An empirical ranking of plasmid stability mechanisms via *in cellulo* competition experiments

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Declaration

I hereby declare that the thesis entitled “An empirical ranking of plasmid stability mechanisms via *in cellulo* competition experiments” has been carried out in the Institute of General Microbiology at the Christian-Albrechts University of Kiel, Kiel, Germany, under the guidance of Prof. Dr. Tal Dagan and Dr. Nils F. Hülter. The work is original and has not been submitted in part or full by me for any degree at any other University. I further declare that the material obtained from other sources has been duly acknowledged in the thesis. My work has been produced in compliance to the principles of good scientific practice in accordance with the guidelines of the German science foundation. I hereby assure that I have not been revoked any of my academic degrees.

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Summary

Plasmids are extrachromosomal genetic elements that initiate their own replication while making use of their hosts replication machinery. They are ubiquitously found amongst bacteria and are seen as a major component of lateral gene transfer. Therefore, they are considered as vector for the spread of antibiotic resistances. The persistence of plasmids within their host population depends on the ability of the plasmid to properly replicate and segregate to daughter cells of the host. Indeed, several systems have been recognized as beneficial for plasmid persistence. This includes active partitioning systems or addiction mechanisms (toxin-antitoxin systems). While such systems are frequently found on plasmids, many plasmids are lacking recognizable stability systems beyond their own replication initiation. The question of this thesis is whether it is more beneficial for a plasmid to be inherently stable or to be unstable but contain a stability mechanism. Answering this question required us to shift our perspective to view plasmids as Darwinian entities; within the Darwinian framework, plasmid genotypes are associated with an individual fitness where plasmid populations within cells experience natural selection (or drift) leading to the survival of plasmids with the highest fitness. Using an experimental evolution approach I simulate competitions amongst plasmid genotypes. The model plasmids used in this thesis are either inherently unstable, inherently unstable and containing a stability mechanism or inherently stable. The model plasmids are put into competitions with each other and the winners of those competitions are identified by a quantification of the proportions of hosts at the end of the experiment. These competitions reveal that the fitness of an unstable plasmid can be increased through addition of plasmid stability mechanisms. The results allowed me to establish a ranking amongst the systems in terms of fitness benefit. A partitioning system is conferring a higher fitness benefit than a toxin-antitoxin system, however when put into a direct competition, the plasmid containing the toxin-antitoxin system prevails. Containing both systems is ideal for an inherently instable plasmid. However, the fitness increase conferred by a stability system is not as advantageous as stability based on proper replication. Further it is shown that plasmid heterogeneity can be maintained over a longer period of time under non-selective conditions. This leads me to theorize that multimerization amongst different plasmid allele-variants may be a driver for plasmid evolution.

Zusammenfassung

Plasmide sind extrachromosomale genetische Elemente, die ihre eigene Replikation initiieren, während sie die Replikationsmaschinerie ihres Wirts nutzen. Sie sind in Bakterien allgegenwärtig und gelten als ein Hauptbestandteil des lateralen Gentransfers und damit als Vektor für die Verbreitung von Antibiotikaresistenzen. Die Persistenz von Plasmiden innerhalb ihrer Wirtspopulation hängt von der Fähigkeit des Plasmids ab, sich ordnungsgemäß zu replizieren und in Tochterzellen des Wirts zu segregieren. Es wurden bereits einige Systeme entdeckt die als vorteilhaft für die Plasmid-Persistenz gelten. Dazu gehören aktive Partitionierungssysteme oder Abhängigkeitsmechanismen (Toxin-Antitoxin-Systeme). Während solche Systeme regelmäßig auf Plasmiden zu finden sind, fehlt es vielen Plasmiden an erkennbaren Stabilitätssystemen jenseits ihrer eigenen Replikationsinitiierung. Diese Arbeit befasst sich mit der Frage, ob eine inhärente Stabilität vorteilhafter für ein Plasmid ist, als die Kompensation der von Instabilität durch Stabilitätsmechanismen. Zur Beantwortung dieser Frage mussten wir unsere Perspektive ändern und Plasmide als darwinistische Einheiten betrachten. Im darwinistischen Rahmen sind Plasmid-Genotypen mit einer individuellen Fitness verbunden, wobei Plasmid-Populationen in Zellen einer natürlichen Selektion (oder Drift) unterliegen, die zum Überleben der Plasmide mit der höchsten Fitness führt. Mit Hilfe eines experimentellen Evolutionsansatzes simuliere ich den Wettkampf zwischen Plasmid-Genotypen. Die in dieser Arbeit verwendeten Modell-Plasmide sind entweder von Natur aus instabil, von Natur aus instabil und kodieren einen Stabilitätsmechanismus oder von Natur aus stabil. Die Modell-Plasmide werden in einen Wettkampf miteinander gebracht, wobei die Gewinner durch eine Quantifizierung der Wirtsanteile am Ende des Experiments ermittelt werden. Diese Wettkämpfe zeigen, dass die Fitness eines instabilen Plasmids durch Hinzufügen von Plasmid-Stabilitätsmechanismen erhöht werden kann. Anhand der Ergebnisse konnte ich eine Rangfolge der Systeme in Bezug auf den Fitnessvorteil aufstellen. Ein Partitionierungssystem bietet einen höheren Fitnessvorteil als ein Toxin-Antitoxin-System, aber wenn man sie in einen direkten Wettkampf stellt, ist das Plasmid mit dem Toxin-Antitoxin-System überlegen. Ein Plasmid, das beide Systeme enthält, ist ideal für ein von Natur aus instabiles Plasmid. Die Fitnesssteigerung durch ein Stabilitätssystem ist jedoch nicht so vorteilhaft wie die Stabilität, die auf einer ordnungsgemäßen Replikation beruht. Außerdem hat sich gezeigt, dass die Plasmid-Heterogenität unter nicht-selektiven Bedingungen über einen längeren Zeitraum erhalten werden kann. Dies veranlasst mich zu der Theorie, dass die Multimerisierung zwischen verschiedenen Plasmid-Allel-Varianten ein Motor für die Plasmid-Evolution sein könnte.

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Glossary

Homogenic host	Host containing isogenic plasmids (a single genotype)
Heterogenic host	Host containing multiple plasmid genotypes
Plasmid replication	Plasmid replication using the host machinery starting from the pBBR1 *oriV* in the backbone of the plasmids
Plasmid segregation	Distribution of plasmid units into the daughter cells of the host following cell division
Segregational loss	Loss of a plasmid genotype due to segregation in single hosts
Segregational drift	Change in the frequency of plasmid alleles following a bottleneck event induced by cell division
Segregants	cells containing no plasmids; in our setting such cells are the result of cell division where no plasmid was located in one of the daughter cells
Multimers	A plasmid entity that contain multiple plasmid backbones (i.e. *oriV*s) and are the result of concatenation of multiple plasmids
Homomultimer	Multimer comprised of plasmids of the same genotype
Heteromultimer	Multimer comprised of plasmids of different genotypes
Plasmid stability trait	A genetic locus that encodes for a function that promotes plasmid persistence in the population
Par-system	Active partitioning system that positions the plasmid at specific locations during cell division
TA-system	A molecular system that includes a toxin and an antitoxin. When encoded in plasmids the system kills or inhibits the growth of segregants (termed also plasmid addiction system)
PCN	Plasmid copy number
WT	Wildtype. In this case used to refer to the *E. coli* K-12 strain MG1655

Glossary of units and quantities

Colony forming units (CFUs) – a measure used to assess the size of bacterial populations

Proportion of hosts – a measure used to assess the prevalence of plasmid-carrying cells in a population, e.g., in plasmid stability experiments

Ratio of hosts to non-hosts – a measure used to assess the relative frequency of plasmid-carrying cells and non-carrier cells in a population, e.g., in fitness experiments

1 Introduction

Evolution is the adaptation of organisms to environmental conditions, or in other words to natural selection. For natural selection to apply to an organism Darwin has set three criteria: First, there must be variation between entities. Second, this variation must be heritable. Third, the genetic variation must be associated with a variation in fitness. The fitness of an organism describes its ability to prevail against other organism in competitions within their environment, struggling to occupy specific ecological niches. The same can be applied on an intraspecies level since individuals compete with each other to pass on their genes to the next generation. To understand the evolution of stability of genetic units it is important to consider them, too, as Darwinian entities. Plasmids for instance are subject to an intraspecies level of competition whenever a new plasmid variant arises, or a new host with an endemic plasmid population is colonized.

1.1 **Prokaryotic plasmids**

Plasmids are extrachromosomal genetic elements which constitute an integral component of prokaryote genomes. They control their own replication and copy number (PCN) while making use of the replication machinery of their host and may encode for beneficial traits (Bernander et al. 1991; Keasling et al. 1991; Blomberg et al. 1992). An example for such plasmid-encoded beneficial traits are antibiotic resistance genes, which enable their host to survive under selection. In the absence of selection for the plasmid-encoded trait, plasmid persistence in its host is linked to the plasmid stability. This stability is the ability of the plasmid to go through the plasmid lifecycle without problems. The plasmids lifecycle starts with the replication of the plasmid, goes on to the resolution of possible multimers and ends with the segregation of the plasmid copies into the daughter cells (see **Fig. 1**). The stability of a plasmid may be inherently high, or it might be enhanced through stability systems. One type of system is the active partitioning system, which affects the stability of the plasmid during the partitioning step by removing stochasticity (Baxter & Funnell 2014). This is especially beneficial for low copy number plasmids that cannot rely on probabilities to ensure that each daughter cell of its host has at least one copy of the plasmid. Another type of stability system is the Toxin-Antitoxin system, which acts as a failsafe. Whenever segregants, or in other words host that have lost the plasmid, arise, the toxin either kills the cell or greatly inhibits its growth. This is possible since the toxin, which is either an RNA or a protein, is more stable than the corresponding antitoxin (Jurėnas et al. 2022). Thus, the antitoxin must be constantly expressed to inhibit the toxin. Once the gene encoding the antitoxin is gone the toxin is no longer prevented from taking effect. The third type of system is the multimer resolution system. This type of system encodes for a resolvase, which may resolve possible multimers that have formed (LeBard et al. 2008).

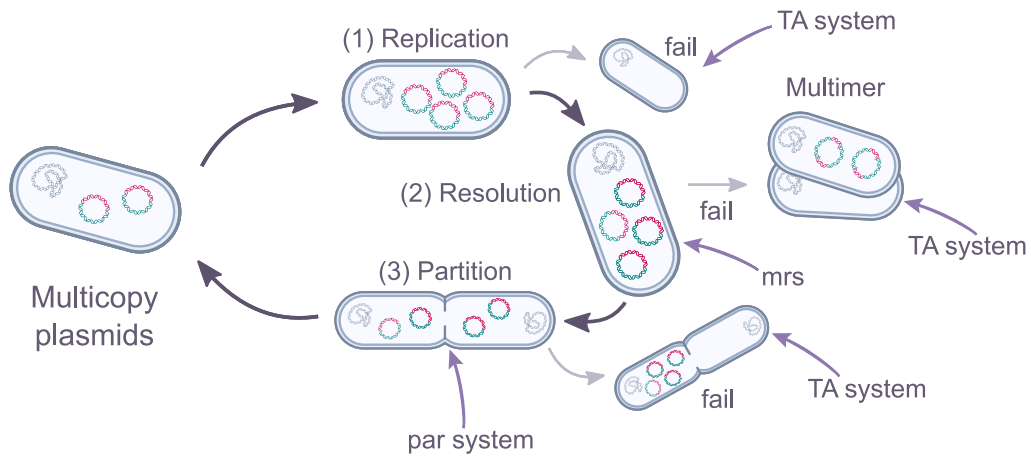

Figure 1. The plasmid lifecycle.

The plasmid life cycle starts with the replication (1) of the plasmids, followed by the resolving (2) of possibly generated multimers and lastly going into the partitioning (3) of the plasmid copies into the two daughter cells to ensure that each daughter cell acquires at least one copy of the plasmid. The points of the lifecycle during which a stability system may have an effect are indicated with the abbreviations for the respective system. Active partitioning systems are labled as par, Toxin-Antitoxin systems as TA and Multimer-Resolution systems as mrs. Modified from Garoña & Dagan (Garoña & Dagan 2021).

1.2 Plasmid fitness

The plasmids used in this study are variants of the model plasmid pCON (Wein et al. 2019). The plasmid pCON consists of the pBBR1 origin of replication, the corresponding *rep* gene and the kanamycin resistance gene *nptII* with its native promoter. This along with the plasmid pCON2 are the ancestral plasmids for all plasmids constructed in this study. The plasmid pCON2 is identical to pCON with the exception of the resistance gene *nptII* which has been replaced with the *cat* gene that confers a resistance to chloramphenicol. Both pCON and pCON2 are characterized by an inherent instability leading to a loss of ca. 10% plasmid hosts per day in a 100% host population. This instability is likely caused by conflicts between transcription of the resistance gene and replication forks that put a physical strain on the plasmid (Helmrich et al. 2013; Lang et al. 2017). The second set of model plasmids that are used in this thesis are the plasmids pCON-S and pCON-S2. The plasmid pCON-S is characterized by increased stability in comparison to pCON and is the result of a longterm evolution experiment conducted with pCON (Wein et al. 2019). In pCON-S2, like in pCON2, the *nptII* gene has been replaced with the *cat* gene. In order to compare two plasmids on an intracellular level, *in cellulo* competitions need to be conducted. These intracellular competitions have been used since at least the 70s to conduct experiments based on

incompatibility between different plasmids (Warren & Sherratt 1978). In a previous study conducted in this lab in order to compare two plasmid variants based on their genetic makeup intra cellular competitions were used (Hülter et al. 2020). The results of the study have shown that the plasmids pCON-S and pCON-S2 are more fit, or likely to displace, the plasmids pCON and pCON2. Here, a great deal of coexistence was observed.

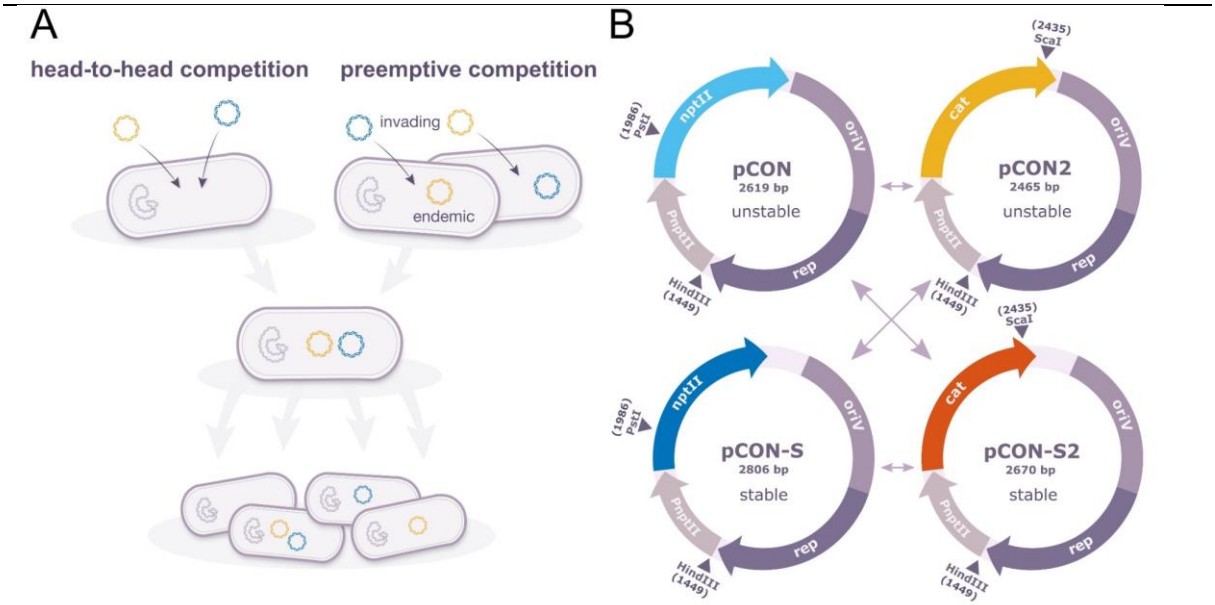

Figure 2: Intracellular competitions.

The setup of the *in cellulo* competitions between the four plasmids pCON, pCON2, pCON-S and pCON-S2 that were conducted in a previous study (Hülter et al. 2020). In subfigure A the general workflow of the competitions is depicted. The competition experiments were either conducted in a head-to-head manner with both plasmids arriving at the same time, or in a preemptive manner where one plasmid was allowed to establish itself within the host population prior to the start of the competition experiment. In subfigure B the different matchups of the plasmids are shown. The unstable plasmids were put into competitions against each other as well as the stable plasmids and *vice versa*. All competitions were conducted in a reciprocal manner, i.e. pCON-S vs. pCON2 and pCON vs. pCON-S2.

Plasmid coexistence is as the term suggests the coexistence of two variants of the same plasmid type, in this case two plasmids with the pBBR1-backbone, within one host cell. This coexistence can either be a result of monomeric plasmid variants inhabiting the same cell being balanced in replication and segregation, or through recombination of the two plasmids resulting in heteromultimers. The occurrence of multimers in the system makes the use of a recombination deficient strain desirable, since multimerization is minimal in the absence of RecA (Bedbrook & Ausubel 1976).

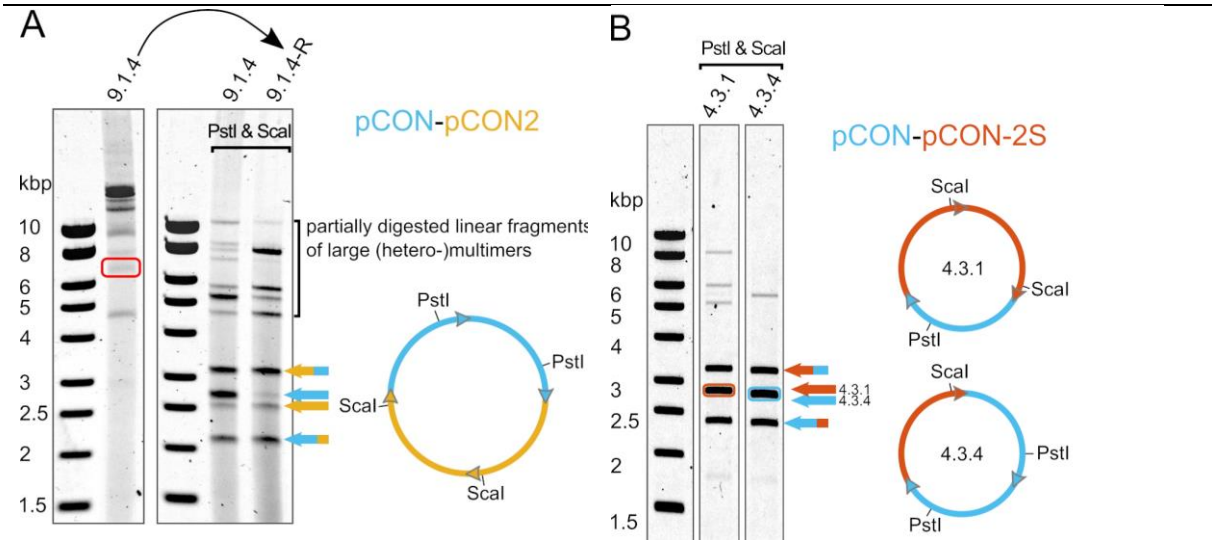

Figure 3: Multimer analysis.

Multimers occurred often in the competition experiments of a previous study conducted in this lab (Hülter et al. 2020). They were shown to be heteromultimers consisting of both plasmids that were put into competition with each other. The multimerization happened to a varying degree. The multimers were confirmed via enzymatic digest and gel electrophoresis.

1.3 Plasmid partition systems

As previously mentioned, par-systems play a prominent role in this study. Active partitioning systems can be categorized in one of three types. The Walker type systems, the actin-like systems and the tubulin-like systems (Brooks & Hwang 2017). An example for a plasmid with a Walker-type par-system is the F plasmid with its Sop-system (Bouet et al. 2007). The Sop system consists of SopA, SopB and *sopC*. The motor-protein of this system is SopA while SopB constitutes the DNA-binding protein targeting the *sopC* site (Mori et al. 1986). Next, for the tubulin-like systems, an example is the repX system of the pXO1 plasmid. The pXO1 plasmid is one of the virulence plasmids found in *Bacillus anthracis* (Tinsley & Khan 2006). The RepX protein of pXO1 has similarities to FtsZ, which is essential for bacterial cell division (Akhtar et al. 2009). In this study the parMRC system of plasmid R1 is used, which has been shown to stabilize mini R1 replicon and pBR322 replicon plasmids (Boe et al. 1987). Lastly, for the actin-like par-systems a well-studied example is the parMRC system of plasmid R1. The parMRC system consists of the actin-like ATPase ParM, the DNA-binding protein parR and the binding site *parC* (Baxter & Funnell 2014).

The parMRC system of plasmid R1 is encoded in the parA locus (Dam & Gerdes 1994). ParR, named for its function as autorepressor of the parMRC system, binds to the centromere like binding site *parC* as dimer. This way two plasmids are linked through the binding of ParR to their respective *parC* sites. The ParM+ATP proteins then bind to the DNA-ParR complex and are able to form filaments through hydrolyzation of ATP to ADP, which pushes the two

plasmids apart (Møller-Jensen et al. 2003). The ATPase activity of ParM is promoted by the binding to the ParR-*parC* complex (Jensen & Gerdes 1997). The parMRC system is the one that is used in this study and termed as parA locus.

1.4 **Toxin-antitoxin systems**

Toxin-Antitoxin systems are thought to have four functions. The first is the prevention of plasmid loss by killing or growth inhibition of segregant cells, the second is for a chromosomally encoded TA-system to counteract the function of a plasmid encoded TA-system, the third is to enable the bacterial cells to survive under stressful conditions and the fourth is the defense against phages (Gerdes et al. 1986; Saavedra De Bast et al. 2008; Maisonneuve & Gerdes 2014; Guegler & Laub 2021). Currently, there are eight different types of TA-systems that are known (Jurėnas et al. 2022). Depending on the type of the TA-system the Toxin and Antitoxin can be either an RNA or a protein. The type I system consists of a toxin protein and an antitoxin, which is a small antisense RNA that prevents the translation of the toxin mRNA. One example for a type I TA-system is the *hok*/*sok* system of the plasmid R1. The functional toxin is the Hok (Host killing) protein that can only be expressed in the absence of the *sok* sRNA, which prevents the translation of the *hok* mRNA (Gerdes et al. 1986). In the case of the type II TA-systems both the toxin and the less stable antitoxin are proteins. An example for a type II TA-system is the *ccd* (coupled cell division) system of the F plasmid which consists of the toxin protein CcdB and the antitoxin protein CcdA which sequesters the toxin protein and thereby inhibits its function (Ogura & Hiraga 1983). Further, the protein complex then serves as an autorepressor for the *ccd* system (Tam & Kline 1989).

The TA-system used in this study is the *hok*/*sok* system of the plasmid R1. The antitoxin is an unstable sRNA which is the result of the transcription of *sok*. The toxin Hok can only be expressed in the absence of *sok* sRNA. Once expressed, Hok targets the cellmembrane and reduces the membrane potential, thereby interfering with cell respiration (Gerdes et al. 1986). The *hok*/*sok* system is referred to as parB system and in this study as parB locus.

1.5 **The plasmid R1**

Plasmid R1 is a conjugative plasmid that was originally isolated from a *Salmonella paratyphi B* strain in 1963 (Datta & Kontomichalou 1965). The plasmid R1 was then extensively studied for various plasmid properties, e.g., the frequency of conjugation (Corliss et al. 1981) and the replication dynamics of plasmid R1 (Bernander et al. 1991; Blomberg et al. 1992; Olsson et al. 2003, 2012). The stabilizing factors of the plasmid R1 parA and parB were also studied to a high degree. The TA-system of R1 is the most well-known TA-system of the type

I systems and is well described (Gerdes et al. 1986). The active partitioning system, namely parA, has also been the subject of many studies and is well described (Dam & Gerdes 1994; Jensen & Gerdes 1997).

Here we use the derepressed derivative of R1, R1*drd*19 (DSM No. 3880), as a source for two tested stability systems parA and parB.

2 Objectives

The aim of this thesis is to determine the impact that stability systems have on the fitness of plasmids. Since stability is an integral part of plasmid fitness, I am trying to answer the question in how far aiding an unstable plasmid during different points of the plasmid lifecycle affects the fitness of this plasmid. Therefore, I want to gauge the difference in fitness benefits conferred by the systems used in this thesis. A further point of interest is to find out whether a stabilized plasmid can have a higher fitness than an inherently stable plasmid, while the stabilized plasmid still possesses the original source of instability. This will answer the question of whether inherent stability is preferable to stability gained through a stability system.

To determine whether these stability systems also improve the fitness of a plasmid the plasmids need to be compared to each other in direct competitions. For these competitions to be viable the plasmids must not have an effect on the fitness of the host since the fitness of a plasmid can be split into two hierarchical levels. The first level is the fitness of the host within its population and the second level is the plasmid within the host. Should the fitness of the host be affected through the carriage of the plasmid it is no longer possible to get a clear picture of the fitness of the plasmid since segregants would overtake the population. Also, the plasmids need to be present in the host cells in a 1:1 ratio. Otherwise, the competition may be biased towards whichever plasmid is present in a higher copy number.

To achieve proper competitions, another aim was to establish an experimental system that fulfills the requirements of balance in the beginning of the competition and an absence of fitness effect on the host. Further, there should be as little multimerization of the plasmids as possible since the formation of heteromultimers prevents a proper view of the fitness differences of the two plasmids in question.

