Effectiveness of CRISPR-Cas in Sensitizing Bacterial Populations with Plasmid-Encoded Antimicrobial Resistance

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The spread of bacteria resistant to antibiotics poses a serious threat to human health. Genes that encode antibiotic resistance are often harbored by plasmids, extra-chromosomal DNA molecules found in bacteria. The ability of plasmids to spread horizontally among bacteria facilitates the emergence and spread of multiresistant strains, outpacing the development of new antibiotics. CRISPR-Cas derived tools with their sequence specificity offer a promising new approach to combating antibiotic resistance. By introducing CRISPR-encoding plasmids that specifically target antibiotic resistance genes encoded on plasmids, the susceptibility of bacteria to conventional antibiotics can be restored. However, genetic variation within bacterial populations can hinder the effectiveness of such CRISPR-Cas tools by allowing some mutant strains to evade CRISPR-mediated cleaving or gene silencing.

In this study, we model the effectiveness of CRISPR-Cas in sensitizing bacterial populations and assess the success probability of a subsequent treatment with conventional antibiotics. We evaluate this probability according to the target interference mechanism, the copy number of the resistance-encoding plasmid and its compatibility with the CRISPR-encoding plasmid.

Our results identify promising approaches to revert antibiotic resistance with CRISPR-encoding plasmids: Cleaving by CRISPR systems on *incompatible* plasmids is most effective against AMR plasmids with a low copy number, while for higher copy numbers, gene silencing by CRISPR systems encoded on *compatible* plasmids offers the superior solution.

CRISPR Cas | antibiotic resistance | gene silencing | plasmid copy number | multidrug resistance | resistance plasmids | CRISPR antimicrobials

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Introduction

The escalating crisis of antimicrobial resistance (AMR) poses a serious global health threat. Projections suggest that the number of deaths associated to AMR could rise to up to 10 million per year over the next 25 years, thereby surpassing cancer-related mortality rates and becoming the world's primary cause of death (O'Neill 2014). Particularly for bacteria, the misuse and overuse of antibiotics has led to the rapid evolution of resistance (Schrader *et al.* 2020; Antimicrobial Resistance Collaborators 2022), resulting in a constant demand for novel effective antibiotics. While the number of multidrug-resistant pathogens increases, the rate of antibiotic discovery has declined in recent years (Luepke *et al.* 2017). Consequently, there is a pressing need for innovative strategies to treat infections with resistant bacteria.

AMR genes are often located on plasmids, which are self-replicating extrachromosomal DNA segments (Rozwandowicz *et al.* 2018; Castañeda-Barba *et al.* 2024). Plasmids self-regulate their replication through negative-feedback loops, and many types of plasmids exist in multiple copies within bacterial cells (del Solar *et al.* 1998; del Solar and Espinosa 2000). The plasmid copy number modulates cellular levels of AMR-conferring enzymes when the corresponding genes are on the plasmid (Friehs 2004; Hernandez-Beltran *et al.* 2024). In addition, higher copy numbers increase the occurrence of novel mutations in plasmid-carried genes (San Millan *et al.* 2016; Santer and Uecker 2020). If two plasmids encode very similar partition and/ or replication functions, their regulatory systems cross-react, leading to the loss of one of the two incompatible plasmids over multiple generations (Ebersbach and Gerdes 2005; Bouet *et al.* 2007). Some plasmids encode a dedicated transfer machinery, allowing them to spread horizontally to neighboring bacterial cells via conjugation. Such self-transmissible plasmids largely drive the spread of multidrug resistance in bacterial pathogens (von Wintersdorff *et al.* 2016; Partridge *et al.* 2018).

One promising alternative to developing new antibiotics is the removal of plasmid-encoded bacterial resistance by employing CRISPR-Cas derived tools, rendering the bacteria sensitive to conventional antibiotics (Buckner *et al.* 2018). CRISPR-Cas are adaptive immune systems that protect their bacterial hosts from infecting mobile genetic elements (MGEs) such as bacteriophages and plasmids. They are highly diverse but generally consist of an array, where short DNA sequences (spacers) are stored between clustered regularly interspaced short palindromic repeats (CRISPR), flanked by variable CRISPR-associated (*cas*) genes. CRISPR-Cas immunity comprises three stages, i) Adaptation, where the spacer sequences are derived from invading MGEs and integrated as immunological memory into the array, ii) Processing, where the array is transcribed, processed, and matured to separate CRISPR RNAs

(crRNAs), iii) Interference, where crRNAs guide the effectors to the complementary target sequence for sequence-specific interference. For CRISPR based sensitizing, ii) and iii) are the relevant stages. The molecular mechanisms by which the ribonucleoprotein complexes/ ribonucleoproteins recognize and interfere with their targets differ greatly, but generally, they provide host immunity by cleaving of or binding to a target nucleic acid (Nussenzweig and Marraffini 2020). Various CRISPR-Cas systems have been exploited extensively to develop versatile genetic engineering tools such as nucleases, base editors, or to control gene expression (Selle and Barrangou 2015; Adli 2018). Thanks to the crRNA-mediated guiding of effectors, these tools can easily be programmed and redirected to desired target sequences, by integrating an artificial array that provides the template for the complementary crRNAs. CRISPR-Cas tools that cleave target sequences, can be directed to the bacterial chromosome for sequence-specific killing, thereby functioning as an antimicrobial (Bikard *et al.* 2014; Citorik *et al.* 2014). When directed toward plasmids, bacteria can be cured of these plasmids and plasmid-encoded functions, such as AMR, can be removed (Citorik *et al.* 2014; Yosef *et al.* 2015; Wang *et al.* 2019; Zhou *et al.* 2023). Alternatively to removing plasmids by target cleaving, effectors that result in transcriptional repression of target genes can be used to silence AMR (Yao *et al.* 2022; Mayorga-Ramos *et al.* 2023).

For CRISPR-Cas-based antimicrobials to be effective, the effector needs to be delivered to the entire target population. Due to the high infectivity of bacteriophages, phage-mediated delivery has been tested extensively (Park *et al.* 2017; Lam *et al.* 2021; Brödel *et al.* 2022; Gencay *et al.* 2024), and plasmid transfer has been deemed not efficient enough (Citorik *et al.* 2014). However, more recent *in vivo* studies highlight the potential of conjugative plasmids and their engineered derivates as potent delivery devices (Ronda *et al.* 2019; Neil *et al.* 2020; Jin *et al.* 2022; Walker-Sünderhauf *et al.* 2023)

However, just as to antibiotics, bacteria can evolve resistance to such CRISPR interference systems, either by mutation in the target sequence on the plasmid or in the CRISPR-Cas tool itself (Mayorga-Ramos *et al.* 2023). Sensitizing the bacterial population followed by antibiotic treatment strongly selects for mutations that evade CRISPR-Cas targeting and consequently result in a renewed rise of antimicrobial resistant bacteria, provided the escape mutation has not compromised the function of the resistance gene.

Although experimental proof-of-concept studies showed promising results (Yosef *et al.* 2015; Wang *et al.* 2019; Zhou *et al.* 2023; Walker-Sünderhauf *et al.* 2023), the specific conditions under which a CRISPR-Cas sensitizing strategy can be effective, even when mutated AMR plasmids can escape CRISPR targeting, remain uncertain. Mathematical models that explain the dynamics of plasmids targeted by CRISPR (Mamontov *et al.* 2022) and the effectiveness of such strategies are still scarce.

Here, we develop a comprehensive mathematical model to explore the effectiveness of CRISPR-Cas tools in removing plasmid-encoded antibiotic resistance. We utilize Moran models and multitype branching processes and consider different design options for the CRISPR-Cas tool. We consider the CRISPR-Cas-encoding plasmid to be either compatible or incompatible with the AMR conferring plasmid, and the CRISPR-Cas effectors to act by either cleaving or by binding/silencing their targets. Our models suggest that the optimal strategy for targeting AMR plasmids depends on their copy number. An incompatible plasmid delivering a target-cleaving CRISPR-Cas tool is most effective in eliminating AMR plasmids with a copy number below a given threshold, which depends on various factors, such as the strength of the antibiotic treatment. Above this threshold, a compatible plasmid delivering a silencing CRISPR-Cas tool is more effective.

