MICROBIOLOGY

A predatory gene drive for targeted control of self-transmissible plasmids

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Suppressing plasmid transfer in microbial communities has profound implications due to the role of horizontal gene transfer (HGT) in spreading and maintaining diverse functional traits such as metabolic functions, virulence factors, and antibiotic resistance. However, existing tools for inhibiting HGT are limited in their modes of delivery, efficacy, and scalability. Here, we present a versatile denial-of-spread (DoS) strategy to target and eliminate specific conjugative plasmids. Our strategy exploits retrotransfer, whereby an engineered DoS plasmid is introduced into host cells containing a target plasmid. Acting as a predatory gene drive, DoS propagates itself at the expense of the target plasmid, through competition or active elimination. Once the target plasmid is eradicated, DoS is removed via induced plasmid suicide, resulting in a community containing neither plasmid. The strategy is tunable and scalable for various conjugative plasmids, different mechanisms of plasmid inheritance interruption, and diverse environmental contexts. DoS represents a new tool for precise control of gene persistence in microbial communities.

INTRODUCTION

Horizontal gene transfer (HGT) is a key mechanism of genetic exchange among bacteria, enabling the spread of genes across different species. Of the mechanisms for HGT, conjugation is particularly important due to the plasmid prevalence (*1*, *2*), broad host range of many plasmids (*3*, *4*), and the abundance of key genes found on plasmids (*5*, *6*). Conjugation involves direct cell-to-cell contact, during which a donor bacterium transfers a plasmid to a recipient via a mating bridge, allowing the plasmid to establish itself in the new host.

A critical yet less commonly discussed aspect of conjugation is retrotransfer. During this process, a mobilizable plasmid in the recipient cell can transfer to the donor cell. This could happen in one step by using the mating bridge formed during the original conjugation event or through secondary transfer. Past studies have provided evidence for both possibilities (*7*–*10*). Regardless of the underlying mechanism, this process represents a unique vulnerability in self-transmissible plasmids, which can be exploited for targeted engineering strategies.

From an engineering perspective, conjugation-mediated plasmid persistence is an effective strategy to stabilize desirable functions in a microbial community. In particular, rapid plasmid transfer has been proposed to program dynamic division of labor, whereby a plasmid-encoded metabolic function can be stably maintained despite its potential burden (*11*–*15*).

Conversely, transferable plasmids play a critical role in the spread and maintenance of antibiotic resistance genes (ARGs). Many pathogens host multiple conjugative plasmids, each often encoding several resistance genes (*16*–*18*). Many of these plasmids transfer at sufficiently high rates to enable the long-term maintenance of ARGs in the absence of positive selection (*19*). To this end, the active suppression of plasmid-mediated gene transfer is critical for combatting the spread of antibiotic resistance.

Many chemical agents have been evaluated for their plasmid-curing efficacy, but they are often not suitable for specific environments, are effective against few targets, or have poorly understood mechanisms (*20*). Compounds that can eliminate conjugative plasmids have only been demonstrated against specific plasmids or hosts (*21*–*26*). For example, plumbagin, a plant-derived dye, can decrease plasmid copy number and offset toxin-antitoxin plasmid maintenance systems (*27*). However, curing success varied across both plasmid target and host, from 100% elimination of plasmids TP181 and R162 in *Escherichia coli* to 4% elimination of a vancomycin-resistant plasmid in *Staphylococcus aureus* (*28*, *29*). Even for the same target plasmid, plumbagin's curing efficacy across different hosts alone varied from 13 to 32% colonies cured of the plasmid (*30*). Fatty acids such as linoleic acid have been used as conjugation inhibitors to eliminate otherwise persisting plasmids, but their efficacies vary considerably depending on the target plasmid or host strain (*19*, *31*, *32*). Last, many microbial communities rely on HGT of various native plasmids to maintain stability, including soil (*33*), gut (*34*–*36*), and other microbiomes (*37*). However, chemical agents could also affect off-target plasmids.

An alternative strategy is to use engineered plasmids, which can allow for more precise control due to sequence-level tunability. Studies have demonstrated the utility of incompatibility at eliminating plasmids belonging to different incompatibility (inc) groups (*38*–*40*). CRISPR-Cas9 has also been used for targeted plasmid cleavage through different guide RNAs (gRNAs). Typically, Cas9 proteins and gRNAs are both delivered into the host via HGT, within which they cut the corresponding target plasmid (*41*, *42*). So far, however, these strategies lack broad applicability to address diverse plasmids or complex communities. For example, engineered plasmids have often relied on conjugation or transformation for delivery into host cells, neither of which control for which cells can receive the plasmid (*38*–*42*). Thus, if all cells are viable recipients regardless of whether they contain the target, then the synthetic plasmids could spread unrestrained throughout a microbial community and interfere with genetically similar nontarget plasmids. Similar to chemical agents, nonspecific disruption of conjugation beyond the specific target can notably destabilize a community (*43*).