A number of options are viable to ensure balance between the plasmids. One of them is the introduction of a split marker to the model plasmids. A split marker describes a marker gene that has been split in two using an intein. In our case the intein used is *dnaE* of *Nostoc punctiforme*, a subunit of the DNA polymerase III. In order to balance the plasmids after transformation into *E. coli* and prior to the intended competition a split marker consisting of the C- and N- terminal end of *dnaE* of *Nostoc punctiforme* and the C- and N- terminal ends of a Hygromycin B (HygB) resistance gene, namely *hph*, split between positions 171 and 172 bp of the gene may be inserted into the plasmids (Jillette et al. 2019; Palanisamy et al. 2019).

In order to correctly assess the fitness of a plasmid they must not form heterodimers with the competing plasmid. This is especially relevant in the case of this study since our plasmids are variants of the same plasmid backbone. Minimizing the rate of multimerization can be achieved by inserting a Multimer-Resolution system into the model plasmids. Another way of

achieving a reduction of the multimerization is to generate a Δ*recA* Host-strain. Since multimerization is driven by recombination of similar sequences, removing the part of the hosts DNA repair system that is responsible for detecting and recombining single- and double-stranded DNA will reduce the amount of multimers that arise through damage and repair of plasmids.

As previously mentioned, it is possible for a plasmid in nature to enter a cell that already inhabits plasmids of the same incompatibility group, in which case a competition between these two plasmid types starts. If we try to emulate this *in vitro* we need to have host cells with an endemic plasmid population that has the same replication initiation mechanism as the invading plasmid. We then need to be aware that applying balancing selection to the hosts might introduce a bias in the competition towards the invading plasmid. Therefore, selection for both plasmids, while necessary to prove that the host contains both plasmid types, must be kept minimal. The illustration below shows the plasmid competitions included in this thesis.

	Par system	TA system	Par and TA	Stable	Random DNA
Par system	-	x	-	x	x
TA system	x	-	-	x	x
Par and TA	-	-	-	x	x
Stable	x	x	x	(Hülter et al. 2020).	-
Random DNA	x	x	x	-	x

This thesis supports the view of plasmids as Darwinian entities and showcases the differences in fitness of plasmid variants based on their features. Further, this thesis highlights the difference in fitness benefits incurred through different sources of stability. These aspects of this thesis will help with understanding the evolution of plasmid stability and thereby the ability of plasmids to persist under non-selective conditions, which is especially relevant for understanding the spread of antibiotic resistances amongst bacteria.

3 Methods

3.1 Materials

Table 1: Materials used

Material	Components	Manufacturer
50x TAE-Buffer	242 g Tris base (Roth) 57.1 ml 100% Acetic acid (Roth) 100 ml 0.5 M EDTA (Na_2EDTA, Applichem) Ad 1 l ddH_2O	
Agarose 0.7%	3.5 g Agarose (VWR Life sciences) 500 ml 1x TAE-Buffer	
Chemically competent *E. coli* DH5α cells	Generated using the protocol of H. Inoue (Inoue et al. 1990)	
Chemically competent *E. coli* MG1655 cells	Generated using the protocol of H. Inoue (Inoue et al. 1990)	
Chloramphenicol		Roth
EcoRI		New England Biolabs
Gel extraction kit		Thermo Scientific
GeneJET Plasmid Miniprep Kit		Thermo Scientific
Glycerol		VWR Chemicals
HindIII		New England Biolabs
Hygromycin B		Roth
Hyperladder™ 1 Kb		Bioline
IPTG		Thermo Scientific
Kanamycin		Roth
LB Agar plates	As LB + 15 g Agar agar	
Lysogeny Broth (LB)	5 g NaCl 10 g Tryptone 5 g yeast extract pH 7.5 (components given per liter)	
MfeI		New England Biolabs
Monarch plasmid extraction kit		New England Biolabs
Nb.BsrDI		New England Biolabs
NcoI		New England Biolabs
NspI		New England Biolabs
Phosphate Buffered Saline (PBS)	8 g NaCl 0.2 g KCl 1.44 g Na_2HPO_4 0.24 g KH_2PO_4	

	pH 7.4 (components given per liter)	
Phusion polymerase		Thermo Scientific
SnaBI		New England Biolabs
Tetracycline		Sigma Aldrich
Trimethoprim		Sigma Aldrich
Tris-HCl		Roth
Wizard DNA extraction kit		Promega

Table 2: Plasmids and relevant genotype

Plasmid	**Relevant genotype**	**Source**
pLC	Art1, AmpR, Art2	(Ilhan et al. 2019)
pCON	*nptII*	(Wein et al. 2019)
pCON2	*cat*	(Hülter et al. 2020)
pCON-S	*nptII*, duplication between ori and *nptII*	(Wein et al. 2019)
pCON-S2	*cat*, duplication between ori and *cat*	(Hülter et al. 2020)
pCON-P	*nptII*, parA locus of plasmid R1	This study
pCON2-P	*cat*, parA locus of plasmid R1	This study
pCON-T	*nptII*, *hok*/*sok* of plasmid R1	This study
pCON2-T	*cat, hok*/*sok* of plasmid R1	This study
pCON-PT	*nptII*, parA locus and parB locus of plasmid R1	This study
pCON2-PT	*cat,* parA locus and parB locus of plasmid R1	This study
pCON-R	*nptII*, Art1, AmpR, Art2	This study
pCON2-R	*cat*, Art1, AmpR, Art2	This study
pCON-C	*nptII*, Hph171-split C-terminal, *dnaE* C-terminal	This study
pCON -N	*nptII*, Hph171-split N-terminal, *dnaE* N-terminal	This study
pGP-Tn7-Tp	*dhfrII*	(Crépin et al. 2012)
pTNS2	*bla*, *tnsABCD* of Tn7 (helper plasmid)	(Choi et al. 2005)
pTKRed	*recA*, λ-Red, ori_{pSC101}	(Kuhlman & Cox 2010)
pTKLP-*tetA*	*tetA*	(Tas et al. 2015)
R1*drd*19	parA, parB	From DSMZ (DSM No. 3880)
pETDuet-1_HygR-split171	N-terminal split marker locus (Hph171-split N-terminal, *dnaE* N-terminal), C-terminal split marker locus (Hph171-split C-terminal, *dnaE* C-terminal)	Acquired from Genscript with synthesized sequences according to (Jillette et al. 2019; Palanisamy et al. 2019)

Table 3: Sequences of genetic loci used

Module name	sequence
ParA	CGGAAAGCTTTCATAACGCTGCATGGGGAGTGCTATCATTGTGGTTTGA ACTCGTCACAAAAATAGATCTGGATATACATAAAAAGAACAGGTATGCAA GTTATGATTTTAGAAGAAAAATCGAAATGCAGCATGAAGAGTTTCAGAAA ATGACAGAGCGTGAGCAAGTGCCTTTATTGAAATTGCCTGAATAACAGA GACAGTGCAAACGATTATTTCATAATAACGATTATGTAATGACTGGTAAG AAAATAACTTGATTGAGTTCCCTTTATCCAGCCTGATAGTGGATAAAGGG AACTCAATAATAATTGAATTAATTTATTAGCTTCATCGCATTTTTTTTGGT CTCTTCATCACTTTTTTGCTCTTGTTGTGAAGAGGATTGAGTGACTATTG AAGAATTAAAATCGGCCATCTCTTTTGGAAATAGCGATCTCAATATATTC ACGATATCTGAAAATGTGGTTTCTTTCGTCAGCAGCTCACATAAAAGGAA AGGGGTCCGGGGATCTTGTCTGTATAAGGCCAGACCTGCCGTCAGTGC GGCCCGGTTAAGGCGGCTTCGTTCCCCTTGCGGGATACTGTCCAGTGT ATCACAAACAATTTTATCTGTTTGATTTACATCTGGATTTAGTTTGAAGGC AATGGTTCTGCGCTTGTCCATCATTAATTACCTATGAGATACATACCGTT AACTAAATCATATTGAGAGTTATTGGTTTTGAAAAAACGTTCATCACGAA TCTGTGTGTGTTTTTTTACTGCATCGCATATTAATTCTGCGCCACCGCCT ATAACCATAACATGAGTATAACCAGAAAATTCATTGAGCGTATTTAATAC ACGTTGCTCAAGTTTACGAAGTGCTTCATTCATTGCTTCGGTGACTATTG ATATTTTGTTCTCATCATTAATTCGTTGCTTCAGATAGTTATTATCTTTTCT GTGAATGATTATATCGTCAGCAAGATAGCTACTTCCTTTTGTTCTCGCAA GAGAAAGGGCATCTTTTACTGCAGATGTAACCAGAGAGACACCAAGAGA TGAGTCTCCGTATATTTTACTGATCCCCGATAATTTCCCCATTACCTGAG AAATATCTAATGTGGTGCCCCCGAGATCTATAATTAATAAAGAATCTAAC TCATCCAGTTCTTGTAGAACTTCATAACCTGCCGGTATAGATTCAGGCAT GACTTTTACATCTTTTATTGTGAATGTATCCCCGCCATTTAATGTAATTTT TTTCCGGAAGTTTGCTTTCTTACGCTCAATATTTTCCGTATTGGGTTGGT TATTTCTGTCGTAATACTCTGTCAGAGGAAGTGTGCAAACAATATCCACT TCGCTTACCGGCAGACCACTGGTCAGTAAGGCGTGATGCACTGCAACG ACATTAACGTCGCTGTATTGCCATGCGATATTGGTTGTGACTACAGCAT CCGGGCTGATTGGATCAAATGAATACTGTTCGCCGTTCAGTGTGTAGTT AAAGACCTTTTTATCACCAAAAGAGACTGCCCACTCGCGTTTGAAGCTG TTCGGGCTAATGTGCTGTTTAATTGTTCCGTCGCTTTCCTGCCACTGTA GTTTGATGTTTGTTGAACCGTCATCAATGAATACCAACATTTATAAAACT CCTTATGGTGTTTTTTTGGTGTGTGTTTGGGTATGTTTTGGGTTTTAAGT GGGTTTGTTTGTCAAGTTTACCCCATTTCAACCATCAATCAATGATTATTT GTCTTGTTTTGGTGTTTTATTGGGTTGGGTATGGGTTGTTTTTGGGTTTT GTTTGGCGGGTAACAAAAGATGTCGCCGGGCTCCTCTTTTTACTGCGCC GGCTGACGCGGCGCGGCAGGAACGCTGCCTGTGGTCAGTGTCCCGCG TCCGGCCGGGCACGGGACGGGCGCTTTTACCGGAATTCGCTGGAAGC TTTGACA
ParB	GTCCGTGAGAAGAAGCTTGGGATGGTGCCGAACAAACTCCGGGAGGCA GCGTGATGCGGCAACAATCACACGGATTTCCCGTGAACGGTCTGAATG

	AGCGGATTATTTTCAGGGAAAGTGAGTGTGGTCAGCGTGCAGGTATATG GGCTATGATGTGCCCGGCGCTTGAGGCTTTCTGCCTCATGACGTGAAG GTGGTTTGTTGCCGTGTTGTGTGGCAGAAAGAAGATAGCCCCGTAGTAA GTTAATTTTCATTAACCACCACGAGGCATCCCTATGTCTAGTCCACATCA GGATAGCCTCTTACCGCGCTTTGCGCAAGGAGAAGAAGGCCATGAAAC TACCACGAAGTTCCCTTGTCTGGTGTGTGTTGATCGTGTGTCTCACACT GTTGATATTCACTTATCTGACACGAAAATCGCTGTGCGAGATTCGTTACA GAGACGGACACAGGGAGGTGGCGGCTTTCATGGCTTACGAATCCGGTA AGTAGCAACCTGGAGGCGGGCGCAGGCCCGCCTTTTCAGGACTGATG CTGGTCTGACTACTGAAGCGCCTTTATAAAGGGGCTGCTGGTTCGCCG GTAGCCCCTTTCTCCTTGCTGATGTTGTACGGGCATGAACAACCTGACT TCAGGAAGGTCGTTTCCCTTCAGGAACGGGGATGAACGCGCGCCTGCG GCGCCCGTTCTTTTCCCCCGAATTCTCTGGTTAAGCTTTGTCAG
R-locus	AATTCAATGACTCAGACGAAGAACTCACTAGGGAATGGCGGTTTCAGTT GCCAATGATGGATTTCAGCGAAACTAGGAAGCGATGTGGCCTCTATGTA TGGCAATCTATGTACGAGGTCTGTCGTAAGACTTAGAAACTAAATATTG GTAGCTTCCTTAGTTAAAGTGCAAGGATTGCACAATTCAGTAGACATTTT CTCTGGTATTCCCGACCGGGGAGCTTTATGAAGACAAAGTTTGATCTCC GACGGGATGAACCACAGGCTCTATGATGTCTGAGAAGCTACTAAACTTC GGGGGTTGGGTACAAGTTAGTTGAACATACAAATCGGAATACGATTGTG TCCTTGGTCGTCTCTGAACAAATCGTAGTGAACCCGTGCAAAGTATATA TATTATTTCATACGGCGTCTCAAAGAGGCAATGGCTTGAGATGAGCTGG ATTTCAGATTATGAGTAACCGCAAGATAGTGTAAACACTGTAATCCATAC TTGATTGAGATCCCTTAAAAATGCTGATACCATTTTCTAAACTCGATGGT TTGTTGACAATTATTTGTACAGCGACGCATCTAGGAATTCGATGGCCGC AAAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACT TGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGA TCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATA ACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATA CCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAG CCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCC TCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGC CAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGT GTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGA TCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCT CCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATC ACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCC GTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAG AATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGG ATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAA ACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCC AGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTAC TTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGC AAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTC CTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGG

	ATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCA CATTTCCCCGAAAAGTGCCACCTGCATCGATCAAGCTTATCGATACCGT CGACCTCGAGGATCGGATAACCTTGTGTTCGTGGAAAGGGTACATTTG GGTCGCAGTAGTGGTGTTACTTCTGAAACTAGCTAGGAATAACGAAAAT CTGTCTTGTTTCTCGACGTCAAACCCTTGTCAGCCACCTGCGGTGGTGC ACGAAAAAACCGATTGGAATATTAAATATTTACAACTCCAAATTTTAAGA GCCCTGCTCTACTTTGAATGCATATATGAGTATAGGCTTCGCAATCTAGT GAATGAGCGGTATGAATTTTCGCGGAGTATGAAAAGCGACTGAGTAATC GAGATACATACCGTTAGATAGATGCTCCAGACTGCATTCTGAATTTTTGA TTTATAACCATCGAAGGAATCAATTTCACGTTCGTAACGTGTTAAGTCGT CAGTAAAAGTGACCAGAAGGGCTCCCCCCTTGAAAGAGTACTGCTTCAA ACTATAGGCTGCCCTACTTGGTGTCCGAGGTACTATAGAGTCTTCGATG ATTTGAAGCCCCCAACCCGTGTTCAATCAACTTCTAGGTTTAGCCTTAAG CTAACACAGGAACCAGCTGAGACTTGCTTAGCATCACTTGCTCGAG
Hph-split171 N-terminal	AGCTTCCAGCCAATTGTCTGTTTCTCGAGCTCAAACCCTTGTGAGGAGA AATTAAGCATGAAAAAGCCTGAACTCACCGCGACGTCTGTCGAGAAGTT TCTGATCGAAAAGTTCGACAGCGTCTCCGACCTGATGCAGCTCTCGGA GGGCGAAGAATCTCGTGCTTTCAGCTTCGATGTAGGAGGGCGTGGATA TGTCCTGCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGATCGTTAT GTTTATCGGCACTTTGCATCGGCCGCGCTCCCGATTCCGGAAGTGCTT GACATTGGGGAATTTAGCGAGAGCCTGACCTATTGCATCTCCCGCCGT GCACAGGGTGTCACGTTGCAAGACCTGCCTGAAACCGAACTGCCCGCT GTTCTCCAGCCGGTCGCGGAGGCTATGGATGCGATCGCTGCGGCCGA TCTTAGCCAGACGAGCGGGTTCGGCCCATTCGGACCGCAAGGAATCGG TCAATACACTACATGGCGTGATTTCATATGCGCGATTGCTGATCCCCAT GTGTATCACTGGCAAACTGTGATGGACGACACCGTCAGT

dnaE N-terminal	TGCTTAAGCTATGAAACGGAAATATTGACAGTAGAATATGGATTATTACC GATTGGTAAAATTGTAGAAAAGCGCATCGAATGTACTGTTTATAGCGTTG ATAATAATGGAAATATTTATACACAACCTGTAGCACAATGGCACGATCGC GGAGAACAAGAGGTGTTTGAGTATTGTTTGGAAGATGGTTCATTGATTC GGGCAACAAAAGACCATAAGTTTATGACTGTTGATGGTCAAATGTTGCC TATTGATGAAATATTTGAACGTGAATTGGATTTGATGCGGGTTGATAATT TGCCGAATTAATAATAACGTATCAGGTATTCGGAATTC
dnaE C-terminal	AAGCTTCCAGCCAATTGTCTGTTTCTCGAGCTCAAACCCTTGTGAGGAG AAATTAAGCATGATCAAAATAGCCACACGTAAATATTTAGGCAAACAAAA TGTCTATGACATTGGAGTTGAGCGCGACCATAATTTTGCACTCAAAAAT GGCTTCATAGCTTCTAACTGC

Hph-split171 C-terminal	GCGTCCGTCGCGCAGGCTCTCGATGAGCTGATGCTTTGGGCCGAGGA CTGCCCCGAAGTCCGGCACCTCGTGCACGCGGATTTCGGCTCCAACAA TGTCCTGACGGACAATGGCCGCATAACAGCGGTCATTGACTGGAGCGA GGCGATGTTCGGGGAT TCCCAATACGAGGTCGCCAACATCTTCTTCTGGAGGCCGTGGTTGGCTT GTATGGAGCAGCAGACGCGCTACTTCGAGCGGAGGCATCCGGAGCTT GCAGGATCGCCGCGGCTCCGGGCGTATATGCTCCGCATTGGTCTTGAC CAACTCTATCAGAGCTTGGTTGACGGCAATTTCGATGATGCAGCTTGGG CGCAGGGTCGATGCGACGCAATCGTCCGATCCGGAGCCGGGACTGTC GGGCGTACACAAATCGCCCGCAGAAGCGCGGCCGTCTGGACCGATGG CTGTGTAGAAGTGCTCGCCGATAGTGGAAACCGACGCCCCAGCACTCG TCCGAGGGCAAAGGAATAGTAATAACGTATCAGGTATTCGGAATTC

Table 4: Oligonucleotides used

Name	**5'-3'**
#98_Constr_pCON-P_H3+ER1	TGTCAAAGCTTCCAGCGAATTCCGGTAAAAGCGCCCGTCC
#99_Constr_pCON-T_H3+ER1	CTGACAAAGCTTAACCAGAGAATTCGGGGGAA
#100_Constr_pCON-T_H3	GTCCGTGAGAAGAAGCTTGGGATGGTGCCGA
#101_Constr_pCON-R	AATTCAATGACTCAG
#102_Constr_pCON-R	CCCCCTCGAGCAAG
#103_Constr_pCON-PT_E+A	GTTTCAGAATTCTGCATGGGCCCTGCTATCATTG
#104_Constr_pCON-R_V2	CTTCGTCTGAGTCATTGAATTGGATGAATGTCAGCTACTGG
#105_Constr_pCON-R_V2	CTTAGCATCACTTGCTCGAGGGGTCAGCACCGTTTCTGCGG
#106_Constr_pCON-P_V2	CGGAAAGCTTTCATAACGCTGCATGGGGAG
#107_pCON/2_HindIII_Inv	TTTGAAGCTTCACGCTGCCGCAAGC
#108_pCON/2_HindIII_Inv	CGTGAAGCTTCAAACATGAGCAGATC
#109_Seq_parA	TTTGATCCAATCAGCCCGGA
#110_Seq_pnptII-- insert	CCTTTAGCAGCCCTTGCGCCC
#111_Seq_parA	GGATACTGTCCAGTGTATCACAAAC
#112_parB_4_GA	GCGCGTTCATCCCCGTTCCT
#113_parB_4_GA	GGAGCGGTGGATGGTGCCGA
#114_GA_pCON-P+T	TCGGCACCATCCACCGCTCCAAACTCTTCATGCTGCATTTCG
#115_pCON-P+T	AGGAACGGGGATGAACGCGCCAGAAAATGACAGAGCGTGAGCAA G
#116_GA_pCON2-T	CTCTGGTTAAGCTTTGTCAGCAAACATGAGCAGATCTACC
#117_GA_pCON2-T	CCCAAGCTTCTTCTCACGGACGCTGCCGCAAGCACTCAGGG
#118_GA_pCON-T	CTCTGGTTAAGCTTTGTCAGGAGCAGATCTACCGGCGCGGCAGC
#119_dhfrII_GA_2	CTACGTGTTCCGCTTCCTTTCTTAGGCCACACGTTCAAGTGC

#120_dhfrII_GA_1	CTCAGGGCGCAAGGGCTGCTCACAAGAAGGATTCGACATGG
#121_GA_dhfrII_1	AGCAGCCCTTGCGCCCTGAG
#122_GA_dhfrII_2	AAAGGAAGCGGAACACGTAG
#123_GA_dhfrII_1	CCATGTCGAATCCTTCTTGTGAGCAGCCCTTGCGCCCTGAG
#124_GA_dhfrII_2	GCACTTGAACGTGTGGCCTAAGAAAGGAAGCGGAACACGTAG
#125_dhfrII_GA_1	TTCTTAGGCCACACGTTCAAGTGC
#126_dhfrII_GA_2	TCACAAGAAGGATTCGACATGG
#127_GA_Insert_pCON-C/N	CAATTGTCTGTTTCTCG
#128_GA_Insert_pCON-C/N	GAATTCCGAATACCTGATACG
#129_GA_Vector_pCON-C/N	CAGGTATTCGGAATTCAAAGGAAGCGGAACACGTAG
#130_GA_Vector_pCON-C/N	GCTCGAGAAACAGACAATTGAGCAGCCCTTGCGCCCTGAG
#131_seq _P/PT_SplitMark	GCAGGAACGCTGCCTGTG
#132_seq_T_SplitMark	CGCCCGTTCTTTTCCCCC
#133_seq _P/PT_SplitMark	GCAGGAACGCTGCCTGTG
#134_seq_C-Split	CTGGAGGCCGTGGTTGG
#135_seq_N-Split	CCTGCCTGAAACCGAACTGC
#136_seq_parB	GGGGGAAAAGAACGGGCG
#137_seq_parB	GACACACGATCAACACACACC
#138_seq_parB	GGTGGTTTGTTGCCGTGTTG
#139_seq_PT_parB	GCACTGTCTCTGTTATTCAGGC
#140_seq_ori_pCON	GGAACGACCCAAGCCTATG
#141_seq_ori/rep_pCON	GATTGCGCATGGTGCCG
#142_seq_rep_pCON	GACACTTTCCAAGCTCATCG
#143_seq_parM-parR	GGTAATTAATGATGGACAAGCGC
#144_Lig-Frag_C/N	CACTCAATTGTCTGTTTCTCG
#145_Lig-Frag_C/N	CGACTGAATTCCGAATACCTGATACG
#146_GA_Bb1+pRep	ATCCTCTACGCCGGACG
#147_GA_Bb2+Split2	GTAGCGGCGCATTAAGCG
#148_GA_pRep1+Bb1	CGTCCGGCGTAGAGGATCTGTAATTGACTGCCAC
#149_GA_pRep+Split1	CATGACCATGGTATATCTCCGCTTATCTCCATGCGGTAG
#150_GA_Split1+pRep	GGAGATATACCATGGTCATG
#151_GA_Split1+pRep2	GCGGCCGTGTACAATAC
#152_GA_pRep2+Split1	GTATTGTACACGGCCGCCTGTAATTGACTGCCAC
#153_GA_pRep2+Split2	CATGAAGATCTGCCATATGCTTATCTCCATGCGGTAG
#154_GA_Split2+pRep2	CATATGGCAGATCTTCATG
#155_GA_Split2+Bb2	CTTAATGCGCCGCTACAGGGCGCGTCCCATTCG
#156_control_GA_pSM	GGGCGCGTCCCATTC
#157_GA_pRep(IHF)+Bb1	CGTCCGGCGTAGAGGATGCGAAATGCGAAAGAC
#158_GA_pRep2(IHF)+Split1	GTATTGTACACGGCCGCGCGAAATGCGAAAGAC
#159_seq_ori-ABr	ACCCGCCTTCCACTTGG
#160_seq_rep-ori	GACTGCGTATTGGACCG
#161_seq_R	CGCAAGGGCTGCTAAAGG
#162_seq_R	CACCACCGCAGGTGG
#163_seq_R	CTGCTATGTGGCGCG

#164_seq_R	GGCTGGCTGGTTTATTGCTG
#165_seq_R	GCACGGGTTCACTACG
NH300	AAACAAGACGATTAAAAATCTTCGTTAGTTTCTTGGCTTCAGGGATGAGG
NH301	GTATTACCCGGCATGACAGGAGTAAAAATGTACGGCCCCAAGGTCCAAAC
art2_F(JI)	CTCGAGCAAGTGATGCTAAGC

3.2 Bacterial strains and culture conditions

All bacterial cultures used were grown at 37 °C at 200 rpm in LB-media or on LB agarplates unless otherwise specified. The strains used include the *E. coli* K-12 strain MG1655 (DSM No. 18039), *E. coli* DH5α (Hanahan 1983), *E. coli* BL21 (DE3) (Studier & Moffatt 1986), *E. coli* MFDpir (Ferrières et al. 2010) and *E. coli* XL-1 (Stratagene).

3.3 Plasmid construction

The plasmid constructions were performed either by standard restriction/ligation cloning procedures or Gibson assembly using *E. coli* DH5α as the primary cloning host.

Insertion of the partitioning system

The plasmids pCON and pCON2 were linearized using HindIII. Afterwards, the parA locus of R1 was amplified using the primers #98 and #106, introducing two HindIII restriction sites at each flank. The amplified fragment was then digested using HindIII and ligated using T5-Ligase with each of the two linearized plasmids, resulting in pCON-P and pCON2-P.