The model

We consider a bacterial population in which each cell contains a combination of three plasmid types:

pAMR: Wild-type (WT) AMR plasmid that provides the host cell with antibiotic resistance.

pAMRmut: Mutated AMR plasmid that evades CRISPR-Cas targeting via a mutation in the target region and – assuming a worst-case scenario – provides the host with the same level of antibiotic resistance during treatment as the WT AMR plasmid.

pCRISPR: Plasmids that encode a CRISPR system that interferes with pAMR but not pAMRmut. We will consider two special cases of pCRISPR with different interference mechanisms: pCleaving, introducing double strand breaks at the target DNA site, and pSilencing, silencing target genes by transcriptional repression.

The combination of these plasmids within a cell defines the cell type, which in turn determines the growth rate of the cell. We assume here that all plasmids are non-transferrable within the population. The influence of incorporating horizontal gene transfer will be addressed in the discussion. However, it is clear that using a transferrable plasmid will increase the effectiveness.

The model comprises three steps:

Step 1: Standing genetic variation prior to any treatment ($t < 0$):

In the initial stage before introduction of pCRISPR and antibiotic treatment, cells can exclusively harbor pAMR and/or pAMRmut. We assume that the CRISPR-Cas evading mutation carries a small cost and that the population is in mutation-selection balance.

Step 2: Sensitizing via pCRISPR introduction ($t = 0$):

At the start of treatment, each cell acquires exactly one pCRISPR in addition to the existing pAMR and pAMRmut. The effect of pCRISPR on pAMR is

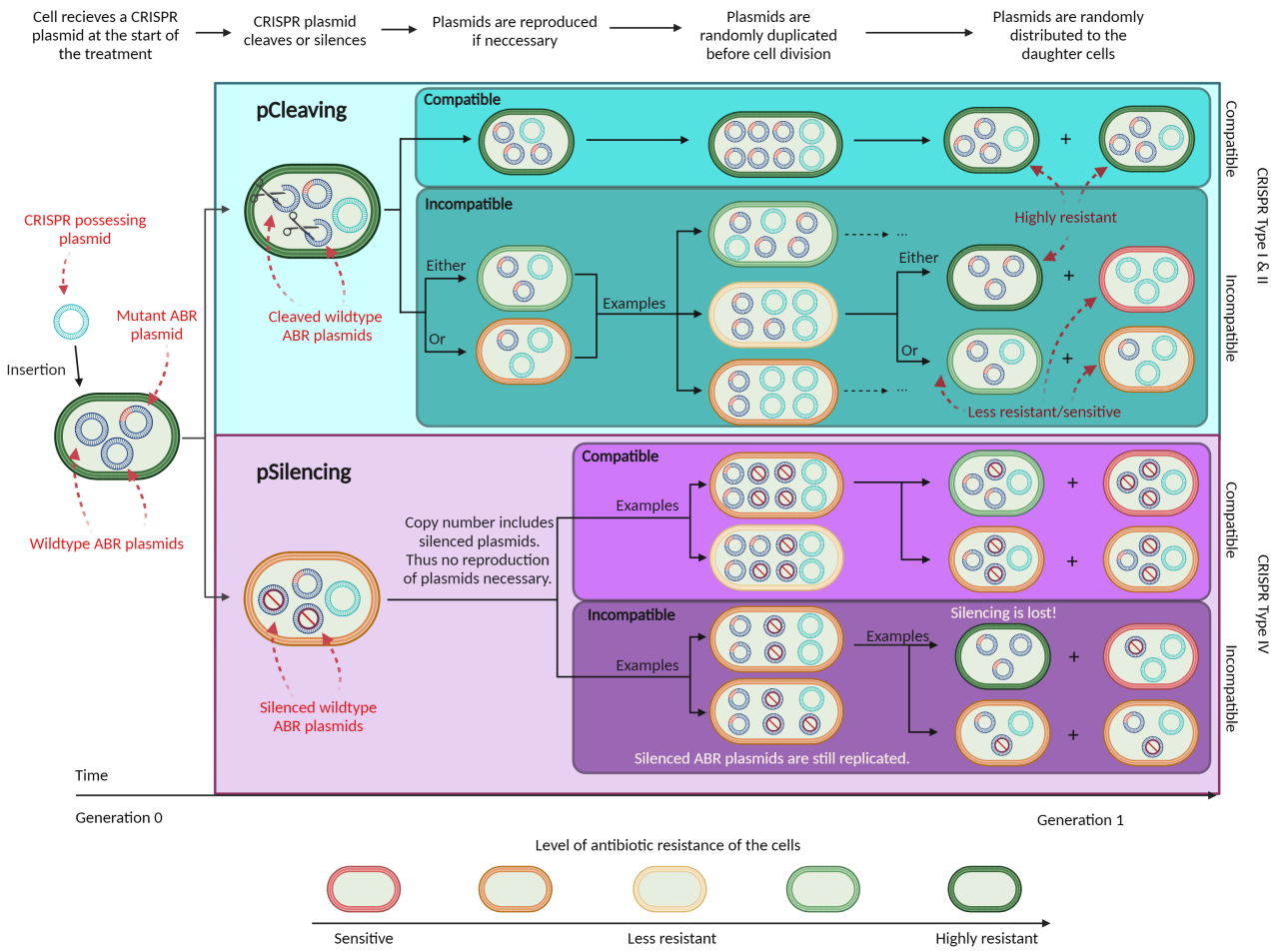


Fig. 1. Sensitizing concept and the interplay of pCRISPR and the AMR plasmid replication and segregation. Each cell in a population of pathogens receives a CRISPR plasmid at the beginning of the treatment (step 2). This CRISPR plasmid is specifically engineered, either to cleave or to silence wild-type antimicrobial resistance (AMR) plasmids. In the event of cleavage, if there are pAMRmut copies in the cell, they undergo replication until the predetermined copy number is attained. If the CRISPR plasmid and the AMR plasmid have the same incompatibility group, both can be replicated, which happens randomly. Conversely, when silencing takes place, there is no imperative for reproduction. Subsequent to these processes, cellular replication proceeds under antibiotic treatment in accordance with individual fitness, determined by the quantity of non-silenced AMR plasmids retained within the cells (step 3). Throughout each cell division, the plasmids are replicated and randomly distributed to the progeny cells. This results in different combinations of mother-daughter cells, introducing variability in the inheritance of plasmids during cell division. Figure created with BioRender.com

assumed to be instantaneous, i.e. the WT pAMR copies are immediately cleaved or silenced. Note that silencing is reversible if pCRISPR is lost from a cell in later generations.

Step 3: Treating the population with antibiotics ($t > 0$): Antibiotic treatment starts as soon as pCRISPR has been added. Cells without pAMRmut are sensitive to antibiotic treatment, as all pAMR are cleaved or silenced. The survival probability of cell lineages that originate from cells with pAMRmut copies depends on demographic stochasticity, the number of pAMRmut copies, the occurrence of *de novo* mutations, and the pCRISPR type and incompatibility group.

With this model, we determine the extinction probability of the bacterial population, or equivalently, the potential for evolutionary rescue through the mutations in pAMR. In the following, we detail considerations for steps 1 - 3.

Step 1: Standing Genetic Variation Prior to any Treatment. Some cells in the bacterial population may already contain pAMRmut or a mixture of pAMR and pAMRmut copies prior to the introduction of pCRISPR. To simulate the frequency of pAMRmut at the time of pCRISPR introduction, we utilize a multitype Moran model, as developed by Santer and Uecker (2020). This model assumes a constant population of N bacterial cells carrying the resistance plasmid, where each cell contains exactly n plasmids, all of which are initially pAMR (i.e., #pAMRmut = 0).

In each Moran model time step, one cell is randomly selected to die, while another one is randomly selected to divide. This mother cell produces two daughter cells, replacing itself and the deceased cell to maintain a constant population size. During each cell division, the plasmids are first replicated up to a total number of $2n$ and then distributed to the daughter cells. (We provide details on plasmid replication and segregation in the section 'Copy number and plasmid incompatibility' below.) At each plasmid replication event, the CRISPR-Cas

target sequence in pAMR mutates with probability v, thereby transitioning from pAMR to pAMRmut. We assume that prior to the introduction of pCRISPR the mutation in pAMRmut has a small cost for the host cell and assume that this selective cost increases linearly with the number of pAMRmut copies up to a factor s. The costs determine the probability that a cell is selected for replication in the Moran model such that cells with j pAMRmut copies have a reduced weight of $1 + \frac{i}{n}s$ (s is negative). Consequently, in the long run the frequency of pAMRmut copies will evolve towards mutation-selection balance, and pAMRmut will be present at a low frequency when pCRISPR is introduced in the next step.