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To overcome these limitations, we present a scalable strategy that exploits the vulnerability in self-transmissible plasmids. The core of our design is an engineered, mobilizable plasmid, termed denial-of-spread (DoS) plasmid, which can propagate by using the transfer machinery encoded by the self-transmissible plasmid. A DoS plasmid enters the target population via retrotransfer, a key vulnerability of conjugation (Fig. 1). During retrotransfer, as a conjugative plasmid transfers from a donor to a recipient cell, a non–self-transmissible but mobilizable plasmid in the recipient cell can transfer back to the original donor through the same mating bridge or secondary transfer. Once in the same cell as the target conjugative plasmid, DoS outcompetes the conjugative plasmid for both horizontal transfer and vertical inheritance. As a result, DoS spreads throughout the population at the expense of the conjugative plasmid until the latter is lost, after which induced DoS plasmid suicide leaves neither the target nor DoS remaining in the system (Fig. 1).

In its operation, the DoS plasmid acts as a predatory gene drive (*44*, *45*). It propagates at an elevated rate to promote its spread, at the expense of the self-transmissible plasmid. Compared to previous plasmid-targeting approaches, our DoS design is modular, scalable, and specific for its designed target. Our results demonstrate the utility of DoS variants to enable specific elimination of a wide variety of plasmids in simple and complex microbial communities.

RESULTS

Design principle of DoS plasmids

Our laboratory previously established a quantitative metric, plasmid persistence potential (ω) to predict the persistence of a plasmid in multistrain, multiplasmid communities (*46*)

$$\omega = \frac{\eta}{\frac{\mu}{\mu-\sigma D}\left(D+\kappa-\frac{D}{1+\lambda}\right)} \quad (1)$$

According to the criterion, the persistence of a plasmid can be suppressed by decreasing its transfer rate (η), increasing its plasmid loss rate (κ), increasing its relative fitness cost (λ), or decreasing species diversity in the community (σ). Previous strategies have shown that modulating these parameters is sufficient to eliminate some transferable plasmids (*19*). We sought to design a modular synthetic plasmid that could achieve all these effects for efficient plasmid curing and be easily tuned for different conjugative plasmids.

To suppress η, we found that, if two plasmids—one conjugative and one mobilizable (i.e., does not express its own conjugation machinery)—within the same cell use the same conjugation mechanism, they will compete with one another for transfer, with the outcome heavily favoring the mobilizable plasmid (Fig. 2A). In essence, simply by encoding the same transfer origin (oriT), an engineered plasmid can suppress its conjugative plasmid counterpart once delivered into the same cell (*47*). The oriT also provides an additional design feature: If a conjugative plasmid transfers from a donor cell into a recipient cell containing a mobilizable plasmid, then that mobilizable plasmid can transfer back into the donor through retrotransfer (Fig. 2B). Thus, retrotransfer can be used to deliver an engineered plasmid into host cells containing the target.

For increasing κ, we use plasmid incompatibility, in which two plasmids using the same replication mechanism cannot stably coexist within the same cell over several generations. Therefore, given a conjugative plasmid in a host, introducing a coresident incompatible plasmid reduces the likelihood the conjugative plasmid is inherited, effectively increasing its segregation error rate and thus its κ (Fig. 2C). Instead of an entire replicon, an engineered plasmid can achieve this feature by encoding the incompatibility determinant sequence (inc) from the original conjugative plasmid. Last, we modulate the λ of the conjugative plasmid by antibiotic selection for our engineered plasmid, giving it a fitness advantage over the target.

Combining these mechanisms, we designed DoS, which encodes an oriT, an inc sequence, and an ARG (Fig. 2D). To evaluate our design, we first built a kinetic model consisting of a set of ordinary differential equations to simulate the different stages of DoS intervention (fig. S1 and see Supplementary Information for model formulation and analysis). In our model, DoS is introduced into a resident community containing a conjugative plasmid. The delivery host, which initially contains only DoS, is chosen to have a slower growth rate to ensure its loss after DoS delivery. Once DoS enters the resident community, it suppresses the transfer and inheritance of the conjugative plasmid. During this phase, a fitness cost is imposed on cells only carrying the target plasmid, while cells containing a DoS plasmid gain a fitness benefit. Demonstrating the feasibility of our design, numerical simulations confirm that DoS could eliminate a conjugative plasmid that would otherwise persist in the system, under a wide variety of conditions (fig. S2 and Supplementary Information). Afterward, DoS can be removed from the system by making it burdensome to cell growth.

In terms of its operation, DoS acts as a predatory gene drive (*48*, *49*). Its propagation depends on the target, self-transmissible plasmid, which it competes with. However, because of its fitness advantage (through the control of growth conditions), DoS can propagate better and lead to the suppression of the self-transmissible plasmid.

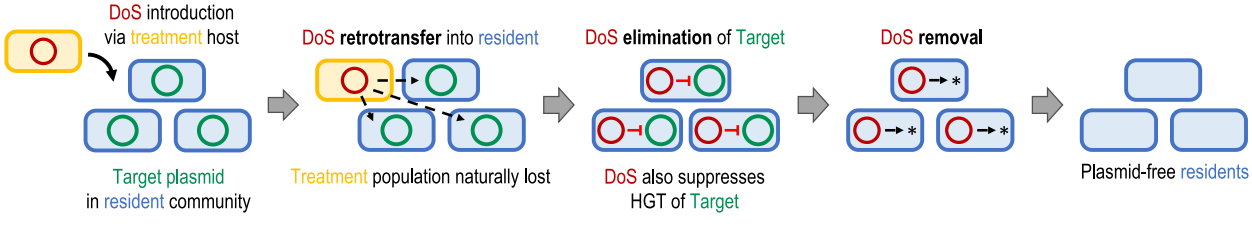

Fig. 1. Targeted suppression of conjugation by an engineered DoS plasmid. Upon delivery of the DoS plasmid (red) in a treatment population (yellow) into a resident community (blue) containing a conjugative plasmid target (green), the DoS plasmid can retrotransfer into the resident population by exploiting the conjugation machinery of self-transmissible plasmid or through secondary transfer. During retrotransfer and DoS-mediated target elimination, antibiotics are administered to give fitness benefit to DoS plasmids. Once in the same cell, DoS eliminates the conjugative plasmid via one of multiple possible mechanisms (incompatibility, HGT suppression, or CRISPR-Cas9). Once the conjugative plasmid is lost, DoS can be removed, leaving a plasmid-free resident population.

