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CENTRO DE CIENCIAS GENÓMICAS

EFECTO DE LA VARIABILIDAD FENOTÍPICA EN LA DINÁMICA EVOLUTIVA DE RESISTENCIA A ANTIBIÓTICOS

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Resumen

Además de portar material genético en el cromosoma, algunas bacterias pueden contar con pequeñas moléculas de DNA adicional, físicamente separadas del genoma, las cuales se replican de manera independiente. Dichas moléculas se denominan plásmidos y pueden contener genes que codifican para funciones que permiten a las bacterias sobrevivir a ambientes hostiles, como metales pesados o antibióticos.

Típicamente, los plásmidos presentan múltiples copias por célula, variando desde 1 o 2 para plásmidos grandes, hasta 200 o más en plásmidos pequeños (Smillie et al. 2010; Münch et al. 2019). Los plásmidos grandes suelen codificar mecanismos para asegurar su permanencia en la población, como partición activa, sistemas toxina-antitoxina o conjugación, siendo esta última de gran interés ya que también promueve la transferencia horizontal de genes y estabiliza a los plásmidos en la población (Stewart and Levin 1977). A su vez, los plásmidos pequeños dependen de su alto número copias para asegurar que ambas bacterias hijas hereden plásmidos al momento de división.

Los plásmidos han sido objeto de estudio no sólo por interferir en las dinámicas de evolución de genes sino también han sido altamente utilizados como vectores de clonación con fines tanto biotecnológicos como de ingeniería genética en el laboratorio (Ensley 1986). Además de esto, debido a la capacidad de transferir horizontalmente genes de resistencia, los plásmidos son parcialmente responsables de la alta prevalencia de aislados clínicos resistentes a antibióticos (San Millan 2018). Por lo anterior, es fundamental entender la dinámica poblacional que emerge de la dinámica de replicación y segregación de plásmidos presentados por cada célula.

En este trabajo presentamos un esfuerzo multidisciplinario, que combina enfoques teóricos y experimentales, tanto en células individuales como a nivel de poblaciones, para analizar distintos procesos evolutivos que pueden ocurrir con plásmidos multicopia. Para esto, utilizamos un plásmido tipo ColE1, que es no conjugativo ni posee mecanismos de partición activa, que autoregula su número de copias con un mecanismo ruidoso y que porta un gen de resistencia a antibióticos y un gen reportero inducible.

En la Figura 1 se muestran los fenómenos relacionados a los plásmidos multicopia abordados en proyectos independientes y compilados como capítulos en esta tesis. En el capítulo 2 discutimos distintos enfoques de modelación de aspectos de la dinámica de plásmidos; desde modelos de replicación y control de número de copias, hasta la dinámica eco-evolutiva de comunidades microbianas. Esta revisión de la literatura fue resultado directo de una actividad académica realizada durante mi posgrado. Posteriormente, en el capítulo 3, utilizamos un modelo de genética de poblaciones para analizar la estabilidad de plásmidos e identificar las condiciones que evitan que un plásmido se pierda en una población.

En el capítulo 4 consideramos que el mecanismo de control de número de copias produce variabilidad de número copias y, por lo tanto, heterogeneidad de fenotipos asociados a los genes portados en los plásmidos. Combinando enfoques de células individuales y a nivel de poblaciones, investigamos las ventajas adaptativas de dicha

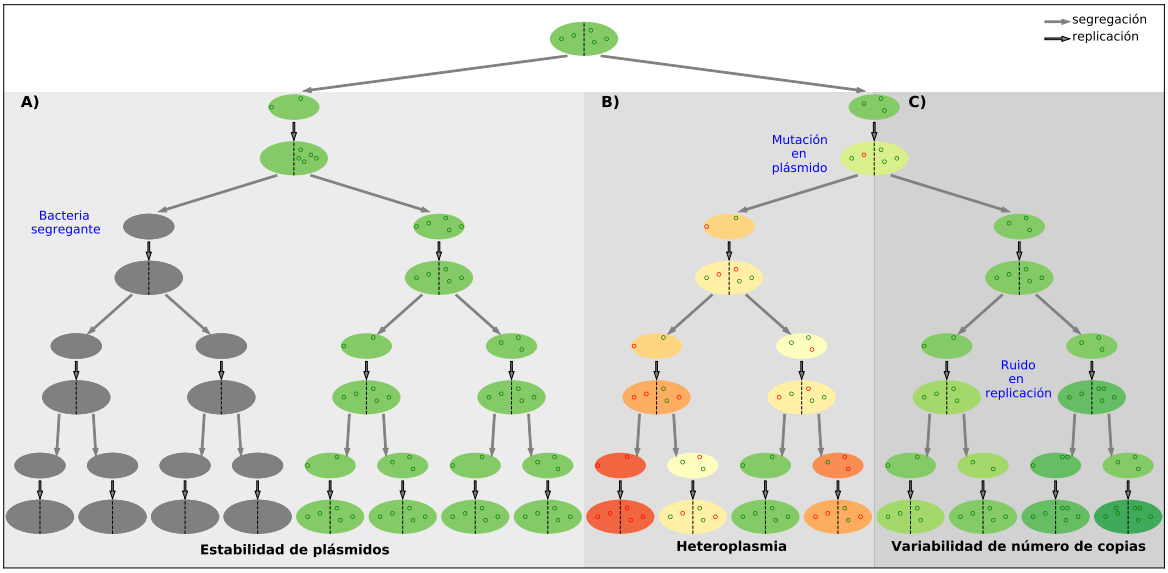

Figura 1: Distintos escenarios de procesos asociados a plásmidos multicopia. **A)** Estabilidad de plásmidos: dinámica de prevalencia o pérdida de plásmidos en una población. Una de las bacterias hijas puede quedar sin plásmidos durante la división celular. **B)** Heteroplasmia: coexistencia de múltiples tipos (o versiones) de plásmidos en una sola célula. Durante la replicación pueden ocurrir mutaciones en una de las copias del plásmido **C)** Variabilidad de número de copias: distintas bacterias de la misma población portan distinto número de copias del plásmido. Ruido en el mecanismo de control de número de copias puede generar diversidad de número de copias

variabilidad en comparación con poblaciones que no presentan variabilidad, es decir, que portan el gen de interés en el cromosoma.

En el capítulo 5 consideramos que durante la replicación de plásmidos pueden ocurrir mutaciones en genes que éste porta y, por lo tanto, producir un plásmido mutante. En plásmidos multicopia, una consecuencia de la replicación y segregación de plásmidos, es que el alelo mutante se propaga verticalmente en la población permitiendo la coexistencia transiente entre plásmidos mutantes y ancestrales a nivel celular, un fenómeno conocido como heteroplasmia. La heterocigosis plasmídica es inherentemente inestable, por lo que utilizamos técnicas de modelación y experimentación que nos permitieron estudiar meticulosamente la dinámica de plásmidos en células individuales con una alta resolución temporal. Este enfoque multi-escalas nos permitió, además, estudiar el efecto que tienen las presiones selectivas específicas a cada alelo en la dinámica de plásmidos subyaciente.

Finalmente, en el capítulo 6 utilizamos un enfoque a nivel de poblaciones para explorar las ventajas evolutivas de la heterocigosis mediada por plásmidos, enfocándonos en las consecuencias del aumento en la diversidad alélica intracelular, lo cual produce redundancia genética y permite aliviar compromisos evolutivos.

Summary

In addition to carrying genetic material in the chromosome, some bacteria may have small extra DNA molecules, physically separated from the genome, which replicate independently. Such molecules are called plasmids and can contain genes that encode for functions that allow bacteria to survive harsh environments, such as heavy metals or antibiotics.

Typically, plasmids are present in multiple copies per cell, ranging from 1 or 2 for large plasmids, to 200 or more for small plasmids (Smillie et al. 2010; Münch et al. 2019). Large plasmids usually encode for mechanisms to ensure their permanence in the population, such as active partition, toxin-antitoxin systems or conjugation, the latter being of great interest since it also promotes horizontal gene transfer and stabilizes plasmids in the population (Stewart and Levin 1977). In turn, small plasmids depend on their high copy number to ensure that both daughter bacteria inherit plasmids at the moment of division.

Plasmids have been object of study not only because they interfere in the dynamics of gene evolution, but they have also been widely used as cloning vectors for both biotechnological and genetic engineering purposes in the laboratory (Ensley 1986). In addition to this, due to the ability to horizontally transfer resistance genes, plasmids are partially responsible for the high prevalence of clinical isolates resistant to antibiotics (San Millan 2018). Therefore, it is essential to understand the population dynamics that emerge from the dynamics of replication and segregation of plasmids presented by each cell.

In this work, we present a multidisciplinary effort which combines theoretical and experimental approaches, both in individual cells and at the population level, to analyze different evolutionary processes that can occur with multicopy plasmids. To do so, we used a ColE1-like plasmid, which is neither conjugative nor does it possess an active partitioning system, that self-regulates its copy number with a noisy mechanism and which carries an antibiotic resistance gene and an inducible reporter gene.

Figure 2 shows different scenarios related to multicopy plasmids addressed in independent projects and compiled as chapters in this thesis. In Chapter 2 we discuss different approaches to modeling aspects of plasmid dynamics; from replication models and control of copy number, to the eco-evolutionary dynamics of microbial communities. This review of the literature was the direct result of an academic activity carried out during my graduate studies. Later, in Chapter 3, we used a population genetics model to analyze plasmid stability and identify conditions that prevent a plasmid from being lost in a population.

In Chapter 4 we considered that the copy number control mechanism produces copy number variability and, therefore, heterogeneity of phenotypes associated with genes carried in plasmids. By combining single cell and population-level approaches, we investigated the adaptive advantages of such variability compared to populations that do not exhibit variability, that is, that carry the gene of interest on the chromosome.

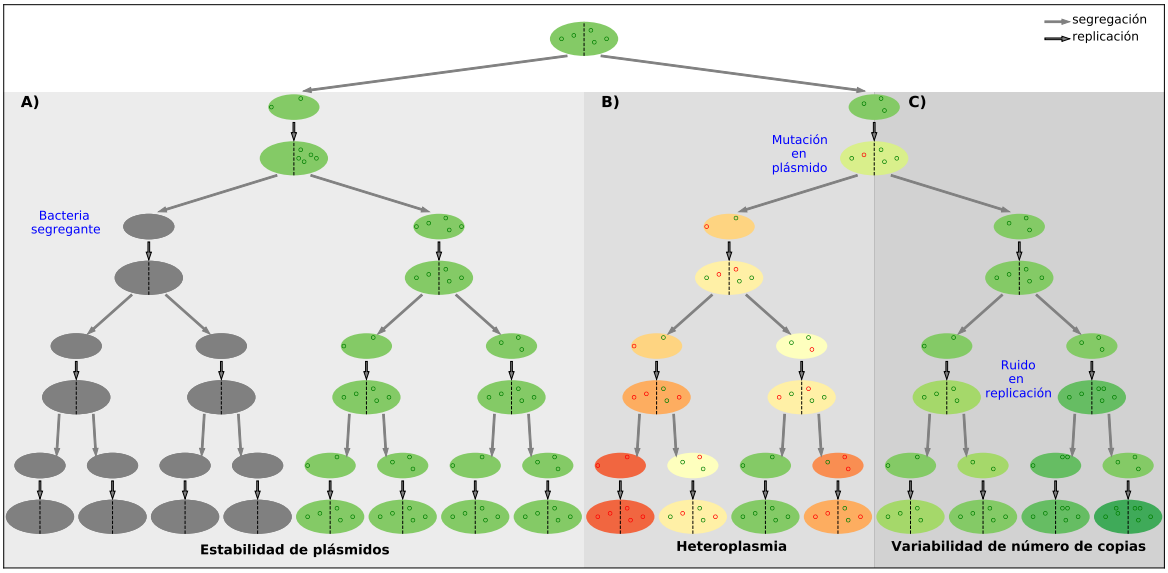

Figura 2: Different process scenarios associated with multicopy plasmids. **A)** Plasmid stability: dynamics of prevalence or loss of plasmids in a population. One of the daughter bacteria can be left without plasmids during cell division. **B)** Heteroplasmy: coexistence of multiple types (or versions) of plasmids in a single cell. During replication, mutations can occur in one of the plasmid copies. **C)** Copy number variability: different bacteria in the same population carry different numbers of copies of the plasmid. Noise in the copy number control mechanism can lead to copy number diversity.

In Chapter 5 we considered that during plasmid replication, mutations can occur in genes that it carries and, therefore, produce a mutant plasmid. In multicopy plasmids, a consequence of plasmid replication and segregation is that the mutant allele spreads vertically in the population, allowing transient coexistence between mutant and ancestral plasmids at the cellular level, a phenomenon known as heteroplasmy. Plasmid heterozygosity is inherently unstable, so we used modeling and experimentation techniques that allowed us to meticulously study plasmid dynamics in individual cells with high temporal resolution. This multi-scale approach also allowed us to study the effect that allele-specific selective pressures have on the underlying plasmid dynamics.

Finally, in Chapter 6 we used a population-level approach to explore the evolutionary advantages of plasmid-mediated heterozygosity, focusing on the consequences of increased intracellular allelic diversity, which produces genetic redundancy and alleviates evolutionary trade-offs.

Índice general

Capítulo 1

Introducción general

Existen distintos mecanismos que permiten a una población de bacterias adaptarse a condiciones ambientales hostiles, por ejemplo la acción de sustancias antimicrobianas. Mutación y regulación son dos mecanismos adaptativos muy bien estudiados (Andersson 2005). Por otro lado, los recientes desarrollos tecnológicos en microscopía de fluorescencia, procesamiento de imágenes y microfluídica nos han permitido estudiar comunidades de bacterias a nivel de células individuales (Balaban et al. 2004; Elowitz et al. 2002), demostrando que la premisa de que una población genéticamente idéntica en un medio ambiente homogéneo tiene un comportamiento uniforme, es falsa. Este fenómeno se conoce como heterogeneidad fenotípica y permite a poblaciones isogénicas de bacterias implementar estrategias colectivas en donde, a pesar de estar asociadas a un costo individual, ciertos comportamientos cooperativos les pueden ofrecer beneficios funcionales a la comunidad (Ackermann 2015).

La variabilidad fenotípica puede tener implicaciones en procesos de infección. Por ejemplo, en comunidades de patógenos se han observado estrategias cooperativas de división del trabajo tales como el comportamiento autodestructivo (Ackermann et al. 2008) y de formación de biopelículas (Chai et al. 2008); estrategias de apuesta-cobertura como la presencia de subpoblaciones que arrestan su metabolismo aumentando la tolerancia de la población a ciertos fármacos y permitiendo infecciones recurrentes (Balaban et al. 2004; Arnoldini et al. 2014), así como estrategias cooperativas de bienes públicos, en donde una subpoblación resistente permite a células susceptibles sobrevivir en ambientes con antibióticos (Lee et al. 2010).

En general, estas estrategias colectivas pueden producir variabilidad en los patrones de susceptibilidad a antibióticos, lo cual puede representar un problema para el diseño de estrategias óptimas de utilización de fármacos, puesto que el problema de optimización resultante es multiobjetivo: la estrategia óptima para eliminar a las poblaciones susceptibles (usar el antibiótico a dosis altas) difiere diametralmente de la estrategia óptima para minimizar la presión selectiva en favor de patógenos resistentes (no usar antibióticos). Estudios clínicos recientes han identificado que la heterogeneidad en la susceptibilidad a antibióticos puede resultar en la falla de tratamientos de antibióticos, un fenómeno conocido como heterorresistencia (Lázár and Kishony 2019; Andersson et al. 2019; Nicoloff et al. 2019).

Entonces, ¿cuál es la estrategia óptima de utilización de antibióticos para combatir una población heterogénea de patógenos? Esta pregunta es, por supuesto, muy difícil de responder, pero una que es interesante y fundamental en la búsqueda de soluciones sustentables al problema de resistencia a antibióticos. En este sentido, el objetivo de este proyecto es estudiar el beneficio funcional que ofrece un perfil heterogéneo de resistencia a antibióticos en la capacidad de una comunidad bacteriana para sobrevivir a medios ambientes hostiles e impredecibles. En particular, nos enfocaremos en estudiar

un mecanismo generador de heterogeneidad fenotípica muy bien caracterizado desde el punto de vista molecular (Cesareni et al. 1991), pero sorprendentemente poco estudiado en el efecto que tiene sobre la dinámica adaptativa: la variabilidad genética -y fenotípica- producida por plásmidos multicopia no conjugativos que contienen un gen de resistencia a antibióticos de relevancia clínica.

Los plásmidos son moléculas extracromosomales de DNA circular que replican independientemente del genoma bacteriano. Los plásmidos portan genes accesorios que proporcionan a su hospedero una ventaja, como la capacidad de sobrevivir en entornos atípicos y hostiles. Ejemplos típicos de esto son la resistencia a las sustancias antimicrobianas, la tolerancia a metales pesados y la fijación de nitrógeno. La estructura de los plásmidos se puede describir en dos categorías: 1) el esqueleto, que lleva genes que codifican las funciones básicas del plásmido, como la replicación, segregación y conjugación, 2) los genes accesorios que codifican rasgos que son beneficiosos para el huésped (Harrison and Brockhurst 2012). Otra funciones codificadas en el esqueleto son las estrategias específicas de cada plásmido para mantenimiento o prevención de pérdidas, como los sistemas de partición activa, la destrucción postsegregacional (PSK) y el mecanismo de control del número de copias.

El número de copias de plásmidos por célula depende del origen de la replicación y puede variar desde 2 copias para plásmidos de copia baja hasta 50 a 800 para plásmidos de copia alta (Münch et al. 2019). El número de copias del plásmido se correlaciona inversamente con su tamaño (y número de funciones). Por esta razón, los plásmidos se clasifican por su tamaño y capacidades generales, particularmente de movimiento. En general, los plásmidos grandes tienen la capacidad de copiarse a otra bacteria a través de la conjugación, mientras que los plásmidos pequeños pueden ser movilizados por los primeros, carecen de la maquinaria para transferirse por sí mismos. Los llamados plásmidos no movilizables son generalmente pequeños, pero un gran porcentaje de ellos son casi tan grandes como plásmidos conjugativos (Smillie et al. 2010).

El estudio de plásmidos ha sido de gran interés en la comunidad científica por intereses biotecnológicos, de estudio de resistencia a antibióticos, o por razones ecológico-evolutivas, ya que algunos son capaces de conjugar, promoviendo la transferencia horizontal de genes (THG) entre especies. Por otro lado, cuando una célula que porta un plásmido multicopia se divide, cada copia se segrega al azar a las células hijas. Esto puede generar que alguna de las bacterias no herede plásmidos, produciendo una bacteria segregante. Las células libres de plásmidos tienen una mayor adecuación dado que portar plásmidos está asociado a un costo metabólico (Baltrus 2013; San Millan et al. 2014), por lo que en ausencia de una presión de selección a favor de genes portados en el plásmido, los plásmidos pueden extinguirse de la población. A este proceso de pérdida o prevalencia de plásmidos nos referiremos como su estabilidad.

Para el estudio de la estabilidad y transferencia horizontal de plásmidos se han utilizado una combinación de sistemas experimentales y modelos matemáticos con gran diversidad de enfoques de modelación. En el Capítulo 2 discutimos una revisión de la literatura sobre modelación de dinámicas ecológicas y evolutivas de plásmidos, una publicación que fue el resultado del trabajo realizado como actividad académica.

En el Capítulo 3, para estudiar el efecto que tiene la interacción entre el número de copias y el medio ambiente en la estabilidad de plásmidos, postulamos un modelo de genética de poblaciones. Con este modelo, argumentamos que la probabilidad de pérdida depende del número de copias y un compromiso entre el costo del plásmido y la frecuencia de selección positiva. Para validar nuestros resultados teóricos, utilizamos un modelo experimental que consisten en *Escherichia coli* MG1655 portando un plásmido tipo ColE1 (previamente caracterizado por San Millan et al. (2016)), no conjugativo, sin mecanismo de partición activa, que en promedio tiene 19 copias por célula. El plásmido porta un gen que codifica para proteína verde fluorescente (GFP) y el gen de resistencia $bla_{\text{TEM-1}}$, el cual codifica para la variante TEM-1 de la β-lactamasa que degrada ampicilina y otros antibióticos β-lactámicos (Salverda et al. 2010; Mira et al. 2015)

A este plásmido lo denotaremos por pBGT y, dado que es de tipo ColE1, autoregula su número de copias por medio de dos RNAs (RNAI y RNAII) que actúan, respectivamente, como represor y activador de la maquinaria de replicación (Lin-Chao and Bremer 1987). Este sistema de replicación es intrínsecamente ruidoso, por lo que dos células que heredan el mismo número de copias pueden presentar distinto número de copias al momento de división y, consecuentemente, exhibir variabilidad en el número de copias en la población. En el Capítulo 4, exploramos las ventajas adaptativas de presentar poblaciones heterogéneas en la distribución de número de copias de un gen portado en pBGT y demostramos, mediante experimentos a nivel de poblaciones y de células individuales, que esta variabilidad puede producir heteroresistencia en la población.

Por otro lado, en un estudio publicado por nuestros colaboradores en el Instituto Ramón y Cajal de Investigación Sanitaria en Madrid (San Millan et al. 2016), sometieron a un población de *E. coli* portadora de pBGT a un experimento de diluciones seriales con un gradiente de ceftazidime, un antibiótico β-lactámico para el cual el gen de resistencia portado en el plásmido ($bla_{\text{TEM-1}}$) no confiere resistencia. Al final del experimento, se encontraron mutaciones específicas en el gen de resistencia (las cuales le conferían resistencia al nuevo antibiótico), pero también en el mecanismo de replicación (las cuales aumentaban el número de copias y, por consiguiente, la dosis génica). Para estudiar esta dinámica evolutiva, postulamos un modelo de agentes individuales donde cada célula se representa mediante un objeto computacional con características específicas y con reglas de actualización que dependen de su estado y del medio ambiente. Este modelo computacional nos permitió realizar simulaciones estocásticas que reprodujeron la dinámica adaptativa observada experimentalmente, pero además identificamos un período donde ambas versiones del gen de resistencia coexisten a nivel intracelular y, por consiguiente, en la población.

Es decir, cuando una mutación ocurre en un gen del plásmido, la naturaleza multicopia del plásmido permite que ambas versiones del gen coexistan a nivel celular. Sin embargo, conforme el plásmido con el alelo mutante se esparce en la población mediante los procesos de segregación y replicación, surge una subpoblación de bacterias heterocigotas. A este proceso nos referiremos como heterocigosis mediada por plás-

midos (PMH, por sus siglas en inglés), un caso particular de heteroplasmia (Novick and Hoppensteadt 1978). La heterocigosis plásmidica es un fenómeno inherentemente inestable, ya que la ausencia de selección por uno de los plásmidos resultaría en su extinción debido a la deriva segregacional (Ilhan et al. 2019). Para estudiar PMH modificamos el plásmido pBGT intercambiando del gen de resistencia $bla_{\text{TEM-1}}$ por la variante $bla_{\text{TEM-12}}$ que confiere resistencia a ceftazidima (CAZ) y el gen de *gfp* por el gen *dsRED* de la proteína roja fluorescente DsRed, llamamos a este nuevo plásmido pBRT12 y renombraremos al ancestral como pBGT1 (para identificar la variante del gen *bla*). Con pBRT12 tenemos tres cepas distintas: la que solo porta el plásmido original (G1), otra que solo porta el plásmido modificado (R12) y una heterocigota que porta ambos plásmidos (HT).

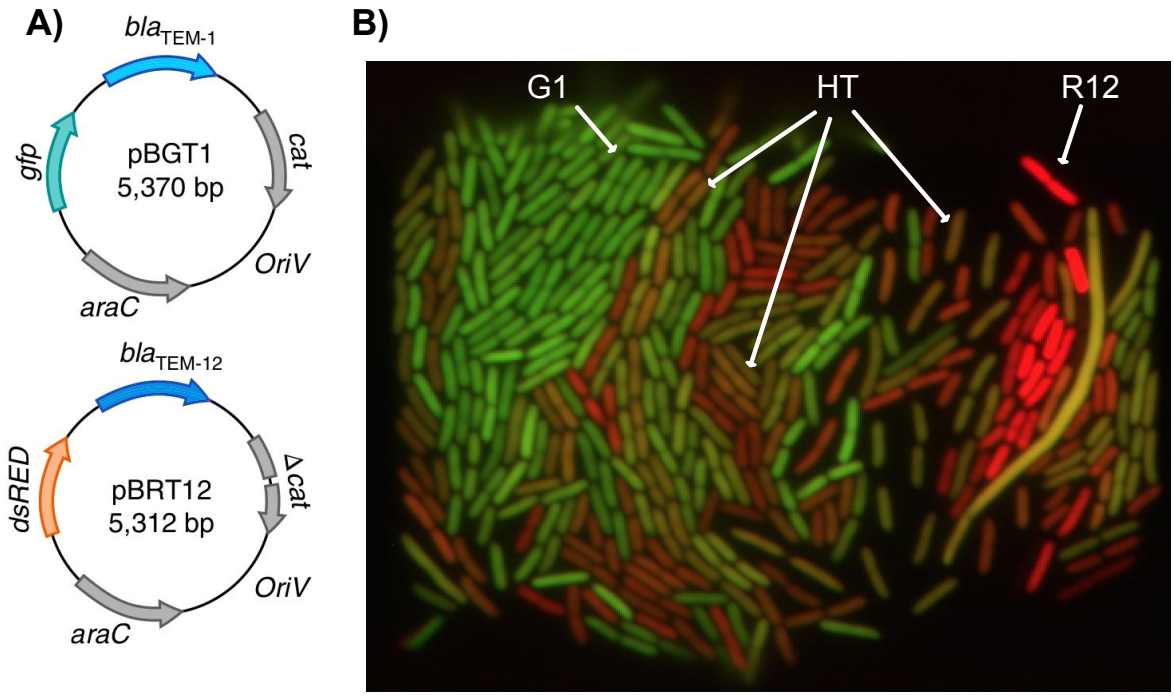

Figura 1.1: Sistema experimental PMH. **A)** Diagrama de plásmidos. **B)** Cepas construidas: G1 denota la cepa homocigota de plásmidos que sólo porta el plásmido pBGT1, R12 la homocigota que porta pBRT12 y HT la heterocigota que porta ambos.

En el Capítulo 5, utilizamos una serie de herramientas bioinformáticas para el análisis de datos de microscopía y citometría para cuantificar la dinámica de plásmidos, tanto a nivel intracelular como a nivel de poblaciones. El objetivo de nuestro estudio era evaluar cómo el efecto de distintas presiones de selección a favor de uno u otro plásmido modulan la distribución de alelos y, de esta forma, determinar las condiciones necesarias para estabilizar la PMH (Hernandez-Beltran et al. 2020).

En el Capítulo 6 exploramos las ventajas evolutivas asociadas a presentar la PMH mediante experimentos y modelado a nivel de poblaciones. El resultado principal de este estudio, publicado en *Nature Ecology and Evolution* (Rodriguez-Beltran et al. 2018), fue demostrar que presentar diversidad genética intracelular aumenta la plasticidad

fenotípica y la evolucionabilidad de la población. Asimismo, encontramos que presentar diversidad alélica a nivel intracelular resulta benéfico para una población respecto a presentar diversidad alélica a través de poblaciones mixtas compuestas por células homocigotas. Esto debido a que la PMH es capaz de mantener la diversidad alélica durante largos periodos de tiempo, permitiendo a la población contender con ambientes fluctuantes y promoviendo la redundancia genética necesaria para resolver compromisos evolutivos.

Capítulo 2

Modelación matemática de la ecología y evolución de plásmidos

2.1 Introducción

Los plásmidos han sido ampliamente estudiados debido a su capacidad de transferir genes horizontalmente, inclusive entre células de distinta especie. La conjugación, junto con la transducción (por bacteriófagos) y la transformación (incorporación de ADN desnudo del entorno), son los procesos a partir de los cuales los procariontes adquieren genes de forma horizontal (Rankin et al. 2012). A pesar del evidente beneficio asociado a transferir información genética horizontalmente, en ausencia de genes seleccionados positivamente, portar un plásmido implica una carga para su hospedero en términos de costo de adecuación con respecto a una bacteria libre de plásmidos. Esta carga se debe al mantenimiento del ADN plasmídico y a la expresión de proteínas codificadas en el plásmido y pueden resultar en la inestabilidad del plásmido en la población.

Por otro lado, aunque los plásmidos sean seleccionados positivamente, a largo plazo, los genes accesorios podrían incorporarse al cromosoma bacteriano. También existen plásmidos crípticos que no cuentan con ningún gen beneficioso para el huésped. En cualquier caso, pareciera que los plásmidos podrían ser redudantes y, al ser costosos metabólicamente, susceptibles a perderse de la población mediante selección purificadora. Por esta razón, los biólogos se preguntan: ¿cómo se mantienen los plásmidos en la naturaleza y en tanta diversidad?

Elucidar la paradoja de plásmidos representa un desafío para la teoría evolutiva y, por esta razón, muchos investigadores se han centrado en estudiar las condiciones ambientales y genéticas que garantizan la estabilidad de plásmidos en la población. Cabe señalar que este no es exclusivamente un problema importante para la biología evolutiva, sino también es relevante para la industria biotecnológica, debido a que los plásmidos son utilizados cotidianamente como vectores para introducir un gen en una bacteria para la biosíntesis de un producto específico. Otro motor para el estudio del comportamiento de los plásmidos en una población ha sido la aparición y propagación de la resistencia a los antibióticos, en el que los plásmidos juegan un papel clave transfiriendo genes de resistencia a cepas patógenas.

En las últimas décadas, se ha demostrado que el modelado matemático es una herramienta útil en el estudio de la dinámica de plásmidos en una población. La importancia de los modelos no sólo depende de su ayuda para comprender un proceso en particular; para que un modelo funcione, el modelo debe reproducir un comportamiento o ajustarse a un conjunto de datos experimentales. En este esfuerzo, se deben hacer

abstracciones y generalizaciones que puedan ayudar a explicar cómo está ocurriendo un proceso en particular. Pero la mayor contribución de los modelos es que pueden usarse para hacer predicciones que podrían probarse en condiciones de laboratorio u obtener datos biológicos o muestras que son difíciles o inclusive imposibles de obtener.

Los orígenes de la modelación de plásmidos se remontan al trabajo de (Stewart and Levin 1977), donde estudiaron la estabilidad de plásmidos conjugativos a través de un modelo de dinámica de poblaciones. Este modelo es utilizado para establecer condiciones suficientes para la estabilidad de plásmidos: la tasa de transferencia horizontal tiene que compensar por la pérdida segregacional y el costo asociado a portar el plásmido. En un trabajo posterior (Levin et al. 1979), el modelo es extendido para considerar plásmidos no-conjugativos, postulando que es necesaria una presión selectiva en favor de los plásmidos para asegurar su permanencia en la población.

Este modelo consiste en ecuaciones diferenciales ordinarias (EDO) que describen la dinámica poblacional que emerge entre subpoblaciones portadoras de plásmidos y subpoblaciones de células segregantes. La popularidad de estos modelos no sólo recae en su simplicidad, sino también a la diversidad de herramientas matemáticas que permiten encontrar soluciones analíticas y, por lo tanto, permiten caracterizar las propiedades dinámicas del sistema. También son relativamente simples de implementar computacionalmente y de esta forma obtener soluciones numéricas. Pero su mayor ventaja, es que se basan en la ley de acción de masas, lo cual permite a los modeladores simplificar los procesos biológicos y consecuentemente las ecuaciones.

Los modelos dinámicos que suponen la acción de masas son aptos para explicar experimentos en condiciones de laboratorio, por ejemplo en quimiostatos donde el medio está "bien mezclado". Sin embargo, esta suposición no necesariamente es válida en ambientes naturales, donde a menudo encontramos estructura espacial. Para modelar estos escenarios, se debe tener en cuenta la componente espacial del sistema y postular ecuaciones diferenciales parciales, las cuales son difíciles de analizar analíticamente y resolver numéricamente. Por esta razón, en escenarios con estructura espacial, las interacciones ecológicas entre poblaciones podrían entenderse mejor utilizando modelos basados en agentes individuales (Sørensen et al. 2005).

Este enfoque de modelación permite integrar características específicas individuales y, a medida que aumenta el número de procesos involucrados, también lo hace la complejidad computacional y de programación. No obstante, este enfoque se vuelve muy útil para estudiar interacciones ecológicas de comunidades espacialmente estructuradas. La ventaja de estos modelos es que pueden capturar de manera eficiente dinámicas poblacionales a partir de las interacciones de los individuos. Estas interacciones están definidas por propiedades que podrían variar o no entre individuos del mismo tipo, facilitando así la incorporación de ruido biológico como diferentes estados del ciclo celular o variaciones metabólicas.

Otra fuente de complejidad emerge a partir del ruido inherente a los sistemas de replicación y segregación de plásmidos. Por ejemplo, (Ponciano et al. 2007) utiliza modelos estocásticos para describir la dinámica de plásmidos a partir de implementar estrategias que incorporan ruido proveniente de los datos observados experimentalmen-

te. Esto promueve la expectativa de que este tipo de modelos se ajusten mejor para estudiar plásmidos cuando no se conoce mucho de su biología.

Es claro que el proceso de diseminación de plásmidos con genes de resistencia es altamente complejo ya que involucra distintas escalas temporales y espaciales, por lo que han surgido algunos nuevos enfoques de modelación que son capaces de integrar dichas escalas. En cada una de estas escalas podrían involucrar variables sociales incontrolables o procesos difíciles de implementar computacionalmente. Una alternativa para estudiar estos procesos multiescala es el uso aprendizaje de máquina donde los algoritmos computacionales optimizan modelos estadísticos entrenados una gran cantidad de datos bajo un objetivo específico. Con estos nuevos métodos se podrían discernir cuáles variables son de mayor relevancia, por ejemplo, en un brote infeccioso y plantear estrategias específicas (o generales) para controlarlos. Este enfoque de modelación ha sido utilizado para evaluar brotes de bacterias patógenas en ambientes clínicos, postulando modelos matemáticos y estadísticos que permiten la identificación de orígenes de las epidemias, así como de eventos de transferencia horizontal.

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Mathematical models of plasmid ecology and evolution

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Abstract

Mobile genetic elements - and in particular plasmids - have a profound effect on the ecology and evolution of microbial populations and therefore have been an important area of research in microbiology. From the onset, mathematical models of plasmid population dynamics have been used in combination with experimental microbiology to increase our understanding of the conditions enhancing plasmid stability, but also providing testable experimental predictions on the complex interaction between plasmids, bacterial hosts, and the environment. With plasmid-driven antibiotic resistance thriving and threatening to become a serious public health problem, it is necessary to make a critical assessment of the existing models describing the spread and maintenance of plasmids in bacterial populations, as well as to evaluate the strengths and limitations of different modeling approaches in the context of recent empirical evidence. In this review, we discuss theoretical models of plasmid dynamics that span from the molecular mechanisms of plasmid replication and segregation occurring in individual cells to their consequences in the ecological and evolutionary dynamics of complex microbial communities.

Introduction

Plasmids are self-replicating, extra-chromosomal DNA molecules widely distributed across bacteria. Plasmids regulate their own replication and copy number in the host cell, propagating vertically within the bacterial population. In addition, plasmids are also able to spread horizontally between different bacterial cells. There are different mechanisms available for the horizontal transfer of plasmids, but conjugation -plasmid transfer between a donor and a recipient cell through a conjugative pilus- is arguably the best-studied and most relevant one (Smillie et al. 2010). Crucially, conjugative plasmids encode all the information required to promote their self-transmission, being able to readily spread across bacterial populations.

Plasmids play a key role in bacterial ecology and evolution because they encode multiple accessory genes that help bacteria to adapt to new environments and stressful conditions (Norman et al. 2009). The repertoire of plasmid-encoded adaptive genes is extremely diverse, ranging for genes allowing bacteria to decontaminate heavy-metal polluted environments to others promoting the establishment of symbiotic relationships between bacteria and plants (Hall et al. 2015; Wang et al. 2018). From a human perspective, plasmids represent a concern for public health, because they encode important traits for pathogenic bacteria, such as virulence factors or antibiotic resistance determinants (Pilla and Tang 2018; Partridge et al. 2018). In fact, plasmids are the main drivers of the spread of antibiotic resistance genes among clinically relevant bacteria, and they have played a fundamental part in the evolution of the current global antibiotic resistance crisis (San Millan 2018).

The relevance of plasmids for bacterial biology and infectious diseases, coupled with the extensive use of plasmid-derived vectors for biotechnological and industrial purposes, have promoted the in-depth study of the molecular mechanisms controlling plasmids life cycle (del Solar and Espinosa 2000; Baxter and Funnell 2014; Ebersbach and Gerdes 2005). This body of work has been paralleled by the development of a wealth of mathematical models aimed at capturing the dynamics of plasmid biology. Mathematical models on plasmid biology are diverse and explore the different processes involved in the

life cycle of plasmids. These models have been fundamental for our understanding of different aspects of plasmid population biology such as, for example, how plasmids are stably maintained in bacterial populations. In this review, we explore the different models available in the field, and we discuss potential future directions for this exciting research topic.

Modelling plasmid replication

Although plasmids can be categorized by their physical structure or their functional properties, their main classification is based on their replication mechanism, as this defines the host range, its compatibility with other plasmids and the number of copies carried by each cell. But plasmid replication and copy-number control are complex processes and, therefore, modeling the molecular mechanisms that control plasmid copy numbers (PCN) within a cell has been an active area of research for decades. In particular for pSC101 and ColE1 plasmids, partly because of the relevance of different PCN in the dosage and stability of cloning vectors used for bioengineering.

Plasmid copy-number control

In 1980, (Nordström et al. 1980) assumed that *n* plasmids were produced in each cell cycle, regardless of the plasmid copy-number of the dividing cell. However, plasmid replication is a tightly regulated process (see (del Solar and Espinosa 2000) for a review on the biology of plasmid replication). For example, some ColE1 plasmids encode for a Rom/Rop (RNA one inhibition modulator/repressor of the primer) small protein dimer that promotes the formation of the kissing complex, thus controlling the upper plasmid copy-number limit. The same year, (Polisky et al. 1980) posed a model for ColE1 plasmid replication based on a control mechanism that recognizes the plasmid content of the cell and halts replication when a maximum plasmid copy-number is achieved, independently of the number of plasmids at birth.

Although precisely regulated, plasmid replication is also a stochastic process with events randomly distributed in time, and therefore can be modeled as a multitype branching process (Seneta and Tavaré 1983; Seneta and Tavaré 1982). (Nordström et al. 1984) introduced a Poisson distributed number of replications with average *n* in each cell cycle, while (Nordström and Aagaard-Hansen 1984) assumed that the probability of replication per unit time is constant throughout the cell cycle and independent of the number of plasmids. In (Bremer and Lin-Chao 1986), the authors modeled copy-number control of ColE1-type plasmids in terms of rate equations depending on cell mass, the replication inhibitor and a plasmid synthesis rate, while (Ataai and Shulert 1987) used an experimental system with different plasmid mutants to parametrize a model and evaluate numerically the average copy number presented by different populations. (Keasling and Palsson 1989) and (Keasling and Palsson 1989) proposed to study intracellular plasmid copy-number as a dynamical system, while (Brendel and Perelson 1993) viewed the copy-number control problem as an optimization process.

Different control mechanisms, hyperbolic and exponential, were analyzed by (Ehrenberg and Sverredal 1995) and formulated in terms of the master equation. In follow-up studies, Ehrenberg and colleagues evaluated the sensitivity of the control mechanism to the transcription frequency of the replication inhibitor (Ehrenberg 1996), as well as its half-life (Paulsson and Ehrenberg 1998) and regulatory efficiency (Paulsson and Ehrenberg 2001). A similar modelling approach based on using stochastic Petri nets to numerically approximate the solution of the master equation was followed by (Goss and Peccoud 1998). By combining Monte Carlo simulations with experimental data, (Kuo and Keasling 1996) explored the interaction between plasmid replication and cell cycle. More recently, (Münch et al.

2019) modeled plasmid replication with a saturating logistic function, while (Rodriguez-Beltran et al. 2019) considered that the probability of plasmid replication at a given time-point in the cell cycle can be expressed in terms of the current number of copies of the plasmid and a parameter representing the maximum number of plasmids cells can carry.

Other studies have studied the controllability and stability of plasmid copy-numbers to extrinsic and intrinsic perturbations. For instance, (Werbowy et al. 2017) studied fluctuations of the plasmid copy number within a population of cells and (Watve et al. 2010) showed that plasmid copy-numbers could be stabilized through population cycles between mutants with low, intermediate and high copy numbers. In (Kentzoglanakis et al. 2013), the authors argued that plasmid copy number control of R1 and ColE1 plasmids are controlled by two processes: a plasmid-borne antisense RNA acting as a policing agent, and the binding affinity of plasmid targets to those inhibitors, a term referred to as obedience. Plasmid replication rate was then modeled by an equation that incorporates a basal replication rate as a function of the concentration and affinity of the transacting replication inhibitor. Plasmid replication has also been shown to be temperature-sensitive through thermal denaturation of a repressor (Leipold et al. 1994). Using a temperature-dependant plasmid replication model, the authors showed that a low-copy plasmid (5-6 copies per cell) can be up-regulated to over 1,000 copies per cell through temperature shifts.

Dimer catastrophe

Another source of complexity of plasmid replication is the production of multimers through homologous recombination in high-copy plasmids, a process referred to as '*dimer catastrophe*' (Summers 1991; Summers et al. 1993). In (Field and Summers 2011), the authors used numerical simulations of a stochastic model of plasmid replication that considered copy-number variability to show that dimers over-replicate compared to monomers in ColE1 plasmids. In consequence, dimers impose a high metabolic cost to the host cell and therefore predicted that multimers should be observed in the population in reduced frequency, an observation validated with experimental data.

Multimerization can also enhance plasmid loss by reducing the units of inheritable plasmids. The formation of dimers was studied by (Paulsson and Ehrenberg 1998) using a mathematical model of ColE1 plasmid replication to argue that copy number variation in ColE1 plasmids is a consequence of the degradation rate of free RNA I, as well as the transcription frequencies of RNA II and RNA I. In consequence, segregational instability of high copy-number plasmids can be reduced by increasing the average copy number or improving replication control (and thereby reducing copy-number variation). In a follow-up paper (Paulsson and Ehrenberg 2001), the authors studied random fluctuations and regulatory efficiency in an inhibitor-dilution copy number control model of the bacterial plasmids ColE1 and R1, concluding that an increased sensitivity amplification due to inhibitor noise can reduce variability in plasmid copy number.

Despite the inherent complexity of copy-number control mechanisms, it is generally assumed by population dynamic models that plasmid replication occurs instantly and therefore the number of plasmids per cell is assumed to be in equilibrium (Bergstrom et al. 2000; San Millan et al. 2014; Cooper et al. 1987; Stephanopoulos and Lapudis 1988; Hsu and Tzeng 2002). This assumption implies that, at the moment of division, all cells in the population exhibit, on average, a fixed number of plasmid copies. In consequence, population-level models based on ordinary differential equations (ODEs) usually describe plasmid loss via segregation as a transition between plasmid-bearing and plasmid-free

compartments occurring at a rate determined by the mean plasmid copy-number of the population. This is, of course, a simplification, but one that is compatible with the resolution of experimental protocols designed to estimate mean plasmid copy-numbers in bacterial populations, either based on flow cytometry (Bahl et al. 2004) or quantitative PCR (Lee et al. 2006).

Plasmid incompatibility

Plasmid incompatibility (also known as plasmid speciation) results from the inability of a plasmid replication mechanism to maintain enough copies to ensure its stability in the presence of other plasmids. In a seminal study, (Novick and Hoppensteadt 1978) proposed a model for the stability of two incompatible plasmids and compared regular replication (for each type of plasmid) with random replication (from a common pool), finding that incompatibility is inevitable and that segregational loss occurs faster when replicating from the random pool. The model was then used to explore the genetic mechanisms that determine plasmid incompatibility, namely the recognition specificity of the origins of replication and partitioning systems, as well as the specificity of the copy-number control mechanism.