Step 2: pCRISPR introduction. When pCRISPR is introduced to sensitize the resistant population, each cell receives exactly one pCRISPR copy at time $t = 0$. For simplicity, we assume that plasmid delivery occurs simultaneously for all cells. After pCRISPR is delivered, the interference activity is immediately effective. As already discussed, we consider two distinct pCRISPR that differ in their mode of target interference. pCleaving employs Cas components that cleave the target DNA (e.g. CRISPR type I and II), and pSilencing employs Cas components that silence expression of the target gene (e.g. type IV). We make the following assumptions for pCRISPR variants:

pCleaving: All pAMR copies within a given cell are cleaved and thus lost before the next cell division. In contrast, pAMRmut copies are protected by their mutation and replicate immediately according to their replication mechanism.

pSilencing: pAMR remains in the cell, but the expression of the AMR gene is silenced. Therefore, pAMR does no longer provide antibiotic resistance. pAMRmut is protected from transcriptional repression. The replication of pAMR and pAMRmut is not affected by target silencing. If all pSilencing copies are lost in later generations, the pAMR copies become functional and provide resistance again.

Step 3: Treatment with antibiotics. Antibiotics are added at the same time as pCRISPR. We assume that pAMRmut confers the same level of resistance as (non-silenced) pAMR, i.e. we only consider CRISPR-Cas evading mutations that do not impair the antimicrobial activity, as mutations that evade CRISPR targeting but no longer confer AMR can be ignored. We simulate the dynamics of the bacterial population using a birth-death process similar to the one used in Santer and Uecker (2020). Each cell divides and dies at rates that depend on the combined number of pAMRmut and non-silenced pAMR copies of the cell denoted by $0 \leq k \leq n$ here. We do not factor in a cost of the total plasmid load, which may differ between cells if pAMR/pAMRmut and pCRISPR are compatible. This decision allows us to compare the successes of different design choices for pCRISPR with each other, focusing on the inherent differences in the design. Accordingly, there are a total of $n+1$ cell types, with different birth and death rates, which we denote by $\lambda_{\min}^{(n)} = \lambda_0^{(n)}, \ldots, \lambda_k^{(n)}, \ldots, \lambda_n^{(n)} = \lambda_{\max}^{(n)}$ ($\mu_k^{(n)}$ analogously), where k is the sum of all functional AMR plasmids.

Since it will turn out that our results depend solely on the ratio of $\lambda_k^{(n)}$ and $\mu_k^{(n)}$, we can assume that the time to cell death is identical for all cell types, while we vary the birth rates for the cell types. Therefore, we set $\mu_k^{(n)} \equiv 1$ and assume $\lambda_0^{(n)} = 0.8$ (if not stated otherwise), which results in a population decline of completely sensitized cells by 50% in 5 to 6 generations. Furthermore, we assume that for cells with the maximum amount of functional AMR plasmids, $\lambda_n^{(n)} > \mu_n^{(n)} = 1$, since the bacterial WT population would face certain extinction otherwise. This can be easily seen when looking at the Malthusian fitness, that is the difference $s_k^{(n)} := \lambda_k^{(n)} - \mu_k^{(n)}$, which describes a growing population when $s_n^{(n)} > 0$.

For cells with $k \in \{1, \ldots, n-1\}$, it is also natural to assume that for the same copy number n, more functional AMR plasmids provide a higher or equal Malthusian fitness, i.e. $\lambda_k^{(n)} \leq \lambda_{k+1}^{(n)}$. This inspires the following fitness functions, which we will observe in detail:

***Linear* fitness:** The fitness increases linearly with the number of functional AMR plasmids, i.e. $\lambda_k^{(n)} = \lambda_0^{(n)} + k/n * \left(\lambda_n^{(n)} - \lambda_0^{(n)}\right)$.

***Dominant* AMR plasmids:** One functional AMR plasmid is sufficient to achieve maximum fitness for the cell, i.e. $\lambda_k^{(n)} = \lambda_n^{(n)}$ for $1 \leq k \leq n$.

***Recessive* AMR plasmids:** The cell needs the maximum amount of functional AMR plasmids, otherwise there is no fitness effect of the AMR plasmids, i.e. $\lambda_k^{(n)} = \lambda_0^{(n)}$ for $0 \leq k \leq n-1$.

When comparing different copy numbers, we assume that the maximum number of functional AMR plasmids, n, always provides the same maximum cell fitness, irrespective of what n is. If we think of the same resistance gene on different plasmids, this assumption is inconsistent with resistance mechanisms such as antibiotic-degrading enzymes for which a higher number of AMR plasmids results in a higher level of AMR, as described in various studies (Stougaard *et al.* 1979; San Millan *et al.* 2016; Santos-Lopez *et al.* 2017). However, it allows us to directly compare the effect of different copy numbers on the success of sensitizing CRISPR treatment without a confounding influence of a gene dosage effect. If we think of different resistance genes on different plasmids, results can be read as 'success probabilities, given a certain copy number and maximum cell fitness'.

Copy number and plasmid incompatibility. Except for the CRISPR-Cas-evading mutation in the AMR gene, pAMR and pAMRmut are isogenic and rely on the same copy number control system and are thus incompatible. For pCRISPR we investigate two scenarios – one where pCRISPR and pAMR/pAMRmut are incompatible and one where pCRISPR

and pAMR/pAMRmut are compatible. For mathematical convenience, we assume that throughout all steps of the model and in both pCRISPR compatibility scenarios, each cell contains exactly n copies of incompatible plasmids, although there is in reality some variation (Shao *et al.* 2021). If we denote the number of pAMR copies within a cell as #pAMR (and accordingly for the other plasmids), this leads, depending on the (in-)compatibility between the CRISPR and the resistance plasmid, to:

Incompatible pCRISPR

$$\#\text{pAMR} + \#\text{pAMRmut} + \#\text{pCRISPR} = n \quad \text{and}$$

Compatible pCRISPR

$$\#\text{pAMR} + \#\text{pAMRmut} = n.$$

Note that the copy number of compatible pCRISPR does not influence the dynamics in our model as long as it is stably maintained. We thus simply assume the copy number of comapatible pCRISPR to be one, which also highlights that the results in this scenario hold also for CRISPR-systems that are encoded on the chromosome. Whether pCRISPR is compatible or incompatible with pAMR/pAMRmut may have drastic consequences for the treatment outcome. If incompatible, the replication of pCRISPR interferes with the replication and segregation of pAMR and pAMRmut because it is perceived as the same plasmid by the regulatory system and thus over time ultimately results in the loss of pCRISPR or pAMR and pAMRmut. If compatible, there is no interference with the replication of pAMR and pAMRmut and thus pCRISPR is never lost. The details of this effect are outlined in the next section.

Plasmid replication and segregation during cell division. Each time a cell divides, all plasmids incompatible with pAMR, including the pAMR copies themselves, replicate to $2n$ copies (as depicted in step 3 of the timeline in Fig. 1). In our calculations, we consider two cases for the plasmid replication system: one in which the system perfectly remembers which plasmid has already been copied, and one where the system is memoryless, as described in Novick and Hoppensteadt (1978); Nordström (2006): If the plasmids replicate *regularly*, all plasmids are replicated exactly one. If the plasmids replicate *randomly*, a random plasmid is chosen for replication and then added to the pool until the copy number has been reached. The random replication model, where the "rich get richer", is known in mathematics as the Pólya urn scheme (Pólya 1930). The frequency of the different plasmid types, pAMR, pAMRmut, and incompatible pCRISPR, among the replicated plasmid copies is then Dirichlet-multinomial (DirMult) distributed. Overall, the deviations between results for the two replication models are minimal, and the overall findings hold true for both. Unless stated otherwise and also for the results depicted in Figures 2 - 4, we utilize the random replication model. The results based on regular replications are depicted in Supplementary Figures S5 - S7.

After replication of the plasmids, the cell divides, and each daughter cell receives the same number of plasmid copies, assuming random assortment. I.e., copies of incompatible plasmids are randomly (hypergeometrically) distributed to the daughter cells, such that each daughter cell receives exactly n copies (Novick and Hoppensteadt 1978).