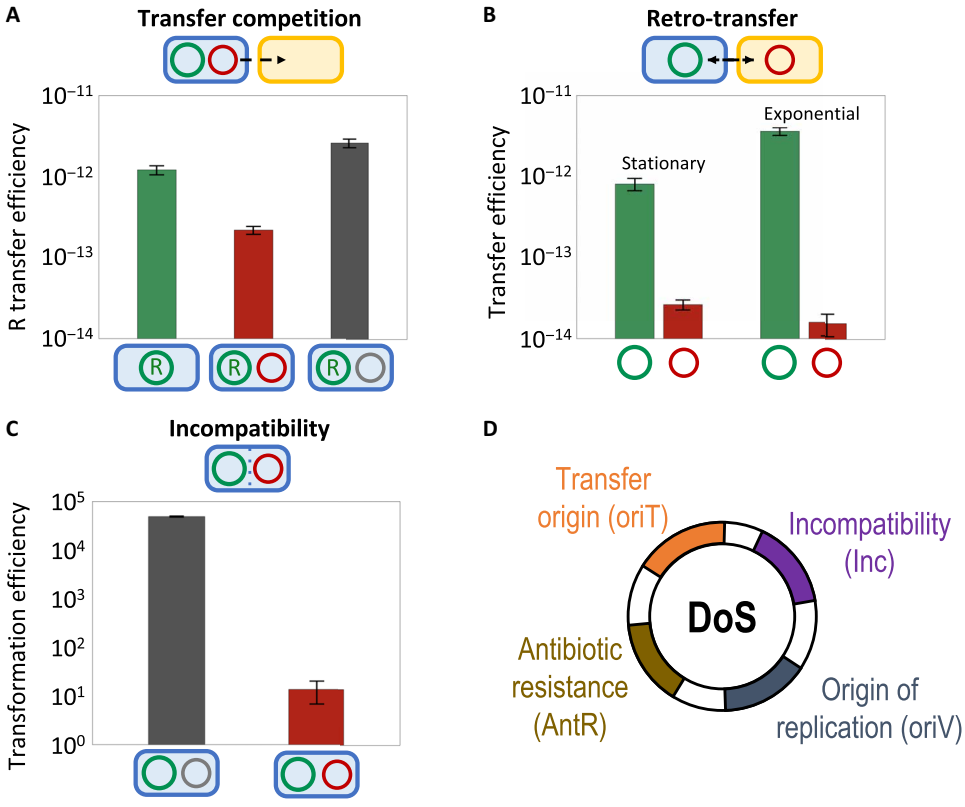

Fig. 2. The foundations of DoS design. (**A**) Competition during cotransfer of two different plasmids. We compared RP4 conjugation rates between MG1655 donors and Top10 recipients. In the first case (left), the donor only contained RP4. In the second case (middle), the donor carried RP4 and a mobilizable (but not self-transmissible) plasmid encoding the RP4 oriT. In the third case (right), the donor contained RP4 and a plasmid lacking the RP4 oriT but otherwise identical to that in the second case. RP4 transfer was suppressed when a competing mobilizable plasmid was present but not in the presence of a nontransferrable plasmid. (**B**) A mobilizable plasmid can enter hosts carrying a conjugative plasmid of the corresponding type through retrotransfer. MG1655 containing the RP4 plasmid was mixed with Top10 containing a mobilizable plasmid encoding the RP4 oriT. MG1655 and Top10 cells containing both plasmids were quantified to measure retrotransfer and conjugation efficiencies, respectively. Conjugation (green) is much faster than retrotransfer (red). (**C**) Incompatibility between plasmids results in segregation. Two engineered plasmids, one encoding the RP4 incompatibility sequence (inc) and the other lacking RP4 inc, were separately transformed into competent cells containing the RP4 plasmid. Transformants containing both plasmids were quantified via dual-selection plates. Few transformants were present containing RP4 and the plasmid with RP4 inc, while numerous transformants were present containing RP4 and the plasmid without RP4 inc. (**D**) DoS design. DoS encodes three key components. (i) An identical oriT to the target plasmid, allowing retrotransfer into the resident population and suppressing further transfer of the target plasmid. (ii) An identical inc, making DoS and the target plasmid incompatible and ensuring segregation during cell division. (iii) A selective marker (e.g., antibiotic resistance), allowing the tuning of DoS benefits and target plasmid burden by adjusting antibiotic concentration.

