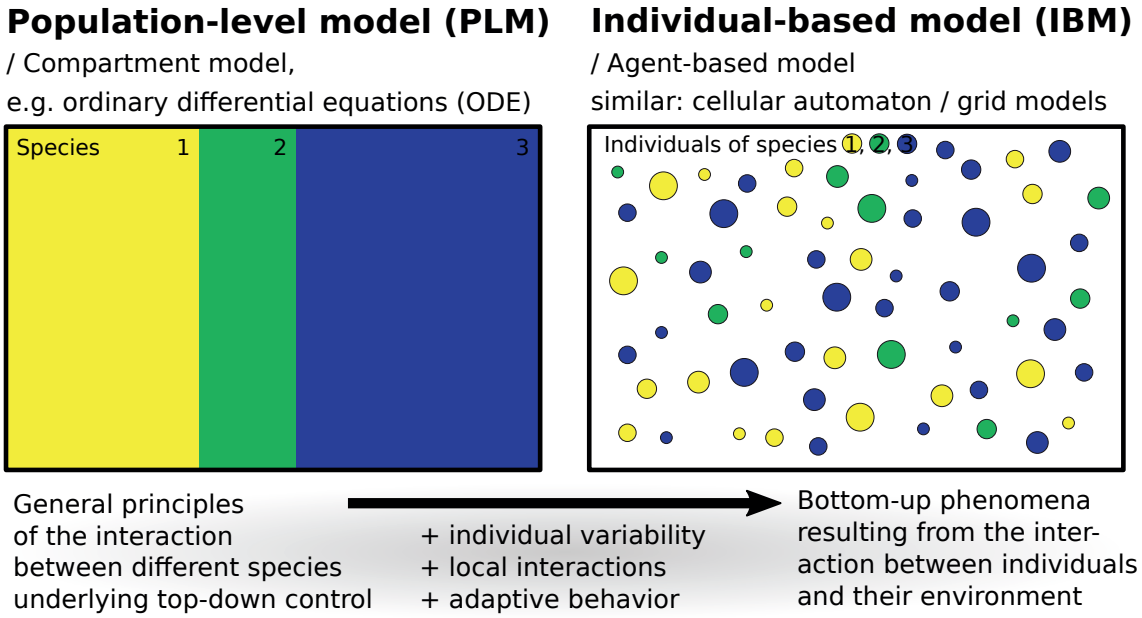

Figure 1.5: Conceptual differences between population-level models and individual-based models.

definitions that are directly described at the population level. This represents a 'top-down' approach, since the observed behaviour is a direct result of the model formulations.

Individual-based models (IBM) represent a fundamentally different approach. They often consists of (1) some individuals (or 'agents') that interact with the world around them and/or with each other and (2) the world in which the individuals 'live' or move around. Often some rules define what every individual is allowed or has to do (Grimm et al., 2006). These rules are repeatedly applied in form of a loop, which allows to repeatedly act or interact.

IBM represent a 'bottom-up' approach, since the changes that are observed at the population level emerge from the activities of lower-level entities. For example, instead of describing the growth of an entire bacterial population, it is considered how individual bacteria take up resources, grow and perform cell fission. This also leads to population growth, but population growth as such is not part of the model formulation. Instead, such characteristics of the population emerge from the behaviour of the individuals, which is determined by some rules. This behaviour can also take into account individual heterogeneity, e.g. that individual bacteria, even those of the same species (which would belong to the same compartment), have different growth rates or perform cell division at different cell sizes. This may also reflect differences in their life cycle that themselves may be important for other processes, such as the ability to perform horizontal gene transfer (see Seoane et al., 2011).

An IBM might also consider that individuals adapt their behavior in order to understand and predict ecosystem complexities (Railsback, 2001). Bacteria can, for example, change their movement direction according to some chemical signal (Taktikos et al., 2013). Thus, bacteria might direct their movement towards higher nutrient availability. Another example are bacteria that carry plasmid-bearing cells, but only shut off the repression of *tra* genes (means they become transfer-competent), when they sense that the conditions to transfer the plasmid are optimal (Koraimann and Wagner, 2014). One of the first real IBM for microbial biofilms is 'BacSim' (Kreft et al., 1998), which assumes, for example, that bacteria perceive local densities and shove each other away, allowing them to remain competitive

for the uptake of local resources.

Although it is not a prerequisite for IBM, they can be spatially explicit. This can be advantageous for a variety of processes that represent local interactions in nature, e.g. conjugation. An individual plasmid-bearing bacterium might therefore be able to perform a transfer attempt only to bacteria in its local neighbourhood (see Fig. 1.2 and Fig. 1.1). Otherwise, it could also be sufficiently detailed to consider that individual bacteria encounter each other randomly in a mixed environment and perform conjugation with a certain probability. Besides conjugation, processes such as consumption of nutrients can be considered to occur local, which means that bacteria directly compete for resources in the local neighbourhood. Such differences in space can also be taken into account by spatially-explicit PLM, i.e. partial differential equations (PDE). This opens the possibility to compare different kind of models in order to reveal the effect of spatial structure (comparing ODE with PDE) or the effect of individual heterogeneity and adaptive behavior (comparing ODE with non-spatial IBM or PDE with spatial IBM), which is best practice (Hellweger et al., 2016).

Good modelling practice and limitations

New laboratory methods that enable to observe and measure the behavior of individual microbes also stimulates individual-based modelling (Kreft et al., 2013), since data on the individual-level is required to parameterize IBM and to compare simulated individual-level characteristics with empirical data on the same scale (Hellweger et al., 2016). Various techniques extract and process information from individual microbes (Leveau et al., 2018). Cell tracking, for example, enables to analyse cell trajectories that can be used to generate a distribution of the turning angle and the swimming speed (before and after a turning event) of bacteria (Theves et al., 2013). Single cell analysis has been used to estimate the probability of successful conjugation in dependence to the donor-recipient orientation as well as distributions for the time required to transfer the plasmid and the delay time between transfer events depending on the individual cells history (Seoane et al., 2011). The application of scanning confocal laser microscopy (SCLM) of fluorescent bacteria revealed the dynamics of the spatial distribution of transconjugants in a flow chamber biofilm (Haagensen et al., 2002). New workflows for the cytometric analysis of pure cultures and complex communities in clear and challenging matrices have been proposed (Lambrecht et al., 2018). Such techniques can sort thousands of cells in a second (Müller and Nebe-von Caron, 2010). This can, for example, be used to determine relative abundances of subcommunities, reveal cell cycle dynamics and to monitor the evolution of microbial communities (Lambrecht et al., 2018). Liu et al. (2018) applied community flow cytometry to characterize single cells of a bacterial community with a high temporal resolution in order to investigate stability properties of complex microbial ecosystems. This opens various opportunities for the field of microbial individual-based ecology (μIBE), which refers to the study of microbial ecology by the combination of such experimental data with individual-based models (Kreft et al., 2013).

A variety of limitations of IBM should be considered (Hellweger et al., 2016). For example, IBM might be used to study the sources of variability between replicates of the same treatment in controlled laboratory experiments, as e.g. Harrison et al. (2015) demonstrated. But when it comes to rare events such as an infinitesimal small probability that a specific mutations is acquired, IBM might be on their limit, since they can usually only simulate rather small populations (in a microbiological context), but the probability that any of them will acquire such a mutation within a certain time depends on the population size. In such a case PLM might be advantageous, since they consider populations that are not

limited in size and can estimate the mean point in time when such a sweeping mutation will take effect.

Apart from the fact that a simulation of too large numbers of individuals is not feasible, computationally expensive models make sensitivity analysis and model fitting more cumbersome (Hellweger et al., 2016). Two methods can overcome this (Hellweger et al., 2016): (1) a simulation of one or several statistically representative volume elements of the larger system in full detail, whereas its size depends on the spatial variation of the features of interest; (2) a simulation of super-individuals that represent a group of similar individuals. In large populations, stochastic differential equation models may better describe heterogeneity. Besides limitations in modelling large-scale systems, IBMs can become too complex to analyse mathematically, understand and communicate (Grimm et al., 2005). As otherwise too simple models might not be representative for natural systems, an intermediate model complexity is supposed to be optimal (Grimm et al., 2005).

Good modelling practice in IBM is to adopt the 'ODD' (overview, design concepts, and details) protocol for the description of IBMs (Grimm et al., 2010), to check structural realism through the application of a pattern oriented modelling approach, which refers to a comparison of multiple simulated patterns with empirical patterns at varying scales, i.e. involving individual- and population-level characteristics (Grimm et al., 2005; Hellweger et al., 2016). The structural realism of models should also be examined by model robustness tests. These involve parameter sensitivity analysis that identifies important parameters, which, if achievable, should therefore be carefully estimated with empirical methods. Other robustness tests focus on the model structure, referring to a sensitivity analysis in which processes are systematically included or omitted (Grimm et al., 2014; Grimm and Berger, 2016). Potential pitfalls of IBM are that individuals have global knowledge, processes that are important for the research question are ignored or behavior that is studied as an emergent property is imposed to the individuals (Hellweger et al., 2016). For example, a single donor cell should not be able to identify the last recipient in a population that is composed of thousands of completely mixed cells. Instead, a donor might only by chance select the 'right' recipient cell for a transfer attempt. Another negative example could be that a study aims to investigate how bacterial interactions lead to spatial clusters, which in reality are induced by a predetermined, fixed spatial distribution of some nutrients.

Example of an IBM

The model of Fox et al. (2008) showed that the invasiveness of plasmids is determined by spatial structure and nutrient levels. Their model represented a 2000×2000 2D- square lattice with 'top' and 'bottom' cells as a simplification for a 3D structure. Reproduction and conjugation were only allowed for cells at the bottom which have access to nutrients. Within their study, they performed some simulations of two scenarios: disturbed (frequent filter transfer changes 'top' or 'bottom' alignment) vs. undisturbed simulations. Each lattice point represented a microcluster of up to $M = 40$ cells with 1 to 2 μm cell size and distances in order of micrometers, resulting in a simulation window of one to several millimetres per side. They considered recipients (R), donors (D) and transconjugants (T) with constant maximum growth rates for each cell type, but a common constant conjugation rate (for D and T). The effective growth rate was assumed to decreases with a reduction in vacant local sites. A local 3×3 neighborhood determined the location for daughter cells and conjugation events. At each model time step, a random focal site was selected and updated according to the probability of certain events. Finally, a comparison of the simulated with the experimentally observed cell counts for varying settings differing in the concentration of nutrients and the disturbance regime revealed the above mentioned

effect. This IBM is just an example that represents a rather strong model abstraction as, for example, compared to the IBM iDynoMiCS, presented in the study by Lardon et al. (2011), further extended and analysed by Merkey et al. (2011), which also demonstrated a growth dependency of conjugation.

1.2 Objectives and content of the dissertation

The main objective of the dissertation is to identify mechanisms and conditions that control the spread of plasmid-encoded antibiotic resistance in aquatic environments. In detail, the following research questions are addressed:

1. Considering a single plasmid-host pair, how can genomic modifications and antibiotics catalyze the spread of plasmid-encoded antibiotic resistance in the aquatic environment?
    a) How do certain ecological conditions affect this catalysis?
2. Considering a simultaneous interaction between frequently co-occurring types of plasmids (non-transmissible and conjugative), how does this change system behaviour and previous predictions?
3. Considering the plasmid diversity found in natural communities, are the previous results still valid?
    a) Do diverse plasmid communities facilitate the persistence of costly plasmid functions such as antibiotic resistance in the absence of abiotic selection?
    b) How do communities evolved under abiotic selection (presence of antibiotics) respond to a change to neutral conditions?

In order to investigate these questions, mathematical models are developed and systematically analyzed. Although these models are supported by empirical data, they are not limited to a specific model organism or plasmid type. Instead, this analysis will deal flexibly with a variety of assumptions and attempt to examine these questions from a rather unrestricted theoretical perspective. This includes, for example, considering the entire spectrum of possible plasmid properties and environmental conditions. Further specific aspects related to the above research questions and covered by the main studies of the dissertation are listed in table 1.1.

Content

The formulation of the objectives and research questions of the dissertation reflect the sequence for the presentation of the content of the dissertation, although Article 2 (Chapter 3) has been published before Article 1 (Chapter 2).

Chapter 2 examines which strength of cost compensation of a mutation on either the chromosome or the plasmid can rescue a plasmid from extinction, considering a single plasmid-host pair. For ancestral plasmid types with different costs, segregation rates and conjugation rates it is shown how they can survive considering a spectrum of environmental conditions, determined by bacterial growth rates (mimicking availability of nutrients, temperature, ...), rates of dilution (mimicking washout, predation and/or natural mortality) and antibiotic action (bactericidal antibiotic that kills a certain proportion of sensitive cells).

Table 1.1: Overview of the specific aspects covered by the main studies of the dissertation (referring to Articles 1-3 presented in Chapters 2-4).

		Article 1	Article 2	Article 3
Evolutionary modifications	Arise of beneficial mutations	x		
	Plasmid community composition			x
Individual heterogeneity	Locally dependent growth conditions		x	x
	Plasmid load (more than 3 levels*)			x
	Adaptive switch of transfer competence		x	
Complex biotic interactions	Intercellular interactions		x	x
	Intracellular interactions		x	x
	Competing plasmid lifestyles		x	x
	Multiple co-occurring plasmid types			x

*related to the number of possible combinations of different plasmid types that co-occur in the same cell

The model that was developed for this purpose represents a set of ordinary differential equations, assuming a mixed population and static environmental conditions. This chapter shows that a plasmid location of a certain compensatory mutation is better at enhancing plasmid survival than a chromosomal location, because the cost compensation can move with the plasmid and is readily provided to any newly infected cell. Assuming that they impose non-zero costs for the host cell, this study indicates that non-transmissible plasmids are not able to survive in such a setup, which is contradictory to their abundance in nature.

Consequently, Chapter 3 examines how the co-occurrence of non-transmissible and conjugative plasmids influences their existence conditions. For this purpose, an individual-based model was set up. It represents a bacterial population attached to a two-dimensional plane. Plasmids spread along with their host bacteria, but impose costs. The conjugative plasmid type can be transferred to local neighbours, but is assumed to be incompatible to the other non-transmissible plasmid type that exists in the population. This study demonstrates that the co-occurrence of incompatible plasmids with such opposing life styles can indeed maintain a non-transmissible plasmid that imposes non-zero costs to its host. In line with the predictions presented in Chapter 2, non-transmissible plasmid types are not able to survive if they are the only plasmid type that interacts with the host, even if local interaction is taken into account. A variety of sensitivity analysis have been examined for this study, including investigations on the dependency of the presented stability mechanism to varying costs of the conjugative and non-transmissible plasmid type, their incompatibility features, varying transfer efficiencies and washout probabilities. This revealed a robustness to assumptions such as global dispersal and a permanent derepression of the conjugative plasmid type. In the default model version a switch of a bacterium's transfer competence between repressed (low costs, but no transfer) and derepressed (transferable, but higher costs) is considered to occur in dependence to the local density of recipient cells, which represents a more complex mechanism similar to quorum sensing. In this study antibiotics and antibiotic resistance is not explicitly considered, but it represents an alarming result that a certain non-transmissible plasmid that imposes a growth rate disadvantage of 7%

(or even more) could persist in the absence of abiotic selection in such a setting.

Chapter 4 continues at this point and demonstrates how the interaction between multiple diverse plasmid types influences their existence conditions and specifically the maintenance of costly traits such as antibiotic resistance. Therefore, an individual-based model similar to that used for the study presented in Chapter 3 was developed. Instead of predetermining a specific set of plasmid types, an initial population comprising 40,000 plasmid types that differ in their costs, transfer ability and incompatibility are considered to interact. Observing the evolution of such a community revealed that the intransitive dynamics of conjugative and less costly non-transmissible plasmid types seems to be an emergent property of a diverse plasmid community and can be simultaneously formed for different incompatibility groups. This shows how the vast genetic repertoire mediated by plasmids could be preserved in nature. The study also demonstrates that such interactions between plasmids allow costly plasmid-encoded antibiotic resistances to persist despite stopped abiotic selection.

After these main studies, Chapters 5, 6 and 7 give an overview of the tasks and results of the student work that was supervised in connection with the thesis. These chapters summarise a Master thesis that focused on the propagation of antibiotic resistances considering migration between microhabitats, a Master thesis on the estimation of the pB10 conjugation rate in Escherichia coli combining laboratory experiments and modelling and the work of a six month long research internship investigating plasmid population dynamics considering individual plasmid copy numbers.

Finally, Chapter 8 summarises and discusses the major findings and provides a critical evaluation of the methodology as well as an outlook on future research directions.

2 Article I (published) – **Mobile compensatory mutations promote plasmid survival**

The persistence of plasmids in nature when they are not beneficial to the bacteria that carry them is a hot topic in plasmid biology, because it has direct implications to human health. What are the mechanisms behind the alarming spread of plasmid-associated antibiotic resistances observed outside the clinical environment, for example in freshwater systems? This chapter focuses on the role of adaptation, which can compensate the costs of carrying plasmids, in the long-term persistence of plasmids and associated antibiotic resistances. Whether the compensatory mutations are to the plasmid itself or to the bacterial chromosome, when they appear, and their consequences, are central questions in microbial ecology and evolution. Although molecular and cell level mechanisms have been characterised, the consequences for the bacterial population are hard to resolve. Here, mathematical methods are used to unravel the differential effects of compensatory adaptation arising from mutation to the host bacterium or to the plasmid itself.

This study is the first demonstrating that a compensatory mutation located on the plasmid can be strikingly more advantageous than a similar chromosomal mutation. We illustrate and explain the consequences for population dynamics and the consequences of varying plasmid properties and environmental conditions. Our study highlights the plasmid's potential to persist in varying levels of antibiotics.

Since transparency in science has never been more important, we provide the full code and a ready-to-run (web) application of our model. This enables users to explore the model by varying any of its parameters, to check the computer code that generated the presented results, and could prove a useful pedagogical tool.

This chapter represents a peer-reviewed publication in *mSystems* from January 2019, entitled „Mobile compensatory mutations promote plasmid survival“. It has been compiled along with Dr. Ellie Harrison, Dr. James Hall and Prof. Dr. Michael Brockhurst, which I worked together during my research stay at the Department of Biology of the University of York in August 2015, and with Prof. Dr. Thomas Berendonk, from the Chair of Limnology at the TU Dresden as well as with Prof. Dr. Uta Berger, the first supervisor of my PhD study.

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RESEARCH ARTICLE
Ecological and Evolutionary Science

Mobile Compensatory Mutations Promote Plasmid Survival

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ABSTRACT The global dissemination of plasmids encoding antibiotic resistance represents an urgent issue for human health and society. While the fitness costs for host cells associated with plasmid acquisition are expected to limit plasmid dissemination in the absence of positive selection of plasmid traits, compensatory evolution can reduce this burden. Experimental data suggest that compensatory mutations can be located on either the chromosome or the plasmid, and these are likely to have contrasting effects on plasmid dynamics. Whereas chromosomal mutations are inherited vertically through bacterial fission, plasmid mutations can be inherited both vertically and horizontally and potentially reduce the initial cost of the plasmid in new host cells. Here we show using mathematical models and simulations that the dynamics of plasmids depends critically on the genomic location of the compensatory mutation. We demonstrate that plasmid-located compensatory evolution is better at enhancing plasmid persistence, even when its effects are smaller than those provided by chromosomal compensation. Moreover, either type of compensatory evolution facilitates the survival of resistance plasmids at low drug concentrations. These insights contribute to an improved understanding of the conditions and mechanisms driving the spread and the evolution of antibiotic resistance plasmids.

IMPORTANCE Understanding the evolutionary forces that maintain antibiotic resistance genes in a population, especially when antibiotics are not used, is an important problem for human health and society. The most common platform for the dissemination of antibiotic resistance genes is conjugative plasmids. Experimental studies showed that mutations located on the plasmid or the bacterial chromosome can reduce the costs plasmids impose on their hosts, resulting in antibiotic resistance plasmids being maintained even in the absence of antibiotics. While chromosomal mutations are only vertically inherited by the daughter cells, plasmid mutations are also provided to bacteria that acquire the plasmid through conjugation. Here we demonstrate how the mode of inheritance of a compensatory mutation crucially influences the ability of plasmids to spread and persist in a bacterial population.

KEYWORDS compensatory evolution, chromosomal mutation, plasmid mutation, plasmid persistence, fitness costs, cost compensation, antibiotic resistance, mathematical modeling, conjugation, horizontal gene transfer

Plasmids accelerate bacterial adaptation by transferring ecologically important functions, such as antibiotic resistance, between lineages. Although plasmids can be advantageous in certain environments, their acquisition normally imposes significant fitness costs upon host cells. This is because the expression, repair, and replication of plasmid genes use up raw materials, occupy the cellular machinery, and can disrupt the cellular environment (1, 2). Coculture studies showed that compensatory mutations

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The genomic location of compensatory mutations can critically affect the dynamics of plasmids: plasmid compensation is better at enhancing plasmid persistence, even when its effects are smaller than those provided by chromosomal compensation.

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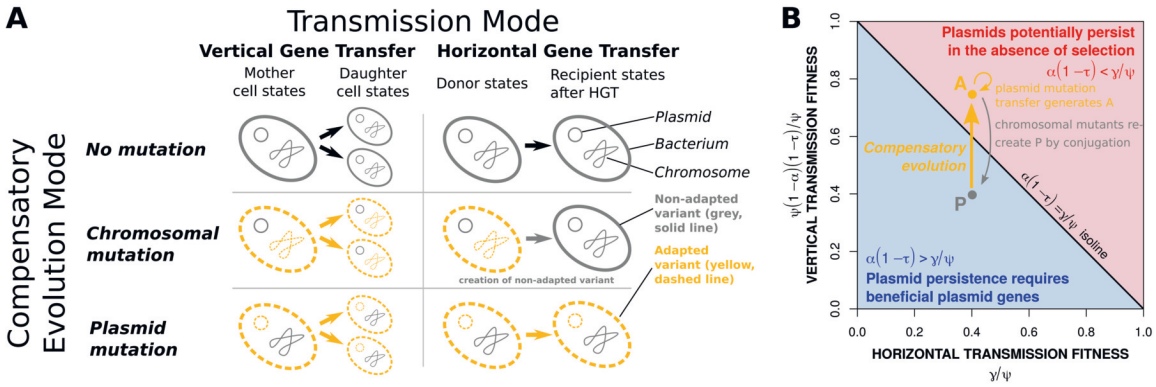

FIG 1 General theoretical concept. (A) The type of compensatory evolution determines the capabilities for the transmission of a compensatory mutation. (B) Plasmid persistence depends on two components characterizing plasmid fitness: (i) the vertical transmission fitness (*y* axis), reflecting the negative effect of the plasmid costs, α, on the host cells' maximum growth rate, ψ, and the rate of segregational loss, τ (characterizing the inability to inherit the plasmid to both daughter cells by binary fission); and (ii) the horizontal transmission fitness (*x* axis), given by the extent of the conjugation rate, γ. In order to enable comparability, both fitness estimates are normalized to the maximum growth rate, ψ. Plasmids potentially persist in the absence of antibiotic-mediated selection when the combined effects of plasmid costs and segregational loss are lower than the relative extent of conjugation. By compensatory evolution, the initial plasmid costs can be reduced—for instance, by modifying the vertical transmission fitness of a notional plasmid, *P*, to the level of *A*, provided by either a chromosomal mutation or a plasmid mutation. As chromosomal mutants cannot transmit the compensatory mutation horizontally, they generate cells with the original fitness level of *P* when they perform conjugation. For simplicity, we neglect that the amelioration could be coupled to a reduction of the conjugation rate.

occurring on plasmids or bacterial chromosomes can reduce these fitness costs of plasmid carriage (3–9), allowing the stabilization of plasmids in the bacterial population (10–12). Because compensatory evolution weakens purifying selection against plasmids, it is likely to increase plasmid persistence even in environments without positive selection for plasmid traits, heightening the risk that plasmid-encoded antibiotic resistances can spread (8, 13, 14).