N	Number of cells before and at CRISPR introduction ($t \leq 0$)
n	Copy number of pAMR and pAMRmut
v	Probability for a pAMR to mutate to pAMRmut during replication
s	Cost of the mutation in the absence of antibiotics ($t < 0$)
$\lambda_k^{(n)}$	Division rate of cells with k functional AMR plasmids (pAMRmut or non-silenced pAMR) and copy number n $\left(\lambda_{\min}^{(n)} = \lambda_0^{(n)}, \lambda_{\max}^{(n)} = \lambda_n^{(n)}\right)$
$\mu_k^{(n)}$	Death rate of cells with k functional AMR plasmids and copy number n

Table 1. Description of all relevant parameters in the model(s)

In summary, for a mother cell with j pAMRmut copies undergoing cell division, this implies that after plasmid replication to $2n$ plasmid copies, the number of newly generated pAMRmut j_d is $\mathrm{DirMult}\,(n,(j,n-j))$ distributed. The number of pAMRmut copies in the resulting two daughter cells $j_1 + j_2 = j + j_d$ are then distributed such that $j_1 \sim \mathrm{Hypergeom}(2n, j + j_{\mathrm{d}}, n)$.

Analysis

Our primary goal is to calculate the extinction probability of a bacterial population undergoing antibiotic treatment after receiving one of the four considered pCRISPR variants: compatible pCleaving, compatible pSilencing, incompatible pCleaving, and incompatible pSilencing.

To compute this probability, we initially need to determine the frequency of pAMRmut copies in our Moran model when CRISPR is introduced into the cells (step 1). Subsequently, we can calculate the frequency of each cell type right after pCRISPR introduction, which takes immediate effect according to its type and incompatibility group (step 2). Then, we can assess the probability of population extinction, denoted as P_{ext} (step 3).

Standing genetic variation before pCRISPR introduction. The multitype Moran model with a constant population size, as described in step 1 of the previous section, has two key factors that affect the frequency of cells carrying a specific number of pAMRmut copies: the probability v of mutation occurrence and the reduction in fitness s for cells carrying pAMRmut. We assume that the population is in mutation-selection balance when the CRISPR plasmid is introduced and describe the composition of the standing genetic

variation (SGV) by the deterministic equilibrium. Computing the equilibrium involves integrating nonlinear systems (see Santer and Uecker (2020), Appendix C and the supplement section for examples of such equilibria). We denote by $f^{(\mathrm{SGV})}(j) = f^{(\mathrm{SGV})}(n-j, j, 0)$ the fraction of cells that carry j copies of pAMRmut and $n-j$ copies of pAMR immediately before introduction of pCRISPR.

Introduction of pCRISPR. For pSilencing, the number of pAMR and pAMRmut copies remains the same after introduction as before, but all pAMR are silenced. In the case of compatible pCleaving, each cell with initially j pAMRmut copies is transformed into a cell with n pAMRmut copies. If pCleaving is incompatible, one pCleaving copy and j pAMRmut copies will compete for the remaining $n-j-1$ spots. In the case of *randomly* replicating plasmids, we use the Pólya urn scheme to fill the remaining places as described in the previous section. For *regularly* replicating plasmids, we repeatedly double the remaining $j+1$ plasmids unless their number would exceed n. The remaining spots are filled by randomly picking plasmids without replacement to replicate.

The resulting distribution of the fraction of cells immediately after the introduction of pCRISPR will be denoted by $f^{(\mathrm{CRISPR})}(i, j, c)$, where i signifies the number of pAMR copies, j represents the number of pAMRmut copies, and c denotes the number of pCRISPR copies.

For compatible pCRISPR we have $i+j=n$, while for incompatible pCRISPR usually $i+j+c=n$ holds. Note that introducing an incompatible pCRISPR will lead to the total number of plasmids within a cell exceeding n, when $j=n$ or when using incompatible pSilencing. In these cases, this state will persist until the next cell division (then under antibiotic treatment), during which pCRISPR, pAMR and pAMRmut will replicate such that there is a total of $2n$ of them. For random replication, this process is straightforward, whereas for regular replication, we simply assume that one random plasmid is not replicated. Subsequently, the plasmids are distributed to the daughter cells, each receiving n plasmids.

Population extinction after pCRISPR introduction during antibiotic treatment. We are now interested in the probability that, once CRISPR has been added for the purpose of sensitization, the entire bacterial population can be eradicated by antibiotics. How can the population escape from extinction? Irrespective of which CRISPR plasmid is used, cells that carried a pAMRmut copy at the time of CRISPR treatment are not (fully) sensitized and can thus leave a growing lineage of progeny. For pCleaving, this is the only way in which the population can survive. For pSilencing, there are additional pathways to population rescue. If pSilencing is incompatible with pAMR, plasmid replication and segregation can lead to loss of pSilencing from cells, in which case the silenced AMR gene becomes functional again. Moreover, irrespective of the compatibility of pSilencing, new mutations from pAMR to pAMRmut can occur during decline of the population. However, loss of incompatible pSilencing has a much higher probability to occur and to lead to treatment failure than rescue from *de novo* mutations or preexisting pAMRmut, which renders incompatible pSilencing useless. In the following, we will thus only consider the case in which pSilencing is compatible and both pCleaving variants.

Importantly, presence of cells with functional resistance genes does not automatically lead to treatment failure since the lineage may still go extinct due to demographic stochasticity. We first focus on pathways to rescue that do not require mutations after pCRISPR introduction – we refer to this as adaptation from the standing genetic variation – and include new mutations from pAMR to pAMRmut after pCRISPR introduction, which we will call *de novo* mutations, afterwards. To calculate the extinction probability for the entire bacterial population, we first calculate the probability for each single cell to not produce a long-term lineage of offspring after the immediate effect of pCRISPR introduction, without taking new mutations from pAMR to pAMRmut into account. Since this probability is the same for cells of the same type, we associate each cell with its respective type, denoted as (i, j, c) as above. We then denote the single-cell extinction probability by $p_{\mathrm{ext}}(i, j, c)$.

The calculation of $p_{\mathrm{ext}}(i, j, c)$ can be done by using the independence of different branches in the birth-death process. In detail, this means that for a single cell the probability of extinction is the probability of immediate extinction, in addition to the probability of both lineages founded by the daughter cells going extinct after a possible cell division. For example, for $c=0$ in the incompatible case and for any c in the compatible case, this simplifies to:

$$p_{\mathrm{ext}}(0, n, c) = \frac{\mu_n^{(n)}}{\lambda_n^{(n)} + \mu_n^{(n)}} + \frac{\lambda_n^{(n)}}{\lambda_n^{(n)} + \mu_n^{(n)}} \, p_{\mathrm{ext}}(0, n, c)^2,$$

which yields $p_{\mathrm{ext}}(0, n, c) = \frac{\mu_n^{(n)}}{\lambda_n^{(n)}}$, which is the known result for the extinction probability of a single-type birth-death process (Allen 2015).

Given a specific copy number n and considering all options for i, j and c, we get a total of $n+1$ quadratic equations for a compatible pCRISPR and $\frac{(n+1)\cdot(n+2)}{2}$ quadratic equations for an incompatible pCRISPR. Due to the high number of equations to solve, we resort to numerical approximations. These approximations are based on properties of the probability generating function of multitype branching processes, as outlined in Appendix A of Santer and Uecker (2020).

To calculate the total extinction probability, we have to consider the escape from extinction based on either existing pAMRmut according to the SGV or *de novo* occurring pAMRmut. (Note that we already excluded the case of incompatible pSilencing, where escape from loss of pSilencing is also relevant.) We thus have to calculate the product of the extinction probabilities

$$P_{\mathrm{ext}}^{(\mathrm{total})} \approx P_{\mathrm{ext}}^{(\textit{de novo})} \, P_{\mathrm{ext}}^{(\mathrm{SGV})}. \qquad \textbf{(1)}$$

To calculate the extinction probability $P_{\mathrm{ext}}^{(\mathrm{SGV})}$ if only the standing genetic variation is taken into account we consider

the resulting distribution of cell types after pCRISPR introduction $f^{(\mathrm{CRISPR})}$ and the extinction probabilities of the corresponding cell lineages:

$$P_{\mathrm{ext}}^{(\mathrm{SGV})} = \prod_{i,j,c=0}^{n} p_{\mathrm{ext}}(i,j,c)^{N\cdot f^{(\mathrm{CRISPR})}(i,j,c)}. \quad \textbf{(2)}$$

Furthermore, we set $P_{\mathrm{ext}}^{(de\ novo)}$ to 1 for pCleaving, since *de novo* mutations can only occur in the pSilencing case.