Elimination of the broad host range plasmid RP4 using DoS 1.0

As a proof of concept, we constructed DoS 1.0 to target the conjugative plasmid RP4, a well-studied IncP conjugative plasmid (Fig. 3A). We chose RP4 due to its fast transfer rate, broad host range, and ability to spread multiple antibiotic resistances (*50*). DoS 1.0 encodes the oriT and 300–base pair inc (iteron) sequence from RP4, a chloramphenicol (Cm) resistance gene, and a ColE1 origin of replication. To evaluate our DoS design, we introduced Top10 *E. coli* cells containing DoS 1.0 into a resident population of MG1655 *E. coli* cells containing RP4, passaged the mixture daily regularly, and measured their long-term population and plasmid dynamics.

As in our modeling analysis, DoS was initially beneficial in the culture via positive selection [Cm (2 μg/ml)], after which it became burdensome by removing the selection. We chose MG1655 and Top10 as our resident and treatment populations, respectively, due to their inherent relative finesses: Because MG1655 grows faster than Top10, the latter will be driven to extinction when both contain the same plasmid(s).

Consistent with model predictions, DoS 1.0 eliminated RP4 (Fig. 3B). Initially, Top10 cells dominated the culture until DoS 1.0 entered sufficient MG1655 cells by retrotransfer. Once in MG1655 cells, DoS 1.0 suppressed RP4 such that MG1655 DoS 1.0 cells eventually dominated the mixture after 19 days. Notably, no subpopulation containing both RP4 and DoS 1.0 arose, meaning that the incompatibility between RP4 and DoS 1.0 was strongly maintained throughout the experiment. After RP4 elimination, DoS 1.0 was lost from MG1655 due to its own fitness cost in the absence of Cm selection, eventually generating a monoculture of empty MG1655 cells after 32 days. The time during which DoS provides a fitness benefit over the target plasmid is critical to its success. If DoS is not given enough time to sufficiently establish itself in the resident population, then the target plasmid will persist in the population as DoS will be lost before it can outcompete said target plasmid (fig. S2E).

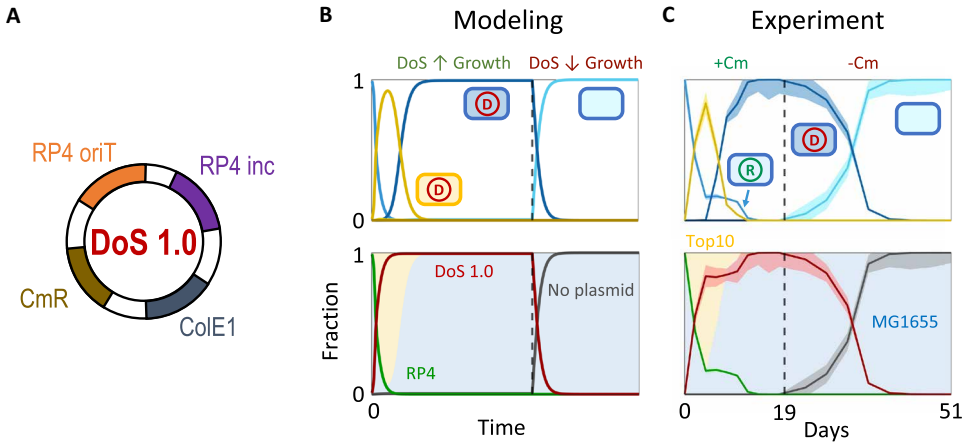

Fig. 3. Elimination of the broad-host conjugative plasmid RP4 by DoS 1.0. (**A**) DoS 1.0 design for eliminating the conjugative plasmid RP4. DoS 1.0 encodes the oriT and inc sequences from RP4, Cm resistance (CmR), and the high copy replication origin ColE1. (**B**) Modeling the elimination of the broad-host conjugative plasmid RP4 by DoS 1.0. We constructed a mathematical model of DoS 1.0 operation (see the Supplementary Materials for full details), whereby DoS inhibits the transfer of RP4 and is incompatible with RP4. During the first half of the simulation, DoS was beneficial to growth (via sublethal Cm selection), while in the second half, DoS was burdensome to growth (no Cm in the culture). (**C**) Experiments mimicking the simulation conditions. We introduced Top10 carrying DoS 1.0 into a resident MG1655 population containing RP4 and passaged the mixture daily. During the first phase, sublethal Cm (2 μg/ml) was added to the culture to provide mild fitness benefit to DoS. As a result, DoS displaced RP4 in the resident MG1655 population after 19 days. Afterward (dotted line), the culture was passaged without antibiotic, making DoS 1.0 burdensome to maintain. As a result, DoS 1.0 was lost from the community due to natural plasmid segregation error after 32 more days. These experimental results are consistent with model simulations.

Each part of our DoS design and experimental protocol was required for RP4 elimination. Moderate Cm selection was necessary for successful curing: Without selection, DoS was too burdensome to maintain before it could eliminate RP4 (fig. S3A). However, Cm alone cannot cure RP4 without a DoS plasmid (fig. S3B). Without oriT, DoS was unable to enter the resident population (fig. S3, C and E), while without inc, DoS stably coexisted with the RP4 plasmid instead of eliminating it (fig. S3, D and G). Moreover, a mismatched oriT (oriT-F from the F plasmid) or inc (F incompatibility sequence) did not confer retrotransfer nor incompatibility, as they behaved similarly to the cases of no oriT or inc (fig. S3, F to H). These results demonstrate that retrotransfer and incompatibility are sequence specific.