Insertion of the toxin antitoxin system

The plasmids pCON and pCON2 were amplified via PCR using the primers #117 and #118 along with Phusion polymerase. The parB locus of R1 was also amplified via PCR using the primers #99 and #100, introducing overhangs that are complementary to the flanks of the linearized plasmids. The plasmids were then assembled with the parB locus containing PCR fragment using the Gibson Assembly mix, resulting in pCON-T and pCON2-T.

Adding the toxin antitoxin system to the partitioning system

Both pCON-P and pCON2-P were amplified via PCR using the primers #114 and #115. The parB locus of R1 was then amplified by PCR using the primers #112 and #113, resulting in the desired fragment with addition of complementary flanks to the linearized plasmids. The two components were then assembled using Gibson Assembly, resulting in pCON-PT and pCON2-PT.

Increasing the size of the model plasmids

The fragment intended for the increase of the plasmid size was amplified from pLC using the primers #101 and art2_F(JI). The plasmids pCON and pCON2 were amplified and linearized using the primers #104 and #105. The fragments were fused using Gibson Assembly, resulting in pCON-R and pCON2-R.

Cloning a split marker into the model plasmids

The N-terminal and C-terminal split marker cassettes were excised from the plasmid pETDuet1-HygR-split171 using the enzymes EcoRI for the acquisition of the N-terminal fragment and MfeI to obtain the C-terminal fragment. Each fragment was then amplified via PCR using the primers #127 and #128 with Phusion polymerase. The plasmid pCON was afterwards amplified using the primers #129 and #130 and Phusion polymerase. The respective PCR products, e.g. the pCON product and the N-Terminal product, were afterwards assembled using Gibson assembly.

Correct clones were first identified via restriction digestion with appropriate enzymes and subsequent gel electrophoresis. Following this, the clones were again confirmed by Sanger sequencing (Eurofins).

3.4 **Preparation of electrocompetent cells**

For the preparation of electrocompetent cells liquid cultures (2 mL) of the respective bacterial strain were inoculated and grown overnight at optimal temperature (37 °C for *E. coli*) with constant shaking (200 rpm in Innova 44). If the strain contained a plasmid, the respective selective pressure for plasmid maintenance needed to be applied. After the overnight incubation 1 ml of the grown liquid cultures was used to inoculate fresh LB medium (1 l) in a 2 l flask, which was afterwards also grown at optimal temperature. After 2 h the OD_{600} of the culture was measured and once an OD_{600} of 0.6 had been reached, the flask was transferred onto ice and incubated while constantly shaking it for 20 min. The culture was afterwards split into four Falcon Tubes and centrifuged in a pre-cooled (4 °C) centrifuge (Heraeus Multifuge X3R with the Fiberlite F15-8x50cy rotor; Thermo Scientific) at 4000 g for 10 min. The supernatant was discarded, and the cells were washed with cold 10% glycerol. The wash step was repeated once. The cells were afterwards pooled again by resuspending them in 1 ml of 10% glycerol, i.e. the cells in the first tube were resuspended in 1 ml of 10% glycerol and this suspension was then used to resuspend the next tube and so on. The final suspension of cells was then transferred into a 2 ml Eppendorf reaction tube. This tube was again centrifuged in a pre-cooled (4 °C) centrifuge (Heraeus Fresco 21; Thermo Scientific) at 4000 g for 10 min and the supernatant discarded afterwards. The cells were afterwards resuspended in 0.4 ml of cold 10% glycerol, aliquoted into 40 µl each and stored at -80 °C (Dower et al. 1988).

3.5 **Transformation**

For the transformations in all conducted experiments or constructions either a heat-shock protocol or an electroporation protocol was used.

Heat-shock:

For the heat shock, chemically competent cells were used (Inoue et al. 1990). The aliquots were thawed, mixed with the DNA and incubated on ice for 30 minutes. The

transformation tubes were shifted to a water bath at 42 °C and incubated between 45 – 90 s, followed by 2 min on ice. Afterwards the transformation mix was then transferred into fresh LB medium and incubated for 1 h at 37 °C and 200 rpm. After this outgrowth period appropriate dilutions and volumes were plated on selective LB agar plates and incubated at the respective temperatures overnight.

Electroporation:

The electrocompetent cells were transferred into cuvettes with a gap of 1 mm and mixed with the respective amounts of DNA, e.g. 1 ng for each plasmid in the intracellular competition experiments. Afterwards the cells were electroporated at 1350 V, 25 uF and 600 Ω using the BioRad Genepulser Xcell. Afterwards the transformation mix was transferred into 1 ml of fresh LB medium and incubated for 1 – 3 h at 37 °C and with 200 rpm. The cultures were then either exposed to positive selection in liquid media or on agar plates.

3.6 **Stability assays**

The stability assays are conducted as follows. First, the plasmid host were given an overnight incubation at 37 °C and 200 rpm in liquid media with selective pressure to keep the host ratio as high as possible. If the cells were taken from a cryostock, an additional day of growth on a selective plate was added prior to the liquid preadaptation. Following the preadaptation, the OD_{600} of the cultures was adjusted to 0.01 and fresh media was inoculated. The ratios of hosts within the populations at t_0 were determined by differential plating after inoculation of the liquid culture used for the assay. Following this step after every 24 h for seven days, i.e. t_0 to t_{168} the ratios were determined by plating on LB plates and on selective plates containing antibiotics that are selective for the respective plasmid (10 ng/µl Km for pCON variants; 10 ng/µl Cm for pCON2 variants). Whenever the culture was plated in order to determine the ratio of hosts, fresh LB liquid media was also inoculated with the previous culture, introducing a 1:100 bottleneck for the population.

3.7 **Construction of Tp^R strains**

The trimethoprim resistant (Tp^R) strains used for the assessment of possible fitness effects of the plasmids on the host were generated through triparental mating of the host strain, in this case either *E. coli* MG1655 or *E. coli* MG1655 *recA*::*tetA*, with the two *E. coli* strains MFDpir pGP-Tn7-Tp (Crépin et al. 2012) and MFDpir pTNS2 (Choi et al. 2005). Liquid cultures for the three strains are set up and incubated overnight at their respective optimal temperature. The next day, all three strains were washed by centrifuging for 5 min at 3000 rpm and resuspension in PBS. This step was repeated once, afterwards the cells were resuspended in 500 µl of PBS mixed in a 1:1:1 ratio based on their OD_{600}. The mix was afterwards spotted onto a non-selective agar plate containing DAP and incubated at 30 °C. After the mating the

conjugation spot was resuspended, the cells plated onto selective agar plates lacking DAP and incubated at 40 °C. This prevents the MFDpir strains from growing due to their requirement of DAP and the plasmids pGP-Tn7-TP and pTNS2 from replicating due to their temperature sensitivity, leaving only the recipient strain alive on the plate. The correct clones were then confirmed by plating on LB agar plates containing Trimethoprim (125 ng/µl), followed by Sanger sequencing.

3.8 **Construction of *E. coli* MG1655 *recA*::*tetA***

To generate the *E. coli* MG1655 *recA*::*tetA* strain the parental strain MG1655 was first transformed with the helper plasmid pTKRED (Tas et al. 2015), harboring genes encoding the λ-Red enzymes for recombineering. The *tetA* gene along with its promoter P_{lacIQ1} was amplified using pTKLP-*tetA* as template. For the amplification of *tetA* with its promoter the primers NH300 and NH301 were used along with Phusion polymerase. After acquiring the MG1655 strain containing pTKRED a liquid culture with IPTG (2 mM) was inoculated and after overnight incubation at 30 °C electrocompetent cells were generated. The electrocompetent cells were then transformed with the cleaned PCR product. The transformed cells were incubated at 30 °C and plated onto LB agar plates containing tetracycline (10 ng/µl). Afterwards the transformants were cured of pTKRED by incubation at elevated temperature (37 °C). The transformants were further confirmed via Sanger sequencing.

3.9 **Plasmid fitness effect on host**

The fitness assays were conducted as follows. The first step was preadaptation in liquid media for a total of three days. The preadaptation was done with and without antibiotics depending on whether the respective strain carries a plasmid. After each day the cultures were transferred to fresh liquid LB media. The plasmid carrying strains and the plasmid-free strain were competed against a plasmid-free strain, that is isogenic except for a Tp^R gene. After the preadaptation the strains were diluted 1:10, the OD_{600} of the strains then measured and adjusted to 0.1. The competing strains were then used to inoculate 1960 µl of fresh LB media, with 20 µl from strain 1 and another 20 µl from strain 2. The competition mix was then divided into two 1000 µl volumes, where one was transferred into the overnight competition and the other was used to determine the ratios of hosts at the starting point t_0. The ratios of hosts were determined by plating the competition mix in appropriate dilutions onto non-selective LB agar plates, LB agar plates with either Km (10 ng/µl) or Cm (10 ng/µl) and LB agar plates with Trim (125 ng/µl). After 24 h the endpoint of the competition was measured by again plating on the respective media plates.

3.10 **Intracellular competitions**

The intracellular competitions were conducted in one of two ways (see **Fig. 4**). The first way was the head-to-head competition in which the host was electroporated and transformed

with two plasmids, 1 ng of plasmid DNA each, at the same time, followed by 3 h of non-selective growth in liquid LB medium to allow the plasmids to start replicating and establish themselves within their host. After this outgrowth period antibiotics were added to a concentration of 10 ng/µl for Km and 3 ng/µl for Cm. The liquid culture is then incubated overnight for ~16 h. Once the incubation had finished, the culture was diluted and then plated on double selective media (Km 10 ng/µl, Cm 10 ng/µl) and incubated at 20 °C for 3 days to ensure that only hosts carrying both plasmids survive, while keeping the growth rate minimal. The colonies that grow on the double selective plate were then excised, resuspended in PBS and diluted.

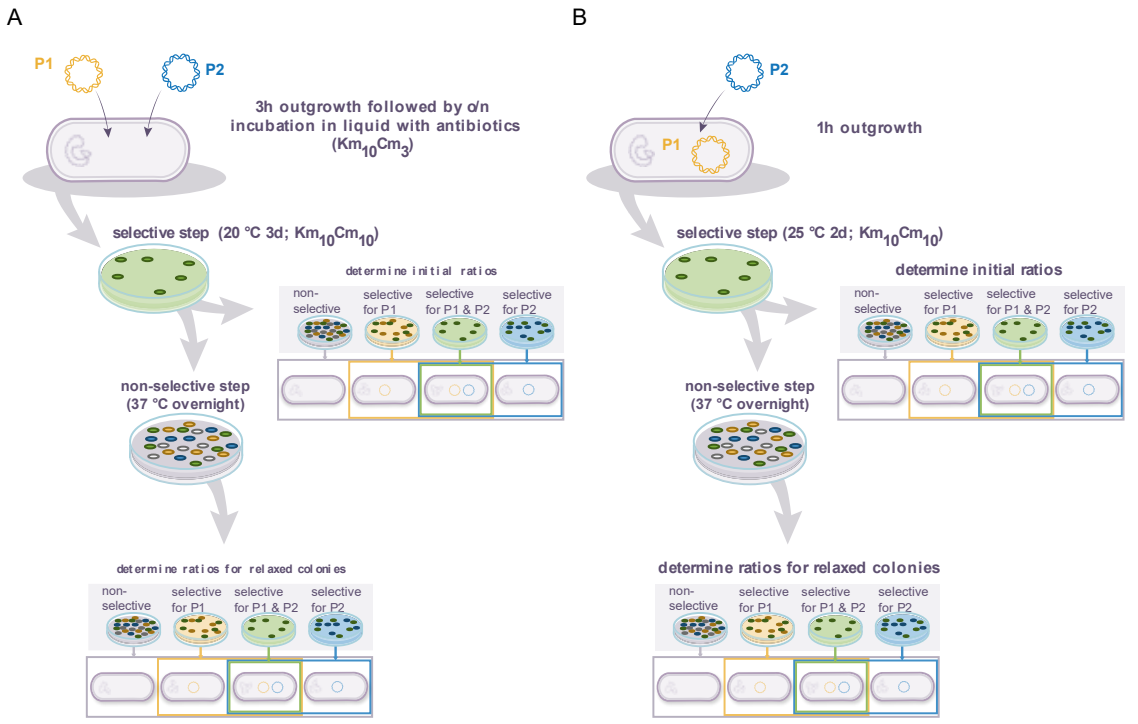

Figure 4: Workflow for the intracellular competitions.

A) Shown here are head-to-head competitions. The plasmids arrive in the cell at the same time which makes it necessary to incubate the host cells for 3 h at 37 °C after the transformation to allow for the expression of plasmid encoded proteins necessary for replication initiation and antibiotic resistance. After this non-selective incubation in liquid media, antibiotics are added to increase the yield of double resistant cells. The selective incubation in liquid media is carried out for 14 h at 37 °C. Following this incubation in liquid media, the cells are seeded on plates selective for both resistance markers and incubated at 20 °C for three days. B) Here, the preemptive competition workflow is shown. In this competition type one plasmid, the endemic plasmid, has been given time to establish itself in the population prior to the arrival of the invading plasmid. This makes a long incubation after the initial transformation unnecessary since the proteins required for the initiation of the replication of the plasmids are already present in a sufficient amount. The workflow after the initial selective step to confirm the presence of double resistant transformants is identical to the head-to-head workflow.

The appropriate dilutions were plated to obtain the proportions of hosts within the original colony and also for the competition under non-selective conditions. The colonies growing on the non-selective plates were again excised, resuspended, diluted and plated for the proportions of plasmid hosts within the colonies.

In the preemptive competitions plasmid 1 was given time to establish itself within the host population prior to the arrival of plasmid 2 (see **Fig. 4**). The host cells were transformed with 10 ng of the invading plasmid. The outgrowth phase was reduced to 1 h since the components necessary for the replication of the plasmids were already supplied by plasmid 1. The double selective step, which was also necessary to apply balancing selection that is not wanted here, has been reduced to 2 days incubation at 25 °C since there was only the necessity to prove that both plasmids were contained within the cell and not to balance them. The colonies grown on the double selective plates after the incubation period were excised, resuspended in PBS, then diluted and plated onto plates to determine the proportions of the plasmid populations within the colony as well as for the non-selective competition. The colonies on the non-selective plates were again excised, resuspended, diluted and plated for quantification.

3.11 **Statistics**

Statistical tests and data analysis were performed in R version 4.2.0 (R Core Team 2022).

4 Establishment of a system for intracellular competitions

As already mentioned in the introduction this type of experiment has been conducted previously (Hülter et al. 2020). There the main findings were that an inherently stable plasmid is unbeatable by an instable plasmid. There it did not matter whether the competitions were conducted as head-to-head style or in a preemptive manner, the stable plasmid always won.

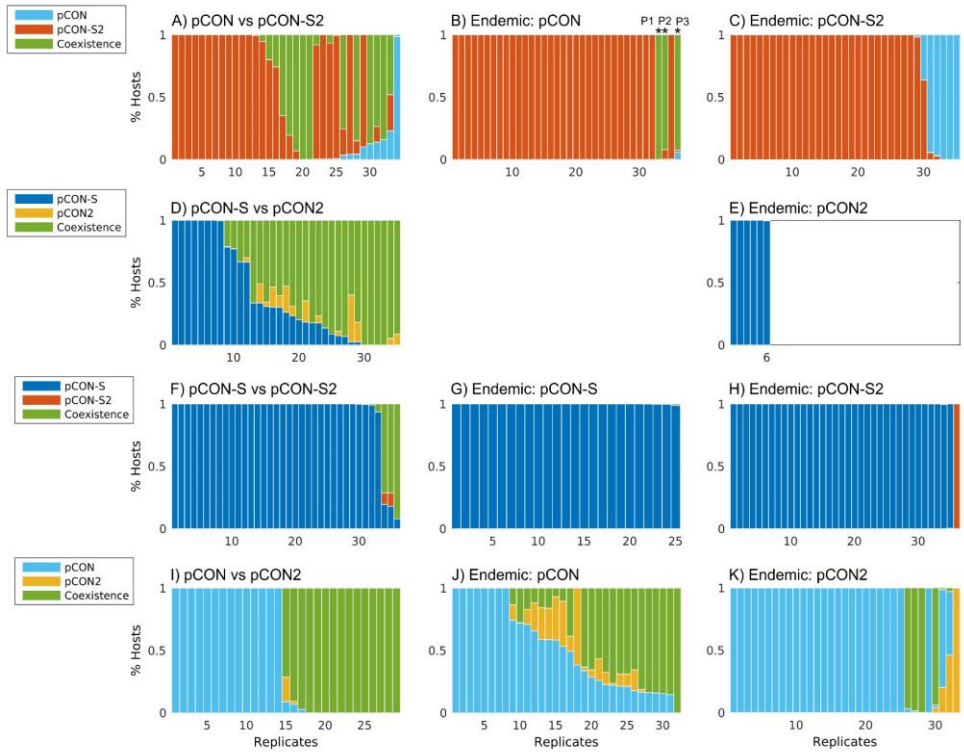

Figure 5: Results of competitions in a previous study.

Depicted here are the results of the competition experiments of a previous study (Hülter et al. 2020). Overall, the stable plasmids pCON-S and pCON-S2 displaced the unstable plasmids pCON and pCON2 when they were put into a competition with each other. The competitions between the stable plasmids ended in favor of pCON-S. These were early attempts at gauging the fitness of plasmid variants, which have already shown that replicative success and the resulting inherent stability is beneficial for the fitness of a plasmid. These competitions were conducted in *E. coli* MG1655 wildtype cells.

The stable plasmid pCON-S is the result of a long-term evolution experiment conducted by Tanita Wein, who was examining the persistence of plasmids under non-selective conditions (Wein et al. 2019). There the plasmid pCON-S emerged after roughly 1000 generations of growth under non-selective conditions at 37 °C. The plasmid pCON-S differs from its ancestral variant in a duplication between the *oriV* and the *nptII* gene. This duplication

led to a reduction of conflicts between transcription and replication, which in turn led to a lower probability of replicating plasmids getting damaged and therefore improved the ability of pCON-S and pCON-S2 to properly replicate, while at the same time reducing the likelihood of multimers to occur.

Consider that an incoming plasmid may not yet be adapted to the host or may encounter different plasmids or variants of the same plasmid that have evolved differently. This prompted the question of whether the inherently stable plasmids can somehow be beaten, what does it take to compete with the S plasmids. Therefore, it made sense to continue with the pCON variants and keep the replicons identical while equipping the plasmids with different systems.

For this we looked at naturally occurring plasmids that are widespread and successful, like the F plasmid or the R plasmids. Both types contain different modules that aid their stability. This had us wonder whether it is preferable for a plasmid to first evolve inherent stability or a stability mechanism.

The system used in our previous study had a few limitations (Hülter et al. 2020). First, the occurrence of multimers, which made it impossible to properly gauge the relative fitness of the competing plasmids, if the multimers were present. Second, the initial population was exposed to primary selection for the transformants and that the colonies already differentiated there. However, the proportions within the initial colonies were not quantified.

To properly answer our questions the system needed to be adjusted/optimized. An appropriate experimental and genetic system had to be constructed. In the following chapters I will guide you through the development of the two systems, which are more intertwined than one would initially think.

4.1 **Model plasmids**

In this thesis the model plasmids pCON and pCON2 are used along with the stable model plasmids pCON-S and pCON-S2. The plasmids pCON and pCON2 are characterized by an intrinsic instability. The plasmids pCON-S and pCON-S2 are inherently stable. The difference between the plasmids pCON and pCON2 is found in the resistance gene that is their cargo. The use of model plasmids with different selection markers allowed for both plasmid types to be put into competitions with each other and enabled selection for each plasmid individually. In a previous study the inherently stable plasmid was found to be fitter than the inherently unstable plasmid, based on the results of intracellular competitions conducted in an *E. coli* MG1655 host strain.

To determine whether an intrinsically instable plasmid can be rescued via the acquisition of a stability mechanism that will increase its fitness, three different variants of the unstable model plasmids pCON and pCON2 were constructed. This included a partition system, toxin-

antitoxin system or the combination of both. The plasmids equipped with a partitioning system were constructed to include the parA locus of plasmid R1. This locus consists of the genes *parM* and *parR* as well as the incC site. The insertion of the locus into our model plasmids pCON and pCON2 generated the plasmids named pCON-P and pCON2-P.

For the construction of model plasmids having a toxin-antitoxin system, we adopted the parB locus of plasmid R1. The parB locus encodes for the *hok*/*sok* toxin-antitoxin system that was cloned into the unstable model plasmids pCON and pCON2 at the same site as the parA locus. The resulting plasmids are named pCON-T and pCON2-T.

In order to see in how far the combination of both systems would affect the fitness of a plasmid, the unstable plasmids were also equipped with both systems, resulting in pCON-PT and pCON2-PT.

Notably, the incorporation of the stability systems in the model plasmids pCON/pCON2 led to a plasmid size increase (see **Fig. 6**). This may have resulted in changes of the plasmid topology. In order to confirm that the results from our competitions were caused by the stability systems, we generated two additional unstable plasmids with an increased size. In those plasmids we integrated a fragment of DNA that led to a similar size increase as integrating the stability systems. The chosen DNA fragment encodes for an ampicillin resistance gene which is flanked both upstream and downstream by a noncoding sequence. The flanking sequences were designed in a previous study and are designed to be noncoding. The entire cloned sequence is contained on the plasmid pLC as, what we termed here, the R-locus. The R-locus was inserted in the same region as the stability systems and resulted in pCON-R as well as pCON2-R. Since the insertion site of the R-locus was in the same region as those of the stability systems, it also enables us to check whether the insertion site had an impact on the stability of the plasmids.

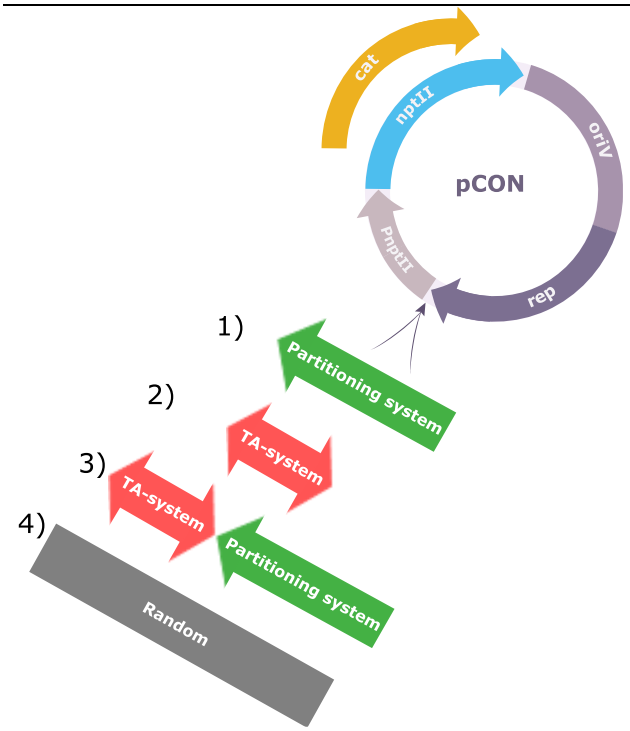

Figure 6: Construction schematic for the modelplasmids.

The model plasmid pCON consisting of the pBBR1 *oriV* and *rep* in addition to *nptII* and its native promoter. pCON2 is identical to pCON with the exception of the resistance gene, which has been exchanged for the *cat* gene. Of both plasmid variants derivates have been generated containing either the parA locus of R1, the parB locus of R1, both loci or a chunk of DNA to increase the size of the plasmid. The direction of transcription is indicated by the arrows.

4.2 Stability in MG1655

The stability of the stabilized plasmids and of the unstable ancestral plasmids was determined through a 5-day stability assay in *E. coli* MG1655. During the assay the cultures were grown under non-selective conditions and transferred to fresh media every day. The results of the assay for the ancestrally unstable plasmids pCON and pCON2 show that the proportion of hosts decreases over time below 100%. Both pCON and pCON2 remain in the population over the course of the 5 days at the level of ca. 85% of the hosts (see **Fig. 7**). At the end of the experiment, pCON maintains the ca. 85% of hosts, while pCON2 shows a stark decline at the very last day to ca. 60% hosts. The variation in the proportion of hosts among the replicate populations of pCON and pCON2 is rather high in comparison to stability assays of the stabilized plasmids. The loss of pCON2 was similar to previous observations (Hülter et al. 2020). Notably, the stability of pCON observed here is much higher compared to the original publication of pCON, where a loss of 10% per day was observed (Wein et al. 2019). Note, however, that a lower loss was recorded for several pCON replicates in the original publication

when cultivated at 20 °C (Wein et al. 2019), in other words, pCON loss rate may depend on the host growth dynamics.

The stability assay of the plasmids equipped with an active partition mechanism pCON-P and pCON2-P shows that the stability of the plasmids is increased compared to the relative pCON variant. The population of pCON-P hosts started with a ratio of 85.7% which first increased to 99,7% and then started to decline. For pCON2-P a starting ratio of hosts of 90.8% was observed. The ratio of hosts then increased to 98.8% and started to decline afterwards. Over the duration of the experiment, there is a constant decrease in the proportion of hosts starting on day one of about 1.75% and 0.75% per day for pCON-P and pCON2-P hosts respectively. The variation in the proportion of hosts per day among the replicate populations is minimal. At the end of the experiment, pCON-P was present in 93% of the hosts and pCON2-P was present in 96% of the hosts. The two plasmid variants show similar loss dynamics.

The plasmid stability assays conducted with the TA-system containing plasmids also show increased stability in comparison to the ancestral unstable variants. The stability essay of pCON2-T shows that the ratio of hosts first decreases from 100% to roughly 80%. Afterwards the ratio of hosts rose from 80.9% on day one to 100% on day two where the ratio of hosts was maintained until day five. In the assay of the pCON-T hosts the proportion of hosts ranged between ca. 70 and 100%. The variation between replicates decreased on day one and the ratio of hosts started to increase from 95.6% on day one to 99% on day three. The ratio of hosts started to decline on day three to 92,5% on day five. The replicates in the assay had shown a small variation with the exception of day zero.