We now turn to the possibility of new mutations from pAMR to AMRmut if the population has received pSilencing at the onset of antibiotic treatment. Due to the high chance that cells lose incompatible pSilencing by segregation, which results in the expression of antibiotic resistance on all pAMR, it suffices to consider the case in which pSilencing is compatible. To calculate the probability that these *de novo* mutations do not lead to population rescue, we consider only the decreasing number of cells that initially, at the start of the treatment, did not carry pAMRmut. (cells that initially carry pAMRmut are covered above, ignoring the possibility of new mutations.) Because mutations can only occur during cell devision in our model, it suffices to consider discrete cell generations or more precisely the number of cell divisions until extinction. Assuming a deterministic decay of the cells carrying only pAMR, the number of cells that split into two daughter cells decreases on average by a factor $(2\cdot\lambda_0^{(n)})\,/\,(\lambda_0^{(n)}+\mu_0^{(n)})$ per generation. We can approximate the number of replications of pAMR during these cell divisions until population extinction by

$$\frac{1}{2}nN\sum_{m=1}^{\infty}\left(2\frac{\lambda_0^{(n)}}{\lambda_0^{(n)}+\mu_0^{(n)}}\right)^m = nN\frac{\lambda_0^{(n)}}{\mu_0^{(n)}-\lambda_0^{(n)}}. \quad \textbf{(3)}$$

The factor $1/2$ is due to only half of the plasmids of a new generation being freshly replicated, while the other half originates from the mother cells.

The number of *de novo* mutations M is then distributed as $\mathrm{Bin}\left(v, nN\frac{\lambda_0^{(n)}}{\mu_0^{(n)}-\lambda_0^{(n)}}\right)$. This results in the following extinction probability arising from *de novo* mutations only, i.e. without considering cells carrying pAMRmut copies when pCRISPR is introduced into the population

$$P_{\mathrm{ext}}^{(de\ novo)} \approx \sum_{m=0}^{\infty} p_{\mathrm{ext}}(n-1,1,1)^m P(M=m), \quad \textbf{(4)}$$

where we ignore the possibility that for a mutation that occurs in an early replication event, the mutant plasmid may be copied again in the *random* replication model (for a treatment of this possibility in a different context, see Dewan and Uecker 2024). Since v is small and N is very large, the binomial distribution can be approximated by a Poisson distribution following the Poisson limit theorem, resulting in $M \sim \mathrm{Poi}\left(vnN\frac{\lambda_0^{(n)}}{\mu_0^{(n)}-\lambda_0^{(n)}}\right)$, i.e.

$$P(M=m) = \frac{\left(v\,n\,N\,\frac{\lambda_0^{(n)}}{\mu_0^{(n)}-\lambda_0^{(n)}}\right)^m}{m!} e^{-vnN\frac{\lambda_0^{(n)}}{\mu_0^{(n)}-\lambda_0^{(n)}}}. \quad \textbf{(5)}$$

Combining equations (4) and (5) then yields

$$P_{\mathrm{ext}}^{(de\ novo)} \approx e^{-v\,n\,N\,\frac{\lambda_0^{(n)}}{\mu_0^{(n)}-\lambda_0^{(n)}}(1-p_{\mathrm{ext}}(n-1,1,1))}, \quad \textbf{(6)}$$

with which we recover the common approach to study evolutionary rescue (Orr and Unckless 2008; Martin *et al.* 2013; Uecker *et al.* 2014; Santer and Uecker 2020).

Results

A. Sensitizing effect. Prior to the pCRISPR introduction (step 1), the overwhelming majority of cells carry solely WT pAMR (referred to as WT cells in the following), a small fraction carries only pAMRmut (referred to as homozygous mutant cells), and some carry both, pAMRmut and WT pAMR copies (referred to as heterozygous cells). This distribution is shown as gray bars in Fig. 2 A.

Upon the introduction of pCRISPR (step 2), the WT cells become fully sensitized to antibiotics – all pAMR copies are cleaved or silenced immediately upon the entry of pCRISPR into the cell. However, this is not the case for heterozygous nor for homozygous mutant cells.

If pCleaving is used, pAMR copies in heterozygous cells are replaced by pAMRmut copies and possibly, if pCleaving is incompatible with the resistance plasmid, also by pCleaving copies. This results in a higher number of homozygous mutant and pCRISPR cells compared to the use of pSilencing. If pCRISPR is compatible, more homozygous mutant cells will arise in comparison to an incompatible pCRISPR, since there is no interference with the replication of pAMRmut. At each cell division, the composition of pAMR, pAMRmut, and incompatible pCRISPR copies will change due to replication and segregation of the plasmids. Fig. 2 A shows the distribution of pAMRmut copies in the population immediately after the introduction of pCRISPR and right before antibiotics are added.

If the population kept proliferating in the absence of antibiotics over multiple generations, the cells with pAMRmut will develop into homozygous pAMRmut and homozygous pCRISPR cells, due to repeated segregation of plasmids at cell division. The expected frequency of both cell types equals the total frequency of pAMRmut and pCRISPR copies. The resulting cell type frequencies are shown in Fig. 2 B, where we ignore *de novo* mutations from silenced pAMR to pAMRmut.

B. Extinction probabilities under treatment with antibiotics. The success probability of an antibiotic treatment that starts directly after the pCRISPR introduction depends on the pCRISPR variant. In the following, we first discuss single-cell extinction probabilities p_{ext} before we determine $P_{\mathrm{ext}}^{(\mathrm{total})}$, the extinction probability of the entire bacterial population for the various CRISPR designs.

B.1. Single cell extinction probabilities. Since WT cells that contain only pAMR are the most prevalent at the start of the

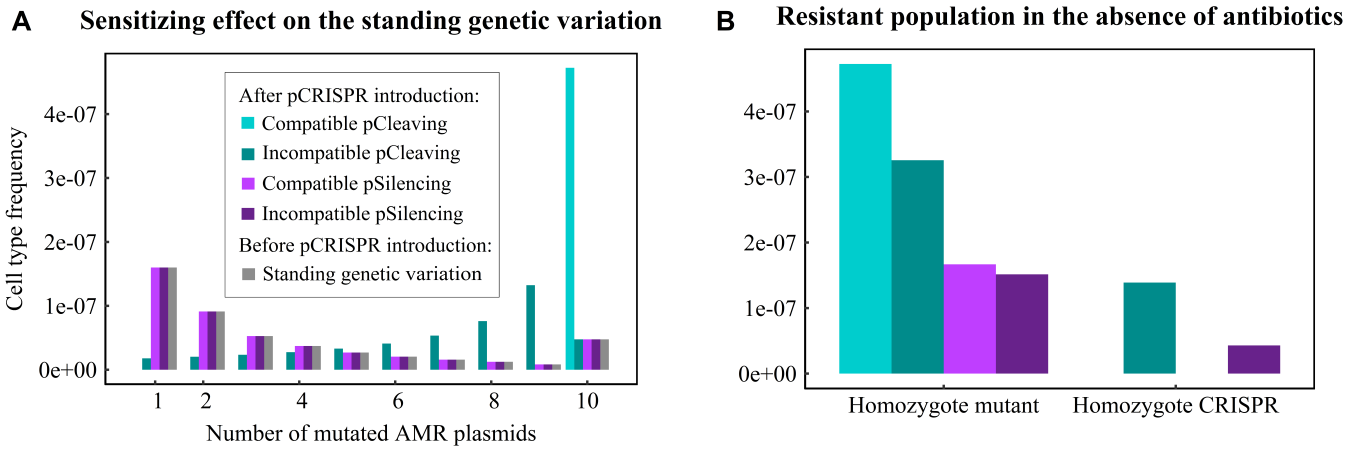


Fig. 2. The standing genetic variation (SGV) before and after the introduction of pCRISPR to the population for copy number $n = 10$. (A) The frequency of cells carrying 1 to 10 pAMRmut copies. The remaining cells are wild-type cells, which are not shown to avoid cluttering the plot. The SGV before the introduction of pCRISPR to the population for copy number $n = 10$ is depicted in grey in A. Here, the other plasmids inside the cell are WT pAMR copies. The other bars show the frequency of all cell types after the introduction of one pCRISPR copy. These frequencies differ greatly between the different options for pCRISPR. Compatible pCleaving transforms all heterozygous cells into homozygous mutant cells, as all pAMR are cleaved and replaced by pAMRmut, regardless of how many pAMRmut copies are initially present in the cell, as long as there is at least one. Similarly, incompatible pCleaving also increases the frequency of cells with high numbers of pAMRmut copies, as pAMRmut will be replicated alongside pCleaving after the pAMR have been cleaved. On the other hand, pSilencing does not change the plasmid composition, resulting in a distribution where most heterozygous cells have a low number of pAMRmut copies. (B) Cell type frequencies many generations after the pCRISPR introduction starting from the cells carrying at least one mutation in the SGV. We excluded the effect of *de novo* mutations and antibiotic treatment. Parameters: $v = 10^{-8}$, $n = 10$, $s = -0.1$.