Elimination of RP4 using the optimized DoS 2.0

While DoS 1.0 was consistently successful across replicates, its long duration for both target curing and DoS removal limits its utility in broader applications. Because of its intrinsically low burden (because it does not express any proteins beyond Cm^R), its loss in the absence of selection was slow. To accelerate this process, we conducted a screening experiment to select optimal conditions. Specifically, we recorded the number of days to cure RP4 using DoS 1.0 while varying dilution rates (D) and Cm concentrations for fitness cost modulation (λ), which are key kinetic parameters governing plasmid persistence as shown in Eq. 1 (fig. S4A). Our result indicated that DoS-mediated plasmid curing can be accelerated with a weaker dilution rate (<10,000×) and a higher Cm concentration (≥6 μg/ml). To this end, we chose the dilution ratio of 1000× and Cm (10 μg/ml) for subsequent experiments.

On the basis of our modeling analysis, DoS can be applied to a broad design space of conjugative plasmids and parameters (fig. S4B). For example, the treatment population used to introduce DoS can have a broad range of growth rates so long as it grows only slightly slower than the resident population. If it grows too fast, then it drives the resident to extinction; if it grows too slow, then DoS does not have sufficient time to invade. Also, elimination will be effective as long as DoS can spread faster than the self-transmissible plasmid. Moreover, although our experimental design relies on DoS to be beneficial to maintain during treatment, DoS can succeed in parameter ranges where it is burdensome so long as the relative growth rate of the treatment population compared to the resident population is modulated appropriately. While DoS is most effective against burdensome conjugative plasmids, it can still eliminate conjugative plasmids that are beneficial at a slower rate. Last, while all versions of DoS impart some burden on their host cells (fig. S4C), the growth effect is not so detrimental as to render DoS ineffective.

Further, we constructed DoS 2.0 by introducing a suicide module containing the restriction enzyme I-SceI from *Saccharomyces cerevisiae* and its cut site downstream of a dual-directional tetracycline promoter (pTet) (Fig. 4A and fig. S5). The tet repressor TetR was encoded on the other side of the pTet. Thus, DoS 2.0 can be induced into plasmid suicide and thus eliminated quickly with exogenously added anhydrotetracycline (aTc).

Under these new conditions, DoS 2.0 eliminated RP4 in 7 days and was then removed in 3 days (Fig. 4B). The higher Cm concentration gave DoS a greater fitness benefit over RP4, promoting faster RP4 elimination. Moreover, in major part due to the induced plasmid suicide, DoS itself was removed 10-fold faster than the natural plasmid loss rate due to segregation error. Thus, our modified experimental parameters and design highlight the importance of selection dynamics on DoS performance. However, a well-mixed system, which we expect maximizes competition between the different subpopulations, is not a limiting constraint. Although DoS relies on direct competition with the target plasmid, DoS 2.0 is just as effective at eliminating RP4 in non–well-mixed conditions, taking similar duration for both RP4 elimination and DoS suicide (fig. S6).

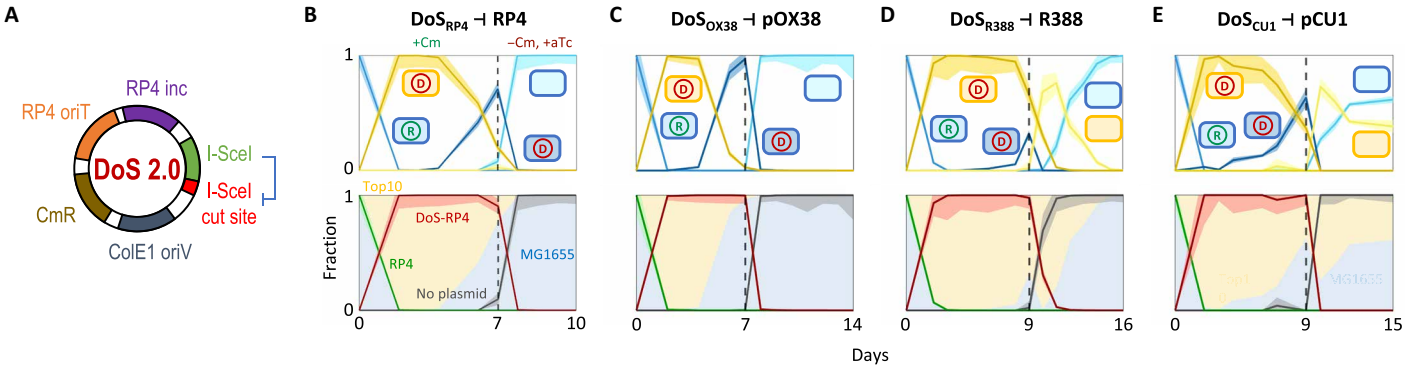

Fig. 4. DoS 2.0 accelerates the curing process. (**A**) DoS 2.0 incorporates the same core design as DoS 1.0 with the restriction enzyme I-SceI and its subsequent restriction site downstream. Induced expression of I-SceI (under control of a pTet promoter) by aTc resulted in DoS 2.0 self-cutting. The mechanism of conjugative plasmid elimination remains the same as DoS 1.0, except now that the elimination of DoS 2.0 is mediated by I-SceI expression. In subsequent panels, DoS2.0 plasmids are named according to their target plasmids. (**B**) Elimination of RP4 by DoS-RP4. We modified the daily dilution rate and Cm concentration for fastest conjugative plasmid elimination according to our screening experiment results (fig. S4). With these optimizations, DoS-RP4 eliminated RP4 by day 7 (table S4). Then, through induced suicide [at aTc (100 ng/ml)], it was eliminated in another 3 days. (**C** to **E**) Broad applicability of DoS 2.0. We designed three additional DoS 2.0 plasmids to target three different conjugative plasmids: pOX38 (MOBF and IncF) (C), R388 (MOBF and IncW) (D), and pCU1 (MOBF and IncN) (E). Each variant encodes the oriT and inc sequences from its corresponding conjugative plasmid. All three variants eliminated their targets, although the time for conjugative plasmid elimination and DoS suicide phases varied between plasmids.