The mechanisms of amelioration of plasmid costs can be various, including changes in host or plasmid gene expression, conjugation rates, or the loss of plasmid genes (1, 2, 15). Moreover, the extent of the amelioration of the fitness cost varies between compensatory mutations (3–7). Overall, however, compensatory mutations can be considered as reducing the metabolic burden of plasmid carriage, allowing improved bacterial growth but not affecting other processes.

We hypothesize that the genomic location of the compensatory mutation, either on the plasmid or the chromosome, will have contrasting effects on plasmid dynamics and persistence: if the compensatory mutation is located on the plasmid, it spreads by both vertical (cell fission) and horizontal (conjugation) transmission. Thus, compensated plasmids acquired by new recipients will impose a reduced cost and be more likely to spread. This likely represents a strong advantage over a chromosomal location (see also the conceptual model presented by Fig. 1A).

In order to comprehensively assess the effects of the genomic location of the compensatory mutation on plasmid dynamics, we developed a mathematical model that simulates the dynamics of plasmid-free bacteria, *F*, nonadapted plasmid bearers, *P*, and adapted plasmid bearers, *A*. The model considers a well-mixed system where (i) bacteria grow with maximal growth rate, ψ, (ii) plasmid bearers suffer according to some plasmids' costs, α, (iii) compensatory mutations provide an amelioration of these costs by strength β, (iv) bacteria are lost through washout and death according to dilution rate ω and by antibiotic killing rate υ, (v) plasmids are lost during bacterial fission by segregation rate τ, (vi) plasmids are horizontally transferred with conjugation rate γ according to a second-order reaction of plasmid-bearing and plasmid-free cells, and (vii) compensatory mutations are acquired (on either the plasmid or the chromosome) with rate χ (see Table 1 for an overview). (The supplemental material provides more details and a link to a web application of the model and its source code.) Since

TABLE 1 Model of differential equations describing the dynamics of plasmid-free bacteria and nonadapted and adapted plasmid bearers[a]

	Bacterial dynamics			
Process	*F*	*P*	*A*	Reaction rate
Growth	1	0	0	$f\psi F$
	0	1	0	$f\psi(1-\alpha)P$
	0	0	1	$f\psi[1-\alpha(1-\beta)]A$
Mortality	−1	0	0	$(\omega+\upsilon)F$
	0	−1	0	ωP
	0	0	−1	ωA
Segregation	1	−1	0	$f\tau\psi(1-\alpha)P$
	1	0	−1	$f\tau\psi[1-\alpha(1-\beta)]A$
Conjugation	−1	1	0	$f\gamma FP$
	−1	1^c	1^p	$f\gamma FA$
Mutation	0	−1	1	$f\chi\psi(1-\alpha)P$
	0	-1^p	1^p	$f\chi\gamma FP$

[a]The model consists of three ordinary differential equations describing the dynamics of plasmid-free bacteria, *F*, nonadapted plasmid bearers, *P*, and adapted plasmid bearers, *A*, respectively. The derivatives of the compartments (*F*, *P*, or *A*) are determined by the reaction rates of the contributing processes, such as growth and mortality. Resource availability: $f = 1 - [(F + P + A)/k]$. The matrix notation indicates these reaction rates as well as the directions of the particular effects (1, positive; 0, no effect; and −1, negative). Model versions for plasmid mutations and chromosomal mutations differ in two ways. (i) In the first way, conjugation initiated by adapted plasmid bearers, *A*, turns plasmid-free cells, *F*, into bacteria of type *A* (indicated as *p* [superscript]), when the compensatory mutation is located on the plasmid ("plasmid mutation"), or into nonadapted plasmid bearers, *P*, when the mutation is located on the chromosome ("chromosomal mutation," indicated as *c* [superscript]). Note that *c* is only valid for chromosomal mutation (otherwise 0), and *p* is only valid for plasmid mutation (otherwise 0). (ii) In the second way, compensatory mutations are acquired proportional to replication events, which occur proportional to bacterial growth (given for "chromosomal mutation" and "plasmid mutation") and conjugation (only given for "plasmid mutation," *p*). For a parameter description, see Table 2. The equation form of each model version (for "no mutation," "chromosomal mutation," and "plasmid mutation") is presented in equations E1, E2, and E3 in Text S1.

multiple host strains or species are not considered, we refer to horizontal gene transfer within a nearly clonal cell population, in which intraspecies competition occurs simply between plasmid-free and plasmid-carrying subpopulations. Antibiotic killing does not affect plasmid bearers, because the plasmid confers resistance. A series of simulations were run in order to encompass the full range of plausible plasmid-host properties and environmental conditions, including various levels of antibiotic action.

RESULTS

Cell-inherent requirements for plasmid survival by conjugation. To disentangle the basic effects of the plasmid- and host-cell-specific characteristics on plasmid survival, we interpret our simulation results in the light of the general theoretical concept presented in Fig. 1B: the competitive disadvantage of plasmid-bearing cells that results from the reduction of the maximal growth rate, ψ, due to the plasmid costs, α, as well as the aligned rate of segregational loss, τ, lead to a decrease of the plasmid-carrying population. The less intense these negative effects are, the higher is a plasmid's vertical transmission fitness: $\psi(1-\alpha)(1-\tau)/\psi$. In contrast, the infection of plasmid-free cells according to conjugation rate, γ, leads to an increase of the plasmid-carrying population. The horizontal transmission fitness controls this process. It increases with the extent of the conjugation rate in relation to the rate at which new plasmid-free cells are generated (γ/ψ). Using these cell-inherent characteristics, we derived a conjugation rate threshold, γ_{low}, that approximates the lowest extent of conjugation that can enable plasmid persistence in the absence of selection for plasmid-encoded traits: $\gamma_{low} = \alpha\psi(1-\tau)$.

Plasmids that spread with conjugation rates lower than this threshold and provide no beneficial genes (in our case, in the absence of antibiotics) will not persist. Compensatory evolution can reduce the initial plasmid costs, α, by some amelioration

TABLE 2 Model parameters and their settings in simulation experiments[a]

Variable	Description	Default value (simulation expt I)	Sampling range (simulation expt II)	Value(s)[b]
k	Carrying capacity (maximal attainable cell density)	1	1	10^9 expt→1 for modeling (28)
ψ	Maximal bacterial growth	1	0–2	0.1–1[c] (39), 0.19–1.23[d] (40), 0.74[e] (22)
α	Plasmid costs	0.2	0–1	0.2[f] (41), 0.14–0.19[g] (4), 0.06–0.21[h] (5), 0.21[i] (8), 0.03–0.58 (28), 0.32–0.64[j] (6)
β	Amelioration strength	0.9	0–1	0.1–0.25[g,l] (4), 0.65–1[i,l] (8), 1[h,l,k] (5, 6)
ω	Dilution rate (washout/mortality/predation)	0.1	0–1	0.1 (26, 41)
υ	Antibiotic action (killing rate)	$0\|10^{-1}\|10^{-2}\|10^{-3}$	10^{-4}–1[p]	0.1 (8)
τ	Segregation rate	0.001	0.5–10^{-6}[q]	10^{-4}[i] (8), 10^{-3}[e] (22)
γ	Conjugation rate	0.02	0–1	0.025[m] (28), 10^{-9}[e,n] (22), ≈10^{-11}–10^{-9}[n] (42)
χ	Mutation rate[r]	10^{-6}	10^{-9}–10^{-3}[p]	10^{-6} (8), ≈10^{-9}–10^{-3} (43), ≈10 − 10[o] (39)

[a]Simulation experiment I was performed using the parameter defaults in combination with one of the rates for antibiotic action. For simulation experiment II, the sampling range of each parameter defines the parameter space that was used for a random sampling, which generated a compilation of 100,000 parameter sets. Each parameter set was used for simulations with the differential equation model described by the model matrix (Table 1) considering "no mutation" ($\chi = 0$), "chromosomal mutation," and "plasmid mutation." To perform simulations in the absence of antibiotics, but under otherwise identical conditions, υ was set to 0, without performing a new sampling.
[b]Reference numbers are shown in parentheses.
[c]Estimated for most bacteria in the wild.
[d]For *Escherichia coli*, growth on glucose at 17.4 to 40°C.
[e]Used as simulation standard.
[f]For plasmid pQBR103.
[g]For plasmid pBR322.
[h]For plasmids R1 and RP4.
[i]For plasmid pNUK73.
[j]For plasmid R1.
[k]For coadaptation of host chromosome and plasmid.
[l]For adaptation of host chromosome.
[m]Considering relative cell densities.
[n]Considering absolute cell densities (which can be converted to relative values as used in this study by a multiplication with the respective maximum attainable cell density, k [e.g., assuming $k = 10^{-9}$]).
[o]Laboratory estimates for different species, but mutation rates in the wild are assumed to be higher (39).
[p]Sampled on a logarithmic scale to prevent an overrepresentation of high values (retransformed by anti-log for simulations).
[q]Results from uniformly sampling x in the range from 1 to 20 and calculating probabilities using function 0.5^x. In this way, τ can also be interpreted as the segregation probability for a certain plasmid copy number x (11).
[r]Referring to a specific compensatory mutation occurring in the first place in the population evolution, but not to neutral, deleterious, or secondary compensatory mutations.

strength, β, to the level of $\alpha(1 - \beta)$, consequently reducing γ_{low}. Nevertheless, plasmids will only persist if cost reduction and conjugation act jointly. Finally, if conjugation rates are higher than γ_{low}, a plasmid can survive under certain environmental conditions (see the next section for further details).

Influence of compensatory evolution on plasmid population dynamics. To initially test how population dynamics depends on the location of the compensatory mutation, the differential equation model (Table 1) was run using typical parameter values found in literature (Table 2, simulation experiment I).

Plasmid-bearing bacteria get outcompeted by plasmid-free bacteria unless the level of antibiotic action is sufficiently high (Fig. 2). This effect is generally reduced when compensatory evolution occurs and adapted plasmid bearers with lowered plasmid costs emerge. Although the reduction of plasmid costs is equal in both cases, plasmid mutation enables persistence at lower antibiotic levels than chromosomal mutation.

This is explained by the fact that at low antibiotic concentrations, positive selection is outweighed by the residual cost of plasmid carriage, even following compensatory evolution. Under these conditions, infectious transmission is required to sustain the plasmid in the population. Where compensatory mutations are linked to the bacterial chromosome, conjugation simply increases the proportion of nonadapted plasmid bearers, which suffer the full cost of plasmid carriage and are readily outcompeted. Where compensatory mutations are linked to plasmids, however, newly formed transconjugants suffer a reduced cost of plasmid carriage. Thus, transconjugants expand the proportion of adapted plasmid bearers which support further infectious

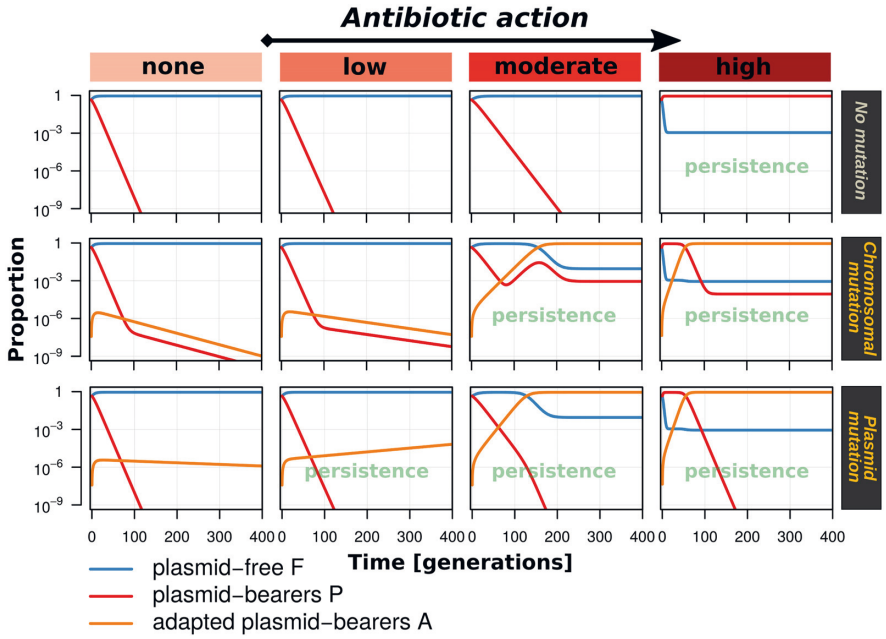

FIG 2 Population dynamics is influenced by the type of compensatory evolution. Each plot refers to a single model run with a set of default parameters (Table 2, simulation experiment I): assuming that plasmid-carriage causes a growth rate disadvantage of 20% ($\alpha = 0.2$) that can be ameliorated by 90% ($\beta = 0.9$), resulting in reduced plasmid costs of only 2% [$\alpha(1\text{-}\beta) = 0.02$] for adapted plasmid-bearers, considering no mutation, chromosomal mutation, or plasmid mutation, as well as four different levels of antibiotic action, υ (none, 0; low, 10^{-3}; moderate, 10^{-2}; high, 10^{-1}).

transmission. The nonadapted plasmid variant is eliminated soon in this case, since it cannot prevail against the more competitive mutant that provides the cost compensation. This feature of a plasmid compensatory mutation represents a fitness advantage (Fig. 1B) that enables plasmid persistence at lower levels of antibiotic-mediated selection.

Influence of compensatory evolution on the conditions favoring plasmid persistence. To test whether the observed advantage of plasmid compensatory evolution is stable under a variety of conditions, the deterministic model (Table 1) was repeatedly run using parameter values randomly drawn within reasonable ranges (Table 2, simulation experiment II). Each model run was performed until the proportional changes of plasmid-free, plasmid-bearing, and adapted plasmid-bearing bacteria were less than 10^{-9} (steady state; considering carrying capacity, $k = 1$).

At first, we looked at the resulting frequencies of plasmid-bearing cells in the steady state. The associated pattern is strongly bimodal: plasmids were either prevalent or became (almost) extinct (Fig. 3, top). When the compensatory mutation was located on the plasmid, more plasmids survived compared to a chromosomal mutation.

We further examined how the result of plasmid extinction or persistence is related to plasmid traits and environmental conditions. In the absence of antibiotics, plasmids could only survive when the threshold γ_{low} was met. In this case, the competitive disadvantage of the remaining plasmid costs, $\alpha(1 - \beta)$, and the proportional rate of plasmid loss, τ, can be outweighed by the relative extent of the conjugation rate, γ: thus, $\{\psi[1 - \alpha(1 - \beta)(1 - \tau)]/\psi\} < \gamma/\psi$ (Fig. 3D and H). Plasmids could not survive by exerting conjugation rates below the threshold γ_{low}, which refers to any conditions below the diagonal line in the Fig. 3D and H. This result is in line with our preliminary theoretical considerations (Fig. 1B or Fig. 3, bottom left). As further expected, plasmids also got lost when the threshold γ_{low} was met (conditions above the diagonal line in

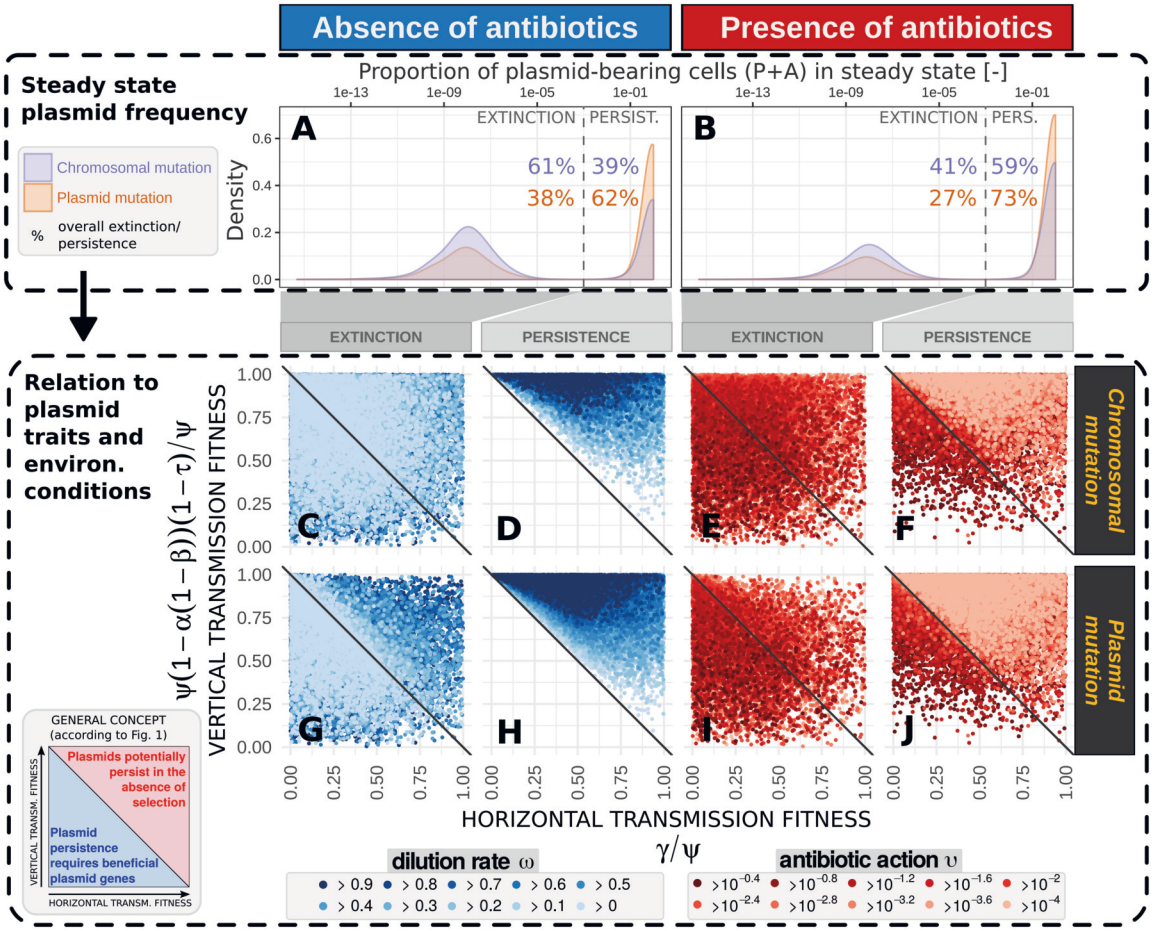

FIG 3 Plasmid compensatory evolution enhances plasmid survival in less favorable environmental settings and fitness contexts. Results refer to the examined global analysis (Table 2, simulation experiment II), where simulations reached a steady state after hundreds to thousands of generations (Fig. S1), characterized by the absence of further substantial changes in the frequencies of plasmid-free bacteria, *F*, plasmid-bearing bacteria, *P*, and adapted plasmid-bearing bacteria, *A*. The prevalence of plasmids in steady state is bimodal (on top). This enabled us to define a threshold (dashed line in plots A and B) to accurately distinguish between long-term plasmid persistence (right from dashed line) and extinction (left from dashed line) and to define the associated overall proportion for chromosomal and plasmid mutation. (Please note that the absolute densities at extinction would all approach $-\infty$, when simulations were not stopped by steady-state computation, i.e., the absence of further substantial changes in the cell proportions.) The conditions that resulted in plasmid extinction and persistence were then further analyzed using the general concept of plasmid fitness introduced in Fig. 1B: Every point in plots C to J refers to the plasmid fitness characteristics (given by the respective parameter values for maximal growth rate, ψ, plasmid costs, α, strength of amelioration, β, segregation rate, τ, and conjugation rate, γ) and the environmental conditions (dilution rate, ω, which refers to washout/natural mortality; and antibiotic action, υ) used in a single model run that resulted either in plasmid extinction or persistence. Since many configurations led to similar results, the degree of overplotting points is high. For this, the order of points is layered from minimal to maximal rates of dilution or antibiotic actions. This feature of the plot reveals the most interesting results regarding plasmid persistence under more unfavorable conditions.

Fig. 3C and G), since this threshold represents an exclusion criterion rather than a stability criterion. With an increased dilution, more bacteria are lost—mimicking washout, natural mortality, predation, or a combination of these processes. This results in decreased bacterial densities that lower the real efficiency of the conjugation rate. The potential of plasmid persistence (even above γ_{low}) therefore increases with lower dilution rates and higher vertical transmission (low plasmid costs and segregation rates), respectively.

In the presence of antibiotics, plasmids were able to persist even below the threshold γ_{low} (conditions below the diagonal line in Fig. 3F and J), at least when

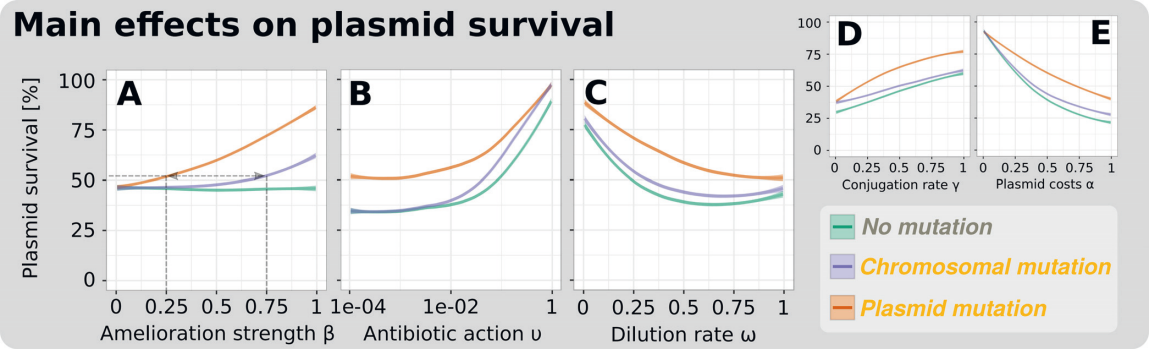

FIG 4 Plasmid compensatory evolution enhances plasmid survival across the full range of tested plasmid properties and environmental conditions. Results refer to the global sensitivity analysis considering the presence of antibiotics (Table 2, simulation experiment II). The single panels depict the main effects of each parameter across its range for each type of compensatory evolution. The higher the difference between the lowest and highest plasmid survival percentages, the stronger is the main effect of this parameter. Antibiotic action (B) and plasmid costs (E) have the strongest main effect. Nonlinear responses indicate that certain parameter ranges are more sensitive than others (when the response is steeper). The slight reversal to increased plasmid survival percentages at high dilution rates results from the extreme conditions for antibiotic-sensitive, plasmid-free cells, when the combined effects of dilution and antibiotic action approach 1. Main effects of the remaining parameters are depicted in Fig. S2.

antibiotic killing rates, υ, were high, representing a strong selection on the resistant plasmid bearers. Nevertheless, some plasmids got lost even if they met the requirements given by the threshold γ_{low} (i.e., by high conjugation rates and/or low plasmid costs) and even if they were supported by high antibiotic action (conditions below the diagonal line in Fig. 3E and I [please consider that the orders of point layers are different between panels F and J and E and I]). This traces back to the effect of dilution, which can therefore also impact plasmid survival in the presence of antibiotics. It suggests that, e.g., higher levels of predation are likely to drive antibiotic resistance genes out, at least in the well-mixed phase.

Most interestingly, plasmid compensatory evolution enabled the persistence of plasmids under less favorable conditions: i.e., inferring higher plasmid costs, exerting lower conjugation rates, and/or facing higher dilution rates or weaker antibiotic-mediated selection. This advantage of a plasmid compensatory mutation over a chromosomal compensatory mutation especially manifests at high conjugation rates, since chromosomal mutants cannot transfer the cost compensation to horizontally infected cells (Fig. 1), whereas plasmid bearers that carry and transfer the cost compensation via the plasmid also to any infected cell directly benefit from conjugation.

To test if the effect of compensatory evolution on plasmid persistence is particularly sensitive to a single parameter of our model, we examined the probability of plasmid persistence for any parameter across the ranges that were used in our simulations. Across the majority of the parameter, space compensatory mutations increased the probability of plasmid persistence (see Fig. 4 for most important parameters and Fig. S2 in the supplemental material for the remaining parameters). This effect was much greater when mutations were linked to the plasmid rather than the chromosome. This demonstrates that the advantage of plasmid mutations is robust within the broad range of tested parameter values. Although the mutation rate does not affect the steady-state results of our model (Fig. S2), it should be noted that it accelerates the increase of adapted plasmid bearers in the short term (see Fig. S3 in the supplemental material) and might represent an advantage of chromosomal mutations (see Discussion).