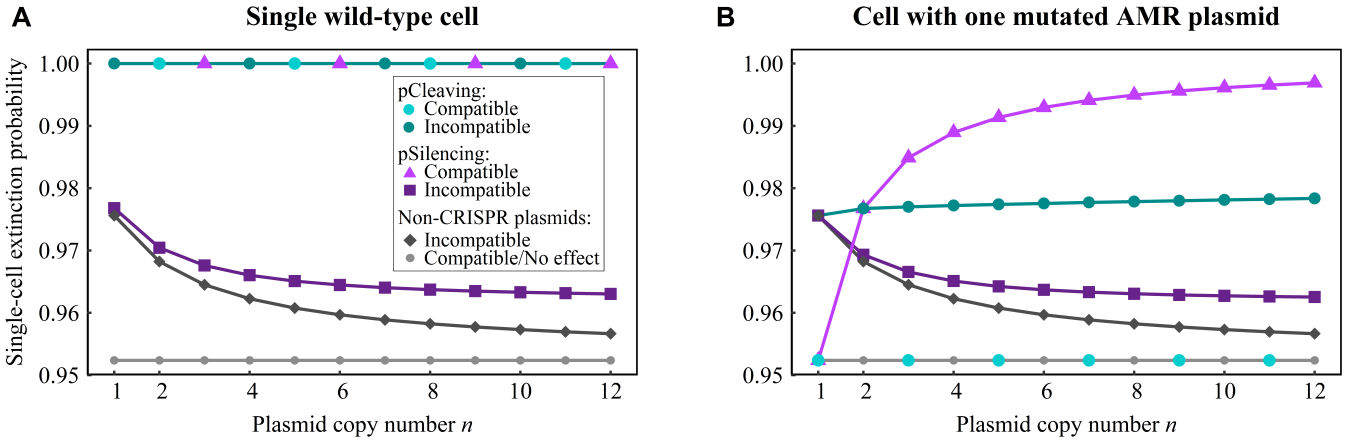


Fig. 3. Extinction probabilities for a single cell lineage due to antibiotic treatment after pCRISPR introduction. The extinction probability for offspring derived from single wild-type cells (A) and cells carrying exactly one pAMRmut and $n - 1$ WT pAMR copies (B) prior to the CRISPR-mediated cleaving or silencing. For comparison, plasmids without a CRISPR-Cas system are also shown. Since a compatible non-CRISPR plasmid has no effect, this scenario is equivalent to the antibiotic treatment of a resistant population. Also note that, in the case of single wild-type cells (A), the extinction probability for pCleaving and also compatible pSilencing is precisely 1, as we exclude the tiny chance that a *de novo* mutation occurs for single cell lineage extinction probabilities and compute the probability of evolutionary rescue due to *de novo* mutations later. For incompatible pSilencing and the non-CRISPR plasmids, a lineage extinction probability of at most 0.98 for WT cells is not high enough to treat a complete population of millions of cells. However, for the much lower number of mutated cells, the probabilities shown in (B) can be sufficient. The analysis uses a linear fitness function with parameters: $\lambda_{\max} = 1.05$, $\lambda_{\min} = 0.95$.

	pCleaving	pSilencing
Incompatible	$j/(j+1)$	$j/(n+1)$
Compatible	$\mathbb{1}_{j \neq 0}$	j/n

Table 2. Relative amount of functional AMR plasmids of a cell with $j\,(\leq n)$ pAMRmut copies at the start of the antibiotic treatment (Step 3). In the incompatible pCleaving case, the specified value is the expected amount, since the plasmids are randomly replicated following the replication model. In the other cases, the values are nonrandom. In the incompatible pSilencing case, the CRISPR plasmid can be lost due to segregation, increasing the relative amount of functional AMR plasmids immediately to 1. $\mathbb{1}$ is the indicator function. The replication model does not influence the outcome.

treatment, efficient sensitization of WT cells is a prerequisite for successful treatment. As can be seen in Fig. 3, only pCleaving and compatible pSilencing lead to high enough extinction probabilities of wild-type cells. Since pCleaving eliminates all pAMR and compatible pSilencing permanently silences all of them, single-cell extinction probabilities are equal to one if we ignore that a new mutation could arise for the calculation of p_{ext}. (We will deal with *de novo* mutations later.) In the case of incompatible pSilencing, plasmid competition, which is independent of CRISPR interference, can lead to the loss of pCRISPR and consequently to the termination of transcriptional silencing of the WT pAMR copies. Therefore, wild-type cells have a considerable chance of gen-

erating a long-term lineage of offspring without the need for *de novo* mutations. This renders incompatible pSilencing similarly inefficient as the introduction of an incompatible plasmid without a CRISPR-Cas system (see Fig. 3 A).

As long as pAMR do not carry mutations, a compatible/ incompatible pCleaving and a compatible pSilencing, followed by antibiotic administration, will result in a successful treatment. However, it is more realistic to assume that cells carrying mutated AMR plasmids pre-exist or in the case of compatible pSilencing arise *de novo* and may prevent the extinction of the population. For cells carrying at least one pAMRmut copy, whether they replicate or die crucially depends on their fitness after pCRISPR introduction, which in turn depends on the relative amount of functional AMR plasmids (Table 2) and the fitness function (dominant, linear, recessive). However, this fitness is not simply "passed on" to the daughter cells but can change since plasmid replication and segregation change the relative abundance of functional AMR copies in the daugther cells with respect to the mother cell.

In the case of compatible pCleaving, all pAMR within a heterozygous cell are cleaved and the number of pAMRmut copies increases to n. The relative amount of functional AMR plasmids thus immediately jumps to one (as stated in Table 2). Therefore, even a single pAMRmut copy fully prevents any sensitizing effect, making this design less suitable to manage pre-existing variation. In contrast, incompatible pCleaving, by sharing a copy number with pAMRmut, prevents the immediate generation of fully resistant cells, and the extinction probability is thus higher than for compatible pCleaving. Likewise, for both incompatible and compatible pSilencing, immediate appearance of fully resistant cells is impossible.

In Fig. 3 B, we specifically analyze the long-term extinction probability of cells containing exactly one pAMRmut and $n-1$ pAMR copies just prior to CRISPR introduction (ignoring new mutations that might appear later). These cells are particularly important, as they are common in the standing genetic variation (compared to cells with more than one pAMRmut copy) and will often arise from *de novo* mutations. Since we have already identified incompatible pSilencing as a poor strategy, and for pCleaving the compatible variant is less effective, we now focus on the remaining two.

Whether compatible pSilencing or incompatible pCleaving is preferable depends on the copy number of pAMR. In the case of incompatible pCleaving, two incompatible plasmids persist post-cleavage: one pCRISPR and one pAMRmut copy, which then replicate until n copies are present in the cell again. Consequently, the expected number of pAMRmut copies after plasmid reproduction is $n/2$. For a compatible pSilencing, pAMR are not cleaved and compete with the pAMRmut copy. Thus, there is only one functional plasmid copy (the pAMRmut) after CRISPR introduction. We see in Fig. 3 B that in line with this consideration, incompatible pCleaving is superior for $n=1$, while compatible pSilencing is superior for $n \geq 3$, see Fig. 3 B. In general, for heterozygous cells with more than one pAMRmut copy, compatible pSilencing leads to a lower average number of functional copies of the antibiotic resistance gene in a cell than pCleaving right after introduction of pCRISPR as long as the number of pre-existing pAMRmut copies in the cell is at most $n-2$ (see Table 2). From this, it seems plausible that those heterozygous cells are better managed by compatible pSilencing than by incompatible pCleaving. When there are initially $n-1$ pAMRmut copies in the cell, both CRISPR variants lead to the same average number of functional copies. Consequently, for copy numbers $n=1$ and $n=2$, incompatible pCleaving is expected to outperform compatible pSilencing in the eradication of heterozygous cells.

B.2. Extinction probabilities of the entire population. By considering single cell extinction probabilities we have identified pCleaving and compatible pSilencing as promising choices for pCRISPR. However, compatible pCleaving will always provide lower success rates compared to incompatible pCleaving. Thus, in the following, we focus mainly on the comparison between incompatible pCleaving and compatible pSilencing.