Elimination of diverse conjugative plasmids with tailored DoS plasmids

To evaluate the generalizability of DoS, we engineered DoS variants to target other plasmids covering broader plasmid diversity. Specifically, we chose the conjugative plasmids pOX38, R388, and pCU1 belonging to the IncF, IncW, and IncN incompatibility groups, respectively, as additional candidate targets. In addition to belonging to different incompatibility groups, these three plasmids also express genetically distinct conjugation machinery compared to RP4 while having distinct oriTs (*51*). Thus, each DoS variant contains a unique oriT and inc sequence(s) matching their respective target. We evaluated these variants using our optimized experimental parameters for DoS 2.0.

Each DoS variant fully eliminated its target conjugative plasmid within 9 days and was itself eliminated within 7 days after inducing DoS suicide (Fig. 4). Similar to RP4 curing, R388 and pCU1 curing were highly reproducible across biological replicates. However, pOX38 elimination exhibited a greater degree of variability, likely due to the reduced efficiency in retrotransfer (table S3). These differences in curing resilience between plasmids suggest that both MOB and Inc group play a key role in plasmid maintenance and may reflect their relative evolutionary fitness. For example, F-type plasmids (MOBF and IncF), from which pOX38 is derived, are among the most predominant type found in antibiotic-resistant pathogenic Enterobacteriaceae (*16*, *52*). Their broad prevalence may be attributed to their robustness against competition by naturally occurring "DoS-like" plasmids that exploit others' conjugative machineries to spread. As a result, while these plasmids might avoid the fitness cost of transfer and thus outcompete their conjugative counterparts, F-type plasmids may be more robust against these "transfer cheaters" according to our results.

These plasmids each displayed unique selection dynamics as well. In the case of R388, the resident MG1655 population did not dominate the system until after the conjugative plasmid was eliminated (Fig. 5, middle). For pCU1, Top10 was never fully removed in any individual replicate. On average, however, MG1655 still ended up as the majority population (Fig. 5, right). This behavior is likely

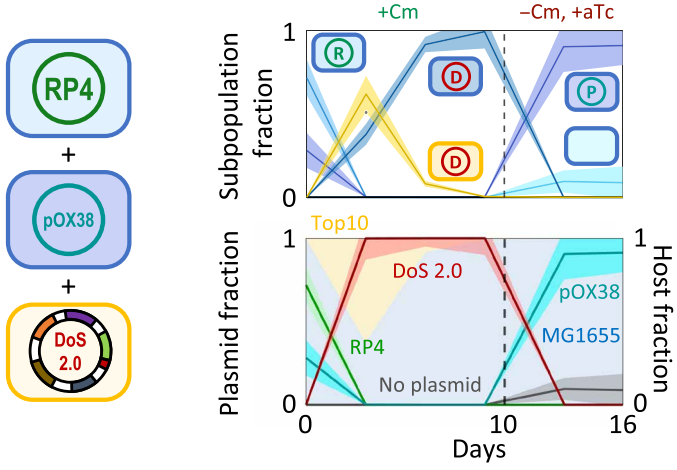

Fig. 5. Selective targeting by DoS. We introduced DoS 2.0 targeting RP4 into a resident MG1655 community containing both RP4 and pOX38 and cocultured the three populations using the same culturing conditions as our DoS 2.0 experiment. During the treatment phase, DoS 2.0 eliminated RP4 in a similar manner to previous experiments, while pOX38 persisted in the community at a low fraction. During the recovery phase, DoS 2.0 was eliminated from the system via induced plasmid suicide, and pOX38 recovered and spread to a majority of MG1655 cells in the system.

related to pCU1's relatively high fitness cost compared to other conjugative plasmids (*19*). Our results would suggest that pCU1 impairs cell growth even after it is eliminated, although the exact mechanism requires further investigation. In addition, the speed of plasmid elimination seemed to correlate with conjugation efficiency, as R388 and pCU1, which took longer to cure than RP4, have lower transfer efficiencies than RP4 (*19*).

Selective curing of target plasmid in mixed communities of multiple plasmids

We then evaluated the ability of DoS to selectively eliminate a target conjugative plasmid without disrupting other plasmids. We introduced

Top10 cells containing DoS 2.0 (DoS-RP4) into a coculture of two MG1655 populations, each containing either RP4 (DoS' target) or pOX38 (nontarget). We passaged this mixed culture using the same dilution factor (1000×) and Cm concentration (10 μg/ml) as DoS 2.0. Under these conditions, DoS-RP4 selectively eliminated RP4 but not pOX38 (Fig. 5). During the treatment phase, the fraction of cells containing pOX38 fell to as low as ~1/10,000 as Cm selection reduced the relative fitness of both pOX38 and RP4. Unlike RP4, however, pOX38 was never lost from the system. Once Cm selection was halted in the recovery phase, pOX38 rebound to more than 90% of MG1655 cells by the end of the experiment.