Incompatibility was believed to be related to the replication mechanism until (Novick and Schwesinger 1976) showed, using a simple combinatorial model of the plasmid partition process, that other genetic mechanisms could also drive incompatibility between autonomous plasmids. (Ishii et al. 1978) explored this hypothesis using a model for two related plasmids that replicate from a common pool with equal but random segregation, concluding that the number of copies carried by each cell determines the number of generations required to generate single-plasmid strains from a heterozygote parental cell. Later, using a simple model for the probability of plasmid loss, (Nordstrom and Austin 1989) showed that the stability of plasmids can be reduced through plasmid clustering and dimer formation, although it can also be enhanced through active partitioning systems that ensure symmetric partition of plasmids upon division. Plasmid incompatibility was also studied by (Sýkora 1992), modeling the formation of cointegrates to show that selection is relaxed on one *rep* gene, promoting the generation of a new *rep* version with new incompatibility specificity. Other mechanisms involving partition systems that lead to incompatibility are discussed in a mini-review (Bouet et al. 2007), with an emphasis in plasmids sharing the same centromere and when one (or both) plasmids partition randomly or form clusters.

In (Hyland et al. 2014), the authors evaluated two related plasmid par systems to show that cross-interactions between them can produce incompatibility. Their experimental system consisted of a resident (low-copy) and a challenging (high-copy) plasmid, and used a first-order kinetics-based model to study the *parC*-ParR association and dissociation reactions for each and cross plasmids elements. The authors then performed stochastic simulations using a Gillespie algorithm to show that plasmids are compatible only when one of the plasmids is Par-. This study also showed that molecular cross-interaction between ParR and *parC* can result in inaccurate plasmid segregation. More recently, (Gama et al. 2020) adapted the models postulated in (Stewart and Levin 1977), (Levin et al. 1979), and (Simonsen 1991) (discussed below) to incorporate multiple interacting plasmids in order to evaluate how the interaction between plasmids impacts the plasmid persistence in bacterial communities. The main result of this study is to postulate a hierarchy in the interaction variables, arguing that epistatic interactions between plasmids produce a stronger impact on plasmid maintenance than other parameters influencing conjugation and plasmid loss.

Modelling plasmid segregation

A consequence of segregational loss is that plasmids do not always persist in bacterial populations in the absence of selection for the traits they encode. Indeed, if the plasmid carries a beneficial gene, then plasmids can be stably maintained in the population through positive selection, although there exists a probability higher than zero that plasmids can be lost at the moment of division. So, if plasmid-free cells have a competitive advantage over plasmid-bearing cells, then the previous increase in frequency in the population and render the plasmid susceptible to being lost through purifying selection.

Molecular mechanisms enhancing plasmid stability

A number of mechanisms are known to circumvent segregational instability in plasmids under non-selective conditions (Salje 2010). Of note, active partition mechanisms, whereby a plasmid-specific DNA sequence segregates plasmid symmetrically upon division, and post-segregational killing (PSK) mechanisms based on toxin-antitoxin systems (TA) that prevent the emergence of plasmid-free cells through the production of a long-lasting toxin and a shorter-lived antitoxin, both encoded on the plasmid. Therefore, if the cell carries the plasmid, antitoxin is produced and the toxin is neutralized, but if a plasmid-free cell emerges in the population, the antitoxin is degraded rapidly and the toxin kills the cell, thus producing a population composed exclusively of plasmid-bearing cells. The evolution of PSK mechanisms appears to be paradoxical because of the detrimental effect they produce on host-cell populations, although theoretical models have shown that PSK can be advantageous for conjugative plasmids (Mongold 1992) or in the presence of competing for genetic elements in spatially structured environments (Mochizuki et al. 2006).

In general, models that include PSK mechanisms consider that segregant cells simply do not produce viable plasmid-free cells (Bergstrom et al. 2000), although a few studies have explicitly modeled plasmid addiction systems. For instance, (Fedorec et al. 2019) extended a plasmid-loss model (Boe et al. 1987) to include PSK by a TA system and evaluate the stability of plasmids as a function of PSK efficacy and the metabolic burden associated with encoding PSK mechanisms. The authors also model another PSK mechanism: the production of bacteriocins that kill plasmid-free populations at a constant rate, instead of exclusively killing segregant cells that have recently lost the plasmid through segregation and, by using Bayesian inference, estimate parameters for PSK efficacy, plasmid loss rates and growth rate differences between plasmid-free and plasmid-bearing strains. Similarly, (Loftie-Eaton et al. 2016) used a Bayesian Information Criterium applied to a population dynamics model to evaluate plasmid loss and fitness costs associated with specific mutations on a putative toxin-antitoxin and a cointegrate resolution system identified after evolving *Pseudomonas sp.* for 1,000 generations in the absence of selection. Interestingly, as evolved plasmids were able to persist for longer in multiple bacterial hosts, mutations in the TA system also resulted in an expanded plasmid host range.

Random segregation of multicopy plasmids

Plasmid addiction systems and the molecular machinery necessary for conjugation are backbone-encoded functions that require several genes and therefore are mostly found in large plasmids (>25 kb) present in low copy-numbers (1-20 copies per cell) (Smillie et al. 2010). As a result, small plasmids lacking active partitioning and post-segregational killing mechanisms can only be

maintained in the population by being present in a large number of copies in each cell, therefore reducing the probability of producing plasmid-free cells when segregating plasmids during cell division.

To model plasmid segregation, (Nordström et al. 1984; Seo and Bailey 1985; Boe et al. 1987) assumed that plasmids are distributed to daughter cells randomly at cell division and that all cells contain N plasmids at division. In contrast, (Müller et al. 1991) posed a Markov chain model whereby segregation and replication of plasmids were modeled as stochastic processes. In general, it is assumed that random segregation of plasmids upon division can be described with a binomial distribution, $B(n,p)$, where n is a variable denoting plasmid copy-number of the mother cell and p the probability of success. If we assume there is an equal probability for each plasmid to be inherited to each daughter cell we obtain $p = 0.5$ and, therefore, the probability of producing a plasmid-free cell is $2^{(1-n)}$ (Summers 1991; Greenhalf et al. 1989). This is a convenient simplifying assumption that enables population dynamic models to describe the segregational loss in terms of a constant rate that depends on the density of plasmid-bearing cells and the mean plasmid copy number in the population (Stewart and Levin 1977; Cooper et al. 1987; Bergstrom et al. 2000; Paulsson and Ehrenberg 2001; De Gelder et al. 2004; Ponciano et al. 2007; San Millan et al. 2014).

Asymmetric partitioning

High-resolution microscopy studies have shown that high-copy plasmids are mainly found in the poles due to displacement by the nucleoid, but migrate inside the cell throughout the cell cycle and therefore segregate randomly upon division (Reyes-Lamothe et al. 2014), a feature consistent with the assumption that multi-copy plasmid segregation can be described as a Poisson process. However, (Hsu and Chang 2019) showed that plasmids can be localized in spatial clusters inside the cell and therefore segregation of plasmids between daughter cells can be asymmetric ($p \neq 0.5$). The authors posed an impeded segregation model whereby plasmids are spatially confined in the intracellular environment, with the aim of showing that segregation in high copy-number plasmids can deviate from the standard random segregation model.

There are other mechanisms that can result in the asymmetric segregation of plasmids (Million-Weaver and Camps 2014). As previously discussed, plasmids do not always replicate as monomers, so the probability of producing a plasmid-free cell also depends on the degree of multimerization of the plasmid (Summers 1991). (Ayala-Sanmartín and Gómez-Eichelmann ...) showed that ColE1-like plasmids that are present in the population mainly as dimers present greater stability than predicted by a random partitioning model of monomers. More recently, (Münch et al. 2019) argued that, while low-copy plasmid bacteria exhibit symmetric segregation, in high copy plasmids one of the daughter cells receives more plasmids, resulting in unequal segregation. By maximizing the average fitness of the population, the authors argued that asymmetric segregation of plasmids could result in an evolutionary stable strategy.

Modelling plasmid cost and segregational instability

Despite the potential benefits associated with plasmid-bearing, mobile genetic elements can also be associated with a fitness burden in the absence of positive selection for plasmid-encoded genes. For instance, if the plasmid encodes for genes that increase the probability of survival in stressful environments (e.g. antibiotics or heavy metals), then carrying plasmids can be detrimental for the host if the stressor is removed. There are a variety of reasons why plasmids can generate a high physiological

burden on the host cell (San Millan and MacLean 2017), for instance, plasmid gene products can inhibit cell division until sufficient plasmid copies are available (Nordström 1985), or drain the cell's resources through replicating additional DNA (Glick 1995) and increasing protein synthesis (Rozkov et al. 2004).

Most population-dynamic models express the fitness burden of carrying plasmids in terms of the relative growth difference between plasmid-free and plasmid-bearing sub-populations. For instance, as a constant rate that multiplies the growth function of the sub-population carrying the plasmid by a factor less than one (Simonsen et al. 1990; Baker et al. 2016) or, if bacterial growth is modelled as hyperbolic growth function, by appropriately selecting the parameters of the Monod-type growth function (Levin et al. 1979; San Millan et al. 2014; Stephanopoulos and Lapudis 1988; Alonso-del Valle et al. 2020). Another possibility is to consider that, from one generation to the next, the number of plasmid-carrying cells doubles from the previous generation (minus the fraction lost through segregation), so the growth rate of the plasmid-free sub-population can be modelled as $2^{1+\sigma}$, where σ denotes the fitness advantage of not carrying the plasmid (De Gelder et al. 2004; Joyce et al. 2005; Ponciano et al. 2007). In (Lau et al. 2013), the authors propose an age-structured model and use it to propose a method for accurate estimation of plasmid-free frequencies based on the probability of plasmid loss per cell and division as well as the subsequent growth of segregant cells. Recently, (Reding 2020) argued that the cost of plasmid-bearing can also be expressed in terms of an increase in lag-phase, with significant differences in the resulting population dynamics.

In any case, plasmid costs are usually considered to be fixed values inherent to each plasmid-host association. However, (Ganusov and Brilkov 2002) argued that a constant reduction in growth rate is not realistic when modelling continuous culture devices, as the selection coefficient is a dynamic property of the system with changes in substrate concentration resulting from diluting plasmid-bearing cells from the system. Similarly, (Ponciano et al. 2007) showed that environmental variability results in a dynamic plasmid burden. By representing the growth rate of the plasmid-free subpopulation as a random variable, the authors were able to capture experimental time-series of plasmid frequencies with higher accuracy than when considering that fitness cost is constant throughout the experiment.

This complex interaction between plasmids, hosts and the environment produces a distribution of fitness effects that can result in increased stability of plasmids in polyclonal microbial communities. In a recent manuscript, (Alonso-del Valle et al. 2020) compared computer simulations of a population dynamics model with pair-wise competition experiments between plasmid-bearing and plasmid-free isolates obtained from clinical samples. Both computational and experimental data presented a wide distribution of fitness effects characterized by mostly neutral effects, but with a right-hand tail expanding towards positive fitness effects.

Population dynamics of plasmid-free and plasmid-bearing populations

In a seminal study (Stewart and Levin 1977), the authors posed a simple ODE model to study the population dynamics of two sub-populations: plasmid-free and plasmid-bearing. The main contribution of this work was to identify a broad range of parametric conditions whereby plasmid-bearing populations can be maintained in the population at high frequencies, even in the absence of selection favoring the genes they carry. Moreover, the authors derived an expression - now referred to in the literature as the *Stewart-Levin criterion* - that determines the equilibrium frequencies of plasmid-bearing cells in terms of key modeling parameters: population growth, conjugational transfer, and segregation rate. In a follow-up study (Levin and Stewart 1980), this model was extended to consider

non-conjugative plasmids and found that the range of conditions that stabilize plasmids are more stringent, therefore concluding that periods of positive selection would be necessary for maintaining costly, non-conjugative plasmids in a population, a result that was later formalized in (Macken et al. 1994).

These results were then followed by two theoretical studies published the same year (Lenski and Bouma 1987) and (Cooper et al. 1987), with both models also based on a system of ordinary differential equations describing changes in the frequency of plasmid-bearing cells from the rate of segregational loss and the selective disadvantage of carrying plasmids. (Cooper et al. 1987) showed that the frequency of unstable microbial populations (in this case, plasmid-bearing cells) decreases exponentially in the absence of selection, while (Lenski and Bouma 1987) obtained an expression that establishes that the effect of selection is higher when the initial plasmid frequency in the population is low. By comparing their model predictions with an experimentally-determined time-series of plasmid frequencies, the authors showed that segregation and selection should be considered simultaneously when addressing the causes of plasmid instability.

In another important study, (Stephanopoulos and Lapudis 1988) analyzed the stability of plasmid-bearing cells growing in a chemostat using the index theory of a singular point in systems of ODEs. The model is constructed under the following assumptions: a mixed population consisting of parental (plasmid-bearing) and negative (plasmid-free) cells, with parental cells producing negative cell variants at a constant rate, while negative variants cannot revert to produce parental cells. An important feature of this model is that the system of ODEs contains an expression for substrate concentration present in the environment, allowing the authors to perform stability analysis and demonstrate the existence of a range of substrate concentrations that allow the co-existence of both sub-populations.

More recently, (Yurtsev et al. 2013) explored theoretically and experimentally the population dynamics of a co-culture of bacteria composed of plasmid-free cells and cells bearing a multicopy plasmid encoding for a drug-resistance gene. By measuring changes in the fraction of resistant and sensitive bacteria in a one-day growth cycle exposed to a lethal dose of antibiotics, the authors showed that the relative fraction between plasmid-free and plasmid-bearing subpopulations reaches an equilibrium. A mathematical model based on difference equations showed that the equilibrium-resistant fraction depends exclusively on the initial cell density and the antibiotic concentration. As expected, the fraction of plasmid-bearing populations increased proportionally to the strength of the selective pressure.

Altogether, these population dynamic studies highlight the existence of two important physiological parameters involved in the generation of plasmid-free cells in a population that composed initially of cells carrying plasmids: growth rate differences between both sub-populations, and the rate at which plasmid-free cells are generated from plasmid-bearing cells. As the previous depends on the environmental conditions, it is theoretically possible to find selective regimes whereby plasmid-bearing cells grow faster than plasmid-free cells, thus stably maintaining plasmids in the population.

There is, however, an apparent paradox associated with frequently beneficial genes: if a plasmid carries genes that confer a benefit for the host, the cell could, in principle, integrate these genes into the chromosome, thus rendering the costly plasmid redundant and susceptible to be lost through segregation. As a result, (Bergstrom et al. 2000) argued that plasmids cannot persist exclusively by carrying genes that are beneficial to their hosts and postulated that plasmids can only persist indefinitely through a series of selective sweeps resulting from transferring to locally adapted

populations with higher fitness, a process termed as the '*hitchhiking hypothesis'.* However, a detailed analysis of this model showed that the conditions for a parasitic plasmid to persist are less stringent than originally thought, showing that plasmids can be maintained indefinitely through oscillations between plasmid-free and plasmid-bearing populations (Lili et al. 2007).

Plasmid stability in fluctuating environments

In any constant environment, the Stewart and Levin criterion states that high rates of horizontal gene transfer (HGT) can maintain plasmids in the population, even when bearing them is associated with a significant fitness cost. But an exhaustive analysis of genomic sequences showed that a large fraction of plasmids found in nature is not mobilizable (Smillie et al. 2010). If plasmids cannot transmit horizontally and the environment does not select for genes encoded in the plasmid, then they can only be maintained in the population by compensating plasmid costs, reducing segregational loss (e.g. increasing plasmid copy-numbers or through plasmid addiction systems) or through sporadic intervals of positive selection.

To study the environmental conditions that maintain plasmids in the population, a series of articles have used ODEs to describe the population dynamics model of plasmid-free and plasmid-bearing cells competing for resources in fluctuating environments. For instance, (Song et al. 2006) proposed a within-host population dynamics model of plasmid-bearing and plasmid-free subpopulations competing for resources in the presence of an inhibitor, while (Svara and Rankin 2011) explored the range of selective pressures that favor plasmid-carried antibiotic resistance genes, focusing on evaluating the effect of different antibiotic doses and the interval between treatments has on plasmid dynamics.

Also, (Yuan et al. 2011) evaluated plasmid stability in a competition model growing in a chemostat in the presence of a substrate and an inhibitor deployed in periodic pulses, showing that the dilution rate and the periodicity of drug input are critical to conditions for the stable coexistence of both sub-populations. More recently, (Rodriguez-Beltran et al. 2018) used stochastic simulations to show that a high rate of environmental fluctuations was correlated with an increase of intracellular genetic diversity, a prediction that was subsequently validated using an experimental model system showing that stochastic environments balance selection for both ancestral and evolved alleles, therefore increasing the probability of survival in environments that alternate selection for both alleles.

Modelling horizontal transmission of plasmids

Although intermittent intervals of positive selection, either in space or in time, could result in enhanced stability of plasmids (Eberhard 1990), selection itself is not sufficient to explain the abundance of plasmids in natural environments. For instance, as an extreme case, cryptic plasmids are ubiquitous in nature although they carry no beneficial genes and thus are never positively selected for. Therefore, another strategy to increase plasmid stability is to overcome the negative demographic effects of segregation and purifying selection by transmitting horizontally into neighbouring plasmid-free hosts. This represents an important problem for the dissemination of antibiotic resistance genes and therefore has been of interest for clinicians (Carattoli 2013) and mathematical modelers (Leclerc et al. 2019).

Horizontal transmission of mobile genetic elements is mediated either via conjugation, transformation or transduction (Smillie et al. 2010), and therefore plasmids can be classified as conjugative (when encoding mechanisms for self-transfer) or non-conjugative (if they are incapable of initiating conjugation for self-transmission). As with other forms of horizontal gene transfer, conjugation has been associated

with promoting evolutionary and ecological innovation, by conferring new phenotypic traits and access to novel ecological niches (Wiedenbeck and Cohan 2011), although it can also be detrimental when selfishly replicating mobile genetic elements do not carry beneficial genes (Vogan and Higgs 2011). Crucially, conjugation can occur between taxonomically distinct bacterial lineages (Fernandez-Lopez et al. 2017), thus confounding phylogenetic relationships and rapidly spreading beneficial genes between members of microbial communities.

Mass action assumption

It has been established that the dynamics of horizontal transfer differ between populations on surfaces and in liquids (Molin and Tolker-Nielsen 2003) but, as most articles studying theoretical population dynamics of plasmids focus on either chemostats or batch culture experiments (i.e. dense cultures in well-mixed environments), conjugative plasmid transfer between donor and recipient cells is generally assumed to occur randomly at a frequency that is jointly proportional to their respective densities (Freter et al. 1983; Stewart and Levin 1977; Simonsen et al. 1990; Clewlow et al. 1990; Macdonald et al. 1992; Smets et al. 1993; Bergstrom et al. 2000; Imran et al. 2005; Svara and Rankin 2011; Peña-Miller et al. 2015; Lehtinen et al. 2020). Indeed, as conjugative transfer imposes a cost to bacterial hosts, the rate of vertical transmission decreases and therefore a trade-off between horizontal and vertical transmission is produced, resulting in higher rates of HGT when plasmid-free cells are present in abundance (Turner et al. 1998).

A consequence of assuming mass action kinetics in the horizontal transmission of plasmids is that HGT only depends on the densities of donor and recipient cells, with conjugation events occurring at a constant rate. However, (Lundquist and Levin 1986) showed that newly formed transconjugants can transiently promote conjugative pili synthesis, resulting in an increased rate of horizontal transfer, while (Levin et al. 1979) fitted a mass action model to experimental data and observed that a constant rate of transmission was only appropriate in stationary phase, thus concluding that plasmid transmission is accelerated during lag phase. (Simonsen et al. 1990) found that the initial donor/recipient ratio and the occurrence of a lag phase have no appreciable influence in the estimation of transfer rates, thus arguing that the rate of horizontal transmission remains constant throughout an experiment.

In contrast, (Ponciano et al. 2007; De Gelder et al. 2007) considered that, as HGT depends on the fraction of donor cells, therefore conjugation rate initially increases steeply as a function of the available recipient and donor cells but subsequently levels off to a saturation limit. Similarly, (Volkova et al. 2012) argued that the rate of conjugation is frequency-dependent and (Philipsen et al. 2010) used stochastic differential equations and a maximum likelihood method to argue that conjugation rates are substrate-dependent that can be described by a sigmoidal function. Transmission rates of some plasmids can also be affected by cell density through quorum-sensing signals (Miller and Bassler 2001), but also by external cues, such as antimicrobial substances (Beaber et al. 2004). However, (Lopatkin et al. 2016) combined a plasmid transmission model with single-cell observations to show that sublethal concentrations of antibiotics do not significantly increase the conjugation efficiency and therefore the contribution of antibiotics to the promotion of HGT may have been previously overestimated.

The interaction between the intensity of positive selection with the rate of environmental change and of horizontal transmission was studied in (Peña-Miller et al. 2015), using computer simulations to show that conditions for the maintenance of a non-transmissible plasmid would be very stringent, therefore

suggesting that apparently non-transmissible plasmids may still experience episodes of horizontal transmission occurring at very low frequencies and that these scattered transmission events are sufficient to stabilize parasitic plasmids in the population. Moreover, for conjugative plasmids, (Lopatkin et al. 2017) showed that plasmids can transfer at sufficiently high rates to be maintained in the absence of antibiotics, thus concluding that reducing antibiotic use alone would not reverse resistance from the population.

For conjugation to occur in well-mixed environments, the plasmid-bearing and plasmid-free cells first need to collide, then attach, and then successfully conjugate before detachment occurs. The mass-action kinetic assumption of most mathematical models of plasmid transfer combine these processes into a single conjugation rate, implicitly ignoring the physiological state of donor and recipient cells and considering that horizontal transmission of plasmids is an instantaneous process. To overcome these limitations, (Massoudieh et al. 2007) proposed a model for conjugative plasmid that included time lags resulting from reversible attachment–detachment of bacteria in a one-dimensional porous environment. Similarly, (Zhong et al. 2010) postulated an ODE model that explicitly considered attachment and detachment dynamics of neighbouring cells, assuming that conjugation can only occur between attached cells. This assumption allowed the authors to explore the differences in the dynamics of plasmid transfer between spatially structured and well-mixed environments.

Stochastic models of horizontal gene transfer

Although mass-action models have been very useful to study the spread and persistence of conjugative plasmids in well-mixed environments (e.g. liquid cultures), the implicit limitations resulting from using ordinary differential equations limits the study of the stochastic interactions between plasmid-bearing and plasmid-free cells. In (Novozhilov et al. 2005), the authors posed a stochastic model to show that horizontally-acquired plasmids can be maintained for long intervals of time in populations when horizontal transmission is comparable to segregational loss, even when the acquired genes are neutral or deleterious. Similarly, (Ponciano et al. 2007) used a stochastic model to argue that relative fitness of plasmid-free cells is considered a random variable and thus changes in plasmid frequency can be understood as a Markovian process, an approach also followed by (Ledda et al. 2020) to study the spread of OXA-48, a plasmid responsible for hospital outbreaks of carbapenem-resistant Enterobacteriales.

Other modeling approaches have also been proposed. For instance, (Tazzyman and Bonhoeffer 2013) studied the fixation probability of a gene that can be horizontally transferred using two modeling approaches: a branching process and a diffusion approximation. By considering a fixed population size and a low initial frequency of mutants, the model predicts a trade-off between horizontal and vertical transmission that results in deleterious alleles having a non-zero probability of fixation, even in the absence of positive selection. In (Billiard et al. 2016), the authors considered two clonal populations of haploid individuals each carrying a different ‘trait', which can be interpreted as a conjugative plasmid. By modeling horizontal transfer as a stochastic process, the authors demonstrated stable coexistence of the traits and then evaluated the magnitude of the fluctuations around the deterministic solution by constructing an Ornstein-Uhlenbeck process to which solution is a stochastic differential equation with Brownian motions related to the birth-death processes of each population and to the horizontal transfer.

Individual-based models of plasmid transmission

As plasmid transfer by conjugation requires physical contact between donor and recipient cells, spatial structure plays an especially important role for the dynamics of horizontal gene transfer (see (Slater et al. 2008) for a review on spatial factors modulating plasmid transmission). However, most studies focus on well-mixed environments and ignore the spatial component of the system. To overcome this limitation, (Beaudoin et al. 1998) posed a model based on partial differential equations (PDE) to study plasmid mobilization in a 1-D biofilm (spatial structure is expressed in terms of the depth of the biofilm). In principle, this PDE approach could be extended to two- or three- dimensional domains, although it would be very difficult to analyze and, as a result, individual-based models (IBM) have been used by multiple studies to study the conjugative transfer of plasmids in spatially-explicit environments.

In short, IBMs assume that each individual is described by a vector of state variables that is updated in response to rules based on local environmental conditions and the state of neighboring cells. This framework has been used successfully to study competitive and cooperative microbial interactions in space and time (Hellweger et al. 2016; Kreft 2014). In the context of HGT, (Lagido et al. 2003) proposed a simple kinetic model of plasmid transfer within microcolonies growing in solid surfaces that assumed that cells are inoculated randomly in a 2-D domain, thus producing circular colonies with a radius that increase exponentially in time until resources are locally exhausted. If a colony of plasmid-bearing cells encounters a colony of plasmid-free cells, the model considers that conjugation occurs and all cells in the recipient colony become transconjugant.

Another IBM used to study the transmission of mobile genetic elements is known as COSMIC (Gregory et al. 2008). The aim of this model was to simulate bacterial evolution in a multi-scale perspective in the sense of incorporating genes, gene products, and their interaction with the environment. This approach is based on HERBY (Devine and Paton 1997), a discrete grid-based environment of plants with herbivores populating the grid, but can also be applied asexual populations. An extension of this model, RUBAM (Vlachos et al. 2004), was developed to simulate adaptive behavior using grid-based environments with nutrient and antibiotic gradients. By integrating COSMIC and RUBAM, (Gregory et al. 2006) proposed COSMIC-Rules, a computer program that works at three organizational levels: genes, cells, and environment, and that can be used to study the transmission of plasmids in a bacterial population. COSMIC-Rules was used by (Gregory et al. 2008) to model conjugational transfer based on the proximity between donors and recipient cells (both moving as a weighted random walk) and their metabolic state (expressed by considering that transconjugant cells have a waiting time before they can act as plasmid donors). Using this approach, the authors showed that plasmid incompatibility is a limiting factor for the spread of plasmids, while compatible plasmids can spread throughout the population and be maintained even in the absence of selection.

Another approach that has been used to study plasmid transfer and persistence in spatially structured bacterial populations is based on discrete-space continuous-time stochastic models (also known as interacting particle systems). Of note, (Krone et al. 2007) used a 2-D square lattice with periodic boundaries, whereby each location of the lattice can contain nutrients, as well as recipient, donor or transconjugant cells. Growth of each cell depends on the concentration of resources in the neighbourhood, while conjugation events occur only between neighbouring donor (or transconjugant) and recipient cells. Using computer simulations, the authors showed that the resulting spatial patterns of plasmid loss and horizontal transfer are consistent with those observed experimentally. Similarly, in (Merkey et al. 2011), an IBM that explicitly considers cells, plasmids and extracellular polymeric

substances was to study plasmid transmission in bacterial biofilms. In this model, segregation and fitness costs, but also conjugation parameters (for example, lag times and pilus reach distance and scan speed) are considered to be dependent on the growth rate of the donor cell. The authors used sensitivity analysis to show that timing and distance between neighbors are important drivers of conjugal plasmid transfer in biofilms, while segregational loss rate and growth rate of the receiver subpopulation are not so relevant.

More recently, (Werisch et al. 2017) evaluated the difference between non-transmissible and transmissible plasmids in biofilms modeled using a lattice-based IBM. In this computational model, conjugation occurs randomly between neighboring cells, while segregational loss and incompatibility occur when both types of plasmids are present in the same cell (incompatible plasmids are unable to persist in the same cell due to cross-reactions between replication control mechanisms). The main conclusion of this study is that non-transmissible plasmids that provide no advantage to the host can still be maintained in the population in co-occurrence with incompatible conjugative plasmids. In (van Dijk et al. 2020), the authors used an IBM to study a population of bacterial cells carrying (or not) a slightly beneficial gene, with the aim of showing that HGT can only evolve if horizontal transmission occurs within spatially localized populations, instead of under well-mixed conditions.

Modelling evolutionary dynamics of plasmid-bearing bacterial populations

As plasmids encode for genes that control their own replication and transmission, it has been argued that they should be viewed as evolving agents subject to natural selection in their own right, with fitness interests that are not necessarily aligned with those of their bacterial host (Harrison and Brockhurst 2012). As a result, the evolutionary dynamics of plasmid-host associations can be understood as a co-evolutionary process, where both conflict and collaboration between bacteria and plasmids can occur. Indeed, it was recently shown that co-evolution between plasmids and hosts promoted the stability of such plasmids by reducing fitness costs associated with plasmid-bearing (Jordt et al. 2020). Interestingly, computational experiments performed using a Gillespie algorithm to simulate a population-dynamics model suggested that compensatory mutations that increase plasmid persistence in one host simultaneously improved plasmid stability in a different host, thus maintaining multidrug resistance for a longer duration if plasmid-host coevolution had occurred. Moreover, (Loftie-Eaton et al. 2016) used experimental evolution and population dynamics modelling to show that evolved plasmids were more stable in their co-evolved hosts than in the ancestral strain due to epistatic interactions that emerged during plasmid-host coevolution.

Fitness cost compensation

Theoretical studies discussed so far suggest there are two key evolutionary routes to increase plasmid stability in the long-term: (1) the evolution of high conjugation rates that allow plasmids to be maintained through horizontal transmission, and (2) compensatory evolution that reduce the cost of plasmid carriage and therefore weakens selection against plasmids. Indeed, experimental evolution studies have shown that selection for horizontal transmission can lead to the evolution of increased conjugation rate in plasmids (De Gelder et al. 2008), but also to the amelioration of plasmid burden (San Millan et al. 2014), with both strategies resulting in an enhancement of the ecological conditions favoring plasmid persistence.

To contrast these evolutionary strategies, (Harrison et al. 2016) used an individual-based model to simulate the evolutionary dynamics of a very costly mega-plasmid with a mercury resistance cassette

carried on a transposon. The model explicitly considers the appearance of compensatory mutations and the transposition of the resistance gene into the chromosome, with numerical simulations showing that plasmid cost compensation can enhance plasmid stability, but also promote the fixation of accessory traits on the bacterial chromosome. In a follow-up study (Hall et al. 2017), the authors used this individual-based model to argue that, due to the inherent costs of increasing conjugation rate, amelioration of plasmid costs is the most likely long-term solution to evolving stable bacteria-plasmid associations.

This result is consistent with a previous study evaluating the effect of compensatory mutations in the stability of plasmids of a costly non-conjugative plasmid (San Millan et al. 2014). By performing a serial dilution stability assay in the absence of selection, the authors found that, after an initial period of exponential decay, the plasmid appeared to stabilize at a low frequency. Competition experiments between the evolved and ancestral strains showed that the cost associated with plasmid-bearing had been substantially reduced in the evolved strains, a feature thereafter included into a population dynamics model to describe the emergence of a sub-population with a reduced fitness cost. In this case, whole-genome sequencing of both plasmids and bacterial hosts confirmed that compensatory mutations were present exclusively in the chromosome (San Millan et al. 2015).

In (De Gelder et al. 2007; Ponciano et al. 2007), the authors compared two models, one with a constant fitness cost and another with a selection coefficient represented as a random variable sampled at every time point from a Normal distribution. The latter model was subsequently used to evaluate if the stability of a self-transmissible plasmid was due to changes in conjugation rate, segregation rate, or plasmid cost. By jointly estimating these parameters and analyzing data from plasmid persistence and competition experiments, (Loftie-Eaton et al. 2016) concluded that plasmid maintenance was improved by the appearance of chromosomal mutations that turned a plasmid cost into a benefit, a theoretical result that was subsequently validated by sequencing the evolved strains.

Another aspect influenced by compensatory chromosomal mutations are pleiotropic effects between different plasmids. Using a combination of mathematical modelling and experimental evolution, (Loftie-Eaton et al. 2017) showed that co-evolution between plasmid and their hosts also not only increased plasmid stability but also enabled mutant cells to retain different plasmids, a feature referred to as plasmid permissiveness. To further evaluate the consequences of genome localization of compensatory mutations in the resulting plasmid dynamics, (Zwanzig et al. 2019) analyzed an ODE model to argue that compensatory mutations occurring in plasmids (spreading both vertically and horizontally) can promote plasmid persistence even when the amelioration effect is less compared to the produced by compensatory mutations found in chromosomes (only inherited vertically).

Plasmid copy-number as an evolvable trait

Another possible strategy to stabilize plasmids is to reduce the probability of plasmid loss by increasing the number of copies carried in each cell. But large numbers of plasmids may seriously affect the growth rate of their bacterial hosts, suggesting the existence of an optimal plasmid copy number susceptible to be tuned by evolution. Based on a model of transcription and translation of repressor and initiator proteins necessary for plasmid replication, (Lee and Bailey, 1984) explicitly evaluated the effect of genetic alterations in regulatory elements controlling copy-number control and, using computer simulations, identified parameter values for the strength of promoters and termination efficiencies

required for the stable maintenance of plasmids, a condition that was achieved by balancing the risk of segregational loss and the cost associated with increasing plasmid content (Lee' and Bailey~ 1984).

More recently, (Mei et al. 2019) combined experiments and mathematical modelling to argue there is a strong selection against increasing the number of plasmids carried in each cell. In their experimental system, the plasmid encodes for a drug-resistance gene and, although an increase in copy-number was associated with an increased level of resistance, the authors found that mutations in the RNA I gene producing an increased plasmid copy-number were only fixed at high antibiotic concentrations, therefore suggesting that the number of copies carried by each cell is robust to fluctuations and a highly optimized evolutionary trait. This result is consistent with another experimental evolution study (San Millan et al. 2016), whereby a plasmid-bearing population was exposed to increasing concentrations of antibiotics, selecting for mutations both in the target resistance gene but also in the RNA I gene controlling plasmid copy-number, with the latter only observed at detectable frequencies at very high drug concentrations.

Another important aspect of plasmid evolutionary dynamics is the interaction between plasmid copy-number and evolvability. Indeed, in a plasmid-encoded gene, the mutational target size is amplified by the number of copies of the plasmid, therefore producing a positive correlation between plasmid copy-number and the probability of mutation (San Millan et al. 2016; Dimitriu et al. 2020). However, mutations on plasmid-encoded genes can be cleared from the population through segregational drift, resulting in a long time to fixation for high-copy number plasmids and a reduced allele frequency of mutations occurring in plasmids compared to those encoded in the chromosome (Ilhan et al. 2019). This complex interaction between plasmid copy-number and random genetic drift (Gillespie 2010) was studied by Ilhan and co-authors using a combination of experimental evolution and a population genetics model based on a standard haploid version of the Wright-Fisher model that incorporates plasmid evolution following an approach previously used to study mitochondrial evolution (Peng and Kimmel 2005).

Besides an increased gene dosage and mutation rate, multi-copy plasmids can also produce genetic redundancy. That is, when a mutation randomly appears in a plasmid-encoded gene, different versions of this gene transiently co-exist in the same cell. This has been termed plasmid-mediated heteroplasmy and was shown to have important consequences in the evolutionary dynamics of bacterial populations (Novick and Hoppensteadt 1978) , for instance by allowing heterozygous cells to evade evolutionary trade-offs and promote evolutionary innovation (Rodriguez-Beltran et al. 2018). Moreover, a consequence of presenting intracellular genetic diversity is that mutations occurring in plasmid-encoded genes can enable evolutionary rescue (Alexander et al. 2014). By modelling plasmid dynamics using a multi-type branching process, (Santer and Uecker 2019) showed that the probability of evolutionary rescue depends on the number of plasmid copies and the degree of dominance of such mutations.

Modelling plasmid-host associations: which genes are carried in plasmids?

Despite the constant gene flow between plasmids and chromosomes, the distribution of genes between them is not random. While certain genes encoding for specific functions are usually present on plasmids rather than chromosomes, others are usually chromosomally-encoded. For instance, plasmids tend to carry genes necessary to catabolize rare substrates or associated with drug resistance and the expression of virulence factors, as well as the production of bacteriocins that kill bacterial competitors, enzymes that detoxify the environment or signalling molecules that nodulate plant roots fix atmospheric

nitrogen. In contrast, genes for most of the cell's structural proteins and fundamental metabolic processes (sometimes referred to as housekeeping genes) are underrepresented on plasmids and usually found in chromosomes.

To explain the over-representation of certain characters encoded in plasmids, several hypotheses have been postulated, with their causes and implications analyzed theoretically. For instance, the local adaptation hypothesis states that many of the characters that tend to be present on plasmids are adaptations to local variations in environmental conditions that occur only sporadically in time or in space (i.e. genes are only useful in certain environments or at certain times). This theory was proposed by (Eberhard 1990) and is based on the observation that sometimes-useful genes linked to horizontally transmissible elements out-compete non-mobile versions of the same genes by associating with bacterial genotypes with increased fitness.

In (van Dijk et al. 2020), the authors used an ODE model of a bacterial population undergoing uptake of genes from a shared DNA pool to evaluate the benefit of HGT based on distinct gene classes of slightly beneficial genes: indispensable (where HGT is not required and does not provide a benefit for the host), enrichable (maintained without HGT, although with increased fitness when acquired horizontally), rescuable (cleared from the population without HGT), unrescuable (also maintained only through horizontal transmission, but presenting reduced fitness in the presence of HGT) and selfish elements (only persisting at high rates of HGT and always decreasing growth of the population). Based on this classification, the authors showed that HGT can be an evolutionarily stable strategy for enrichable and rescuable genes, although the absence of gene-carrying donor cells renders HGT evolutionary inaccessible for rescuable genes. Moreover, using an individual-based model, the authors showed that spatial structure constrains the maintenance of slightly beneficial genes and that, once stable communities have evolved, selfish genetic elements can be stably maintained in the population.

The repertoire of genes carried in plasmids also depends on their degree of dominance, as discussed in a recent manuscript (Rodriguez-Beltran et al. 2019). By combining experiments with stochastic simulations of a fluctuation assay, the authors found a positive correlation between PCN and the frequency of phenotypic mutants for mutations of high dominance, and a negative association for mutations of low dominance. A conclusion of this study is that the repertoire of genes carried in plasmids is determined by the degree of dominance of the genes it carries. Interestingly, the degree of dominance of plasmid-carried alleles has been shown to depend on gene dosage and the environment, with important consequences in the adaptive evolution of bacterial populations carrying non-transmissible multi-copy plasmids (Santer and Uecker 2019).

The role of plasmids in social evolution

Plasmids have been shown to carry a disproportionate amount of genes involved in bacterial virulence and cooperation, suggesting a key role for plasmids in bacterial social evolution. This hypothesis was explored by (Smith 2001) using a within-host mathematical model to argue that mobility is beneficial because it enforces cooperation between neighbouring cells. As secreted proteins are costly to produce (the authors focus on virulence factors, but this argument could also be applied to nitrogen fixation, micro-environment detoxification and other proteins secreted to the extracellular environment), then a microbial community would be susceptible to the invasion of cheaters that fail to produce the public good (and therefore avoid the metabolic cost associated with the production), but still obtain the benefit resulting from other members of the community producing and exporting the public good. As a result,

cheaters would increase in frequency and render the cooperative behaviour unstable. In theory, HGT could force cheaters to produce the virulence factor, resulting in a stable cooperative behaviour.

This hypothesis addresses a fundamental problem in sociobiology: how can cooperation persist? In (Nogueira et al. 2009), the authors studied the interaction between cooperation and mobile plasmids and showed that, when the production of public goods is costly, HGT via plasmids increases local relatedness by infecting previously unrelated neighbours, therefore promoting cooperation. The model used to support this argument is based on a standard recursion equation for relatedness in a patch-structured population to describe horizontal gene transfer and gene loss occurring at a constant rate. The main result of this study is that invasion of cheaters in a population of cooperators could be prevented if the social trait was encoded in a conjugative plasmid, through the re-acquisition of the cooperative trait. Indeed, as relatedness increases through horizontal transmission, the authors suggest that, in theory, cooperative traits (e.g. secreted and outer membrane proteins) should be preferably encoded in mobilizable regions of genomes (Rankin et al. 2011). However, (Giraud and Shykoff 2011) argued that local transmission of uninfected cells would be enough to maintain the production of public goods, without the need for invoking kin selection.

The interaction between plasmid compatibility and the benefit of carrying public-good genes was studied by (Mc Ginty et al. 2011), showing that invasion by non-producers is prevented when competing (incompatible) plasmids arise in a population that does not carry the gene for production of the public good. Furthermore, (Mc Ginty and Rankin 2012) used a neighbor-modulated fitness approach that extends the Price equation (Price 1970) to describe changes in gene frequency in terms of both selection and transmission, with the aim of evaluating the interaction between relatedness (measured at the locus of interest) and horizontal gene transfer in the evolution of plasmid-borne cooperative traits. This model considers that segregation is negligible and concludes that when a plasmid carries genes involved in cooperation, there is the potential for genomic conflict between the plasmid and the host chromosome. A similar population genetics approach that includes horizontal transfer between local hosts was used to evaluate the effect of both transmission and relatedness as factors in the evolution of plasmid-borne public goods production (Mc Ginty et al. 2013). In this study, the authors showed the existence of a positive feedback between transmission and relatedness: if individuals are less related in a patch, there would be an increase in available cells to infect, resulting in an increase in overall transmission and therefore increasing relatedness.

The social aspect of plasmid-mediated antibiotic resistance (e.g. cross-protection) was also studied by (Yurtsev et al. 2013). In this manuscript, the authors postulated a simple population-dynamics model to show that the cooperative nature of antibiotic inactivation enables co-existence between sensitive and resistant cells, even in the absence of spatial structure. More recently, (Lehtinen et al. 2020) used a competition model between resistant and sensitive bacteria (with resistance carried either in a plasmid or in the chromosome) to show the existence of positive frequency-dependent selection on gene location, a property that emerges from considering that having both chromosomal and plasmid-borne copies of the gene provides no additional benefit than carrying a single copy of the gene (i.e. the increase in resistance resulting from carrying a second copy of the gene is less than the cost of carrying it).

Plasmids host-range

In a mini-review on broad-host-range plasmid replication, (del Solar et al. 1996) discussed that plasmid promiscuity is increased by two factors (initiation independence and versatile communication between plasmid and host initiation factors), and that versatility is constrained by host-specific adaptation. The relevance and interaction between these factors, however, remains an open question. (Tokuda et al. 2020) argued that plasmids are likely to have similar oligonucleotide (*k*-mer) composition to their host, and that this distance could be used to evaluate a plasmid's host-range. Using this approach, the authors predicted the host-range of the novel plasmid isolated from cow manure and found that, as expected, it is more similar to transconjugant hosts than those from other genera. In (Beaudoin et al. 1998), the authors studied the transference of a broad-host-range plasmid pDLB101, transferring from *Pseudomonas putida* into a *Bacillus azotoformans* biofilm. Species invasion to an established biofilm and the spatial distribution of each species was simulated by modifying a biofilm simulation program based on partial differential equations (AQUASIM) to incorporate plasmid transmission (modeled as a function of a limiting resource).

(De Gelder et al. 2007) analyzed the stability of another broad-host-range IncP-1 plasmid by experimentally introducing the plasmid to 19 species from the Alpha-, Beta- or Gammaproteobacteria and evaluating its stability under non-selective conditions. Interestingly, the authors found a great diversity of generation times to plasmid loss and, using a difference equation model, argued that this variation could be due to compensatory mutations, with stability patterns resulting from considering different replication and segregational loss rates, as well as plasmid costs and plasmid reuptake. In a follow-up study, (De Gelder et al. 2008) considered the two strains for which the plasmid was less stable and evaluated plasmid adaptation using experimental evolution, either in a single species or when alternating hosts. The experimental results were consistent with those predicted by the mathematical model, namely that horizontal transfer between unfavorable hosts can hamper plasmid adaptation. Finally, (Loftie-Eaton et al. 2016) combined a discrete plasmid population dynamics model with experimental evolution to show that the acquisition of a TA system can expand the long-term range of a plasmid in a single step.

Modelling plasmid dynamics in natural environments

Most of the theoretical studies discussed so far have focused on analyzing plasmid dynamics on novel host–plasmid combinations under controlled laboratory conditions, whereas in nature HGT occurs in much more complex environmental and community contexts, for instance in the soil or in the mammalian gut. However, studying MGEs in multi-species microbial communities is a difficult and ongoing problem that spans multiple levels of complexity and presents intrinsic limitations associated with obtaining data (most microbial species cannot even be grown in laboratory conditions). Another difficulty arises when trying to estimate prokaryotic diversity in natural microbial populations or to infer phylogenetic relationships between species. Actually, plasmids are at the core of this complexity, as they can obscure taxonomic classifications and diffuse the boundaries between bacterial species (Doolittle and Zhaxybayeva 2009). Altogether, these limitations have hindered the application of classic ecological theory to study microorganisms (Prosser et al. 2007), although a few studies have tried to extend simple population dynamic models to study plasmid transmission in complex ecological settings.