Overall, plasmid compensatory evolution increased the proportion of conditions enabling plasmid persistence far more by increased amelioration strength (Fig. 4A). Even by providing only 25% reduction of the plasmid costs, plasmid mutation allowed persistence for the same proportion of conditions as mediated by chromosomal mutations, with a cost compensation of 75%. The advantage of plasmid mutations that is given by the ability to transfer the cost compensation with the plasmid to both daughter and infected cells also manifests by higher survival rates at very low antibiotic action.

DISCUSSION

In this article, we examined the role of two different types of compensatory evolution, namely chromosomal mutations versus plasmid mutations, for the long-term persistence of plasmids. We demonstrated that

1. Compensatory evolution can have a significant effect, allowing plasmid-encoded antibiotic resistances to persist for a much longer time, even in the absence of antibiotics.
2. The genomic location (chromosome or plasmid) can play a pivotal role for the success of a compensatory mutation. Since chromosomal mutations cannot be transmitted to infected bacteria, the benefits that such plasmid-bearing bacteria could gain from conjugation are reduced.
3. Plasmid mutations facilitate plasmid persistence even when the direct amelioration effects are far less effective than those provided by chromosomal mutations.

If the plasmid adapts, it is also very likely that the nonadapted plasmid variant will be eliminated soon, as it cannot prevail against the more competitive mutant that provides the cost compensation. Of course, this may not occur, if a more specific mechanism of compensatory evolution is considered, e.g., the reduction of the conjugation rate or the loss of functional traits (1), which would reshape the fitness differences between nonadapted and adapted plasmid bearers. A deletion of the type 4 secretion system (T4SS) or a part of it can decrease the costs associated with plasmid carriage (16), but any increase of vertical transmission to the detriment of horizontal transmission implies a shift in a plasmid's survival strategy. This can sustain a resistance plasmid in its current host, even in the absence of selection (5), but likely limits its dissemination potential to new hosts. Understanding this trade-off in interplay with the genomic location of the compensatory mutation represents a further challenge, which can also be addressed with mathematical methods analogous to those used in this study.

It should be noted that compensatory mutations are discrete events that arise on the single-cell or plasmid level. In small bacterial populations, the resulting stochasticity needs to be considered (17). Although it is hard to predict which mutations might be available to ameliorate the plasmid costs, chromosomal mutations might occur much more frequently than plasmid mutations, considering their different amounts of genes. This timing can be important to stabilize the plasmid before it is lost from the population (17) and might explain why chromosomal mutations are so common in experimental evolution.

Our results indicate that a single nontransmissible plasmid cannot persist in the absence of selection of plasmid-borne traits, but this might not hold considering a more diverse plasmid community, since conjugative plasmids can promote the survival of co-occurring, less costly nontransmissible plasmids of the same incompatibility group (18). It is also important to note that the availability of alternative hosts, which is not considered in our model, can have evolutionary consequences on plasmid population dynamics. This is because fitness effects of the same plasmid can be really variable in different hosts (19), although some plasmid compensatory mutations have been shown to increase plasmid persistence even in other plasmid-host pairs (20). Another effect of a multispecies (or nonclonal) host environment is that interspecific plasmid transfer can allow plasmids to survive in host species which cannot sustain the plasmid in monoculture (21). Furthermore, we neglected that bacteria experience spatiotemporal fluctuating environmental conditions during their lifetime, which could help to sustain a resistance plasmid by rare antibiotic exposure (8).

Our model considers average conditions in notional habitats. Such simple mass action models have been successfully applied to various research questions related to plasmid biology (22–28) as they allow a fast computation of highly comprehensive simulation experiments, which can be used to draw general conclusions (29). Our

results are in line with model-supported empirical studies highlighting the importance of conjugation-assisted persistence for costly plasmids (28) and provide further insights into the role of compensatory evolution for the empirically found persistence of antibiotic resistances at concentrations of antibiotics far below the MIC of the susceptible strain (30, 31).

We believe that a more thorough exploration of this issue, especially regarding to the mechanisms that underlie the costs of plasmid carriage and the conditions in natural microbial communities, will be an important further step toward an improved understanding of the population dynamics and evolutionary biology of plasmids. This will also help to develop strategies against the dissemination of antibiotic resistance genes.

MATERIALS AND METHODS

Mathematical model. Our model consists of a system of three ordinary differential equations. It describes the dynamics of the following compartments: plasmid-free bacteria, *F*; plasmid-bearing bacteria, *P*; and adapted plasmid-bearing bacteria, *A*. The latter originates from the evolution of the plasmid and its host and is provided either by a mutation of the chromosome or a mutation of the plasmid (in the meaning of a compensatory mutation occurring in the first place in the population evolution; neutral, deleterious, or secondary compensatory mutations are not considered). To compare both types of compensatory evolution, we developed two different model versions. They are described as a model matrix (Table 1). All parameters and their settings in the simulation experiments are given in Table 2.

Please note that this study considered relative cell densities instead of absolute ones. This enables a direct comparison of the extent of plasmid costs and conjugation. If absolute cell densities would be used, the conjugation rate has to be scaled in relation to the carrying capacity, *k*. Assuming, for example, $\gamma = 0.2$ and $k = 10^9$ cells/ml, an adjusted conjugation rate, $\gamma_{adjusted}$, of $\gamma/k = 2e{-}11$ would be obtained. This means the model could also be run with typical conjugation rates to those generated by the endpoint method (32). Model performance would remain the same as long as robust estimates for *k* are available.

Our model does not consider that the viability of resistant cells (plasmid bearers *P* or *A*) can be reduced by the antibiotic (33). We assume this to be a valid simplification, since the bactericidal antibiotic killing rate was for instance reported to be 55 times higher for sensitive bacteria than for resistant bacteria (8). As we consider antibiotic action to range only from no inhibition ($\upsilon = 0$) to full inhibition of the plasmid-free bacteria ($\upsilon = 1$), we never achieve those levels that cause, with respect to dose-response experiments (8), a significant inhibition of resistant bacteria. Our model predictions are therefore valid in this range.

The supplemental material (Text S1) provides further details of the model and the link to a web application that enables the reader to explore the model behavior with default or self-defined parameter estimates. (Please, consider the discussion on relative and absolute conjugation rates above.)

Simulation experiments. Two different types of simulation experiments were carried out. While simulation experiment I focused on the general dynamics of the model system with a reference parameter set, simulation experiment II scanned the whole parameter space in order to reveal which conditions favor the long-term persistence of plasmids.

Both experiments have in common that the initial frequency of plasmid-free bacteria, *F*, and nonadapted plasmid bearers, *P*, was defined as half of the proportion of bacteria that are approached in steady state, which was approximated by $k(1 - \omega)$. Since the latter depends on the particular parameter settings, the initial frequency could differ in absolute values, but the $F/P_{initial}$ ratio remained the same. Adapted plasmid bearers, *A*, were not present in the initial state. Please note that model results are insensitive to the particular initial conditions (see Fig. S4 in the supplemental material for validation).

(i) Simulation experiment I: general dynamics of the model system. Simulation runs were carried out with default parameter values (Table 1), considering the absence of antibiotics as well as their presence at low, moderate, and high levels. The time horizon of one simulation run corresponded to a time course of 4,000 h (here equal to approximately 400 generations).

(ii) Simulation experiment II: conditions favoring the long-term persistence of plasmids and associated antibiotic resistances. Simulation experiments were carried out within the parameter space of reasonable maxima and minima (Table 2, third column) mimicking a broad spectrum of possible plasmid-host properties and environmental conditions. Each parameter was randomly sampled generating 10,000 parameter sets by an adjusted Latin hypercube approach (34).

The objective of any of these simulations was to examine if the proportion of plasmid bearers ($P + A$) was still higher than 10^{-3} when the system reached a steady state. A steady state was assumed when the proportional change in the population composition was less than 10^{-9} h^{-1}. This corresponds to a change of the bacterial composition of less than 1 cell per hour for an absolute carrying capacity of 10^9 cells.

Long-term persistence of plasmids was assumed when the proportion of plasmid bearers ($P + A$) was still higher than 10^{-3} after reaching the steady state. The threshold of 10^{-3} was arbitrarily chosen but served well for the distinction between settings promoting persistence and leading to extinction since the vast majority of simulation experiments leading to the latter ended up with plasmid bearer proportions of less than 10^{-6} (see Fig. S1 for a validation of the system's behavior).

Model implementation. The model was implemented in the software environment R version 3.4.2 (35) and executed using aligned packages deSolve (36) and rootSolve (37) for solving and analyzing the steady state of ordinary differential equations. Sampling in simulation experiment II was performed with function randomLHS from R package lhs (34). Graphics were generated using R package ggplot2 (38) and core functions of R. The source code is available in the supplemental material (Text S2).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mSystems.00186-18.

TEXT S1, PDF file, 0.1 MB.
TEXT S2, TXT file, 0.1 MB.
FIG S1, TIF file, 3.0 MB.
FIG S2, TIF file, 0.7 MB.
FIG S3, TIF file, 0.7 MB.
FIG S4, TIF file, 1.0 MB.

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M.Z., M.A.B., E.H., and J.P.J.H. designed research. M.Z. built the mathematical model. M.Z. and U.B. designed the simulation experiments. M.Z., J.P.J.H., E.H., M.A.B., T.U.B., and U.B. analyzed the results and reviewed the manuscript.

The authors declare no conflict of interest.

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Supplemental Material

Mobile compensatory mutations promote plasmid survival

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Supplemental text

This text provides a more detailed description of the model system. Please note that we deployed our model using the *R*-package shiny (1) and the hosting service 'shinyapps.io', which allows the user to parameterize, execute and explore our model and the following simulation results by an interactive web app (https://martin20.shinyapps.io/plassim/). Since we want to be fully transparent, we also provide the full code, which enables you to check the model and to run it on your local machine (source code S1).

Additional Background information

The conditions enabling plasmid persistence have been addressed in a series of simulation studies reflecting theoretical as well as empirical considerations (2-10). Although there was a long debate about horizontal transfer rates, recent studies demonstrate that common conjugative plasmids are indeed transferred at sufficiently high rates to stabilize the plasmid in the population, even if plasmid costs are high and the environment does not select for plasmid-encoded traits such as mercury resistances (8) or antibiotic resistances (10).

In this study we consider the whole continuum of plasmid traits and environmental conditions, including high or low infectivity, plasmid costs and selection regimes. This allows us to obtain general insights and to explore the 'plasmid fitness space' (Fig. 1 B), which we refer to both components of a plasmid's fitness (11): (i) its vertical transmission fitness, given by the ability to spread within the same cellular lineage and (ii) its horizontal transmission fitness, given by the ability to infect new hosts through conjugation.

Common fitness estimates resulting from empirical measurements often either reflect a comparison of single growth rates or they compare initial and final population sizes of two competing strains or populations (12). In this study we like to adapt this concept and define fitness as a relative estimate by comparing both (i) the growth rates of plasmid-bearers and (ii) their conjugation rates to the growth rates of plasmid-free cells. This estimates how much the single components affect the proliferation of the plasmid-bearers in the next generation. Whereas the relative contribution of conjugation to plasmid fitness often remains inconclusive from empirical studies (12, 13), our mathematical methods enable an exact definition.

Details of the mathematical model

Our model represents a system of ordinary differential equations describing the dynamics of plasmid-free bacteria *F*, non-adapted plasmid-bearers *P* and adapted plasmid-bearers *A* according to growth, dilution (by washout/mortality/predation), segregation, conjugation and compensatory evolution. Equations are presented in a succinct matrix in Table 1 and parameters are described in Table 2 in the main text. The differential equations considering each type of compensatory evolution ('no mutation', 'chromosomal mutation' and 'plasmid mutation') can also be written as follows:

Considering no compensatory evolution ('no mutation'), only plasmid-free cells *F* and (non-adapted) plasmid-bearers *P* compete (the mutation rate χ is assumed to be 0 and no adapted plasmid-bearers *A* are generated):

$$\frac{dF}{dt} = f\left(\psi F + \tau\psi(1-\alpha)P - \gamma FP\right) - (\omega+\upsilon)F$$
$$\frac{dP}{dt} = f\left(\psi(1-\alpha)P - \tau\psi(1-\alpha)P + \gamma FP\right) - \omega P \quad \text{(Equ. E1)}$$

with resource availability $f = 1 - \frac{F+P}{k}$, carrying capacity k, maximal growth rate ψ, plasmid costs α, segregation rate τ and conjugation rate γ.

Considering compensatory mutations located on the chromosome ('chromosomal mutation'), adapted plasmid-bearers emerge proportional to fission with process rate $\chi\psi(1-\alpha)P$ and grow with plasmid costs reduced by amelioration strength β, whereas the compartment of non-adapted plasmid-bearers P benefits from the infection of plasmid-free cells by A (γFA), since A cannot transmit the chromosomal mutation by conjugation:

$$\frac{dF}{dt} = f\left(\psi F + \tau\psi(1-\alpha)P + \tau\psi(1-\alpha(1-\beta))A - \gamma FP - \gamma FA\right) - (\omega+\upsilon)F$$
$$\frac{dP}{dt} = f\left(\psi(1-\alpha)P - \tau\psi(1-\alpha)P + \gamma FP + \gamma FA - \chi\psi(1-\alpha)P\right) - \omega P$$
$$\frac{dA}{dt} = f\left(\psi(1-\alpha(1-\beta))A - \tau\psi(1-\alpha(1-\beta))A + \chi\psi(1-\alpha)P\right) - \omega A \quad \text{(Equ. E2)}$$

with resource availability $f = 1 - \frac{F+P+A}{k}$, and, in addition to those listed for Equ. E1, amelioration strength β (of compensatory mutation) and mutation rate χ.

Considering compensatory mutation located on the plasmid ('plasmid mutation'), the compartment of adapted plasmid-bearers A benefits from the rate γFA (transmission of the compensatory mutation with the plasmid to the infected plasmid-free cells F) and $\chi\gamma FP$ (mutation proportional to plasmid replication after conjugation):

$$\frac{dF}{dt} = f\left(\psi F + \tau\psi(1-\alpha)P + \tau\psi(1-\alpha(1-\beta))A - \gamma FP - \gamma FA\right) - (\omega+\upsilon)F$$
$$\frac{dP}{dt} = f\left(\psi(1-\alpha)P - \tau\psi(1-\alpha)P + \gamma FP - \chi\psi(1-\alpha)P - \chi\gamma FP\right) - \omega P$$
$$\frac{dA}{dt} = f\left(\psi(1-\alpha(1-\beta))A - \tau\psi(1-\alpha(1-\beta))A + \gamma FA + \chi\psi(1-\alpha)P + \chi\gamma FP\right) - \omega A \quad \text{(Equ. E3)}$$

The features of the biological system we like to capture with our mathematical model are those of a well-mixed aquatic system. In laboratory experiments this is often mimicked by the use of chemostats, which enable a stabilization and homogenization of certain environmental conditions. In such systems bacteria can grow in a physiological steady state, with a specific growth rate that depends on a species- and substrate-dependent maximal growth rate, here denoted as ψ. Furthermore, washout and death of bacteria determines the loss of bacteria from the system domain, here denoted as the dilution rate ω. If $\psi < \omega$ all bacteria will be washed out, because the bacterial population cannot sustain itself. Otherwise, if $\psi > \omega$ the bacterial population will reach a constant specific growth rate at steady state that is equivalent to the dilution rate ω, because then, when the system approaches the stationary phase, growth is limited by the proportion of bacteria that become washed out or die. In our model, this competition for resources is considered explicitly. Any resource-dependent processes are assumed to be limited by a carrying capacity *k*, modeled according to the standard logistic law, given by the factor *f* in the reaction rates in Table 1 and equations E1, E2 and E3. This approach has been previously applied for growth in a plasmid population model (6), but we also account for a resource-dependency of conjugation, which has been highlighted in many studies (3, 14-21), and the influence of resource-dependent growth and conjugation on segregation and compensatory evolution. It is important to account for a resource dependency of conjugation, since we would otherwise assume that bacteria are infected with the same rate, even if bacterial growth approaches zero, when resources are exhausted.

For convenience, we refer to relative instead of absolute densities in our model. This means bacterial densities of *F*, *P* and *A* range between 0 and 1 and the carrying capacity *k* is always 1, i.e. equals the maximal relative cell density.

Growth

Plasmid-free bacteria *F* grow with respect to a maximal growth rate ψ, whereas plasmid-bearers *P* suffer according to some plasmid costs α, that can be compensated to a certain extent for adapted plasmid-bearers *A* by amelioration strength β.

Mortality

All three compartments *F*, *P* and *A* are influenced by a dilution rate ω, which represents an unspecific homogeneous reduction of the entire population that can be referred to natural mortality, bacterial washout, predation pressure or the combination of these effects. It is assumed that only plasmid-free bacteria *F* are sensitive to the action of a bactericidal antibiotic, given by the antibiotic killing rate υ. This implies that plasmids remain the sole source of antibiotic resistance genes or that only plasmid carriage and the associated multiplicity of resistance genes enables a sufficient degree of resistance.

Segregation

The probability to produce a plasmid-free daughter cell during bacterial fission is considered to be a random event, represented by the probability τ. In nature this probability can be linked to the plasmid copy number, in the following denoted as *c*. Assuming that segregation is totally random, the probability for segregation could also be calculated by 0.5^{c-1} (11). Although this is an oversimplification, as certain mechanisms can prevent plasmid loss, it enables us to evaluate the effect of certain degrees of plasmid instability. For the sake of simplicity, and because the test of an extended model version revealed that the associated effect is marginally, it is further assumed that all plasmid-bearers that lose their plasmid turn into plasmid-free cells, even those bacteria that acquired a chromosomal mutation.

Conjugation

Plasmid transfer in mixed cultures can be described by simple mass-action models (2, 4, 14, 22, 23). Whereas growth, mortality and segregation are first-order reactions, conjugation follows the law of mass action as a second-order reaction of plasmid-bearing and plasmid-free cells. This assumes that (i) mating occurs at random with a frequency proportional to the joint frequency of bacteria, (ii) the ratio of plasmid-free to plasmid-bearing bacteria is not significant, (iii) the time since last receipt or transfer has no effect on transfer rates and (iv) all bacteria or plasmids of the same type have

identical properties. Although these are simplifying model assumptions, they have already been demonstrated to be applicable to both broth and chemostat experiments for varying plasmid types (14). Similarly, the conjugation rate in our model is defined by a single parameter γ (Table 1).

The results presented in the main text were generated using relative cell densities, which also assumed that the carrying capacity *k* equals one. If instead absolute values for cell densities and the carrying capacity are given, for instance in cells / ml, the conjugation rate has to be scaled relative to the carrying capacity *k* in order to generate comparable results:

$\gamma_{abs} = \gamma / k$

The resulting estimates for γ_{abs} reflect typical values as those generated by the end-point-method (22). For example utilizing our default parameter estimates (Table 2) and assuming $k = 10^9$ we obtain a conjugation rate $\gamma = 2e^{-11}$.

Nevertheless, in this study we considered relative cell densities to account for the relative extent of both plasmid costs and conjugation. This enabled us to directly compare the conjugation rate γ with the plasmid costs α in relation to the hosts maximal growth rate ψ.

Adaptation

In the initial state, only plasmid-free bacteria *F* and non-adapted plasmid-bearers *P* are present. Compensatory evolution by chromosomal or plasmid mutations leads to the emergence of adapted plasmid-bearers *A*. It is assumed that beneficial mutations are acquired with a rate χ in the course of replication events. Whereas the bacterial chromosome replicates during fission, plasmid replication takes place according to fission and conjugation.

In this study, the conjugation rate γ does not change as an effect of compensatory evolution. This means γ is fixed at the same level for both non-adapted plasmid-bearers *P* and adapted plasmid-bearers *A* within a single simulation run. Changing this feature might convey interesting results in further investigations.

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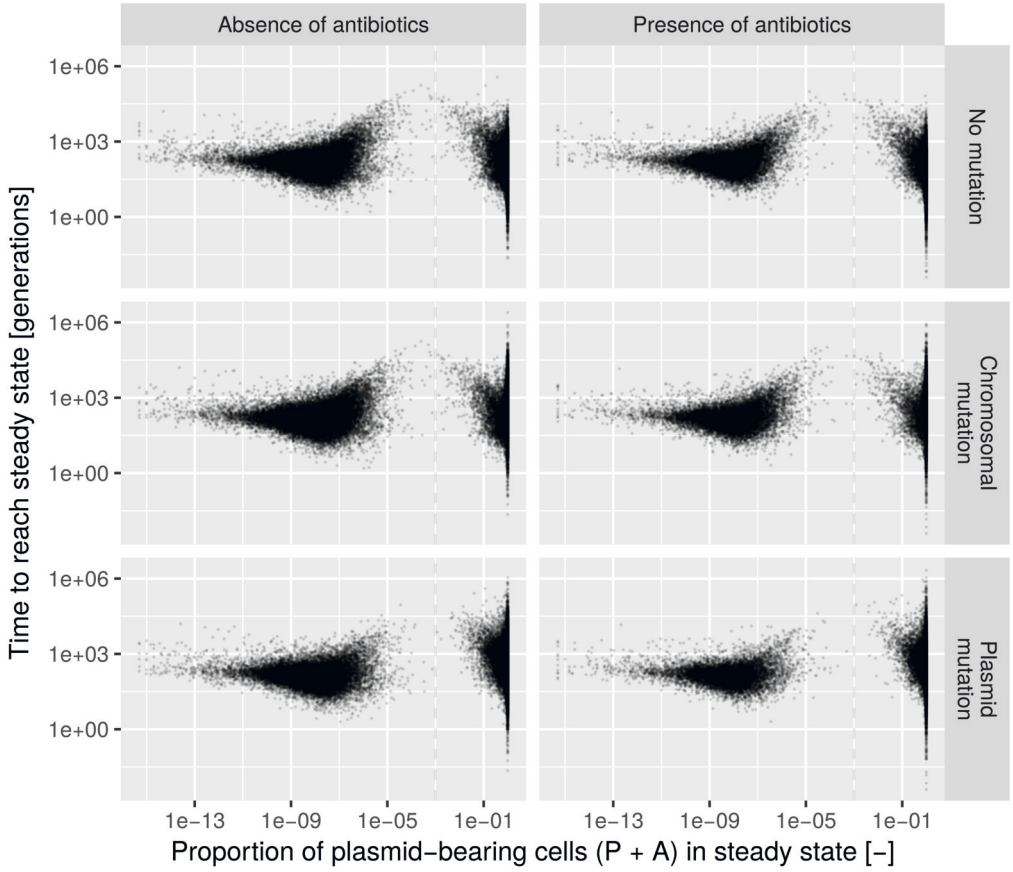


FIG S1 Characterization of the steady state of all simulations run in simulation experiment II (Table 2) by persistence time and proportion of plasmid-bearing cells. Each point reflects the conditions when a numerical steady state is reached for a given combination of plasmid-host properties and environmental conditions. Columns indicate the results for simulations considering the absence (left) or presence (right) of antibiotics. Rows distinguish the results for the types of compensatory evolution ("no mutation," "chromosomal mutation," and "plasmid mutation"), which differ only slightly on this scale. It can also be seen that the prevalence of plasmids in steady state is bimodal, which enabled us to define a threshold (dashed line) to accurately distinguish between long-term plasmid persistence (right from dashed line) and extinction (left from dashed line).

Download FIG S1, TIF file, 2.9 MB.