The total extinction probability of the bacterial population depends on the different fractions of pAMRmut copies in the cells in the standing genetic variation and the risk of *de novo* mutations from pAMR to pAMRmut during population decline. The fraction of heterozygous or homozygous mutant cells in the standing genetic variation varies, depending on the copy number and the selection pressures prior to CRISPR introduction. While higher copy numbers increase the occurrence of mutated plasmid variants, their fixation probability decreases. Therefore, higher copy numbers lead to larger fractions of heterozygous cells, as also illustrated in Supplementary Fig. S8.

Since compatible pSilencing is more effective than incompatible pCleaving for heterozygous cells, it provides a higher extinction probability $P_{\text{ext}}^{(\text{SGV})}$ if the copy number is high, while incompatible pCleaving leads to higher extinction probabilities if the copy number is low and cells that are homozygous for pAMRmut are rare at the time of pCRISPR introduction. The example in Fig. 4 A demonstrates this effect, showing that compatible pSilencing is superior for copy numbers $n \geq 5$ for these specific parameter choices. Throughout the examples in this manuscript the threshold was between 4 and 10 but this will strongly depend on the concrete parameters.

As long as we ignore *de novo* mutations of silenced pAMR copies, there always exists a threshold copy number above which compatible pSilencing is preferable over incompatible pCleaving. However, a large number of *de novo* pAMRmut copies can considerably decrease the extinction probability. The existence of a threshold, if we account for *de novo* mutations, depends on the fitness function (see Supplementary Note 1). In brief, for a linear and recessive fitness function, the threshold always exists. For dominant fitness functions, the existence of the threshold is possible, but not guaranteed; in the latter case, pCleaving is superior for all copy numbers n.

B.3. The Effectiveness of pCleaving and pSilencing across various conditions. We next investigate the population extinction probability under various conditions and focus partic-

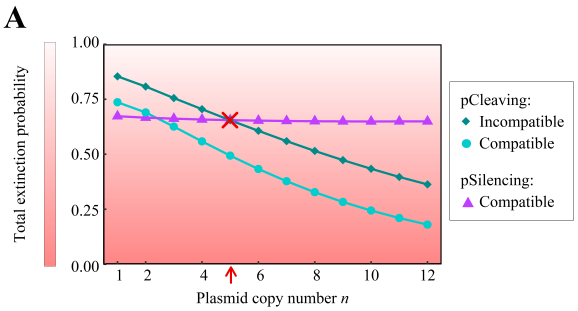
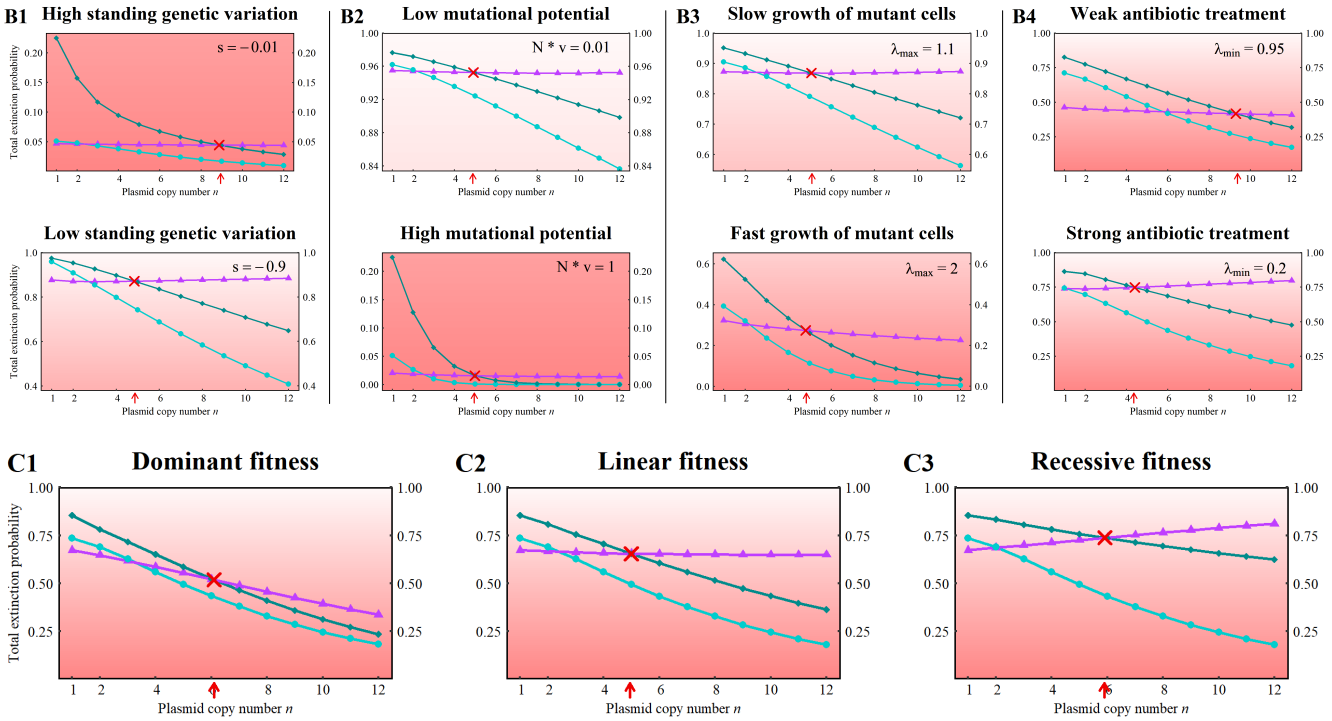


Fig. 4. Total extinction probability in a pathogenic population. The focus is only on the following three primary cases: compatible and incompatible pCleaving, and compatible pSilencing. Other cases from Fig.3 are omitted due to their limited potential to eradicate the population. (A) Influence of the copy number on the population extinction probability for all three cases. The parameters are $\lambda_{\max} = 1.3$, $\lambda_{\min} = 0.8$, $v = 10^{-8}$, $s = -0.1$, $N = 10^7$ assuming a linear fitness function. (B) illustrates the effect on (A) when a specific parameter is adjusted to a higher or lower value as indicated in the top right-hand corner. All other parameters remain as in (A). In (C), the fitness function is varied while all parameters are the same as in (A). The red arrow and cross indicate the point of intersection of the two curves, representing compatible pSilencing and incompatible pCleaving. Below this intersection point, incompatible pCleaving is consistently superior, while above it, compatible pSilencing is more effective. Note the different ranges on the y-axis across panels. The gradient of the background color from white to light red visually represents the success of the treatment.



ularly on the threshold copy number above which compatible pSilencing is preferable in comparison to incompatible pCleaving. In Fig. 4 B, we alter a single model parameter with respect to the scenario shown in Fig. 4 A.

A pCRISPR targeting a more conserved DNA segment (i.e. strong selection s against pAMRmut) leads to fewer cells carrying pAMRmut copies in the standing genetic variation, thereby enhancing the probability of population extinction. As compatible pSilencing is most effective for cells with a low proportion of pAMRmut copies, this enhances the efficacy of compatible pSilencing compared to incompatible pCleaving, as the fraction of cells possessing a low count of pAMRmut copies increases. Consequently, the threshold occurs at a lower copy number than for weak negative selection against pAMRmut, as depicted in Fig. 4 B1.

A higher mutational potential, which is the product of the mutation rate and the population size, increases the occurrence of cells carrying pAMRmut copies, which leads to a lower chance of extinction of the bacterial population. Likewise, the extinction probability decreases with the growth rate of mutant cells. As can be seen in Fig. 4 B2 and B3, the threshold copy number is not strongly affected by either of these factors. This is because changes in the growth rate, the mutation rate, and the population size only alter the total number of cells with pAMRmut but not the composition of the standing genetic variation at the start of treatment.

The effectiveness of the antibiotic treatment, i.e. the rate of decline of wild-type cells, has a particularly strong effect on the extinction probability. This is especially the case for compatible pSilencing, where the faster population decline leads to a lower expected number of *de novo* mutations of silenced pAMR into functional pAMRmut copies. A more effective treatment thus particularly increases the extinction probability for compatible pSilencing, resulting in a threshold at a lower copy number, as can be seen in Fig. 4 B4.