Plasmid transfer in the gut microbiota

An important issue driving plasmid-mediated dissemination of antibiotic resistance is that commensal bacteria can harbor resistance genes that can be potentially transferred to pathogenic bacteria. A theoretical study focusing on the role of antibiotic resistance in the microbiome was published by

Tepekule and co-authors (Tepekule et al. 2019). This model consists of a system of ordinary differential equations that describes the rate of change of different species over time and was used to argue that treatment history has a significant impact on the prevalence of resistance.

In order to study how antibiotic resistance is influenced by the presence of other species, (Klümper et al. 2019) postulated a discrete-time mathematical model in the presence (or absence) of a natural gut microbial community. In particular, the authors evaluated the interaction between *E. coli* and a pig-gut microbiota and showed that selection against resistance occurs at higher antibiotic concentrations when in the presence of other strains. Similarly, (Freter et al. 1983) postulated a dynamical model of plasmid transfer in a mouse intestine, and (Ahmad et al. 2015) studied the effect of different antibiotic regimes and composition of the gut microbiota in the levels of resistance observed in a pig gut.

While most studies focusing on the kinetics of plasmid transfer in the animal gut consider that the intestine can be approximated by a continuous-culture device (ignoring the spatial structure and assuming perfect mixing of donor and recipient cells), (Licht et al. 1999) considered compartmentalization of the intestinal environment. Likewise, (Grover and Wang 2019) evaluated the environmental dynamics of a gut colonized by two bacterial populations, each carrying a non-conjugative plasmid with a TA system, competing in a spatially extended habitat referred to as a flow reactor. Using numerical simulations of a system of partial differential equations, the authors explored the conditions that allowed for co-existence between plasmid-bearing and plasmid-free subpopulations, concluding that segregation rate and fitness cost of plasmid carriage must be relatively low for both strains to co-exist.

Plasmid transmission in the environment

It has been argued that the environment may be a source of resistance genes and, therefore, a series of articles have focused on transmission and maintenance of plasmids in bacterial populations living in the gastrointestinal tract of livestock or their associated farm environment, as well as to perform risk assessments on genetically engineered organisms released into the environment (Landis et al. 2000). Pharmacokinetic–pharmacodynamic models have also been used to study the dissemination of antimicrobial resistance genes at the farm level (Lanzas et al. 2011) and to estimate the length of a drug withdrawal period before slaughter, with the aim of reducing resistance levels before meat consumption (Cazer et al. 2018).

(Baker et al. 2016) used a simple mathematical model to evaluate the spread and selection of antimicrobial resistant bacteria in a slurry tank of a dairy farm. This model is based on (Volkova et al. 2012) and uses ODEs to describe growth of each subpopulation and the inflow of fresh slurry, containing both sensitive and resistant bacteria, as well as gene transfer and selection for plasmid-encoded resistance genes. By performing a global sensitivity analysis and numerical simulations, the authors showed that the rate of horizontal transmission and the length of time that slurry is stored in the slurry tank (without outflow and therefore considering the tank increases in volume) are the most significant parameters driving plasmid maintenance and therefore good targets for preventing antimicrobial resistant pathogens entering the human food and water supply chains.

Epidemiology of plasmids

The epidemiological dynamics of infectious diseases can be described mathematically, either using within-host models to study the evolution of resistance within a treated host, or between-hosts models

to describe the spread of resistance in a community of hosts (Blanquart 2019). Between-host epidemiological models of antibiotic resistance are usually based on simple compartmental models whereby individuals can be susceptible, recovered or colonized by drug-sensitive or drug-resistant bacteria, with transitions between these compartments resulting from transmission events or as a consequence of drug treatment. Although only a few epidemiological studies have explicitly focused on plasmid-bourne resistance, a few have evaluated the role of horizontal transmission in the emergence and maintenance of drug resistance. For instance, (Levin et al. 2014) postulated an epidemiological model to argue that the frequency of resistance is maintained due to the presence of plasmids, although it can also be reduced by decreasing antibiotic use, constraining the development of resistance during treatment or by restricting invasion of resistant pathogens into the community.

In (de Been et al. 2014), the authors studied extended-spectrum beta-lactamase resistance in *E. coli* strains isolated from humans and retail chicken meat. A statistical predictive model found significant similarities between human and chicken isolates, therefore concluding that chicken is a significant contributor to drug resistance in human infections. Similarly, (Jiang et al. 2020) used statistical models to analyze the prevalence of colistin resistance gene mcr-1 found in clinical isolates of *E. coli*. This study concludes that the outbreak was not a consequence of clone dissemination, but that conjugative plasmids were likely to have played a critical role in mcr-1 transmission.

Other studies have also combined high-throughput data and statistical models to study dissemination routes in hospital settings. In (Leon-Sampedro et al. 2020), the authors analyzed epidemiological data from 9,000 patients and whole genome sequencing data from 250 enterobacteria clones to study the spread of a carbapenemase-encoding plasmid (pOXA-48) in a hospital over a two-year period. A model selection approach based on a case-specific probabilistic model that implements a Hamiltonian Markov chain Monte Carlo algorithm was used to make inferences about the dissemination routes observed in the data. The source of the outbreak was identified using SCOTTI, a structured coalescent-based tool for reconstructing bacterial transmission. pOXA-48 also produced a hospital outbreak of carbapenem-resistant Enterobacteriales in the United Kingdom, which was studied by (Ledda et al. 2020) using a mathematical model for conjugation (modeled as a homogeneous Markov process between bacterial hosts). This modeling approach allowed the authors to identify the founder strain responsible for the outbreak and to calculate the number of conjugation events that occurred during the outbreak.

Although the aforementioned studies focus on localized outbreaks, more general models have also been proposed to study plasmid-driven dissemination of antibiotic resistance genes. For instance, (Ledda and Ferretti 2014) proposed a model for plasmid fitness depending on its length, arguing that the use of antibiotics can increase both the length of plasmids and the number of antibiotic resistance genes carried by each plasmid. Another modeling approach (referred to as membrane computing) was recently used to simulate dynamics of antibiotic resistance at multiple complexity levels: genes, phenotypes, cells, populations, communities, and ecosystems. In short, this computational approach consists of a multi-scale individual-based system where individuals are confined within membranes, which in turn are organized in compartments in tree-like structures. In (Campos et al. 2019), the authors used membrane computing to simulate multiple study cases, including the introduction of a resistance plasmid and a conjugative element (which transfers a resistance gene into the chromosome) into a hospital community compartment. This study concluded that, in the long-term, dissemination of traits would be more effective if they are encoded in plasmids rather than in the chromosome.

Plasmid dynamics in polymicrobial synthetic communities

To overcome the complexities associated with studying plasmid ecology and evolution in natural environments, a few studies have used *in vitro* model systems consisting of synthetic communities composed of multiple species co-existing in controlled environmental conditions. For instance, (Jordt et al. 2020) studied the co-evolution of host-plasmid pairs in a mixed-species culture of *Klebsiella pneumoniae* and *Escherichia coli,* showing that pleiotropy enhances plasmid stability in microbial populations when the plasmid had previously co-evolved with one of the members of the community. Similarly, (Alonso-del Valle et al. 2020) studied the distribution of fitness effects of a clinically-relevant plasmid in different *Klebsiella* and *E. coli* isolates obtained from clinical samples. Using a population-dynamics model, the authors showed that variability in the cost of plasmid-bearing between different hosts can stabilize plasmids in polymicrobial communities and reduce the critical conjugation rate necessary to maintain plasmids in the population. Moreover, using computer simulations, it was shown that plasmid frequency is a decreasing function of community complexity when plasmid-bearing is associated with a constant fitness cost, but an increasing function of the number strains in the community when the distribution of fitness effects presents a large variance.

In (Lehtinen et al. 2020), the authors postulate a stochastic model of resistance acquisition and transfer in a bacterial community that considers that a beneficial gene is transferable between n possible bacterial species, either through transformation (horizontal transfer between species of chromosomal resistance) or through conjugation (resistance transfer between species). The main result of this study is that the probability of finding genes on plasmids increases with higher rates of inter-species plasmid transfer and with a higher number of species between which the gene can be shared. The consequence of this frequency-dependence is that moderately beneficial genes can be maintained on plasmids, despite segregation loss, if they are present at a higher frequency.

Finally, an aspect that is usually overlooked is the influence of viruses in the ecology and evolution of plasmid-bearing populations. (Dionisio 2005) proposed a chemostat model whereby a male-specific phages that can only infect only donor cells and showed that heterogeneity in the rate of transfer is a critical parameter influencing the stability of conjugative plasmids in bacterial communities when conjugative plasmid–dependent phages are present. Also, by combining experimental observations with mathematical modeling and computer simulations of an individual-based model, (Harrison et al. 2015) studied the interaction between lytic bacteriophages and the persistence of conjugative plasmids and showed that the population is under strong indirect selection pressure from lytic bacteriophages, therefore limiting the ecological conditions whereby plasmids can persist.

Discussion

Plasmids, along with other mobile genetic elements like conjugative transposons, bacteriophages, and integrative conjugative elements, are important drivers of the ecological and evolutionary dynamics of bacterial populations (Slater et al. 2008). In particular, as plasmids can be viewed as self-replicating units of selection, understanding the impact of different plasmid replication and segregation dynamics have on the population dynamics has been an active area of research for several decades. In this context, mathematical models and computer simulations have proved to be important tools to unravel the interplay between replication, segregation, and horizontal transmission, as well as to evaluate the role that different selective pressures have on the stability of plasmids.

Notably, mathematical modeling has played a critical role addressing the plasmid paradox, by establishing sufficient conditions for the maintenance of plasmids in populations, namely that the rate of horizontal transmission had to be large enough to compensate for segregational loss and fitness costs. The relevance of theoretical models, however, was also evident in later theoretical studies aiming at understanding the limits of the Levin and Stewart criterion and also to propose other possible mechanisms that increase plasmid stability, from spatial structure and fitness-cost compensation to plasmid-host coevolution and complex ecological interactions.

Most of these studies, however, consider simple experimental microcosms, like batch cultures or chemostats, thus allowing mathematical modelers to parametrise their models and to contrast predictions emanating from these models with experimental data obtained *in vitro,* but also for modeling convenience (e.g. limited sub-populations interacting at high densities in homogeneous environments). However, recent advances in genomic technologies and bioinformatic algorithms are beginning to shed light on the influence of plasmids and other MGEs in natural environments (Smalla et al. 2015; Li et al. 2020), a progress that contrasts with the scarcity of theoretical models aimed at providing a mechanistic understanding of the role of plasmids in polyclonal populations.

This is, of course, a very difficult problem, partly because it spans multiple levels of complexity (Paulsson 2002). Indeed, W. Eberhardt stated more than thirty years ago: *"As already noted by other authors (Hardy 1975; Dawkins 2016; Broda 1979), analyses of plasmid evolution entail simultaneous consideration of selection acting at several different levels of reproduction, including genes, transposons, plasmids, chromosomes, cells, and clones"* (Eberhard 1990). Decades later, many conceptual advances have been made, including the realization of the importance of another level of selection: plasmids as ecological drivers of mixed bacterial populations and as promoters of community-level evolution (Doulcier et al. 2020; van Vliet and Doebeli 2019).

Plasmid segregation and replication are inherently stochastic processes, so another fundamental problem in plasmid biology is to understand how noise resulting from the stochastic segregation and replication of plasmids drives the evolutionary dynamics of bacterial populations? Interestingly, most of the studies discussed in this review focus on mechanisms that reduce noise in PCN (therefore increasing the stability of plasmids, a useful feature of genetic circuits and cloning vectors used in bioengineering). However, the high-frequency of plasmid-bourne resistance genes found in heterorresistant clinical isolates (Andersson et al. 2019; Lázár and Kishony 2019) suggests that plasmids could be providing a platform that produces gene copy-number variability and heterogeneity in cellular responses to multiple antibiotics, therefore increasing the probability of treatment failure.

Furthermore, as recent in vivo studies are beginning to track evolution in real-time inside complex communities (Ramiro et al. 2020) and to identify DNA transmission events directly from the microbiota (Munck et al. 2020), then another challenge for the future is to produce data-driven models that incorporate high-throughput data obtained with high temporal and spatial resolution into predictive models of plasmid evolution. We believe that the mathematical and computational tools necessary to include this data are yet to be developed, but most likely will not result from scaling-up systems of ordinary differential equations to consider thousands of equations with millions of parameters. The reason most studies discussed in this review have restricted to study simple model systems is not because of lack of interest or computational power, but because, independently of the modeling framework used, it is unrealistic to estimate kinetic parameter values for individual strains within

polymicrobial communities, in part because of empirical constraints (bacteria are not culturable, metabolic interactions are complex and distributions of fitness effects are heterogeneous), as well as due to theoretical limitations (interactions are highly non-linear and parameters have identifiability issues).

Finally, as discussed previously, the introduction of mobile elements encoding for drug resistance genes into clinical environments can produce plasmid-related outbreaks of antimicrobial-resistant pathogens (Mayer 1988). In this scenario, it could be argued that the responsibility for the outbreak lies not on a particular bacterial strain but on a plasmid that is shared between different hosts, even from different species. In consequence, it has been suggested that, in order to control the spread and evolution of resistant pathogens, it is necessary to view the problem from a plasmid-centric perspective. We believe that mathematical modeling and computer simulations provide powerful tools to address this challenge and, in the future, maybe even to propose new therapeutic avenues that control plasmid-driven antibiotic resistance.

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Capítulo 3

Estabilidad de plásmidos multicopia: un modelo de genética de poblaciones

3.1 Introducción

En la revisión previa hemos analizado una gran cantidad de trabajos sobre modelación de plásmidos. Los enfoques de modelación, así como sus objetivos son muy amplios; desde reproducir y entender experimentos de laboratorio, optimizar producción de proteínas recombinantes, atacar problemas asociados a resistencia a antibióticos o entender procesos evolutivos. En este último caso, la coexistencia de subpoblaciones con plásmidos y libres de plásmidos puede ser entendida como una estrategia evolutiva conocida como apuesta-cobertura (Bayramoglu et al. 2017), ya que la subpoblación que porta plásmidos paga un costo con el fin de contender a futuros ambientes hostiles. Sin embargo, bajo la luz de la evolución, el fenómeno más intrigante, quizá, es la paradoja de plásmidos, ya que su elucidación implica múltiples procesos y sus consecuencias tienen aplicaciones en biotecnología y salud.

En este capítulo nos enfocaremos a estudiar la estabilidad de plásmidos mediante un modelo de genética de poblaciones, una aproximación que sorprendentemente no se ha utilizado para estudiar este problema. En particular nos centraremos en estudiar la estabilidad de plásmidos multicopia, utilizando como modelo experimental un plásmido previamente caracterizado (San Millan et al. 2016). Este plásmido, pBGT, es de tipo ColE1, por lo que replica por medio de RNAI y RNAII y, al no poseer mecanismos de muerte post-segregación ni de partición activa, es segregado al azar.

El modelo postulado es resultado de una colaboración con colegas matemáticos del Instituto de Matemáticas y del Instituto de Investigaciones en Matemáticas Aplicadas y Sistemas de la UNAM. Dicho modelo nos permitió encontrar condiciones específicas que permiten aumentar la estabilidad de los plásmidos a largo plazo, por ejemplo el rango de números de copias que para plásmidos con un costo especifico maximiza su permanencia en ambientes sin selección. De igual manera, se propone una estrategia para optimizar regímenes de selección que garanticen que un plásmido con características específicas no se extinga la población, o bien, encontrar la dosis de antibiótico que mantiene a una población en determinada proporción de plásmidos.

Nota: este trabajo está en progreso. Hace falta realizar un análisis profundo de la exploración de parámetros.

Population genetics of multi-copy plasmids

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ABSTRACT

Multicopy antibiotic resistance plasmids can confer some selective advantage to bacteria carrying them but are costly to maintain. We consider a type of plasmids without horizontal transfer, for which the number of copies per cell is very well conserved. Here we use a population genetics model to argue that the probability of plasmid loss depends on its copy number and a trade-off between the plasmid cost and the frequency of positive selection. Moreover, our model provides into how antibiotics have to be administrated in order to promote plasmid maintenance or loss. We show some experimental results that allow us to validate our model.

KEYWORDS Multicopy plasmids, Wright-Fisher model, Experimental microbiology

Introduction

Prokaryotes transfer DNA at high rates within microbial communities through mobile genetic elements such as bacteriophages (Chen *et al.* 2018), transposons (Chen and Dubnau 2004) or extra-chromosomal DNA molecules known as plasmids (Funnell and Phillips 2004). Crucially, plasmids have core genes that allow them to replicate independently of the cellular chromosome but also encode for accessory genes that provide their bacterial hosts with new functions and increased fitness in novel or stressful environmental conditions (Groisman and Ochman 1996). In particular, as plasmids often carry antibiotic-resistance genes, they have been identified as significant factors contributing to the current global health crisis that represents drug resistance of clinically-relevant pathogens (San Millan 2018).

It is generally assumed that, in the absence of selection for plasmid-encoded genes, most plasmids represent a burden for their bacterial hosts (San Millan and Maclean 2017). As a result, plasmid-bearing populations have a competitive disadvantage against plasmid-free cells, thus threatening plasmids to be cleared from the population through purifying selection (Vogwill and MacLean 2015; Baltrus 2013). To avoid extinction, some plasmids overcome segregational instability by transmitting horizontally to lineages with increased fitness. Previous theoretical results have established sufficient conditions for plasmid maintenance, namely that the rate of horizontal transmission has to be larger than the combined effect of segregational loss and fitness cost (Stewart and Levin 1977; Bergstrom *et al.* 2000). Also, some plasmids encode molecular mechanisms that increase their stability, for instance, toxin-antitoxin systems that kill plasmid-free cells (Mochizuki *et al.* 2006), or active partitioning mechanisms that ensure the symmetric segregation of plasmids upon division (Salje 2010).

However, active partitioning and post-segregational killing mechanisms, and in particular the molecular machinery necessary to conjugate, require many genes and therefore are only found in large plasmids (>20Kb). For small plasmids to be maintained in the population, they tend to be present in many copies per cell, therefore decreasing the probability of producing a plasmid-free cell when randomly segregating plasmids at the moment of division. But this reduced rate of segregational loss is not

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sufficient to explain the stable persistence of costly plasmids in the population, and therefore previous theoretical and experimental studies have argued that persistence of non-conjugative plasmids can also be explained with intermittent periods of positive selection (Stevenson *et al.* 2018; Wein *et al.* 2020) or through compensatory mutations that ameliorate the fitness cost (San Millan *et al.* 2014; Harrison *et al.* 2012). Interestingly, these small, multi-copy plasmids represent a large fraction of all plasmids identified in genomic sequences (Smillie *et al.* 2010), suggesting they must provide an evolutionary benefit for bacterial communities, even if they are not mobilizable.

Indeed, recent studies have shown that multi-copy plasmids can also have important consequences in the evolutionary dynamics of bacterial populations. For instance, as multi-copy plasmids are present in 10-100 copies per cell, the mutational target is increased several orders of magnitude. Moreover, once a beneficial mutation appears, multi-copy plasmids can subsequently amplify its expression, resulting in an accelerated rate of adaptation to adverse environmental conditions (San Millan 2018) and promoting evolutionary rescue (Santer and Uecker 2020). Also, multi-copy plasmids increase the genetic diversity of the population, thus enhancing survival in fluctuating environments and allowing bacterial populations to circumvent evolutionary trade-offs (Rodriguez-Beltran *et al.* 2018). Furthermore, random segregation and replication of plasmids produce a complex interaction between plasmid copy-number, genetic dominance, and segregational drift, with important consequences in the fixation probability of beneficial mutations (Ilhan *et al.* 2019) and the repertoire of genes that can be carried in mobile genetic elements (Rodriguez-Beltran *et al.* 2019).

While the benefits of carrying plasmids may be clear under certain circumstances, it is also patent that their maintenance can be associated with a considerable energetic cost. For instance, increasing plasmid copy-number can be associated with an increase in fitness and a reduced probability of segregational loss, but can also be associated with a larger fitness cost in the absence of selection for plasmid-encoded genes. This trade-off poses the following fundamental questions: when is it evolutionary optimal for cells to carry plasmids? What is the optimal copy-number? To address these questions, we will use population genetics approach to evaluate the stability of multi-copy plasmids as a function of the strength and duration of selection in favour of the plasmids. In particular, our model is based on a classic Wright-Fisher approach with two cell types: plasmid-bearing (type 1) and plasmid-free cells (type 0).

To determine environmental conditions that stabilize plasmids in populations, we will consider that plasmids encode for a drug resistance gene that allows bacteria to survive exposure to an otherwise lethal concentration of bactericidal antibiotic. We then consider intermittent periods of positive selection where every T generations type 0 individuals are killed with probability $p \in (0, 1]$. Our main result is that, when the frequency of antibiotic exposure is high, type 1 individuals will prevail and thus the plasmid is stabilized in the population. To validate our theoretical results, we will use an experimental model system consisting on *Escherichia coli* with a multi-copy plasmid (~ 19 copies per cell) encoding a GFP fluorescent marker and $bla_{\text{TEM-1}}$, a drug-resistance gene that produces a β-lactamase that degrades ampicillin and other β-lactam antibiotics (Salverda *et al.* 2010; San Millan 2018). By using population-level measurements, we obtain fluorescence intensity data on the fraction of plasmid-bearing cells present in the population after exposure to a range of selective pressures.

We conclude by arguing that, as the existence of plasmids in natural environments requires intermittent periods of positive selection, the presence of plasmids contains information on the environment on which a population has evolved. Indeed, the number of copies n associates the frequency of selection with the energetic costs of plasmid maintenance. That is, there is a minimum frequency of drug exposure that allows multiple copies to persist in the population, and, for each environmental regime, there is an optimal number of plasmid copies.

A Wright-Fisher model for multi-copy plasmid dynamics

We consider a discrete-time Wright-Fisher process to model the evolution of a population with fixed population size N. There are 2 types of individuals. Type 1 individuals contain n copies of the plasmid. Type 0 individuals contain no plasmids. Let us denote the number of plasmids carried by a type 1 cell as n and argue that this is an important parameter; in one hand, the selective disadvantage of type 1 individuals due to the costs of carrying plasmids is assumed to be a constant multiplied by the number of copies of plasmids. On the other hand, the number of copies determines the heritability of type 1 individuals.

Moreover, we will consider that, at the moment of division, each copy of the plasmid is randomly segregated into one of the two new cells, and, once in the new host, the plasmids replicate until reaching n copies. If, however, one of the two new cells have all the n copies, the other one will become type 0, meaning that it will not carry any copy of the plasmids. This means that the daughter of a type 1 cell is type 1 with probability $1 - 2^{-n}$. As a result, plasmids are readily cleared from the population when the number of copies is low, with the probability of transmitting this trait to their progeny increases exponentially with n. We model the fitness cost associated with carrying plasmids by considering that type 0 individuals have a selective advantage in $T - 1$ generations out of T. This, together with the loss of plasmids during cell division, results in type 1 individuals driven to extinction in the population.

Let $X_i^{(N)}$ be the proportion of type 1 individuals at generation i. This type of individuals has some selective disadvantage $\kappa_{N,n}$ due to the cost of the plasmid maintenance. This means that each individual at generation $i + 1$ chooses a type 1 parent with probability

$$\frac{(1 - \kappa_{N,n})X_i^{(N)}}{(1 - \kappa_{N,n})X_i^{(N)} + 1 - X_i^{(N)}}.$$

We assume that $\kappa_{N,n}$ is proportional to n. At the moment of a cell division, we suppose that plasmids of the mother are uniformly spread between the two daughters. Of course, if a new born individual receives no plasmid from her type 1 mother, she switches to type 0. This can be modeled as a mutation from type 1 to type 0, occurring with probability $\mu_{N,n} = 2^{-n}$.

To see the accumulated effects of the selection, the "mutation" and the genetic drift, we need $\kappa_{N,n}$ and $\mu_{N,n}$ to be of order $1/N$.The first condition is fulfilled if the cost per plasmid is very low, for example when $\kappa_{N,n} = \kappa n/N$. The second one stands if n is of order $\log_2(N)$, which is the case, for example, if $n = 20$ and $N = 10^6$, or if $n = 15$ and $N = 10^5$. In that case we set $\mu = N2^{-n}$. Under this setting, when time is accelerated by N, the frequency process of individuals with plasmids can be approximated by the solution of the stochastic differential equation (SDE)

$$dX_t = -\mu X_t dt - \kappa X_t(1 - X_t)dt + \sqrt{X_t(1 - X_t)}dB_t. \tag{0.1}$$

This is known as the Wright-Fisher diffusion with mutation and selection.

When the cost of plasmid does not scale with N, i.e. $\kappa_{N,n} = \kappa_n$, we are in the case of strong selection. The frequency process of type 1 individuals converges, when $N \to \infty$, to a discrete time deterministic sequence defined in a recursive way as

$$X_{i+1} = f(X_i) := \frac{(1 - \kappa_n)X_i}{(1 - \kappa_n)X_i + 1 - X_i}(1 - \mu_n), \qquad i \geq 1,$$

where $\mu_n = 2^{-n}$. Observe that this approximation does not require any time rescaling.

Modeling the introduction of antibiotics

Additionally, the population is treated with antibiotic pulses. Individuals with no plasmids are more exposed to this treatment and at each pulse we observe an increment of the relative frequency of the type 1 population. To model this phenomenon, we assume that, in the presence of antibiotic, type 0 individuals have some selective disadvantage $\alpha_n \in [0,1]$. Let $T, 2T, \dots$ be the times of the pulses (in the rescaled time). At time kT, only a fraction

$$\frac{(1-\alpha_n)(1-X_{kT-})}{(1-\alpha_n)(1-X_{kT-})+X_{kT-}}$$

of type 0 individuals survives. Then (0.1) modifies to

$$dX_t = \sum_{k\geq 1} \frac{\alpha_n X_{kT-}(1-X_{kT-})}{1-\alpha_n(1-X_{kT-})}\mathbf{1}_{kT\leq t} - \mu X_t dt - \kappa X_t(1-X_t)dt + \sqrt{X_t(1-X_t)}dB_t \tag{0.2}$$

In the strong selection case, if the antibiotic pulse occurs at generation i, type 0 individuals die with probability α_n so that the fraction of type 1 individuals becomes

$$g(X_i) := \frac{X_i}{X_i + (1-\alpha_n)(1-X_i)}.$$

Then, after reproduction, the frequency of type 1 individuals at the next generation is

$$X_{i+1} = f(g(X_i)).$$

Finally, considering that the pulses occur at generations $T, 2T, \dots$, the frequency process becomes

$$X_{i+1} = \begin{cases} f(g(X_i)) = \frac{(1-\kappa_n)X_i}{1-\alpha_n+X_i(\alpha_n-\kappa_n)}(1-\mu_n) & \text{if } i = kT,\ k = 1, 2\cdots \\ f(X_i) = \frac{(1-\kappa_n)X_i}{(1-\kappa_n)X_i+1-X_i}(1-\mu_n) & \text{otherwise.} \end{cases} \tag{0.3}$$

Serial dilution protocol

Following the set up of Casanova *et al.* (2016), we consider that each generation in the Wright-Fisher model corresponds to one day in the experiments. We also refer the reader to Baake *et al.* (2019) and Chevin (2011). We now detail how this correspondence is made.

Intraday dynamics Day i starts with N founder individuals ($N \sim 10^5$ in the experiment) and reproduction stops when saturation is reached, which corresponds to a population size of γN ($\gamma N \sim 10^7$ in the experiment). Among the founder individuals, a fraction x is of type 1. We assume that, in the absence of antibiotic, the population evolves as a continuous time multi-type branching process $\mathbf{Z}_t = (Z_t^0, Z_t^1)$ where the reproduction rate (or *Malthusian fitness*) of type 1 (resp. type 0) individuals is r (resp. $r+\rho$), with $\rho > 0$ (since type 1 individuals have some disadvantage due to the cost of plasmid maintenance).

Following Casanova *et al.* (2016), we assume that $\rho \sim N^{-b}$ for some $b \in (0, 1/2)$ (this regime is known as *moderate-strong selection*). In every branching event, an individual splits in two. Type 0 individuals only split in two type 0 individuals. Type 1 individuals can split in one type 0 individual and one type 1 individual with probability 2^{-n} (if all the plasmids go to one of them) or they can split in two type 1 individuals with probability $1-2^{-n}$. Let $M(t) = \{M_{i,j}(t) : i, j = 0, 1\}$ be the mean matrix given by $M_{i,j}(t) = \mathbb{E}_{\mathbf{e}_i}(Z_t^j)$, the average size of the type j population at time t if we start with a type i individual. According to Athreya and Ney (Athreya 2006, Section V.7.2), $M(t)$ can be calculated as an exponential matrix

$$M(t) = e^{tA} \qquad \text{where} \qquad A = \begin{pmatrix} r+\rho & 0 \\ r2^{-n} & r(1-2^{-n}) \end{pmatrix}.$$

More precisely,

$$M(t) = \begin{pmatrix} e^{(r+\rho)t} & 0 \\ \frac{r2^{-n}}{r2^{-n}+\rho}(e^{(r+\rho)t} - e^{r(1-2^{-n})t}) & e^{r(1-2^{-n})t} \end{pmatrix}.$$

Let σ be the duration of the growth phase. Since N is very large, one can assume that reproduction is stopped when the expectation of the number of descendants reaches γN, i.e. that σ satisfies

$$\begin{aligned}\gamma N =& (1-x)N(M_{0,0}(\sigma) + M_{0,1}(\sigma)) + xN(M_{1,0}(\sigma) + M_{1,1}(\sigma)) \\ =& Ne^{r\sigma}\left(e^{\rho\sigma} + \rho x\,\frac{e^{-r2^{-n}\sigma} - e^{\rho\sigma}}{r2^{-n}+\rho}\right).\end{aligned}$$

Since $\rho \sim N^{-b}$, we have for large enough N that

$$\sigma \simeq \frac{\log \gamma}{r}.$$

Interday dynamics. We consider a discrete time model, with fixed population size N, in which generation i corresponds to the founder individuals of day i. Suppose that the frequency of type 1 individuals in generation i is x. To form generation $i+1$ we sample N individuals from the γN individuals present at the end of day i . Since γ is large enough, we can assume that individuals are sampled with replacement. The probability that an individual in the sample is of type 1 is equal to

$$\frac{xM_{1,1}(\sigma)}{(1-x)(M_{0,0}(\sigma) + M_{0,1}(\sigma)) + x(M_{1,0}(\sigma) + M_{1,1}(\sigma))} = \frac{xe^{-(r2^{-n}+\rho)\sigma}}{(1-x) + x\frac{r2^{-n}+\rho e^{-(r2^{-n}+\rho)\sigma}}{r2^{-n}+\rho}} \tag{0.4}$$

This is equal to the probability of choosing a type 1 parent in the Wright-Fisher model introduced in teh previous section, with

$$\kappa_n = \frac{\rho(1-e^{-(r2^{-n}+\rho)\sigma})}{r2^{-n}+\rho} \qquad \text{and} \qquad \mu_n = 1 - \frac{r2^{-n}+\rho}{r2^{-n}e^{(r2^{-n}+\rho)\sigma}+\rho}.$$

Model parametrization

In order to parametrize our model and validate its predictions, we used *Escherichia coli* MG1655 carrying, pBGT, a well-characterized multi-copy plasmid (San Millan *et al.* 2016). pBGT is a ColE-1 like plasmid with ∼19 plasmid copies per cell, lacking the necessary machinery to perform conjugation or any other strategy to enhance stability or symmetric segregation of plasmids. As a result, each plasmid is inherited to a daughter bacteria randomly upon division with probability $1/2$, therefore producing a plasmid-free cell with probability $1/2^{19}$. This plasmid also carries a GFP reporter under an arabinose inducible promoter and $bla_{\text{TEM-1}}$, a a gene that encodes a β-lactamse that efficiently degrades β-lactam antibiotics, particularly ampicillin (AMP). The minimum inhibitory concentration (MIC) of the plasmid-bearing population is 8,192 mg/l, while the plasmid-free strain (WT) has an MIC of 4 mg/l.

It is generally assumed that plasmids entail a metabolic burden in the absence of selection for plasmid-encoded genes(Andersson and Levin 1999; San Millan and MacLean 2019). As a result, plasmid-free bacteria present competitive advantage with respect to cells carrying plasmids in the absence of selection against genes carried in the plasmid. In contrast, in the presence of antibiotics, plasmid-free cells are cleared from the system and the frequency of plasmid-bearing cells increases. To measure the plasmid

cost we performed growth kinetics experiment in titration plates for the two strains growing in lysogeny broth (LB) rich media and measured the growth rate following the protocol in (Hall *et al.* 2014), with results summarized in table 1.

We found that the selective disadvantage of the plasmid bearing strain, κ_n, to be 0.272. We also measured this cost directly by comparing maximum growth rates and found that the metabolic burden entailed by the plasmid is 0.108 ± 0.067. This difference in values may come by the fact that the growth rate is only one component of fitness Vasi *et al.* (1994). Yet our model allowed us to estimate fitness cost more accurately than from simple growth dynamics.

Segregational instability in the absence of selection

Although, as expected, in the absence of selection plasmids are always cleared from the population, the loss rates shown in Figure 1-A is considerably smaller than Figure 1-B showing the selective disadvantage of the plasmid bearing strain is due to the metabolic cost. Now, Figure 1-A shows the dynamics for plasmid loss for strains that would carry a pBGT-like plasmid with different plasmid copy numbers.

For all copy numbers, plasmids are lost (the plasmid frequency tends to 0) but at different rates. We can notice that the loss rate does not correlate linearly with the copy number, which is the case if the plasmid does not entail a burden as shown in Figure 1-B.

Taken together, these observations indicate the existence of a non-linear relationship between the plasmid copy number, the rate for generation segregant cells and the plasmid cost. This suggests that, in drug-free environments, there must be an optimal plasmid copy number that maximizes plasmid maintenance.

We then numerically explored this relationship in order to determine the time to plasmid loss for a range of plasmid costs. Figure 1-C shows that, if plasmid cost is large, plasmid loss is fast regardless of the plasmid copy number. However, we can surprisingly notice that there is a short range of plasmid copy numbers that for different costs ensures the persistence of plasmids for long periods of time.

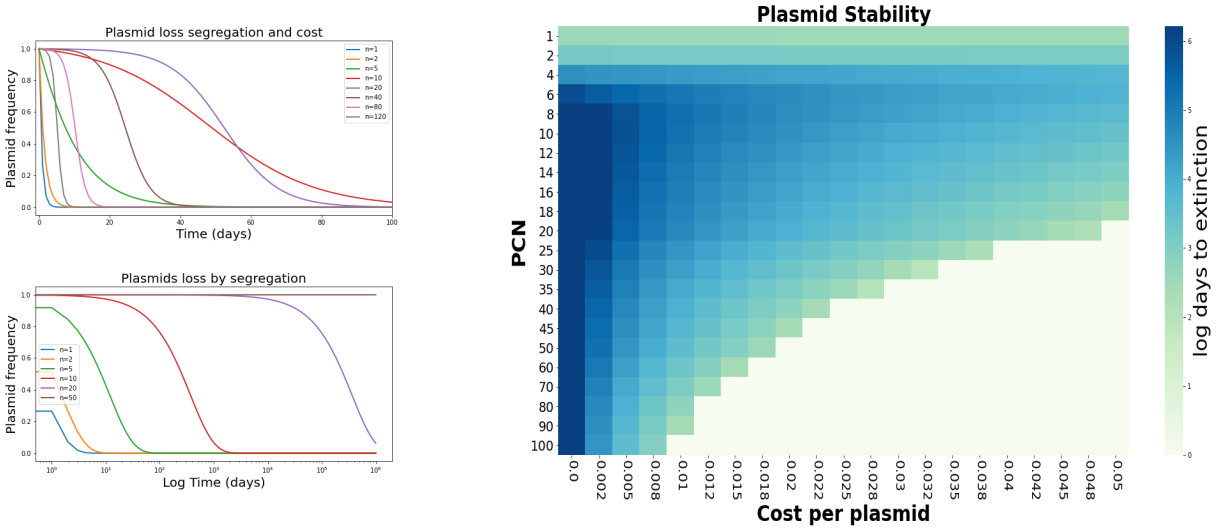

Figure 1 Simulations without antibiotics, n represents the plasmid copy number. **A** Times for plasmid loss for strains carrying different copies of the same plasmid. **B** Time for plasmids loss considering only plasmid loss by segregation. **C** Time for plasmids loss considering different plasmid copy numbers with multiple plasmid costs.

Evaluating the role of selection in plasmid maintenance

It is clear that selective pressure in favor of plasmid encoded genes is needed for plasmid maintenance, which is often the case of antibiotic resistance and our study plasmid. In our experimental system we will use ampicillin to kill plasmid-free bacteria and fix the plasmid in the population. Once the antibiotic clears from the environment plasmid-free bacteria begin to appear and increase in fraction, and if enough time passes without antibiotic, plasmids may be completely depleted from the population.

Before plasmid extinction, we could find a populations on all the range of subpoplations fractions. With the aim to address how the strength of selection affects this fractions we used the model defined by equation (0.3) and made some simulations starting from different population fractions to explore the effects of multiple selection strengths, αs, in the dynamics of fractions over time. Figure 2 shows the fixation dynamics over time for αs 0, $\kappa_n/2$, κ_n, 0.25, $1.5\kappa_n$, and 1 using κ_n measured in the previous section and applying selection everyday, we can notice that regardless the initial fraction for $\alpha > \kappa_n$ plasmid population always fixes (the plasmid frequency tends to 1) and that the rate of fixation is proportional to value of α. Similarly, for $\alpha < \kappa_n$ the plasmid population always extinguishes meaning that there is an effective antibiotic dose required to maintain the plasmid. Mathematically this critical dose is reached when $\alpha = \kappa_n$, and as shown in the simulations in Figure 2, this α stabilizes the plasmid frequency. In fact, this finding is proven in the functional analysis section of the appendix

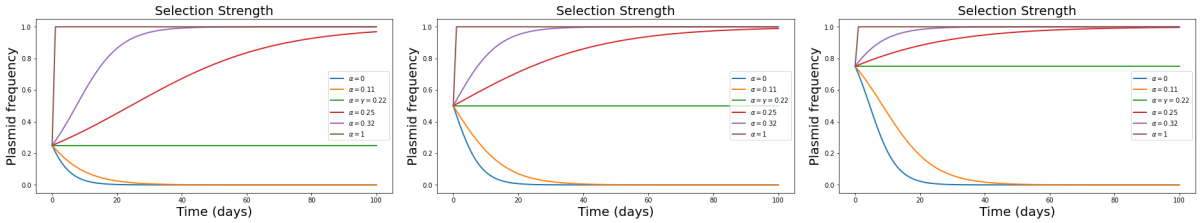

Figure 2 Simulations showing the effect of selection strength. We fixed κ_n with value measured experimentally and varied the parameter α for different initial fractions applying selection everyday (T=1). Note that for $\alpha > \kappa_n$ plasmid population always fixes and the rate of fixation is proportional to value of α. For $\alpha < \kappa_n$ the plasmid population always extinguishes. For $\alpha = \kappa_n$ the plasmid frequency stabilizes.

In the laboratory, we could perform an stability experiment starting with a plasmid-bearing strain, make serial dilution transfers and measure the fraction changes every day, and at a given moment (specific fraction) submit the population to an antibiotic peak which, depending on the dose and the previous fraction, would end differently. However, experimental evolution produces changes such as compensatory mutations (Peña-Miller *et al.* 2015) or plasmid genes integration to the chromosome, to avoid this traits, we design the a one-day experiment using a difference equation map system. To estimate the dynamics of plasmid loss and recovery of the plasmid-bearing subpopulation, we constructed mixed populations of bacteria with different fractions of plasmid-bearing and plasmid-free strains, and exposed them to a range of antibiotic concentrations.

As the plasmid used in this study encodes for a GFP protein, this allows us to use a spectrophotometer to distinguish between our two strains and to determine the fractions of each subpopulation (see Methods). With this approach we in fact, found a linear correlation between plasmid fraction and fluorescence intensity (R^2=0.995).

Figure 3 shows growth dynamics of populations composed of different initial frequencies of plasmid-bearing cells. For antibiotic concentrations below the plasmid-free MIC, curves are below the identity line, indicating a decrease in frequency of plasmid-bearing strain. As antibiotic concentration increases the plasmid-free strain is eliminated from the population and curves move above the identity line indicating antibiotic indeed increases the proportion the plasmid bearing strain

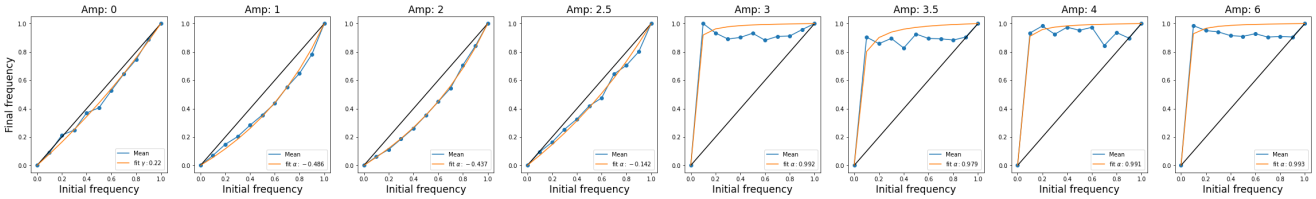

Figure 3 Gradient of antibiotic concentration is presented in columns. Blue lines indicates the mean normalized data from 4 replicates, while the yellow line represents the projections of our parameterized model.

Note that for small antibiotic doses (1 and 2 mg/l) the curves moves farther down from the identity than the antibiotic-free curve. This could be due an Eagle effect Eagle *et al.* (1948) on the plasmid-free strain or augmented OD from the non-fluorescent strain derived from elongation. This paradoxical effect yield us negative α for this concentrations.

Plasmid stability in fluctuating environments

In the previous section we have stated how the antibiotic dosage affects the population plasmid fraction. Now need to understand how often the antibiotic shocks are needed to avoid plasmid loss and how this periodicity is affected by the antibiotic dose.

Similarly than the simulations before, we performed simulations using the plasmid bearing strain measured parameters but instead applying antibiotics daily, we varied the frequency of the antibiotic peaks to see whether the plasmids are fixed in the population or not. In Figure 4-B, we fixed $\alpha = 0.99$ and varied the frequency of the antibiotic peaks. We observe that for $T \leq T^* = 15$ the plasmids are maintained whereas for $T > T^*$ the plasmids are lost . In Figure 4-A we fixed $\alpha = 0.5$ and found that for this mild selection the plasmid was lost every time unless antibiotics are used at least every two days.

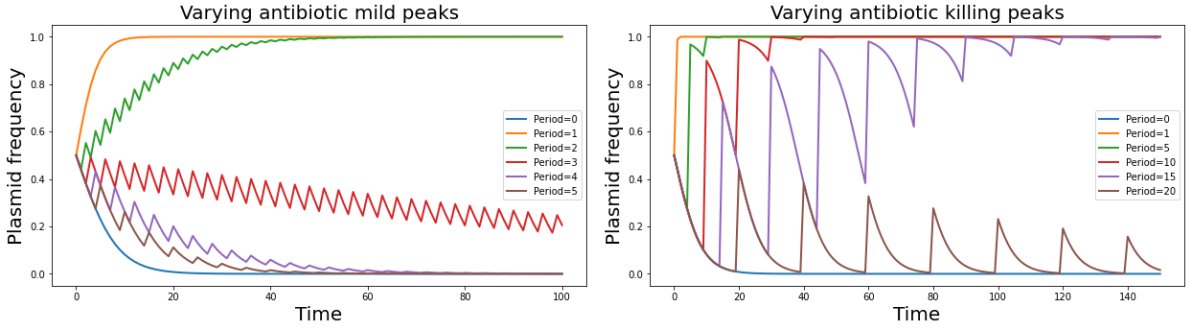

Figure 4 Simulations with multiple antibiotic peaks. **A** Mild selection $\alpha = 0.5 \approx 2\kappa_n$. **B** Strong selection, $\alpha = 0.99$. Period, T, is the time period between two antibiotic peaks. Observe that for mild selection, rapid plasmid rescue is needed whereas with strong selection stability can endure longer periods.