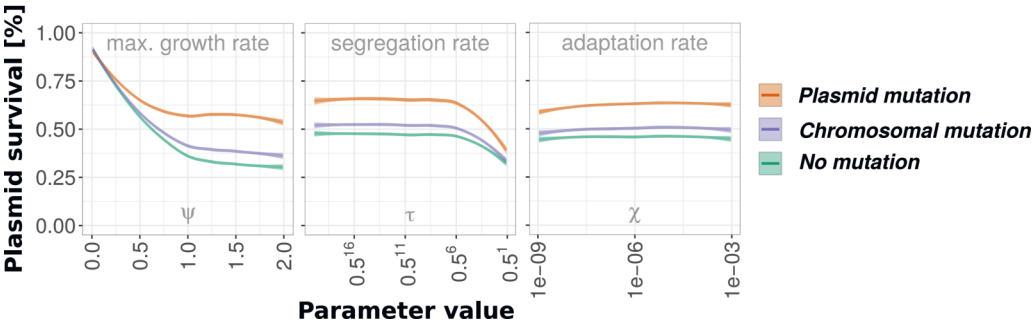


FIG S2 Nonlinear main effects of remaining model parameters not shown in Fig. 4 in the main text. Results refer to the global analysis considering the presence of antibiotics (Table 2, simulation experiment II). Here, it is consecutively examined how the variation of a single parameter within its predefined range affects the overall result of all tested conditions.

Download FIG S2, TIF file, 0.7 MB.

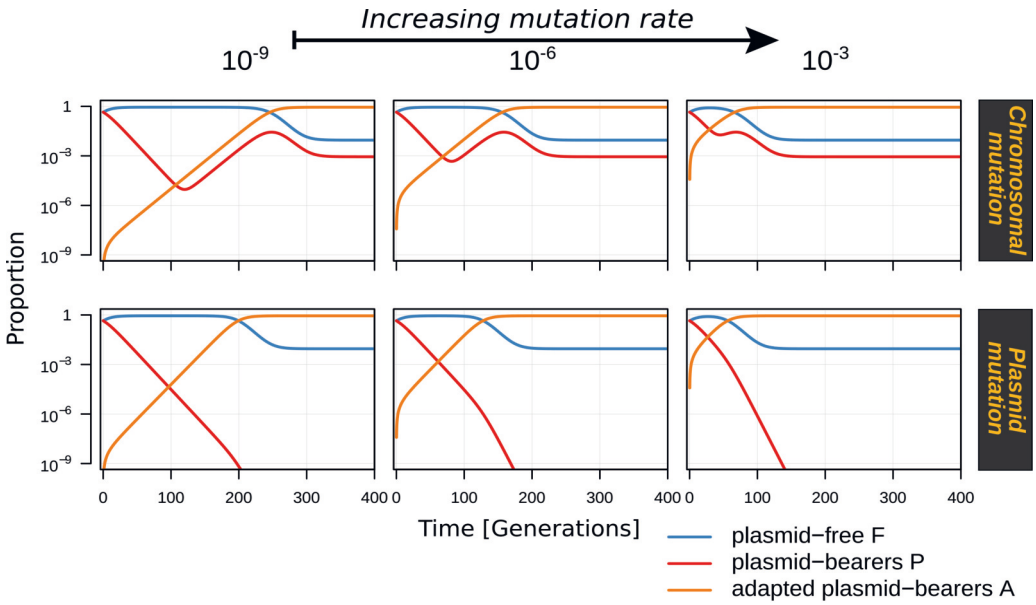


FIG S3 Increasing mutation rates accelerate plasmid prevalence. Rows refer to the types of compensatory evolution ("chromosomal mutation" and "plasmid mutation") and columns to increasing mutation rates, χ, namely 10^{-9}, 10^{-6}, and 10^{-3}. All other parameters are as the default (Table 2), with antibiotic action as $\upsilon = 10^{-2}$. Overall, an increase of χ by as much as 10^6 results only in a 3-fold increase (here around 160 generations) of the time until plasmids reach their most prevalent state.

Download FIG S3, TIF file, 0.7 MB.

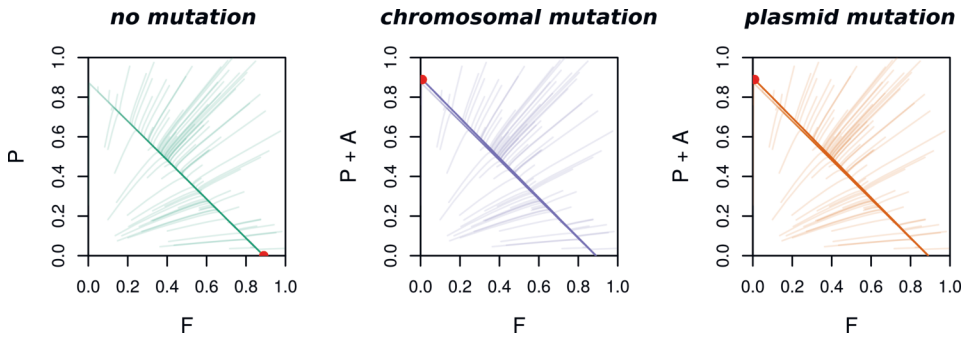


FIG S4 Mathematical phase planes for the population dynamics considering "no mutation," "chromosomal mutation," and "plasmid mutation." Curves show the trajectories from random initial relative frequencies of plasmid-free cells, *F*, and nonadapted plasmid bearers, *P*. Red dots mark the stable fixed points to which the trajectories are attracted, which demonstrates that the steady-state results are independent from the initial conditions. Parameters are as the default (Table 2), and antibiotic action is set to a moderate level ($\upsilon = 10^{-2}$). All model versions show the same behavior and turn to the same attraction point in the beginning, but the evolution of adapted plasmid bearers, *A*, given by a compensatory mutation located on the chromosome or the plasmid, causes the movement to a reverse attraction point and enables the persistence of the plasmid.

Download FIG S4, TIF file, 0.9 MB.

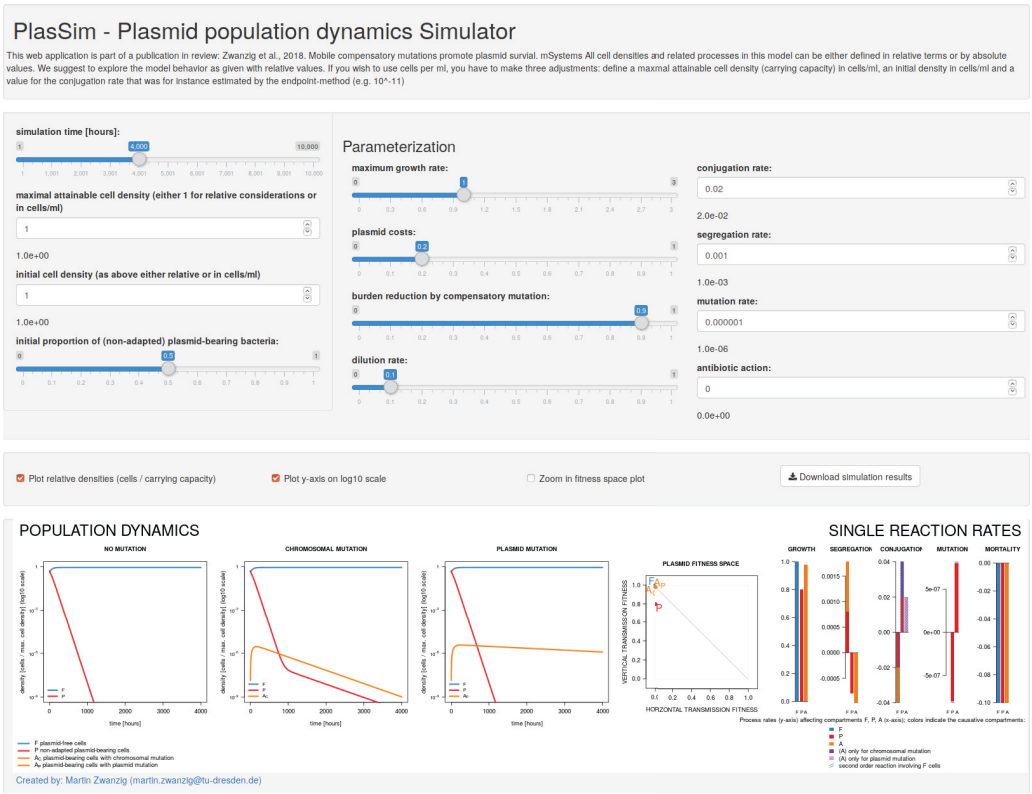
TEXT S2

The computer code of the mathematical model, representing a platform to perform simulations with our model called PlasSim (for plasmid population dynamics simulator), equal to the interactive web app at https://martin20.shinyapps.io/plassim/, which can also be used to download the results of simulations that were run with self-defined parameters.

Download Text S2, TXT file, 0.02 MB.

SHINY APP

https://martin20.shinyapps.io/plassim/



Screenshot of the PlasSim model as a browser-based simulation tool for plasmid dynamics

3 Article II (published) – **Conjugative plasmids enable the maintenance of low cost non-transmissible plasmids**

Non-transmissible plasmids, that means those that can neither be transmitted by conjugation nor mobilization, are frequent plasmid types, but their maintenance is supposed to depend on positive selection of plasmid-encoded traits. Here it is shown that the co-occurrence of incompatible and more costly conjugative plasmids facilitates the persistence of non-transmissible plasmids in a bacterial population, even if they are not beneficial to their host. To demonstrate and test this mechanism, all relevant information about bacteria bearing different plasmid types were compiled and theories for their behavior were formulated by means of an individual-based simulation model providing a comprehensive test of the contrasting hypotheses. The results demonstrate that the proposed mechanism for the maintenance of non-transmissible plasmids is robust. Although the model represents a simplification of the real world, it is considered to be structurally realistic. Its general behavior is in line with empirical observations.

This study is the first that demonstrates such a mechanism for the maintenance of non-transmissible plasmids. It is also the first that considers that transfer genes of transmissible plasmids are turned on or off in dependence to the local conditions (mimicking conjugation pheromones) and that simultaneously accounts for plasmid incompatibility not as a mechanism as surface exclusion, but the probability that arising daughter cells may contain only one type of plasmid. It is believed that this study improves the understanding how local adaptation through conjugation pheromones and interactions of co-occuring plasmid types affect plasmid population dynamics.

This chapter represents a peer-reviewed publication in *Plasmid* from April 2017, entitled „Conjugative plasmids enable the maintenance of low cost non-transmissible plasmids“. Co-authors are Prof. Dr. Thomas Berendonk from the Chair of Limnology at the TU Dresden and Prof. Dr. Uta Berger, the first supervisor of this PhD study. Please note that this work was published before my surname changed from Werisch to Zwanzig with my marriage in June 2017.

This research article was published in *Plasmid* 91 in May 2017 and is not open access:

Werisch, M., Berger, U., Berendonk, T.U. (2017). Conjugative plasmids enable the maintenance of low cost non-transmissible plasmids. Plasmid 91, 96-104.

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4 Article III (submitted) – **The autopoiesis of plasmid diversity**

The persistence of plasmids in nature when they are not beneficial to the bacteria that carry them seems to be paradox and represents currently one of the most important research topics in microbiology and environmental medicine, because it has direct implications to human health. What are the mechanisms behind the alarming spread of plasmid-mediated antibiotic resistances recently observed outside the clinical environment, for example in freshwater systems? This study focuses on the role of biotic interactions that originate from the diversity of plasmid types themselves. Although this is close to the natural conditions, this has hardly been investigated so far, since empirical studies, including lab experiments, are limited in their logistics. By means of simulation experiments, however, it is shown that interactions between plasmid types control their diversity and maintain antibiotic resistance even when abiotic selection by antibiotics stopped. These findings thus change existing theory about the existence conditions of plasmids, which assumes that costly traits can only persist through frequent positive selection by the abiotic environment. Although alarming, the provided mechanistic insights can help to improve plasmid curing and anti-plasmid approaches that are currently developed in order to actively support the reversal of plasmid-mediated antibiotic resistances.

This study is the first demonstrating that the plasmid diversity found in nature matters for the autopoiesis of the system. It illustrates and explaines the consequences for population dynamics and for the probability of varying plasmid types to survive. Since transparency in science has never been more important, the full computer code of the simulation model is provided. This comes with an app that allows the user to study the model's behavior by varying each of its parameters with easy-to-use sliders. In order to validate the generality of the proposed mechanism, simulation results were compared to empirical patterns and manifold robustness tests were performed to check whether the results strongly depend on the underlying model assumptions or not. The work combines current knowledge on mechanisms at cell and population level and will hopefully stimulate new empirical studies.

This chapter represents a manuscript close to submission for peer-review and is entitled „The autopoiesis of plasmid diversity“. Co-author of this manuscript is Prof. Dr. Uta Berger, the first supervisor of my PhD study.

The autopoiesis of plasmid diversity

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ABSTRACT

The diversity of plasmids found in nature represents a phenomenon. Although abiotic selection of plasmid-encoded traits might explain how plasmids could persist in a limited spatiotemporal frame, the mechanisms maintaining plasmids as ubiquitous and extremely diverse mobile genetic elements are yet poorly understood, particularly concerning regarding the spread of antibiotic resistance. Here we show that inter- and intracellular competition enables plasmid diversity to be self-preserving and thus autopoietic. Without any fine-tuning of the parameters of our model, a network of intransitive substructures emerged, which lack strict competitive hierarchies and maintain plasmid types varying in costs, transmissivity and incompatibility. These features determine the differentiation of plasmid niches, which can be occupied by different plasmid types in each local community of a fragmented population. Our findings explain how the huge genetic repertoire of plasmids could be preserved in nature; and demonstrates how costly plasmid-mediated antibiotic resistances are maintained despite stopped abiotic selection.

In the face of a global spread with plasmid-mediated antimicrobial (or antibacterial) resistance, new plasmid curing and anti-plasmid approaches have been developed. These promising tools could help to combat antibiotic resistance genes in humans, animals and the environment[1], but more research is needed to uncover how safe and effective such methods are *in vivo*[2] and *in natura*[3]. This requires a better understanding of the importance of interactions between different plasmids, which is an open challenge[4].

Natural communities that generate subpopulations with varying plasmid content are at an advantage, since genetic variation is key for bacteria to cope with environmental uncertainty[5]. In addition to the stable core genome, bacteria gain and loss genes which are highly mobile between bacterial cells[6] and can make up 90% of a species pan-genome[7]. In this context, the plasmidome, that refers to the entire plasmid DNA of an environmental sample[8], is an important current object of study[8–14]. It has been shown that the number, size and functions of the plasmids found in different genera[15], single species[9,13], strains[16] or in environmental samples[8,10–12] can vary remarkably, including many plasmids encoding selfish traits as stability and conjugation[17]. Meta-analysis of sequence data reported that associations between small and large plasmids are specifically frequent[18], but it is still not completely clear why. It also remains unsolved, how a high diversity of plasmids that encode no or unknown accessory functions persist, for example, in freshwater habitats[12].

The existence conditions of plasmids have so far been considered mainly from the perspective of pairwise plasmid-host interactions. According to this, plasmid survival basically relies on their own fitness, which has two dimensions[19]: vertical transmission fitness (spread to the daughter cells of the host by binary fission) and horizontal transmission fitness (spread to new host cells, e.g. by conjugation). High conjugation rates[20], compensatory evolution[21,22] as well as high or low frequency pulses of positive selection of accessory functions such as antibiotic resistances[23,24] can slow down or prevent plasmid-loss. If bacteria can incorporate the beneficial plasmid gene into their chromosome, plasmids might only persist because they are able to transfer back and fourth between noncompeting species or ecotypes[25].

Understanding how species interactions modulate biodiversity is a central aim in ecology that benefits from a recognition of the multidimensional nature of a species niche[26]. A high level of within-population genetic diversity can also be maintained by dynamic cycling of two co-evolving species or populations, similar to the mechanisms associated with Van Valen's "Red Queen hypothesis"[27]. The theory emphasizes that biotic conflicts likely drive selection by frequent evolutionary changes, even if abiotic factors put strong environmental selection to a community. Within this study, we do not consider explicit evolutionary changes at the gene level, but we observe how dynamic rearrangements of the plasmid composition on the bacterial level alter the competition between various plasmid-host associations. Thus we extend the concept of Red Queen dynamics from the original two species (or populations) model to diverse communities with more than two players, which can provide a new perspective for understanding inter- and intraspecific coevolutionary dynamics[27].

A model of the plasmidome

We compiled all relevant information about plasmids and formulated theories for their interaction in a clonal bacterial population by means of an individual-based simulation model of the plasmidome. It aims to investigate the role of biotic interactions on

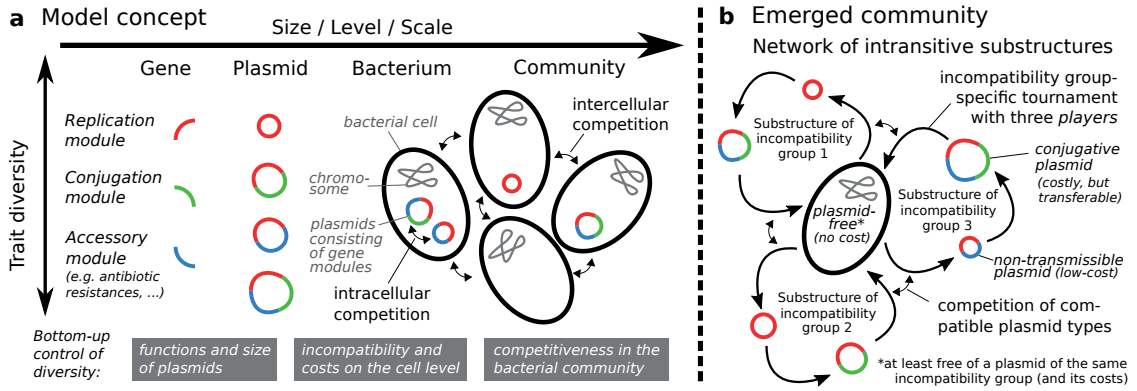

Figure 1. Concept and emerging community characteristics of the individual-based plasmidome model. a: Plasmid traits are determined by their gene modules. Larger plasmids are considered to be more costly for the host, but might also be able to secure their own propagation by conjugation and/or the provision of additional functions such as resistance to antibiotics. **b**: Simulating evolutionary selection reduced plasmid content, but led to the emergence of a stable community, representing a network of intransitive substructures that promote the maintenance of at least one conjugative and one less costly non-transmissible plasmid type per incompatibility group.

plasmid community dynamics and the fate of antibiotic resistance. In brief, the model considers that plasmid types spread by cell fission and horizontal transfer (conjugation) with respect to their individual plasmid costs associated with replication, conjugation and accessory genes (Fig. 1a). Plasmids get lost due to competition for space, whereas (i) the host cell's ability to compete with neighboring bacteria decreases with increasing costs for all resident plasmids, (ii) entry-exclusion prevents a co-residence of incompatible plasmid types in the same host cell, and (iii) the host cell can be killed through the action of an antibiotic (if not carrying a resistance plasmid, but antibiotics are present). Robustness tests show that the model is flexible in dealing with a wide range of assumptions. They are presented in the methods section along with a more detailed model description.

We performed two different simulation experiments that show the dynamics of a diverse plasmid community (i) in 'non-polluted environments', which refers to the complete absence of abiotic selection, and (ii) in 'polluted and restored environments', where the action of an antibiotic that kills sensitive cells stops after some time and no longer benefits costly resistance plasmids.

Diversity in non-polluted environments

To test if at all, how long or to which degree plasmid diversity can be maintained by intra- and inter-cellular interactions alone, we run our model assuming that the environment does not select for any plasmid-encoded trait. The initial plasmid community was constructed by ca. 40,000 different plasmid types, each belonging to one of the given incompatibility groups and associated with random costs for replication (α_{rep}), accessory genes (α_{acc}) and conjugation (α_{con}). Half of the plasmids were considered to be 'non-transmissible' (by horizontal gene transfer) and to impose no costs for conjugation.

In a first step, we performed single model runs to assess the basic model behavior when either 1, 3 or 5 different plasmid incompatibility groups are present. Our results show that, although the enormous initial plasmid diversity declines quickly, a small final set of diverse plasmid types is actually maintained in the evolved communities (Fig. 2). This is induced by various consequences of the given biotic interactions.

Local interactions influence the spatial distribution of plasmid types. An initially random spatial distribution of diverse plasmid types rapidly evolves to a highly structured form, characterized by an aggregation of plasmid-free bacteria and plasmid types that reside alone or in conjunction with other plasmid types on bacteria (Fig. 2a). The more incompatibility groups are present, the greater the number of diverse plasmid types and small-scale changes in their configuration. The proportion of plasmid-free bacteria decreases, the more plasmid incompatibility groups are present in the plasmid community.

Opposing survival strategies lead to cyclical changes in the community composition (Fig. 2b): a rapid proliferation of plasmid-bearing cells when plasmid-free cells are common, followed by a fast decline when they are rare. This is associated by an enhanced spread of highly infectious conjugative plasmid types, if they can access big aggregates of plasmid-free cells. When the latter have been infected and the remaining plasmid-free cells are distributed rather uniformly, the number of

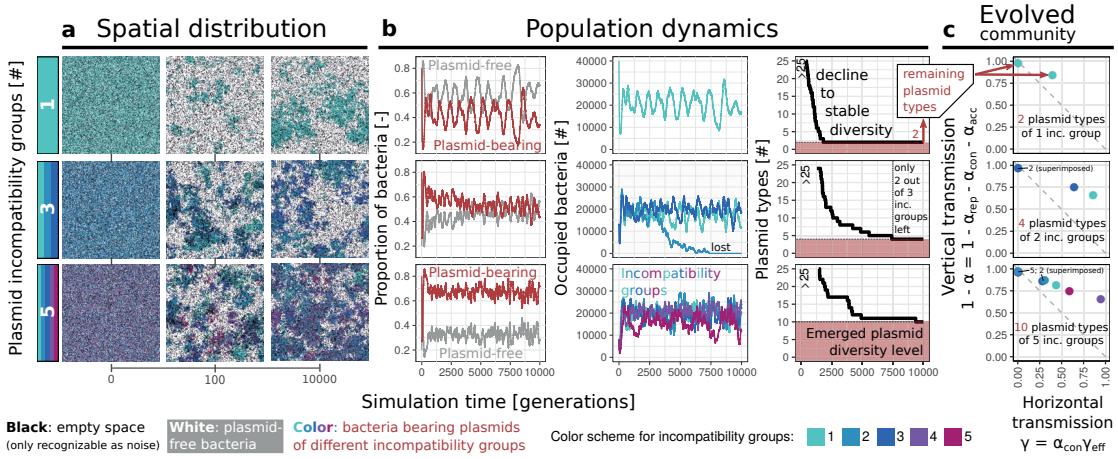

Figure 2. Evolution of plasmid communities. Each row represents a single simulation run considering either 1, 3 or 5 initially present plasmid incompatibility groups. **a**: The emergence of an aggregated spatial distribution; only incompatibility groups are distinguished, not plasmid types; if multiple incompatibility groups are present in a bacterium, only one color is shown. **b**: Oscillatory population dynamics and the decline of plasmid diversity to a stable state that depends on the number of present incompatibility groups. **c**: Emerged communities comprising one conjugative and one less costly non-transmissible plasmid type per remaining incompatibility group.

successful horizontal gene transfer events drops. Especially in this state, when the conditions for horizontal transmission are poor, non-transmissible plasmid types can play out their advantage over conjugative plasmid types due to their higher vertical transmission fitness, which is related to the absence of the conjugation module and consequently associated with lowered costs. As the local abundance of non-transmissible plasmid types increases to the detriment of conjugative types, the chance of a successful (re-)proliferation of plasmid-free cells increases, since without any plasmid load they have a higher fitness than cells bearing one or more non-transmissible plasmid types. Thus, the cycle starts again, since a rise of plasmid-free cells accelerates conjugation.

During evolution, competitive exclusion minimizes plasmid diversity to a locally sustainable level. Plasmid types, who are not able to take their chance, are quickly outcompeted. The oscillatory dynamics intensifies this eradication process, especially when plasmid diversity is still high. Even all plasmid types of an incompatibility group can get lost due to competitive exclusion. This is particularly likely when cycle-relevant interactions are disturbed by a fragmented spatial distribution of the associated plasmid types. If, for example, the remaining non-transmissible plasmid type of an incompatibility group cannot reach conjugative plasmid types of the same incompatibility group, it can only compete with plasmid types it is compatible to. This is less favorable than the opportunity to directly replace the conjugative counterpart, since this is likely more costly and cannot prevail by horizontal transmission over plasmids it is incompatible to.