In addition to the parameters, we varied in Fig. 4 B, the fitness of the heterozygous cells is also important. If the maximum and minimum fitness remain the same, the fitness function (*dominant*, *linear* and *recessive*) only affects the fitness of cells where at least one but not all plasmid copies are functional pAMR or pAMRmut. Fig. 4 C shows that a dominant function results in the lowest extinction probability, while a recessive function leads to the highest. Compatible pSilencing is of particular interest here, since with increasing

copy number, the fraction of cells in the SGV with only a few pAMRmut copies increases, while the fraction of homozygous mutant cells decreases (see Supplementary Fig. S8). The influence of the copy number on the overall extinction probability therefore depends on how many cells with a few pAMR copies are necessary to outweigh the establishment probability of the homozygous mutant cells. Consequently, the success rate of compatible pSilencing decreases monotonically with dominant fitness, is almost constant with linear fitness, and increases monotonically for a recessive fitness function.

In contrast to compatible pSilencing, compatible pCleaving is completely unaffected by the fitness function, while the extinction probability in incompatible pCleaving does not change the monotonic trend, but extinction is most likely for a recessive fitness function. How the fitness function influences the population extinction probabilities is illustrated in Supplementary Figure S11.

Discussion

In our study, we have developed a mathematical model to explore strategies for combating antimicrobial resistant bacteria using CRISPR-Cas technology. The model incorporates the dynamics of multicopy AMR plasmids and their interaction with different compatible and incompatible CRISPR-Cas-encoding plasmids, which either cleave or silence the targeted AMR genes. The success probability of the subsequent treatment is contingent on various factors, some of which are included in our analysis, while other potentially important factors are discussed in this section.

Our results suggest that target cleaving by a CRISPR-Cas system encoded on a plasmid incompatible with the AMR plasmids is most effective if this pAMR is of low copy number. To remove pAMR of higher copy numbers, a CRISPR-Cas system for gene silencing encoded on a plasmid compatible with pAMR offers the superior solution because silencing effectively slows down the spread of CRISPR-resistant AMR plasmids. In the case of a pSilencing that is incompatible with pAMR, plasmid competition could result in the loss of pSilencing, which would stop transcriptional repression and restore AMR. It would therefore be advantageous to enhance pSilencing competitiveness and stability. This could be achieved by equipping pCRISPR, whether it acts by silencing or cleaving, with a toxin-antitoxin (TA) systems (Kamruzzaman *et al.* 2017). This acts as a plasmid addiction system by post-segregational killing of cells which have lost the TA system encoding plasmid (Hayes 2003). On the other hand, TA systems on the resistance encoding plasmids could enhance the effectiveness of cleaving pCRISPR, as bacteria that have lost the plasmid are readily killed (Citorik *et al.* 2014; Reuter *et al.* 2021).

The choice of the specific genetic target gene seems particularly important. Targeting AMR genes directly is useful because escaping requires deleting/mutating this target sequence and arising pAMRmut copies are likely to have no or non-functional AMR genes. This is in contrast to our model assumptions, where we ignore such nonfunctional mutations as they are not able to rescue the population from extinction. We thus included only mutations that evade CRISPR targeting while maintaining full functionality in our model. To further decrease the occurrence of pAMRmut with a functional AMR gene, several spacers can be multiplexed to simultaneously target different sites within the AMR gene or also other loci in the AMR plasmid. The effect of this strategy is covered by our modeling approach by setting a lower mutational potential. Our results show that using multiple targets can dramatically increase the extinction probabilities (Fig. 4). Targeting other regions of the AMR plasmid in addition to or instead of the AMR gene is an interesting option. First, with the addition of more targets, mutating these simultaneously and thus escaping becomes increasingly unlikely. Second, depending on the chosen loci, targeting or escaping can result in decreased fitness and thus the loss of pAMRmut. A suitable target may be the plasmid replication machinery, which might hinder the proper replication of pAMRmut or slow down their replication. However, targeting the AMR plasmid backbone alone is likely not a promising strategy: AMR genes on plasmids are frequently encoded within mobile elements such as insertion sequences or transposition units, which can integrate into the chromosome or other plasmids (Partridge *et al.* 2018). Targeting the plasmid would select for AMR genes that have jumped to other replicons, whereas targeting the AMR gene would sensitize the cell independently of its location.

Mutations in the target sequence are not the only mechanisms leading to failure of the antibiotic treatment. Several experimantal studies have demonstrated how the application of antibiotics following CRISPR-Cas interference selects for defective constructs, for example by spacer deletion or inactivation of the nuclease (Bikard *et al.* 2014; Citorik *et al.* 2014; Reuter *et al.* 2021; Mamontov *et al.* 2022). This poses a real risk for treatment success, especially for applications with pSilencing, which needs to remain functional throughout antibiotic treatment. As a result, in reality the actual probability of *de novo* mutations for pSilencing may be higher than assumed in our model. Reintroducing functional pCRISPR might reduce this risk (Uribe *et al.* 2021). In Supplemental Figure S9, we explore the scenario in which cells constantly acquire additional pCRISPR copies after the initial introduction. However, as we assume that initially every cell receives a pCRISPR, this has minimal impact on the results, even at high pCRISPR reinsertion rates.

For target interference by cleaving, Mamontov *et al.* (2022) discussed the existence of equilibria between CRISPR interference and pAMR replication rates. When both rates are equal, this can result in stable equilibria at numbers of copies below the actual copy number. However, experimental studies have shown that interference by cleaving was efficient even with plasmids present in up to 300 copies per cell (Citorik *et al.* 2014; Tagliaferri *et al.* 2020) and thus, in our model, we assume that CRISPR interference outweighs the pAMR replication rate.

While the assumption that the elimination of pAMR copies in cells which contain pCleaving is fast because of a high cleaving rate is reasonable, it is unlikely that the remain-

ing pAMRmut and pCRISPR copies replicate at the same rate. A difference between the replication rates of pAMR and pCRISPR influences only the efficacy of the incompatible pCRISPR (Supplementary Fig. S10). This is because the replication rate only matters if the CRISPR encoding plasmid and the AMR plasmid compete with each other. If incompatible pCRISPR can replicate itself very quickly, it can be more effective than compatible pSilencing, even for higher copy numbers. Conversely, if pCRISPR replicates slower than the AMR plasmid, this reduces the extinction probability.

Horizontal gene transfer was not taken into account in our analysis. We assume that each cell receives a pCRISPR via an unspecified delivery mechanism. However, efficient delivery of a CRISPR-Cas tool in situ is a main bottleneck for the application of CRISPR-Cas as novel antimicrobials. One promising avenue is to use self-transmissible conjugative plasmids for CRISPR-Cas delivery (Neil *et al.* 2020). Horizontal transfer is an important consideration for not only the delivery plasmid but also the AMR-encoding plasmid, which are frequently self-transmissible and can transfer to plasmid-free cells within the populationon. This suggests that plasmids that have escaped CRISPR-Cas interference (pAMRmut) could transfer to cells that have lost the targeted plasmid pAMR, thereby lowering the extinction probability. The compatible pCleaving approach would be most affected by this, as any wild-type cell acquiring a pAMRmut via horizontal gene transfer would replicate these genes up to the copy number n, providing the former wild-type cell with maximal antimicrobial resistance. In contrast, incompatible pCleaving and compatible pSilencing would be less affected due to the lower number of pAMRmut copies and the presence of plasmids in the same incompatibility group within the cell. However, the exact influence of horizontal gene transfer on the success probability of the treatment remains to be explored further in future studies.

Although our model has been formulated in terms of plasmid-plasmid interactions, the results extend to chromosome-plasmid and plasmid-chromosome interactions. A compatible CRISPR-encoding plasmid that interacts with an AMR plasmid mirrors the dynamics of a chromosomal CRISPR-Cas system targeting an AMR plasmid. Vice versa, an AMR plasmid with a copy number of one mimics AMR genes that are encoded on the chromosome, as in our case, removal of the pAMR in a cell guarantees the extinction of its cell lineage, which is equivalent to killing the cell directly by cleaving the chromosome. For AMR genes on the chromosome, pCleaving is thus predicted to be more effective than pSilencing.

In conclusion, sensitizing bacteria with CRISPR-encoding plasmids and subsequently treating them with antibiotics appears to be a promising strategy for combating resistant bacteria. We find that, in our model, treatment with incompatible CRISPR plasmids is effective only when the targeted DNA sections are cleaved, whereas compatible pCRISPR treatment demonstrates efficacy regardless of whether the targeted DNA section is cleaved or silenced. Generally, silencing mechanisms are more effective for higher copy numbers, while cleaving is preferable for lower copy numbers. Therefore, our results suggest that considering silencing mechanisms may offer a promising alternative approach to cleaving when combating antimicrobial resistant strains, particularly for strains with higher copy numbers.

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