We designed our DoS-RP4 to be nonmobilizable by any plasmid other than RP4. However, we observed a minor subpopulation of cells containing both pOX38 and DoS-RP4 (<1 cell per 500,000 cells) in the mixture of RP4, pOX38, and DoS-RP4. While the presence of this population initially suggests pOX38-mediated DoS-RP4 retrotransfer, this subpopulation instead was likely generated in a different manner. For example, pOX38 could have transferred into MG1655 cells containing DoS-RP4 via normal conjugation, or DoS-RP4 could have retrotransferred into MG1655 cells containing both conjugative plasmids. Then, DoS-RP4 could have eliminate RP4 from the cell via transfer competition and incompatibility. In either case, DoS-RP4 was unable to transfer into cells without RP4, and pOX38 was not eliminated by DoS-RP4. Thus, DoS-mediated curing was specific for its intended plasmid target.

Modular and versatile DoS design for RP4 elimination

To demonstrate the modularity and generalizability of our DoS design, we constructed DoS 3.0 by replacing the inc sequence from DoS 2.0 (DoS-RP4) with CRISPR-Cas9 targeting the β-lactamase on RP4. We switched I-SceI promoter to pBAD to encode CRISPR-Cas9 under pTet (Fig. 6A). Although we removed the inc sequence, DoS 3.0 can still compete against RP4 for transfer machinery.

To evaluate DoS 3.0 efficacy, we used similar experimental protocols to DoS 1.0 and 2.0 experiments but with a four-phase treatment program (Fig. 6B). During the first phase (24 hours), we allowed the cells retrotransfer in the absence of Cm selection. We supplemented LB media with 0.4% glucose to suppress basal I-SceI–mediated self-cutting. On the following day, the cells were diluted 1000× in LB supplemented with Cm (25 μg/ml) and 0.4% glucose. The previous screening data showed that a strong Cm selection could accelerate the curing process (fig. S4A). Because we allowed retrotransfer in the absence of Cm during the first 24 hours, the delayed strong Cm treatment maximized DoS selection without killing off the sensitive resident population.

When the culture reached 100% MG1655 cells (after 3 days of Cm treatment), we induced CRISPR-Cas9–mediated cutting with aTc (100 ng/ml). After another 3 days, we confirmed the elimination of RP4 (day 7). DoS was subsequently removed via I-SceI–mediated self-cutting by adding 0.2% arabinose and, on day 20, we were left with the plasmid-free resident community. During the DoS self-curing phase, we did not add aTc and did not observe RP4 relapse, which indicates the complete elimination of RP4 from the community. DoS 3.0 successfully eliminated the RP4 plasmid at a similar efficacy to earlier DoS versions, demonstrating the flexibility of our DoS design.

DISCUSSION

Gene drives, wherein certain genes propagate at a biased rate to promote their spread throughout a population, have been proposed as effective tools for disseminating or removing traits from target

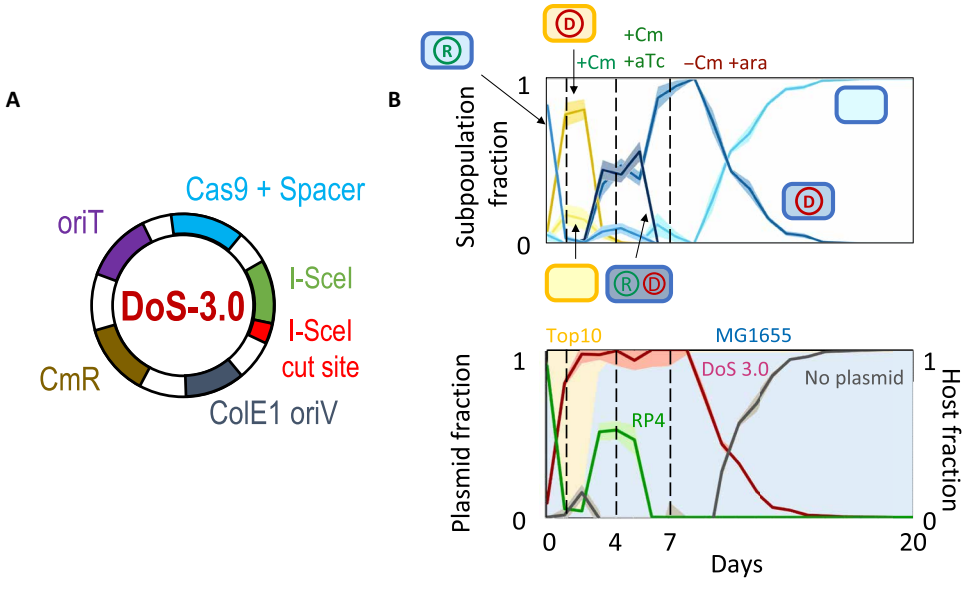

Fig. 6. DoS 3.0 demonstrates the modularity and versatility of the DoS design. (**A**) DoS 3.0 was constructed by replacing the inc sequence on DoS 2.0 with inducible CRISPR-Cas9 targeting the β-lactamase. (**B**) Targeted elimination of RP4. In the first phase (first 24 hours), the cell mixture was cultured in plain LB to allow DoS establishment. In the second phase, Cm was administered to select for DoS-carrying cells. During this phase, the MG1655 carrying DoS had fitness advantage over Top10 carrying DoS. Once we completely removed TOP10, we induced CRISPR-Cas9–mediated RP4 cutting (+aTc) in the third phase. When RP4 was eliminated from the community, DoS suicide was induced in the last phase.

populations (*44*, *45*). Most gene drives have so far been developed in insects to combat the spread of insect-borne pathogens (*53*).