In the assays with plasmids containing both stability systems – pCON-PT and pCON2-PT – a stabilizing effect was observed. At the start of the experiment the pCON-PT containing host strain had a ratio of ca. 85% within the population which declined to 70% on day one. The ratio of hosts then rose to ~95% on day two which is around the level at which it was maintained until day five. The pCON2-PT host strain constituted 95% of the population which dropped to roughly 60% on day one and increased again to ca 100% on day two which is the level at which it was maintained until day five. The variation among the replicates is on single days higher than in the assays of the pCON-P and pCON-T variants but is in general lower than in the assays of the ancestral plasmid variants.

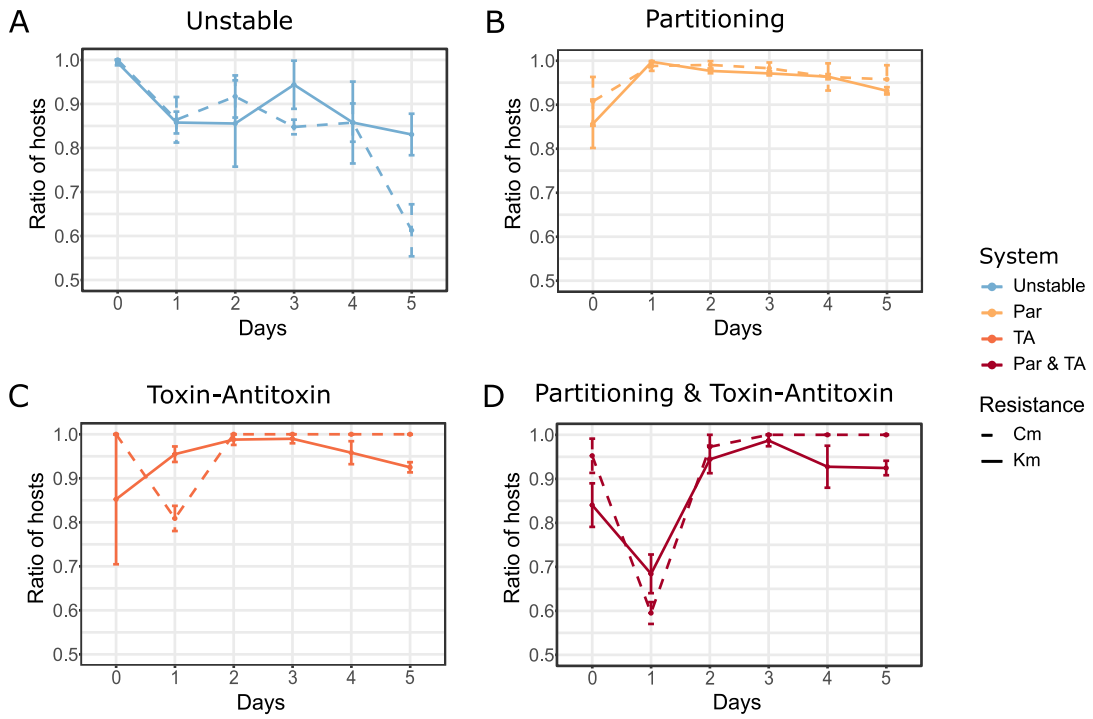

Figure 7: Stability of the model plasmids in WT *E. coli* MG1655.

A) Shown here is the ratio of hosts of the unstable plasmids pCON (solid line) and pCON2 (dashed line) as quantified over five days. B) Shown here are the ratio of hosts measured over five days of pCON-P (solid line) and pCON2-P (dashed line). The plasmids are derived from pCON and pCON2 and contain an active partitioning system while being identical otherwise. C) The ratio of hosts of pCON-T (solid line) and pCON2-T (dashed line) is shown here. Both plasmids are derivates of the unstable plasmids shown in subplot A and contain a toxin-antitoxin system while otherwise maintaining sequence identity. D) The ratio of hosts of pCON-PT (solid line) and pCON2-PT (dashed line), derivates of pCON and pCON2 that contain both an active partitioning system and a toxin-antitoxin system, in their respective populations over the course of five days.
P-variants (n = 8); T-variants (n = 3); PT-variants (n = 3; Day 0 pCON2-PT n = 2); pCON-variants (n = 3)

4.3 Fitness effect on E. coli MG1655 hosts

The assessment of plasmid fitness requires us to validate that the changes in plasmid frequency in the population are due to plasmid segregation dynamics rather than differences in growth dynamics among the plasmid hosts. Consequently, it was tested whether the model plasmids have an effect on the MG1655 hosts fitness (see **Fig. 8**). The plasmid fitness effect on the host was quantified through competitions between the plasmid hosts and plasmid free MG1655::Tp^R (see methods). Additionally, the fitness of MG1655 compared to MG1655::Tp^R was quantified in a similar manner. The results show that the relative fitness (*w*) of plasmid-free MG1655 was markedly lower compered to MG1655::Tp^R (Median (*w)* = 0.61). The relative fitness of the MG1655 hosts compared to MG1655::Tp^R show a similar trend to MG1655. The

fitness difference between MG1655 hosts and MG1655 WT were similar. Thus, I suspected that the WT strain was somehow compromised. At the same time, the results observed for all plasmid hosts are quite similar suggesting some regularity in the results (average median (*w)* = 0.69). Note that the same source MG1655 population was used in all experiments. Nonetheless, comparing the results of the plasmid hosts with the plasmid-free MG1655 suggests that they have similar growth dynamics (all compared to MG1655::Tp^R), hence it could be concluded that the plasmid carriage has either a negligible or even positive fitness effect on MG1655.

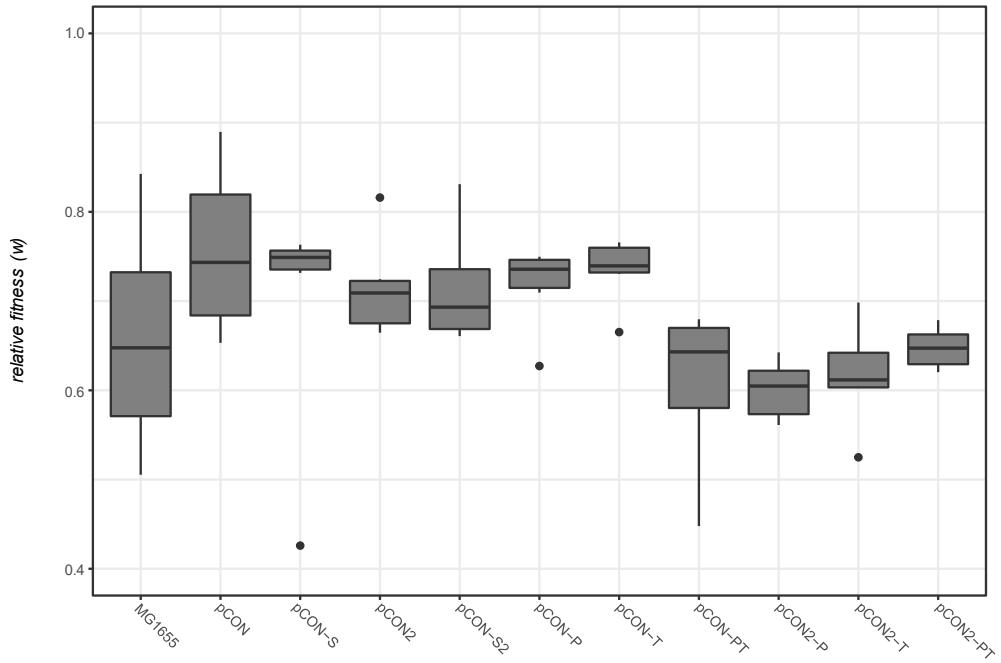

Figure 8: Relative fitness of *E. coli* MG1655 with and without the model plasmids measured against MG1655 Tp^R.

In this plot the relative fitness (*w*) of the wildtype MG1655 strain and host MG1655 strains containing our model plasmids can be seen. The strains noted on the x-axis were competed against an MG1655 Tp^R strain and the relative fitness (*w*) of the strains was determined against the Tp^R strain. (n = 6).

4.4 Splitmarker endeavors put last

In order to conduct proper competitions between the plasmids the proportions within the host needed to be adjusted to be roughly 1:1. An attempt at achieving balanced proportions in the initial colonies was the construction of plasmids containing a split antibiotic marker. The split marker consists of the N-terminal parts of the *hph* gene and the intein, as well as the C-terminal parts of the intein and the marker gene. The marker gene *hph* confers a resistance to Hygromycin B (HygB) through phosphorylation of the antibiotic, while the intein is responsible

for completion of the protein after translation within the cell. As intein *dnaE* of *Nostoc punctiforme* was used.

The genes were put in an operon with the *rep* gene so as to not impact the transcriptional load of the plasmid, which might have introduced new sources of instability into the system. To test whether this approach was feasible the *dhfrII* gene from the Tn7 transposon was inserted at the site where the split markers were to be introduced. The resulting plasmid, pCON-Trim, was then transformed into the MG1655 host and the resistance to trimethoprim along with the susceptibility of the wildtype confirmed by plating both strains onto LB agar plates containing trim 125 ng/µl.

The function of the split marker was confirmed while they were contained on a single plasmid. At the same time the susceptibility of the MG1655 wildtype to HygB was verified. The plasmid hosts were able to grow on concentrations of HygB 100 ng/µl while the wildtype was impaired starting with concentrations of HygB 50 ng/µl.

However, the minimal inhibitory concentration for the cells containing plasmids with the complementary marker-halves was not suitable for our use. The intended use was to select immediately after the transformation so that both plasmids would reach an equilibrium since the selective stress would be applied to both plasmids. The number of transformants acquired through this selective regime would have been lower than the number of cells with spontaneous resistance.

4.5 **Balance in MG1655**

After putting the splitmarker endeavors to rest, balance was still necessary so another way had to be found. This way presented itself when referencing the stability data for pCON from the time the plasmid was first constructed. Comparing stability at 37 and 20 °C showed more plasmid retention at lower temperatures. This approach was used to balance pCON vs. pCON2-P and pCON2 vs. pCON-P after the respective transformation. The initial proportions in the transformant colonies of pCON vs. pCON2-P obtained after three days of incubation at 20 °C have shown a high ratio of coexistence. After the relaxation phase at 37 °C the colonies stemming from initial colonies two and three have differentiated, whereas the colonies stemming from initial colony one have maintained the coexistence which suggested possible multimerization. In the initial colonies of the competition between pCON2 vs. pCON-P have shown very high proportions of coexistence between 75 and 80%. The relaxed colonies have then shown differentiation to mainly homogenic hosts in all cases.

The high initial proportions in the initial colonies followed by the differentiation in the relaxed colonies indicate that the balancing selection was successful and was incorporated in the competition protocol in the way it was conducted here. However, the occurrence of multimers was still a hinderance in the conduction of the competitions.

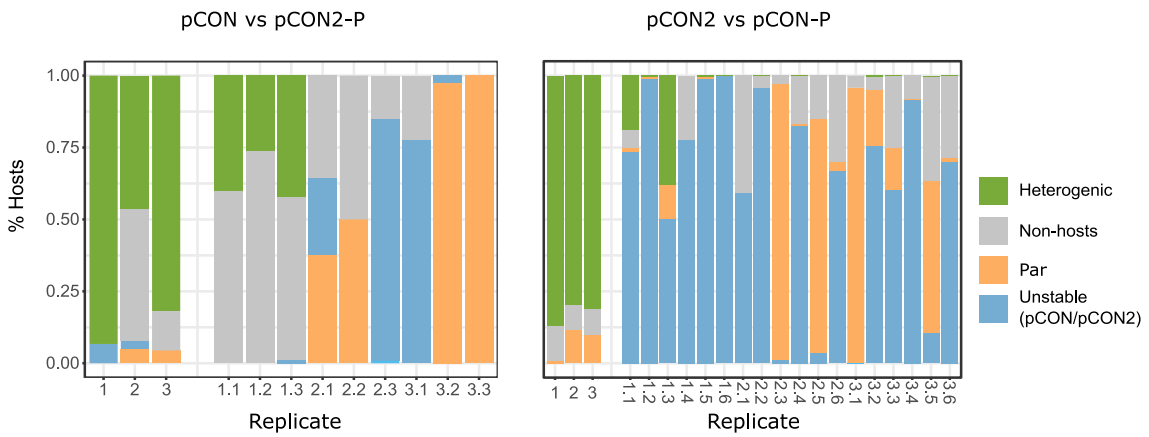

Figure 9: Balancing selection in WT *E. coli* MG1655.

The proportions of hosts contained within colonies that underwent balancing selection at 20 °C after transformation. The left plot shows colonies that were transformed with pCON and pCON2-P with the initial proportions of hosts shown on the left side of the plot. The colonies were then put through the experimental workflow of the head-to-head competitions. The proportions of hosts were again quantified after a non-selective incubation at 37 °C for which the results are depicted on the right side of the plot. The right plot is set up identically to the left plot and shows the results for cells that were transformed with pCON2 and pCON-P.

4.6 Construction of MG1655 *recA*::*tetA*

One issue that was encountered when using the wildtype MG1655 strain was the high abundance of multimers. The multimers could reach varying degrees of multimerization (**Fig. 10**). The existence of these multimers and their occurrence during competitions made it hard to impossible to gauge the fitness of the plasmids since they are one entity rather than two individuals.

Therefore, the potential for multimers to arise needed to be minimized. This was achieved by constructing a *recA* deficient strain. This strain was generated by replacing the *recA* gene with the *tetA* gene using the pTKRED system. Subsequent experiments showed a decreased potential for multimerformation, as intended (**Fig. 11**). The use of the newly generated strain MG1655 *recA*::*tetA* made it necessary to quantify the stability of the plasmids and their potential fitness effect on their hosts again.

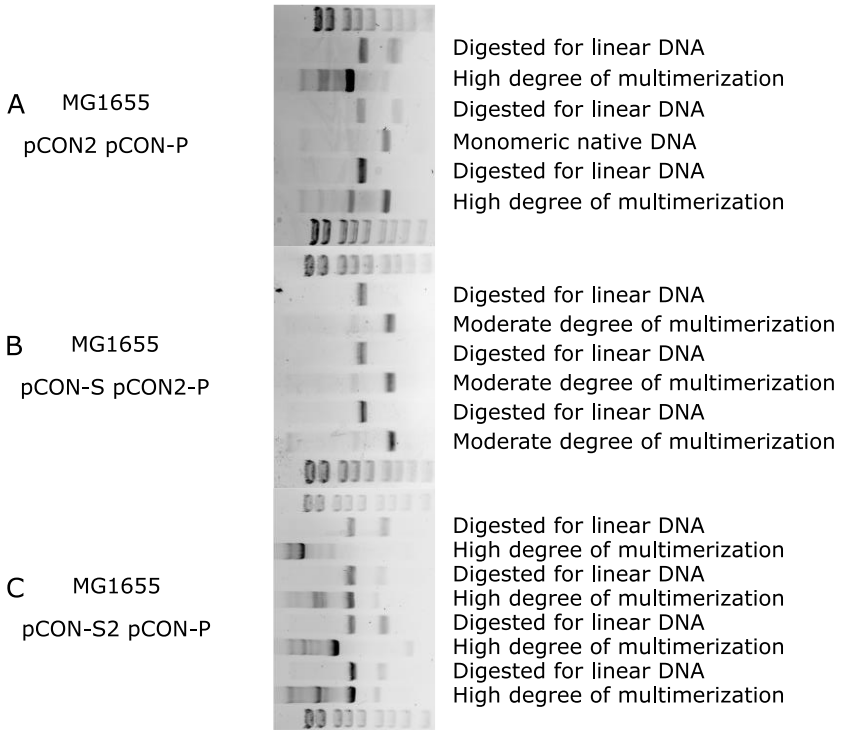

Figure 10: Multimers in WT *E. coli* MG1655

Depicted here are three agarose gels loaded with various isolated plasmid DNA which is either digested or native along with Hyperladder™ I (Bioline). A) plasmid DNA isolated from three different *E. coli* MG1655 strains containing pCON2 and pCON-P can be seen here. The plasmid DNA has been loaded onto the gel starting with native DNA and the corresponding linearized DNA revealing different degrees of multimerization in the isolates. B) As in A) the isolated plasmid DNA from *E. coli* MG1655 strains containing both pCON-S and pCON2-P is shown. Here no multimerization could be observed. C) Isolated plasmid DNA of four different *E. coli* MG1655 strains were loaded onto the gel in the same fashion as in A) and B). Here, varying degrees of multimerization can be seen.

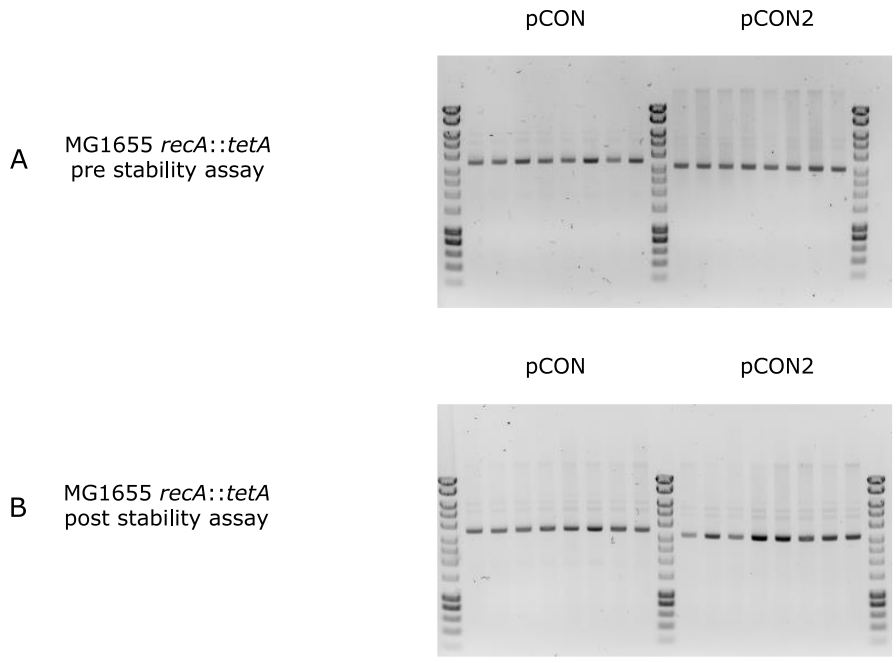

Figure 11: Multimerization in *E. coli* MG1655 *recA*::*tetA*

A) Extracted plasmid DNA from the eight cultures for both MG1655 *recA*::*tetA* pCON and MG1655 *recA*::*tetA* pCON2, prior to them being put into a seven-day stability assay, is shown. B) Here, the extracted plasmid DNA from the eight cultures of both MG1655 *recA*::*tetA* pCON and MG1655 *recA*::*tetA* pCON2 is shown after they underwent seven-days of growth without selection.

4.7 Balance in MG1655 *recA*::*tetA*

After confirming that the occurrence of multimers has been reduced the balancing regime was applied once more to confirm its viability in the newly generated *recA* deficient strain (see **Fig. 12**). The balancing selection was applied with incubation at 30, 25 and 20 °C. In all balancing regimes coexistence was observed, with the highest proportions in the colonies grown at 20 °C. The proportions after a non-selective incubation phase at 37 °C were quantified for cells stemming from the colonies that had experienced the balancing regime at 30 °C. In all of the quantified evolved colonies differentiation was observed. This indicates that no heteromultimers arose.

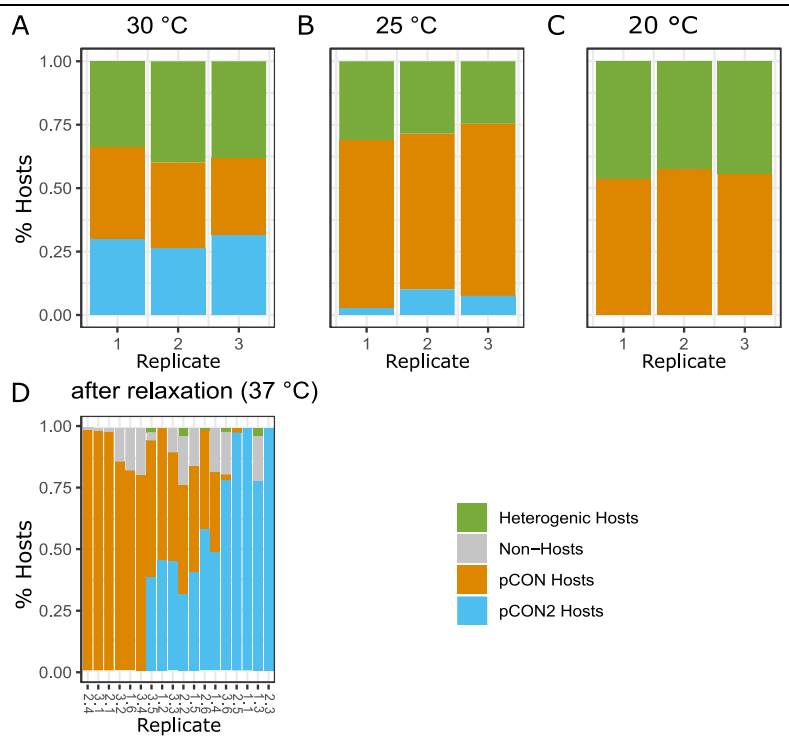

Figure 12: Balancing selection in the *recA*::*tetA* MG1655 strain

In this figure three different approaches to achieving balancing selection for the first step of the head-to-head competition protocol can be seen. All incubations were continued until microcolonies were visible. A) Depicted here are the proportions within colonies that experienced incubation at 30 °C overnight on double selective agar plates. B) Here the proportion of hosts for the transformant colonies incubated for two days at 25 °C are shown. C) The proportions of hosts observed in transformant colonies incubated at 20 °C for three days. D) The proportions of hosts after the three colonies shown in A) were plated on a non-selective plate and incubated at 37 °C overnight sorted by success of the single plasmids.

4.8 Stability and fitness-effect in MG1655 *recA*::*tetA*

Next, the stability assay was repeated using eight replicates of each plasmid carried by the newly generated MG1655 *recA*::*tetA* strain (see **Fig. 13**).

Here, the loss dynamics have shown a loss of 9.96% for pCON and 12.21% for pCON2 per day on average. The host population started at ca. 100% and dropped over the course of 5 days to ca. 50% for pCON and 33% for pCON2. The loss rate was more or less constant over the length of the assay. The stability of the plasmids pCON and pCON2 observed here was more like what we would expect based on previous data (Hülter et al. 2020).

The assays conducted with the hosts of pCON-R and pCON2-R resulted in loss rates of 7.21% for pCON-R and 8.19% for pCON2-R. says of the pCON-R variants the host populations started at 90 or 70%. The pCON-R hosts lost roughly 40 % after five days, while the pCON2-R host population lost ~25%. The stability of the pCON-R plasmid was similar to that pCON

which confirms that the insertion of the R-locus had no impact on the stability of the plasmid. The stability of the plasmid pCON2-R was similar to that of pCON-R.

After evaluating the results of the stability assay for the plasmids pCON-S and pCON-S2 the following observations were made. During the assays of pCON-S the host population, which started at 80%, reached 90% over the course of 5 days and had on average no loss. The pCON-S2 host population started at 90% and maintained that percentage over the course of five days with an average loss of 0.77%. The observed stability of the pCON-S variants was on a level consistent with previous observations.

In the assays conducted with the host strains of the plasmids pCON-P and pCON2-P a stabilizing effect could be observed for both plasmids. The pCON-P and pCON2-P hosts constituted 90% of the population at t_0 of the assay. The pCON-P host population had dropped to 80 % on the final day of the assay, resulting in an average loss rate of ca. 1.78%. The pCON2-P hosts dropped to 80% of the population on day 2 and rose back up to 90% on day 5. This corresponded to a loss of 0% per day on average.

The next modified plasmid variants, the T-variants, have also displayed a stabilizing effect. In the assays of the pCON-T hosts and pCON2-T hosts the starting populations contained roughly 90% hosts and maintained that ratio over the course of five days. The loss rates of both variants came down to 0.29 and 0.13% per day on average respectively.

Lastly, the loss rates of the PT-variants were determined. The assays of the pCON-PT variants started with populations constituted of 80 to 90% of hosts, which after five days reached nearly 100% of the population. The loss rates for both plasmid variants amounted to an average of 0% per day.