Finally, the composition of the evolving plasmid community can reach a quasi stable state. Thus, intransitive dynamics stably maintains a certain number of diverse plasmid types in local communities. This is at least one non-transmissible and one conjugative plasmid type per (remaining) incompatibility group (Fig. 2c). Although the survival of individual plasmid types is driven by chance, they do represent specific traits. Whereas the remaining non-transmissible plasmids are those representing a maximum vertical transmission (lowest costs), the remaining conjugative plasmids reflect a variable horizontal transmission efficiency, but are more costly than the non-transmissible plasmids (of the same incompatibility group). Since our model does not impose any of such relations, it is rather surprising that this fulfills the conditions of a tournament equal to the children's game rock-paper-scissors: non-transmissible plasmids can outcompete the more costly conjugative type, which themselves can infect the plasmid-free cells that would otherwise outcompete the cells bearing the non-transmissible type. If more than one incompatibility group is present, such an intransitive dynamic occurs more than once, representing a multi-layered interaction network. The evolution of a diverse plasmid community is therefore characterized by the emergence of a network of intransitive substructures (Fig. 1b), which enables the maintenance of plasmid diversity itself. This suggests that plasmid diversity is a result of autopoiesis.

To test the generality of the observed pattern, we made a series of repetitions and compiled some summarized statistics

(Fig. 3a), assuming that any of these repeated simulations represents a local community of a fragmented population, e.g. similar to the conditions in soil[28]. These simulations confirmed our previous results and showed that randomness of purifying selection causes differences in plasmid content of local communities. Competition rapidly eliminates plasmid types with the lowest fitness regarding their vertical and horizontal transmission efficiency. Only those plasmid types that represent an optimized trade-off between costs and mobility can persist. Very costly plasmids are maintained, but only by high conjugation rates. Non-transmissible plasmids are only maintained if they have very low costs, since selection effectively eliminates all non-transmissible plasmids of the same incompatibility group that exert higher costs, but do also not provide any beneficial function for the host cell. When more incompatibility groups are present, more costly non-transmissible plasmids are able to persist, which shows that such purifying selection appears to be less effective considering a higher degree of interaction. Nonetheless, the increase of plasmid diversity with the number of incompatibility groups is limited by the size of the local population (Fig. S2).

Diversity in polluted environments

To investigate the associated effects of antibiotic-mediated plasmid selection, we extended the model considering that an antibiotic is constantly present and kills sensitive cells with a certain probability υ, but some plasmids confer resistance to this antibiotic.

Our results show that plasmid diversity can be maintained in the presence of antibiotics if the present plasmid types belong to more than one incompatibility group (Fig. 3b). Moreover, even when abiotic selection by antibiotics stops, plasmids encoding costly antibiotic resistance can persist. This holds as long as the conditions for intransitive dynamics are still met. If there is only one incompatibility group, plasmid diversity is more easily lost, since only one plasmid conferring resistance at the lowest costs could remain from the time of abiotic selection. As a result, no other plasmids are present that could help to preserve the costly plasmid providing antibiotic resistance, when this is no longer beneficial.

We believe that two mechanisms are central for the niche differentiation of plasmids, which enables the preservation of plasmid diversity and the persistence of plasmid-mediated antibiotic resistance even in the absence of abiotic selection. First, this is the plasmid survival strategy that is either focused on vertical transmission, usually represented by non-transmissible plasmids that are optimized to be less costly, or on horizontal transmission, usually represented by horizontally transferable plasmids that exert higher costs, e.g. by conjugation. Second, this is the inability of plasmids of the same incompatibility group to persist in the same host cell. A certain combination of survival strategy and incompatibility group therefore represents a potential niche for a plasmid that shares the same characteristics. Nonetheless, the survival of a specific plasmid is not deterministic, since there are likely many other plasmids that aim to occupy the same niche and are equally suited. The higher the plasmid fitness, the more likely a plasmid will succeed this competition, but the survival in a local community, as represented by a single simulation run with our model, is still to a certain extent random. This stochasticity on a local scale increases the overall plasmid diversity of a population, analogous to natural communities consisting of fragments that are limited in size and degree of interaction, e.g. as in soil[28].

The properties of the plasmids that are able to survive as part of the evolved communities in our simulation experiments (Fig. 3c) match key characteristics reported from bioinformatic analysis of plasmids in the GenBank database[29,30]: Non-transmissible plasmids are usually smaller than conjugative plasmids, i.e. less costly due to the smaller range of functions they provide. Since some non-transmissible plasmids carry costly antibiotic resistances, their distribution has a bimodal form. This has also been described for empirical data, explained with the presence of external forces, e.g. antibiotic-mediated selection, which allow the survival of more costly non-transmissible plasmids encoding resistances. Our model demonstrates that this pattern also emerges considering restored environments that lack any present abiotic control. Considering the paradigm of pattern-oriented modelling[31], we were able to design a structurally realistic model, although we did not apply any parameter fitting to force this result.

Discussion and conclusions

In this study we examined the consequences of plasmid diversity for plasmid population dynamics and persistence. We constructed an individual-based model of the plasmidome. Assuming a constant, homogeneous abiotic environment and a single constant genetic background in the host, we observed which population level characteristics emerge, when multiple plasmid types that differ in cost, transfer probability and incompatibility interact inter- and intra-cellularly with each other. We found that these biotic interactions between diverse plasmid types lead to the evolution of a system lacking strict competitive hierarchies, which promotes the persistence of plasmid diversity itself and extents the conditions of plasmids coexistence to a so far unrecognized level. This is due to the nature of the intransitive system, which can provide niches even for plasmid types such as non-transmissible plasmids conferring costly antibiotic resistance that could otherwise not survive. Such intransitive competition has long been overlooked, but is now recognized to shape species coexistence for a variety of taxa at various ecological scales[32], including microbial communities[33]. This also fits well with the observation that interactions in more

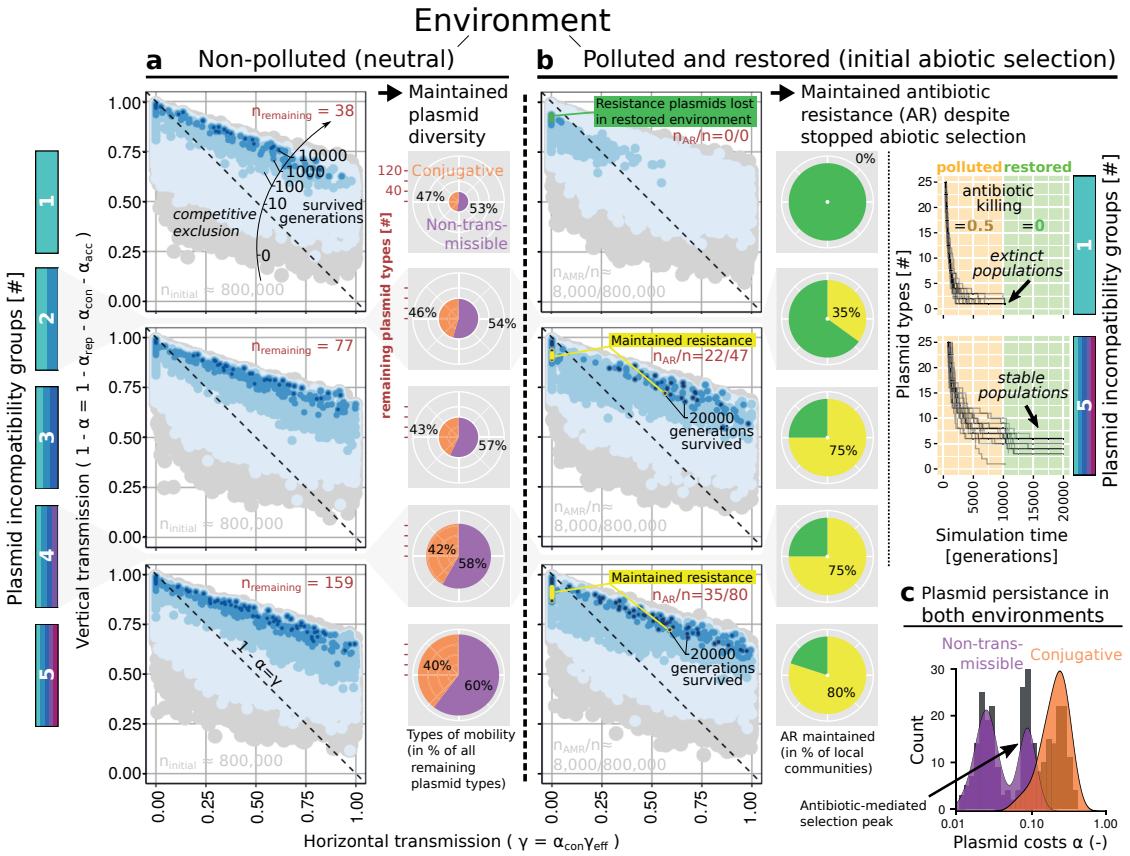

Figure 3. Plasmid content of multiple independently evolved local communities. Each subfigure depicts the outcome of 20 repetitions for the respective setting (environment, incompatibility groups). Plasmid diversity increases with the number of incompatibility groups. Interactions between plasmids belonging to different incompatibility groups maintain (**a**) a high plasmid diversity in the absence of abiotic selection of plasmid-encoded functions and (**b**) plasmids conferring costly antibiotic resistance (AR) even though selection by an antibiotic that kills sensitive cells is stopped (here after 10000 generations). **c**: Association between costs and mobility of plasmid types persisting in all presented scenarios ('non-polluted' and 'polluted and restored'; see Fig. S1 for more details).

complex communities may limit the response to abiotic selection, as recently demonstrated for competitive bacterial species[34] and across trophic levels[35].

The insights provided by this article might be used to improve strategies that aim to actively support the reversal of plasmid-mediated antibiotic resistance. For example, a resistance plasmid might be pushed out of its niche through the introduction of a synthetic plasmid that belongs to the same incompatibility group and follows the same survival strategy, but is more competitive due to a higher fitness considering both vertical and horizontal transmission. The obvious next step is to perform laboratory experiments to tests whether the mechanisms presented here promote plasmid diversity and the persistence of plasmid-mediated antibiotic resistance in practice. We call for studies that further investigate the role of biotic interactions, involving varying plasmids, bacterial species and other mobile genetic elements, because only if we try to be a little as complex as nature we will get results that are also valid there.

Methods

An individual-based model (IBM) was developed, since this allows to account for individual variability, local interactions and adaptive behavior. These features are important for the study of plasmids co-existence in microbial communities and provide insights that cannot be obtained with classic population level models[36]. Finally, we applied a series of robustness tests to check how strong our results depend on the underlying model assumptions, which is essential for the understanding of the model itself and the identification of 'robust theories' about the functioning of the ecological system[37].

Model description

Here we present a model description following the Overview, Design concepts and Details (ODD) standard protocol for individual- and agent-based models[38]. It is proposed to facilitate evaluation and comparison of IBM and consists of seven elements, which will in turn be used to address general and specific aspects. The model was implemented using the software platform NetLogo 6.0.2[39] that works on a Java virtual machine.

Purpose

The mechanisms enabling the diversity of plasmids observed in nature are not fully understood. Although plasmids can be beneficial if they encode for any environmentally selected function, they may otherwise represent a burden to their host cell and can become eliminated within a few hundred generations. Frequent environmental selection can maintain single, sufficiently beneficial plasmids, but this mechanism does not explain the diversity of plasmids in size and function as observed in nature. The model primarily addresses whether intrinsic mechanisms, i.e. those that originate from plasmid diversity itself, are capable to regulate and maintain it.

Entities, state variables and scales

The model composes two entities: lattice cells and plasmids. Each lattice cell is either empty or occupied by a single bacterium. A bacterium may bear a number of plasmid types that reside as individuals on the respective cell.

State variables of lattice cells are location (integer x- and y-coordinates of the lattice point) and occupation (representing a bacterium or an empty lattice cell). The state variables for plasmids are the association with a certain bacterium (=location), and costs of certain gene modules related to replication, conjugation and accessory functions. The variable efficiency of conjugation determines how costs of the conjugation module are related to the realized plasmid transfer probability. The incompatibility group defines those plasmid types that cannot co-occur in the same cell. All state variables are listed in Tab. 1. During initialization, random states are assigned to the plasmids, which - besides location - do not change over time and depend on distribution parameters that are used exclusively for initializing the model. (Tab. 2). Other constant, but global model parameters are mortality, antibiotic action, as well as segregation.

The model world consists of a grid with a dimension of 250×250 lattice points and periodic (or 'wrap-around') boundaries. It represents thus a surface-attached population of maximal 62,500 bacteria. The model world may correspond to one or a half square millimeter of a biofilm surface, assuming an average bacterial cell size of about 1 or 2 μm and distances between bacteria in the same order of magnitude. Each model time step represents one hour. A certain number of time steps is required to generate as much new cells by bacterial fission as are given for an existing population. This is defined as the time of one generation. Experiments simulating 'non-polluted environments' lasted for 10000 generations and for 'polluted and restored environments' 20000 generations.

Process overview and scheduling

At each time step, lattice cell states are updated sequently in random order, following the models decision rules (Fig. S3). For each lattice cell update a random lattice cell is chosen (the so called "focal cell"). If the focal lattice cell is not occupied by a bacterium, nothing happens. If it is occupied either mortality, fission or horizontal gene transfer happens according to the probabilities for these events. The probabilities depend on the particular bacteria-plasmid association characterizing the current

Table 1. Model entities and associated state variables.

ENTITY	ATTRIBUTE	STATES (/SYMBOL)	MEANING
Lattice cell (/Bacterium)	Location	x-y-coordinates	Lattice position
	Occupation	Empty | Bacterium	Considered as bacterium or not
Plasmid	Location	x-y-coordinates	Association with bacterium xy
	Replication module costs	α_{rep}	Costs associated with the expression, repair and replication of genes from these single plasmid modules
	Accessory module costs	α_{acc}	
	Conjugation module costs	α_{con}	
	(overall) plasmid burden*	$\alpha = \alpha_{rep} + \alpha_{acc} + \alpha_{con}$	Overall plasmid costs reducing the host cells growth rate◇
	Conjugation efficiency	γ_{eff}	Determines how costs are related to the real probability γ
	Plasmid transfer probability*	$\gamma = \alpha_{con}\gamma_{eff}$	Probability to perform a transfer attempt◇
	Plasmid incompatibility group	η	Plasmids belonging to the same group use similar replication mechanisms and cannot co-occur in the same bacterial cell (in reality, this assumption holds at least in the long term)

* Indirect parameters that do only represent an aggregation of other parameter values (as indicated in the table)

◇ Further influenced by the proportion of empty neighboring cells (mimicking the local resource availability)

state of the focal cell. Whereas mortality is fixed for each bacteria-plasmid association, the probabilities for fission and transfer depend furthermore on the local resource availability as well as the transfer probabilities and the costs that are associated with all plasmids of the bacterium (see 'Submodels' for further details).

If a bacterium dies, the lattice cell becomes empty and all plasmids die too. If a bacterium performs fission, a random empty lattice cell is occupied by a copy of the bacterium and its residing plasmids, whereas plasmids can get lost according to a fixed segregation probability. If plasmid transfer happens, one of the plasmids that reside concurrently on the host is randomly selected according to its relative capability to perform the transfer. It will then attempt to transfer a copy to a random, non-empty lattice cell in its local neighborhood (Figure S4 illustrates the spatial context). Transfer is prevented if the target bacterium already contains the identical plasmid type (always considered for technical reasons: a plasmid type represents a set of traits that is either present or absent, whereas plasmid copy numbers are neglected). The same applies for plasmids of the same incompatibility group.

Design Concepts

Basic principles The mutual exclusion of plasmids belonging to different incompatibility groups leads to the emergence of an intransitive system, which lacks strict competitive hierarchies. This is similar to models that describe coexistence as the result of the so-called rock-paper-scissors concept (e.g.[40]). Biotic interactions allow niche differentiation that can preserve biodiversity.

Emergence The temporal dynamics, spatial distribution and (long-term) composition of the plasmid community emerges from the intra- and inter-cellular interaction of plasmid types varying in costs, transfer-rates and incompatibility. Each initial plasmid type has a unique combination of features (costs, horizontal transfer probability and incompatibility group) which may or may not be advantageous in competition with other plasmid types or plasmid-free cells. The evolution of conflict within the plasmidome might be understood in the frame of the red queen hypothesis[27], here extended to intraspecific interactions: different plasmid-plasmid associations compete within the same host species, resulting in a survival of the fittest that represent an optimized strategy regarding plasmid costs and horizontal transmission. Low-cost plasmids survive because they have a small effect on vertical transmission, even in conjunction with another more costly conjugative plasmid. More costly conjugative plasmids survive as long as they are infectious enough to compensate for the loss they impose on host fitness. Nonetheless, this balance between costs and function is disturbed, when multiple conjugative plasmids are present in the same host cell. This is because the remaining host fitness gets too low compared to the fitness of (neighboring) cells that carry only less costly (non-transmissible) plasmids belonging to the same incompatibility group. On the other hand, if less costly (non-transmissible) plasmid-plasmid associations displace more costly ones, the fraction of plasmid-free cells grows, since they are infected less often. This opens a new chance for the rise of more costly (conjugative) plasmid-plasmid associations. The fundamental differences of the two plasmid survival strategies (non-transmissible and less-costly vs. conjugative and more costly) results in oscillatory dynamics. Similar to the rock-paper-scissor concept mentioned above, each member of the evolving community has a more competitive and a less competitive opponent, which has been suggested to be of central importance for

microbial diversity[33]. One of the major differences in our model is that this principle of non-hierarchical competition occurs simultaneously for varying incompatibility groups, forming a network of intransitive substructures (Fig. 1b).

Adaptation The model does not consider adaptation of individuals, since plasmids cannot adapt their costs, conjugation efficiency and incompatibility group influencing their behavior and fitness. Individual bacteria can also not directly adapt their own fitness, as this is influenced by the residing plasmids. However, the community adapts indirectly over time as only some of the plasmid types can survive which, in common presence with the other remaining compatible plasmid types, do not cause excessive fitness costs on their bacterial hosts.

Prediction Bacteria and plasmids are not able to predict their behavior and thus optimize it.

Sensing Plasmids are able to sense if a bacterium already bears a plasmid of the same incompatibility group. If so, any attempt to perform horizontal transfer to this bacterium fails. Bacteria do not sense other bacteria in their neighborhood, but the number and costs of plasmids residing on themselves.

Interaction Interaction of plasmid types occurs by inter- and intra-specific competition. The latter is given when neighboring host cells carry the same plasmid type and consequently compete for the same local resources namely empty neighboring lattice cells. Inter-specific competition is given when neighboring host cells carry different plasmid types. In addition, inter-specific competition of compatible plasmid types occurs on an intra-cellular level, since the fitness of the host and thus the probability of fission and plasmid spread decreases with the number of hosted plasmids.

Stochasticity The initial composition of traits in the plasmids (costs, horizontal transfer rate and incompatibility group) are drawn from random distributions. All processes are handled by probabilities (Fig. S3). Cell fission, for example, depends (1) on the availability of empty cells in the local neighborhood from which one of them is randomly chosen, and (2) on the host-fitness described as a probability which decreases with the costs of all plasmids carried. Although bacteria are considered to be clonal, the stochasticity leads to demographic noise in the bacterial population.

Initialization

The model is initialized by a random assignment of lattice-point states. The default probability that a lattice point becomes occupied by a bacterium corresponds to 1 minus the probability for mortality ω, which approaches the expectable population density of such a system. Each plasmid created receives values for its state variables, defining replication module costs α_{rep}, conjugation module costs α_{con}, accessory module costs α_{acc}, efficiency of conjugation γ_{eff} and plasmid incompatibility group η. These parameters are plasmid type-specific and drawn from a normal or uniform distribution with some constraints on the lower limit (see Table 2). Half of the initial plasmid population is non-transmissible ($\alpha_{con} = 0$). The default probability of a bacterium to carry a plasmid is 0.8, which is similar to some reported frequencies of plasmid-bearing strains of 73% in *Lactobacillus helveticus*[11]. This value is high enough to ensure that the initial diversity of plasmid traits is comparable among simulations. Considering the default parameterization, ca. 40,000 different plasmid types were initially generated.

Input data

The model does not use input data to represent processes that change over time.

Submodels

The following paragraphs describe in detail the single model processes and the calculation of the used probabilities $P(cell\ lysis)$, $P(cell\ fission)$, $P(plasmid\ transfer)$ and $P(no\ action)$ (see schedule given in Fig. S3). For the latter, local conditions, state variables (Table 1) and fixed parameters (Table 2) are taken into account. In general, parameters are chosen in a way that $P(cell\ lysis) + P(cell\ fission) + P(horizontal\ plasmid\ transfer) < 1$. The probability that nothing happens when a lattice cell is considered for an update in a single time step is given as follows:

$$P(no\ action) = 1 - P(cell\ lysis) + P(cell\ fission) + P(horizontal\ plasmidtransfer)$$

Cell lysis A bacterium and all its residing plasmids die according to a global probability for mortality. They are then removed from the model domain, which can occur due to natural mortality but also due to other processes such as washout or predation. In addition, bacteria that do not host a plasmid with the relevant trait can die with a certain probability under antibiotic action. The probability for cell lysis can thus be denoted as:

$$P(cell\ lysis) = \omega + \upsilon$$

with the global probability for mortality ω and the antibiotic action υ. Cells that died leave an empty lattice cell that can become recolonized through 'cell fission' by other bacteria.

Table 2. Values assigned to global parameters and state variables during the model initialization.

PARAMETER	VALUE	MEANING
Mortality ω	0.2	Probability of each bacterium and all its residing plasmids to die (/be removed from the system domain, e.g. according to washout, natural mortality and/or predation)
Antibiotic action υ	$0^{\diamond}$	Probability that a sensitive bacterium is killed due to the action of an antibiotic
Segregation τ	0.001	Probability to inherit a plasmid type only to one of both daughter cells during fission (in addition to potential effects of incompatibility)
Immigration ϖ	$0^{\diamond}$	Probability that a lattice cell is replaced by an immigrating bacterium (with random plasmid load)
Initial bacterial density ρ_B	1-ω	Proportion of lattice cells that are occupied by a bacterium at the beginning of a simulation
Initial plasmid density ρ_P	0.8	Proportion of plasmid-bearing cells of the initial bacterial population (only one plasmid per bacterium is generated
Initial resistance density ρ_{ARP} *	$0^{\diamond}$	Proportion of plasmids conferring resistance to antibiotics
Initial α_{rep} distrib.	$0.03 \pm 50\%^{\star}$	Replication module costs (normal distribution mean $\pm$ coefficient of variation > minimum size) of the initial plasmid population
Initial α_{acc} distrib. *	$0 - 0.25$	Accessory module costs (uniform distribution minimum and maximum) of the initial plasmid population
Initial α_{con} distrib.	$0.2 \pm 50\%^{\star}$	Conjugation module costs (normal distribution mean $\pm$ coefficient of variation) of the initial plasmid population
Initial γ_{eff} distrib.	$2 \pm 50\%$	Conjugation efficiency (normal distribution mean $\pm$ coefficient of variation) of the initial plasmid population
Initial # inc. groups	1, 2, 3, 4, 5 (up to 25)	The number of different plasmid incompatibility groups each plasmid of the initial population can belong to (represent single scenarios)

* considering a lower limit: costs > 0.01

$^{\diamond}$ in default model application, otherwise $0 - 1$ * if antibiotic resistances are considered, those plasmids with nearly mean α_{acc} ($mean_{\alpha_{acc}} \pm sd_{\alpha_{acc}}$) are considered to carry the resistance gene

Cell fission Plasmids can be inherited within a cell lineage (also called 'vertical gene transfer'), depending on the growth of their host. A bacterium performs cell fission according to a probability that depends on its local resource availability and the costs that are imposed by all residing plasmids. The local resource availability represents a factor that is given by the proportion of empty cells in the 8 cell Moore neighbourhood. Thus, the less neighboring cells are occupied the higher the nutrient availability for the focal cell. The probability for cell fission is further determined by the costs of plasmid carriage given as the sum of costs that are imposed by each residing plasmid. Let us denote the factor given by the local resource availability as f_{res} and the factor given by the costs of plasmid carriage as $f_{\alpha_{sum}}$, than the probability for cell fission follows:

$$P(cell\ fission) = \frac{neighbors_{empty}}{neighbors_{total}} \cdot \left(1 - \sum_{i=1}^{\infty} \alpha_i\right)$$

whereas α_i represents the costs imposed by the i-th plasmid on the host cells growth rate; $neighbors_{total}$ refers to the surrounding cells (=8), and $neighbors_{empty}$ represent the number of empty cells among them.