Now we wanted to simulate the utopian experiment proposed so we made use of the experimental frequency changes maps and performed a parameter fitting using our model. We first estimated the κ_n using the function $f(X_i)$ from equation 0.3 from the antibiotic-free experiment. Once we had this κ_n=0.215, we proceeded to estimate the corresponding α for each antibiotic concentration using function $f(g(X_i))$. Allowing us to predict the dynamics of the populations during several days under distinct fluctuating regimens.
Figure 5 shows estimated dynamics of plasmid stability under different antibiotic concentrations during a serial dilutions experiment. Note that in Figure 5-A for small antibiotic dose plasmids are loss around day 100. For concentrations higher than 3 mg/l plasmids are preserved within a range of periods without seeing antibiotics and after periods of 50 days stability begins to decay. This results are expected

taking into account the results shown in figure 3.
The matrix rows for concentrations 1,2, and 2.5 showing less stability than the are a consequence of negative αs caused by the Eagle effect discussed above.

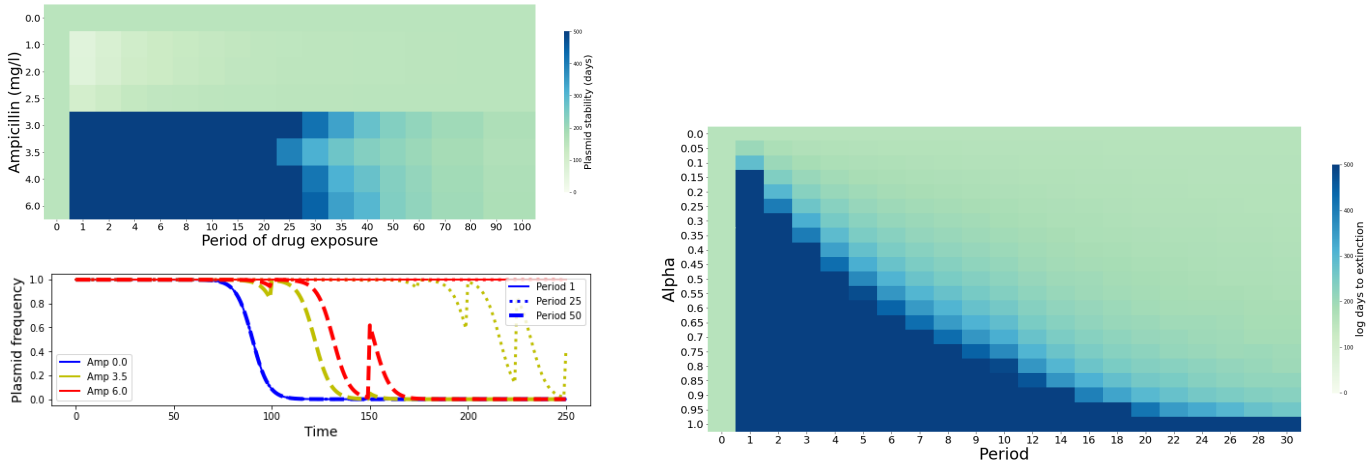

Figure 5 Stability experiment predictions **A** Simulations using experiment αs. **B** Sample trajectories using experiment αs. **C** Simulations plasmid stability using abstract αs. Colormap indicate the duration of the stability, dark blue color indicate period-α combinations that lasted 500 days or more and light-green color present the base time to plasmid loss.

Adding antibiotics should imply positive αs, we then disregarded the αs estimated from our experiment and performed simulations using the pBGT plasmid cost and its copy-number for a range of αs and periods. As expected, we found a strong correlation between the periodicity of the antibiotic pulses and the killing rate. 5-C shows the times to plasmid loss of our simulations using multiple combinations of periods and αs, dark-blue zones represents combinations that showed greater stability while light-green color indicates the time to plasmid loss of the plasmid population under a no-selection regime. Stability areas indicate that for small antibiotic doses is required rapid plasmid recovery. Conversely, for strong doses the plasmid is stable for longer periods without antibiotic exposure.

Plasmid copy-number and stability

In the fitness cost section, we found that there is an optimal range plasmid copy-numbers that enhance stability without selection, and that this property is not linear with respect to the copy-number. In this section, by means of our model, we will explore how plasmid copy-number affects stability under selective regimes.
First, we analyzed the effect of the selection strength over plasmid copy-number. We assumed that $\kappa_n = 0.215$ is proportional to n and that the number of plasmids per cell in the experiment was 35, so that the cost of the plasmid (measured) is $0.00615 \times n$. We then made simple simulations varying the number of plasmids n for daily mild and strong selection treatments.

In Figure 6-A and 6-B we added antibiotic peaks daily with different strengths ($\alpha_n = \alpha = 0.5$ or 0.99). We observed that, if the peaks are not very strong ($\alpha = 0.5$), the plasmids are only maintained for values of n (<=81) that yields a total cost less than the α used, as we have previously stated. Now note that if n is too low they are not completely lost but the mutation rate is so high that maintains the plasmid

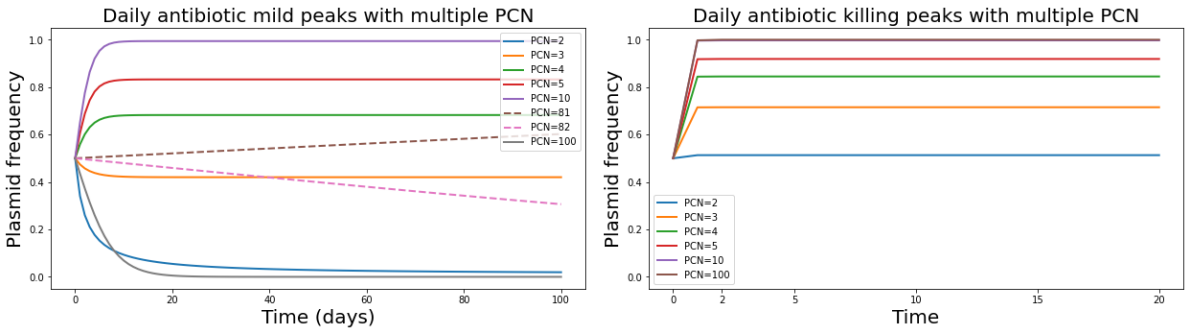

Figure 6 Simulations for multiple PCNs with antibiotic peaks very day **A** Mild selection $\alpha = 0.5, n = 2, 3, 4, 5, 10, 81, 82, 100$. Dashed lines indicates copy-numbers where the total cost is less or greater than α showing the fixation or loss of plasmids. When $n = 2$, the probability of losing a plasmid in a reproduction even is so high that the plasmid fraction stays low. **B** Simulations with antibiotic killing peaks every day $\alpha = 0.99$, $n = 2, 3, 5, 10, 100$. The plasmid is never lost and the dynamics fixes quickly to a fraction ruled by the segregational rate.

is low frequency and if n is too high they are lost because of the cost is too high regardless of a low segregational rate, μ_n. However, if the antibiotic peaks are very strong ($\alpha = 0.99$), the plasmids are maintained and the dynamics stabilizes quickly to a fraction govern by both the plasmid segregational rate and selection strength.

Then we look for joint effect on stability of plasmid copy-number, selection strength, periodicity of peaks, and costs. We performed simulations using 100 values for each the parameters ($n, T \in [0, 100]$, $\alpha \in [0, 1]$, and $\kappa \in [0, 0.05]$) and look for the time it took for the plasmid bearing population to extinct. For convenience, we limited simulations to 500 days and set the time to plasmid loss to -1 when plasmids total cost was greater or equal to 1, because it does not have biological meaning. We focused in three interesting cases:

1) We fixed the cost per plasmid of pBGT ($\kappa = 0.011$) and explored the parameter space for range of values of alphas, plasmid copy number and periods. Simulations results are summarized in Figure 7-A, as expected, the simulations that showed major stability, the upper dark-blue zone, are those with strong antibiotic dosage and short periods without antibiotics diminish only for low plasmid copy numbers. Additionally, another zone of stability is reached for high copy numbers for a range of αs and periods until the total cost is too high and the model does not cope, this zone es marked by the lower-right little red triangle.

2) In simulations of 7-B we applied daily antibiotic peaks and varied plasmid copy-number, selection strength and the cost per plasmid. We observe a great area of stability that propagates from high values of αs and low costs (upper corner) to low α values with low costs and high copy-numbers (lower right corner). It also propagates to high cost values with low copy-numbers and low α values but with less time to plasmid loss.

3) We fixed $\alpha = 0.99$ and explored stability for a multiple copy-numbers, costs and periods, Figure 7-C We can see three zones of high stability at the three corners joined by another high stability zone for high copy-numbers, low costs and all range of periods. We can also notice a medium stability zone for copy-number less than 10, and equally than the case 2, the red zone only indicates the region discarded by cost.

Finally, we performed exhaustive simulations to analyze all the parameters combinations using the parameters ranges described in the example cases simulations above. The overall results are shown in Figure 8-A, but let us first analyze the sample heatmap in 8-B resulting from fixing period to 25 and α to 0.3. The region covering low copy-numbers and high cost (lower-left corner) is showing stability

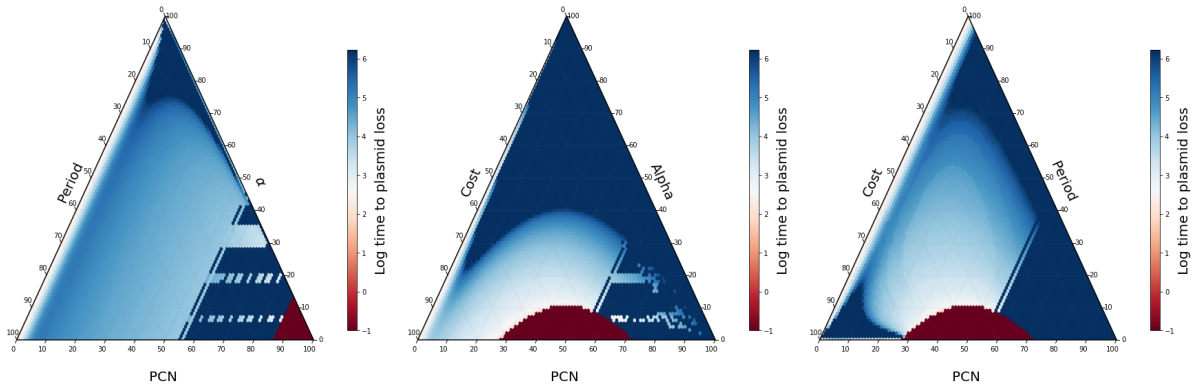

Figure 7 Multiple parameters simulations. Color indicate the time required to lose the plasmid. To improve visualization times are shown in log scale and in the cases when total cost is greater than 1, times are set to -1. **A** Simulations using pBGT cost per plasmid. **B** Simulations using daily peaks. **C** Simulations using an antibiotic killing dose. Dark-blue areas represent simulations reaching maximum stability (500 days), red zones indicate total costs greater than 1.

accompanied by a small region of smaller copy-numbers values for low costs. The red area on the region for high copy-numbers and high cost on the lower-right corner indicates that the total cost of the plasmid is greater than 1 and finally the for high copy-numbers and low costs we can observe the plasmids are lost. Another remark that can be seen in Figure 8-B is that when dealing with really low costs stability increases with the cost regardless of the copy number. Now if sum up the effects of fluctuating selection and the selection strength, we can notably see how frequent antibiotic peaks and high doses stabilizes most of copy-number and cost relations, as shown in the sub-figures on the lower-left corner of Figure 8-A. In fact, only is this zone the sub-corners for high copy-number and low cost are stabilized. If we look the individual effects of selection strength or frequency of peaks, columns and rows in Figure 8-A respectively, we can clearly notice that the strength of selection, as well as frequent antibiotic peaks increases stability for some the copy-number and cost combinations.

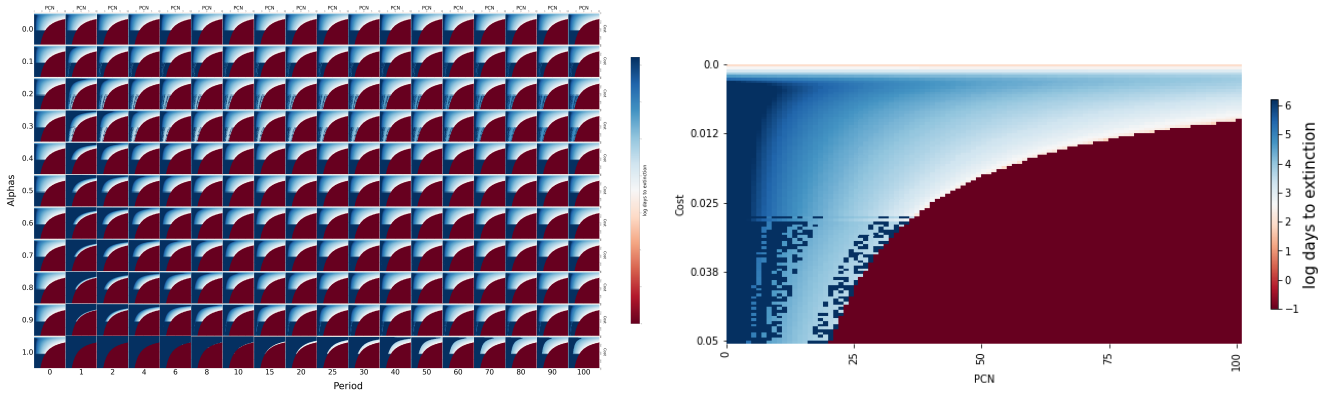

Figure 8 **A** Matrix of heatmaps for all parameter combinations **A** Sample heatmap from **A** for $\alpha = 0.3$ and period period $T = 25$. Color indicate the time required to lose the plasmid. To improve visualization times are shown in log scale and in the cases when total cost is greater than 1, times are set to -1. Dark-blue areas represent simulations reaching maximum stability (500 days), red zones indicate total costs greater than 1.

Discussion

In this work we have used a novel approach to study multi-copy plasmids stability, a population genetics Wright-Fisher model. This a 2-types model were types whether carry plasmids or not. To properly asset our goal, we consider biological implications: 1) Plasmids encode for accessory genes that confer an advantage in harsh environments, for instance antibiotic resistance genes. 2) Bearing plasmids is associated with a fitness cost in absence of selection. 3) Each plasmid is segregated randomly to a daughter cell upon division. Thus, 4) plasmid bearing bacteria can produce plasmid-free cells with a probability of $1/2^n$, were n is the plasmid copy number. 5) Selection kills plasmid-free bacteria with a variable rate.

We validated our model using a well characterized model plasmid used in (San Millan *et al.* 2016; Rodriguez-Beltran *et al.* 2018; Hernandez-Beltran *et al.* 2020). By analyzing growth kinetics of the plasmids-bearing and the plasmids-free strains we estimated our model parameters and calculated a fitness cost which differed from the typically calculated from growth rates. This issue could be due to the fact that growth rates is only one component of fitness. By performing simulations without selection we found that for a given cost per plasmid there is a range of plasmid copy numbers that enhance plasmid maintenance.

We also performed simulations with different antibiotics concentrations found that selection is always necessary to stabilize the plasmid for long periods, and that the strength of selection is highly correlated with the final fraction of plasmids in the entire population. Consequently, we also found that there is a trade-off between selection strength and plasmid cost, i.e. the cost per plasmid and the plasmid copy number.

Our approach also allows for seasonal treatments, a situation more likely to occur in natural environments. By performing simulations we found that foretold trade-off can be ameliorated by the frequency on the antibiotic peaks.

Whether plasmids are maintained or lost in the long term is a complex interplay between the plasmid copy number, the cost per plasmid, the intensity, and frequency of the antibiotic peaks. This relationships are not necessarily linear, as seen in the exhaustive simulations exploring a wide-range of parameters combinations. We argue then that this model could be easily applied to make informed decisions for a specific plasmid maintenance, for instance in biotechnology whereabouts.

Acknowledgements

We thank A San Millan for the generous gift of the strains and for useful discussions. JCRHB is a doctoral student in Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México, and received fellowship 59691 from CONACYT. RPM was supported by PAPIIT-UNAM (grant IN209419) and by CONACYT Ciencia Básica (grant A1-S-32164).

Appendix

Function analysis

Let $\overline{f} = f \circ g$. We want to analyse the behaviour of $\overline{f}$ in terms of κ_n and α_n. Observe that $\overline{f}$ is increasing since

$$\overline{f}'(x) = \frac{(1-\kappa_n)(1-\alpha_n)}{(1-\alpha_n + x(\alpha_n - \kappa_n))^2}(1-\mu_n) > 0.$$

If $\alpha_n = \kappa_n$, then $\overline{f}(x) = x$. If $\alpha_n \neq \kappa_n$, then

$$\overline{f}''(x) = \frac{-2(1-\kappa_n)(1-\alpha_n)(\alpha_n - \kappa_n)}{(1-\alpha_n + x(\alpha_n - \kappa_n))^3}(1-\mu_n).$$

Since $x \in [0,1]$, we have that $1 - \alpha_n + x(\alpha_n - \kappa_n) > 0$. Therefore, if $\alpha_n > \kappa_n$ then $\overline{f}$ is concave and if $\alpha_n < \kappa_n$, $\overline{f}$ is convex.

Bacterial strains and media

For plasmid free strain we used *E. coli* K12 MG1655 and for plasmid bearing strain we used the strain MG/pBGT (San Millan *et al.* 2016) carrying the multicopy plasmid pBGT with the β-lactamase $bla_{\text{TEM-1}}$ which confers resistance to ampicillin and the fluorescent protein GFP under an arabinose inducible promoter.

Overnight cultures were grown in flask with 20 ml of lysogeny broth (LB) (Sigma L3022) with 0.5 % w/v L-(+)-Arabinose (Sigma A91906) for fluorescence induction, in a shaker-incubator at 220 RPM. and 37 deg *C*, for plasmid bearing strain, 25 mg/l of ampicillin (Sigma A0166) were added to eliminate segregants. Ampicillin stock solutions were prepared at 100 mg/ml directly in LB and sterilized by 0.22 μm (Merck Millipore/Millex-GS SLGS033SB) filtering. Arabinose stock solutions were prepared at 20% w/v in DD water and sterilized by filtration.

To construct our inoculation plate, overnight cultures of the parental strain and the pBGT plasmid bearing strain were adjust to 1 OD (630 nm) using a BioTek ELx808 Absorbance Microplate Reader diluted with fresh ice cooled LB. Appropriate volumes were mixed to make co-cultures at fractions 0, 0.1, 0.2, ..., 1 and set column-wise on a 96 well plate (Corning/costar CLS3370) .
Competition experiments were performed using 96 well plates with 200 μl of LB with 0.5% w/v arabinose, and respective ampicillin concentrations: 0, 1, 2, 2.5, 3, 3.5, 4, and 6 mg/l was implemented by plate rows. Antibiotic plates were inoculated using a 96 Pin microplate replicator (Boekel 140500), flame sterilization was made before each inoculation. Four replicates plates were grown in static incubator at 37 deg *C*. After 24 hours growth, plates were read in a fluorescence microplate reader (BioTek Synergy H1) using OD (630 nm) and eGFP (479,520 nm) after 1 minute shaking. Growth kinetics measurements were performed in the same media conditions, plates were sealed using X-Pierce film (Sigma Z722529), each well seal film was pierced in the middle with a sterile needle to avoid condensation. Plates were grown at 37 deg *C* and reading for OD and fluorescence were made every 20 minutes after 30 seconds linear shaking.

Model kinetics parameters were estimated using the R R Core Team (2020) package growthrates Petzoldt (2019). Exponential phase duration,σ, was calculated by finding lag phase duration using the linear model and the time to reach carrying capacity, found using the non-linear growth model Baranyi. Maximum growth rates, r and $r + \rho$, were estimated using the smooth spline method.

Plasmid fraction determination

To calculate fluorescence intensity values, we first subtracted the background signal of LB for fluorescence and OD respectively, then the debackgrounded fluorescence signal was scaled dividing by the debackgrounded OD. The measurements for our inoculation plate showed a strong linear correlation ($R^2 = 0.995$) between co-cultures fractions and fluorescence intensity. This allowed to directly approximate the populations plasmid fractions from the readings of our competition experiments. We normalized the data independently for each antibiotic concentration taking the average measurements of the 4 replicates. Plasmid fractions, PF, were inferred by normalizing the mean fluorescence intensity for each well, f_i, to the interval [0,1] using the following formula: $PF_i = f_i - f_{min}/(f_{max} - f_{min})$ were f_{max} and f_{min} are the mean fluorescence intensities of the fractions 1 and 0 respectively.

Tables

Parameter	Measured value	Formula	Estimated value	Description
r	0.43543	NA	NA	plasmid strain growth rate
ρ	0.05233	$\rho = (1 - k_n)/(e\sigma)$	0.05397	WT growth rate advantage
σ	6.07408	$\sigma = log(\gamma)/r$	6.07408	exponential phase duration
γ	100	$\gamma = e^{r\sigma}$	14.08165	population growth factor
μ_n	x	$\mu_n = 1 - (1 - 2^{-n})^d$	1.267208e-05	1-day fraction of segregants
k_n	0.10888	$k_n = (\rho(1 - e^{-(r2^{-n}+\rho)\sigma}))/(\rho + r2^{-n})$	0.27231	fistness cost
d	x	d=$log(\gamma)/log(2)$	6.64385	number of divisons per unit of time
n	19	NA	NA	plasmid copy number

Table 1 Model kinetic parameters

Amp	gamma	alpha
0.0	0.21515091329917516	0.0
1.0	0.21515091329917516	-0.4859447524378162
2.0	0.21515091329917516	-0.437254204890177
2.5	0.21515091329917516	-0.1415237417290935
3.0	0.21515091329917516	0.9923556690497974
3.5	0.21515091329917516	0.9785392221689677
4.0	0.21515091329917516	0.9914541602258449
6.0	0.21515091329917516	0.9932394293058193

Table 2 Selection parameters

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Capítulo 4

Heterogeneidad fenotípica producida por plásmidos multicopia

4.1 Introducción

En el capítulo anterior hemos abordado teóricamente el problema de estabilidad de plásmidos multicopia. Establecimos que, la naturaleza multi-copia de los plásmidos pequeños puede producir, bacterias segregantes a través de ruido en el proceso de segregación de plásmidos.

Por otro lado, si centramos nuestra atención en el proceso de replicación, éste no sólo es responsable de mantener bajo control el número de copias por célula, sino también es una fuente generadora de ruido, probablemente de mayor relevancia para la dinámica adaptativa. Este ruido proviene del hecho que el mecanismo de control (autoregulación por RNAI y RNAII) no es preciso y, por ejemplo, dos bacterias que heredan el mismo número de plásmidos pueden terminar con distintas copias. Si extrapolamos estos dos procesos a una población, encontramos que existe una gran variabilidad entre número de copias entre distintas células. Si un plásmido de este tipo porta un gen benéfico, entonces distintas células portarán distinto número copias del gen y por lo tanto presentarán distintos fenotipos. Debido a que el modelo de estudio utiliza un plásmido multicopia con un gen de resistencia a antibióticos, esto podría resultar en heterogeneidad en los perfiles de resistencia de las células que componen una población. En este capítulo nos enfocamos en estudiar la dinámica de plásmidos en la población, con el objetivo de explorar las ventajas ecológico-adaptativas que esta variabilidad conlleva.

El enfoque de este capítulo es multiescala. A nivel de poblaciones, realizamos experimentos evolutivos donde expusimos a antibióticos a distintas poblaciones de bacterias portando un gen de resistencia, tanto en un plásmido multicopia como en el cromosoma.

Utilizando citometría de flujo pudimos evaluar cómo los antibióticos modulan la distribución de plásmidos que presentan distintas poblaciones. A nivel de células individuales, utilizamos un sistema experimental de microfluidos que nos permite controlar las condiciones ambientales y realizar observaciones en tiempo real mediante microscopia de fluorescencia. Este enfoque experimental se complementa con un modelo estocástico de agentes individuales, donde simulamos computacionalmente cambios temporales en la distribución de plásmidos en una población ante pulsos de antibióticos.

En específico, evaluamos explícitamente las ventajas adaptativas de que una población presente variabilidad de número de copias en comparacación con poblaciones que no presentan variabilidad, concluyendo que los plásmidos pueden actuar como vehículos que facilitan la rápida adaptación a ambientes hostiles al aumentar la plasticidad

fenotípica de la población. Conjuntamente, los resultados teóricos y experimentales nos permiten concluir que, al ser portado en un plásmido multicopia, la variabilidad en número de copias del gen de resistencia se encuentra correlacionada con la resiliencia de la población a cambios ambientales súbitos.

4.2 Modelo de agentes individuales

Este es un modelo mecanístico con el cual simulamos bacterias como un objeto computacional. Estas bacterias cuentan con distintas propiedades como un identificador, nivel de energía, parámetros metabólicos, y tres genotipos, contamos con el silvestre, una que porta el gen de resistencia en cromosoma y una que porta el gen de resistencia en un plásmido multicopia. El número de copias asociado a cada bacteria se obtiene a partir de una distribución normal con media μ y desviación estándar σ, asociadas a la población en general.

Para modelar el crecimiento poblacional, consideramos un recurso limitante ambiental R y cada bacteria incorpora recursos considerando parámetros metabólicos individuales. La incorporación de recursos sigue una dinámica tipo Michaelis-Menten mediante una función de crecimiento $G(R) = c \cdot u(R)$ donde c representa la eficiencia de conversión de recursos y está asociada al número de copias de cada bacteria, y $u(R)$ es la función de incorporación de recurso general y tiene la forma $u(R) = (V_{max} \cdot R)/(K_m + R)$ con V_{max} representando la máxima tasa de incorporación y K_m la constante de saturación media.

Para tomar en cuenta la acción del antibiótico, consideramos el perfil de resistencia de las cepas que portan el plásmido; así como también los perfiles de resistencia de las cepas que portan el gen de resistencia en cromosoma y para la cepa silvestre. Con esta información asociamos el número de copias de cada gen a su nivel de resistencia mediante una regresión lineal. Así, calculamos el perfil de susceptibilidad/resistencia considerando el número de plásmidos que cada bacteria porta.

Con estos supuestos, una realización del modelo consiste en la siguiente heurística: las bacterias incorporan recursos hasta que alcanzan un nivel de energía crítico (cATP), cuando éste es alcanzado entran en un proceso de división el cual consiste en dividir su energía inequitativamente entre la célula madre y la hija, repartir los plásmidos entre la célula madre y la hija, tomando en cuenta que cada plásmido se segrega al azar con una probabilidad de 0.5; después entrar en un proceso de replicación de plásmidos donde para cada una de las bacterias, se calcula el número final de plásmidos obteniendo un número al azar de la distribución Normal(μ, σ).

Como los antibióticos son β-lactámicos, consideramos que estos tendrían más efecto en bacterias que crecen con una mayor velocidad. Para ello establecimos otro umbral para el máximo ATP ganado el cual se modifica con ruido. Entonces consideramos la concentración de antibióticos en el ambiente y el perfil de resistencia descrito anteriormente para decidir si la bacteria sobrevive o muere, también con otro nivel de ruido.

También tomamos en cuenta que las bacterias tienen la capacidad de degradar el antibiótico a nivel intracelular y mediante esta degradación reducir la concentración de los antibióticos en el ambiente.

Iteramos este proceso para cada bacteria en la población en cada momento del tiempo. Usualmente cada realización consta de 24 horas y con esto somos capaces de realizar experimentos evolutivos donde cada día tomamos una muestra de la de la realización anterior y la sometemos a una nueva concentración de antibióticos.

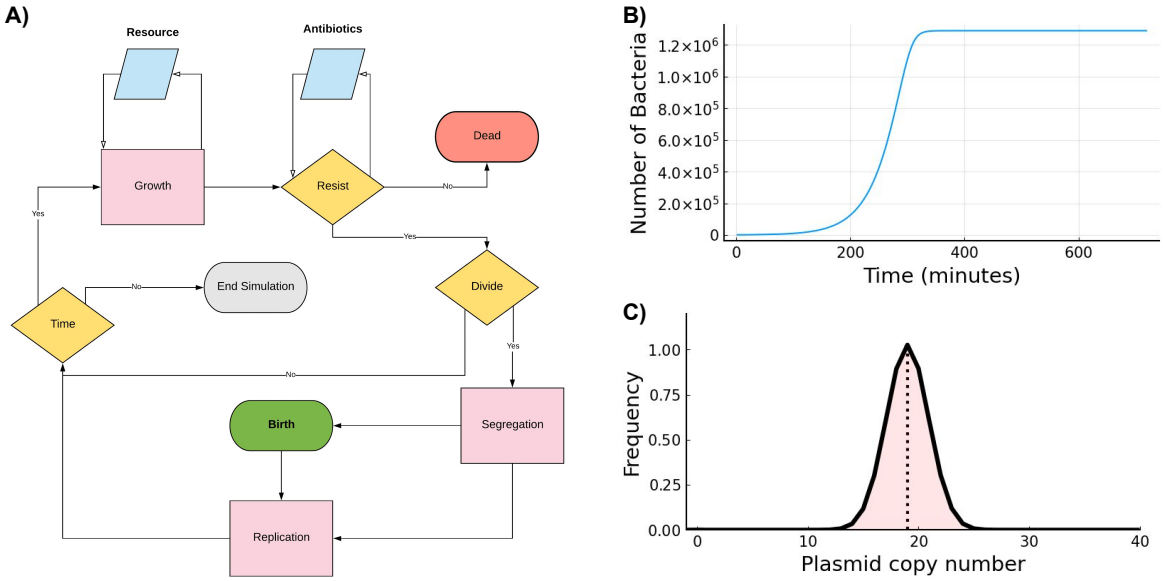

Figura 4.1: Modelo de agentes individuales de plásmidos multicopia. **A)** Diagrama de flujo de la heurística del modelo. Los rombos (amarillo) indican toma de decisiones, los rectángulos (rosa) indican procesos, los paralelogramos (azul) indican entrada y salida de datos, y los óvalos indican inicios y términos. **B)** Simulación de crecimiento poblacional. Simulación con un recurso limitante y sin antibióticos, con una población inicial de 1 000 bacterias, al cabo de 7 horas la población deja de crecer alcanzando 1 289 163 células. **C)** Distribución del número de copias del plásmido en la población. En la simulación se considera una media poblacional de 19 copias con un coeficiente de variación de 0.05.

En la Figura 4.1-A se muestra el diagrama de flujo de la heurística del modelo, la Figura 4.1-B muestra el crecimiento poblacional que llega a fase estacionaria dado que consideramos un recuerso limitante. En la Figura 4.1-C podemos observar la variabilidad de número de copias en la población, para estas simulaciones hemos utilizado una $\mu = 19$ y coeficiente de variación de 0.05 ($\sigma = 0{,}95$)

Nota: este trabajo está en progreso. Falta realizar un mayor análisis de los experimentos de microfluídica.

Antibiotic heterorresistance to β-lactam antibiotics generated by multicopy plasmid dynamics

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ABSTRACT

Bacterial communities implement different strategies to survive in unpredictable and hostile environments. Sensing the surroundings and triggering stress response mechanisms to regulate the metabolic machinery accordingly is a pervasive strategy that increases microbial survival in dynamic environmental conditions. But precise responsive regulation may not be optimal if environmental perturbations are frequent or environmental cues are unreliable. Indeed, if the environment rapidly turns for the worse, persistence of the bacterial community may rely on the preexisting diversity of the population. Here we argue that heteroresistance in plasmid-bearing populations is a consequence of the stable co-existence of cells with high- and low-plasmid-copy numbers that emerge from the underlying replication and segregation dynamics of multicopy plasmids. By using a combination of stochastic simulations of a computational model with high-throughput single-cell measurements of $bla_{\text{TEM-1}}$ expression, we show that multicopy plasmids can provide *Escherichia coli* MG1655 with transient resistance to a lethal concentration of β-lactam antibiotics. Using fluorescence microscopy, single-cell microfluidics, and image analysis, we demonstrate that cell-to-cell differences in the expression of $bla_{\text{TEM-1}}$ produce an asynchronous stress response that increases survival of plasmid-bearing populations to fluctuating environmental conditions. Our results provide further support to the tenet that plasmids are more than simple vehicles for horizontal transmission of genetic information between cells, as they can also drive bacterial adaptation in dynamic environments by providing a platform for rapid gene amplification that can lead to treatment failure in the clinic.

Keywords: antibiotic heteroresistance, stochastic plasmid dynamics, phenotypic heterogeneity

Introduction

The evolution and spread of antimicrobial resistance in clinical pathogens represent a major public health problem that threatens to become a global crisis.[1] In general, drug resistance is considered to be the consequence of stable genetic mutations**?** or antibiotic resistance genes acquired through horizontal gene transfer.[2] But treatment failure can also result from a sub-population with an increased level of resistance compared with the mean resistance of the population.[3] This is known as heteroresistance[4,5] and has been identified in different bacterial species and to a wide range of antimicrobial classes.[6–8]

Multiple genetic and metabolic mechanisms can produce heterogeneous resistance levels in the population. For instance, increased tolerance to bactericidal molecules can be achieved through a subset of dormant cells, known as persisters, that survive drug exposure and resume growth once the antibiotic is withdrawn.[9] It has also been reported that cooperative resistance mechanisms, for example based on the production of drug-degrading enzymes[8] or signaling molecules,[10] can generate multiple sub-populations with varying degrees of resistance. Also, genes that encode for intrinsic antibiotic-resistance mechanisms, for example efflux pumps, can be stochastically expressed and yield phenotypically-diverse populations.[11,12]

Previous laboratory studies[8,13,14] have demonstrated that rapid adaptation to antibiotics can be achieved through genomic duplications that increase dosage of known drug-resistance genes, for example efflux pump operons[15,16] or genes encoding for drug-modifying enzymes.[17,18] Genomic amplifications are known to scale-up with the strength of the selective pressure[13] and are unstable in the absence of selection due to the fitness burden associated with the duplication of large-scale chromosomic regions.[13,19,20] So, at least in laboratory conditions, rapid amplification and attenuation of gene copy-number provides an effective strategy to increase adaptation in fluctuating environments.

In the clinic, heterorresistance due to spontaneous tandem gene amplifications has been proposed as a plausible cause for treatment failure,[21] with incidences most likely underestimated due to intrinsic limitations of standard microbiology assays.[5] A recent large-scale analysis of heteroresistant clinical isolates found a high incidence of genomic amplifications that increased resistance to multiple antibiotics.[22] Interestingly, whole genome sequencing revealed that, while some duplications were found in large chromosomal regions containing known drug resistance genes, a considerable fraction of sequence amplifications were found in extrachromosomal DNA. This observation is consistent with experimental evolution studies that have suggested that plasmids can enhance genetic diversity in the population[23] and accelerate bacterial adaptation by increasing the probability of appearance of beneficial mutations and subsequently amplifying their expression.[24]

Here we show that cell-to-cell differences in plasmid copy number can promote heterorresistance in a population of *Escherichia coli* MG1655 carrying a non-conjugative, multicopy plasmid with a fluorescent reporter and $bla_{\text{TEM-1}}$, a gene that confers resistance to ampicillin and other β-lactam antibiotics. Using a combination of computer simulations with single-cell and population-level experiments, we will argue that plasmid-driven phenotypic noise can increase adaptation of bacterial populations to stringent and rapidly changing environmental conditions, like those imposed by antimicrobial treatment.

Results

Plasmid segregation and replication dynamics produce heterogeneous populations

Plasmids are extra-chromosomal DNA molecules that replicate independently of the bacterial genome and can harbor drug resistance and virulence genes and, as a result, are considered significant drivers of the evolutionary dynamics of antibiotic resistance.[25] As illustrated in Figure Figure 1A, the number of plasmid copies carried by each cell is controlled by an inherently stochastic process with two main sources of intrinsic noise: plasmid replication and plasmid segregation.

Plasmid replicate in discrete events that occur throughout the cell cycle, until reaching an upper limit that varies from a few copies to several hundred for high copy-number plasmids. While large plasmids (20-500kB) contain the molecular machinery necessary to conjugate, small plasmids (1-10kB) tend to be non-mobilizable and present in multiple copies.[26] We therefore assume that the probability of plasmid replication decreases as plasmids accumulate inside the cell.

Although plasmids are excluded from the nucleoid and tend to accumulate at the poles, single-molecule microscopy studies have shown they can travel across the cell body and segregate randomly upon division.[27,28] As a result, in plasmids lacking active partitioning or post-segregational killing mechanisms, the probability that a plasmid is inherited to a given cell can be seen as a random process that follows a binomial distribution (although for very high-copy plasmids, the resulting asymptotic copy-number distribution can be bimodal[29]).

We used a multi-scale computational model that couples the intracellular plasmid dynamics of individual cells with the extracellular drug concentration to obtain a population-level PCN distributions in response to environmental perturbations of different duration and intensity. A description of the model and numerical implementation can be found in the Methods, but, in short, is an agent-based model that implements explicitly cellular processes and their interaction with the environment: cell duplication, resource-dependent growth, antimicrobial-induced death, as well as plasmid replication and segregation. Propensities of each process are determined from the concentrations of limiting resource and a bactericidal antibiotic present in a homogeneous environment (see Supp Fig: Diagram-ABM).

Figure 1B shows numerical realizations of the agent-based model, simulating an exponentially-growing population of cells from a single mother cell (black line). Parameter values were determined from experimental data and reported in Table 2. Note how, as the cell grows and divides, plasmids replicate and segregate randomly, producing a heterogeneous distribution of plasmids in the population. Figure 1C shows the PCN distribution obtained after growth is approximated by a Normal distribution. Furthermore, we estimated the coefficient of variation of the synthetic data as a function of the maximum number of plasmids per cell and observed that mean and variance of the PCN distribution are correlated, resulting in a constant coefficient of variation.

Now, if we consider that the plasmid carries a drug-resistance gene, then a consequence of presenting a heterogeneous PCN distribution is the existence of different subpopulations composed of cells with a range of resistance levels. In the simplest case, we could consider a linear relationship between gene dosage and level of resistance, as observed in genes encoding for drug-modifying enzymes and efflux proteins.[8,30–32] In this case, the likelihood of death of each cell can be estimated from the cumulative

distribution function of the PCN distribution and the extracellular concentration of antibiotic.

For instance, if we assume that every individual in a clonal population is phenotypically identical, then there exists a drug dose, referred to as the Minimum Inhibitory Concentration (MIC), that would kill all cells simultaneously. Therefore the survival probability function of a homogeneous population would be a step-wise function that switches from 1 to 0 at the MIC (black dotted line in Figure 1E). However, if we consider a heterogeneous population characterized by a PCN distribution with large variance, then the population would contain cells with fewer or more gene copies than the expected value (blue lines in Figure 1D). As a result, the survival probability of the plasmid-bearing population should be lower than the expected for sub-MIC concentrations, and larger for high drug concentrations, as illustrated in Figure 1E.

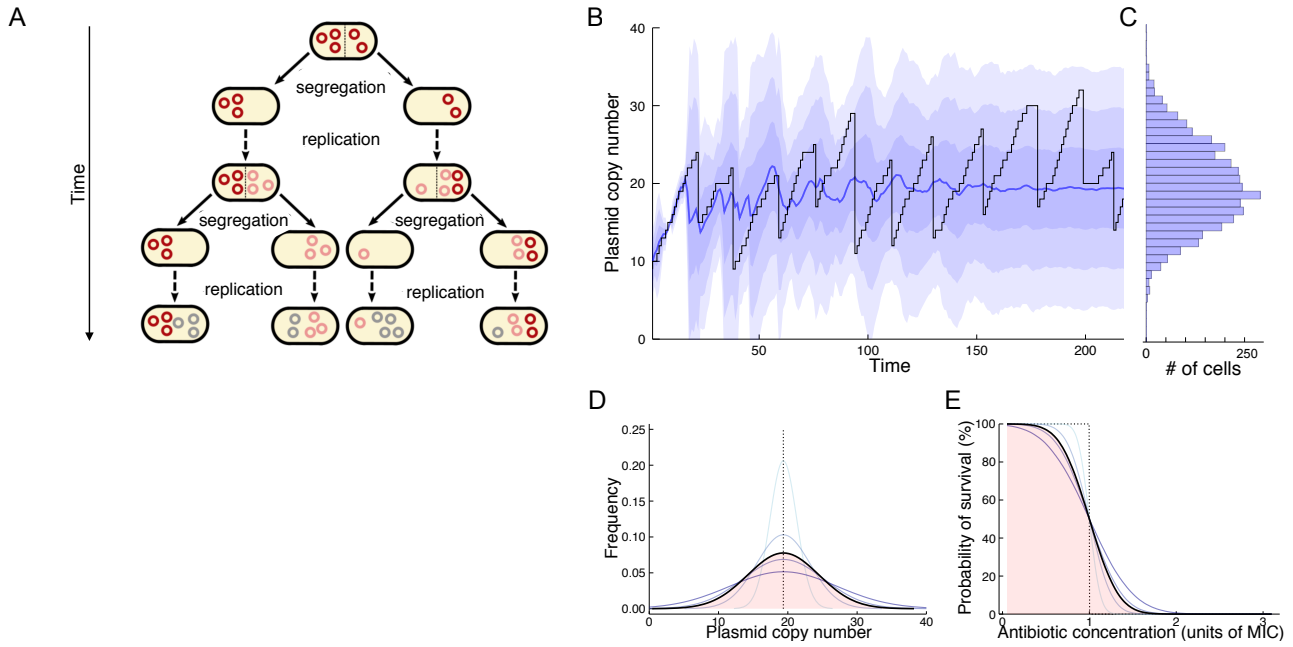

Figure 1. A) Plasmid replication and segregation dynamics diagram. B) 1-day realizations of the model, with color-coded variance of the ensemble of trajectories. C) population-level distribution after T units of time. D) Probability density functions of Normal distributions with a fixed mean ($\hat{\mu}$) and increasing standard deviations ($\hat{\sigma}$). E) Probability of survival as a function of the antibiotic concentration obtained from the cumulative density function of the PCN distribution.

Selection modulates plasmid copy-number distributions in bacterial populations

The simple model described in the previous section predicts that the stochastic nature of the underlying segregation and replication dynamics of multicopy plasmids produces heterogeneous populations composed of cells exhibiting a range of drug-resistance levels. To test this prediction, we used an experimental model system that consists on *Escherichia coli* MG1655 carrying a non-conjugative, multicopy plasmid containing a GFP fluorescent marker (*GFPmut2*) and $bla_{\text{TEM-1}}$, a gene that encodes for β-lactamase, an enzyme that inactivates β-lactam antibiotics by hydrolyzing the β-lactam ring.[33] We will denote this strain MG:pBGT (average copy-number=19.12, s.d.=1.53, n=3[24]) and, as a control,

we used a fluorescently-tagged chromosomally-encoded $bla_{\text{TEM-1}}$ that we represent as MG/GT.

We used a micro-chemostat microfluidic device to expose a population of MG:pBGT to a lethal dose of ampicillin (see Methods). We let cells grow in antibiotic-free rich media (LB) then we switched growing media to LB with a lethal concentration of ampicillin and the red fluorescent marker, rhodamine. With this fashion, bacterial cell-wall is compromised by the action of ampicillin allowing rhodamine to enter the cell and functions as death-marker. Figure 2A shows a montage of green and red channels overlayed. Note that cells that are killed turn from green to red, consistent with a loss of cell-wall integrity.

First note in Figure 2A (top) that some cells have higher GFP intensity than others. In contrast, this cell-to-cell heterogeneity is not observed in MG/GT, suggesting that this variation comes indeed from cell-to-cell differences in PCN.

Also note in Figure 2A how cells that survive for longer are those that present high levels of fluorescence previous to antibiotic exposure, suggesting that fluorescence and β-lactamase are correlated. This is expected, as the plasmid encodes for both *GFPmut2* and $bla_{\text{TEM-1}}$ and therefore the concentration of GFP and β-lactamase molecules depends on the number of plasmid copies carried in the cell.[8,31]

To further validate this association, we used a library of strains obtained in a previous experimental evolution study whereby MG:pBGT was transferred daily to environments that doubled the concentration of a β-lactam antibiotic, ceftazidime.[24] Whole genome sequencing revealed mutations in the origin of replication, two G to U changes at positions 54 and 55 of RNAI placed in the loop of the central hairpin affecting the RNAI-RNAII kissing complex and, resulting in a high mean plasmid copy-number and increased hydrolase production.