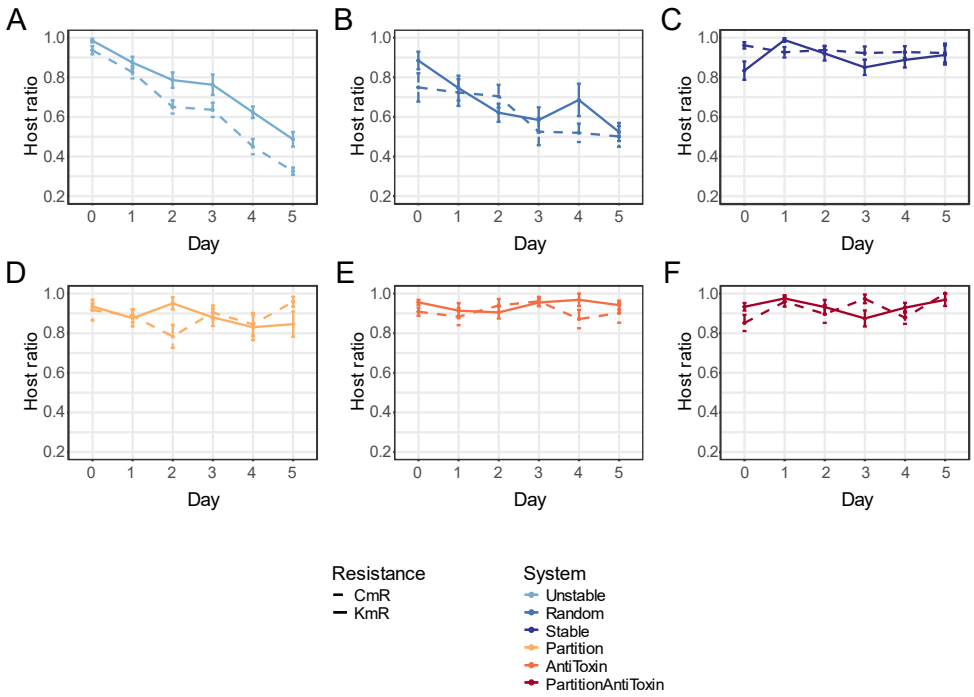

Figure 13: Stability of the model plasmids in *E. coli* MG1655 *recA*::*tetA*

Stability assay of different plasmids in the host *E. coli* MG1655 *recA*::*tetA* over the course of five days. Each plot shows the average plasmid host ratio within the population along with the standard error. A) Shows the ratio of hosts of the unstable plasmids pCON and pCON2 over the course of five days. B) Here, the ratio of hosts of pCON-R and pCON2-R is shown. The two plasmids are derivates of pCON and pCON2 respectively which both contain a large chunk of DNA with no benefit for stability. C) The ratio of hosts tracked over five days of the inherently stable plasmids pCON-S and pCON-S2 can be seen here. D) Shown here are the ratio of hosts of the plasmids pCON-P and pCON2-P, stabilized plasmids based on pCON and pCON2 containing an active partitioning system. E) This plot shows the ratio of hosts of pCON-T and pCON2-T quantified over the course of five days. The plasmids contain a toxin-antitoxin system for stabilization. F) Here the ratio of hosts of pCON-PT and pCON2-PT followed over the course of five days can be seen. Both plasmids contain a combination of the two stability systems used in this study. (n = 8 in all experiments except that for pCON2-PT where n=7).

The next plasmid trait that needed to be reevaluated in the MG1655 *recA*::*tetA* strain was the fitness effect of the plasmids on their host (see **Fig. 14**). As before, the strains were competed against a marker strain to determine their relative fitness (*w*). Here, no notable differences between the plasmid-free strain and plasmid host strains could be observed (Median (*w*) for MG1655 *recA*::*tetA* = 0.92; average median (*w*) for plasmid carrying strains = 0.98). Another observation that could be made was that the relative fitness of the strains revealed no inherently reduced fitness in contrast to the previously conducted assays with the wildtype MG1655 strains. This difference can be explained by the fact that in the previous assays the marker strain was not generated by modifying the MG1655 strain used as plasmid

host, but another MG1655 strain that may have a different history. The results were obtained as part of the bachelor thesis of J. Obermeyer und my supervision.

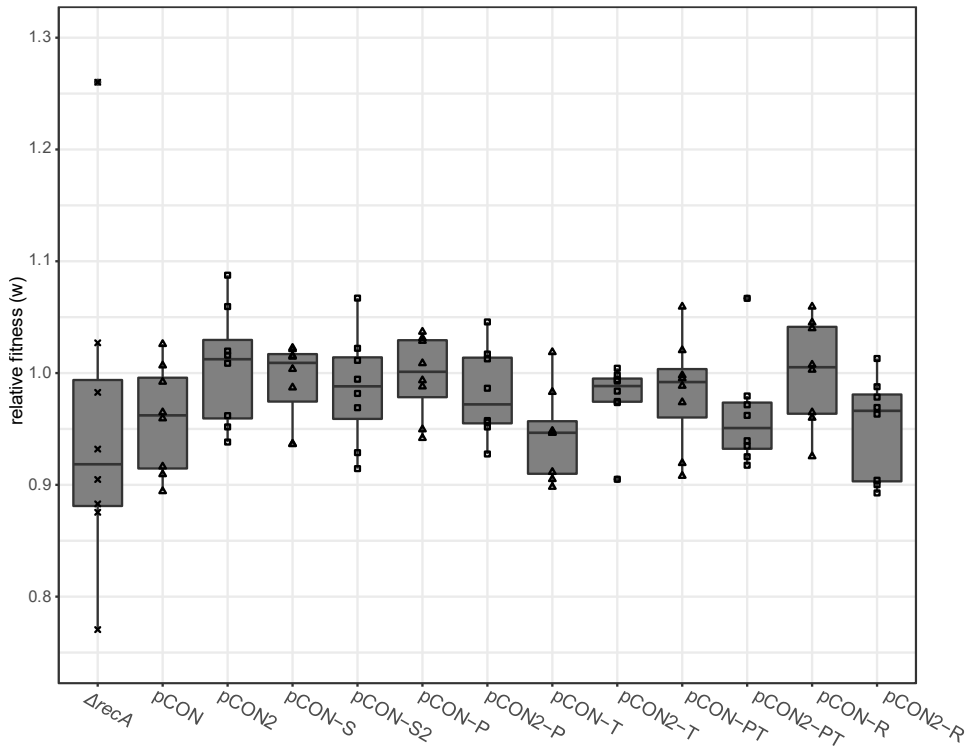

Figure 14: Quantification of relative fitness of *E. coli* MG1655 *recA*::*tetA* with and without model plasmid against MG1655 *recA*::*tetA* Tp^R.

Fitness-assays of MG1655 *recA*::*tetA*. The "wildtype" and the host strains were competed against a MG1655 *recA*::*tetA* Tp^R strain. The relative fitness of each strain was similar to or higher than that of the wildtype strain. (n = 8)

4.9 **Intracellular competitions**

Since all requirements for the experimental system were met, a pipeline was now established (see **Fig. 15**). The head-to-head competitions were conducted as follows. The competing plasmids were used to transform the host strain at the same time. After transformation, the host cells were subjected to an outgrowth phase, which was initially carried out without antibiotics. After one hour of incubation antibiotics were added, in order to increase the amount of transformants that could be detected. In the second step the transformation mix was plated onto double selective LB agar plates and incubated for three days at 20 °C to balance the plasmids within the cells. Then the resulting colonies were excised from the plates, resuspended, the resuspension diluted and plated onto plates that were non-selective, selective for either of the plasmid hosts and selective for hosts containing both plasmids. The resuspension was also plated onto other non-selective plates which were incubated at 37 °C. During this step the competitions took place. The colonies grown on these plates were also

excised and differentially plated, as described before to get the proportions of hosts within the populations after the competitions.

The preemptive competitions were conducted similarly to the head-to-head competitions. Here the difference lies within the fact that one plasmid had already established itself in the population. Therefore, the incoming plasmid no longer needed a long outgrowth time to express the RepA protein to start the replication of the plasmid. Additionally, we did not want to achieve balancing selection, rather we wanted to select for double plasmid carriage. Therefore, the selective step on plates was adjusted to 25 °C with less time between plating and harvesting of the colonies. The rest of the protocol remained identical to the head-to-head protocol.

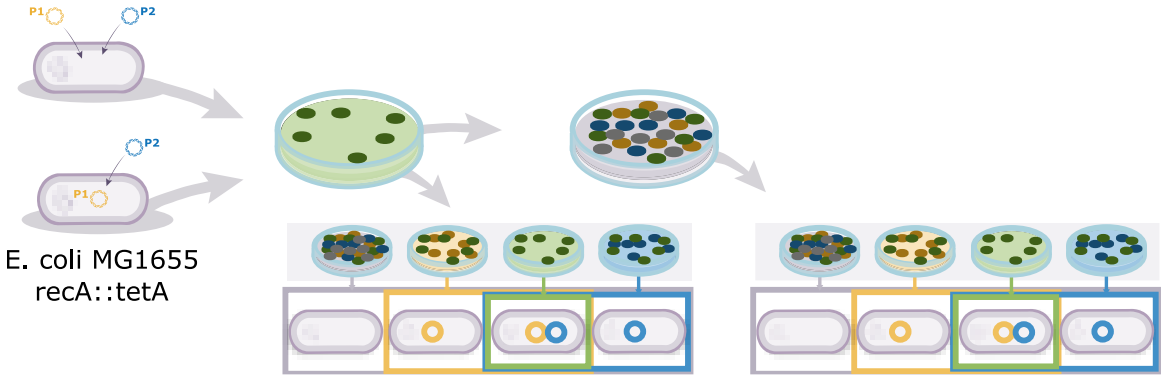

Figure 15: Workflow for intracellular competitions based on the results of this study. Depicted here is the version of the workflow for the intracellular competitions that was used in the competition experiments. The competitions were conducted in my *E. coli* MG1655 *recA*::*tetA* strain. Starting from the top left side of the figure the workflow for the head-to-head competition experiments is shown, while starting from the bottom left side the workflow for the preemptive competition experiments can be seen.

5 Head-to-head competitions against an inherently unstable plasmid

According to the previously established protocols competitions between the modified plasmids and the respective competing plasmids, either unstable or stable, were conducted. To start these competitions plasmid DNA was used to transform the host cells (see **Fig. 16**). Although multimeric fractions were present in some of the isolates, they were deemed to be unproblematic. This is based on the lower transformation efficiency of larger plasmids (Hanahan 1983; Szostková & Horáková 1998) and the relatively low occurrence of these multimeric fraction in comparison to the monomeric fractions in the isolates. The plasmid DNA was added to the competent cells in equal proportions.

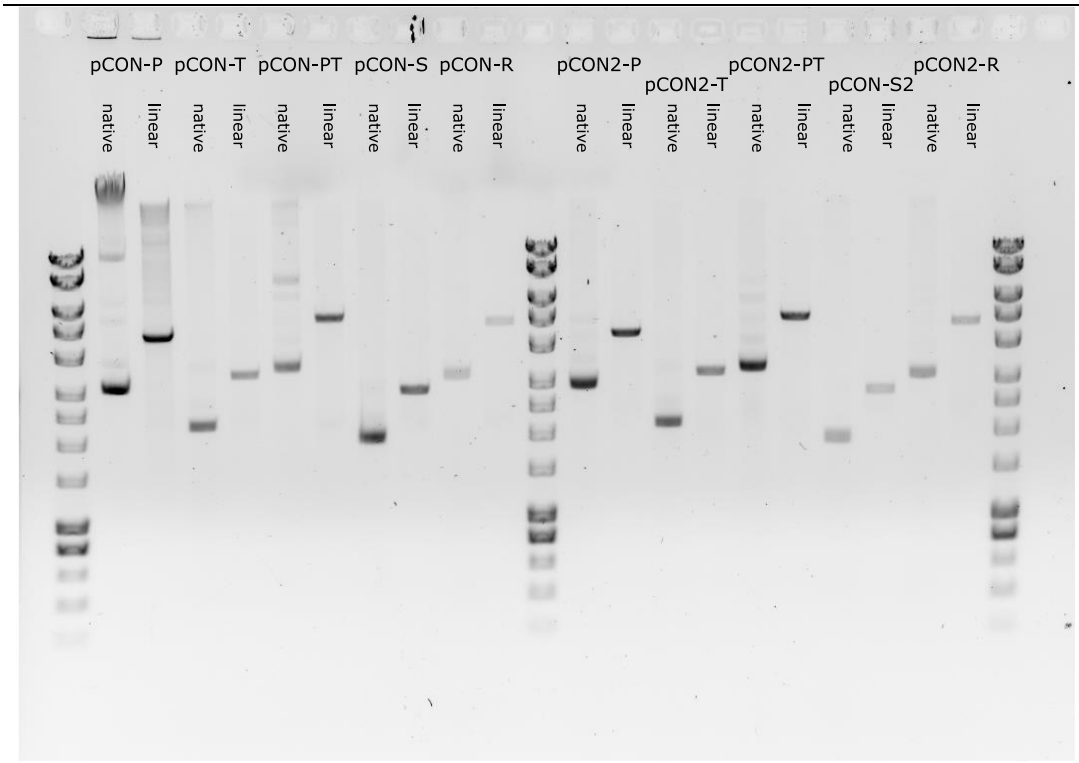

Figure 16: Agarose gel with the plasmid DNA used to start the *in cellulo* competitions. Here the isolated plasmid DNA used for the competition experiments can be seen. The isolated plasmid DNA was loaded onto an agarose gel starting with the native DNA and followed by DNA that was linearized with Nco I.

5.1 **Inherent advantage of the pCON2 variants**

In this competition the unstable plasmids pCON-R and pCON2-R were pitched against each other to determine whether one of the plasmids has an inherent advantage over the other. In the initial colonies we could observe even proportions of roughly 1/3 double hosts, 1/3 pCON-R hosts and 1/3 pCON2-R hosts. This indicates that the balancing selection had worked and that the plasmids segregate evenly under selective conditions. In the evolved colonies we then saw that pCON2-R won 16 of the 25 competitions, while pCON-R had won 8 and a tie was observed in one of the competitions. The average proportion of homogenic pCON-R hosts amounted to 28.56 ± 6.42% and the average proportion of homogenic pCON2-R hosts was 52.78 ± 6.15% (see **Fig. 17**). The average proportion of pCON2-R hosts was higher than that of the pCON-R hosts however the difference was not significant (P = 0.06932) From this we could conclude that the pCON2 variants had an inherent advantage over the pCON variants.

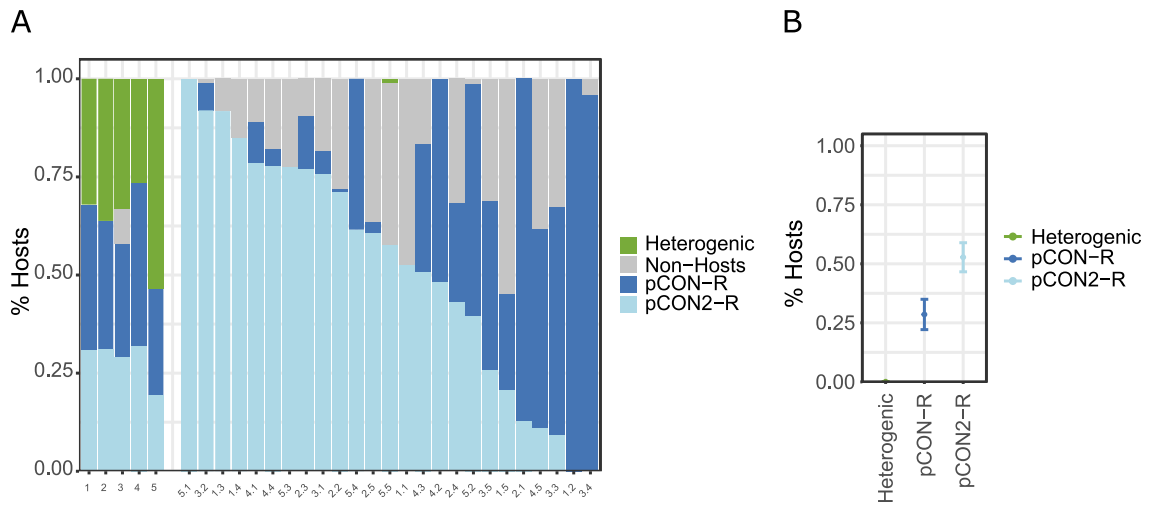

Figure 17: Head-to-head competition between pCON-R and pCON2-R.

A) The proportions of host genotypes within each colony (see legend; Heterogenic hosts harbor both plasmids). The left bars (1..5) show the proportion of hosts within the initial colonies. The right bars show the proportions in the evolved colonies. Evolved colonies can be traced back to the ancestral colonies according to their number: left of the dot is the initial colony number and right of the dot is the evolved colony number. The bars are sorted by the proportion of hosts. B) The mean proportion of the heterogenic and homogenic plasmid hosts are shown. The error bar shows the standard error of the mean. The mean proportion of hosts were compared using Wilcoxon test. The results of the comparisons are reported: pCON-R vs. pCON2-R P = 0.06932; pCON-R vs. Heterogenic P = 0.0001299; pCON2-R vs. Heterogenic P = 2-889e-5 (n=25).

Note: the results of all plasmid competitions in the thesis are reported in the format as presented in Figure 17. Variants among the experiments are possible due to differential number of initial or evolved colonies.

The preemptive competition experiment between pCON-R and pCON-S2 started with five initial colonies. Three of these initial colonies had some proportions of heterogenic, homogenic pCON-R and homogenic pCON-S2 hosts, while one colony consisted only of pCON-S2 hosts and in another one only heterogenic hosts remained. Not all initial colonies yielded colonies after the non-selective phase. However, for the evolved colonies that could be observed mostly homogenic pCON-R hosts were present with an average proportion of 77.03 ± 7.40%. Only in one evolved colony homogenic pCON-S2 made up 100% of the hosts. This colony descended from initial colony 3 showing that this colony descended from a cell in which both plasmids were present. The average ratio for the homogenic pCON-S2 hosts was 10.66 ± 8.14% (see **Fig. 18**).

These results indicate that even though the plasmid pCON-R is not as efficient as pCON-S2 in regard to replication, the initial advantage due to the higher initial copy number cannot be easily overcome.

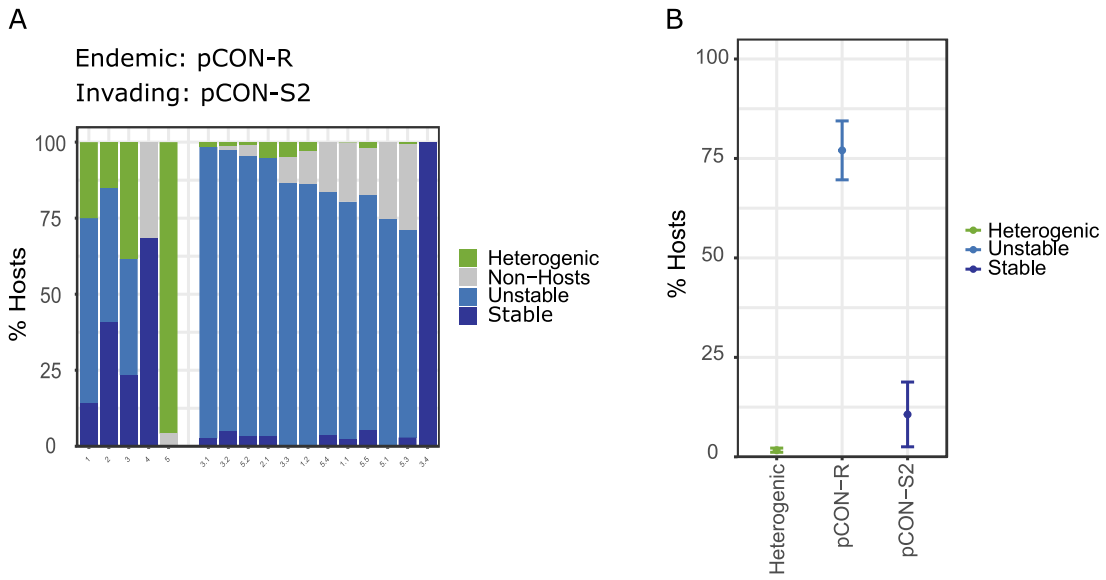

Figure 18: Preemptive competition between endemic pCON-R and invading pCON-S2

A) The proportions of hosts within the colonies in the preemptive competitions between pCON-R and pCON-S2. the proportions in the initial colonies can be seen on the left side, while the proportions in the evolved colonies can be seen on the right. B) The mean proportion of each type of plasmid host in the evolved colonies. The error bars indicate the standard error. A comparison was conducted using the Wilcoxon test. The results are reported here: pCON-R vs. pCON-S2 P = 0.03763 (n = 12)

5.2 The impact of partitioning on the fitness of an unstable plasmid

In the first set of competitions conducted, the variants carrying the *par* locus were pitched against the unstable R-variants. As already observed in the competition between the two instable plasmids pCON-R and pCON2-R (chapter 5.1), the double selection regime for the initial colonies yielded again four categorial classes of plasmid carriage configurations: heterogenic host cells with the two plasmids present in coexistence, homogenic host cells carrying either one of the plasmids and plasmid-free segregant cells. In the initial five colonies selected for the competition between pCON-P and pCON2-R, the proportion of double resistant cells, i.e., the proportion of cells that showed phenotypically the heterogeneous presence of both plasmids, ranged between 25 and 50%. Between 25 and 75% of cells carried only pCON-P and a minimal proportion of cells were only pCON2-R hosts or segregants (see **Fig. 19**).

The existence of the double resistant cells suggested that the competition indeed had started with cells that contained both plasmids when they were seeded on the plate. However, the fact that plasmid loss was observed in the initial colonies indicates that the spatial structure of the bacterial colony apparently attenuates the antibiotic selection pressure, that is supposed to act on each cell during colony formation, to such a substantial extent that cells which experience loss of one of the plasmids survive and proliferate within the colony anyway. The loss likely occurred due to an antibiotic gradient caused by the spatial structure of the colonies. The cells most proximate to the selective agar plates still experienced the intended selective pressure while distal cells were protected by the layers of cells beneath them which allowed them to differentiate. This gives room for the plasmids to gain an advantage, presumably based on their replicational frequency or successful segregation. These proportions showed that the competition between the two plasmids started directly after arrival in the same cell and that segregation dynamics already took place during selective conditions, as was the case during the balancing step of the experimental pipeline.

To allow segregation dynamics in absence of selection for plasmid carriage, aliquots of the five initial colonies of the competition between pCON-P and pCON2-R were individually seeded onto the non-selective plates as detailed (see section 4.10). Following the non-selective growth passage, the proportions of the different plasmid host type cells were assessed for five colonies, resulting in a total of 25 replicates.

The proportions in the evolved colonies of this experiment revealed that the segregation dynamics of heterogenic pCON-P and pCON2-R hosts under non-selective conditions were in strong favor for pCON-P; plasmid pCON-P “won the competition” in 15 out of a total of 25 replicates, while the unstable plasmid pCON2-R had “won” in ten replicates (see **Fig. 19**). In all of the evolved colonies the average host proportion of pCON-P was 43.19 ± 6.09% while the average proportion of pCON2-R was 34.86 ± 6.14%. The maximum proportion in the competitions where one plasmid has won was 97% as maximum and 39% as lowest proportion for pCON-P. For pCON2-R the highest observed proportion in won competitions was 87% and the lowest 51%. Application of a Wilcoxon test has resulted in a P value of 0.6474 which shows that while the mean proportion of homogenic pCON-P hosts was higher than that of homogenic pCON2-R hosts there was no significant difference. However, the average proportion of hosts of pCON-P was improved in comparison with the results of the head-to-head competition between pCON-R and pCON2-R (28.56 ± 6.42% to 43.19 ± 6.09%). These results supported that the fitness of pCON-P is higher in comparison to pCON-R.

In the reciprocal competition experiment between pCON-R and pCON2-P (**Fig. 19**) we saw in the proportions of the initial colonies that we observed a moderate number of cells that contained both plasmids while most cells were homogenic pCON2-P hosts. This strengthened

the assumption that both plasmids start to compete for their respective host cell immediately upon arrival under selective conditions and that this competition depends on the intrinsic fitness properties of each of the respective plasmids and not on the resistance marker gene carried by the respective plasmids. The evolved colonies revealed in their proportions that pCON2-P won every set of the competitions with an average host proportion of 87.7 ± 2.72%, a maximum proportion of 100% and a minimum proportion of 54%. While pCON-R did not win any of the competitions and no coexistence could be observed the plasmid was still present in the population with an average proportion of 4.23% ± 0.18%, a maximum of 4% and a minimum of 1% (in the colonies where plasmid presence was detected). The proportions of hosts in the initial and evolved colonies both indicated that the trend seen in the competition between pCON2-R and pCON-P, namely that the plasmid containing the par-system is more successful starting from the transformation, could also be observed here in an amplified manner. For example, in the evolved colonies 4.3 and 4.2 frequencies of 99% for homogenic pCON2-P hosts could be observed while homogenic pCON-R hosts had a proportion of 1%. In the initial colony 4, from which both of these evolved colonies descended, the proportions of homogenic pCON2-P and pCON-R hosts were 61% and 4% respectively while heterogenic hosts had a proportion of 35%. This comparison shows that the par-system encoding plasmid had a higher success rate under selective as well as under non-selective conditions. The means of the homogenic plasmid hosts were compared and a significant difference was observed ($P = 1.211e-5$)

The results observed in the two experiments suggest that a lack of replicative success can be somewhat alleviated by an active partitioning system. Since the plasmids pCON-P and pCON2-P were still not efficient in their replication, the increased proportions allowed for the conclusion that the stabilizing effect of the par-system had a direct impact on the fitness of the plasmids.