Considering the exemplary conditions for the focal lattice cell depicted in Figure S4, $neighbors_{empty} = 3$, $\alpha_{i=1} = 0.21$, $\alpha_{i=2} = 0.02$, resulting in $P(cell\ fission) = (3/8)(1 - 0.21 - 0.02) \approx 0.29$.

Plasmid heritage during cell fission is controlled by two subroutines: *Plasmid incompatibility* and *Segregation*.

Plasmid incompatibility: In the standard model version, surface exclusion is considered. This means that plasmid incompatibility already has an effect during the horizontal gene transfer and prevents the co-occurrence of incompatible plasmids in the same cell. A robustness test (see next section) revealed that this default model version behaves similar to another, computationally more demanding model version that considers incompatibility as follows: a daughter cell that arises from fission of a bacterium that harbored more than one plasmid of the same incompatibility group will not carry more than one plasmid type of each group. To process the loss of incompatible plasmids in the arising daughter cells, it was assumed that each daughter cell has a random combination of plasmids that all belong to different incompatibility groups, but are still originating from the mother cells plasmid load. This means that random incompatible plasmids are removed and that the arising daughter cells can by chance even have identical plasmid loads.

Segregation: A plasmid type can get lost by chance in one of both daughter cells that arise during fission. This accounts for the fact that a plasmid type might not be inherited to both daughter cells, even when no other (incompatible) plasmids are present.

Horizontal plasmid transfer Plasmids that are conjugative can also be transferred to cells of another cell lineage by horizontal gene transfer. In this submodel, a focal cell attempts to transfer a plasmid copy to a randomly chosen neighboring cell. The overall probability that the focal cell performs a plasmid transfer attempt is given by:

$$P(horizontal\ plasmid\ transfer) = \frac{neighbors_{empty}}{neighbors_{total}} \cdot \sum_{i=1}^{\infty} \gamma_i$$

whereas γ_i represents the transfer probability of the i-th plasmid that resides on the focal cell; $neighbors_{total} = 8$ refers to the local neighbourhood, and $neighbors_{empty}$ to the number of neighbouring cells which are not occupied.

This procedure couples plasmid transfer rates to the growth conditions, which has been reported by various experimental and simulation studies[41–44]. Nevertheless, our model neglects that transiently derepression of newly transferred plasmids may initiate a cascade of conjugative transfer events, which has been described as the epidemic spread phenomenon[45,46].

Considering that $i > 1$, which means that the focal cell bears more than one plasmid type, the single transfer probabilities γ_i are considered to randomly select the i-th plasmid for a transfer attempt. The transfer attempt will only be successful, if the receiving, randomly selected, neighboring lattice cell is neither empty nor occupied by a bacterium that already harbors the same plasmid type. In the default model version transfer is also prohibited when the receiving bacterium already bears another plasmid type from the same incompatibility group.

Considering the exemplary conditions for the focal lattice cell depicted in Figure S4, $neighbors_{empty} = 3$, $\gamma_{i=1} = 0.21$, $\gamma_{i=2} = 0$, resulting in $P(plasmid\ transfer) = (3/8)(0.71 + 0) \approx 0.27$, which refers in this case to the probability of plasmid $i = 1$ to perform a transfer attempt. The probability that this is successful is further reduced according to the proportion of potential recipients (non-empty, not already bearing the same plasmid type) in the local neighborhood, here denoted as $neighbors_{pot.recipients} = 2$, which results in $P(horizontal\ plasmid\ transfer_{real}) = P(horizontal\ plasmid\ transfer) \cdot (neighbors_{pot.recipients}/neighbors_{total}) = 0.27 * (2/8) \approx 0.07$.

Robustness tests

We tested if the observed results are sensitive to certain model settings and competing model versions.

Population size and more than five incompatibility groups It was tested if the evolution of a stable state that maintains a certain number of diverse plasmids is affected by the model world dimension and a larger number of incompatibility groups. This test revealed that the evolving local communities are able to preserve a larger number of diverse plasmid types, the larger the model world size and the number of incompatibility groups. Smaller populations are only able to sustain a smaller diversity of both plasmid types and incompatibility groups (Fig. S2).

Simulation time To test if the community composition that emerged after 10000 generations significantly changes in an increased time horizon, we run a couple of simulations for 30000 generations. The results show that the number of surviving plasmid types does not further or not substantially change in an increased time horizon (Fig. S5). Along with these simulations a model version considering another mechanism of incompatibility was tested, as our next robustness test shows.

Incompatibility mechanism In the standard model version it is assumed that incompatibility prevents the entry of plasmids into a cell that already contains another plasmid of the same incompatibility group. We tested this simplifying assumption and performed simulations with a model version that neglects such a surface-exclusion mechanism. Instead, incompatible plasmids might co-occur in the same cell, but are easily lost during cell fission. We included this feature in our model using probabilities to generate daughter cells that are free of one of both incompatible plasmid types, when a cell harboring more than one incompatible plasmid type performs cell fission. This imitates that replication is usually disrupted in such cases, resulting in decreased copy numbers and increased segregation rates. Incompatible plasmids are thus also excluded from the same cell in this way, only with a slight delay in which horizontal gene transfer can potentially take place. Our test results show that this alternative model definition does not seriously affect the model behaviour (Fig. S5). This enabled us to opt for the computationally less demanding mechanism of surface exclusion.

Immigration To test how the immigration of bacteria that bear new plasmid types influences the stability of the evolved community, we extended the model by a subroutine for immigration. Within this submodel, a random lattice cell can be occupied by an immigrating bacterium with a probability ϖ, for the presented test cases fixed at 10^{-5} per lattice cell per time step (corresponds to one hour). Immigrants can be plasmid-free or plasmid-bearing according to the same probabilities that were used to set up the initial state of the model (Table 2). First, it was tested if the invasion of diverse plasmid types affects the stability of the evolved local community. Second, it was tested if the same plasmid community evolves when the model is initialized with a single plasmid type rather than a diverse community, but considering that new plasmid types can immigrate with bacteria. Fig S6 shows exemplary plasmid population dynamics for both above mentioned cases. These results confirm that plasmid diversity can be maintained for evolved communities and evolves in initially clonal populations considering immigration. Both the stability of the proposed plasmid diversity mechanism and the behavior of the more simplified model version that neglects immigration is therefore robust, since the resulting plasmid communities are similar.

Well-mixed, unstructured environments Another robustness test focused on local interaction and spatial structure. To observe if plasmid diversity can be maintained for the same reasons in a mixed population, we extended the model by a subroutine for mixing. This procedure relocates any cell after each time step, which prevents the emergence of a spatially clustered community structure. Even in this case, plasmid diversity was maintained, but antibiotic resistance got lost in restored environments (Fig. S7). The presence of a structured environment such as biofilms can therefore be seen as an important factor that supports the persistence of costly traits such as antibiotic resistance, which might get lost in a well-mixed environment such as the planktonic phase of water bodies.

Absence of plasmid diversity - pairwise plasmid-host interactions As a validation of the general model behavior, model runs were performed considering only a single plasmid type. It was found that, in the absence of plasmid diversity, highly infectious conjugative plasmids can persist, but non-transmissible plasmids not (Fig. S8), which is in line with the predictions of previous studies assuming similar conditions (neglected co-occurrence of diverse plasmid types), as summarized in the introduction.

Distribution of antibiotic resistance among plasmid types Antibiotic resistances cannot persist after stopped antibiotic use when they are conferred by a single non-transmissible or weakly to moderate infectious conjugative plasmid (Fig. S9). This is because such systems are hierarchical during the timecourse of antibiotic use, since all other plasmids or at least those of the same incompatibility group that do not confer the antibiotic resistance gene are likely eliminated. Therefore, the conditions of the non-hierarchical tournament cannot be fulfilled, when antibiotic-mediated selection stops, which facilitates resistance reversal. Conjugative plasmids that are infectious enough to compensate for the huge costs imposed by the non-beneficial resistance might still persist, even in the absence of abiotic selection. However, this only works as long as it does not have to cope with other less costly plasmid types of the same incompatibility group. Otherwise, the requirements for the non-hierarchical tournament maintaining the resistance can also be established, when a plasmid mutant arises that confers the resistance, but lost or at least repressed its conjugation genes, making this mutant less costly than the other conjugative type that is still present.

The investigation of such conditions require a consideration of transiently derepression, which has been previously approached using a similar model[47]. Since such adaptations are likely, we argue that the maintenance of antibiotic resistance by single plasmid types is probably less restricted than these tests suggest.

Code availability

Our model, the PlasmidomeIBM, was implemented in the software environment NetLogo[39]. The Supplementary Data provide its full code, embedded in a ready-to-run application. This allows it to reproduce our simulation experiments, stored as setups in the 'BehaviorSpace' as well as to run simulations with self-defined parameters. Figures were generated with the software environment R (https://www.R-project.org/). Researchers interested in the computer code used to generate the figures are invited to contact the corresponding author.

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Author contributions M.Z. designed the study, developed the model and did the simulations; M.Z. and U.B. analysed the results and wrote the paper.

Author information The authors declare no competing financial interests. We provide the full computer code of the model that was used to generate the presented results. The data supporting the findings of this study can be generated by the model and are available upon request from the corresponding author (martin.zwanzig@tu-dresden.de).

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Supplementary Information (SI)

Supplementary Data

The authors provide the full model called PlasmidomeIBM, which is programmed and executable in NetLogo, a free software environment (https://ccl.northwestern.edu/netlogo/).

Supplementary Figures / Extended data

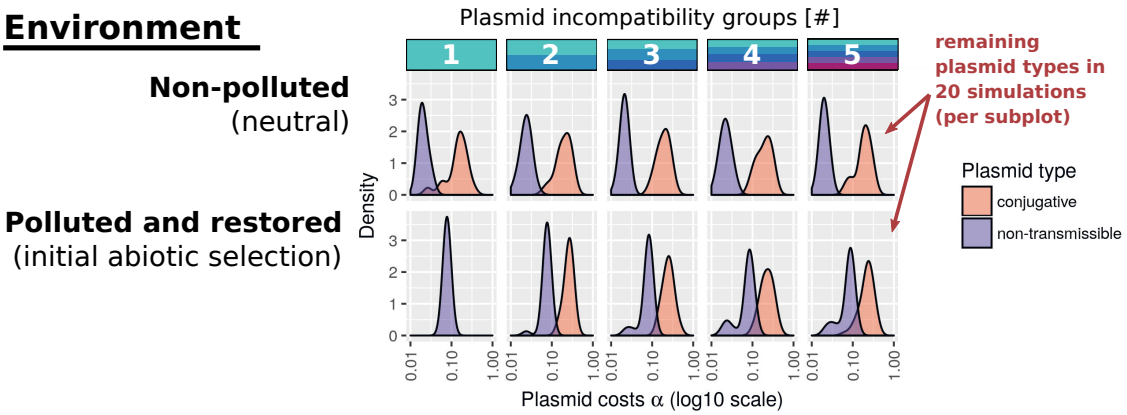

Figure S1. Association between plasmid costs, mobility and the number of incompatibility groups in non-polluted as well as polluted and restored environments. Results refer to the communities that evolved in our simulation experiments considering the absence ('non-polluted' environment) or initial presence (for 10000 generations; 'polluted and restored' environment) of antibiotics.

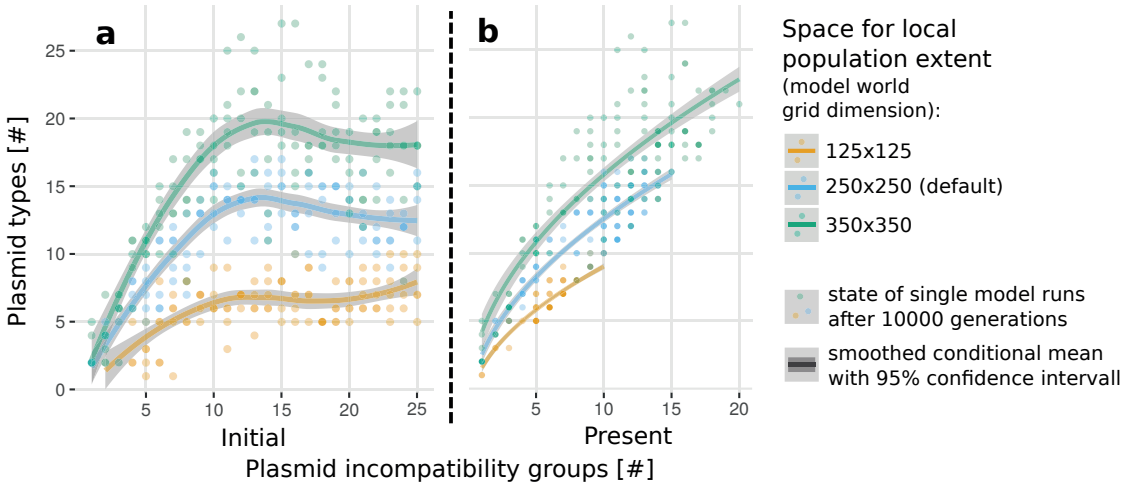

Figure S2. Plasmid diversity in evolved communities considering varying population sizes and numbers of incompatibility groups. The initial number of plasmid incompatibility groups is determined by initialization, whereas the present number reflects the state of each local community (single model run) after 10000 generations.

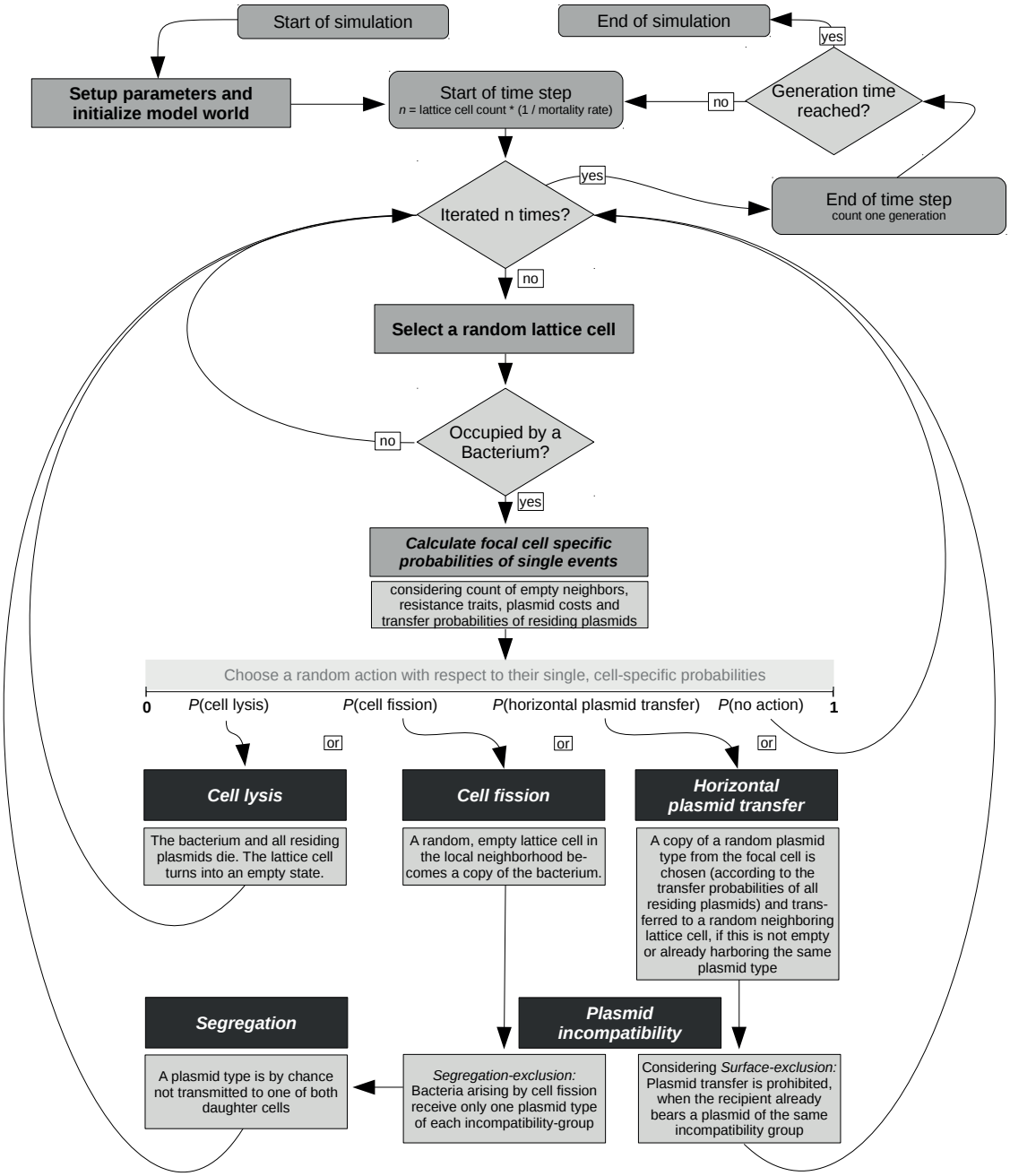

Figure S3. Flow chart of the individual-based plasmidome model. Stochasticity is a major principle in our model: the individual cell that becomes updated is selected at random as well as the action that is chosen regarding to its cell-specific probabilities. Although plasmid types are considered as individuals that can solely be inherited or transfered to other lattice cells, they only perform actions in the context of their host cell environment (the occupied lattice cell (bacterium) they reside on possibly in conjunction with other plasmid types).

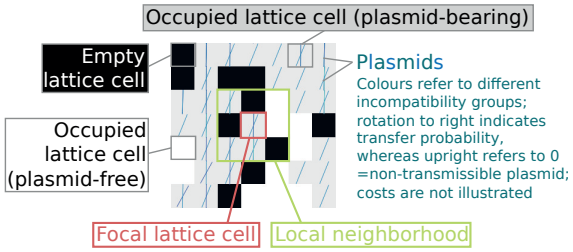

Figure S4. Spatial representation of model entities. Graphics represent a random clipping of the model world, generated by a snapshot from the user interface of NetLogo (the software providing the platform for the development and simulation of the presented individual-based model).

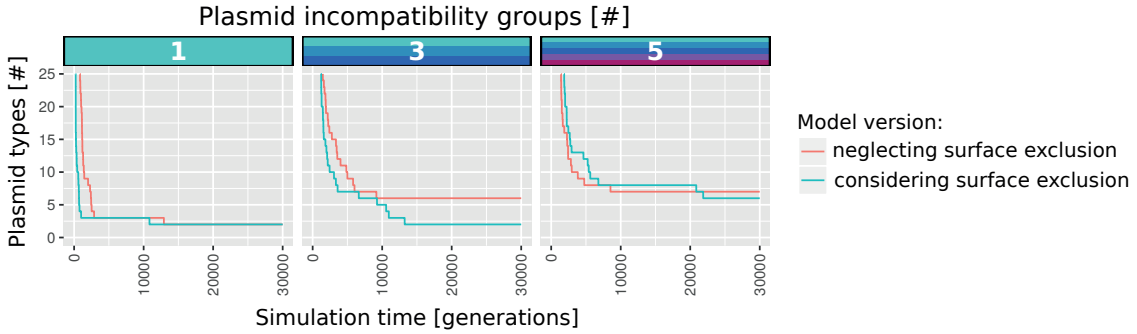

Figure S5. Model behaviour considering varying mechanisms of incompatibility. Each line depicts plasmid diversity dynamics for a single model run either assuming surface exclusion of incompatible plasmids (model's default) or random exclusion during cell fission, when incompatible plasmids co-occur in the same cell. Neglecting the effect of surface exclusion, more plasmids can enter cells, enhancing plasmid maintenance and the stability of the evolved community. In reality, some plasmids actively promote surface exclusion whereas others don't. But as both mechanisms alone show a similar behavior, this appears to be unimportant for our main findings.

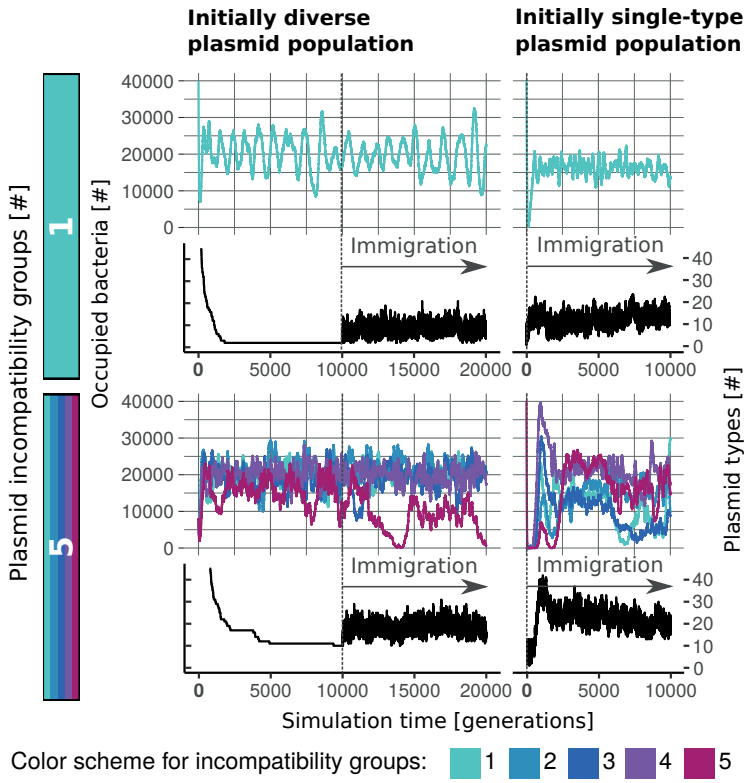

Figure S6. Plasmid diversity can be maintained for evolved communities and evolves in initially clonal populations considering immigration. Each row represents the observations for a single simulation run assuming initially 1 or 5 incompatibility groups and a probability to become occupied by an immigrant of 10^{-5} per lattice cell per hour. Immigrants are random plasmid-free or plasmid-bearing bacteria. Although plasmid diversity is constantly raised by a kind of noise resulting from immigration, this does not substantially affect the frequencies of the prevalent plasmid types.