Figure 2B) shows a positive correlation between plasmid copy-number (measured using qPCR[34]) and fluorescent intensity (quantified using a fluorescence plate reader). Of course, there are caveats associated with inferring gene copy-number from fluorescent intensity; maturation times of fluorescent proteins,[35] phenotypic delays,[36] phototoxicity and photobleaching,[37] to mention a few. So, to further validate the PCN-GFP correlation and use fluorescent intensity as proxy for PCN, we used a cell sorter to group the population of plasmid-bearing cells based on their fluorescent intensity. In particular, we partitioned the population into clusters with low-, medium- and high- fluorescence and estimated the mean PCN of each subpopulation. As expected, we found a positive correlation between fluorescence and PCN.

As the plasmid also encodes for $bla_{\text{TEM-1}}$, then the concentration of β-lactamase molecules depends on the number of plasmid copies carried in the cell.[8,31] We measured the Minimum Inhibitory Concentration (MIC) of different plasmid-bearing strains and validated previous data showing that PCN and resistance are indeed correlated. Altogether,we conclude that PCN is positively correlated with GFP fluorescence and drug resistance (Figure 2C).

Now, the segregation-replication model described previously predicts that plasmid-bearing populations should produce GFP distributions with a higher mean than the strain with chromosomally-encoded *GFPmut2*. Indeed, fluorescent intensity histograms obtained using flow cytometry show that GFP intensity increases proportionally to the mean PCN. Also, as predicted by the theoretical model, Supp Figure 2 shows how mean and variance of the distribution of GFP intensities for different strains

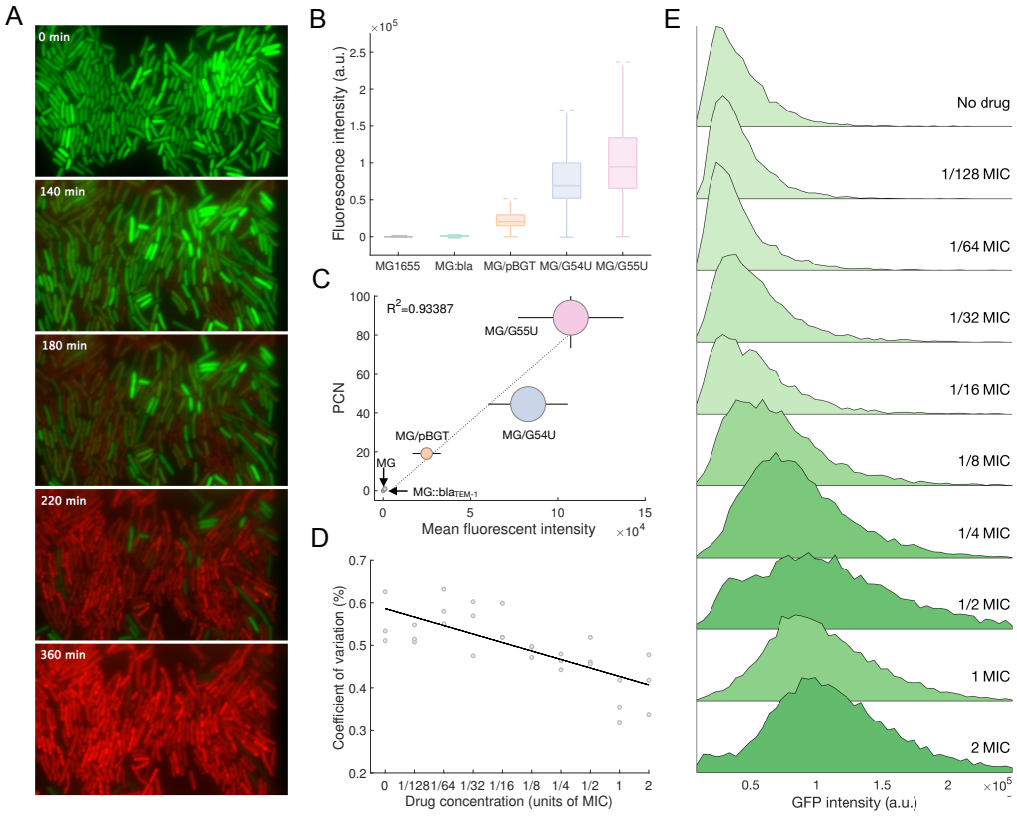

Figure 2. A) Microscopy time-lapse image of an AMP shock experiment. B) Boxpolot of GFP fluorescent intensity obtained for strains with different mean copy numbers. C) Mean fluorescence, mean plasmid copy number and antibiotic resistance are positively correlated in plasmid-bearing populations. D) Coefficient of variation of PCN distributions decreases as the strength of the selective pressure increases. D) Normalized? GFP distributions of MG:pBGT under different antibiotic concentrations. Note that as antibiotic concentration increases, fluorescence distributions skews to the right and CoV is reduced, meaning that antibiotics selects for cells having higher PCN.

are positively correlated, resulting in a constant coefficient in strains with different mean plasmid copy-number.

To evaluate the effect of selection on the shape of the PCN distribution, we exposed MG:pBGT to a range of antibiotic concentrations and used flow cytometry to obtain GFP distributions after 24 hours of drug exposure. Figure 2E shows how as the strength of selection increases, GFP distributions shift towards higher fluorescent values. This is expected, as we have established that resistance and fluorescence are correlated and, as argued using the replication-segregation model, the probability of survival of each cell can be derived from the cumulative density function of the PCN distribution. Also, the coefficient of variation decreases as a function of drug concentration Figure 2D. As a control, we repeated the experiment with MG/GT and observed that mean fluorescence remained constant, independently of the concentration of ampicillin.

Phenotypic noise increases resilience of the population to intermittent drug exposure

We exposed MG:pBGT to a range of ampicillin concentrations (Figure 3A, green line) and, after 12 hours of drug exposure, transferred a sample of each population into a drug-free environment (black line). As expected, at sub-MIC concentrations both strains recovered growth, while at high drug concentrations the population was driven to extinction. Crucially, one of the replicates in MG:pBGT (out of 88) presented growth (i.e. we have heteroresistance).

After growing 88 populations overnight, we transferred them into microtiter plates with a lethal dose of ampicillin. After 30 minutes, we sampled each well using a 96-well pin replicator into a new plate with LB and incubated for 24 hours. We repeated this sampling process every 30 minutes. For each time-point, we counted how many populations were able to survive treatment and recover growth (measured using optical density at 630nm and validated by plating and counting CFUs in drug-free media).

Figure 3B presents Kaplan-Meier plots showing that, as predicted by the model, MG:pBGT exhibits increased resilience to fluctuating environments than MG/GT. We then tested MG:G54U and MG:G55U, also resulting in higher survival rates than MG/GT. We performed a pair-wise Log-rank tests and found significant differences between all plasmid bearing strains and MG/GT, p-value < 0.005. Additionally we only found a significant difference between G54U and G55U.

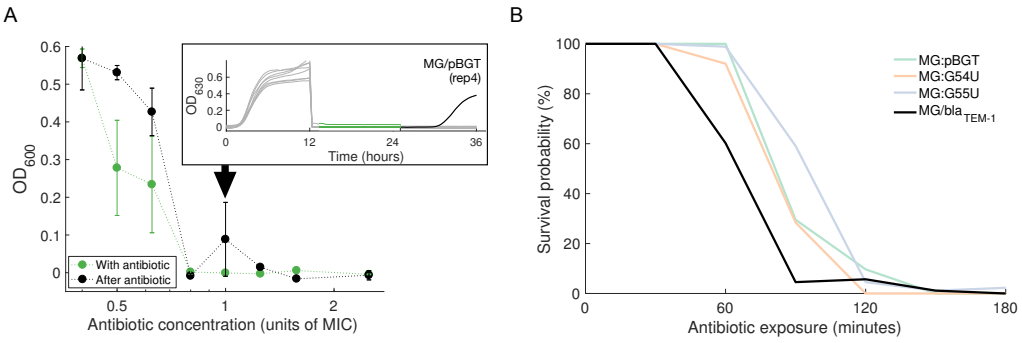

Figure 3. A) Re-growth at lethal doses of antibiotics, 12h experiment. B) Kaplan-Meier: plasmid encoded vs chromosomal encoded resistance.

Now, it is known that plasmid-bearing can be associated with a reduction in growth rate and therefore result in a competitive disadvantage with respect to plasmid-free bacteria.[38–40] To confirm that the increased survival rate in plasmid-bearing populations was not a consequence of the decrease growth rate associated to the inherent metabolic burden of carrying plasmids (instead of resulting from selection in favor of a subpopulation with more copies of $bla_{\text{TEM-1}}$), we repeated the survival experiment for MG:pBGT under the MG/GT amp MIC in the presence of 256 $\mu g/mL$ of sulbactam, a β-lactamase inhibitor. As expected, cells were unable to survive antibiotic exposure and fluorescence remained constant independently of the concentration of ampicillin.

Based on these results, we conclude that plasmid-driven $bla_{\text{TEM-1}}$ variability increases resilience of the population to intermittent ampicillin exposure. But we have not identified the mechanism yet. There are two possibilities for the observed patterns of heterorresistance, first, pre-existing genetic diversity in the

population, or, second, a heterogeneous response to stress consequence of cell-to-cell heterogeneities. Note that we cannot use population-level experiments to contrast both options so, in the next section, we will use single-cell microfluidics to study the response of individual cells to an antibiotic pulse when $bla_{\text{TEM-1}}$ is located in the chromosome or in a multi-copy plasmid.

Heterogeneity in $bla_{\text{TEM-1}}$ expression generates an asynchronous stress response

We have shown that plasmid-bearing populations present heterogeneity in the expression of $bla_{\text{TEM-1}}$ and that selection imposed by antibiotics modulate the shape of the PCN distribution, suggesting the existence of cells producing β-lactamase at a higher rate and hence presenting increased tolerance to the antibiotic. Therefore, even if all cells are exposed to the same environmental concentration of antibiotics, we would still expect heterogeneity in the intracellular concentration of ampicillin between different cells, producing variability in peptidoglycan damage and, therefore, responding to the stress differently. To test this, we used single-cell microfluidics and fluorescent microscopy to measure the level of expression of $bla_{\text{TEM-1}}$ in individual cells (see Methods).

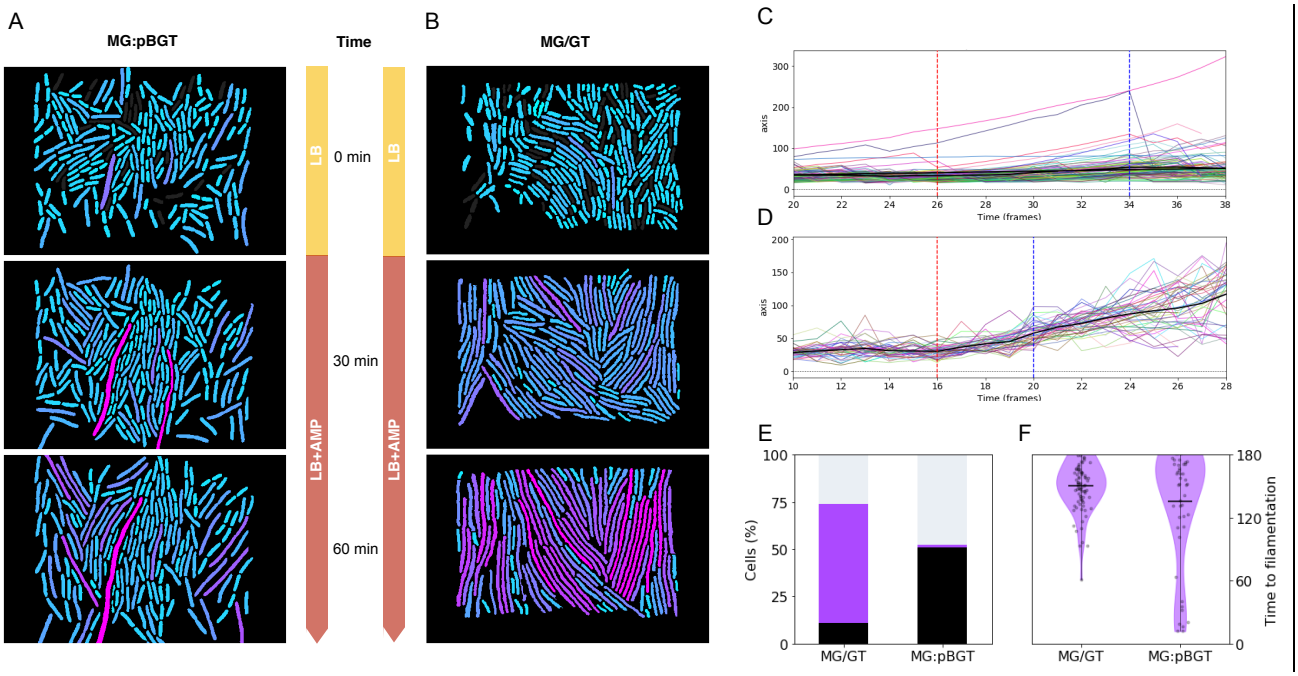

Figure 4. Microscopy montage: cell length vs. time. A) $bla_{\text{TEM-1}}$ carried in a multicopy plasmid (MG:pBGT). B) chromosomally encoded $bla_{\text{TEM-1}}$. C) Cell length as a function of time for MG/GT. D) Cell length as a function of time for MG:pBGT. E) Fraction of dead (black) and live (frey) cells after intermittent antibiotic exposure. Purple area represents the fraction of cells that produced bacterial filaments and survived drug exposure. F) Time elapsed between introduction of the antibiotic and cell elongation. Note how MG:pBGT has a higher variance than MG/GT.

First, note that, similar to our flow cytometry data, distributions of fluorescent intensity obtained using a fluorescent microscopy in plasmid-bearing strains present increased mean and variance compared to MG/GT.

After growing in exponential phase for 8 hours, we introduced a short pulse of a lethal concentration

of ampicillin into the device. Critical concentrations of antibiotics used were determined using dose-response experiments, 32 mg/mL for MG:pBGT and 2 mg/mL for MG.

Figure 4-D illustrate an experiment where a population of MG/GT cells produced filaments simultaneously in response to the presence of ampicillin. Note how, as the antibiotic is introduced, bacterial cells stop dividing but continue growing, thus producing elongated cells. Drug-induced filamentation is a consequence of a general stress response that regulates the expression of over 50 genes,[41] including genes directly involved DNA repair, DNA damage tolerance or the induction of a DNA damage checkpoint that transiently suppresses cell division. This stress response mechanism is known as the SOS system and is known to increase resistance to heavy metals[42,43] and antimicrobial substances,[44,45] as well as to increase genetic variation[46] by promoting bacterial mutagenesis[47] and enabling the horizontal transmission of virulence factors[48] and antibiotic resistance genes.[49]

In particular, binding of β-lactamase molecules to the penicillin-binding protein 3 (PBP3) can trigger the SOS response system through DpiBA, a two-component signal transduction system[50] that induces sulA, which in turn inhibits septation by blocking FtsZ polymerization. As a result, cell division is suppressed and bacterial filaments are produced.[51,52] These bacterial filaments are known to increase tolerance to cell wall damage produced by the antibiotics we use in this study.[53] Crucially, once the antibiotic is removed, filamented cells reorganize the FtsZ ring, divide and resume normal growth,[54] as can be seen in Figure 4D right side.

Using image processing algorithms (see Methods), we also obtained time-series of cell length as a function of time. This allowed us to estimate the time elapsed since the introduction of the antibiotic and the formation of filaments (see Figure 4C,D . This data is consistent with previous studies reporting that the temporal expression of genes in the SOS system is tightly regulated[55]

However, MG:pBGT produces a very heterogeneous response: some cells die, some produce filaments and others continue to grow and divide (Figure 4E). Moreover, from the fraction of cells that produced filaments, the timing of SOS activation varied considerably (Figure 4F).

Discussion

Recent studies have argued that presenting heterogeneity can provide functional benefits for bacterial communities in fluctuating environments,[56] with examples including asynchronous sporulation,[57] metabolic diversification[58,59] and increased tolerance to adverse conditions imposed by phages[60] or antimicrobial substances.[61] Moreover, phenotypic heterogeneity also enables isogenic populations to perform complex tasks through division of labour, with examples ranging from metabolic cooperation[62,63] and biofilm production[64] to virulence[65] and drug resistance.[10]

While the benefits of phenotypic noise may be clear, the underlying molecular mechanisms that stably maintain heterogeneous populations are not. Previous studies have shown that transitions between multiple phenotypic states can be driven by noise in promoters,[66] asymmetry in the cell division process[67] or stochastic fluctuations in the concentrations of proteins and mRNAs present at low-copy numbers in the cell.[68,69] Similarly, stochastic[70,71] and deterministic[72,73] features of gene regulatory networks can also produce phenotypically-diverse populations.

Here we have shown that , even without genetic mutations, multicopy plasmids can generate heteroresistant populations composed at all times of susceptible and resistant cells, therefore increasing tolerance of the population to a sudden increase in drug concentration and recovering fitness once it has been withdrawn.

Materials and Methods

Bacterial strains and culture conditions

In this study we used an *Escherichia coli* MG1655 bearing different versions of the pBGT plasmid. This versions differ on point mutations on the RNAI leading to different plasmid copy numbers. Plasmids and strains were previously characterized on.[24] The plasmid encodes for the β-lactamase resistant gene $bla_{\text{TEM-1}}$ that confers resistance to ampicillin and GFP gene under an arabinose inducible promoter and the araC repressor. Plasmid bearing strains average copy numbers are 19, 44 and 88 for pBGT, G54U and G55U respectively. We also used a strain that has one copy of the same gene, fluorescently tag, but in the chromosome, MGGT.

All experiments were conducted in Lysogeny Broth - Lenox (LB) (Sigma-L3022) supplemented with arabinose 0.5% w/v, antibiotics were supplemented as specified in each experiment. Stocks solutions at 20% of Arabinose (Sigma-A91906) were prepared by diluting 2g of arabinose in 10ml DD water sterilized by filtration. AMP stock solutions (100mg/ml) were prepared by diluting ampicillin (Sigma-A0166) directly in LB and adding arabinose (0.5% w/v)

Antibiotic susceptibility determination

Standard dose-response curves were used to determine each strain minimum inhibitory concentration (MIC). Ampicillin MICs were 2,000, 32,000, 43,000, and 46,000 μg/l for strains MGGT, pBGT, G54U, and G55U strains respectively. Dose-response curve were performed in 96-well plated (Costar CLS3370), plates were sealed using X-Pierce film (Sigma Z722529), each well seal film was pierced in the middle with a sterile needle to avoid condensation. Plates were grown at 37 deg$C$ and reading for OD (630 nm) and fluorescence for eGFP (479,520 nm), were made every 20 minutes after 30 seconds linear shaking using a fluorescence microplate reader (BioTek Synergy H1).

β-lactamase inhibitor experiment

We used Sulbactam (Sigma-S9701-10MG) for β-lactamase inhibition. For this experiment we fixed ampicillin concentration equal to the MIC of the MGGT strain, that is 2,000 μg/l. And then performed a sulbactam dose-respose curve for the pBGT strain and found total growth inhibition with 256 μg/l. Dose-response was performed in 96-well plates We used this concentration to perform the serial dilution experiment of LB-LB+AMP+Sulbactam-LB. Samples of four experiment replicates were used for flow cytometry and, independently another four replicate samples we used for plasmid quantification.

Plasmid copy-number determination

Sample preparation were performed following the protocol in.[74] 100 μl culture samples were centrifuged at 16,000 g for 60", the supernatant was removed and the pellet was resuspended in an equal volume of milliQ water. Then, samples were boiled at 95 °C during 10' using a thermoblock.

Plasmid copy-number were determined by qPCR using the ΔΔCT method following the protocol in.[24] We used the chromosomal monocopy gene *dxs* as reference and specific primers for the $bla_{\text{TEM-1}}$ gene were used as plasmid targets. qPCR reactions were performed using SYBR Select Master Mix (Applied biosystems - 4472908) in a Bio-Rad CFX Real-Time PCR Detection System.

Flow cytometry

Population-level experiment measurements were performed by sampling 1ml of overnight culture and measured their fluorescence using an imaging flow cytometer (Amnis ImageStream Mark II by Luminex). INSPIRE software was used to acquire data discriminating by area, aspect ratio, focus, and side scatter features. GFP fluorescence was excited at 488 nm with 25 mv intensity. Populations data files were processed using IDEAS 6.2 software and feature values were exported and analyzed using bespoke scripts implemented in Python programming language and MATLAB.

Plasmids costs determinations

Growth rates analysis was made using the R package GrowthRates using non parametric smoothing splines fit. Strains were grown in 96-well plates in LB supplemented with arabinose 0.5% w/v. Growth kinetics were done at $37\,\text{deg}C$ for 24 h, reading every 20 min after 30 s shaking in a Synergy H1 microplate reader.

Single-cell microfluidics

We used a microfluidic device built-in PDMS (polydimethylsiloxane) from molds manufactured using soft photolithography (SU-8 2000.5). In particular, we used a micro-chemostat (adapted from**?**) that contains two media inputs and 48 rectangular chambers (40x50x0.95μm^3). Each confinement chamber traps approximately 500 cells in the same focal plane, enabling us to use time-lapse microscopy to follow in time thousands of individual cells.

Drug was introduced into the microfluidic device using a dynamic pressure control system developed with a signal generator and robotic linear actuators.[75] Media inputs are mixed at different proportions using a red fluorescent dye (rhodamine) diluted in one of the media inputs. Growth media was loaded into 60ml-syringes connected to the PDMS chip through Tygon tubes and assorted Luer connectors. The pressure inside the chip is controlled with vertical linear actuators and a digital signal generator that controls the height of each syringe. Initial inoculates of cells were grown overnight in the presence 25 $\mu g/l$ for palsmid maintenance. A sample was then transferred to 200ml of fresh LB and grown at 30°C until reaching an $\text{OD}_{600} = 0.3$. After centrifuging, cells were re-suspended in 5ml of LB, and this dense culture was used to inoculate the microfluidic chip. For all microfluidics experiments, we used LB media supplemented with arabinose at 0.5% and Tween20 (Sigma-P2287) at 0.075%. In all cases, cells were allowed to grow and divide multiple cell cycles in a drug-free environment, allowing us to determine the baseline fluorescent intensity of the population. We later use this value to normalize our data and compare fluorescent intensities during the experiments.

Image analysis

Microscopy time-lapse movies were analyzed using a semi-automated ImageJ**?** analysis pipeline that implements a deep-learning algorithm for image segmentation.[76] Cell tracking and lineage

reconstruction were performed in Python using standard numerical and geometric libraries (NumPy, Shapely, Pandas, Scipy, Matplotlib). Data and code are openly distributed and available for download at https://github.com/ccg-esb-lab/uJ.

In short, the image processing pipeline consists on 1) organizing TIF files generated by NIS Elements, 2) aligning traps and using rigid motion transformation to correct for x-y drift in time-lapse images, 3) using *Parallel Interactive Deconvolution* with a theoretical point spread function generated with PSF Generator to produce a segmentable image, 4) using *DeepCell*[76] to segment images and obtain binary masks, 5) cell detection and automatic correction of ROIs, 6) manual correction of a binary mask, 7) data acquisition by overlapping masks in different fluorescent and bright field channels, 9) cell tracking using a feature-aided nearest-neighbor algorithm and lineage reconstruction and, finally, 10) data analysis and visualization. The goal of this image bioinformatics pipeline is to acquire time-series of fluorescent intensity and other morphological properties of individual cells, as well as to obtain population-level statistics to estimate, for instance, the mean cell duplication rate of the population or changes in the shape of the fluorescence distribution.

Computational model

We developed a stochastic individual based model were individual cells are modelled as computational objects. Each cell may have specific plasmid copy-number derived from a Normal distributions with a specific mean and standard deviation. Cells growth incorporating a limiting Resource with a Michaelis-Menten function, this functions takes into account the remaining common resource and the cost entailed by the number of plasmids. Cells divide when they reach a threshold of "ATP" and upon division, plasmids are segregated randomly to the daughter cells, which began to replicate plasmids following a probability determined by $1 - \mu_i(t)/\hat{\mu}$, where $\mu_i(t)$ denotes the number of copies of a plasmid of type i at time t, and $\hat{\mu}$ the cell-specific maximum plasmid copy number. The action of the antibiotic is implemented using individual resistance/susceptibility profile derived from a linear approximation of the population MIC and population copy-number, determined experimentally.

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Capítulo 5

Heterocigosis Mediada por Plásmidos

En un estudio previo realizado por nuestros colaboradores en el Departamento de Microbiología del Hospital Universitario Ramón y Cajal en España (San Millan et al. 2016) se realizó un experimento evolutivo en el cual se sometieron 48 poblaciones de la cepas MG/pBGT (portadora del plásmido), MG:blaTEM1 (que porta el gen de resistencia integrado en el cromosoma) y MG1655 (silvestre) ante otro antibiótico β-lactámico para el cual el gen $bla_{\text{TEM-1}}$ no confiere resistencia, ceftazidime (Caz). El experimento se realizó durante 16 días, iniciando con 1/4 de la respectiva CMI de cada cepa y cada día se duplicó la concentración de Caz con el fin de ejercer una presión selectiva fuerte y analizar el porcentaje de supervivencia por día.

Se encontró que la cepa que porta el gen de resistencia en el plásmido fue capaz de sobrevivir por mucho más tiempo en el experimento como se muestra en la Figura 5.1-A. El número de copias del plásmido se incrementa significativamente ante la presión selectiva, esto es debido a que ocurren mutaciones en el mecanismo de control de número de copias, además de eso, para que las poblaciones puedan sobrevivir ante presiones selectivas tan altas es necesario que ocurran mutaciones en el gen de resistencia. Se secuenciaron aislados de cada uno de los días y se encontraró que las mutaciones causantes del aumento de número de copias, así como la mutación en el gen de resistencia son mutaciones puntuales, siendo esta última la variante $bla_{\text{TEM-12}}$ que proporciona resistencia a Caz (Mira et al. 2015).

A partir de este experimento, postulamos un modelo de agentes individuales y realizamos simulaciones *in silico* del experimento. Este modelo capturó con precisión cuantitativa los comportamientos poblacionales de las distintas cepas (como se muestra en la Figura 5.1-B), pero también nos permitió analizar el perfil de mutaciones que confieren un mayor número de copias de plásmidos, así como las dinámicas poblacionales de mutaciones en el gen de resistencia (Figura 5.1-C,D).

Interesantemente, el modelo teórico predice que los plásmidos multicopia producen periodos en los cuales el alelo ancestral coexiste con el alelo mutante. Esta coexistencia de alelos ocurre, no solamente entre subpoblaciones portadoras de un solo alelo, sino también a nivel intracelular, debido a que los alelos en cuestión son portados en plásmidos multicopia.

Estas células portadoras de ambos alelos, son en sí, heterocigotas. La coexistencia de plásmidos distintos dentro de una misma bacteria ha sido descrita con anterioridad, se le conoce como heteroplasmia y ha sido estudiada con el objetivo de analizar la compatibilidad de plásmidos, una característica que ha servido para clasificarlos. La coexistencia de distintas versiones del mismo plásmido podría entenderse como un caso particular de heteroplasmia pero no había sido descrito con anterioridad por lo que nombramos a este fenómeno, heterocigosis mediada por plásmidos. Así como para mantener un plásmido con genes no esenciales es necesario mantener una selección

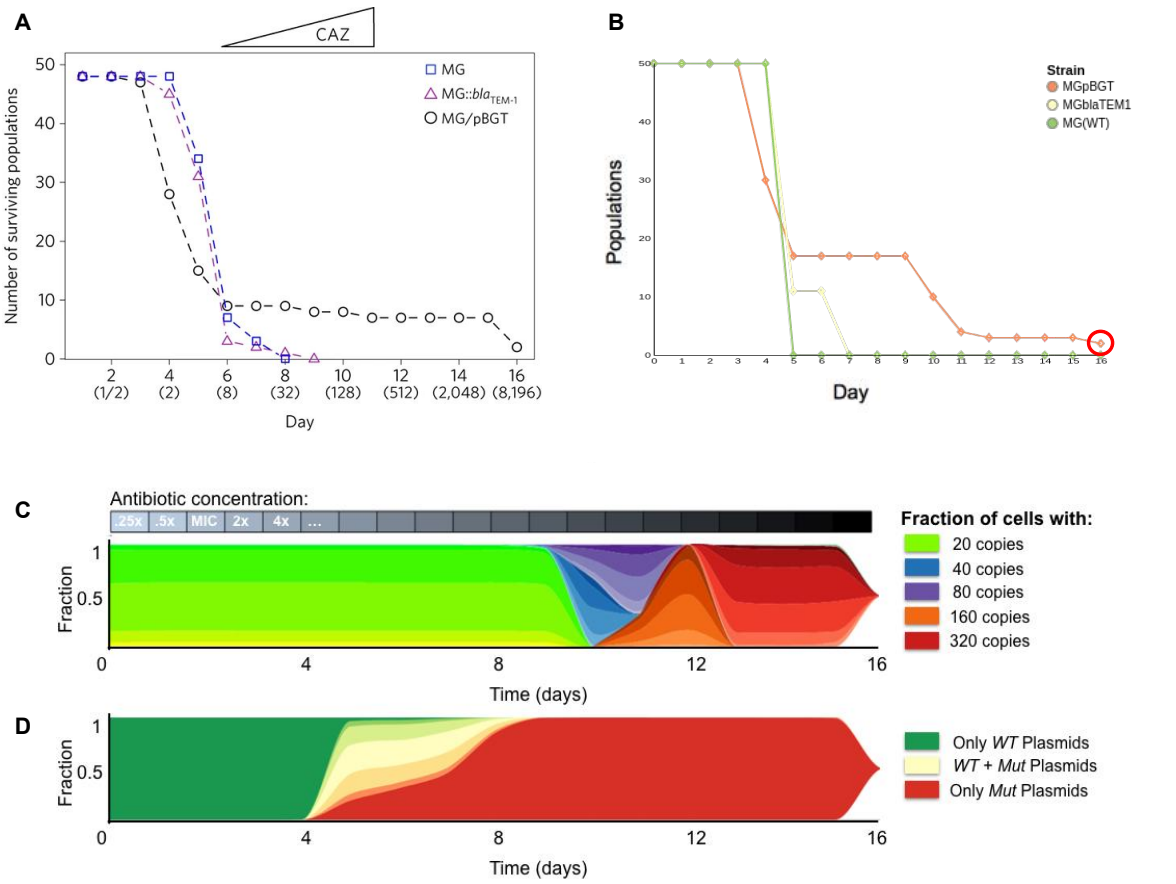

Figura 5.1: Experimento de supervivencia en una rampa de antibióticos. A) Resultados Experimentales. La población que porta el plásmido es capaz de sobrevivir por mucho más timepo al tratamiento. B) Simulaciones con un modelo de agentes individuales. Observamos el mismo comportamiento introduciendo mutaciones que aumentan el número de copias y mutaciones en el gen de resistencia C) Dinámica incremento de número de copias del plásmido de una población que subsistió hasta el final del experimento. Observe cómo surgen poblaciones dominantes cada vez con mayor número de copias. D) Dinámica de frecuencia de alelos mutantes de la misma población. Note que antes de que el alelo mutante (rojo) se fije en la población existe un periodo considerable donde ambos alelos coexisten, además esta coexistencia de alelos se da mayoritariamente a nivel intracelular (zona en amarillo). Note también que la zona amarilla es en realidad un gradiente de color correspondiente a subpoblaciones con distintas proporciones de ambos alelos. Al final del experimento sólo las poblaciones que acumularon las mutaciones necesarias sobreviven al tratamiento.

positiva (Peña-Miller et al. 2015), mantener la heterocigosis mediada por plásmidos requiere también de una presión selectiva, y se requiere que esta presión sea dinámica.

En ese capítulo, suponemos que los comportamientos observados a nivel de poblaciones son consecuencia de la dinámica de plásmidos a nivel intracelular (enten-

diendo como dinámica de plásmidos el producto de los procesos replicación, mutación, división celular, segregación, costo metabólico, adecuación, etc). El análisis de células individuales lo realizamos mediante experimentos de microfluídica similares al protocolo descrito por Mather et al. (2010). Estos experimentos producen series de tiempo en imágenes que resultan espectaculares al ojo humano, pero que además permiten obtener datos temporales de intensidad de fluorescencia en células individuales, por lo que fue necesario crear herramientas computacionales de análisis y procesamiento de imágenes que nos ayudaran en la tarea. En particular, desarrollamos μJ (`https://github.com/ccg-esb-lab/uJ`), un pipeline de análisis de imágenes con el cual analizamos las imágenes de microscopía para obtener información sobre el comportamiento de las bacterias en los distintos ambientes de selección y, de esta forma, discernir las dinámicas de plásmidos a nivel intracelular y con alta resolución temporal.

5.1 Modelo de agentes individuales para PMH

Este modelo es una extensión del modelo descrito en el capítulo anterior en el cual integramos otro plásmido con consideraciones apropiadas.

Primero añadimos mutaciones tanto en el gen de resistencia, consideramos que estas mutaciones pueden ocurrir con cierta probabilidad ε_P durante la replicación de cada plásmido. Luego incluimos mutaciones en el mecanismo de control de número de copias que ocurren con probabilidad ε_C y que cuando una bacteria adquiere una de estas, todos sus plásmidos aumentan en número. Estas mutaciones son acumulables y cada una duplica el número de copias. También consideramos que las ambas mutaciones pueden ser reversible y las tasas de mutación son ajustadas a los tamaños poblacionales de las simulaciones.

Los antibióticos son considerados conjuntamente tomando en cuenta los perfiles resistencia de las cepas homocigotas y calculamos el perfil de susceptibilidad/resistencia considerando el número de plásmidos de cada tipo que cada bacteria porta.

Tomando en cuenta estas modificaciones, las realizaciones del modelo se realizan de la misma manera que la versión anterior.

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Quantifying plasmid dynamics using single-cell microfluidics and image bioinformatics

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ABSTRACT

Multicopy plasmids play an important role in bacterial ecology and evolution by accelerating the rate of adaptation and providing a platform for rapid gene amplification and evolutionary rescue. Despite the relevance of plasmids in bacterial evolutionary dynamics, evaluating the population-level consequences of randomly segregating and replicating plasmids in individual cells remains a challenging problem, both in theory and experimentally. In recent years, technological advances in fluorescence microscopy and microfluidics have allowed studying temporal changes in gene expression by quantifying the fluorescent intensity of individual cells under controlled environmental conditions. In this paper, we will describe the manufacture, experimental setup, and data analysis pipeline of different microfluidic systems that can be used to study plasmid dynamics, both in single-cells and in populations. To illustrate the benefits and limitations of microfluidics to study multicopy plasmid dynamics, we will use an experimental model system consisting on *Escherichia coli* K12 carrying non-conjugative, multicopy plasmids (19 copies per cell, in average) encoding different fluorescent markers and β-lactam resistance genes. First, we will use an image-based flow cytometer to estimate changes in the allele distribution of a heterogeneous population under different selection regimes. Then we will use a mothermachine microfluidic device to obtain time-series of fluorescent intensity of individual cells to argue that plasmid segregation and replication dynamics are inherently stochastic processes. Finally, using a microchemostat, we track thousands of cells in time to reconstruct bacterial lineages and evaluate the allele frequency distributions that emerge in response to a range of selective pressures.

1. Introduction

Plasmids are significant drivers of microbial ecology and evolution by horizontally transmitting beneficial genes and providing recipient cells with access to novel ecological niches (Wiedenbeck and Cohan, 2011). But the contribution of plasmids to bacterial evolutionary dynamics is not reduced to merely acting as vehicles for the horizontal dissemination of genetic information between strains and species. Recent studies have enlisted a series of benefits associated with carrying genes in plasmids, as opposed to encoding them in the chromosome.

The fixation probability of beneficial mutations and the rate of segregational loss are determined by the number of plasmid copies carried by each cell (Stewart and Levin, 1977) and, therefore, copy number control, as well as timing and mode of plasmid segregation, are important factors influencing the population genetics of plasmid-bearing populations (Paulsson, 2002; Rodriguez-Beltran et al., 2019; Ilhan et al., 2019). As a result, the stochastic nature of replication and segregation of multi-copy plasmids has been studied extensively, both in theory (Ishii et al., 1978; Nordström, 1984; Keasling and Palsson, 1989; Paulsson and Ehrenberg, 2001) and in laboratory conditions (Nordström et al., 1984; Novick, 1987; Del Solar and Espinosa, 2000), showing that copy-number control is a noisy process with events randomly distributed in time (Seneta and Tavaré, 1983) and, therefore, intracellular fluctuations in copy numbers can be seen as a stochastic dynamical system (Keasling and Palsson, 1989).

Similarly, plasmid partition is a random process such that, in the absence of plasmid addiction systems (Mochizuki et al., 2006; Baxter and Funnell, 2015), results in an equal chance for each plasmid to be inherited to each daughter cell and, therefore, in a binomial probability of producing a plasmid-free cell upon division. Of course, this is a simplification, as high-copy plasmids can produce dimers through homologous recombination (Summers, 1991) and intracellular spatial

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structure can result in the asymmetric segregation of plasmids upon division (Wang et al., 2016). Also, low-copy plasmids tend to have active partitioning mechanisms that organize plasmids around a centromere-like site to segregate plasmids symmetrically between daughter cells at division (Salje, 2010). In contrast, plasmids that do not encode partition systems decrease the probability of segregational loss by being present in high-copy-numbers.

But carrying multiple plasmid copies not only increases plasmid stability but can also have important consequences in the adaptive dynamics of plasmid-bearing populations, for instance increasing the rate of fixation of beneficial mutations (Galitski et al., 1995) and accelerating the rate of adaptation to deteriorating environmental conditions (San Millan et al., 2016). Furthermore, once a beneficial mutation appears in a plasmid-borne gene, multicopy plasmids can provide a platform for rapid gene amplification (Nicoloff et al., 2019). By increasing gene dosage, bacterial cells have been reported to transiently enhancing the level of resistance to antibiotics (Santos-Lopez et al., 2017), allowing the population to increase in size and, as a result, increasing the probability of appearance of secondary drug-resistant mutations (Sun et al., 2018).

Moreover, multicopy plasmids produce genomic regions of local polyploidy that can generate heterozygous cells where different alleles coexist at a cellular level, a phenomenon also referred to as heteroplasmy (Novick, 1987). As a result, individual cells can increase their genetic diversity and enable populations to circumvent evolutionary trade-offs (Rodriguez-Beltran et al., 2018). It has also been reported that the multicopy plasmids can increase standing genetic variation in the population, thus enabling bacterial populations to escape extinction following a sudden environmental change (Santer and Uecker, 2019). In the absence of selection, however, random genetic drift of multicopy plasmids during cell division (also known as segregational drift (Ilhan et al., 2019)) can reduce the rate of adaptation, despite high-copy plasmids having increased mutational supply.

By focusing on large population sizes, laboratory studies have been able to characterize the interaction between genetic dominance and strength of selection, and correlate these traits with the probability of fixation of mutant alleles and the horizontal transmission of plasmid-borne genes (Rodriguez-Beltran et al., 2019). Other plasmid evolution studies have focused on compensatory adaptation (San Millan et al., 2014; Wein et al., 2019; Hall et al., 2020), horizontal transmission (Lopatkin et al., 2017) and co-evolution between hosts and plasmids (Harrison et al., 2015). Altogether, these results highlight the complex interaction between the intracellular plasmid dynamics and the evolutionary dynamics of bacterial populations.

1.1. Single-cell microfluidics

In the past decades, biology and medicine have been rapidly evolving towards using quantitative tools to study complex biological systems. Interdisciplinary studies use statistical, mathematical, and computational tools, combined with experimental and molecular biology, to understand the behavior of individual cells within a population (Artemova et al., 2015) and to predict their response to environmental change (El Meouche and Dunlop, 2018). The benefit of implementing a *bottom-up* approach is that we can follow the life history of individual cells, instead of averaging large populations and making inferences about cellular processes from population-level observations.

Fluorescence microscopy has been previously used to estimate plasmid copy-numbers (Ng et al., 2010; Løbner-Olesen, 1999), as well as to visualize conjugation (Babić et al., 2008), to study horizontal transmission of plasmids (del Campo et al., 2012) and to explore the range of different plasmid-host associations (Shintani et al., 2014). It has also been used to evaluate in situ conjugation in bacterial plant endosymbionts (Bañuelos-Vazquez et al., 2019) and horizontal gene transfer in microbiomes (Pinilla-Redondo et al., 2018). Moreover, with the use of fluorescent probes, microscopy studies have been able to study with great detail the spatio-temporal distribution of plasmids inside a cell, as well as the segregation dynamics occurring upon division (Wang et al., 2016; Reyes-Lamothe et al., 2013; Hsu and Chang, 2019). These single-molecule studies, however, only consider a small set of cells and are constrained to short-term experiments in constant environments.

On the other hand, microfluidic devices have been used in combination with fluorescence to obtain time-series of gene expression of individual cells (Young et al., 2012; Tomanek et al., 2020). Microfluidic devices can be fabricated using soft lithography (Zhang et al., 2012; Pan et al., 2011), micro-droplets (Boedicker et al., 2009) and protein-based micro-3D printing (Connell et al., 2014). In general, the goal of these devices is to restrict the movement of bacterial cells to observe them for long time intervals with the aim of studying, for instance, gene expression dynamic (Young et al., 2012; Baumgart et al., 2017; Bennett and Hasty, 2009; Locke and Elowitz, 2009), as well as to evaluate the consequences of asymmetric division and cell-to-cell variability of key cellular processes (El Meouche and Dunlop, 2018; Mosheiff et al., 2017; Bergmiller et al., 2017). A brief overview of different microfluidic devices and their uses can be found in (Bennett and Hasty, 2009; Potvin-Trottier et al., 2018).

A myriad of computer vision algorithms have been developed to analyze time-lapse movies acquired using a fluorescent microscope (Young et al., 2012; Van Valen et al., 2016; Berg et al., 2019a; Balomenos et al., 2017; Arnoldini et al., 2014; Sachs et al., 2016; Lugagne et al., 2019; Kamentsky et al., 2011). Of note, *Schnitzcell (Young et al., 2012)* was designed to study colonies of rod-shaped bacteria (e.g. *Escherichia coli* and *Bacillus subtilis*) growing in agar pads, although it is not longer maintained. Recently, novel computational techniques based on machine learning have been successfully incorporated into bio-image analysis pipelines. For example, *DeLTA (Lugagne et al., 2019)* and *DeepCell (Van Valen et al., 2016)* implement deep convolutional neural networks to perform accurate segmentation and lineage reconstruction, while *Ilastik (Berg et al., 2019b)* provides a user-friendly suite for image segmentation and cell tracking.

Besides quantifying single-cell fluorescent intensity, imaging algorithms can also be used to record division events and to estimate duplication and elongation rates of individual bacterial cells. In consequence, microfluidics have been used to study cell growth and homeostasis (Wallden et al., 2016), senescence (Ackermann et al., 2003; Lindner et al., 2008) and bacterial adaptation to stress (Łapińska et al., 2019; Mathis and Ackermann, 2016; Patange et al., 2018). By correlating physiological and morphological properties of individual cells with the level of expression of a gene of interest, previous studies have shown that phenotypic heterogeneity can provide functional benefits for bacterial populations, for instance allowing the implementation of division of labor strategies or increasing survival of the population to fluctuating environmental conditions (Ackermann, 2015). Another benefit of single-cell microfluidics is that it allows us to estimate growth rate differences and survival rates in response to environmental change. Therefore microfluidics has been proposed as a strategy for rapid antimicrobial susceptibility determination (Baltekin et al., 2017; Aroonnual et al., 2017) and to study gene regulatory changes that emerge in response to genetic and environmental perturbations (Rochman et al., 2016; Chait et al., 2017)..