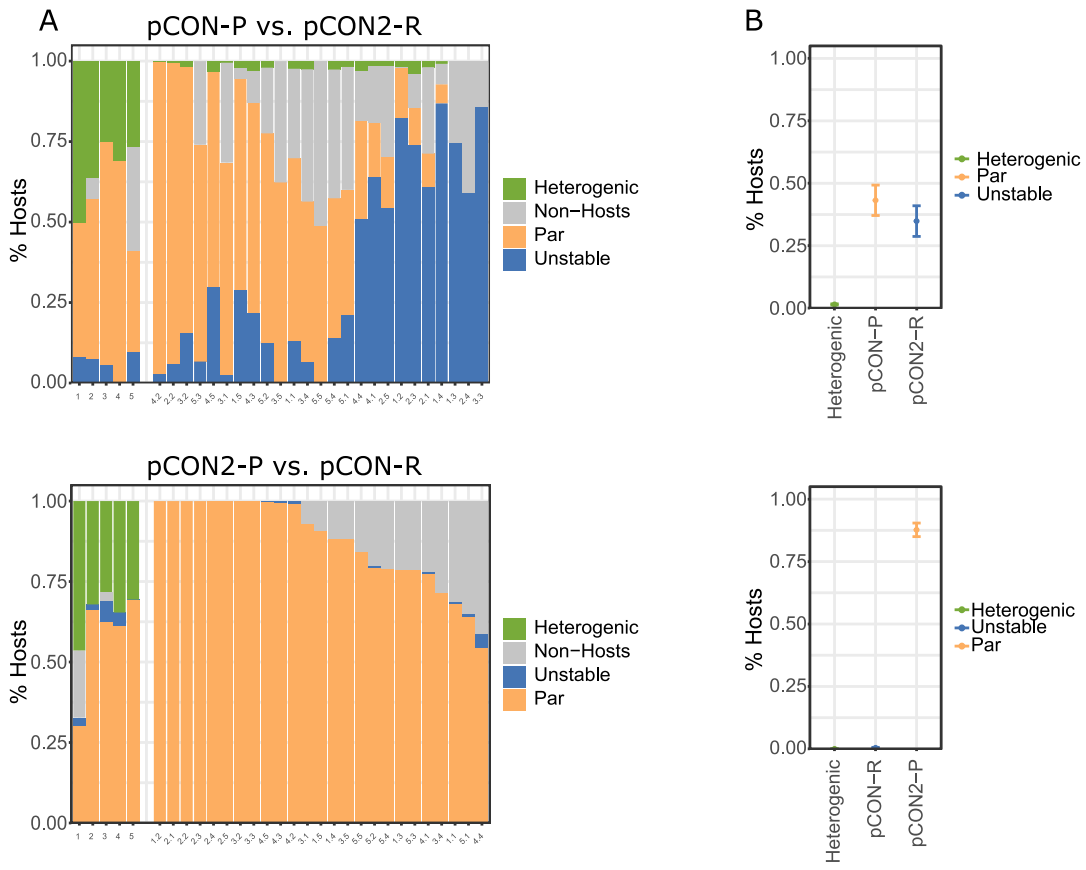

Figure 19: Head-to-head competitions between the variants of pCON-P and pCON-R.

A) Shown in these plots are the results of the quantifications of plasmid host proportions in the initial and competition populations. On the left most side of the plots the proportions within the initial colonies can be seen, while on the right the proportions of hosts in the evolved colonies are shown. The upper figure shows the results for the competition between pCON-P and pCON2-R, while the lower plot shows the results for the reciprocal competition between pCON2-P and pCON-R. B) Shown here are the average proportions of hosts of the evolved colonies. The standard error is shown as error bar. The order of the plots is identical to A). The results of the conducted Wilcoxon tests are reported here: pCON-P vs. pCON2-R P = 0.6474 (n = 25); pCON2-P vs. pCON-R P = 1.211e-5 (n = 25)

Our results so far were in agreement with the ability of partitioning systems to increase plasmid stability and fitness (see **Fig. 19**). Nonetheless, many plasmids are lacking an active partitioning mechanism. Is it possible that naturally stable plasmids are more stable than plasmids that harbor an active partitioning mechanism? To test this, the P plasmids were put into competition with the pCON-S variants in a head-to-head competition using the established protocol. The initial colonies of hosts harboring both pCON-S2 and pCON-P revealed a high proportion of hosts where the plasmids coexist (see **Fig. 20**). The plasmid coexistence was maintained in most of the evolved colonies with an average proportion of 75.93 ± 3.71%. Here the single plasmid hosts had an average proportion of 11.13 ± 1.89% in the case of pCON-P and 7.53 ± 4.03% for pCON-S2. Only one of the 25 tested colonies showed evidence for

outperformance of one of the competing plasmids – the pCON-S2 – that colonized 100% of the hosts. While the result of this experiment did not allow us to draw conclusions on the fitness on the respective plasmids, they did showcase the role of multimers in saving plasmid variants since here the pCON-P plasmid was maintained in the populations by homologous recombination with pCON-S2. The multimerization was confirmed for one colony via sequencing (see **Fig. 20**).

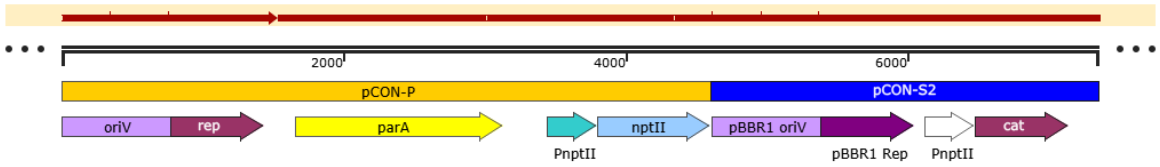

Figure 20: Alignment of sequencing results with the sequence of a multimer.
The plasmid map of a multimer consisting of pCON-P and pCON-S2. The corresponding parts of the two plasmids are indicated underneath the sequence (two black bars with vertical markers indicating position on the plasmid). Underneath the plasmid indicators are the features of the respective plasmids, e.g. the parA locus of pCON-P as a yellow arrow. The through sequencing acquired data was aligned to and is shown above the sequence of the multimer as red arrow in a tan box.

The competition between pCON2-P and pCON-S showed a very different result. The initial colonies already showed a high proportion of cells containing only pCON-S (four out of five colonies). Only colony 4 has shown, as in the reciprocal competitions, roughly 70% of plasmid coexistence. The relaxed colonies continued this trend, with a majority of colonies harboring only pCON-S (avg. proportion 77.71 ± 6.93%) and five colonies, all originating from initial colony 4, having maintained plasmid coexistence (avg. proportion 17.06 ± 7.02%; based on all 25 replicates). Note that the pCON2-P has been practically lost in all replicate populations in this competition.

The results of this set of experiments led to the conclusion that the inherently stable plasmids are still fitter than the modified plasmids. Therefore, high fidelity during the replication was more important than during segregation.

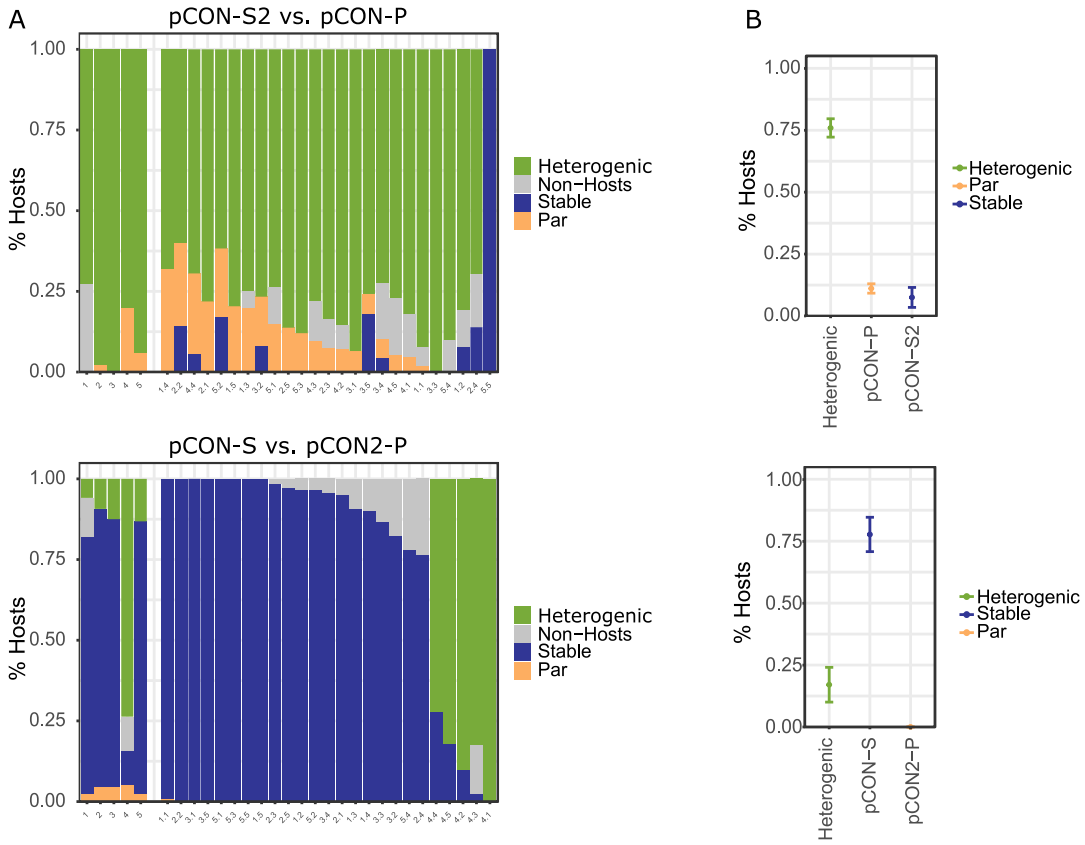

Figure 21: Head-to-head competition between the pCON-S and pCON-P variants.

A) Shown here are the proportions of hosts within the initial (left most) and evolved colonies (right side). The upper plot shows the quantified proportions for the competition between pCON-P and pCON-S2, while the lower plot shows the results for the competition between pCON2-P and pCON-S. B) The plots shown here depict the average proportion of hosts in the evolved colonies along with the standard error as error bar. The order of the plots is identical to the order in A). Wilcoxon tests were conducted and their results are reported here: pCON-P vs. pCON-S2 P = 0.01998 (n = 25); Heterogenic hosts vs. pCON-S2 P = 0.0002157 (n = 25); Heterogenic hosts vs. pCON-P P = 1.942e-5 (n = 25); pCON2-P pCON-S P = 1.838e-5 (n = 25); pCON2-P vs. heterogenic hosts P = 0.05917 (n = 25); pCON-S vs. heterogenic hosts P = 0.0006338 (n = 25)

The final step in the assessment of the fitness of the pCON-P variants was the competition of the endemic pCON-P versus an invading pCON-S2. This pairing was decided since pCON-P had shown that it is less fit than the chloramphenicol resistance-encoding pCON2-P. Also, pCON-S was perceived as fitter than pCON2-P. Only three colonies could be harvested after transformation. Two of the colonies have shown that pCON-P hosts constituted the majority of the populations while the third colony consisted of mostly pCON-S2 hosts. The proportions within the evolved have shown the same trend seen in the initial colonies with an average proportion for pCON-P of 48.5 ± 14.1% and 44.15 ± 15.06% for pCON-S2 in a total of 11 colonies.

The results suggests that the segregation of the plasmids takes place rather rapidly after the initial transformation. In contrast to the head-to-head experiment, no multimerization was observed here. This suggests that the time of arrival and saturation of the PCN might play a role in the formation of multimers.

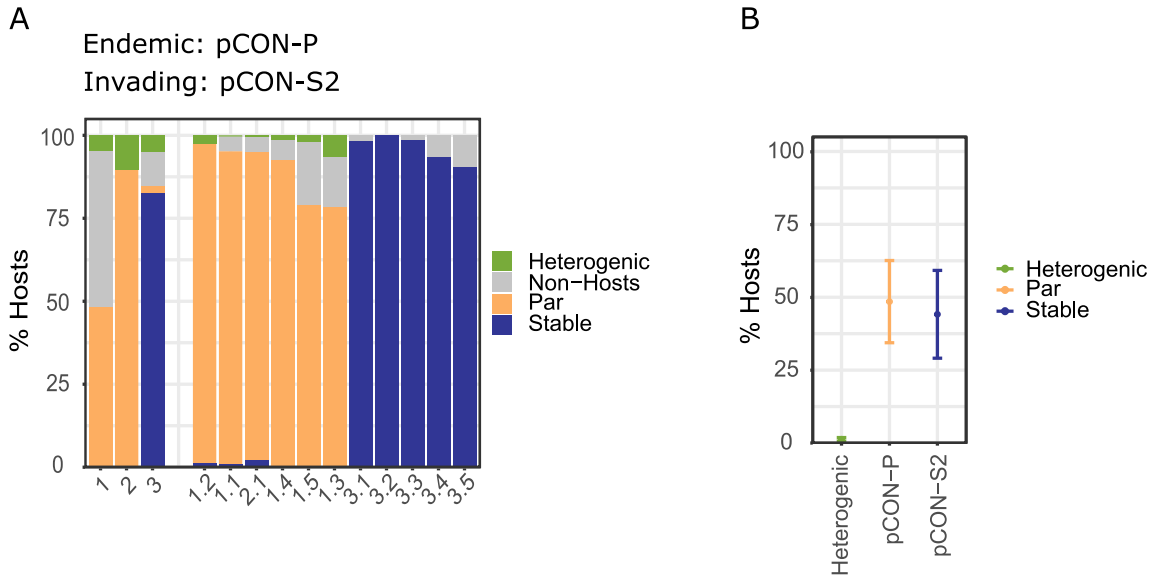

Figure 22: Preemptive competition between pCON-P and pCON-S2.

The proportions of hosts as quantified during the preemptive competition experiment between endemic pCON-P and invading pCON-S2. A) the proportions observed in each colony can be seen here. On the left side of the plot the proportions within the initial colonies and on the right side the proportion of hosts in the evolved colonies are shown. B) shows the average proportion of plasmid hosts along with the standard error of the evolved colonies. A Wilcoxon test for the proportions of pCON-P hosts and pCON-S2 hosts was done, the result is reported here: pCON-P vs. pCON-S2 P = 0.6248 (n = 11)

5.3 The story of addiction on plasmids

The second set of competitions conducted were the competitions between the TA system-containing plasmids and the unstable pCON-R variants. In the first competition, between pCON-T and pCON2-R, I observed that the initial colonies had between 100% and 58% of coexistence of the two plasmids (see **Fig. 24**). This indicated the existence of multimers. For the plasmids contained in initial colony 1 the presence of plasmid multimers was confirmed using enzymatic digests (see **Fig. 23**). This assumption was further supported by the proportions within the evolved colonies. However, no attempt at confirming the presence of multimers was made for the evolved colonies. The evolved colonies were comprised of

mostly heterogenic hosts (avg. proportion 70.65 ± 3.41%). This finding resembled the outcome after balancing selection commonly observed in the competitions conducted in the *recA*-proficient MG1655 wildtype strain (see chapter 1.2). The remaining proportions in the evolved colonies were mostly homogenic pCON-T hosts (avg. proportion 17.4 ± 2.53%). The coexistence observed in the evolved colonies may be a result of plasmid coexistence, however it appears likely that the high proportion of heterogenic hosts is a result of multimerization given that multimers were observed in one of the initial colonies.

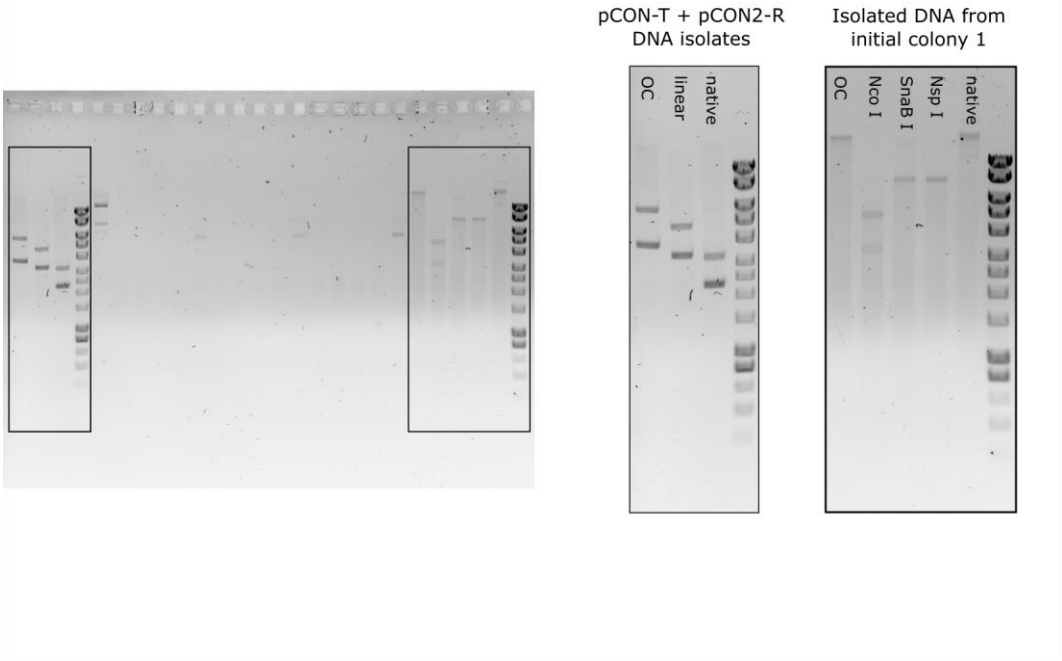

Figure 23: Multimers in an initial colony.
This figure shows an agarose gel loaded with isolated plasmid DNA of single plasmids which was mixed together and treated with enzymes (left box). The image also shows isolated plasmid DNA from initial colony 1 of the competition between pCON-T and pCON2-R in its untreated (native) form as well as linearized with enzymes cutting in pCON-T (NspI), pCON2-R (SnaBI) or both (NcoI) and also shows the open circle (OC) form produced by nicking with Nb.BsrDI (right box).

In contrast, the reciprocal competition revealed proportions of hosts in the initial colonies that one would expect in the absence of multimers (see **Fig. 24**). In the competitions between pCON2-T and pCON-R, the proportion of heterogenic host cell was between 21% and 50%. For homogenic hosts proportions between 37% and 52% for pCON2-T and between 4% and 52% for pCON-R were observed. In the colonies which were exposed to the non-selective

conditions, mostly pCON2-T was present in the cells with an average proportion of 88.95 ± 4.19%. Only in one colony we observed 100% carriage of pCON-R. This indicated that we picked a colony that originated from a cell that after the balancing selection already contained only pCON-R and also that the TA-system is not perfect.

The experiments show, that the fitness of a plasmid variants containing a TA-system is increased in comparison to a plasmid variants that contains no plasmid stability system. This can be explained by the function of the TA-system which increases the plasmid ability to persist within the population while not altering its inherent stability.

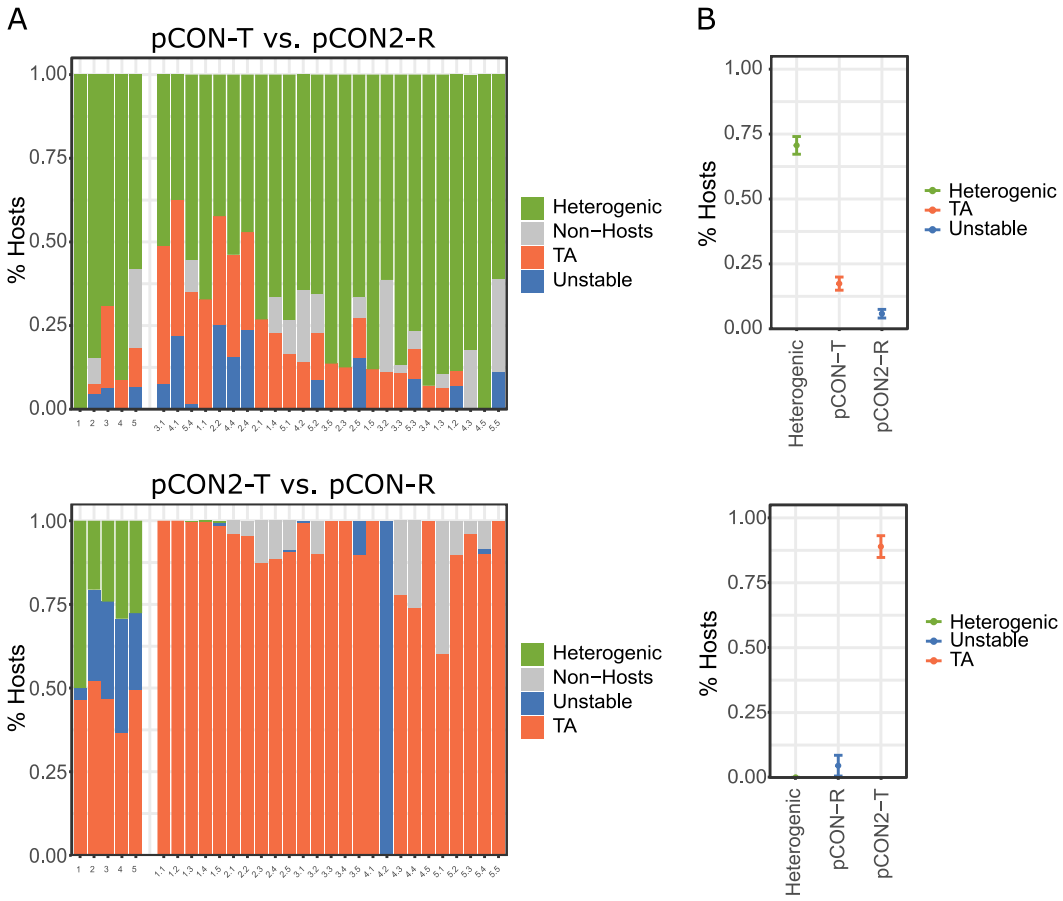

Figure 24: Head-to-head competition between pCON-T and pCON-R variants.

A) These figures show the initial proportions of plasmid hosts (left) and the proportions after the respective competitions (right) pCON-T vs. pCON2-R (upper) and pCON2-T vs. pCON-R (lower). B) The average proportions of hosts within the evolved colonies of the competitions. the upper and lower plot correspond to the competitions shown in A) at the same position. The standard error is shown as error bar. Wilcoxon tests were conducted and the results are reported here: pCON-T vs. pCON2-R P = 0.0002147 (n = 25); pCON-T vs. heterogenic hosts P = 1.476e-5 (n = 25); pCON2-R vs. heterogenic hosts P = 1.307e-5 (n = 25); pCON2-T vs. pCON-R P = 0.0001477 (n = 25))

The high proportion of heterogenic hosts in the competition between pCON-T and pCON2-R had raised the question whether this coexistence is stably maintained for a longer period than the observed duration of the competition. Therefore, the dynamics of the maintenance of the plasmid coexistence were observed for a longer period of time (see **Fig. 25**). Indeed, over the course of seven days the coexistence of the plasmids was maintained. The average proportion of heterogenic hosts in all replicates over seven days was 79.38 ± 5.03%.

This showed that the coexistence of the plasmids, whether it was in monomeric or multimeric form, was highly stable.

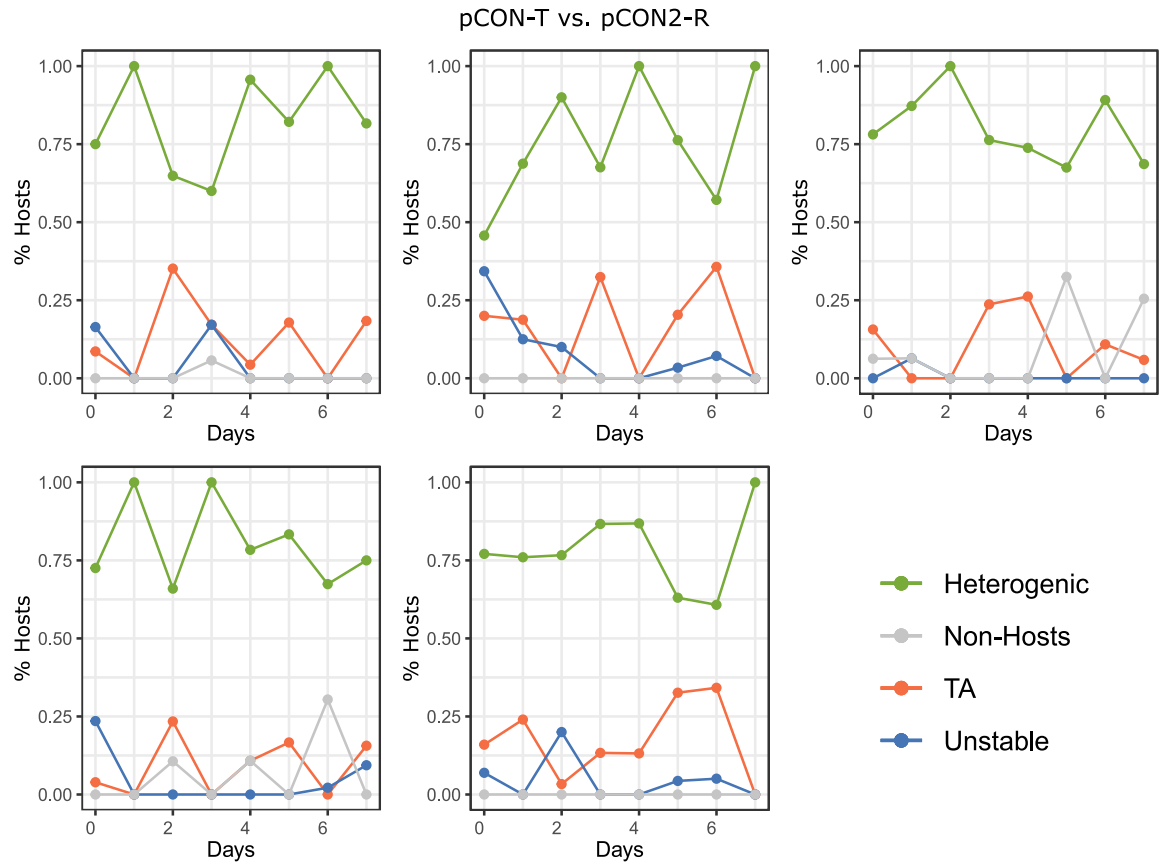

Figure 25: Seven-day assay of heterogenic hosts.
Shown here are the five replicates of the seven-day assays of colonies stemming from the heterogenic host fraction of the initial colonies of the head-to-head competition experiment between pCON-T and pCON2-R. The proportion of hosts can be seen for each day.

To exclude that the observed stability was a result of the adaptation of the host to the plasmid or *vice versa*, a naïve host was transformed with the plasmids obtained at the end of the longterm assay. For this, plasmid DNA was extracted from replicate 2 of CS pCON-T vs. pCON2-R after the last measured timepoint. Five colonies of the transformed naïve hosts were chosen for observation (see **Fig. 26**). The proportion of homogenic hosts did not surpass that

of the heterogenic hosts at any point during the assay. The proportion of heterogenic hosts was on average 86.11 ± 6.63% including all measured timepoints of all replicates.