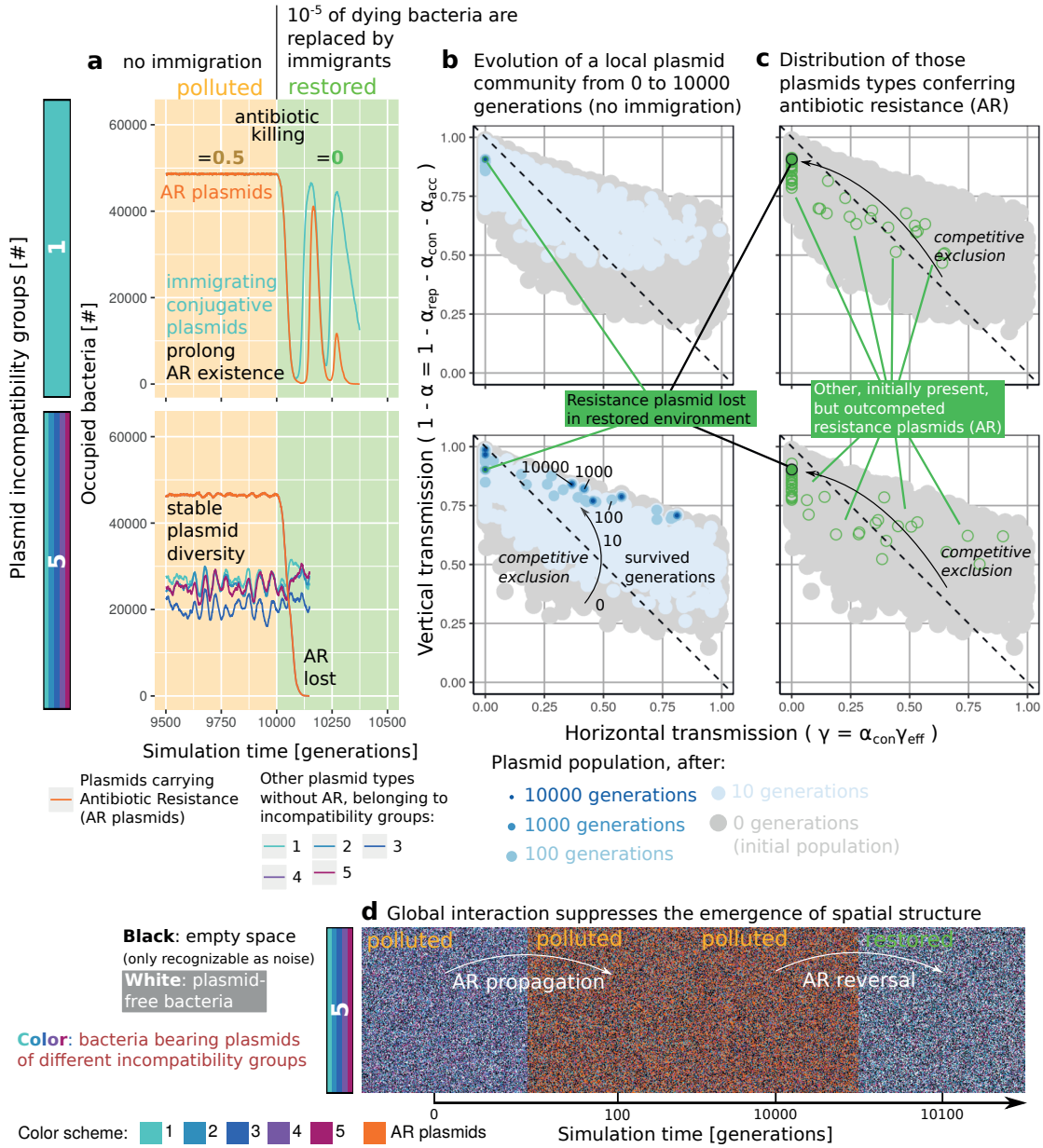

Figure S7. Plasmid persistence considering environmental mixing. **a**: Plasmid diversity can be maintained in polluted environments when more than one incompatibility group is present; Antibiotic resistance (AR) is lost in restored environments, even considering immigration that could help to reestablish a stabilizing community structure; **b**: Depending on the number of plasmid incompatibility groups, only some of the most competitive plasmids can be sustained in each local plasmid community; **c**: Although multiple plasmid types provide AR, competitive exclusion reduces such redundancy by a selection of one of the most competitive plasmid types that confer this function to the local community; **d**: Constant mixing of bacteria prevents an evolving community to become spatially structured

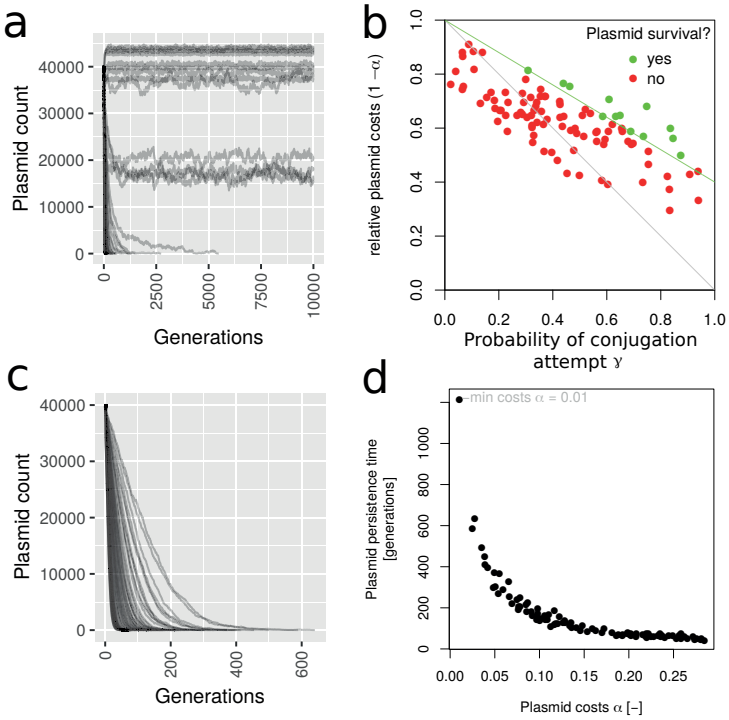

Figure S8. Plasmid persistence in the absence of plasmid diversity. Considering the presence of either one single conjugative or one non-transmissible plasmid type, conjugative plasmids are able to survive, when the probability to perform a conjugation attempt is far greater than the associated plasmid costs (a, b). Non-transmissible plasmids are not able to survive in the long-term in a non-diverse community, given that their costs are greater than zero and they are not supported by abiotic selection (c, d); Each line (a, c) and point (b, d) refers to a single simulation run.

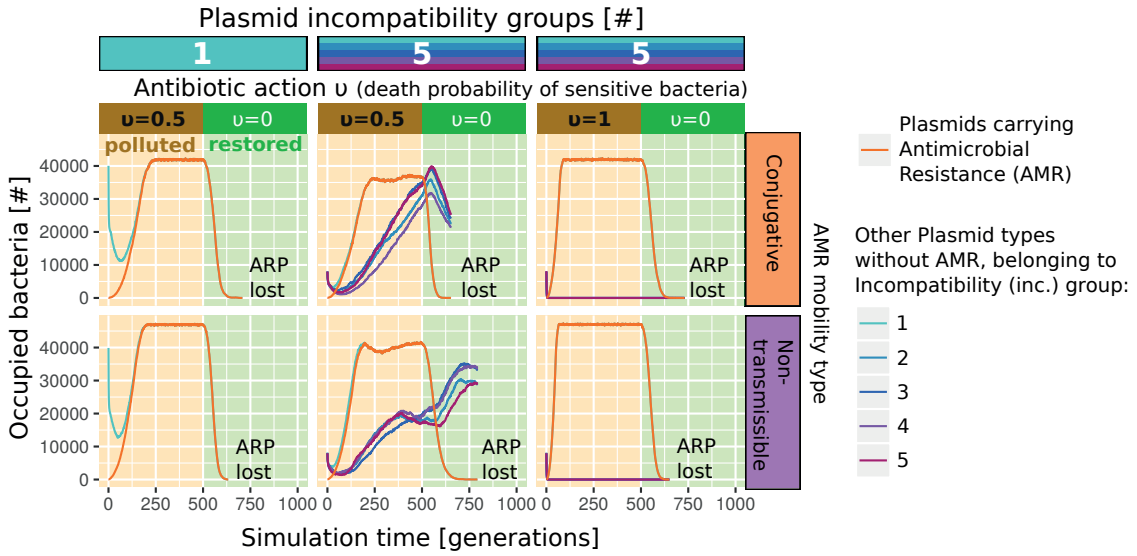

Figure S9. Resistances are lost considering single plasmid types conferring antibiotic resistance genes. Each plot depicts the results of a single simulation, initialized with an antibiotic resistance plasmid (ARP) that has the mean properties of the initial plasmid population (see table 2 for a list with means for plasmid module costs, efficiency of conjugation etc.)

5 Supervised Master thesis I – **The propagation of antibiotic resistances considering migration between microhabitats**

This study aims to consider metapopulation theory to investigate the spread of antibiotic resistances in aquatic environments such as rivers or lakes. It has been designed and supervised by the author of this doctoral study. In the following, (1) a summary of the thesis is given by the author of this doctoral study, (2) the outline of tasks, written by the author of this doctoral study, is presented (in german) and (3) the front page and abstract of the Master thesis (in both german and english), written by the Master student himself, is shown.

Summary The spread of antibiotic resistance is a serious global problem, but the underlying mechanisms are not yet sufficiently understood. The carriage of plasmids promoting antibiotic resistances can suffer a high cost to the host. Although it has been shown that coevolution of bacteria is able to reduce this cost, establishment of adapted traits takes time. In nature, especially in hydrodynamic aquatic systems, processes related to fluid dynamics could counteract the adaptation of a population, since environmental conditions do not remain constant. This Master thesis examines how the combination of microhabitats influences the spread of resistance. Therefore, an individual-based model was set up, which tries to simulate the dynamics of bacteria in connected biofilms. In simulation experiments the influence of submodels (subprocesses) in interaction with different model assumptions was investigated.

It was shown how antibiotic-resistant bacteria might spread through migration between microhabitats, where they can face strong competitive pressure by high growth rates of plasmid-free bacteria in environments without or only a low antibiotic concentration. This can have a strong negative influence on their abundance. The importance of antibiotic concentration was emphasized in this context. Nevertheless, spread to less polluted environments is enhanced, since polluted environments sustain a high number of plasmid-bearing bacteria that have enough time to acquire compensatory mutations that provide a strong benefit in less polluted environments.

Insitut für Waldwachstum und Forstliche Informatik

Professur für Forstliche Biometrie und Systemanalyse

Datum: 01.04.2016

Aufgabenstellung zur Masterarbeit

Betreuung: Martin Werisch M.Sc.
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Thema: *Das Ausbreitungspotenzial von Antibiotikaresistenzen unter Berücksichtigung der Verbindung von Mikrohabitaten*

Antibiotikaresistenzen verbreiten sich weltweit in Bakterien und ihrer Umwelt. Dies stellt eine der größten Gefahren für die menschliche Gesundheit und die Gesellschaft dar, weil Antibiotika oft der einzige Weg sind Infektionskrankheiten zu behandeln. Die Erforschung der Ausbreitung von Resistenzen gegen diese Antibiotika ist demnach unerlässlich für unsere künftige Fähigkeit Infektionen zu bekämpfen.

Einige Bakterien sind intrinsisch resistent. Bakterielle Plasmide bilden jedoch die bevorzugte Platform von Resistenzgenen, da sie über den horizontalen Gentransfer eine schnelle Anpassung der Bakterien ermöglichen. Es wird angenommen, dass die Präsenz von Antibiotika die Verbreitung resistenter Bakterien befördert. Kläranlagen wird dabei oft die Rolle von Hotspots zugeschrieben, da diese Antibiotika, Resistenzen und umweltrelevante Bakterien zusammenbringen.

Ohne einen selektiven Vorteil wird das Plasmid zum Parasiten für die Wirtszelle. Plasmid-tragende Bakterien sind dann nur noch schlecht in der Lage sich im Wettbewerb um Nahrung und Raum gegenüber Plasmid-freien Bakterien zu behaupten. Koevolution von Bakterien und Plasmiden kann jedoch zu einer Reduzierung der Plasmid-Kosten für das Wirtsbakterium führen. Dieser Prozess ist allerdings an Mutationen gebunden und in der Regel stochastischer Natur. In Laborexperimenten waren für eine Ausbreitung solcher Anpassungen hunderte Generationen nötig. Dies wirft die Frage nach dem Ausbreitungspotenzial von Resistenzen auf, die mit hohen Kosten für das Wirtsbakterium verbunden sind und keinen beständigen Selektionsvorteil besitzen.

In der Masterarbeit soll dieser Frage mithilfe eines gitterbasierten Modells nachgegangen werden. Die allgemeine Funktionsweise soll sich sich an einem bestehenden Gittermodell zur

Koevolution von Bakterien und Plasmiden orientieren. Dieses Modell soll jedoch in seiner räumlichen Dimension umgebaut und um einzelne Konzepte und Komponenten ergänzt werden. Letztendlich soll ein Modell aufgebaut werden, welches die Plasmid-Dynamik im Sediment von Fließgewässerabschnitten abbildet. Jeder Abschnitt stellt demnach ein Mikrohabitat dar, dessen Standorteigenschaften sich von denen anderer Abschnitte unterscheidet. Neben Wachstum, horizontalem Gentransfer und Anpassung der Bakterien innerhalb eines Abschnittes soll der Transport von Bakterien (und ihrer Resistenzen) zwischen den Abschnitten berücksichtigt werden. Eine Parametrisierung des Transports soll in Anlehnung an bestehende Fließgewässermodelle durchgeführt werden. Zur Bestimmung des Effekts der Antibiotikakonzentration auf die bakterielle Fitness werden bereits vorliegende Ergebnisse von Laborexperimenten genutzt (Functional-Response-Curves).

Es bestehen folgende Hypothesen:

1. Die dynamische Verbindung von Mikrohabitaten befördert die Ausbreitung von Antibiotikaresistenzen
2. Anhaltend hohe Selektion in einzelnen Abschnitten ermöglicht die Entstehung von Anpassungen, die eine Dominanz des Plasmids in weiteren Abschnitten ermöglichen
3. Hohe Transferraten zwischen den Abschnitten steigern die Dynamik dieses Prozesses, sind aber keine Notwendigkeit

TECHNISCHE UNIVERSITÄT DRESDEN
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Institut für Waldwachstum und Forstliche Informatik
Professur für Forstliche Biometrie und Systemanalyse

MASTERARBEIT

»Das Ausbreitungspotenzial von Antibiotikaresistenzen unter Berücksichtigung der Verbindung von Mikrohabitaten«

von

Louis Georgi
geboren am 11. November 1990 in Dresden

zur Erlangung des akademischen Grades

MASTER OF SCIENCE
(MSc.)

Tag der Einreichung: 06. Juni 2016
Betreuer der Masterarbeit: MSc. Martin Werisch
Verantwortliche Hochschullehrerin: Prof. Dr. rer. nat. Uta Berger
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Kurzfassung

Die Ausbreitung von bakteriellen Antibiotikaresistenzen stellt eine große Gefahr für die menschliche Gesundheit dar. Durch die anthropogene Kontaminierung von Fließgewässern mit Antibiotika können in darin lebenden Bakterien Resistenzen entstehen und verbreitet werden. Abschnitte von Fließgewässern können als Mikrohabitate betrachtet werden, in denen verschiedene, variable Antibiotikakonzentrationen (c_A) herrschen. Der Einfluss des Bakterientransports zwischen diversen Mikrohabitaten auf die Ausbreitung von Resistenzgenen ist bisher nicht bekannt. In der vorliegenden Arbeit wird mit Hilfe eines in netLogo erstellten Individuen-basierten Modells der Effekt von diversen, dynamisch verbundenen Mikrohabitaten auf die Ausbreitungsdynamik von Antibiotikaresistenzen untersucht. Dabei werden die Prozesse Zellwachstum, Mutation, horizontaler Gentransfer, Bakterientransport sowie die Änderung der c_A in eindimensionalen Biofilmen des Flusssediments simuliert.

Es wird gezeigt, dass mit steigender c_A eines Systems die Verbreitung von Antibiotikaresistenzen zunimmt. Zudem fördert eine Anpassung der resistenten Bakterien deren Verbreitung. Weiterhin wird der uneinheitliche Effekt des Bakterientransports sowie der c_A-Änderung aufgezeigt.

Abstract

The spread of antibiotic resistance in bacteria poses a crucial threat to human health. Anthropogenic contamination of rivers with antibiotics may cause the development and spread of bacterial resistances. Sediment sections in rivers can be regarded as microhabitats. The concentration of antibiotics varies between microhabitats and is variable over time. Until today, the influence of bacteria transport on the spread of antibiotic resistance remains unknown. With an individual-based model implemented in netLogo, the presentet work analyses the effects of dynamically connected microhabitats on the dispersal dynamics of antibiotic resistances. The established one-dimensional riverbed biofilm model simulates cell growth, mutation, horizontal gene transfer, transportation of bacteria as well as the change in antibiotic concentration.

It is demonstrated that a systems antibiotic concentration has a clear effect on the dispersal of antibiotic resistances. Moreover, adaption of resistant bacteria promotes their distribution. Furthermore, the inconsistent effect of the transportation of the bacteria and the dynamic antibiotic concentration change caused is shown.

6 Supervised Master thesis II – **Estimation of the pB10 conjugation rate in *Escherichia coli* combining laboratory experiments and modelling**

This study aims to examine and improve approaches to derive conjugation rates from laboratory experiments with mathematical models. It has been designed and supervised by Dr. Veiko Voolaid for the conduction of laboratory experiments and the author of this doctoral study. In the following, (1) a summary of the thesis is given by the author of this doctoral study, (2) the outline of tasks, written by the author of this doctoral study, is presented and (3) the front page and abstract of the Master thesis, written by the Master student himself, is shown.

Summary This Master thesis investigates how horizontal gene transfer can be estimated with the help of simulation models. Different models were subsequently fitted to data acquired by laboratory experiments. The work covers a broad spectrum of methods, which allows a more thorough understanding of this supposedly simple system. This analysis shows the influence of the utilization of prior information on the model calibration results. It is further evaluated how sensitive the model reacts to slight changes of single model parameters or changes in model structure. Based on the estimates generated by a fitting to three independent datasets, a distribution for each parameter estimate is generated. These distributions are used to perform Monte-Carlo Simulations.

The work demonstrates that a consideration of bacterial type specific maximal growth rates and of the lag phase are important factors, able to improve the goodness of the model fit. Furthermore, the analysis reveals that the frequency of transconjugants is considerably driven by growth and not only by conjugation, which is often neglected in the evaluation of laboratory data. Finally, the Monte-Carlo simulations show which uncertainties in the model output are associated with uncertainties in the model input.

Insitut für Waldwachstum und Forstliche Informatik

Professur für Forstliche Biometrie und Forstliche Systemanalyse

Date: July 22., 2016

Definition of tasks for master thesis

Supervisor: Martin Werisch M.Sc.
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Topic: Estimation of the pB10 conjugation rate in *Escherichia coli* combining laboratory experiments and modelling

Antibiotic resistances spread worldwide in bacteria and their environment. This has been recognised as one of the major challenges for human health and society, since antibiotics are often the only way to treat infectious diseases. The study of the examination of antibiotic resistances is therefore essential for our future ability to fight against infectious diseases.

Horizontal gene transfer is the ability to accept and express genetic material from sources external to the cellular lineage. This process broadens the genetic pool to the whole microbial community, instead of a restriction to the parent genome. It is considered to be one of the most important evolutionary forces within microbial populations. For the dissemination of antibiotic resistance genes plasmids are the preferred platform. Nevertheless, conjugation rates estimated by laboratory experiments can differ to the order of magnitudes. To some extend this reflects the poor consideration of non-static processes driving population dynamics.

In preparation to this study three independent repetitions of a laboratory experiment have been carried out to examine the conjugation rate of pB10 in *Escherichia coli*. Several effects regarding the realisation of plate counting of conjugation experiments have been identified. A treatment with a bactericidal antibiotic eliminated the effect of on-plate counting, at least after 6h. For the conjugation experiment, densities of recipients (R), donors (D) and transconjugants (T) could be achieved at 0h, 0.5h, 1h, 2h, 3h and 6h. Further experimental conditions were an initial population of donors and recipients of 1:1, a temperature of 37řC and a mixing intensity of 150 rpm. The transfer frequency as the ratio of transconjugants to recipients was calculated to be 3.7×10^{-6}

within 3h and 4.1×10^{-6} within 6h. But this measure is only a crude approximation, since it does not take into account the effects of growth and can not be used for model predictions.

The task of this master thesis will be to set-up an appropriate simulation model, which is able to capture the observed patterns in R, D and T densities. A parametrization will incorporate own experimental results, literature data and parameter fitting. The final model will account for mating pair formation, since previous studies found this to be a very important feature for horizontal gene transfer.

Based on this, the master thesis addresses the following hypotheses:

1. A consideration of mating pair formation provides better fits of the experimental results (R, D and T densities)

2. Bacterial type specific growth rates deliver better model fits and a more reliable approximation of the intrinsic conjugation rate

3. Parametrizing the model with values carefully taken from laboratory experiments or the literature has the potential to cause questionable model predictions and fits of the remaining unknown parameters.

4. Independent model fits to repetitions of the same experiment reveal uncertainties in the laboratory procedure, but enable the estimation of a distribution for each parameter estimate, which allow a better fit of the single realisations than parameter estimates resulting from a fit to the combined data.

In the first step a traditional compartment model including the compartments of R, D and T will be set up. It will account for a resource dependency of growth and conjugation. A parametrization will consider the measured maximum growth rates of the R, D and T strains and the estimate of the systems carrying capacity. Segregation and cell death will be neglected.

In the second step the formulation of the conjugation process of the first model will be extended. Instead of assuming a single conjugation rate parameter the model will describe conjugation through mating pair formation by the attachment and detachment of cells and intrinsic conjugation rates.

In the third step both models will be fitted to (i) all single realisations of the experiment one after the other and (ii) to all datasets together. Simulated R, D and T densities will be compared to the mean values of the respective observations of R, D and T densities. Fitting will take place for logarithmic values to prevent an over-fitting to the last time point with the highest cell density. In general different 'scenarios of prior knowledge' will be considered: (i) no knowledge about specific parameter estimates, but knowledge about possible parameter ranges and (ii) prior knowledge of some parameter estimates given by laboratory experiments or the literature and knowledge about possible parameter ranges of (remaining) unknown parameters. For example values for attachment, detachment and intrinsic conjugation rates for pB10 and *Escherichia coli* can be taken from the literature and tested for their performance. It can be expected that the simulated results do not match our observations, since the parameters in the literature were fitted by a model which did not consider differences in the growth rates of R, D and T. Thus, this effect will also be evaluated and accompanied by a sensitivity analysis.

In the fourth step the results of the model fits will be further analysed with statistical methods. Uncertainties in the estimation of the conjugation rate relating to the model structure, the fitting procedure and the laboratory data will be revealed. As a final result distributions of parameter estimates will be generated from the fits of the scenario excluding prior knowledge to the independent repetitions of the laboratory experiment.

In the last step Monte-Carlo-Simulations will be performed. Parameter values randomly drawn from the generated distribution of the parameters will be repeatedly used to make model predictions. This predictions will be evaluated by a comparison to the fit to all datasets (which generated one single parameterset) and the three single realisations of the experiment.

TU Dresden
Fakultät Umweltwissenschaften
Institut für Hydrobiologie

Estimation of the pB10 conjugation rate in *Escherichia coli* combining laboratory experiments and modelling

MASTERARBEIT

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Studiengang: *Masterstudiengang Hydrobiologie*

Betreuer: *Prof. Dr. Uta Berger*

Martin Werisch M.Sc

Dr. Veiko Voolaid

Abgabedatum: *15.01.2017* .....

Unterschrift des Diploma

Abstract

In recent years increased research effort has been directed towards the estimation of conjugation rates in aquatic environments or biofilms. However, there is a lack of one consensus concerning the best approach to describing the extent of plasmid transfer efficiency. Besides, deterministic, process-oriented models describing the dynamics of donor, recipient and transconjugant *E. coli* in laboratory settings have not taken account of the growth rate differences between resistant and susceptible strains and the different transfer ability of donor and transconjugant. This Master's thesis has introduced a mechanistic model based on aggregation model which assumes plasmid pB10 transmission from resistant to susceptible cells to be dependent upon bacterial density and metabolic activity.

The model is fitted to laboratory data on bacterial growth within 6 hours after inoculation in Lysogeny broth(LB) medium. Various scenarios of the aggregate model are run in order to understand the correlations within the analyzed system. A sensitivity analysis is conducted to determine which parameters essentially influence the model output.

The fitting results imply that the modified aggregation model has a similar performance as the modified mass-action based model although it allows one to account for experimental and environmental effects such as mixing intensity. Sensitivity analysis and model uncertainty calibrations highlight the growth rates of the three strains and the length of lag phase as the key factors that affect the population dynamics. They also prove that the transconjugant-to-recipient plasmid transfer efficiency plays little role in the population dynamics of transconjugant. The predicted population distribution of three strains using Monte-Carlo-Simulation method implies a possible on-plate-conjugation effect in the laboratory data. However, this result can serve as a reference which requires further improvement.