In this paper, we combine microfluidics, fluorescent microscopy, and computer vision algorithms to study the interaction between multicopy plasmids and bacterial fitness in dynamic environments. To achieve this goal, we use a previously characterized experimental system consisting of plasmid-mediated β-lactam resistance evolution in *Escherichia coli (Rodriguez-Beltran et al., 2018)*. The objective of this manuscript is to describe the use of fluorescence and multiple image-based technologies to identify the source of noise in the replication and segregation dynamics of multicopy plasmids, as well as to evaluate the effect of selection imposed by antimicrobial substances on the distribution of plasmids exhibited by single-cells and bacterial

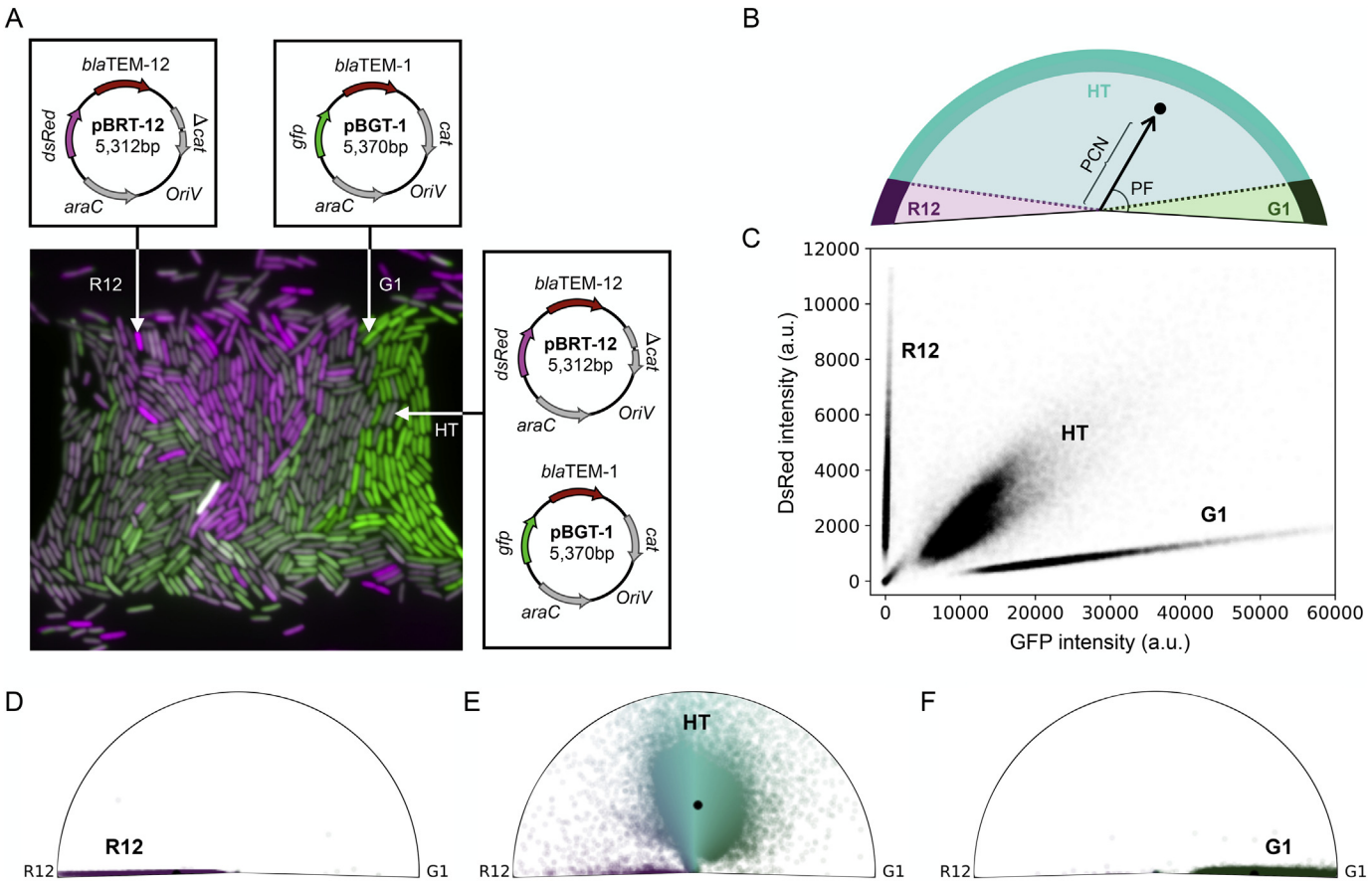

Fig. 1. A) Maps of plasmids used in this study. Composite microscopy image shows a heterogeneous *E. coli* population composed of cells carrying only pBRT-12 (denoted as R12, in magenta), pBGT (G1, in green) and a combination of both plasmids (HT). B) Diagram illustrating a polar representation of multi-channel fluorescent data. After normalizing fluorescence intensities obtained in GFP and DsRed channels, each cell can be represented as a point in a two-dimensional polar coordinate system, where the relative fluorescence between DsRed and GFP channels can be approximated by an angle and the absolute fluorescence intensity from its distance to the origin. We argue that absolute fluorescence is correlated with plasmid copy-number (PCN) and relative fluorescence to the plasmid fraction (PF). C) Raw cytometry data of a heterogeneous population illustrates that a flow cytometer can be used to identify different subpopulations, namely R12, G1 and HT. D-F) Polar representations of different plasmid-bearing populations n drug-free media: D) cells carrying only pBRT-12, E) a heterozygous population where both plasmids co-exist at a cellular level, and F) homozygous cells with only pBGT-1. Black dotes denote the expected value of the corresponding plasmid copy-number distribution. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

populations.

2. Material and methods

2.1. Bacterial strains and plasmids

In this study, we use derivatives of *Escherichia coli* MG1655 strains previously published (San Millan et al., 2016; Rodriguez-Beltran et al., 2018). In short, a β-lactamase gene $bla_{\mathrm{TEM-1}}$ that confers resistance to ampicillin (AMP) was inserted into a small non-transmissible multi-copy plasmid p3655 derived from pSU18T and pBADgfp2, with a ColE1-like (p15a) origin of replication (Le Roux et al., 2007), a plasmid referred to in this study as pBGT-1. This plasmid also contains an eGFP gene under an arabinose promoter with an *araC* repressor. Another plasmid, pBRT, was derived from pBGT by replacing the $bla_{\mathrm{TEM-1}}$ gene with $bla_{\mathrm{TEM-12}}$, a TEM variant that confers resistance to ceftazidime (CAZ) and mild resistance to ampicillin (AMP). Also, eGFP was replaced by a DsRed gene, and the native *cat* resistance gene was knocked-out. In this study, we refer to heterozygous cells simultaneously carrying both plasmids as HT, while G1 and R12 denote homozygous cells bearing pBGT-1 and pBRT-12, respectively (see Fig. 1A for maps of these plasmids). It is important to emphasize that pBGT-1 and pBRT-12 share an origin of replication and only differ in the fluorescent marker and the TEM variant carried. Therefore the plasmid copy-number control mechanism regulates the maximum number of plasmids carried in each cell, independently of the plasmid type.

2.2. Media and growth conditions

Experiments were performed using Lysogeny Broth- Lenox (LB) (Sigma-L3022) supplemented with arabinose (0.5% *w/v*). Antibiotic minimum inhibitory concentrations (MIC) were calculated using standard dose-response curves. To balance HT cells, we prepared the overnight culture using LB media supplemented with 15 mg/l of chloramphenicol and 0.5 mg/l ceftazidime. Stocks solutions at 20% of Arabinose (Sigma-A91906) were prepared by diluting 2 g of arabinose in 10 ml DD water sterilized by filtration. AMP stock solutions (100 mg/ml) were prepared by diluting ampicillin (Sigma-A0166) directly in LB and adding arabinose (0.5%). Stock solutions of ceftazidime (Sigma-A6987) were diluted in water at 10 mg/ml and sterilized by filtering. Chloramphenicol (Sigma-C0378) stock solutions were prepared at 50 mg/ml in ethanol (97%). Population-level experiments were performed using 20 ml of LB media in 125 ml titration flasks. Three replicates of HT cells cultures were grown in balancing media for 24 h in a shaker incubator at 37 °C and 200 rpm.

2.3. Imaging flow cytometry

Population-level experiment measurements were performed by sampling 1 ml of overnight culture and measured their fluorescence using an imaging flow cytometer (Amnis ImageStream Mark II by Luminex). INSPIRE software was used to acquire data discriminating by area, aspect ratio, focus, and side scatter features. GFP fluorescence was excited at 488 nm with 25 mv intensity, and DsRed fluorescence was excited at 561 nm with 200 mv intensity. Populations data files were processed using IDEAS 6.2 software and feature values were exported and analyzed using bespoke scripts implemented in Python programming language.

2.4. Microfluidic devices

We used PDMS (polydimethylsiloxane) microfluidic chips obtained from wafers manufactured using soft photolithography (SU-82000.5, micro resist technology GmbH). In particular, we used a micro-chemostat (Mondragón-Palomino et al., 2011) that contains multiple inputs (a cell-loading input, a shunt, and two for different growth media), as well as two waste outputs. Media inputs are mixed at different proportions using a red fluorescent dye (rhodamine) diluted in one of the media inputs. This device contains 48 chambers of $40x50x.95\ \mu$m, capable of trapping approximately 500 bacterial cells in the same focal plane. Each chamber is open to a big channel on two sides and, when chambers are filled, bacteria in the edges are pushed out of the chamber and washed away. We used this device to obtain high-throughput population-level data derived from single-cell measurements at different time-points. We also used a dual-input mother-machine (Kaiser et al., 2018) device in which bacteria are trapped in a comb-like channels architecture. Mother cells restrained at the bottom of each channel and, as they grow and divide, daughter cells are pushed downwards to a larger channel and washed away. We used this microfluidic chip to keep track of mother cells for very long periods of time, while quantifying fluorescence and other morphological attributes.

2.5. Cell loading and environmental control

Growth media was loaded into 60 ml-syringes connected to the PDMS chip through Tygon tubes and assorted Luer connectors. The pressure inside the chip is controlled with vertical linear actuators and a digital signal generator that controls the height of each syringe. This Dial-A-Wave (DAW) system (Ferry et al., 2011) enables precise control over the extracellular environmental conditions. For the purpose of this paper, we use this DAW to introduce antibiotics into the chip gradually, until reaching a maximum dose that is then maintained until all cells are killed, a protocol we refer to as a ramp experiment. Later we will introduce antibiotics following a sinusoidal signal to alternate selective pressures periodically.

Initial inoculates of HT cells were grown overnight in the presence of sub-lethal doses of chloramphenicol and ceftazidime, in order to clear homozygous cells from the culture and obtain a well-balanced HT culture. A sample was then transferred to 200ml of fresh LB and grown at 30 °C until reaching an $OD_{600} = 0.3$. After centrifuging, cells were re-suspended in 5 ml of LB, and this dense culture was used to inoculate the microfluidic chip. For all microfluidics experiments, we used LB media supplemented with arabinose at 0.5% and Tween20 (Sigma-P2287) at 0.075%. In all cases, cells were allowed to grow and divide multiple cell cycles in a drug-free environment, allowing us to determine the baseline fluorescent intensity of the population. We later use this value to normalize our data and compare fluorescent intensities obtained for different channels.

2.6. Microscopy and image acquisition

Time-lapse images of microfluidic experiments were acquired using a Nikon Eclipse Ti-E epifluorescence microscope equipped with differential interface contrast (DIC), a motorized stage and a perfect focus system that allows us to obtain long-time time-lapses. The microscope was controlled by the Nikon NIS-Elements AR 4.20 program and is equiped with a Lexan Enclosure Unit with Oko-touch temperature control that allows us to incubate the microfluidic chips. The experiments are conducted at 30 °C. For all experiments, time-lapse movies were acquired with a 100× Plan APO objective without analog gain and with field and aperture diaphragms as closed as possible to avoid photobleaching. DIC images were taken at 9v DIA-lamp intensity with exposure of 200 ms, DsRed channel (excitation from 540 to 580 nm, emission from 600 to 660 nm filter) with exposure of 600 ms, GFP channel (excitation from 455 to 485 nm, emission from 500 to 545 nm) with 300 ms exposure. Images were taken every 5 or 10 min, depending on the experiment.

2.7. Image processing and analysis

Microscopy time-lapse movies were analyzed using a semi-automated ImageJ (Schneider et al., 2012) analysis pipeline that implements a deep-learning algorithm for image segmentation (Van Valen et al., 2016). Cell tracking and lineage reconstruction were performed in Python using standard numerical and geometric libraries (NumPy, Shapely, Pandas, Scipy, Matplotlib). Data and code are openly distributed and available for download at https://github.com/ccg-esb-lab/uJ.

In short, the image processing pipeline consists on 1) organizing TIF files generated by NIS Elements, 2) aligning traps and using rigid motion transformation to correct for x-y drift in time-lapse images, 3) using *Parallel Interactive Deconvolution* with a theoretical point spread function generated with PSF Generator to produce a segmentable image, 4) using *DeepCell (*Van Valen et al., *2016)* to segment images and obtain binary masks, 5) cell detection and automatic correction of ROIs, 6) manual correction of a binary mask, 7) data acquisition by overlapping masks in different fluorescent and bright field channels, 9) cell tracking using a feature-aided nearest-neighbor algorithm and lineage reconstruction and, finally, 10) data analysis and visualization. The goal of this image bioinformatics pipeline is to acquire time-series of fluorescent intensity and other morphological properties of individual cells, as well as to obtain population-level statistics to estimate, for instance, the mean cell duplication rate of the population or changes in the shape of the fluorescence distribution.

2.8. Estimation of plasmid copy-number and plasmid frequency

There are two quantities we estimate from fluorescent data: plasmid copy-number (PCN) and plasmid frequency (PF). Previous studies have established that gene copy-number and fluorescent intensity are positively correlated, both when carried in chromosomes (Bergmiller et al., 2017) or in plasmids (Rodriguez-Beltran et al., 2018; Ghozzi et al., 2010). Therefore we will use flow cytometry and fluorescent microscopy to determine the relative intensity of individual cells with respect to the population-level mean (it was previously determined that the plasmids used in this study are carried, on average, ~19 copies of the plasmid per cell) (San Millan et al., 2016).

Also, we estimate the proportion of each plasmid type carried in each cell from the relative fluorescent intensity measured on different channels. Let us define Φ_g and Φ_r the fluorescent intensity of cell i measured in the green and red channels, respectively. As fluorescent proteins have different maturation times and intrinsic brightness (Balleza et al., 2018), we normalized the data by dividing every channel measurements by the maximum intensity and obtained relative intensity values for each channel, quantities that we will denote as $\hat{\Phi}_g$ and $\hat{\Phi}_r$.

We argue that, in this case, a polar representation of fluorescent

data is more appropriate, as illustrated in the diagram shown in Fig. 1B. That is, to estimate plasmid fraction from flow cytometry or microfluidic data we will transform the $\widehat{\Phi}_g/\widehat{\Phi}_r$ ratio into polar coordinates:

$$PF \sim \theta := \arctan(\widehat{\Phi}_r/\widehat{\Phi}_g).$$

This expression allows us to estimate the fraction of plasmids (PF), pBGT-1 plasmid with respect to the pBRT-12 plasmid, a quantity we refer to as *relative fluorescent intensity* and denote as θ. Similarly, we can use r to approximate the absolute plasmid copy number (PCN) by assuming that fluorescent intensity is proportional to the number of copies of the gene carried by each cell,

$$PCN \sim r := \sqrt{\widehat{\Phi}_g^2 + \widehat{\Phi}_r^2}.$$

However, the assumption of a linear relationship between PCN and absolute fluorescent intensity does not always hold and, as discussed extensively in (Tal and Paulsson, 2012), prevents from using absolute fluorescent intensities as a proxy for plasmid copy number. For this reason, in this paper we will restrict our analysis to using θ to evaluate changes in the relative abundances of each allele (i.e. the plasmid fraction) in response to different environmental conditions.

3. Results

3.1. Using flow cytometry to study population dynamics of heterogeneous populations

A fundamental problem in plasmid biology is to determine environmental conditions that enable costly plasmids to be stably maintained in bacterial populations (Harrison et al., 2015; Loftie-Eaton et al., 2016; Porse et al., 2016), This problem is of particular interest for bioengineers and synthetic biologists, as genetic manipulations of microorganisms generally use plasmids as cloning vectors, despite being metabolically costly and, therefore, susceptible to be lost through purifying selection. In contrast, as drug-resistant genes tend to be carried in plasmids (Alekshun and Levy, 2007; San Millan, 2018), it is also a problem of interest for biomedical scientists to determine conditions that cure drug-resistant plasmids of pathogenic populations (Boucher et al., 2009) and to evaluate the probability of fixation of drug-resistance mutations (Ilhan et al., 2019; Rodriguez-Beltran et al., 2018).

Independently of the motivation, experimental studies routinely estimate the fraction of plasmid-bearing cells within a bacterial population by replicating bacterial colonies from non-selective agar plates onto plasmid-selective and non-selective media. In recent years, other studies have used a combination of flow cytometry (FCM) and real-time quantitative PCR (qPCR) to estimate the mean plasmid copy number of the population (Ng et al., 2010) and to determine the relative abundance of plasmid-bearing cells (Bahl et al., 2004). The benefit of the FCM and qPCR is that both are cultivation-independent and provide precise estimations about the mean plasmid copy number of the population.

Here we use an image-based FCM (see Methods) to study the resulting PCN distribution that emerges from exposing genetically-diverse populations to different selection regimes. We focus on a well-characterized experimental system of drug resistance evolution: plasmid-mediated TEM-1 evolution towards ceftazidime resistance in *Escherichia coli*. The numerous ways in which TEM has evolved suggests that it can respond very specifically to each β-lactam, and therefore has been used extensively to study the molecular evolution in response to different selection regimes, both when TEM is encoded in the chromosome (Barlow and Hall, 2002; Barlow and Hall, 2003) or in plasmids (Santos-Lopez et al., 2017; Rodriguez-Beltran et al., 2018). Indeed, nearly every β-lactamase that has been identified as a resistance determinant among clinical bacteria has experienced molecular evolution in response to the use of different β-lactam antibiotics, with over 215 variant TEM β-lactamases identified with differences in amino acid sequence and susceptibility to β-lactam antibiotics (Barlow and Hall, 2002).

3.1.1. Relative allele frequencies are modulated by selection and segregational drift

Our experimental systems consists of a bacterial population containing small (5.3Kb), non-conjugative, multicopy plasmids (pBGT-1 with mean PCN = 19.12 ± 1.56, and pBRT-12 with 21.1 ± 0.85 plasmids in average (San Millan et al., 2016)), different fluorescent markers (GFP and DsRed respectively, both under the *araC* promoter) and TEM genes that produce different variants of β-lactamase, an enzyme that hydrolyzes the active portion of β-lactam antibiotics (Knox, 1995).

It has been established there is a fitness cost associated with synthesizing fluorophores, in this case, expressed in terms of a reduced growth rate in the presence of arabinose with respect to the same strain growing in arabinose-free environments (two-tailed t-test, p-value < 0.05, $N = 4$). For this reason, all experiments described in this study were performed in the presence of arabinose. Crucially, both fluorescent proteins impose a similar fitness burden and therefore no significant differences in growth rate were observed in populations producing either GFP or DsRed proteins (two-tailed t-test, p-value = 0.512, $N = 6$) (San Millan et al., 2014), therefore allowing us to associate changes in fluorescent intensity to differences in fitness of the corresponding TEM alleles, and not due to differential cost of producing fluorescent proteins.

Fig. 1A displays a composite microscopy image showing that a population of HT cells presents high levels of heterogeneity; while some cells are only detected in DsRed or GFP channels (corresponding to cells with high proportions of either plasmid), other cells exhibit analogous levels of fluorescence in both channels (corresponding to heterozygous cells bearing both pBGT-1 and pBRT-12, mean PCN = 22.3 ± 4.7). Fig. 1C shows raw fluorescent intensity determined with flow cytometry of cells in a heterozygous population, revealing the existence of three main clusters, corresponding to homozygous cells (R12 and G1) and the heterozygous population (HT). All flow cytometry experiments were performed in triplicate, with fluorescence distributions obtained by sampling 20,000 cells from the corresponding population.

Fig. 1D and F show that clonal populations of G1 and R12 are only present in the corresponding region of the polar coordinate system when measured after 24 h of growth. In contrast, HT cells carry both plasmids and are therefore scattered throughout the polar plane. This large dispersion in PCN and PF has been predicted by theoretical models of multicopy plasmid dynamics (Peña-Miller et al., 2015; Münch et al., 2019), suggesting that populations bearing multicopy plasmids can present cell-to-cell differences in total plasmid copy-number and in plasmid frequency. Indeed, recent clinical studies have suggested that gene amplification and copy-number variability in drug-resistance genes yield heteroresistant populations (Andersson et al., 2019), potentially leading to treatment failure in clinical settings (Nicoloff et al., 2019; Wang et al., 2014; Band and Weiss, 2019).

A consequence of bearing plasmids with different variants of TEM is that heterozygous populations exhibit heterogeneous profiles of resistance. In this case, $bla_{\mathrm{TEM-1}}$ provides resistance to ampicillin (AMP), while $bla_{\mathrm{TEM-12}}$ to ceftazidime (CAZ) and partially to AMP (Rodriguez-Beltran et al., 2018; Mroczkowska and Barlow, 2008). So, to determine how different environments modulate the distribution of plasmids, we inoculated a population of HT cells in drug-free media and, after 24 h, used a flow cytometer to obtain the distribution illustrated in Fig. 1E. Similarly, we exposed a heterozygous population to a sub-lethal concentration of ampicillin (8 *mg/ml*) and estimated the resulting plasmid distribution after 24 h (see Fig. 2A). We then repeated this assay with ceftazidime (8 *μg/ml*) and, analogous to AMP, HT cells exhibited large dispersion, while R12 and G1 showed variability in PCN, but not in PF.

We then clustered the population based on their relative fluorescent intensities and counted the number of cells in each group. The relative abundances of each subpopulation are illustrated in Fig. 2E. Note how,

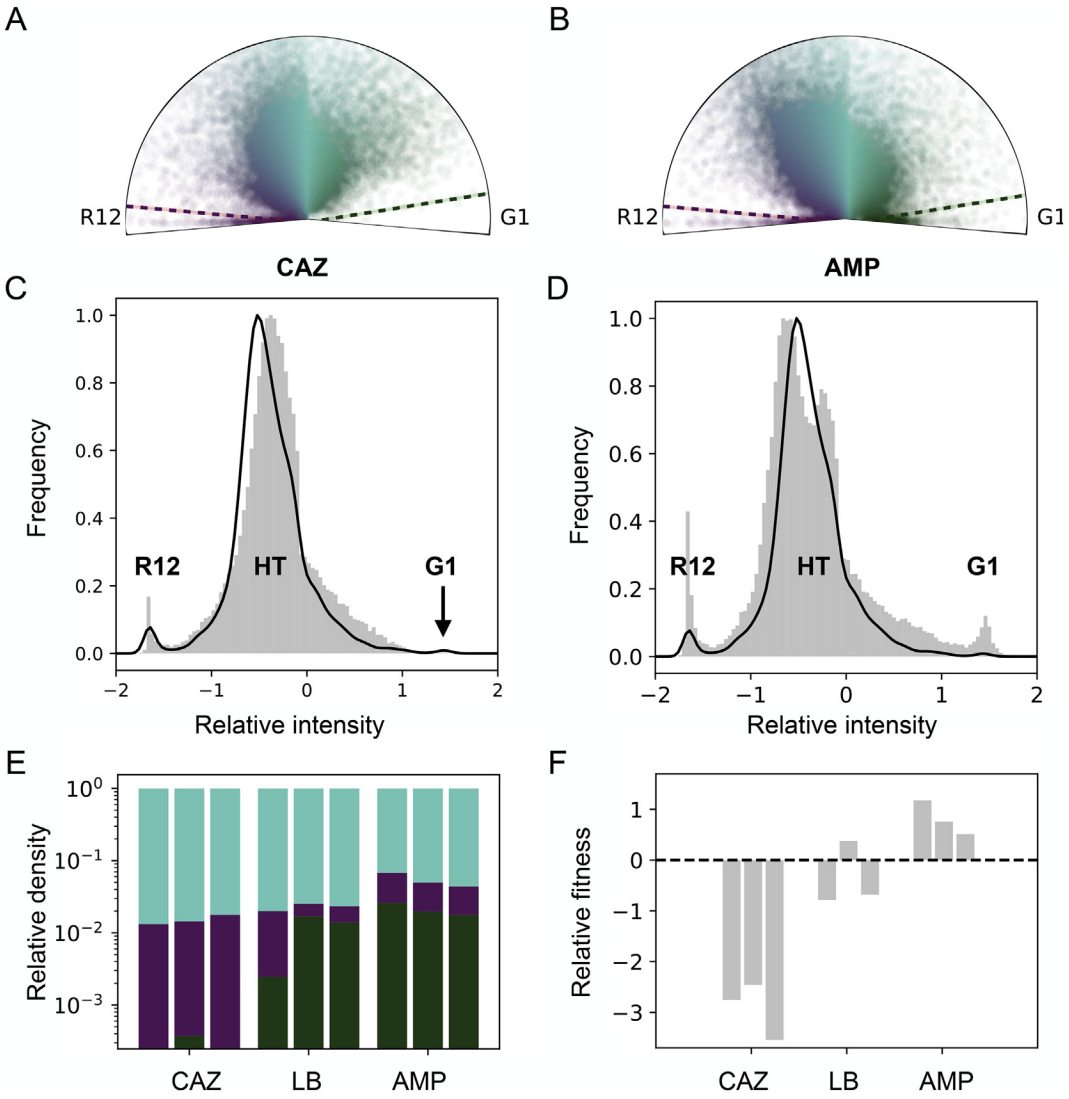

Fig. 2. Polar representation of fluorescent intensities obtained using flow cytometry of HT populations exposed to A) ceftazidime, and B) ampicillin. Distributions were obtained by sampling 60,000 cells from three independent biological replicates. C-D) Histograms of relative intensity for both selection regimes, CAZ and AMP, respectively. Note how AMP maintains a subpopulation of G1 cells, while in CAZ, only HT and R12 cells are present at the end of the experiment (the arrow in C points towards relative intensity values that correspond to G1). This is a consequence of R12 cells being resistant to both antibiotics and G1 only resistant to AMP. In drug-free media, both homozygous populations are present, resulting from the segregation of HT cells into G1 or R12. E) The relative density of each subpopulation under different environmental conditions, determined by clustering cells according to their relative fluorescent intensity. Each bar corresponds to a replicate experiment in each environment ($N = 3$). F) Relative fitness of G1 with respect to R12, after 24 h of growing under different environmental conditions (N = 3). As expected, AMP provides a fitness benefit for G1, while CAZ positively selects for R12.

in drug-free media, segregational instability produces homozygous subpopulations, either carrying $bla_{\mathrm{TEM-1}}$ or $bla_{\mathrm{TEM-12}}$. As $bla_{\mathrm{TEM-1}}$ is susceptible to ceftazidime, we did not observe any G1 cells when HT was exposed to CAZ (left bar in Fig. 2E). The absence of cells with relative fluorescent intensity values in the range corresponding to G1 cells can also be seen in Fig. 2A and C (arrow in 2C shows the location of the G1 subpopulation). In contrast, as $bla_{\mathrm{TEM-12}}$ confers resistance to CAZ, then R12 increased in abundance relative to G1 when exposed to ceftazidime.

Similarly, in environments that positively select for cells carrying plasmids encoding $bla_{\mathrm{TEM-1}}$, the resulting distribution shows an increase in the relative abundance of G1 (right bars in Fig. 2E). Note that, in this case, R12 cells were able to survive treatment with ampicillin, a consequence of a previously reported cross-resistance to both AMP and CAZ provided by the $bla_{\mathrm{TEM-12}}$ gene (Rodriguez-Beltran et al., 2018; Salverda et al., 2010). We also performed statistical tests to analyze the PF distributions of the populations under each selective regime and found that they are significantly different (Kruskal-Wallis H statistic = 649.6, p-value < 0.001). Similarly, pair-wise Kolmogorov-Smirnov tests demonstrated significant differences when performing direct comparisons between AMP-CAZ, AMP-LB, and CAZ-LB distributions (p-values < 0.001).

Based on the relative abundances of each strain, we estimated the relative fitness of G1 with respect to R12 under different selection regimes. Fig. 2F shows that using ampicillin increases the relative frequency of G1 and, as a result, produces an increase in the relative fitness of G1 compared to R12. Conversely, CAZ positively selects for R12, and therefore G1 was suppressed in the population.

Altogether, by analyzing the distribution of fluorescent intensities in different environments, we conclude that selection imposed by antibiotics modifies the relative frequency of different alleles in the population. There are, however, two possible explanations for this behavior: selection acting on populations (this would mean that population-level dynamics is a consequence of changes in the relative abundances of

different subpopulations) or at a level of single-cells (implying that segregation and replication may not be completely stochastic). Using a flow cytometer does not help us differentiate between these possibilities, so, in the remainder of this paper, we will use microfluidic devices that allow us to correlate selection with changes in allele frequency, both at a level of single-cells and in bacterial populations.

3.2. Using microfluidics to analyze plasmid dynamics of individual cells

We have shown that flow cytometry can be used to evaluate the effect of selection in the frequency of heterozygous cells in the population. However, flow cytometry data does not provide time-resolved information about the rate of fixation of different alleles or about the stochastic nature of segregation and replication of plasmids. To overcome these limitations, we used microfluidics to perform long-term observations of individual cells and, with the aid of fluorescent microscopy and image processing algorithms, quantified segregational drift in heterozygous populations.

In particular, we will use a microfluidic device known as a dual-input mother-machine, designed to precisely control the environmental conditions while trapping individual cells in narrow channels under controlled environmental conditions. As cells grow and divide, daughter bacterial cells are pushed downwards to the channel opening and washed away of the device. We will use this microfluidic chip to perform long-term observations of single-cells and quantify temporal changes in the fraction of pBRT-12 and pBGT-1 plasmid, with the aim of studying segregational drift resulting from the stochastic segregation and replication of multi-copy plasmids.

3.2.1. Intracellular plasmid dynamics is stochastic and not influenced by antibiotic selection

The benefit of mother-machine microfluidic devices is that they allow us to culture individual cells for hundreds of generations under the microscope, in contrast to microscope culture protocols which do not actively remove progeny during growth and therefore get rapidly saturated. Multiple mother-machine devices have been proposed (Taheri-Araghi and Jun, 2015; Long et al., 2013), but we will we use a dual-input mother machine (Kaiser et al., 2018), as it allows us to precisely control the concentration of antibiotic inside the microfluidic chip.

First, we performed a long-term experiment consistent on introducing HT cells into the device and observing them for a period of 72 h. We observed four device positions with ~13 microchannels per field of view, leading to 244,249 single-cell measurements, with mean fluorescent intensities of 212 ± 81 for GFP and 155 ± 51 in DsRed, normalized relative intensity of 1.42 ± 0.53, and normalized absolute intensity of 0.46 ± 0.14. By acquiring images in multiple channels (GFP represented in green and DsRed in magenta) we can follow changes in fluorescence intensity between division events. Fig. 3A shows a montage of mother cells at specific time-points, with their corresponding time-series represented in Fig. 3B (black line corresponding to the cell illustrated in Fig. 3A, while grey lines show the relative intensity time-series obtained for other representative cells in the device).

It is important to highlight that time-series shown in Fig. 3B are very long time-series (72 h, up to 99 cell cycles), allowing us to quantify the difference in relative fluorescent exhibited by each cell at the moment of division and to estimate the difference in fluorescence between consecutive cell cycles, a quantity we refer to as Δ *relative intensity*. Fig. 3E shows how the time-series of Δ relative intensity produces increases in one fluorescent channel as frequently as increases in the other direction. As a result, the difference between relative intensity values estimated in consecutive time-points is approximated by a Normal distribution with $\mu = 0.00057$ a $\sigma^2 = 0.0092$ (see Fig. 3D).

Fig. 3F shows the partial autocorrelation function obtained for time-series of relative intensity in a drug-free environment. Note lags > 0 are within the 95% confidence interval, suggesting that changes in plasmid frequency are generated by an auto regressive process of first order, consistent with the tenet that random segregation and replication of plasmids are inherently stochastic processes. Another interesting feature of our data is that intracellular plasmid diversity can be maintained for many generations in individual bacterial cells. Of course, phenotypic delay (Sun et al., 2018) and fluorescent protein stability (Balleza et al., 2018) could also stabilize fluorescence, but only for a few cell cycles.

However, a consequence of the random segregation of plasmids is that there is a probability larger than zero of segregating plasmids unevenly between mother and daughter cells. In our experimental system, this would be reflected as large jumps in Δ relative intensity. Fig. 3C shows a kymograph obtained from a time-lapse movie (Supplementary Movie S1), whereby the cell in the top of the channel (marked with a black triangle) corresponds to the time-series shown in Fig. 3B. Top and middle images correspond to GFP and DsRed channels, while the bottom image illustrates masks obtained after image segmentation, colour-coded to represent the relative intensity value obtained after normalizing both fluorescent channels. Note how, in general, fluorescence between mother and daughter cells appears to be correlated but, occasionally, a mother cell segregates plasmids unevenly upon division, producing daughter cells with different plasmid configurations (for example the magenta lineage in the kymograph). In the extreme scenario, HT inherits only plasmid of one type to the daughter cell, producing R12 or G1 cells with a probability that can be estimated from a binomial distribution.

In summary, we have established that, as generally assumed by theoretical models of plasmid dynamics (Ilhan et al., 2019; Rodriguez-Beltran et al., 2018; Santer and Uecker, 2019; San Millan et al., 2014) segregation and replication of multicopy plasmids are noise-driven stochastic processes. Now we would like to evaluate if plasmid frequencies are under selection at the level of single cells. To precisely control the concentration of antibiotics inside the microfluidic chip, we developed an automated pressure control system (Ferry et al., 2011) that allows us to introduce different antibiotics into the device and quantify changes in intracellular plasmid frequency in response to environmental change. So we introduced HT cells into the device and observed them for 15 h previous to the introduction to the antibiotic following a ramp protocol: linearly increasing the concentration of antibiotic until reaching a lethal dose and maintaining that concentration constant until all cells are dead.

When introducing ampicillin, we found that the distribution of Δ relative intensity remained symmetric with respect to zero, implying that AMP is not selecting for pBGT-1 plasmids at the level of individual cells. We repeated this microfluidic experiment, now introducing CAZ to select for pBRT-12 plasmids, and confirmed that the shape of the resulting distribution of Δ relative intensity was not skewed towards DsRed. Fig. 3G illustrates violin plots of Δ relative intensity for different selective pressures. Note that, independently of the environmental condition, the shape of the distributions is qualitatively the same (for AMP a Normal distribution with $\mu = -0.00073$, $\sigma^2 = 0.0129$, and for CAZ with $\mu = 0.01298$, $\sigma^2 = 0.0098$). We performed a non-parametric Kolmogorov-Smirnov normality test comparing each distribution against a theoretical Normal distribution with the corresponding μ and σ^2 (H_0: the distribution is not Normal, p-values = (0.527, 0.493, 0.8017) for LB, AMP, and CAZ respectively). In conclusion, regardless the selection regime, the distribution of Δ relative intensities follows a Normal distribution, indicating that changes in relative abundances of different plasmids in single-cells are driven by random noise and not by selection.

3.3. Using microchemostats to study plasmid dynamics in bacterial colonies

We have established that selection can modulate plasmid frequency distributions of heterozygous bacterial populations, and also that

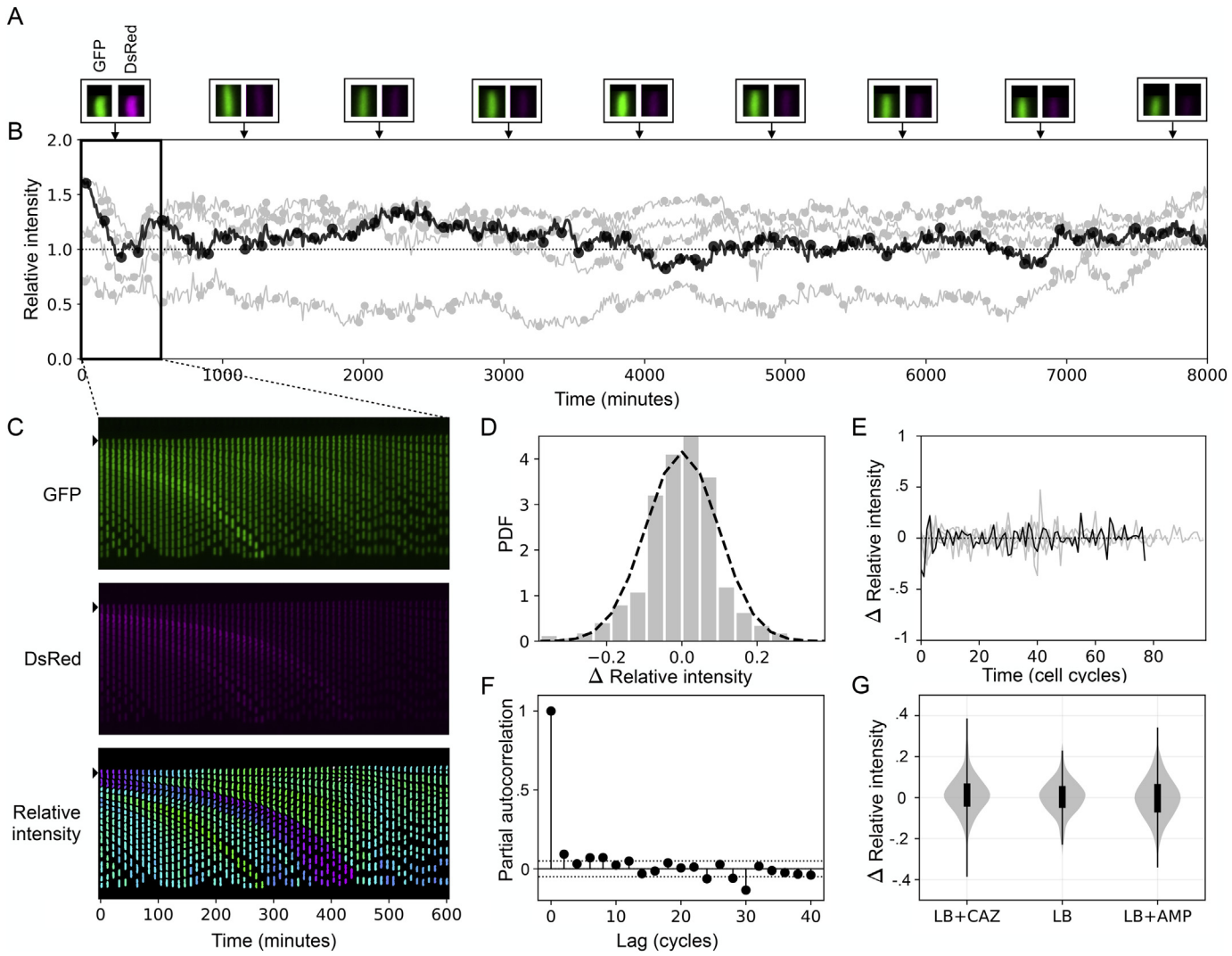

Fig. 3. A) Mother cells at different time-points. Note how the intensity in GFP and DsRed channels changes in time. B) Time-series of relative intensity for individual cells in a long-term experiment. In black data obtained from the mother cell illustrated in A), while 4 other cells are shown in grey. Circles represent division events. C) Kymograph showing the progeny of the mother cell shown in A). From the images obtained in GFP (top) and DsRed (middle), we can use image processing to estimate the relative intensity of each cell (bottom). D) Probability density function of the difference in relative fluorescence of an individual cell between consecutive frames. This distribution can be approximated by a Normal distribution with mean near zero and $\sigma^2 = 0.096$. E) Δ relative intensity as a function of time for cells shown in B). F) Partial autocorrelation function of Δ relative intensity. G) Distributions of Δ relative intensity are normally distributed. A symmetric distribution suggests a random walk that is not correlated with the selective pressure imposed by the environment (left: ceftazidime, middle: drug-free, and right: ampicillin).

intracellular plasmid dynamics is a noise-driven process that does not seem to be affected by selection. Therefore we argue that the shift in fluorescence observed at a population-level must be a consequence of antibiotics selecting for subpopulations with different plasmid configurations. To evaluate this hypothesis and to study the effect on selection in heterozygous populations, we used a different microfluidic device that provides high-throughput time-resolved information about thousands of individual cells simultaneously.

Microchemostats are designed to cultivate bacterial colonies for long periods of time in controlled and well-mixed environments (Mondragón-Palomino et al., 2011; Moffitt et al., 2012; Lopatkin et al., 2016; Li et al., 2019). In particular, here we use a microchemostat adapted from (Mondragón-Palomino et al., 2011) that consists of two parts: the signal generator and the cell confinement region. In the confinement section there are 48 rectangular chambers distributed in four rows. Each containment chamber measures $40 \times 50 \times 0.95\ \mu m^3$, with two sides open to a large channel where media is introduced and cells are washed out of the device. Since *E. coli* cells are approximately 1 μm in diameter, confining them in these microfluidic traps allows the simultaneous observation of a colony of approximately 500 cells in the same focal plane. Furthermore, as with the dual-input mother machine, we can use a signal generator to dynamically control the extracellular concentration of antibiotics.

3.3.1. *Heteroplasmy is unstable in environments with constant selection*

Fig. 4 illustrates an experiment where a population of HT cells was cultured in drug-free media for 6 h, followed by the introduction of antibiotics using a linear ramp. When drug concentration reached a lethal dose (4 *mg/ml* for AMP and 8 *μg/ml* for CAZ), the concentration of antibiotics was maintained constant until all cells were dead (see Supplementary Movies S2 and S3). Fig. 4C shows montages of selected traps at different time-points (CAZ in the top and AMP at the bottom).

We used our image processing pipeline to analyze all traps containing cells growing exponentially after growing overnight inside the device. As in the flow cytometry data, we measured the relative and absolute intensity of each individual cell but, as opposed to flow cytometry data, our microchemostat allows us to track cells in time and perform lineage reconstruction. In particular, we obtained 557 lineages,

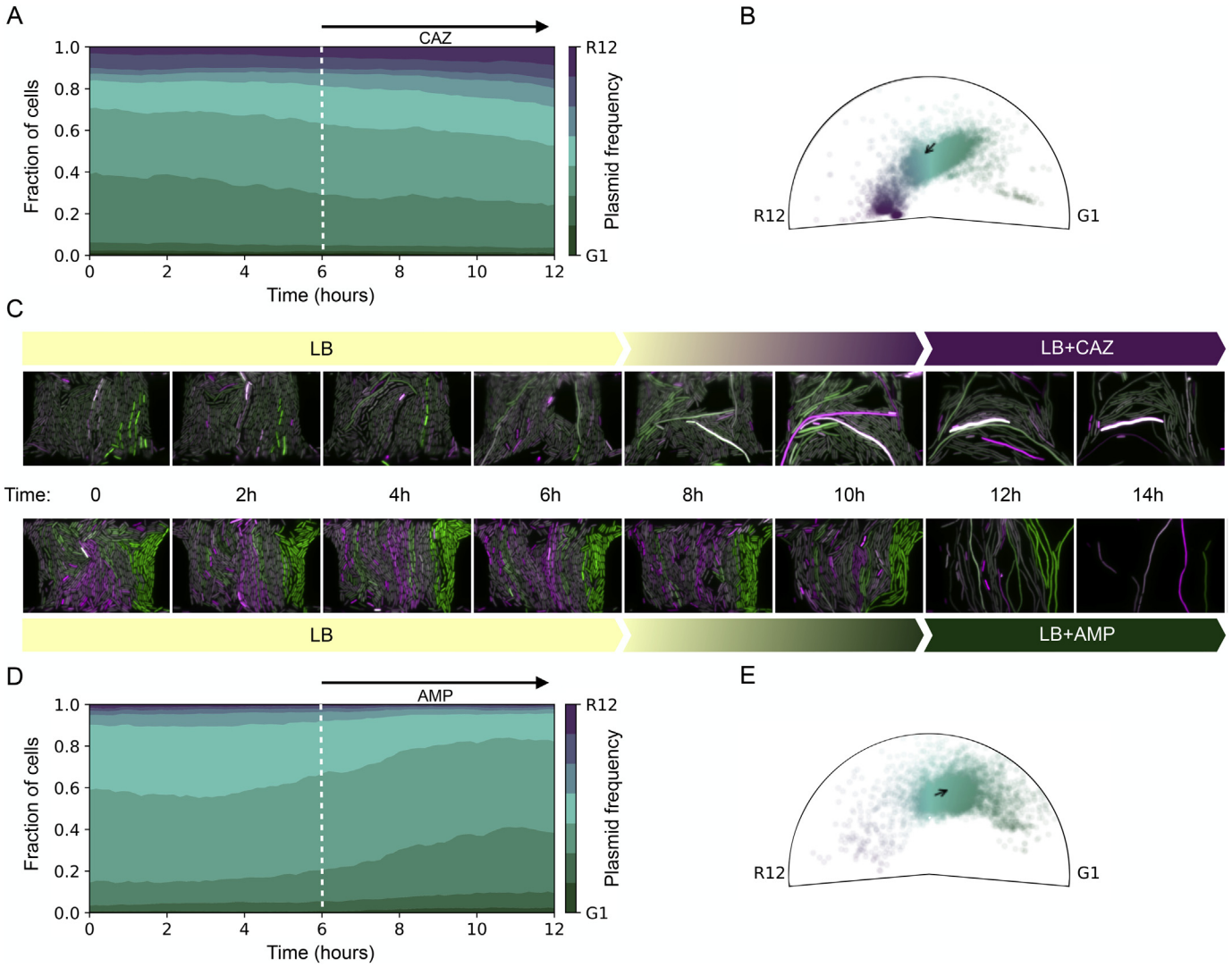

Fig. 4. A) Plasmid fraction as a function of time for a population of HT cells exposed to a ramp of CAZ. B) Polar distribution of cells at the end of the experiment. The black arrow represents changes in the mean plasmid frequency of the population, before and after antibiotic exposure. C) Montage of microscopy images (GFP channel in green, and DsRed in magenta, with both channels overlaid). D) Fraction of cells with a higher proportion of pBGT-1 plasmids is increased when AMP is introduced into the device. *E*-F) Population-level distribution at the end of the experiment. Note how the black arrow points towards higher values of GFP, suggesting that the mean plasmid frequency of the population moved towards cells carrying relatively more copies of pBGT-1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

corresponding to 5,870 cells in the CAZ experiment and 498 lineages of 5,754 cells for AMP. Of course, as the colony is growing exponentially, most cells are pushed out of the trap and washed out of the device, so only a few lineages were observed from start to end of the experiment. We recovered 48 complete lineages for CAZ and 46 for AMP and, consistent with the results shown in Fig. 3G, the resulting time-series were not correlated with the selective pressure imposed by the environment.