Since the heterogenic hosts remained high in proportion and stable within the population the adaptation occurred likely on the side of the plasmid and not the host.

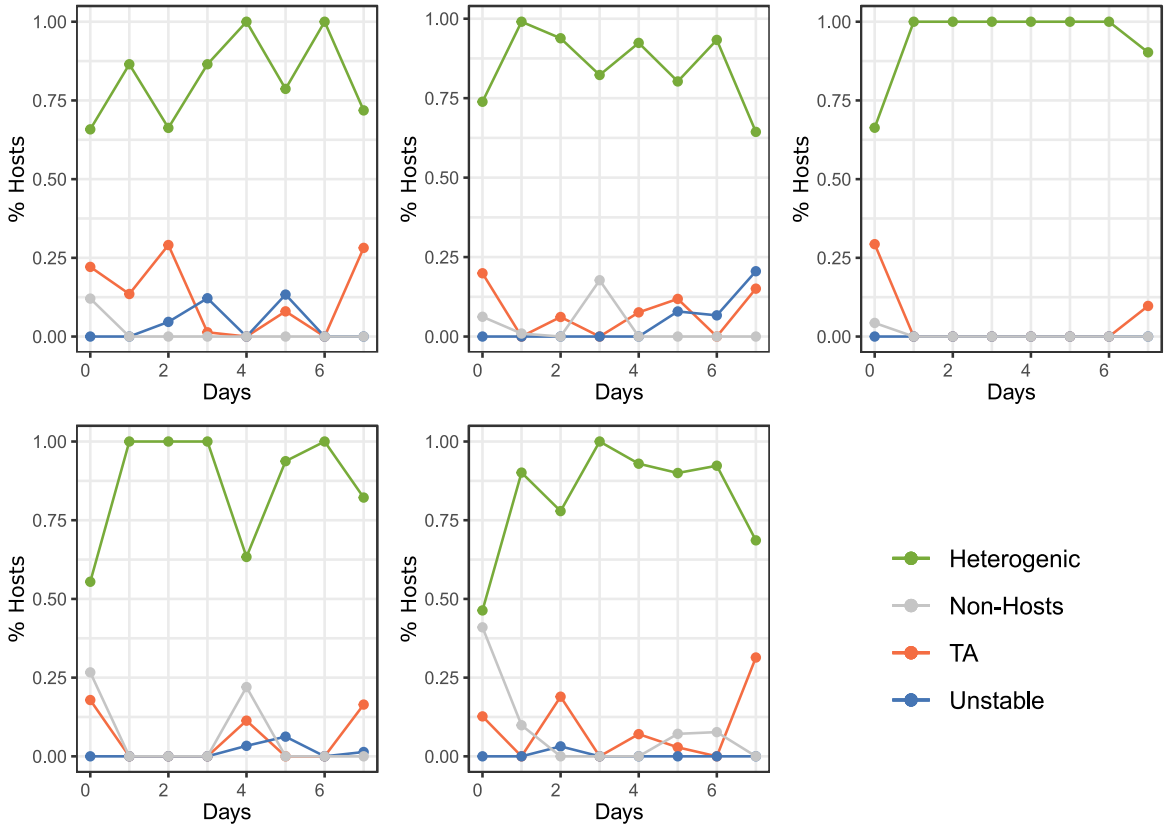

Figure 26: Naïve host assay.
Here five populations that originated from transformant colonies, which were transformed with the plasmid DNA acquired from replicate 2 of the seven-day assay at the last timepoint, are shown. The proportion of host within the populations is shown for each day.

Up until this point, the results have shown that the TA-system helps the plasmid to be maintained within the population and have indicated a benefit for the fitness of the plasmid. The next point of interest was whether a TA-system enables an inherently instable plasmid to compete with an inherently stable plasmid. To answer this question, another pair of competitions was conducted where the R-plasmids were replaced with the S-plasmids. The initial proportions in the competition between pCON-T and pCON-S2 could only be determined for two out of five colonies (see **Fig. 27**). These two colonies have shown a high proportion of heterogenic hosts. This heterogeneity was maintained with an average proportion of 44.52 ± 8.2%. The heterogenic hosts had a maximum proportion of 98% and a minimum

proportion of 64% in the colonies where heterogeneity was detected. However, homogenic pCON-S2 hosts were also present in a high proportion with an average of 43.23 ± 8.29% and an overall minimum proportion of 1%. In colonies where only homogenic pCON-S2 hosts were present a minimum proportion of 71% and a maximum proportion of 100% was observed. In none of the colonies homogenic pCON-T hosts could be observed.

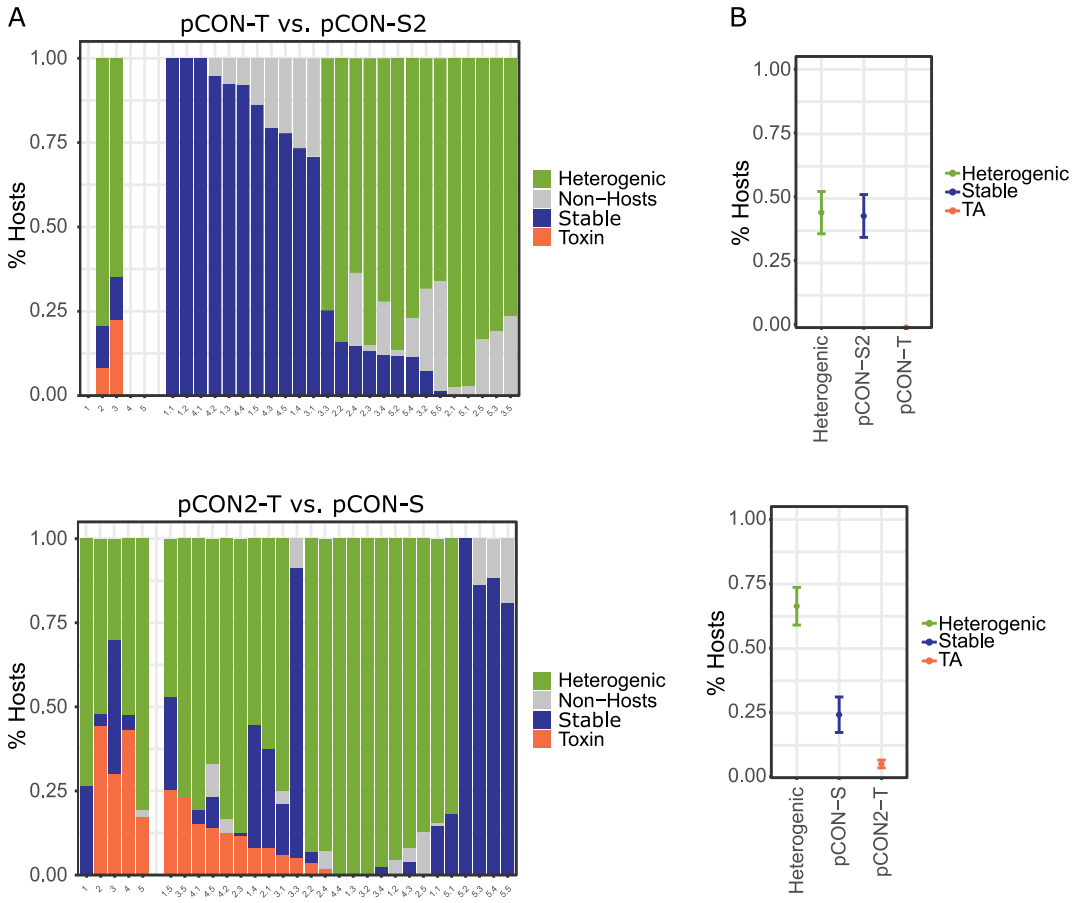

Figure 27: Head-to-head competitions between pCON-T and pCON-S variants.

A) The proportions observed in the competition experiments are shown for each colony. The plots show on the left side the proportions within the initial colonies. On the right the proportions within the evolved colonies can be seen. The results for the competition between pCON-T and pCON-S2 is shown in the upper plot, while the lower plot shows the results for the competition between pCON2-T and pCON-S. B) Shown here are the mean proportions along with the standard error as error bar. In the upper plot the mean proportions of the competition experiment pCON-S2 and pCON-T are shown. The lower plot depicts the mean proportions for the competition experiment between pCON2-T and pCON-S. Wilcoxon tests were conducted. The results of the comparisons are reported here: pCON-T pCON-S2 P = 9.516e-5 (n = 25); pCON-T vs. Heterogenic hosts P = 0.4511 (n = 25); pCON-S2 vs. Heterogenic hosts P = 0.001097 (n = 25); pCON2-T vs. pCON-S P = 0.03822 (n = 25); pCON2-T vs. Heterogenic hosts P = 7.384e-5 (n = 25); pCON-S vs. Heterogenic hosts P = 0.02293 (n = 25)

In the competitions between pCON2-T and pCON-S four out of five initial colonies have shown frequencies between 81% and 52% heterogenic hosts while one colony had roughly equal proportions of the population for homogenic and heterogenic plasmid hosts (40% for pCON-S hosts, 30% for pCON2-T hosts, 30% for heterogenic hosts). The evolved colonies have mostly retained the coexistence of the plasmid except for one colony descendent from colony 3 and four colonies originating from colony 5. The average proportion for pCON-S was 24.38 ± 6.87%, while the average proportion for pCON2-T was 5.31 ± 1.51% and for heterogenic hosts 66.4 ± 7.2%.

The results of these competitions suggest that while the TA-system containing plasmids can maintain themselves in the population, they cannot outcompete the inherently stable plasmids. The heterogenic host fraction were multimers in both experiments. This was confirmed through whole plasmid sequencing.

The final experiment conducted to assess the fitness impact of the TA-system was the preemptive competition between pCON-T and pCON-S2. Here we could observe in only one of two colonies that heterogenic hosts were still present after the initial selective phase (see **Fig. 28**). In that colony proportion of 32% for pCON-T and 45% for pCON-S2 as well as 24% for heterogenic hosts were observed. In the other colony homogenic pCON-S2 hosts were observed with a frequency of 53%. In all of the 10 quantified replicates only two still contained homogenic pCON-T hosts with proportions of 75% and 2%. The colony with a proportion of 2% homogenic pCON-T hosts, homogenic pCON-S2 hosts had a proportion of 22% of the colony while the rest of the colony consisted of segregants. Additionally, one colony was found that consisted entirely of segregants. The remaining seven colonies consisted mostly of homogenic pCON-S2 hosts with a maximum proportion of 100% and a minimum proportion of 93%.

These results further exemplify that a TA-system is not perfect. They also showcase that inherent stability plays a large part in the fitness of a plasmid, since an endemic plasmid population of unstable plasmids containing a TA-system were displaced by an incoming inherently stable plasmid variant.

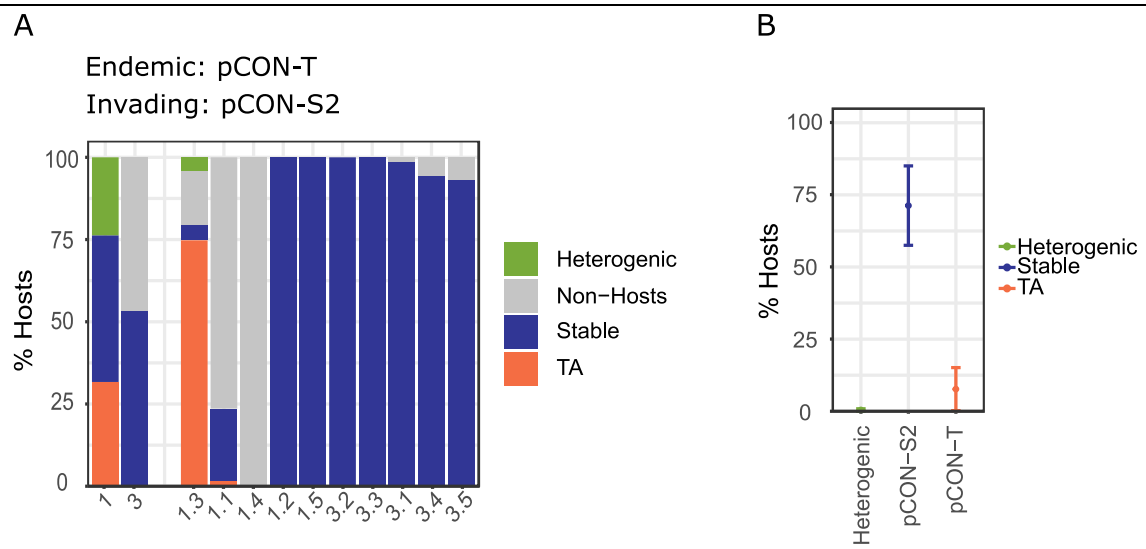

Figure 28: Preemptive competition between pCON-T and pCON-S2.

A) Shown here are the proportions of hosts within colonies for the initial (left) and the evolved colonies (right). B) Here, the average proportions of hosts observed in the evolved colonies of the preemptive competition can be seen. The proportions of hosts in the evolved colonies were compared in a pairwise manner using the Wilcoxon test and the results are reported here: pCON-T vs. pCON-S2 P = 0.01829 (n = 10)

5.4 Partitioning versus Addiction

The third set of competition experiments were conducted between the par and TA plasmids to determine whether it is preferable for a plasmid to have either of the systems over the other. In the head-to-head competitions between pCON-T and pCON2-P we observed proportions of homogenic pCON-T hosts between 69% and 86% in the initial colonies (see **Fig. 29**). The colonies obtained after the competition carried on with this trend of high proportions of homogenic pCON-T hosts with an average proportion of 83.48 ± 4.56%. Only one of 24 colonies contained homogenic pCON2-P hosts. This was the only colony where pCON2-P had won. However, even within that colony the homogenic pCON2-P hosts took a proportion of 59%, while the proportion of segregants was 41%.

In the reciprocal competition between pCON-P and pCON2-T the initial colonies showed higher proportions of cells with both plasmids, between 35 and 68%, while the rest of the cells contained mostly pCON2-T (see **Fig. 29**). In the evolved colonies the vast majority contained homogenic pCON2-T hosts with an average proportion of hosts of 90.86 ± 2.27%, while only 4 colonies still maintained a minor part of homogenic pCON-P hosts. The proportions of the homogenic pCON-P hosts amounted to an average of 1.33 ± 1.06% while the maximum value was 27% and the minimum 1% where the homogenic hosts were present.

These results indicate that when in direct competition with a plasmid that contains a par-system, a plasmid encoding a TA-system has a higher retention rate. This may be caused by

the function of the par-system that now works against the plasmid. The plasmids compete for the PCN in the host cell and the par-system aids the corresponding plasmid during cell division. The TA-system, however, increases the ability of the corresponding plasmid to persist within the population without increasing its stability. This may cause homogenic pCON-P hosts to arise which are in turn killed by the toxin.

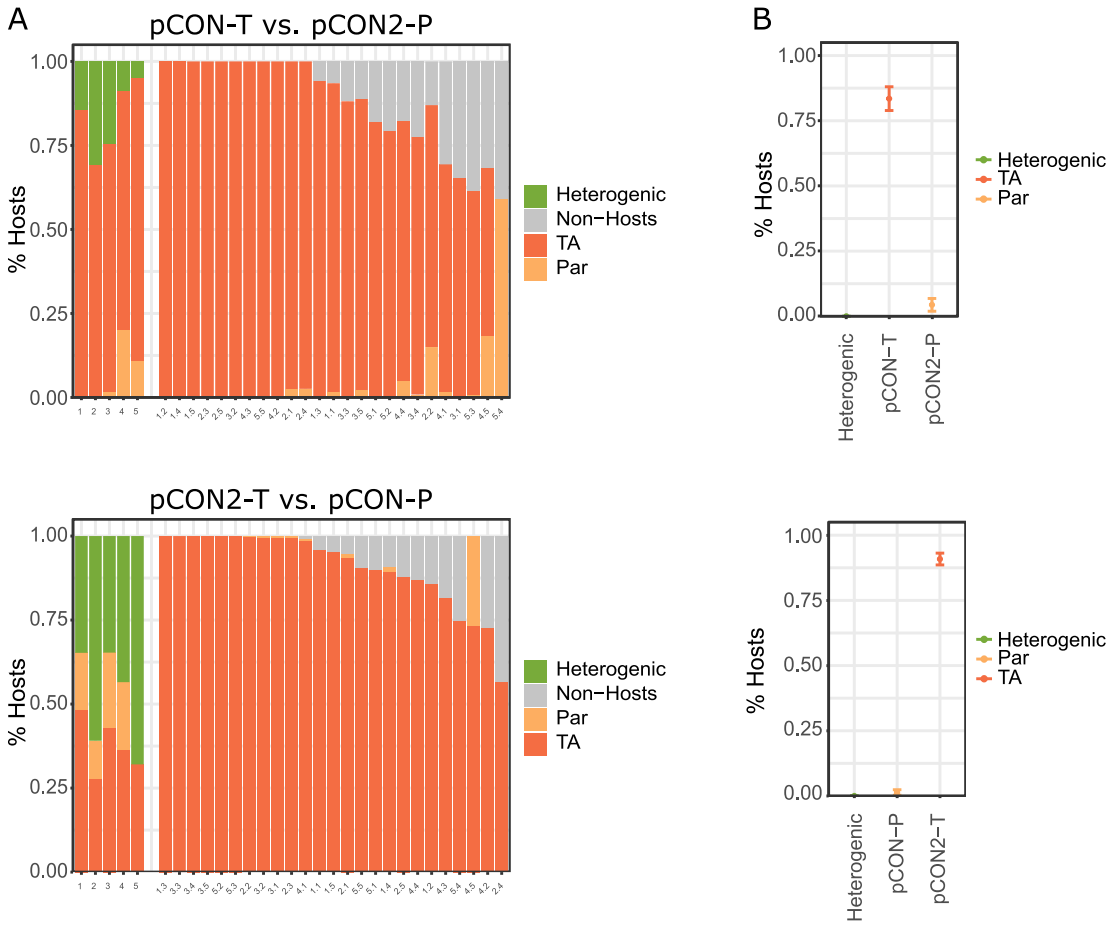

Figure 29: Head-to-head competition between pCON-P and pCON-T variants.

A) Depicted here are the proportions of hosts within the initial colonies (seen on the left side of the plot) and the evolved colonies (right side of the plot). The upper plot shows the results for the competitions between pCON-T and pCON2-P, while in the lower plot the results for the competitions between pCON2-T and pCON-P can be seen. B) Here the average proportions of plasmid hosts are shown for the evolved colonies. The upper plot shows the means for the experiment between pCON-T and pCON2-P while the lower plot shows the mean proportions for the experiment with pCON2-T and pCON-P are shown. The means are depicted along with the respective standard error as error bar. The proportions of hosts were compared using the Wilcoxon test and the results are reported here: pCON-P vs. pCON2-T P = 1.266e-5 (n = 25); pCON2-P pCON-T P = 1.75e-5 (n = 25)

5.5 **Two is better than one**

In this set of competitions between the R-variants and the stabilized plasmids the combination of partitioning and addiction was tested. In the first competition between pCON-PT and pCON2-R the initial colonies showed that a majority of the cells had retained both plasmids (see **Fig. 30**), while roughly 1/3 had the PT plasmid. In the evolved colonies we then saw that homogenic PT hosts had an average proportion of 55.36 ± 6.55% and won most of the competitions. Homogenic pCON2-R had a proportion of 28.39 ± 5.19% within the colonies. In six of the colonies pCON2-R constituted the majority of hosts while in 19 colonies had a majority of homogenic pCON-PT hosts.

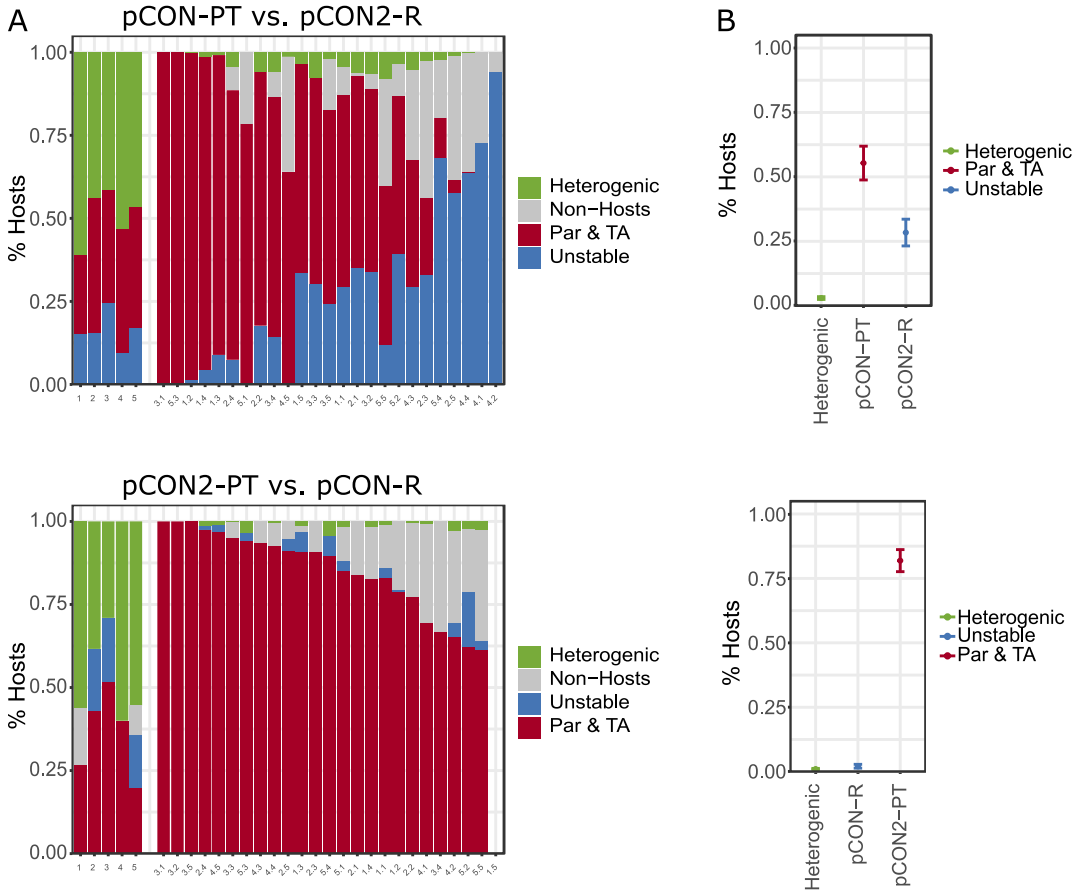

Figure 30: Head-to-head competition between pCON-PT and pCON-S variants.
A) Here the proportions within the initial colonies (left) and the colonies after the conducted competitions (right) are shown. The competition between pCON-PT and pCON2-R is shown in the upper plot and the competition between pCON2-PT and pCON-R in the lower plot. B) In these plots the average proportion of hosts for each host type can be seen along with the respective standard error as error bar. The arrangement of the plots is identical to A). The proportions of hosts were compared using the Wilcoxon test and the results are reported here: pCON-PT pCON2-R P = 0.02735 (n = 25); pCON2-PT pCON-R P = 1.935e-5 (n = 24)

The competition between pCON2-PT and pCON-R also showed that a high number of cells maintained both plasmids throughout the balancing selection or had a majority of pCON2-PT in the cells of the colony (see **Fig. 30**). The evolved colonies then revealed that the competitions were won in all of the examined colonies by pCON2-PT with an average proportion of 81.89 ± 4.28%. Only minimal proportions of homogenic pCON-R and heterogenic hosts remained (2.12 ± 0.74%; 1.02 ± 0.25%).

These results suggested that the combination of two stability systems is slightly more effective than a single system when comparing the average proportions of homogenic pCON-PT hosts to the average proportion of homogenic pCON-P hosts (55.36 ± 6.55% to 43.19 ± 6.09%). When comparing the average proportion of homogenic pCON2-PT with the average proportion of homogenic pCON2-T hosts and pCON2-P hosts in the respective competition experiments (81.89 ± 4.28%; 88.95 ± 4.19%; 87.7 ± 2.72%) the fitness benefit for the pCON2-PT plasmid seemed to be slightly lower than for the plasmids containing a single stability system. However, this may be caused by the dynamics of the combination of the par-system and the TA-system.

To further explore these dynamics seven-day-assays were conducted with colonies stemming from the heterogenic host population of the initial colonies of the competition experiment.

In the assays conducted with cultures that originated from colonies of the competitions between pCON-PT and pCON2-R the same trend could be observed in all five replicates (see **Fig. 31**). In each assay the proportion of pCON-PT hosts rose to over 90% on day 3. Whenever the proportion of homogenic pCON-PT hosts declined only segregants were observed while the proportions of heterogenic and homogenic pCON2-R hosts remained stable on a low level.