Key word: conjugation rate, aggregation model, *Escherichia coli*, pB10, predicted population dynamics

7 Supervised research internship – **Plasmid population dynamics considering individual plasmid copy numbers**

San Millan et al. (2014b) elucidated the importance of an acquisition of both: compensatory adaptations and general beneficial mutations. Whereas the first may eliminate the absolute cost of a plasmid, the latter are necessary to keep abreast with general fitness improvements of plasmid-free cells. The compartment model of San Millan et al. (2014b) performed well predictions of the relative proportions of plasmid-bearing bacteria with compensatory adaptation, but their model failed to predict the experimentally observed long-lasting high frequency of plasmid-bearing bacteria without compensatory adaptation. What are the mechanisms behind this plasmid stabilization? Are we able to provide more accurate calculations of plasmid half-lifes as a function of days before antibiotic exposure?

This study aims to examine the role of the costs to generate a certain number of plasmid copies for plasmid persistence. It has been designed and supervised by the author of this doctoral study in the frame of a research internship lasting for six months. In the following, (1) a summary of the outline of tasks and the research report is given by the author of this doctoral study and (2) the front page and table of contents of the Research report, written by the student himself, is shown.

Summary The dynamics of bacteria and plasmids as independent, reproducible entities were investigated by means of an individual-based model (IBM). The developed model is based on an existing differential equation model describing the dynamics of non-transmissive plasmids conferring antibiotic resistance. It explicitly considers the conflict between bacteria and plasmid in the use of shared limited resources: low plasmid replication rates increase the growth of the plasmid-bearing cell, but reduce the number of plasmid copies in it and thus increase the probability of segregation during cell division. Thus, the persistence of plasmids depends on two states: the proportion of plasmid-bearing bacteria in the total population and the distribution of the number of plasmid copies per bacterium. The following steps were carried out during the practical course: (1) Model development and description according to ODD standard. (2) Detailed testing of the model for logical and implementation errors. (3) Sensitivity analysis (e.g. Morris screening) to evaluate the model behaviour. (4) Application of the model in simulation experiments to identify critical states of plasmid frequencies for different velocities of plasmid replication. How are these states balanced and how does it change with the boundary conditions (e.g. antibiotics)?

Although the developed model versions have not been validated by empirical data, they allowed to test competing hypothesis. The presented results provide valuable insights into mechanisms potentially driving the evolution of the copy number distribution.

Report of the Practical Internship

Module MHYB05

Plasmid Population Dynamics Considering Individual Plasmid Copy Numbers

Institute of Forest Growth and Forest Computer Sciences

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1

Contents

8 Discussion

8.1 Major findings

This work adds to a series of previous studies investigating the persistence conditions of plasmids either in a single plasmid - single host (Stewart and Levin, 1977; Merkey et al., 2011; del Campo et al., 2012; San Millan et al., 2014b; Stevenson et al., 2018; Harrison et al., 2018, i.a.) or single plasmid - multiple host environment (Bergstrom et al., 2000; Gelder et al., 2007; Heuer et al., 2007; Hall et al., 2015, 2017, i.a.) as well as to those few studies that started to exmanie the role of a co-occurrence of multiple plasmid types, either in a single (Cooper and Heinemann, 2000; Haft et al., 2009; Cooper et al., 2010; Silva et al., 2011; Platt et al., 2014; Morton et al., 2014; San Millan et al., 2014a; Gama et al., 2017a,b, 2018, i.a.) or multiple host (Lopatkin et al., 2017) framework. It has been shown that the number, size and functions of the plasmids found in different genera (Shintani et al., 2015), single species (Boronin, 1992; Kwasiborski et al., 2015) or in environmental samples (Ricci et al., 2006; Smalla and Sobecky, 2002; Brown et al., 2013; Dib et al., 2015) can vary remarkably, including many plasmids encoding selfish traits as stability and conjugation (Li et al., 2012). Both ecological and genetic factors were reported to affect plasmid distribution (Medaney et al., 2016).

The first study of the thesis, which was presented in Chapter 2, focussed on the following research question, first mentioned in the section 'objectives and content of the dissertation':

1. Considering a single plasmid-host pair, how can genomic modifications and antibiotics catalyze the spread of plasmid-encoded antibiotic resistance in the aquatic environment?
    a) How do certain ecological conditions affect this catalysis?

Considering a single plasmid-host pair, it was found out that compensatory evolution facilitates plasmid persistence and can allow plasmid-encoded antibiotic resistance to persist for a much longer time. This general finding is in line with previous studies reporting that high conjugation rates (Stewart and Levin, 1977) as well as high or low frequency pulses of positive selection of accessory functions such as antibiotic resistances (Stevenson et al., 2018) can slow down or prevent plasmid-loss. It is also in agreement with a study showing that positive selection can help to maintain non-transmissible plasmids, because the resulting high frequency of bacteria promotes compensatory evolution to ameliorate the plasmid cost (San Millan et al., 2014b). Although many other studies investigated the role of an amelioration of plasmid costs (Harrison and Brockhurst, 2012), the work presented in Chapter 2 is the first demonstrating the relevance of the genomic location for the success of a compensatory mutation. It shows that plasmid mutations facilitate plasmid persistence even when the direct amelioration effects are far less effective than those provided by chromosomal mutations. This is because chromosomal mutations cannot be transmitted to infected bacteria. The chromosomal-adapted variant does therefore not gain the same benefit from conjugation as the plasmid-adapted variant, which can directly propagate through conjugation as it readily provides the cost compensation to any infected cell. In addition to this, the study suggests that in the investigated well-mixed phase higher

levels of predation are likely to drive antibiotic resistance genes out, but even low antibiotic killing rates can represent a strong selection on the resistant plasmid bearers.

Nonetheless, bioinformatic analysis revealed that co-infection with multiple plasmids is common across a wide range of bacterial phyla (San Millan et al., 2014a). For example, the large horizontal gene pool of rhizobia indicates that the pool of plasmids rather than an individual plasmid is stable (Turner et al., 2002). Meta-analysis of sequence data reported that associations between small and large plasmids are specifically frequent (San Millan et al., 2014a), which has also been found in ecological communities (Medaney et al., 2016), but it is still not completely clear why. It also remains unsolved, how a high diversity of plasmids that encode no or unknown accessory functions persist in freshwater habitats (Brown et al., 2013). Consequently, instead of considering only a single plasmid-host pair, the study presented in Chapter 3 addressed the following research question, also first mentioned in the section 'objectives and content of the dissertation':

2. Considering a simultaneous interaction between frequently co-occurring types of plasmids (non-transmissible and conjugative), how does this change system behaviour and previous predictions?

It was found that the co-occurrence of another plasmid type plays a pivotal role for the system behaviour. In detail, it has been shown that conjugative plasmids are able to maintain less costly non-transmissible plasmids, if both belong to the same incompatibility group. This finding readily enhances the persistence conditions of plasmids, since it demonstrate a mechanism by which even cryptic plasmids that do not provide any obvious benefit might persist. This adds to findings reported by Hoeven (1984) and Hoeven (1986), but shows that this also holds for a surface-attached population and considering a much more detailed and realistic description of the associated mechanism of plasmid incompatibility. This study is also the first considering that transfer genes of transmissible plasmids are turned on or off in dependence to the local density of recipients, mimicking conjugation pheromones. It suggests that the constellation of conjugative and non-transmissible plasmids might represent a general mechanism maintaining plasmids and associated antibiotic resistances.

However, plasmid communities might seldom comprise only two different plasmid types. Chapter 4 therefore addresses the next and final research questions that were initially mentioned in the section 'objectives and content of the dissertation':

3. Considering the plasmid diversity found in natural communities, are the previous results still valid?
    a) Do diverse plasmid communities facilitate the persistence of costly plasmid functions such as antibiotic resistance in the absence of abiotic selection?
    b) How do communities evolved under abiotic selection (presence of antibiotics) respond to a change to neutral conditions?

Observing the evolution of a population comprising various plasmids varying in costs, transfer ability and incompatibility by means of an individual-based model revealed that the intransitive dynamics of conjugative and less costly non-transmissible plasmid types seems to be an emergent property of a diverse plasmid community and can be simultaneously formed for different incompatibility groups. This demonstrates how the vast genetic repertoire mediated by plasmids could be preserved in nature and remained an open challange in the field (San Millan and MacLean, 2017). The evolved communities comprise a varying amount of plasmid-free bacteria and bacteria harboring one or more plasmid

types. This has also been observed for *Escherichia coli* populations (Medaney et al., 2016). Similar to experimental results reported for microbial communities run in steady state (Liu et al., 2019), the plasmid communities that evolve in parallel setup in our simulation experiments are variable, but rather robust to immigration of new members. The poor effect of immigration can be referred to a founder effect that reduces the chance that a new gene is established when similar genes (here all without benefit) are already given (Martínez et al., 2015). Consistent with the conclusions of Liu et al. (2019), the variability of the communities that evolved in parallel suggests that neutral forces have a serious impact on community assembly. However, the findings presented in Chapter 4 indicate that not just random plasmid types are able to survive. Only plasmid types that, in comparison to the other plasmid types of the same incompatibility group, optimised their survival strategy - either focused on vertical or horizontal transmission - have a rather high probability to occupy a niche in the plasmid community that enables it to be maintained through non-hierarchical competition to another plasmid type of the same incompatibility group that follows the opposite survival strategy. Compartmentalisation by the presence of species that have a higher probability of interacting with each other than with other species has been proposed to promote community stability, because compartments act to buffer perturbations (Griffiths and Philippot, 2013). Assuming that plasmids belonging to different incompatibility groups likely interact more strongly, as they can occupy the same bacterium, these findings suggest that incompatibility determines the compartmentalisation of a plasmid community and therefore plays a pivotal role for its stability. Since a maintenance of genetic variation is key for bacteria to cope with environmental uncertainty (Erkus et al., 2013), this might also explain why incompatibility groups exist at all.

Further simulation experiments showed that such diverse plasmid communities can also maintain antibiotic resistance, even when abiotic selection by antibiotics stopped. This adds to other studies that suggest that biotic interactions likely play a more significant role than has been previously assumed (Cairns et al., 2018b), especially regarding the occurrence of intransitive competition (Soliveres et al., 2018; Kelsic et al., 2015). Another surprising recent finding in this context is that wastewater treatment plants do neither increase nor decrease the proportion of antibiotic resistance in a bacterial population (Mahfouz et al., 2018). Accordingly, environmental factors such as a high cell density and antibiotic-mediated selection that should theoretically enhance the spread of antibiotic resistances does indeed appear to be less important in natural populations.

It can also be argued that the existence conditions of plasmids in a diverse community that is presented in Chapter 4 represent a conservative prediction, since the study neglects positive epistasis between co-occurring plasmids, which has been previously reported to promote plasmid survival (San Millan et al., 2014a). This means, if fitness costs are buffered by epistatic interactions with another co-residing plasmid-type, a plasmid might also persist if it imposes higher 'single' fitness costs or experiences a lower degree of antibiotic-mediated selection.

Some plasmids encode more than one stabilization module, often including a postsegregational-killing system, as it has been reported for resistance plasmids from a wastewater treatment plant (Schlüter et al., 2007). This may benefit plasmid persistence under some conditions, but will not work when some still emerging plasmid-free cells are able to outcompete the plasmid-bearers by much higher growth rates.

Still, it remains unclear what it means to consider multiple hosts. It has been reported that conjugative plasmids may only be stably maintained in one of the hosts, but this can act as a reservoir for other hosts that become continuously infected (Hall et al., 2016).

Moreover, it can become even more complex considering that such source-sink dynamics might be affected by intracellular interactions between two (Gama et al., 2017a) or three (Gama et al., 2017b) distinct conjugative plasmids, since such a co-occurrence can affect the conjugation efficiency, with a trend towards a reduction of the conjugation rate. Besides, interactions with other mobile genetic elements such as bacteriophages can affect plasmid fitness directly, when they specifically invade those cells performing conjugation (Harrison and Brockhurst, 2017), or indirectly, when they impose fitness costs on the host bacterium, potentially resulting from interactions with the plasmid itself (San Millan and MacLean, 2017). All of these points direct to future research opportunities that are discussed in the 'Outlook'.

8.2 Methods evaluation

Of course, some caveats are necessary when interpreting the presented results. In this section, points of criticism are expressed which refer in particular to the assumptions of the simulation models developed within the framework of the dissertation. Please note that this criticism is incomplete here, as the following "outlook" mentions further points in the form of a description how these problems, which relate to the methodology applied and have already been mentioned or not, can be solved.

The population level model presented in Chapter 2 neglected that in the real world much bacteria exist as microcolonies on surfaces and biofilms (Summers, 1996). Such natural populations are for example not completely mixed and consist of several different bacterial species and plasmid types that occur at once, perform more complex interactions and experience various evolutionary trajectories simultaneously (Martínez et al., 2007). It is also important to note that plasmid-bearing bacteria in this model are either adapted or not. It does not consider heterozygosis, which refers to the occurrence and competitions of both types (adapted and not-adapted) within the same cell. Although this might only represent a transient state, if the adapted plasmid is quite more competitive, it would impede its spread.

Another point of criticism that might be mentioned is that none of the models presented in the thesis considered the emergence of chromosomal cells, which incorporated the focal plasmid gene into the bacterial chromosome. Thus, plasmids are assumed to be the sole source of perhaps beneficial genes such as antibiotic resistance. The consequences related to a co-occurrence of so called 'chromosomals' have been investigated by Bergstrom et al. (2000). They report that plasmids might only persist because they are able to transfer back and fourth between noncompeting species or ecotypes. In their analysis, it is considered that the so called 'S-L-criterion', which refers to Stewart and Levin (1977), holds. It states that, in the absence of selection of plasmid-borne traits, the effect of conjugation should never exceed the negative effects imposed by the plasmid. In other words, plasmids are not infectious enough to overcome their own costs according to this definition. In the studies presented within this thesis, conjugation rates of plasmids were not assumed to be constrained according to this definition. This allowed to obtain more general predictions and to explore the whole 'plasmid fitness space', as presented in Chapter 1. Considering only pairwise plasmid-host interactions plasmids that exceed this 'S-L-criterion' might infect a whole population. If such results are generalized, they may indeed appear unrealistic. But the reason for this is not the assumption of how infectious a plasmid could be or not, but the assumption that a natural population is so simply constructed. If instead a more diverse population is considered, conjugative plasmids are likely not able to take such a big advantage from horizontal transfer in competition to plasmids

with other optimized 'plasmid life styles'. Considering this, conjugative plasmids might, besides physical constrains, indeed not be 'endless' infectious, since evolutionary selection likely favours a limitation of this costly process to a level that is sufficiently beneficial to maintain a plasmid in a population composed of diverse plasmid types, but this would still exceed the 'S-L-criterion'. Considering such conjugation rates, chromosomal variants might emerge, but could not outcompete the plasmids, since they are continuously infecting the chromosomal variant, which means that they have to carry the resistance gene twice, eventually without an additional advantage, making this constellation less competitive.

Another simplification has been made in Chapters 2 and 4 regarding horizontal gene transfer. Although some permanently derepressed plasmid types have been reported, most natural occurring individual bacterial cells that carry a conjugative plasmid are not by default transfer competent (Koraimann and Wagner, 2014). Only a fraction of the donor population undergoes a transition towards this state, which indicates a form of social behavior (Koraimann and Wagner, 2014). This is not explicitly considered in the models presented in Chapters 2 and 4. Otherwise, the effect of conjugation on the population level could also be addressed to the effect of a certain, invariable proportion of transfer competent cells.

Another constrain of all presented models is that bacterial gene expression and the viability of plasmid-bearing cells is not triggered by the concentration of the antibiotic (Martinez, 2008). This might represent a fair generalization that is less important for the general conclusions, since, for example, the bactericidal effect on the sensible bacteria can be 55 times higher than on the resistant bacteria (San Millan et al., 2014b).

The approaches presented in this thesis considered some of the guidelines that were proposed for predictions of the dissemination of antibiotic resistance genes (Martínez et al., 2007), but the studies presented in this thesis have neglected that bacteria experience fluctuating environmental conditions during their lifetime. Instead, the average conditions for populations in notional habitats were addressed. Considering the population level model presented in Chapter 2, the fictitious habitat could be large enough to reduce the variability of conditions so as to approach these assumptions. Nevertheless, environmental variations could impose evolutionary bottlenecks, that can impede the persistence or prevalence of plasmids. The opposite effect could be given by frequent selection, i.e. by short periods of antibiotic action, which facilitates plasmid maintenance (Stevenson et al., 2018).

8.3 Outlook

This section picks up some of the criticisms mentioned and presents various future research directions. Some of them have already been investigated by some preliminary analysis during this doctoral thesis. The following first example is particularly detailed, because it relates to the topic of a project within the framework of which this doctoral thesis was started.

Improving waste water treatment plant models Wastewater treatment plants (WWTP) are considered to be hotspots for the spread of antibiotic resistances (Rizzo et al., 2013). Whereas higher loads of antibiotic resistance genes have been found to coincide with antibiotic prescriptions (Caucci et al., 2016), recent studies observed that WWTP-influent and -effluent do not substantially differ in resistance and pan genome size (Mahfouz et al., 2018). An integration of our findings and model approaches to WWTP models could help to uncover the role of diversity in basic questions such as the following: (1) How does the flow regime, availability of nutrients and the dynamics of antibiotic concentration influence

the abundance of resistant bacteria? (2) What influence does the influx of resistant bacteria have on the abundances in the wastewater treatment plant?

Current models are often at odds with the diversity in the treatment plant (or that of natural populations). They usually divide the population only into plasmid-free and plasmid-containing bacteria. These models tend to have a threshold behavior: if the horizontal gene transfer is above a certain threshold value, the plasmid-bearing bacteria dominate the whole system, if it is below it they die out. The same applies to antibiotic concentration: if it is above a certain threshold, the plasmid-bearing bacteria dominate the whole system, if it is below it they die out. Thus, in the long run the system tends to be plasmid-free or plasmid-bearing only. The closer the parameterization is to the threshold value, the longer it will take. Only if the antibiotic concentration fluctuates around the threshold value it might be stabilised. Among other things, this neglects the fact that (i) in bacterial populations, several plasmids usually occur next to each other, (ii) plasmids of the same incompatibility group cannot survive in the same host bacterium and (3) approx. 50% of the plasmids are non-transmissive (not horizontally transferable).

Modified model approaches could take plasmid diversity into account and at the same time counteract dependence on the above-mentioned threshold behaviour. On the one hand, a model approach that includes not only plasmid-free (F) and bacteria bearing conjugative plasmids (T - transmissive) but also a subpopulation of plasmid-bearing bacteria that bear a non-transmissive plasmid (N). If the plasmids belong to the same incompatibility group, N cannot be infected with T due to surface exclusion, or at least cannot occur stably with it, because increased segregation rates occur due to the use of the same replication mechanism. As a result, T might not spread unhindered and is also displaced by N, assuming that the cost-of-carriage of the non-transmissive plasmid type is lower. Without abiotic selection N has a lower growth rate than F, which can therefore displace N. All this takes place in an alternation that leads to a flow equilibrium. Thus, if N is incorporated into the WWTP model, plasmid diversity could also be maintained without a perfect fluctuation of antibiotic concentration.

Another model approach would be to consider the simultaneous occurrence of plasmids that belong to different incompatibility groups and have different costs for the host bacterium or even different bacterial species. They all compete with each other in the system. In principle, this would be an extension of the model presented in Chapter 4 to include the consideration of various species and the fluctuation of different environmental conditions (nutrients, antibiotic concentration). This might contribute to the preservation of plasmid diversity over hundreds of generations and could explain how resistance and pan genome size are kept despite other variations. However, the integration or coupling of this diversity into existing WWTP models, which represent compartment models, is rather challanging, but already existing hybrid models, which, for example, couple an individual-based approach and differential equations (Ofiţeru et al., 2014), provide insights how this could work.

Models as a test bed for plasmid-curing and anti-plasmid approaches Based on the results of this work, in particular with respect to the model presented in Chapter 4, a model platform could be developed to help investigate the outcome of different anti-plasmid approaches aimed at reversing antibiotic resistance. This might be used to examine how different plasmid types are suited to replace the resistance plasmid in a bacterial population with certain characterisitics.

Approaching natural diversity at multiple scales: considering the role of bacterial diversity, plasmid host-ranges as well as interactions with other mobile genetic elements and between trophic levels Both previous examples refer at least in part to the model presented in Chapter 4. This model itself, and therefore also the approaches suggested in the previous points, might be extended considering that natural communities likely compose varying bacterial species that can be associated with specific costs and transfer abilities for different plasmid types. This can also be influenced by the presence of other mobile genetic elements that interact with the plasmid (Dionisio et al., 2019). In addition to the consideration of a bacterial population that evolves 'only' in dependence to the within population interactions, a scenario closer to a natural setting might also account for interactions between trophic levels, as shown for instance by Bellanger et al. (2014) and Cairns et al. (2018b). The presence of predators such as *Paramecia* or predatory bacteria and/or bacteriophages could, for example, be implemented in one of the models presented in this thesis in order to investigate how plasmid and/or resistance dynamics is affected by such biotic interactions.

Exploring the consequences of more specific mechanisms of compensatory adaptation In Chapter 2 it has been demonstrated that compensatory mutations promote plasmid survival. For this, it has been considered that either the plasmid or the chromosome acquires some mutation that reduces the costs of plasmid carriage. No other effects were considered, meaning that rather unspecific changes in gene expression that do not alter other processes occurred. Nevertheless, amelioration can also be associated with the loss of plasmid genes, changes in the conjugation rate or the plasmid copy number (Harrison and Brockhurst, 2012). Both, an extension of the system of differential equations presented in Chapter 2 as well as an individual-based model have already been setup to investigate the associated effects. Preliminary experiments show that these are substantial, making this a potential topic for deeper analysis.

Mechanisms controlling the regulation of bacterial conjugation Several studies showed that some plasmids can be highly infectious, leading to the rapid horizontal transfer of the conjugative plasmid to the whole bacterial population. But why then do most naturally occurring plasmids regulate bacterial conjugation? This is often explained by a form of social behavior, as only a fraction of the population carries the high burden that is associated with the expression of transfer genes.

The major focus of Chapter 3 is on the role of a co-occurrence of different plasmid types, but the model that was used to investigate these effects also provides a platform to address basic questions related to the regulation of bacterial conjugation by induction or repression of transfer genes, which has been multiple times reported to be tightly linked to the pheromone system (Dunny and Berntsson, 2016).

What is the dynamics and the ecological potential that is associated with a regulation of transfer gene expression through pheromones that are either produced by plasmid-free cells, plasmid-bearing cells or both? And regarding the duration of such pheromones, is it advantageous for the regulation of conjugation when a durable and a fragile quorum sensing signal is combined, as it has been reported in another study (Cornforth et al., 2014)?

8.4 Conclusions

It has been found that evolutionary modifications and biotic interactions have a major influence on the spread of plasmid-encoded antibiotic resistance, in a way that explains how plasmids observed in nature could persist. Individual-based models have proven to be advantageous, since they allow to study the resolution of conflict from a focal perspective: the individual cell. The combination of such mathematical tools with the increasing number of laboratory methods that provide detailed insights into the dynamics of individual cells opens a way to answer open research questions related to the complexity of natural environments.

List of publications presented as thesis chapters

- **Zwanzig, M.**, Harrison, E., Brockhurst, M.A., Hall, J.P.J., Berendonk, T.U., Berger, U. (2019). Mobile compensatory mutations promote plasmid survival. mSystems 4:e00186-18. http://doi.org/10.1128/mSystems.00186-18

- **Werisch, M.**, Berger, U., Berendonk, T.U. (2017). Conjugative plasmids enable the maintenance of low cost non-transmissible plasmids. Plasmid 91, 96-104. http://doi.org/10.1016/j.plasmid.2017.04.004

Publications apart the doctoral thesis

- **Zwanzig, M.**, Schlicht, R., Frischbier, N., Berger, U. (in press 2019). Primary Steps in Analyzing Data – Tasks and Tools for a Systematic Data Exploration. In: Levia, D.F. (Editor), Carlyle-Moses, D.E. (Co-Editor), Iida, S. (Co-Editor), Michalzik, B. (Co-Editor), Nanko, K. (Co-Editor), and Tischer, A. (Co-Editor), Forest-Water Interactions. Ecological Studies Series, No. 240, Springer Nature, Switzerland AG, 624 p. [ISBN: 978-3-030-26085-9 (Print); 978-3-030-26086-6 (eBook); DOI: 10.1007/978-3-030-26086-6]

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