Now, by clustering the population according to their relative fluorescent intensity, we determined the fraction of cells with different plasmid frequencies. As illustrated in Fig. 4A, exposing a population of HT cells to CAZ produces an increase in the fraction of cells with high levels of DsRed and low intensity values of GFP, implying that selection favours cells with a higher proportion of pBRT-12 plasmids. The black arrow in the polar distributions shown in Fig. 4B denotes changes in the mean relative intensity of the population after 6 h of exposure to CAZ and, as expected, points towards R12. In contrast, when introducing AMP into the device, the fluorescent intensity distribution appeared to be shifted towards GFP, consequence of G1 cells being positively selected for, a feature that can be seen in Fig. 4D and in the polar distribution shown in Fig. 4E.

Notably, the shift is larger when using CAZ than in the presence of AMP. This is explained by $bla_{\mathrm{TEM-1}}$ providing partial resistance to AMP and therefore the relative fitness (and thus the rate of fixation) is larger for R12 in ceftazidime than G1 in ampicillin. In any case, HT cells reduced in frequency and are destined to be outcompeted by homozygous subpopulations: R12 if using CAZ or G1 in an AMP environment. This is consistent with previous studies showing that heteroplasmy is unstable in constant environmental conditions (Rodriguez-Beltran et al., 2018). It has also been reported that fluctuating environments can stably maintain intracellular genetic diversity for long-time intervals, so in the following section we will evaluate this hypothesis using microchemostats.

3.3.2. *Fluctuating environments stabilize genetic diversity*

By alternating both antibiotics periodically, we experimentally explored if fluctuating environmental conditions can stabilize plasmid-mediated heterozygosis. Specifically, we introduced HT cells into the device and observed them in LB for about 3 h before introducing antibiotics. To implement a fluctuating selection regime, we generated a

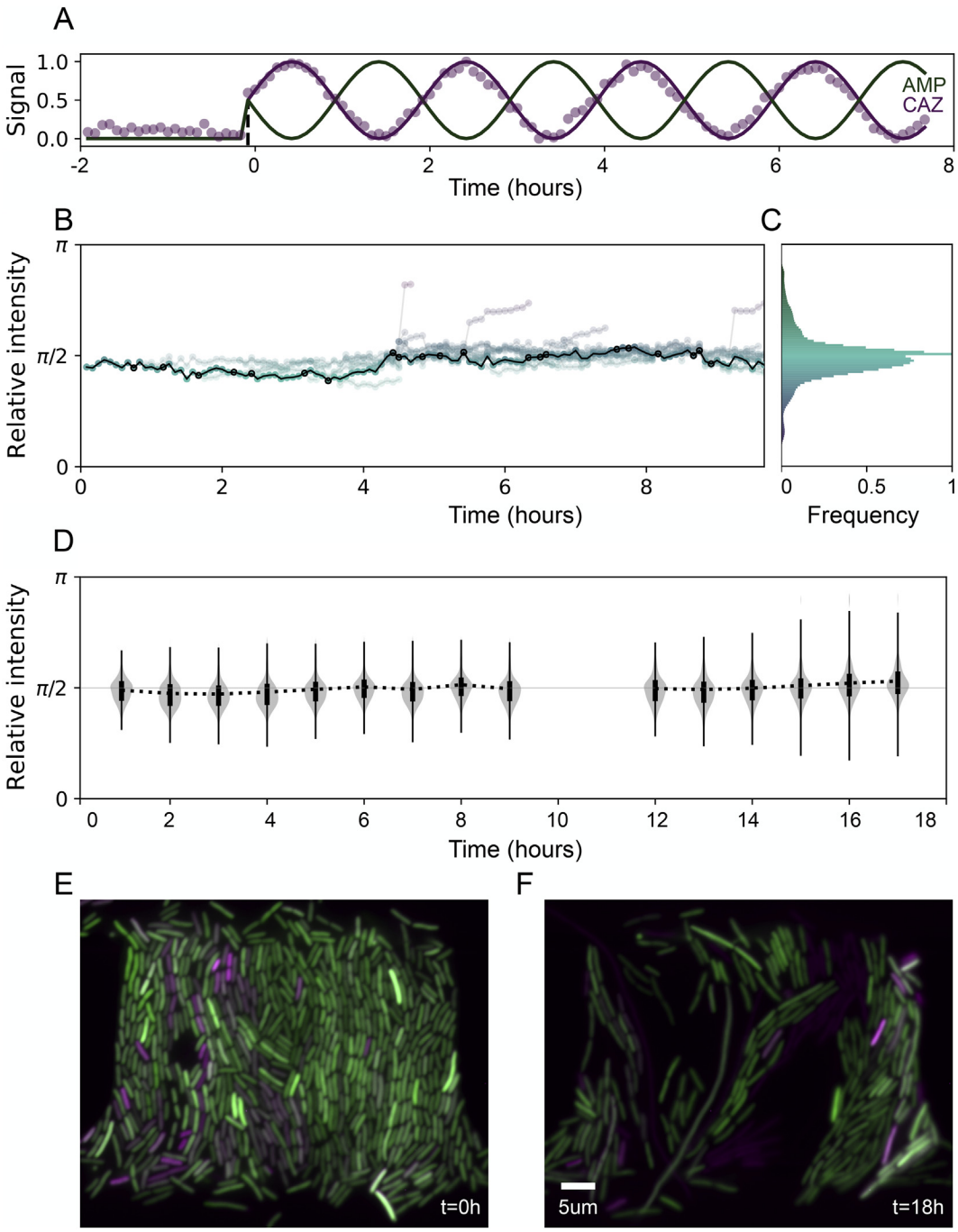

Fig. 5. A) Oscillatory drug deployment protocol consisting on CAZ (magenta) and AMP (green) being alternated every two hours. Magenta circles correspond to measured values of fluorescent dye also introduced into the device together with CAZ. B) Black line represents a single-cell lineage obtained from a time-lapse movie of a microchemostat. Black circles represent division events and relative fluorescence of daughter cells is illustrated in cyan. C) Relative fluorescent distribution obtained after 18 h of exposure to fluctuating CAZ and AMP selective pressures. D) Population-level relative intensity distributions at different time-points. A consequence of alternating selection for both alleles is that intermediate values of relative fluorescence are maintained for long time periods, suggesting that genetic diversity can be stabilized in fluctuating environmental conditions. E) Microscopy images at the beginning (left) and at the end (right) of the experiment. Note how, after 18 h of fluctuating selection, the resulting population is composed of R12 and G1 cells, but also of HT cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sinusoidal signal of period 2 h such that, when CAZ concentration is at 100%, then AMP is at 0%, and vice versa, as illustrated in Fig. 5C (in green the concentration of AMP and in magenta of CAZ, both normalized to the same critical concentrations used before). We diluted a fluorescent dye to one of the antibiotic inputs to calibrate the height of the syringes, but this also allowed us to use the fluorescent microscope to validate that cells are exposed to the expected proportion between both antibiotics. Magenta dots represent DsRed measurements in a cell-free area of the device and correspond very precisely with the drug-deployment protocol sent by the signal generator.

Fig. 5B shows a lineage reconstruction where the black line corresponds to an individual cell observed for the complete duration of the experiment, while other cells in the lineage are illustrated in cyan. Note how, as previously shown in the mother-machine, the intracellular plasmid dynamics appears to be random and is not correlated with the environmental signal. A consequence of the random segregation and

replication of plasmids is that, after only a few generations, the distribution of alleles in the population presents a large variance, as shown in Fig. 5C.

As we have previously argued, we cannot make inferences about the stability of plasmid-mediated heterozygosis from single-cell data. So we included the remaining cells to our analysis and estimated relative intensity distributions at different time-points. Fig. 5D shows violin plots representing the distribution estimated every hour. As opposed to the constant drug environment discussed previously, in the alternating selection regime, the mean relative intensity is centered around HT throughout the duration of the experiment (although the variance increases in time). Fig. 5E and F show composite images at $t = 0$ and at $t = 18$ h, extracted from Supplementary Movie S4, revealing the presence of G1 and R12 cells at the end of the experiment and, crucially, of cells still bearing both plasmids.

4. Discussion

The rate at which pathogenic bacteria evolve resistance to antibiotics is dramatically decreasing the efficacy of current antimicrobial treatments. It may seem a surprising statement but, after more than a century of using antimicrobials in the clinic, some of the evolutionary forces that drive the emergence and spread of drug resistance in pathogenic bacteria are still poorly understood. For instance, most of our understanding of drug resistance adaptation assumes that clonal populations growing in constant environments present similar susceptibility and resistance profiles to antibiotics, while actually clinical isolates can present a high degree of heterorresistance generated, in many cases, by heterogeneous expression of plasmid-borne resistance genes (Andersson et al., 2019).

In a previous paper (Rodriguez-Beltran et al., 2018), we used mathematical modelling and experimental evolution to argue that multi-copy plasmids can provide a platform to increase intracellular genetic diversity and, in consequence, enhance the probability of survival to dynamic environmental conditions. Here we used microfluidics and fluorescence microscopy to study, with single-cell resolution, the effect of selection in the relative abundance of incompatible plasmids carrying different versions of an antibiotic resistance gene and a fluorescent marker. As expected, in the absence of selection, the stochastic nature of plasmid replication and segregation renders plasmids unstable and decreases allele frequency in the population. In contrast, positive selection for plasmid-encoded genes stabilizes plasmids at high copy-numbers, increasing the frequency of the corresponding allele and promoting resistance to the antibiotics used.

Of course, natural environments are not constant but alternate selection between subpopulations with different genetic configurations. Therefore, in dynamic environments, it may be optimal for bacterial populations to present genetic heterogeneity, thus increasing the probability that some individuals are pre-adapted to future environmental conditions (Ackermann, 2015). Indeed, in agreement with previous laboratory studies (Rodriguez-Beltran et al., 2018), we showed that fluctuating selection - in this case, alternating the extracellular concentration of different β-lactam antibiotics - maintained intracellular genetic diversity in the population for longer than constant environmental regimes.

Although previous studies have successfully deployed a combination of experimental evolution (MacLean and San Millan, 2015; Harrison and Brockhurst, 2012; Holloway et al., 2007), genome sequencing (San Millan et al., 2014; Harrison et al., 2015; Porse et al., 2016) and mathematical modeling (Stewart and Levin, 1977; Santer and Uecker, 2019; San Millan et al., 2014; Wein et al., 2019; Yurtsev et al., 2013) to evaluate the population dynamics that emerge in response to different environmental conditions, the intrinsic resolution of flow cytometers and qPCR machines do not allow us to dissect stochastic plasmid dynamics (generated by randomly replicating and partitioning plasmids) from deterministic population-level effects (e.g. differences in relative fitness associated with expressing multiple alleles). So, in this paper, we used single-cell microfluidics to generate high-throughput fluorescent intensity data of heterozygous bacterial populations exposed to a range of selective regimes.

In particular, we used computer vision algorithms to analyze time-lapse movies acquired in multiple fluorescence channels, allowing us to characterize the allele distribution in the population in terms of the relative and absolute fluorescent intensities of its constituent cells. This allowed us to evaluate directly the contribution of selection and random genetic drift in the rate of fixation and extinction of different plasmid variants. We showed, using a mother-machine to restrain individual cells and follow them for very long periods, that changes in plasmid frequency are the consequence of a noise-driven process that is not correlated with the direction and strength of selection imposed by the environment.

We conclude by arguing that imaging and microfluidics can provide a potentially useful approach to study the interaction between intracellular plasmid dynamics and selection imposed by the environment, and therefore could be used to increase our understanding of the complex interaction between mobile genetic elements, their bacterial hosts, and the environment.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plasmid.2020.102517.

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Capítulo 6

Dinámica Evolutiva de Heterocigosis Mediada por Plásmidos

6.1 Introducción

En los capítulos anteriores observamos que portar genes en plásmidos multicopia puede producir heterogeneidad fenotípica, confiriendo la capacidad de implementar una estrategia de apuesta-cobertura a partir de mantener subpoblaciones con menor y con mayor número de copias del plásmido y, por consiguiente, con menor o mayor tolerancia a antibiótico. En este capítulo indagaremos las ventajas evolutivas que la heterocigosis mediada por plásmidos le confieren a las poblaciones de bacterias.

Resulta evidente que portar genes benéficos en plásmidos multicopias incrementa la dosis génica; diversos estudios han demostrado que las amplificaciones genéticas y sus modificaciones juegan un papel esencial en la evolución de nuevos fenotipos bacterianos (Ochman et al. 2000; Näsvall et al. 2012; Andersson and Hughes 2009). Entonces portar genes benéficos en plásmidos multicopia podría ayudar a la bacteria a producir nuevos fenotipos a través de generar variantes alélicas en copias que no están bajo selección. En el contexto de plásmidos, esto podría generar una gran diversidad de polimorfismos y por lo tanto de fenotipos.

En este capítulo nos limitaremos a estudiar dos variantes alélicas de genes portados en plásmidos utilizando el sistema experimental presentado en el capítulo anterior. Hemos establecido que aún con dos variantes alélicas distintas células pueden tener diferentes proporciones de plásmidos y, por lo tanto, generar un rango de fenotipos. Mediante experimentos a nivel de poblaciones comparamos poblaciones que portan ambos plásmidos dentro de una misma célula con poblaciones que cuentan con la misma diversidad celular pero mediante subpoblaciones que portan un solo tipo de plásmido. Encontramos que las poblaciones con diversidad intracelular son capaces de mantener la diversidad genética por largos periodos aún ante selección negativa, produciendo poblaciones más resilientes ante cambios de ambientes hostiles.

Tomando en cuenta estas características, postulamos un modelo de dinámica de poblaciones fundamentado en ecuaciones diferenciales ordinarias para corroborar nuestros resultados y realizar experimentos *insilico* para explorar los regímenes de selección que mantengan PMH.

6.2 Modelo de dinámica de poblaciones

El objetivo del modelo es estudiar poblaciones en respuesta a diferentes condiciones ambientales, (recurso limitante y antibióticos). Por ello se considera que la población está compuesta de una subpoblación libre de plásmidos con densidad al tiempo t denotada por $B_{PF}(t)$, una subpoblación heterocigota con densidad $B_{HT}(t)$ y subpoblaciones homocigotas G1 y R12 representadas por $B_{G1}(t)$ y $B_{R12}(t)$, respectivamente.

Se considera que el crecimiento de cada población depende de la concentración del recurso limitante ambiental $R(t)$, por lo que podemos modelar el crecimiento de una subpoblación i como $G_i(R) = r_i U_i(R)$, donde ϱi es un parámetro constante y $U_i(R) = V^i_{max} R/(K^i_m + R)$ una función de incorporación de recursos con parámetros V^i_{max} y K^i_m que denotan respectivamente la máxima tasa de crecimiento y la constante de saturación de la subpoblación i. Para tomar en cuenta la acción de los antibióticos (Amp y Caz) representados por D^A y D^C respectivamente, consideramos el perfil de susceptibilidad de la cepa B_i a Amp y Caz por un término lineal con un parámetro de inhibición k^A_i y k^C_i respectivamente. También δ^A_i y δ^C_i denota la tasa de degradación de D^A y D^C respectivamente para la cepa B_i.

Como estos plásmidos se segregan al azar, la probabilidad de que una célula que porta plásmidos produzca una célula libre de plásmidos es un proceso de Poisson que depende del promedio de número de plásmidos, esto es $\Sigma = 2^{1-\mu}$ donde μ denota el promedio de número de plásmidos en la población (San Millan et al. 2014). De igual manera, la probabilidad de que una célula heterocigota produzca una homocigota, asumiendo que HT tiene la misma proporción de plásmidos, puede ser estimada por $\sigma = 2^{1-\mu/2}$. En nuestro sistema experimental el promedio de número de copias del plásmido es 19 y por ello la tasa de pérdida de heterocigosis es 1/362 mientras que la tasa de pérdida de plásmidos es considerablemente menor, 1/524,288. Finalmente la dinámica evolutiva del sistema considera que la transición de B_{G1} y B_{R12} a B_{HT} ocurre con una tasa ε >0, representando la tasa de mutación puntual en los alelos $bla_{\text{TEM-1}}$ o $bla_{\text{TEM-12}}$. En resumen, el sistema de ecuaciones diferenciales ordinarias que describe la dinámica de plásmidos a nivel de poblaciones, puede escribirse de la siguiente manera:

$$
\begin{aligned}
\frac{d}{dt}R &= -\left(U_{PF}(R)B_{PF} + U_{G1}(R)B_{G1} + U_{HT}(R)B_{HT} + U_{R12}(R)B_{R12}\right) \\
\frac{d}{dt}D^A &= -D^A\left(\delta^A_{PF}B_{PF} + \delta^A_{G1}B_{G1} + \delta^A_{HT}B_{HT} + \delta^A_{R12}B_{R12}\right) \\
\frac{d}{dt}D^C &= -D^C\left(\delta^C_{PF}B_{PF} + \delta^C_{G1}B_{G1} + \delta^C_{HT}B_{HT} + \delta^C_{R12}B_{R12}\right) \\
\frac{d}{dt}B_{PF} &= G_{PF}(R)B_{PF} + \Sigma(G_{G1}(R)B_{G1} + G_{HT}(R)B_{HT} + G_{R12}(R)B_{R12}) - B_{PF}(\kappa^A_{PF}D^A + \kappa^C_{PF}D^C) \\
\frac{d}{dt}B_{G1} &= (1-\varepsilon-\Sigma)G_{G1}(R)B_{G1} + \sigma G_{HT}(R)B_{HT} - B_{G1}(\kappa^A_{G1}D^A + \kappa^C_{G1}D^C) \\
\frac{d}{dt}B_{HT} &= (1-\Sigma)G_{HT}(R)B_{HT} + \varepsilon(G_{G1}(R)B_{G1} + G_{R12}(R)B_{R12}) - B_{HT}(\kappa^A_{HT}D^A + \kappa^C_{HT}D^C) \\
\frac{d}{dt}B_{R12} &= (1-\varepsilon-\Sigma)G_{R12}(R)B_{R12} + \sigma G_{HT}B_{HT} - B_{R12}(\kappa^A_{R12}D^A + \kappa^C_{R12}D^C)
\end{aligned}
\tag{6.2.1}
$$

Con parámetros estimados utilizando un método de parametrización bayesiano que implementa un método Monte Carlo de Cadenas de Markov con muestreo Metropolis-Hastings. Este método es similar al publicado por San Millan et al. (2014). Los parámetros estimados y mayores detalles pueden encontrarse en el material suplementario del trabajo presentado por Rodriguez-Beltran et al. (2018).

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Multicopy plasmids allow bacteria to escape from fitness trade-offs during evolutionary innovation

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Understanding the mechanisms governing innovation is a central element of evolutionary theory. Novel traits usually arise through mutations in existing genes, but trade-offs between new and ancestral protein functions are pervasive and constrain the evolution of innovation. Classical models posit that evolutionary innovation circumvents the constraints imposed by trade-offs through genetic amplifications, which provide functional redundancy. Bacterial multicopy plasmids provide a paradigmatic example of genetic amplification, yet their role in evolutionary innovation remains largely unexplored. Here, we reconstructed the evolution of a new trait encoded in a multicopy plasmid using TEM-1 β-lactamase as a model system. Through a combination of theory and experimentation, we show that multicopy plasmids promote the coexistence of ancestral and novel traits for dozens of generations, allowing bacteria to escape the evolutionary constraints imposed by trade-offs. Our results suggest that multicopy plasmids are excellent platforms for evolutionary innovation, contributing to explain their extreme abundance in bacteria.

The ability of a species to expand its ecological niche and thrive in new environments depends directly on the development of novel adaptive traits through evolutionary innovation. Evolutionary innovation, in turn, relies on the repurposing of old traits to serve new roles[1,2]. Examples of this process are abundant and include the development of important traits such as new metabolic capabilities[3], the control of bacterial division[4], and the evolution of multicellularity[5]. However, mutations providing a new role are usually detrimental to native gene function. These negative correlations between ancestral and evolved traits are called trade-offs. Trade-offs are common during evolutionary innovation and have been identified in a plethora of natural and experimental settings[2]. Crucially, the interplay between trade-offs and selective pressures for the ancestral and new activities determines the fate of innovative mutations. For instance, strong selection to maintain the native activity leads to the purging of variants with adaptive potential, hampering innovative evolution[6,7]. Hence, trade-offs are arguably one of the major constraints on evolutionary innovation.

Several models have been proposed to explain the ready occurrence of innovation despite the restraints imposed by trade-offs. Most of these models are based on the emergence of genetic amplifications that provide genetic redundancy[1,8–11]. Amplifications, such as duplications, alleviate trade-offs because they allow the coexistence of different alleles of the same gene. In this way, extra gene copies might acquire new functions while others retain their original role[8]. However, genetic amplifications might be unstable as they are usually a target for homologous recombination, resulting in the deletion of one of the duplicated regions[10,12]. Furthermore, the majority of amplifications have been found to be neutral or deleterious in the absence of selection[13]. Nevertheless, stable amplifications have been reported for genes involved in antibiotic resistance[14], metabolism[15] or encoding weak secondary functions[11,16].

Among the most paradigmatic examples of genetic mechanisms providing genetic redundancy in prokaryotes are multicopy plasmids (MCPs): small and highly prevalent genetic elements in bacteria[17], that typically range from 10 to 30 copies per cell and lack active segregation and partition systems[18]. Therefore, MCPs maintain a population of the same genes within a cell, and thus can be regarded as an extreme example of stable genetic amplification. Here, we propose that MCPs could alleviate trade-offs during the evolution of innovation. This hypothesis is based on two predictions. First, the multicopy nature of these plasmids allows different versions of the gene (alleles) to coexist in the same cell under heterozygosity, and this coexistence will alleviate fitness trade-offs. Second, once the evolved allele appears, plasmid segregation and replication dynamics will maintain heterozygosity at the cellular and population level for a prolonged period of time, even under strong selection for one of the alleles (Fig. 1a). To test these predictions, we used a well-characterized model of antibiotic resistance evolution in which trade-offs have profound effects on adaptive trajectories[7,19,20]. By combining simple mathematical models with experimental work, we demonstrate that MCPs promote the maintenance of genetic diversity against strong selection, alleviating trade-offs and promoting evolutionary innovation.

Results

Experimental system. TEM-1 is a β-lactamase that confers high-level resistance to penicillins such as ampicillin (Amp) but negligible resistance to third generation cephalosporins such as ceftazidime (Caz). The evolution of TEM-1 toward conferring Caz resistance has been shown in laboratory and natural settings to occur predominantly via a single mutation (Arg164Ser) that gives rise to TEM-12[19,21]. The evolved allele ($bla_{\text{TEM-12}}$) confers high-level resistance to Caz at the expense of a reduced activity against Amp compared with the ancestral allele ($bla_{\text{TEM-1}}$), thus demonstrating a

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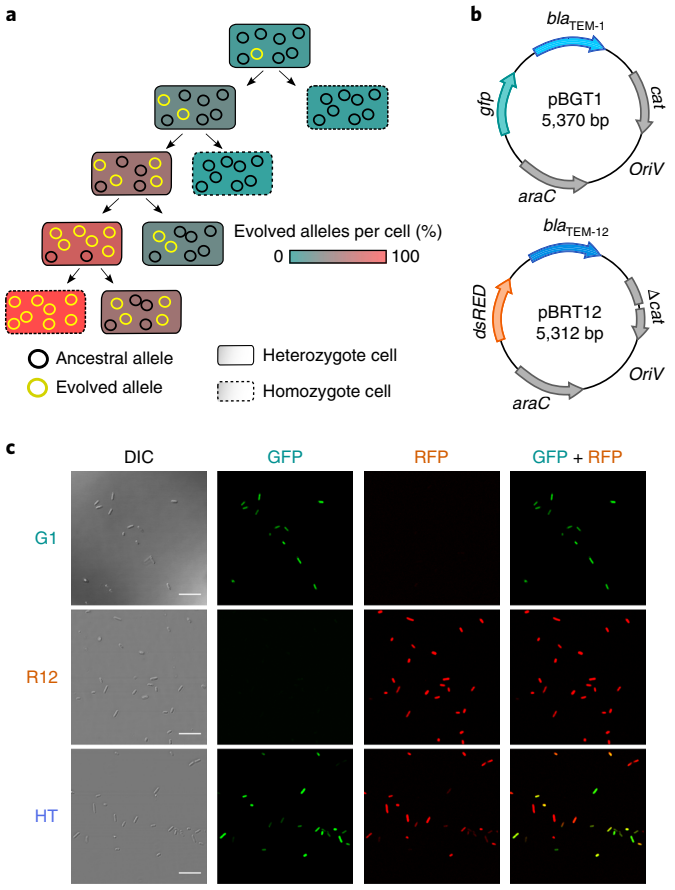

Fig. 1 | Conceptual and experimental models. a, Scheme of the plasmid replication and segregation dynamics that drive the co-existence of ancestral and mutated alleles under heterozygosity. In every generation, plasmids are randomly segregated to daughter cells and then replicated. Following this simple model, heterozygosity is maintained for several generations as an emergent property of plasmid replication and segregation dynamics. **b**, Maps of the plasmids used in our experimental model. **c**, Confocal microscopy images showing representative fields of bacterial populations of the G1 and R12 homozygote strains, and the HT strain. Scale bar, 10 μm.

trade-off between Amp and Caz resistance[19,21]. In a recent study, we detected TEM-12 mutation arising in response to Caz pressure in an experimental model using plasmid pBGT-1; an MCP carrying $bla_{\text{TEM-1}}$ (ref. [21]). Crucially, sequencing of evolved clones revealed pBGT-1 plasmid variants differing only in the TEM Arg164 residue coexisting under heterozygosity (see supplementary file 2 of ref. [21]). To test the ability of MCPs to alleviate trade-offs, we reproduced the TEM-1 evolutionary scenario using a traceable MCP system. We developed two plasmids coding TEM-1 or TEM-12. As the ancestral allele carrier we used plasmid pBGT-1, which occurs at ~19 copies per bacterium and includes a $bla_{\text{TEM-1}}$ gene and a tightly controlled *gfp* gene[21]. In parallel, we constructed the pBRT-12 plasmid by replacing $bla_{\text{TEM-1}}$ with $bla_{\text{TEM-12}}$ and the *gfp* gene with *dsRED* to allow plasmid identification by fluorescence (Fig. 1b). We also constructed plasmids with interchanged fluorescent markers (pBGT-12 and pBRT-1; Supplementary Fig. 1) and used them to show that the reporter genes did not influence the results obtained hereafter (Supplementary Table 1, Supplementary Figs. 2–4). We transformed *Escherichia coli* MG1655 with the pBGT1 and pBRT12 plasmids individually, respectively generating the G1 and R12 strains, and also together to generate a heterozygote strain (HT) carrying both plasmids (Supplementary Table 1). Direct observation of cells under confocal microscopy (Fig. 1c) and whole-genome sequencing of the genomes of all strains confirmed their isogenic nature and the presence of the different plasmids.

Multicopy plasmid-mediated heterozygosity alleviates trade-offs. It is reasonable to expect that, by maintaining copies of the ancestral and the evolved alleles within individual cells, MCPs would alleviate trade-offs emerging during evolutionary innovation. To test this possibility, we measured the ability of the G1, R12 and HT strains to colonize an array of 48 liquid environments containing varying concentrations of Caz or Amp alone or in combination (Fig. 2a). We also included a 1:1 mixed population of the G1 and R12 homozygotes as a control. The G1 and R12 strains were unable to grow at high concentrations of Caz or Amp, respectively, demonstrating the anticipated trade-off (Fig. 2a). By contrast, both the HT and the mixed population colonized a significantly higher number of antibiotic environments than the ancestral (G1) and evolved (R12) strains (Fig. 2b; one-way ANOVA $F=290.7$, d.f. $=3$, $P<0.001$ followed by Tukey's multiple comparison of means $P<0.001$). This result indicated that the presence of both alleles, either at the population level (the mixed population) or at the cellular level (the HT strain), allowed bacterial populations to persist in the presence of Caz and Amp.

Because the plasmids used in this study were engineered to carry distinguishable fluorescence reporters (Fig. 1b), we were able to estimate the relative abundance of $bla_{\text{TEM-1}}$ and $bla_{\text{TEM-12}}$ alleles by simply measuring the GFP/RFP ratio in each environment (Methods and Supplementary Fig. 5). As expected, both the HT and the mixed populations—but not the homozygotes—showed variation in the plasmid proportions in every environment in response to different antibiotic combinations (Fig. 2c). In general, higher concentrations of Caz selected for higher proportions of pBRT12, biasing fluorescence toward red (Spearman's rho −0.58 and −0.71 for Mix and HT, respectively; $P<0.01$). Higher Amp concentrations selected for pBGT1, and therefore produced a higher GFP/RFP ratio (Spearman's rho 0.92 and 0.69 for Mix and HT, respectively; $P<0.03$).

To quantify allelic richness across environments, we counted the environments that contained both plasmids at detectable levels (that is, environments with fluorescence ratios between those of the homozygote populations; shaded region on Fig. 2d). Although the HT and Mix populations were able to colonize the same number of environments, HT maintained both alleles to a greater extent than the mixed population (45% [38/84] versus 23% [18/78] of the colonized environments, respectively; $\chi^2=7.8293$, d.f. $=1$, $P<0.01$).

Multicopy plasmids maintain genetic diversity against selection. Our second prediction was that once an adaptive mutation appears in an MCP, random plasmid segregation will maintain heterozygosity at the cellular level (and consequently at the population level) for a prolonged period, even under strong selection for one of the alleles. To study the fixation dynamics of the innovative allele $bla_{\text{TEM-12}}$ under strong Caz selective pressure, we performed invasion experiments in which a small (~1%) HT population invades a G1 population. As a control, we performed the same experiment with R12, instead of HT, invading the G1 population (Fig. 3). The experiment, which ran for eight days with four replicates per treatment, started with the minimal selective concentration[22] of Caz for the $bla_{\text{TEM-12}}$ allele (0.0625 mg l^{-1}; Supplementary Fig. 4). Every day, 0.1% of each population was transferred to fresh medium containing double the Caz concentration of the preceding day. The relative abundance of each genotype was tracked by selective plating, and the frequency of the $bla_{\text{TEM-1}}$ and $bla_{\text{TEM-12}}$ alleles in every population was determined by qPCR.

The results showed that the G1 strain was rapidly outcompeted both by the R12 and HT strains at the same Caz concentration (25%

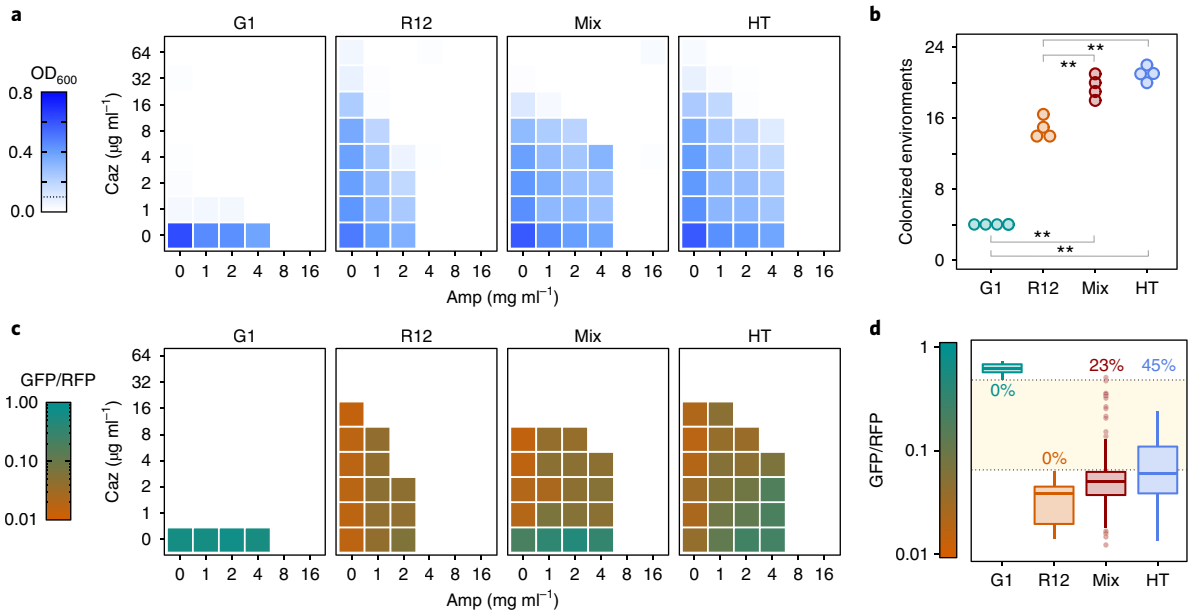

Fig. 2 | Bacterial growth and allelic content in the antibiotics array. a, The optical density (OD_{600}) of populations growing in a range of concentrations of Caz and Amp alone or in combination is colour-coded as indicated in the legend. We concluded that a particular environment in the array was colonized if, after 20 h of incubation, OD_{600} was greater than 0.1 (dotted line in the legend). **b**, The number of colonized environments of the four replicate arrays plotted for every genotype and the mixed population. The asterisks denote significant differences (Tukey's test $P < 0.001$ after significant ANOVA). **c**, The GFP/RFP fluorescence ratio (arbitrary units) offers insight into the allelic content of every environment and is plotted according the colour-code shown in the legend. **d**, Boxplots showing the distribution of fluorescence ratios in four replicate arrays for each genotype. The yellow-shaded region denotes fluorescence levels between those of the G1 and R12 strains and thus those populations carrying both plasmids at detectable levels. The numbers designate the percentage of environments colonized by a population containing both plasmids. For the sake of clarity only one of four replicates is shown in **a** and **c**. All four individual replicates can be found in Supplementary Data 1, under the label 'day 1'.

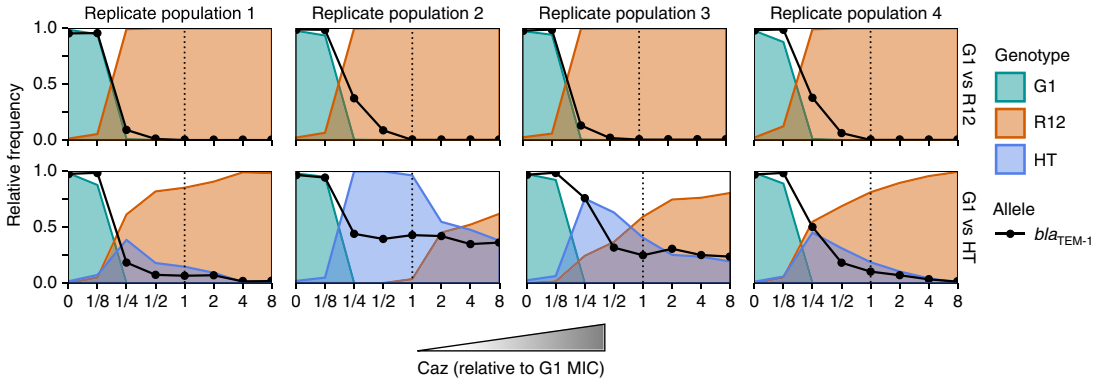

Fig. 3 | Invasion experiments. The upper panels show invasion experiments in which G1 population was invaded by a rare R12 population (1%); the lower panels show invasion experiments in which the HT strain invaded instead of R12. Experimental results obtained with four replicate populations (columns). Invasion experiments were carried out for eight consecutive days in which the Caz concentration in the medium was doubled daily. Each panel shows, for an individual invasion, the relative frequency of every genotype on each day, calculated by selective plating (filled areas). The solid black line shows the frequency of pBGT-1/pBRT-12 (bla_{TEM-1}/bla_{TEM-12}) calculated by qPCR. The black dotted vertical line denotes the day on which the MIC of Caz for G1 was reached.

of the G1 minimal inhibitory concentration; MIC). In the control invasions this meant the rapid fixation of the R12 genotype. However, in the HT invasions, the HT strain displaced G1 but never reached fixation as it was gradually replaced by R12 cells that emerged as segregants through intracellular fixation of pBRT-12. Even so, HT cells were still detectable at the end of the experiment (mean 14.8%; range 0.005–37.9%) after approximately 77 bacterial generations under increasing selective pressure for the bla_{TEM-12} allele. qPCR results confirmed that bla_{TEM-1} is maintained throughout the experiment when HT is invading. Interestingly, these results showed that HT cells are able to maintain bla_{TEM-1} in the population well above the MIC of Caz conferred by this allele, increasing the allelic diversity of these populations throughout the experiment. This result is demonstrated by the fact that the Simpson diversity index calculated from qPCR data over the experiment is significantly higher when the invading strain is HT than when it is R12 (repeated-measures ANOVA invading genotype by time interaction $F_{7,21} = 4.767$, $P < 0.003$). Taken together, these results demonstrate that by slowing down the fixation of beneficial mutations multicopy plasmids preserve genetic diversity against strong selective pressures.

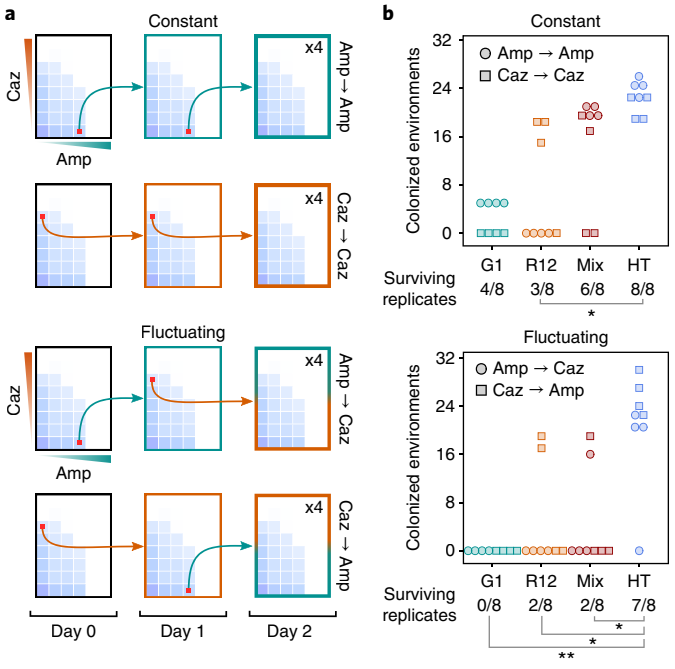

Fig. 4 | MCPs alleviate trade-offs under strong selective pressures. **a**, Experimental design. Each plate represents an antibiotic array (identical to those shown in Fig. 2) from which the bacterial populations growing in either high Caz or high Amp concentrations (red filled squares) were diluted and used to sequentially seed new antibiotic arrays over a two-day course. This gives rise to four selective regimes; two in which selection was held constant (Caz → Caz and Amp → Amp) and two with fluctuating selection (Caz → Amp and Amp → Caz). **b**, Dot plot showing the number of colonized environments by each replicate line at the end of the experiment (day 2). Each dot represents a replicate line, and the symbol denotes the antibiotic treatment. Populations that became extinct colonized zero environments. Note that the results are grouped by the type of selection regimen. Under each panel, the number of surviving replicates is shown together with asterisks denoting statistical significance (*$P<0.05$, **$P<0.002$; χ^2 test). Raw data of all replicates can be found in Supplementary Data 1.

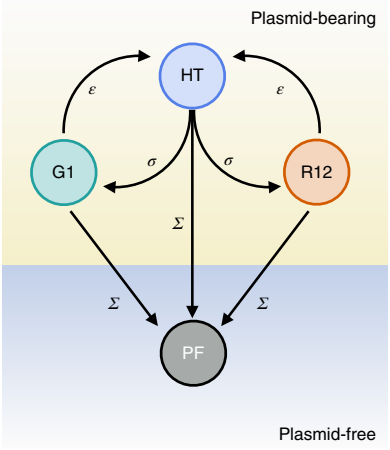

Fig. 5 | Theoretical model. Diagram illustrating the population dynamics of homozygote cells (G1 and R12), heterozygote cells (HT) and plasmid-free cells (PF). As described in the Methods, we assume that plasmids are uniformly distributed in the cell and therefore the rate of segregational loss (Σ) can be derived from the population-level mean plasmid copy number (see Methods and ref. [62]). Transitions between HT to G1 and R12 are also segregation-driven processes that occur at a rate σ, a parameter that can be estimated by assuming that HT cells have, in average, equal allelic proportions. The HT cells are produced by single-point mutation (occurring at a rate ε) in either G1 or R12 strains.

Multicopy plasmids promote adaptation under constant and fluctuating selective pressures. We have shown that MCPs allow populations to invade new environmental conditions and maintain genetic diversity over time in bacterial populations. These results suggest that MCPs may provide an important evolutionary advantage by alleviating trade-offs over time and promoting adaptation in bacterial populations under strong selective pressures. To test this possibility, we designed a short-term selection experiment in which we propagated G1, R12, HT and mixed populations (G1 + R12) under constant or fluctuating strong selective pressure with Amp (4,096 mg l^{-1}) and Caz (16 mg l^{-1}) over a period of two days (Fig. 4a). Although below the MIC, these concentrations are the highest at which growth of any of the genotypes was observed and therefore imposed an extremely stringent selective pressure for the bla_{TEM} alleles. This approach led to four possible antibiotic treatment routes that can be grouped into two constant selection regimes (Amp → Amp and Caz → Caz) and two fluctuating selection regimes (Amp → Caz and Caz → Amp). Every day, the populations able to survive the antibiotic treatments were inoculated into a fresh complete array of 48 Caz/Amp environments. With this experiment, we were able to measure two adaptive traits for each genotype: (i) survival under strong selective pressure and (ii) the ability to grow in 48 Caz/Amp environments after selection. We predicted that the HT population would be able to escape trade-offs over time better that any other genotype, leading both to higher survival and to better colonization of Caz/Amp environments after the antibiotic treatments, especially under fluctuating selection regimes.

These predictions were confirmed with four experimental replicates for each genotype and treatment (Fig. 4b and Supplementary Data 1). The extinction patterns showed that although the HT strain was relatively insensitive to the antibiotic treatment, the homozygotes and the mixed populations faced massive extinctions. This pattern was especially true under fluctuating selection regimes, where all G1 replicates became extinct and only 2 of 8 replicate lines of the R12 homozygote population and 2 of 8 of the mixed population survived; in contrast, 7 of 8 HT strain replicates survived (HT versus Mix, HT versus R12 and HT versus G1; Fisher exact test $P<0.04$). In addition, the surviving HT populations were able to colonize more environments at the end of the experiment than those from the homozygote and mixed populations regardless of the treatment (Tukey HSD $P<0.00005$ after ANOVA effect of genotype on the number of colonized environments, $F=25.116$, d.f. = 3, $P<0.001$).