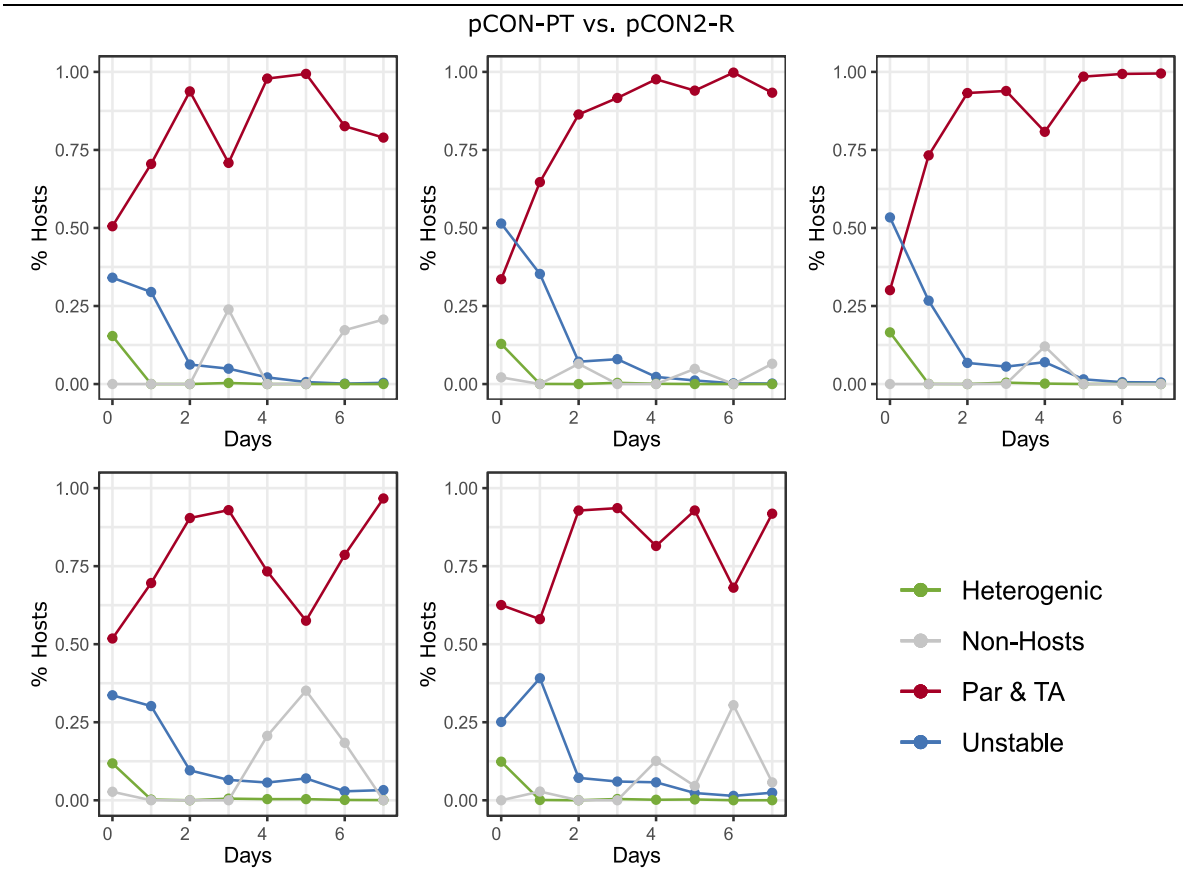

Figure 31: Seven-day assay of hosts containing pCON-PT and pCON2-R.
Depicted here are the proportions of hosts as quantified during the seven-day non-selective assays for five replicates. The assays were conducted as a follow-up of the head-to-head competitions between pCON-PT and pCON2-R starting from colonies that were part of the heterogenic host fraction.

Similar trends were observed in the two assays conducted with cultures originating from two colonies of the competitions between pCON2-PT and pCON-R (see **Fig. 32**).

These results demonstrate that the inherently unstable plasmids modified with two stability systems still experience loss when observed over a longer period of time. However, the plasmid is capable to maintain itself within the population very efficiently.

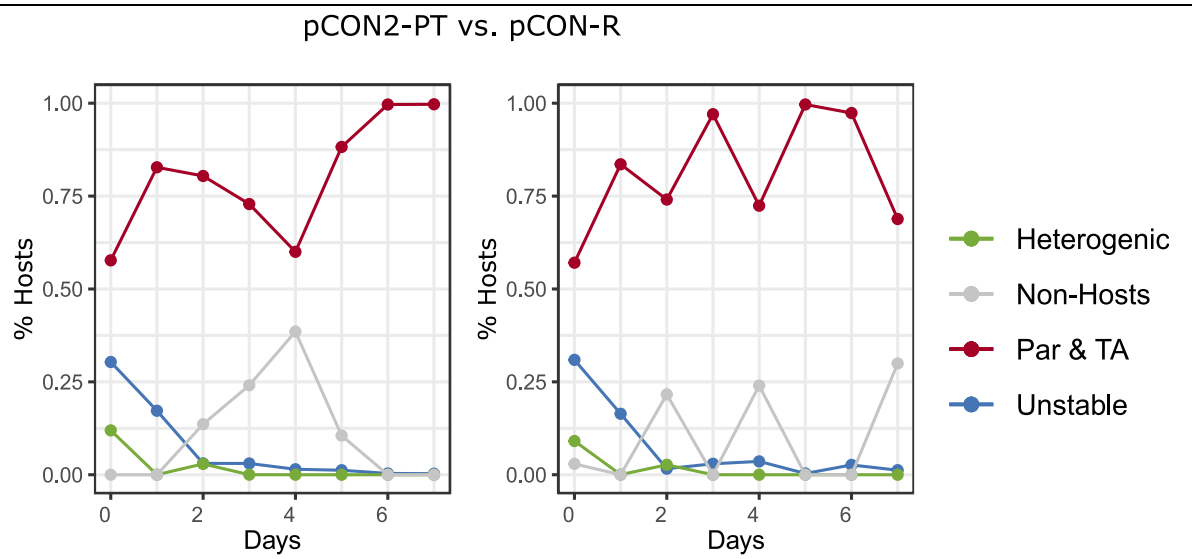

Figure 32: Seven-day assay of hosts containing pCON2-PT and pCON-R.
Here the proportions of hosts tracked over the course of seven days for two replicates are shown. The two replicates originated from the heterogenic host fraction of the initial colonies of the competition experiment between pCON2-PT and pCON-R. The cultures were incubated under non-selective conditions during the experiment.

After observing the dynamics of the modified plasmids containing both a par-system and TA-system, the modified plasmids were put into head-to-head competitions with inherently stable plasmids (see **Fig. 33**).

In the match up of pCON-PT vs. pCON-S2 the proportions in the initial colonies could only be quantified in two of five colonies. There high proportions of heterogenic plasmid hosts were observed. The evolved colonies then showed a more mixed picture with average proportions of heterogenic, homogenic pCON-PT and homogenic pCON-S2 hosts of 30.45 ± 7.2%, 32.21 ± 4.96% and respectively 33.27 ± 5.33%.

The reciprocal setup of pCON2-PT vs. pCON-S has shown similar results. The initial colonies could not be completely quantified in this match up either and the proportions were only determined for three colonies. The evolved colonies have shown that the competitions went overall in favor of pCON2-PT which had an average proportion of 38.04 ± 8.95%, while the average proportion of pCON-S was 14.9 ± 5.12%.

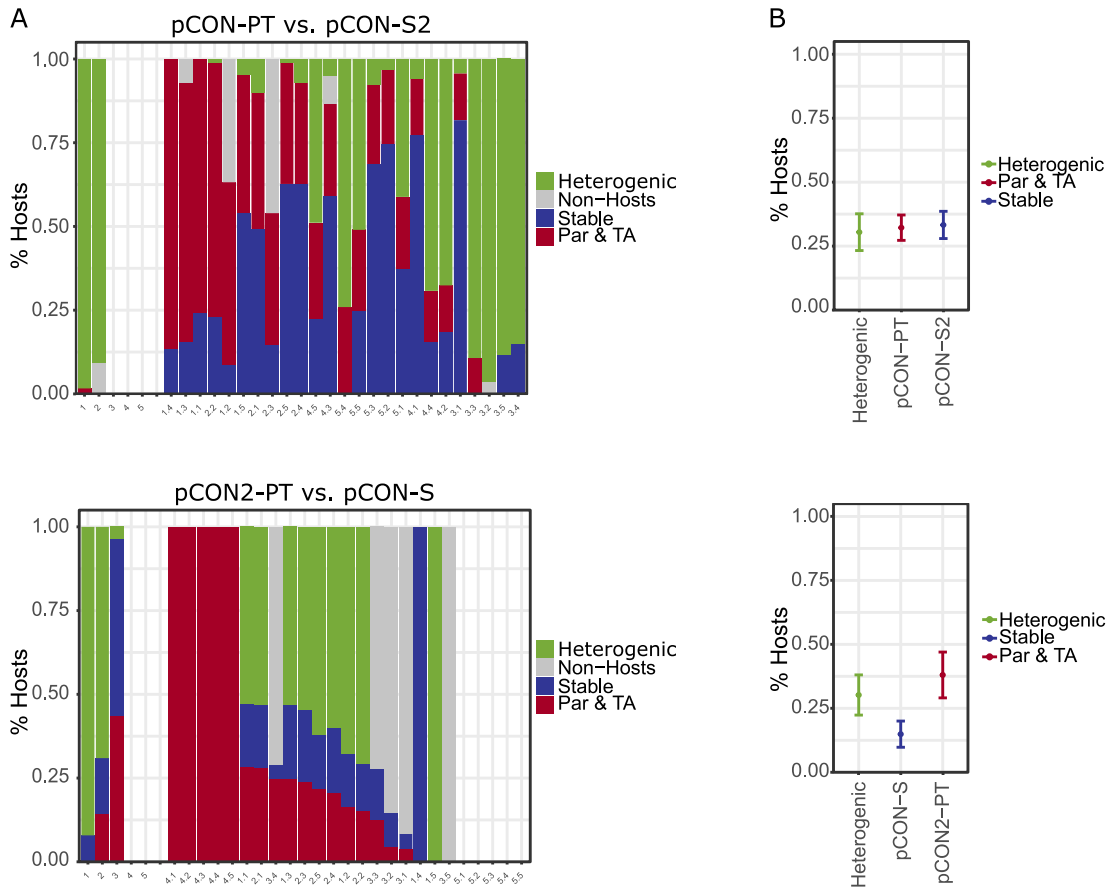

Figure 33: Head-to-head competition between pCON-PT and pCON-S variants.
Shown here are the results of the competition experiments between the pCON-PT variants and the pCON-S variants. A) Shown here are the proportions of hosts in each colony quantified in the competitions between pCON-PT and pCON-S2, while the lower plot shows the same for the colonies of the competitions between pCON2-PT and pCON-S. on the left side of the plots the proportions of hosts within the initial colonies of the competitions can be seen, while the right side shows the proportions observed in the evolved colonies. B) Depicted here are the average proportion of hosts within the evolved colonies of the competition experiments. The upper plot shows the mean proportions for the competitions between pCON-PT and pCON-S2, while the lower plot shows the average proportions in the evolved colonies of the competitions between pCON2-PT and pCON-S. The average proportions are shown along with the corresponding standard error as error bar. Wilcoxon tests were conducted for the evolved colonies and the results are reported here: pCON-PT vs. pCON-S2 P = 0.7701 (n = 25); pCON-PT vs. heterogenic hosts P = 0.893 (n = 25); pCON-S2 vs. heterogenic hosts P = 0.8296 (n = 25); pCON2-PT vs. pCON-S P = 0.03041 (n = 19); pCON2-PT vs. heterogenic hosts P = 0.9477 (n = 19); pCON-S vs. heterogenic hosts P = 0.07352 (n = 19)

These results suggested that the fitness of an unstable plasmid that contains both a par- and a TA-system is similar to that of an inherently stable plasmid. However, this also suggests that inherent stability has a major impact on the fitness of a plasmid. The heterogenic hosts in both experiments turned out to contain multimers, which was confirmed via whole plasmid sequencing (Illumina, see **Fig. 34**).

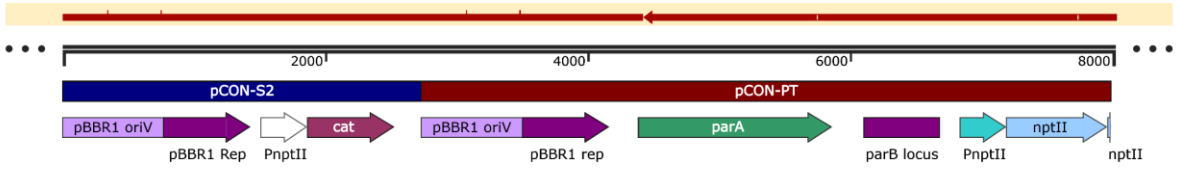

Figure 34: Alignment of sequenced plasmid DNA to the sequence of a multimer consisting of pCON-S2 and pCON-PT.
Shown here is the alignment of sequenced plasmid DNA acquired from an evolved colony of the competition experiment between pCON-PT and pCON-S2. The red arrow within the tan box (top) corresponds to the sequencing result, while the sequence of a theoretical multimer consisting of pCON-S2 and pCON-PT is shown as two black lines along with position indicators from position 1 to 8000 bp in steps of 2000 bp. Beneath that the respective plasmid components (pCON-S and pCON-PT) are indicated in a red and blue box. Lastly, the relevant genetic loci are indicated in the lowest layer.

The last experiment conducted with the double modified plasmids was the preemptive competition experiment with MG1655 *recA*::*tetA* pCON-PT with an invading pCON-S2. Here the initial colonies have shown that the colonies originated from heterogenic hosts since homogenic hosts of both plasmid types were present. In the evolved colonies the majority of cells were homogenic pCON-PT hosts with an average proportion of 85,08 ± 4.78%. The homogenic pCON-S2 hosts were present in 6.94 ± 4.73% (see **Fig. 35**).

This shows that a replication impaired plasmid that is stabilized with both a par- and a TA-system is not easily displaced by an invading plasmid that is inherently stable.

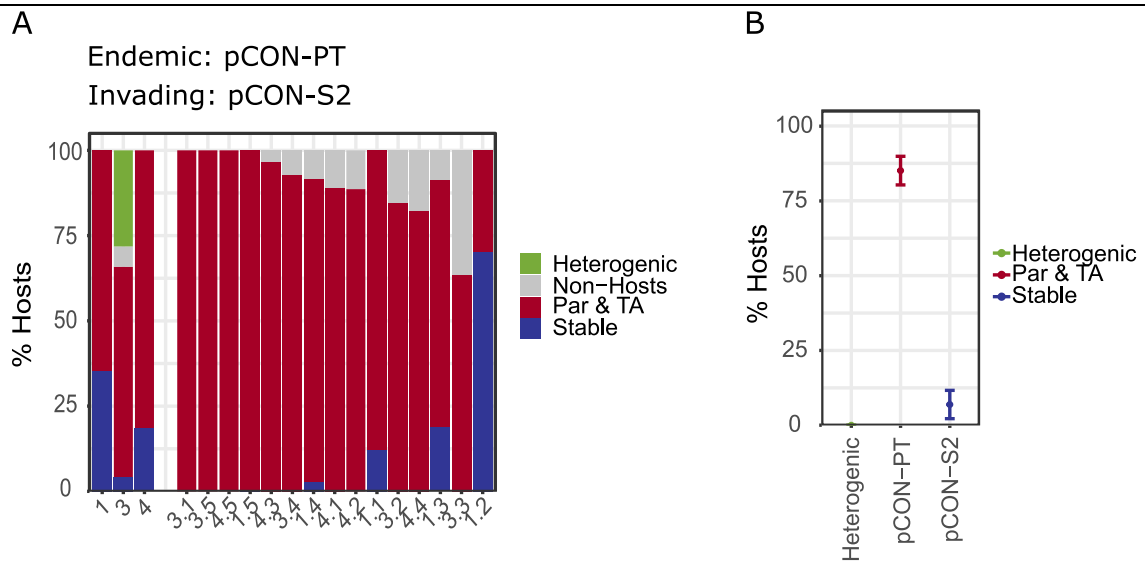

Figure 35: Preemptive competition between pCON-PT and pCON-S2.

A) Here, the proportions of hosts within the initial (left) and the evolved colonies (right) can be seen. The proportions of hosts are shown for each individual colony. B) The average proportions of hosts for the evolved colonies are depicted here. The standard error is shown as error bar for the respective average proportion of hosts. The proportions of hosts in the evolved colonies were compared using Wilcoxon tests. The results of said comparisons are reported here: pCON-PT vs. pCON-S2 P = 0.00008834 (n = 15).

5.6 The conducted match ups

The head-to-head competitions have shown that the pCON2 variants had an inherent, albeit not significant, advantage over the pCON variants as could be seen in the competitions between the plasmids pCON-R and pCON2-R. The competition experiments between the modified pCON2 variants and the pCON-R plasmid were always won by the modified plasmid. On the other hand, the competitions between the modified pCON variants and pCON2-R had a clear winner in only one match up, namely pCON-PT vs. pCON2-R. When looking at the head-to-head competitions between the pCON-S variants and the modified plasmids in only one match up, the competitions between pCON-S and pCON2-P, a clear winner was seen with pCON-S. In the other match ups, mostly coexistence of the plasmids or similar proportions between homogenic and heterogenic hosts were observed. The match ups of the conducted competition experiments and the respective winner can be seen in **Figure 36**.

Head-to-head competitions	pCON-P	pCON-T	pCON-PT	pCON-R	pCON-S
pCON2-P		pCON-T		pCON2-P	pCON-S
pCON2-T	pCON2-T			pCON2-T	Coexistence
pCON2-PT				pCON2-PT	pCON2-PT/ Coexistence
pCON2-R	pCON-P/pCON2-R	Coexistence	pCON-PT	pCON2-R	
pCON-S2	Coexistence	pCON-S2/Coexistence	pCON-PT/ Coexistence/pCON-S		

Figure 36: Match ups of the head-to-head competition experiments.
The winners of the respective match up (row vs. column) are shown in the respective box with the name of the plasmid (Coexistence in case of heterogenic hosts) and the respective color. The color corresponds to either the feature (stable in blue, unstable in light blue), the system (Par in yellow, TA in orange, Par & TA in red) or coexistence of the plasmids (green).

All planed preemptive competition experiments were conducted and quantified (see **Fig. 37**). The preemptive competition experiments have shown that the outcome may have been decided shortly after the successful transformation of the host cells. The results have also demonstrated that TA-systems are not 100% efficient. Further it was shown that a combination of both par- and TA-systems enables an instable plasmid to completely outcompete an incoming inherently stable plasmid. However, the same result was observed in the preemptive competitions between pCON-R and pCON-S2, since the inherently unstable plasmid also won in this case.

		Endemic			
	Preemptive	pCON-P	pCON-T	pCON-PT	pCON-R
Invading	pCON-S2	pCON-P/pCON-S2	pCON-S2	pCON-PT	pCON-R

Figure 37: Match ups of the preemptive competitions.
Here, the match ups in the preemptive competition experiments are shown. The invading plasmid pCON-S2 (row) is pitched against the endemic plasmids (columns). The winning plasmid of each match up is indicated in the respective box with the plasmid name and color for the feature (stable in blue, unstable in light blue) or system (Par in yellow, TA in orange, Par & TA in red) of the plasmid.

6 Discussion

In this thesis plasmids are considered as Darwinian entities. By definition a Darwinian entity needs to be subject to natural selection. This can only apply to an entity with variation amongst individuals and this variation must in turn be heritable. Lastly, the genetic variation needs to be associated with a variation in fitness. Therefore, the fitness of a plasmid needs to be assessed by competing them with other plasmids that belong to the same incompatibility group. Here we have put variants of our unstable model plasmids pCON and pCON2 into competitions with each other and our stable model plasmids pCON-S and pCON-S2. The modified plasmids contain either a stability system or a DNA fragment with no stabilizing function.

The intracellular competitions were already established in a previous work (Hülter et al. 2020). However, the experimental set up used in said work was not ideal. The pipeline used in the intracellular competitions in this thesis is an improved version of the previous work. The heterogenic hosts obtained after transformation are selected for with double antibiotic pressure and temperature limitation. The temperature limitation has a stabilizing effect on the plasmids has been observed in the study during which the model plasmid pCON was constructed (Wein et al. 2019). The issues encountered during the first of our studies using intracellular competitions regarding multimerization were still a problem in the beginning of this doctoral project (Hülter et al. 2020). Since the cause for the high degree of multimerization is likely recombination of the plasmids after damage incurred through conflicts between transcription and replication which is mediated by RecA. The *recA* deficient host strain shows minimal multimerization over a period of seven days, which confirms that the absence of RecA minimizes the occurrence of multimers.

The impact of a partitioning system on the fitness of a plasmid is evident when viewing the results of the competition experiments between the pCON-P variants and the pCON-R variants. Here, the pCON-P variants were more successful in persisting within the host population than the pCON-R variants. This suggests that while the inherent source of instability is present in both plasmids the higher success during segregation of the plasmid copies into the daughter cells of the host grants the pCON-P variants a fitness benefit. However, the benefit that a par-system conveys is not as high as inherent stability. This suggests that an increase in replicative success is a preferred way of stabilization to higher success during segregation.

A Toxin-Antitoxin system does not contribute to the stability of a plasmid when defining plasmid stability as the ability of a plasmid to successfully complete the plasmid lifecycle. It does, however, increase the persistence of a plasmid within the host population. This is achieved by killing segregants or inhibiting their growth through a stable toxin, which is inhibited

in the plasmid hosts through the less stable antitoxin. In this case the *hok*/*sok* system of plasmid R1 is used (Gerdes et al. 1986). The increase in persistence of the plasmid alleviates the negative impact on the fitness incurred by the low replicational success. However, the persistence may not lead to single plasmids in monomeric form being present in the host cells. If a plasmid, that in the absence of the TA-system would be more fit than the TA-system containing plasmid, is in direct competition with the TA-system containing plasmid, then the TA-system might promote the formation of multimers. One of these cases can be seen in the case of initial colony 1 and the evolved colonies which descended from it (ref 5.3). There, in the initial colony a heteromeric plasmid consisting of pCON-T and pCON2-R is present. Whether or not the heteromeric form was kept was not confirmed, however the heterogeneity within the plasmid hosts persists in a large proportion of the evolved populations. This can also be seen in the head-to-head competition experiments between the inherently stable pCON-S variants and the pCON-T variants. Here the stable variants contribute a higher proportion of copies to the allele pool in the daughtercells of the hosts, however in many replicates heterogenic hosts constitute the majority of the cells within the examined colonies. These results show that the TA-system used is not 100% efficient and that the inherently stable plasmid is fitter than an unstable plasmid with a TA-system.

In a direct comparison between to plasmid variants that are identical except for their antibiotic resistance marker gene and the stability system that they contain, the TA-system containing plasmid has a higher fitness than the par-system containing plasmid. A possible explanation for this is the way active partitioning systems work. The par-system containing plasmids are not dependent on stochastic distribution into the daughter cells, unlike the TA-system containing plasmids. This may lead to fast segregation into homogenic plasmid hosts of each type, where the hosts containing the par-system containing plasmid are subjected to the toxin left behind by the TA-system containing plasmid. In this hypothetical scenario the stability of the par-system containing plasmid is higher, however the increased persistence of the TA-system containing plasmid effectively leads to a higher contribution of the TA-system containing plasmid to the next generation of host cells.

When both systems are present in combination on one plasmid it further enhances the fitness of the plasmid. However, this is not very evident in the competition experiments of the modified plasmids with the pCON-R variants. This may be a result of the dynamics caused by the interplay of the par- and TA-system. When the competition experiments are observed over a longer period, the advantage of the combination becomes more apparent. There, the modified plasmid outcompetes the unstable plasmid and even though the proportion of homogenic pCON-PT variant hosts may fluctuate only the proportion of segregants varies. In the competition experiments between the modified plasmids and the inherently stable plasmids

the positive effect of the combination of the two systems is more apparent. In a direct competition between inherently stable plasmids and the pCON-PT variants the average proportions are either equally split between heterogenic hosts and homogenic hosts of either kind or with a higher proportion of homogenic PT hosts.

Recombination between plasmids may stabilize the resulting multimeric plasmid. This can be seen in the seven-day observation of the competition between pCON-T and pCON2-R. It was previously observed that multimeric plasmids are a possible way to keep an antibiotic resistance within a plasmid population (Mazin et al. 1996). This dimerization in the absence of selective pressure was hypothesized to be a 'catastrophe' for a plasmid relying on stochastic distribution within its host due to over replication of the multimeric plasmids (Summers et al. 1993). The multimeric plasmid reduces the effective PCN of the plasmids by one or more, which in turn reduces the likelihood of distribution into both daughter cells. However, this scenario does not apply when stability systems are involved. The relatively frequent occurrence of multimers within the Δ*recA* host strain used in this thesis suggests that multimers may be a way for plasmids to increase their stability and prolong the time during which they can evolve inherent stability. Alternatively homologous recombination between plasmids of the same backbone with different alleles can be a way to transfer traits beneficial for plasmid maintenance to an already stable plasmid.

When aiming to rank the different systems used in this study, one can only reach the conclusion that a combination of both the par- and the TA-system is ideal. The next system in this ranking would be the par-system, however this depends on the context, since a direct competition between a plasmid containing a par-system and a plasmid containing a TA-system leads to the displacement of the partitioning plasmid. Nevertheless, it can also be observed that inherent stability is the biggest factor in plasmid fitness, since no modified plasmid was able to completely outcompete a stable plasmid in a head-to-head experiment.

Since in this study the focus was on the comparison of two specific stability systems, which are found on the same plasmid, future studies may be conducted using stability systems that do not originate on the same plasmid, or between stability systems of the same type, e. g. a TA-system found on one plasmid may be compared to that found on another. It is also possible to include further stability mechanisms in these comparisons, i.e. determine the benefit of multimer resolution systems for the fitness of a plasmid. To help decide on specific systems to be used, comparative genomics can be included to find the most widespread type of systems. Comparative genomics can also be applied to further validate the results of such competition experiments by determining the most commonly found combination of stability systems amongst plasmids.

The knowledge gained from the results of this thesis may improve our understanding of the evolution of stability on plasmids. It may also serve to further improve the construction of vectors used for biotechnological applications if the aim is a long-term persistence of the plasmids within the bacterial population. This may not be useful when looking at simple protein expression, where it is enough to have a vector that is unstable but has a high copy number, e.g. pUC, to express as much of the protein as possible over a short period of time. However, if the expression of proteins needs to occur over a longer period with constant expression, as may be the case for probiotics, then a vector with the highest possible stability would be ideal.

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