Given the short duration of the selection experiment, new antibiotic resistance mutations arising in the populations are unlikely. Hence, our results strongly suggest that the better survival of the HT strain and its markedly improved ability to thrive in the range of environments after selection are a consequence of the preservation of allelic richness by MCPs. Consistent with this view, we found that the HT strain maintained both alleles in a significantly higher number of environments than the mixed populations, both in constant regimes (35/181 versus 6/118 of colonized environments, respectively; $\chi^2=11.088$, d.f. = 1, $P<0.001$) and in fluctuating regimes (57/167 versus 1/35; $\chi^2=12.341$, d.f. = 1, $P<0.001$). Collectively, these results show that the maintenance of genetic diversity promoted by multicopy plasmids provides an evolutionary advantage in the colonization of new environments, especially when selective pressures rapidly shift.

Exploring the maintenance of plasmid-mediated heterozygosity under different selection regimes. A question that arises from our

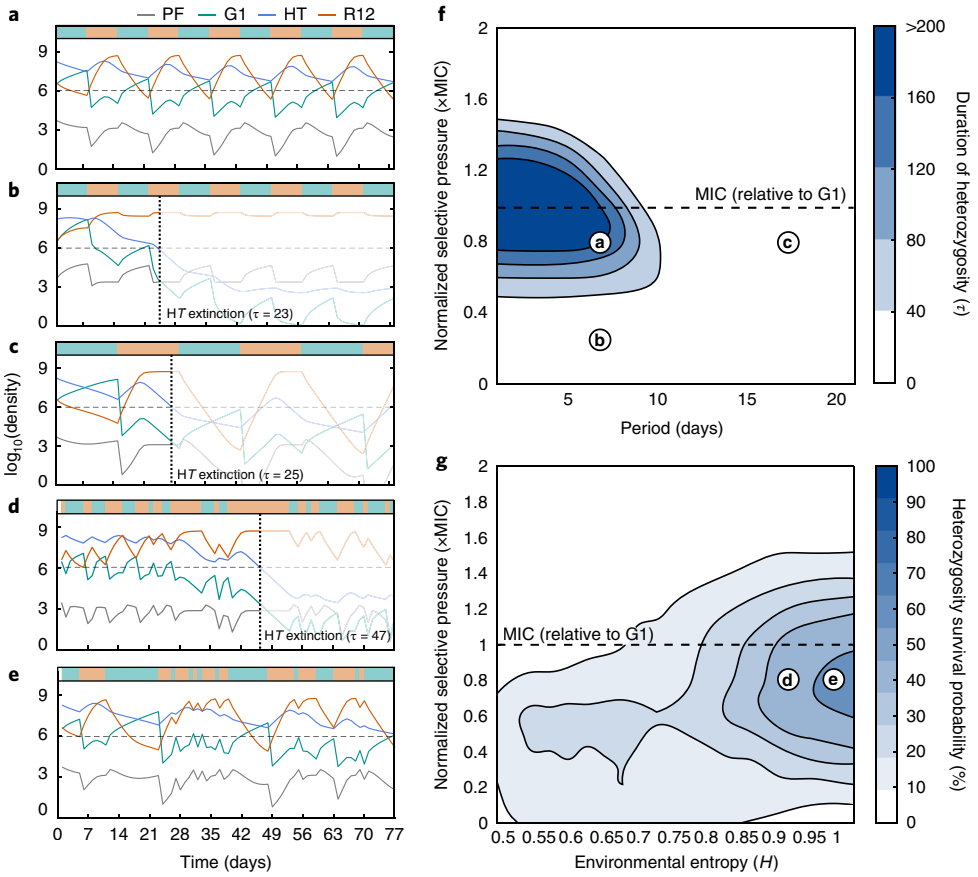

Fig. 6 | Numerical examples predicting the duration of plasmid-mediated heterozygosity under a range of fluctuating antibiotic conditions. **a**–**c**, Population dynamics in response to periodic selection regimes annotated in **f**. For clarity, only the densities at the end of each day are shown. **d**–**e**, Realizations of the model under the specific stochastic selection regimes annotated in **g**. **f**, Heat map representing the duration of heterozygosity under a range of normalized drug concentrations and periods of alternating drug exposure. Note how the regime **a** deploys drugs at 0.8×MIC in a seven-day period and is able to maintain the density of HT above the extinction threshold (in this case 10^6 cells). However, if the dose (as in regime **b**) or the frequency of environmental switching (as in regime **c**) is reduced, then the HT subpopulation is driven towards extinction. **g**, Heat map representing the survival probability of the HT population in response to different antibiotic concentrations and the entropy of the environment, a property that represents the uncertainty of each sequential treatment protocol.

work is to what extent plasmid-mediated heterozygosity (PMH) can be maintained in bacterial populations. As the homozygotes or plasmid-free cells will eventually replace the HT strain in any possible constant environment, PMH is inherently unstable. However, PMH may be stabilized in dynamic environments that alternately select for each allele, as in drug cycling protocols[23,24].

To test this possibility, we developed a simple compartmental population-dynamics model (Fig. 5) with parameter values obtained from experimental data (Methods and Supplementary Table 3). Our model accurately reproduces the evolutionary dynamics that emerge from the interaction between heterozygote and homozygote populations competing for limiting resources and exposed to the action of Caz and Amp (Supplementary Figs. 6,7). By systematically exploring a range of fluctuating environmental regimes, we obtained relevant statistics about the population dynamics that arise as a consequence of different patterns of selection.

First, we considered treatments that alternate periodically between Caz and Amp at normalized drug dosages (by adjusting the concentration of each drug so that they achieve equal inhibitory effect). We simulated a range of concentrations and periods of cycling and computed, for each selection regime, the time elapsed until the density of HT cells is less than the initial bacterial density, a value we refer to as duration of heterozygosity. The realizations of the model showed that PMH can last for hundreds of days under regimes that present high frequencies of environmental switching and moderate drug concentrations (relative to the MIC of G1; Fig. 6).

However, fluctuations in nature are expected to be not periodic, but stochastic. To assess if the increased stability of PMH observed in periodic regimes is also present in stochastically switching environments, we generated $N = 10{,}000$ environmental regimes consisting of random sequences of both antibiotics. Each environmental regime is numerically generated by considering that the distribution of elapsed times between environmental switches follows an exponential distribution (see Methods). We also evaluated environmental regimes that randomly switched between three discrete states (Caz, Amp and a drug-free environment) and obtained qualitatively the same results. The Shannon entropy[25], H, of each previously determined environmental regime was used to quantify the uncertainty and stochasticity of each sequential treatment protocol. As shown in Fig. 6, environments with $H \ll 1$ have large single-drug time intervals that select for one of the homozygote strains and thus render PMH unstable. Conversely, duration of heterozygosity is maximized when exposed to selection regimes associated with high entropy values and moderate selective pressures (Supplementary Fig. 8).

Together, our computational study predicts that heterozygosity can be stabilized for relatively long periods of time in fluctuating environments with high temporal heterogeneity and intermediate selective pressures.

Discussion

In this study, we investigated the role of MCPs in the alleviation of trade-offs during evolutionary innovation in bacteria. We developed a traceable model system using plasmid-mediated bla_{TEM} genes that reproduces the evolutionary dynamics toward Caz resistance[21]. Our results demonstrate that the intrinsic properties of replication and segregation dynamics of MCPs allow bacterial populations to escape the restraints imposed by trade-offs on the colonization of new environments by facilitating the co-existence of the ancestral and evolved alleles within the same cell (Fig. 2). Critically, this co-existence was maintained over tens of generations in the presence of increasingly strong pressures for the evolved allele (Fig. 3), suggesting that MCPs are able to delay the loss of non-adaptive allelic variants and preserve allelic richness for generations. Allelic richness, in turn, translates into increased phenotypic robustness to withstand stringent fluctuating selective pressures (Fig. 4). Collectively, our experimental and theoretical models establish that when the evolution of the plasmid resident loci is constrained by fitness trade-offs, the heterozygote has an advantage compared to the homozygotes. Hence, the population is under balancing selection for the maintenance of both ancestral and evolved alleles, especially when the environments fluctuate[26]. Although examples of selection favouring heterozygotes are abundant[27,28], it comes as no surprise that these examples refer to polyploid eukaryotic organisms, in which new mutations are always heterozygous. In bacteria, MCP are able to provide heterozygote advantage because they represent an island of polyploidy in an otherwise haploid genome.

Previous studies have addressed the importance of MCPs in the evolution of new functions[21,29,30], and the co-existence of mutant and ancestral allele variants encoded in the same plasmid has recently been demonstrated in laboratory evolution experiments[21,31] and clinical settings[32]. However, the prevalence of plasmid-mediated heterozygosity in nature remains largely unknown. At least three arguments support the idea that plasmid-mediated heterozygosity could be pervasive in bacterial populations. First, natural MCPs encode important genes for bacterial physiology that are under strong selective forces such as antibiotic resistance genes[33], determinants of ecological interactions such as colicins[34], and even indispensable metabolic genes as in the aphid endosymbiont *Buchnera aphidicola*[35]. Second, MCPs are excellent platforms for the generation of genetic variation. Their multicopy nature increases the mutational supply and hence the chance of the resident loci acquiring mutations[21]. Additionally, this genetic variation can be further enriched by horizontal gene transfer[18] and inter- and intraplasmid recombination[36,37]. Third, MCPs are also excellent platforms for the preservation of genetic diversity. Our experimental results show that allelic richness can be maintained in the face of increasingly strong selective pressures for more than 75 bacterial generations, and our theoretical model indicates that it could be maintained for much longer periods under a range of conditions (Fig. 6 and Supplementary Fig. 10). Considering that natural *E. coli* populations are estimated to undergo 100–300 generations per year[38,39], we propose that the maintenance of genetic diversity by MCPs will persist long enough to profoundly affect bacterial evolvability. In view of these arguments, we predict that deep sequencing of bacterial clones (not populations) and careful examination of plasmid sequences from genomic data will increase the number of examples of plasmid-mediated heterozygosity in both experimental and natural scenarios.

Most plasmids cause a fitness cost to their host that should, in theory, constrain plasmid existence to very particular situations[40,41]. However, plasmids are extremely abundant in natural bacterial populations. This 'plasmid paradox' is particularly challenging in MCPs, as they lack mechanisms to increase their frequency such as active transfer or partitioning systems[42]. Several studies have offered compelling explanations for plasmid maintenance. For instance, epistatic interactions have been shown to buffer plasmid cost[43,44]. Moreover, this cost can be ameliorated by compensatory mutations in either the plasmid or the bacterial chromosome[45–48]. Other possible explanations involve the increase in gene copies for the resident loci, which in turn translates into increased gene expression that could be beneficial under certain circumstances[21,35]. Our results raise the possibility that MCPs, as promoters of bacterial evolution, could be maintained by second-order selection, a process by which evolution indirectly selects for the systems that create adaptive mutations[49]. We propose that although the conditions for the maintenance of plasmids by second-order selection will be stringent, they are plausible in bacterial populations[49].

In conclusion, the ability of MCP-encoded genes to overcome the constraints imposed by trade-offs during evolutionary innovation could play a key role in the evolution of novel traits in bacterial populations and in the colonization of new environments. Additionally, our results may have implications for antibiotic therapies designed to slow the evolution of resistance by taking advantage of resistance trade-offs, such as drug cycling[24] or the exploitation of collateral sensitivity networks[50]. Future work will be needed to test the extent to which MCPs can jeopardize the success of these promising antimicrobial strategies.

Methods

Strains, media and plasmid construction. The plasmids used in this study (Supplementary Table 1) are derivatives of the pBGT and pBGT R164S plasmids that were previously used to reconstruct the plasmid-mediated evolution of Caz resistance[21]. For the sake of clarity, these plasmids were named pBGT-1 and pBGT-12 after the bla_{TEM} allele present. These plasmids also carry a *gfp* gene under the control of an L-arabinose inducible promoter, the gene coding for the repressor of this promoter *araC*, and a chloramphenicol (Chl) resistance acetyltransferase gene, *cat*. Variants of both plasmids in which the *gfp* gene was precisely replaced by the *dsRED* gene, encoding a red fluorescent protein, were generated by the Gibson assembly method[51] using the primers listed in Supplementary Table 2. To allow appropriate selection of heterozygote cells, a selective marker was needed to distinguish between plasmids. To this end, *cat* gene of the plasmids encoding bla_{TEM-12} was inactivated by creating an in-frame deletion (Δ574–591 in *cat* open reading frame) by site-directed mutagenesis using the primers $Cat_{SDM}F$ and $Cat_{SDM}R$ (Supplementary Table 2) and the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) following manufacturer instructions. This deletion completely abolishes *cat* function by eliminating six essential amino acids[52] and renders the cells Chl sensitive. All possible combinations of the two bla_{TEM} alleles with both fluorescent markers were generated. All plasmids were introduced by transformation into chemically competent MG1655 cells giving rise to the strains shown in Supplementary Table 1. Transformants were selected in Chl (15 mg l^{-1}) or Caz (1 mg l^{-1}) for plasmids carrying bla_{TEM-1} and bla_{TEM-12}, respectively. To generate the heterozygote strains, two plasmids were simultaneously co-transformed and selected in both antibiotics. Transformants were checked by PCR using the primers listed in Supplementary Table 2 and examined by confocal microscopy (Leica TCS SP5 multispectral confocal system) after induction of the fluorescent reporters by incubating the samples in Lysogeny broth (LB) supplemented with 0.1% L-arabinose for 4 h. All strains were routinely grown in LB medium supplemented with the appropriate antibiotics at 37 °C.

In agreement with previous reports[21], all plasmid carriers showed a reduced growth rate compared with the plasmid-free strain although there were no significant differences among plasmid-bearing strains (Tukey's multiple comparison of means $P > 0.27$; see Supplementary Table 1). No significant differences in plasmid copy number (estimated by qPCR, see below) were found in the new constructs compared to G1 (Multiple comparisons of means, $P > 0.05$), discarding any confounding effect in the interpretation of the data. Furthermore, genomic DNA of all the strains was isolated using the Wizard genomic DNA purification kit (Promega), following the manufacturer's instructions. Whole genome sequencing was conducted at the Wellcome Trust Centre for Human Genetics using the Illumina HiSeq4000 platform. Possible mutations were predicted using the breseq 0.30.0 pipeline[53] and using MG1655 genome (NC_000913.3) and plasmid sequences as references. Whole genome sequencing discarded the presence of unexpected mutations in coding regions of the chromosome or the plasmids in all the strains used in this study.

Antibiotic array. The antibiotic array was prepared in 96 well flat-bottom plates (Falcon) using the checkerboard technique[54]. To diminish inter-plate variability, all plates were prepared in the same day from a single stock solution using a Liquidator 96 manual pipetting system (Mettler Toledo) and stored at −20 °C prior to use. All plates were used within a week and appropriate controls were included

to check antibiotic stability over time. Bacteria were inoculated at a final dilution of 1:2,000 and incubated for 20 h at 37 °C. After incubation, optical density at 600 nm (OD_{600}) was determined after strong shaking in a Synergy HTX microplate reader (Biotek). We interpreted that a particular environment of the array was colonized if, after incubation, OD_{600} was greater than 0.1. Appropriate samples were then collected to inoculate the next day's plate. At the end of the experiment, plasmid DNA from samples of the surviving populations was extracted (Accuprep plasmid mini extraction kit; Bioneer) and the possible presence of plasmid hybrids containing both bla_{TEM} alleles was discarded by gel electrophoresis of undigested and PvuI (New England Biolabs) digested plasmid extractions. To determine the fluorescence ratio (GFP/RFP), L-arabinose to a final concentration of 0.1% was added to each well to induce the expression of the fluorescent reporters present on the plasmids. After 4 h of incubation at 37 °C to allow protein expression, the plates were further incubated for 18 h at 4 °C. This step favours dsRED maturation, which is slower than that of GFP[55]. The GFP/RFP ratio was calculated by dividing green fluorescence (485/528 nm) over red fluorescence (540/590 nm) signals in a Synergy HTX microplate reader (Biotek). After collection of all data, the GFP/RFP ratio was rescaled between 0.01 and 1 to make the results more amenable to interpretation.

Competition and invasion experiments. Minimal inhibitory concentrations (MIC) of Caz and Amp were determined for every strain by the microdilution method following CLSI guidelines[56] (Supplementary Table 1). Pairwise competitions between strains carrying $bla_{\mathrm{TEM\text{-}1}}$ and $bla_{\mathrm{TEM\text{-}12}}$ alleles were performed in media containing sub-inhibitory Caz concentrations and without antibiotics. Briefly, overnight cultures were mixed 1:1 and diluted 1:2,000 in fresh media. After 20 h of incubation at 37 °C with shaking every 15 minutes in a Synergy HTX Multimode Reader (Biotek), appropriate dilutions were plated in LB agar plates containing Chl (15 mg l^{-1}) to select G1 and R1 strains or Caz (1 mg l^{-1}) to select G12 and R12 strains. The fitness of strains carrying $bla_{\mathrm{TEM\text{-}12}}$ relative to the strains carrying $bla_{\mathrm{TEM\text{-}1}}$ was determined using the formula:

$$W_{bla_{\mathrm{TEM\text{-}12}}} = \frac{\ln(N_{\mathrm{final};bla_{\mathrm{TEM\text{-}12}}}/N_{\mathrm{initial};bla_{\mathrm{TEM\text{-}12}}})}{\ln(N_{\mathrm{final};bla_{\mathrm{TEM\text{-}1}}}/N_{\mathrm{initial};bla_{\mathrm{TEM\text{-}1}}})}$$

where $W_{bla_{\mathrm{TEM\text{-}12}}}$ is the relative fitness of the $bla_{\mathrm{TEM\text{-}12}}$ bearing clone; $N_{\mathrm{initial};bla_{\mathrm{TEM\text{-}12}}}$ and $N_{\mathrm{final};bla_{\mathrm{TEM\text{-}12}}}$ are the numbers of $bla_{\mathrm{TEM\text{-}12}}$ cells before and after the competition, and $N_{\mathrm{initial};bla_{\mathrm{TEM\text{-}1}}}$ and $N_{\mathrm{final};bla_{\mathrm{TEM\text{-}1}}}$ are the numbers of cells carrying $bla_{\mathrm{TEM\text{-}1}}$ plasmids before and after the competition. The minimal selective concentration (MSC) of Caz, defined as the minimal antibiotic concentration that produces a detectable fitness benefit for the $bla_{\mathrm{TEM\text{-}12}}$ carrying strains[22], was used as starting concentration for the invasion experiments.

Invasion experiments were started by mixing overnight cultures of either HT or R12 and G1 strains at a 1:100 proportion in fresh LB. Immediately, the mixes were diluted 1:2,000 into 96-well flat-bottom plates (Falcon) containing 0.2 ml of fresh media supplemented with the MSC of Caz. A checkerboard pattern was used to avoid cross-well contamination. After 20 h of growth at 37 °C (approximately 11 bacterial generations per day), the cultures were further diluted 1:2,000 and inoculated into fresh media containing doubled antibiotic concentrations. Samples were plated in LB agar supplemented with either Caz (1 mg l^{-1}), Chl (15 mg l^{-1}) or Caz + Chl (1 mg l^{-1} + 15 mg l^{-1}) to quantify the number of cells corresponding to each genotype in the competitions. To verify that the selective plating procedure was specific and to discard the presence of new mutations in the $bla_{\mathrm{TEM\text{-}1}}$ gene, we sequenced the bla_{TEM} allele of four independent clones per replicate obtained on Caz + Chl plates at day five using the primers shown in Supplementary Table 2. All chromatograms consistently showed a double peak (C + A) at the nucleotide 484 of the bla_{TEM} open reading frame assessing the presence of both $bla_{\mathrm{TEM\text{-}1}}$ and $bla_{\mathrm{TEM\text{-}12}}$ alleles in these clones. No other mutations were found. We additionally ruled out the possible presence of plasmid hybrids containing both bla_{TEM} alleles by digestion as described above. To assess allelic proportion, samples were also stored at −20 °C for subsequent qPCR analysis.

Quantification of plasmid copy number and allele frequencies. Quantitative polymerase chain reaction (qPCR) was used to determine relative plasmid copy number and allele frequency using an Applied Biosystems 7300 Real-Time PCR System and SYBR select master mix (Applied Biosystems). Specific primers for the pBGT plasmid backbone and the *dxs* chromosomal gene were used as previously described[21]. Additionally, specific primers for the *gfp* and *dsRED* genes were developed. All primer sequences can be found in Supplementary Table 2. Samples were prepared as previously described[57]. Briefly, bacterial cultures were pelleted (9,000*g*, 1 min), resuspended in molecular biology grade water and boiled (95 °C, 10 min). After brief centrifugation to spin down cellular debris, samples of the supernatant were diluted and subsequently used as template. Amplification conditions were as follows: initial denaturation for 2 min at 95 °C, followed by 40 cycles of denaturation for 15 s at 95 °C, annealing and extension for 1 min at 60 °C. After the amplification was completed and to control for the specificity of the reaction, a melting curve analysis was performed by cooling the reaction to 60 °C and then heating slowly to 95 °C. Inter-run calibration samples were used to normalize the results from different plates of each qPCR. Relative copy number was calculated using the $\Delta\Delta C_{\mathrm{T}}$ method as previously described[58]. All reactions showed similar efficiency values (92.7–93.8%, $R^2 > 0.998$) and produced amplicons of similar size (112–114 b.p.). Control reactions were carried out to ensure that relative plasmid copy number was comparable when using pBGT or GFP and RFP targeting primers.

Calculation of Simpson's diversity index. When the relative abundance of each allele could be quantified by qPCR, Simpson's diversity index was used. Simpson's index gives the probability that two randomly selected alleles from the population are equal and can be calculated as $\sum (P^2_{bla_{\mathrm{TEM\text{-}1}}} + P^2_{bla_{\mathrm{TEM\text{-}12}}})$ were P_i represents the relative proportion of each alelle[59]. Simpson's diversity index is an integrative measure that takes into account both population richness and evenness. In preliminary experiments, the GFP/RFP fluorescence ratio was found to strongly correlate with the allelic ratio estimated by qPCR (Pearson's product-moment correlation $t = 26.103$, d.f. $= 16$, $R^2 = 0.977$, $P < 10^{-13}$; Supplementary Fig. 5), demonstrating that it can be used as a proxy to assess the proportion of each allele. However, direct interpolation of allelic proportions from fluorescence data demonstrated to be unreliable. Instead, we used a more conservative approach in which the presence of both alleles in a particular environment was assessed by simply determining if the GFP/RFP ratio was comprised between that of the homozygotes. In this way, allelic richness of an environment can be confidently determined. We note, however, that this method underestimates the real environmental richness as populations carrying a small number of one plasmid type would show undetectable levels of fluorescence. In that sense, for an environment to be classified as having both alleles, it must have substantial amounts of both plasmids. We argue that this is indeed a strength of this approach, because an environment must show some extent of allelic evenness to be cataloged as rich by our method.

Population-level plasmid dynamics model. The objective of our model is to study the population dynamics that emerge in response to different environmental concentrations of resource and multiple bactericidal antibiotics. First, we will assume that the bacterial population is composed of a plasmid-free subpopulation (with density at time t denoted by $B_{\mathrm{PF}}(t)$), a subpopulation of heterozygote cells (with density represented by $B_{\mathrm{HT}}(t)$) and homozygote subpopulations G1 and R12 (with densities represented with $B_{\mathrm{G1}}(t)$ and $B_{\mathrm{R12}}(t)$, respectively). We will consider that growth of each subpopulation depends on the environmental concentration of a limiting resource, $R(t)$, and therefore we can model growth rate of subpopulation i with a standard Monod term, $G_i(R(t)) = \rho_i U_i(R(t))$, where ρ_i is the metabolic efficiency of the cell and $U_i(R) = V_i R/(K_i + R)$ a sigmoidal resource uptake function characterized by parameters V_i and K_i denoting, respectively, the maximum growth rate and half-saturation constants of bacterial type i. In order to model the action of antibiotics (in this case drugs Amp and Caz, with an additive interaction and concentration represented by D^A and D^C respectively) we consider that the susceptibility of strain B_i to Amp and Caz can be modelled with killing rates κ_i^A and κ_i^C. Conversely, δ_i^A and δ_i^C denote the rates of drug inactivation by the degrading enzyme produced by bacterial type i.

Note that the plasmids used in this study replicate as monomers and do not possess any molecular mechanisms that increase its stability (for example, active partitioning or post-segregational killing mechanisms). Therefore, we can assume that plasmids are uniformly distributed in the cell at the moment of division and, as a result, the probability of a plasmid-bearing cell producing a plasmid-free cell through segregation can be described by a Poisson process and thus the rate of plasmid loss via segregation is approximated by $\Sigma = 2^{1-\mu}$, where μ denotes the mean plasmid copy number in the population[47,60]. Similarly, the probability of a heterozygote cell giving rise to a homozygote cell can be estimated by assuming that, in average, HT cells have equal fractions of both plasmids and thus the rate of heterozygosity loss can be written as $\sigma = 2^{1-\mu/2}$. Therefore, our model makes the critical assumption that HT cells carry equal proportions of each plasmid. Although this simplification may underestimate the complex distribution of intracellular allele frequencies, our model accurately reproduces the experimental results (Supplementary Figs. 6,7). In our experimental system, the mean per-cell plasmid copy number is 19 and therefore the rate of heterozygosity loss is 1/362 while the rate of plasmid loss is considerably lower, at 1/524,288. Finally, the evolutionary dynamics of the system considers that the transition from B_{G1} and B_{R12} into B_{HT} occurs at a rate $\varepsilon > 0$ representing the probability of a single-point mutation in the $bla_{\mathrm{TEM\text{-}1}}$ or $bla_{\mathrm{TEM\text{-}12}}$ locus.

In summary, the system of ordinary differential equations that describe the plasmid dynamics at a population-level can be written as:

$$\frac{\mathrm{d}}{\mathrm{d}t}R = -(U_{\mathrm{PF}}(R)B_{\mathrm{PF}} + U_{\mathrm{G1}}(R)B_{\mathrm{G1}} + U_{\mathrm{HT}}(R)B_{\mathrm{HT}} + U_{\mathrm{R12}}(R)B_{\mathrm{R12}}) \quad (1)$$

$$\frac{\mathrm{d}}{\mathrm{d}t}D^A = -D^A(\delta^A_{\mathrm{PF}}B_{\mathrm{PF}} + \delta^A_{\mathrm{G1}}B_{\mathrm{G1}} + \delta^A_{\mathrm{HT}}B_{\mathrm{HT}} + \delta^A_{\mathrm{R12}}B_{\mathrm{R12}}) \quad (2)$$

$$\frac{\mathrm{d}}{\mathrm{d}t}D^C = -D^C(\delta^C_{\mathrm{PF}}B_{\mathrm{PF}} + \delta^C_{\mathrm{G1}}B_{\mathrm{G1}} + \delta^C_{\mathrm{HT}}B_{\mathrm{HT}} + \delta^C_{\mathrm{R12}}B_{\mathrm{R12}}) \quad (3)$$

$$\frac{\mathrm{d}}{\mathrm{d}t}B_{\mathrm{PF}} = G_{\mathrm{PF}}(R)B_{\mathrm{PF}} + \Sigma\left(G_{\mathrm{G1}}(R)B_{\mathrm{G1}} + G_{\mathrm{HT}}(R)B_{\mathrm{HT}} + G_{\mathrm{R12}}(R)B_{\mathrm{R12}}\right) - B_{\mathrm{PF}}\left(\kappa^{A}_{\mathrm{PF}}D^{A} + \kappa^{C}_{\mathrm{PF}}D^{C}\right) \quad (4)$$

$$\frac{\mathrm{d}}{\mathrm{d}t}B_{\mathrm{G1}} = (1-\varepsilon-\Sigma)G_{\mathrm{G1}}(R)B_{\mathrm{G1}} + \sigma G_{\mathrm{HT}}(R)B_{\mathrm{HT}} - B_{\mathrm{G1}}\left(\kappa^{A}_{\mathrm{G1}}D^{A} + \kappa^{C}_{\mathrm{G1}}D^{C}\right) \quad (5)$$

$$\frac{\mathrm{d}}{\mathrm{d}t}B_{\mathrm{HT}} = (1-\Sigma)G_{\mathrm{HT}}(R)B_{\mathrm{HT}} + \varepsilon\left(G_{\mathrm{G1}}(R)B_{\mathrm{G1}} + G_{\mathrm{R12}}(R)B_{\mathrm{R12}}\right) - B_{\mathrm{HT}}\left(\kappa^{A}_{\mathrm{HT}}D^{A} + \kappa^{C}_{\mathrm{HT}}D^{C}\right) \quad (6)$$

$$\frac{\mathrm{d}}{\mathrm{d}t}B_{\mathrm{R12}} = (1-\varepsilon-\Sigma)G_{\mathrm{R12}}(R)B_{\mathrm{R12}} + \sigma G_{\mathrm{HT}}B_{\mathrm{HT}} - B_{\mathrm{R12}}\left(\kappa^{A}_{\mathrm{R12}}D^{A} + \kappa^{C}_{\mathrm{R12}}D^{C}\right) \quad (7)$$

with parameters values estimated using a parametrization approach that implements a Markov Chain Monte Carlo (MCMC) method with Metropolis–Hastings sampling. A similar Bayesian approach was published previously[47] and allows us to obtain maximum likelihood estimates for the specific affinity V_i/K_i and the resource conversion rate, ρ_i, from experimental growth curves of each strain grown in isolation (Supplementary Fig. 9 shows the MCMC diagnostics plots). The inhibition parameters of each strain were estimated using single-drug dose–response experiments. Numerical solutions of the system of equations (1)–(7) were obtained using standard ODE solvers in Matlab, with parameter values summarized in Supplementary Table 3.

Computational experiments in fluctuating environments. In order to numerically simulate an N-day experimental serial dilution protocol, we considered that each day ($0 \le n < N$) resources are replenished at the beginning of each season, that is $R_n(0) = R_0$. The initial bacterial density on the first day ($n = 0$) can be written as $B_0\Gamma$, where $B_0 = 1\times10^6$ denotes the total initial bacterial density and $\Gamma = (\gamma_{\mathrm{PF}}, \gamma_{\mathrm{G1}}, \gamma_{\mathrm{HT}}, \gamma_{\mathrm{R12}})$ a vector containing the initial relative frequencies of each strain and thus $\gamma_{\mathrm{PF}} + \gamma_{\mathrm{G1}} + \gamma_{\mathrm{HT}} + \gamma_{\mathrm{R12}} = 1$. For subsequent seasons, $n > 1$, we consider the initial population density of each subpopulation to be a function of the terminal condition of the previous season, that is $\eta(B_{\mathrm{PF}}(T), B_{\mathrm{G1}}(T), B_{\mathrm{HT}}(T), B_{\mathrm{R12}}(T))$, whereby $\eta = 0.01$ denotes the dilution parameter and $T = 24$ the duration of each season.

To quantify the stability of single-cell genetic diversity, we use the duration of heterozygosity, τ, defined as the time elapsed until the density of B_{HT} is below an extinction threshold, that is $\tau > 0$ such that $B_{\mathrm{HT}}(\tau) < B_{\mathrm{ext}} = 1\times10^6$ and $B_{\mathrm{HT}}(t) \ge B_{\mathrm{ext}}$ for all $t \in [0, \tau)$. In particular, we considered fluctuating environmental regimes that alternate selection between both drugs, so if $\widehat{D}^{A}$ and $\widehat{D}^{C}$, represent the concentrations of each drug normalized to achieve equal inhibitory effect then on day n we can represent the treatment used as $D_n = \phi_n\widehat{D}^{A} + (1-\phi_n)\widehat{D}^{C}$. Here, we used $\phi_n = 0$ or $\phi_n = 1$ and therefore the environmental regime could be represented as a random sequence of N bits, allowing us to compute the information entropy (Shannon–Wiener Index) associated with each environmental sequence using the following expression: $H = -\sum P\log_2(P)$ where P represents the proportion of days where each drug was used. Note that low-values of H correspond to constant, predictable environments, whereas high entropy values are associated with unpredictable, rapidly switching environmental conditions.

The $bla_{\mathrm{TEM\text{-}1}}/bla_{\mathrm{TEM\text{-}12}}$ trade-off explored in this work can be considered 'weak' in the sense that the gain in the new function is big (64-fold increase in Caz MIC), whereas the loss in the ancestral function is small (twofold reduction in Amp MIC). In natural and laboratory evolution most trade-offs are weak[2] (>10-fold gain in new function versus loss of the existing one) and hence the trade-off of TEM evolution used in this work can be deemed representative. Nevertheless, we decided to theoretically explore various trade-off strengths to extend our computational analysis to other possible scenarios. As can be seen in Supplementary Fig. 10, stronger trade-offs increase the range of fluctuations and antibiotic pressures that render PMH stable. In contrast weaker trade-offs render PMH unstable in most conditions, as the acquisition of a new genetic activity barely involves any loss in the ancestral function.

Statistical analysis. Analyses were performed using R (v. 3.4.2)[61]. One way ANOVA and Tukey's multiple comparison of means were used to assess differences in colonization in the antibiotic array. Spearman's rank correlation coefficient was used to assess the monotonic relationship between antibiotic concentrations and the GFP/RFP ratio. A chi-squared (χ^2) test was used to analyse the frequency of populations conserving both alleles after growth in the antibiotic array. Repeated-measures ANOVA was used to ascertain the variation of Simpson's allelic diversity index over time in the invasion experiments. Fisher's exact test was used to analyse the differences in survival in the short-term selection experiment. A two-tailed two-sample t-test with Bonferroni correction was used to compare fitness data. Pearson's product-moment correlation was used to assess the linear relationship between fluorescence and plasmid proportion determined by qPCR.

Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The sequence data that support the findings of this study have been deposited in the European Nucleotide Archive with the accession code PRJEB25055.

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Author contributions

J.R.B., A.S.M. and R.C.M. were responsible for the conceptualization of the study; J.R.B., A.S.M. and J.A.E. designed the methodology; R.P.M., J.C.R.H.B. and A.F.H. postulated and analysed the mathematical model; J.C.R.H.B., A.F.H., J.A.E., J.D. and J.R.B. performed experiments and contributed to data analysis; J.R.B. and A.S.M. analysed data and prepared the original draft of the manuscript and undertook the reviewing and editing; all authors supervised and approved the final version of the manuscript. A.S.M. was responsible for funding acquisition and supervision.

Competing interests

The authors declare no competing interests.

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Capítulo 7

Conclusión general

En este trabajo hemos analizado a los plásmidos multicopia como un sistema generador de heterogeneidad fenotípica. Esta variabilidad fenotípica es generada primordialmente por la naturaleza estocástica de los procesos de replicación y segregación que presentan los plásmidos multicopia. El objetivo de la tesis fue abordar, a través de múltiples niveles de complejidad, los fenómenos que dichos procesos pueden generar, así como las consecuencias que tienen en la dinámica evolutiva.

Primero estudiamos la estabilidad de nuestro plásmido modelo con un enfoque de genética de poblaciones utilizando un modelo Wright-Fisher multi-tipo. Las aportaciones de este proyecto no recaen plenamente en los resultados encontrados sobre las relaciones de factores que afectan la estabilidad de estos plásmidos (número de copias, costo, frecuencia y fuerza de selección), sino en el enfoque matemático utilizado; a pesar de que la genética de poblaciones y el estudio de plásmidos tienen una gran tradición, nunca se habían conjuntado.

También exploramos las consecuencias adaptativas de que las distintas bacterias de una población que porta plásmidos multicopia tengan distinto número de copias. Este simple hecho le confiere a la población una gran resiliencia ante pulsos de antibióticos. Este mecanismo de resistencia transiente sugiere un mecanismo generador de heteroresistencia, que no sólo es un problema en el tratamiento de infecciones, sino que también puede ayudar a explicar la prevalencia de plásmidos con genes de resistencia a antibióticos en ambientes sin selección.

Otro hecho relevante qué resaltar de este trabajo, es el uso de modelos para ayudarnos en la comprensión y diseño de experimentos. Nuestro modelo de agentes individuales jugó un papel crucial en la identificación del periodo de heterocigosis, mediante éste fuimos capaces de visualizar y comprender que antes de que el alelo mutante bajo selección se fije, coexisten subpoblaciones de bacterias con distintas fracciones de plásmidos ancestrales y mutantes. Dejando por demás claro, que los plásmidos mutantes y ancestrales pueden cohabitar dentro de una misma célula por largos periodos.

Aunque podemos decir que PMH es un caso particular de heteroplasmia, la visión de utilizar este sistema experimental como un mecanismo generador de variabilidad genética resulta novedoso. Mediante una perspectivas de células individuales y el uso de un innovador sistema de cuantificación de plásmidos fuimos capaces de observar y cuantificar por primera vez la deriva segregacional, es decir, las dinámicas intracelulares de plásmidos.

Estas dinámicas intracelulares repercuten en los comportamientos generales de la población, que son los hechos de relevancia ecológica. En el último proyecto aquí mostrado, realizamos experimentos a nivel de poblaciones para explorar las posibles consecuencias evolutivas de PMH y encontramos que resulta ventajosa en varios aspectos:

permitiendo la redundancia genética (lo cual permite evitar compromisos evolutivos) y manteniendo la diversidad genética en la población (y por ende la posibilidad contender con nuevas adversidades). Estos resultados fueron corroborados con un modelo de dinámica de poblaciones y realizamos exploraciones acerca de la duración de la heterocigosis en ambientes estocásticos. Los resultados obtenidos nos dan nociones sobre las condiciones requeridas para mantener la heterocigosis, pero así mismo de cómo perderla, información que podría ser de gran utilidad, en el futuro, para el diseño de tratamientos antibacterianos en la clínica.

Capítulo 8

Perspectivas

8.1 Exploración exhaustiva del modelo de agentes individuales

Hemos visto que nuestro modelo de agentes individuales es capaz de capturar efectivamente los comportamientos poblacionales pero por cuestiones relacionadas a los tiempos y enfoques de las publicaciones, no se ha incluido en ninguna publicación. Contamos ya con una gran cantidad de simulaciones de los experimentos realizados en los proyectos de heterocigosis que podrían incluirse y extenderse para producir una artículo completamente computacional.

8.2 Un modelo de agentes individuales con conjugación

Una perspectiva natural de nuestro enfoque es introducir el fenómeno de conjugación en a nuestro sistema, ya sea mediante la introducción de otro plásmido conjugativo que sea capaz de movilizar a los plásmidos aquí utilizados o modificar estos plásmidos para introducir los genes requeridos para expresar la maquinaria de conjugación. El estudio de los efectos del proceso de conjugación en la dinámica y estabilidad de plásmidos se vuelve aún más interesante cuando el enfoque incluye estructura espacial. Los modelos de agentes individuales son convenientes para implementar esta característica a través de matrices en dos o tres dimensiones.

8.3 Un modelo de agentes individuales para estudiar dinámicas de fijación de variantes alélicas de la β-lactamasa TEM portada en plásmidos

En los distintos proyectos hemos utilizado nuestro modelo de agentes individuales para estudiar la variabilidad fenotípica generada por los plásmidos multicopia. Se ha propuesto que la variabilidad en número de copias puede establecer una estrategia de heteroresistencia, por otra parte, hemos visto que los plásmidos ayudan a contender con los compromisos evolutivos, para ello utilizamos un modelo de dos variantes alélicas del gen bla_{TEM}. Ahora, podemos postular una extensión del modelo de agentes individuales a un modelo multi-alélico, el cuál se pueda equiparar con todos los polimorfismos de la β-lactamasa. En trabajos previos se han explorado los caminos evolutivos entre estos polimorfismos (Mira et al. 2015), con el modelo propuesto se podrían explorar las dinámicas de fijación en ambientes libres de antibióticos. Si además integramos las propiedades fenotípicas de dichos polimorfismos, es decir, los niveles de resistencia asociados a los múltiples antibióticos β-lactámicos, se podría establecer la correlación

entre un antibiótico y la diversidad alélica, y así encontrar alelos óptimos en ambientes complejos (cócteles de antibióticos o ciclos) y proponer terapias específicas o más efectivas.

8.4 Efectos del retardo fenotípico en rescate evolutivo

Hemos explorado las consecuencias en las dinámicas evolutivas y adaptativas que conlleva el hecho de que ocurran mutaciones en genes portados en plásmidos multicopia. En esta visión se ha asumido una relación casi directa entre el genotipo y el fenotipo, pero sabemos que esto no es necesariamente correcto, es decir, por ejemplo si una célula heterocigota se vuelve homocigota, ¿Qué tanto tiempo ocurre en que la descendencia de esa célula continúe teniendo proteínas del gen perdido? En la figura 8.1 se muestran simulaciones de una célula que porta dos plásmidos y uno de estos es perdido por deriva segregacional. Observe que hay un tiempo diferencial entre el evento de pérdida del gen y la perdida total de proteínas. Más aún, la presión selectiva actúa sobre los fenotipos, si pudiéramos alterar estos retardos fenotípicos, ¿cuál sería el efecto de este retardo fenotípico en la dinámica de supervivencia de la población y sobre todo en las dinámica de plásmidos en ambientes fluctuantes?

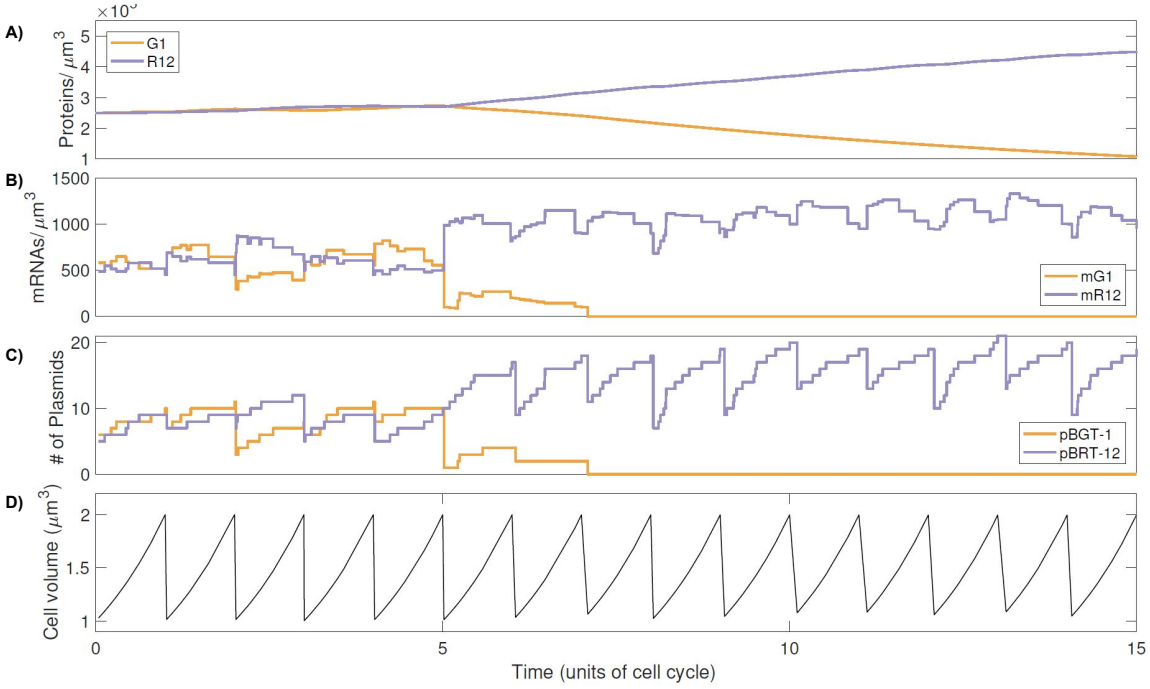

Figura 8.1: Simulación multiescala de retardo fenotípico. En la figura se ejemplifica la dinámica de 2 variantes de plásmidos durante crecimiento y división de una sola célula. Los plásmidos aumentan en ńumero (C) conforme la célula aumenta en volumen (D). Las caídas en volumen (D) representan eventos de división y en (C) podemos observar el número de plásmidos con los que se quedó la célula en el mismo momento. En (B) observamos la concentración correspondiente de mRNA al número de alelos en el momento, la correlación es casi directa dado a las altas tasas de degradación y producción. En (A) observamos la correspondiente proporción de proteínas. Observe que los cambios en frecuencia de alelos están amortiguados por la lenta degradación de proteínas. La concentración de proteínas está mayormente es afectada por dilución entre divisiones.

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