Our work demonstrates the exploitation of conjugation as a vehicle for gene drives to achieve targeted elimination of self-transmissible plasmids, a task that has been challenging in efficacy and specificity (*20*). Chemical suppression often lacks specificity, indiscriminately affecting HGT in a community. Similarly, current plasmid-based strategies have relied on delivery via regular conjugation. As a result, these strategies risk unintended spread of synthetic plasmids into other hosts, thereby disrupting nontarget plasmids.

By design, DoS cannot enter cells without the corresponding conjugative target (fig. S3). This property allows DoS to selectively suppress or eliminate its target plasmid with minimal disruption to other plasmids (Fig. 5). The induced suicide mechanism adds another layer of containment. If DoS is observed to disrupt the community stability too much, then induced suicide allows for its rapid removal, thus minimizing long-term disruption.

Determining the effect of pathogen hosts on plasmid maintenance is essential to understanding and controlling the spread of antibiotic resistance. However, ascertaining this relationship even in vitro is nontrivial. While separating a copy of the plasmid from its original host is simple, isolating the host without plasmid has been challenging (*40*, *54*, *55*). To this end, DoS provides a platform for selective removal of target plasmids, creating plasmid-free host populations. This capability is critical for disentangling plasmid-host interactions and understanding their respective contributions to phenotypic traits.

A potential limitation of DoS for natural contexts is the need for sufficient sequence information to create an optimal matching DoS plasmid (*47*). However, many conjugative plasmids share mobilization type and exhibit high sequence similarity, potentially enabling the development of tailored libraries of DoS variants (*2*, *17*, *56*, *57*). To eliminate poorly characterized plasmids, a library of DoS variants could be evaluated in parallel. Another challenge of natural plasmids is the presence of toxin-antitoxin systems, which promote plasmid maintenance by killing cells that lose the plasmid and are commonly found on plasmids of clinical interest (*58*). To overcome this challenge, DoS can be engineered to express antitoxins to remove the host's reliance on the target plasmid for survival.

Also, retrotransfer, a cornerstone of DoS, exhibits variability across different plasmid systems and generally occurs at slower rates compared to direct conjugation. Natural barriers to HGT that reduce conjugation rates could also hinder retrotransfer and limit the initial establishment of DoS. A DoS system with insufficient retrotransfer rates could lose plasmid elimination capability (fig. S4). For example, slow retrotransfer may reduce the efficacy of DoS in eliminating pOX38, as demonstrated by the limited presence of cells containing both pOX38 and DoS (table S3). In addition, DoS 1.0 and 2.0 rely on plasmid incompatibility for exclusion and elimination, requiring a deep understanding of the target plasmid's mechanism of replication. DoS 3.0 alleviates this challenge by leveraging the power and specificity of Cas9, but the expression of CRISPR machinery could create additional burden to the host. These tradeoffs underscore the need for modeling-guided optimization to tune circuit-specific and host-specific parameters (fig. S4).

In conclusion, the DoS system represents a gene drive–inspired platform for targeted control of mobilizable genes in microbial communities. By combining modularity, precision, and containment, the system can be engineered to promote or suppress traits that are not associated with plasmid persistence per se. For instance, the persistence of ARG carried by a mobilizable (but not self-transmissible) plasmid can be considered an example of gene drive maintaining a costly trait. Conversely, a recent study demonstrates the use of a gene drive to inactivate β-lactam resistance (*59*). As the DoS platform evolves, it holds promise for broad applications in plasmid biology, medicine, and microbiome engineering.

METHODS

Strains, growth conditions, and plasmid construction

For a complete list of strains and plasmids used in this study, see table S1. To create *E. coli* strain MG1655 containing different conjugative plasmids, empty MG1655 cells were mixed at 1:1 ratio with the original bacterial hosts containing their respective conjugative plasmids and grown for at least 1 hour at 37°C with shaking (250 rpm) in LB broth (Genesee Scientific, SKU 11-120) containing no antibiotics. Cultures were then plated on LB agar (Genesee Scientific, SKU 11-122) plates containing Xgal (100 μg/ml), 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and appropriate antibiotics to select for blue MG1655 transconjugants. In the cases where the original host was also blue, the conjugative plasmid was transferred first to Top10 then MG1655.

To construct DoS 1.0, we introduced the RP4 (Genbank BN000925) oriT (coordinates 51090-51463) and inc (coordinates 12364 to 12666) sequences into R+ from Lopatkin *et al.* (*60*) via polymerase chain reaction and Gibson assembly, respectively. Unless otherwise stated, all DoS plasmids were transformed into Top10 cells. DoS 2.0 and other variants (DoS-pCU1, DoS-R388, and DoS pOX38) were ordered via gene synthesis from GenScript and were identical except for their respective oriT and inc sequences: R388 (Genbank, NC_028464) oriT (coordinates 16018 to 16470) and inc (coordinates 27176 to 27351), pCU1 oriT (Genbank, M81668) and inc (Genbank, M182622), and pOX38 (modified from Genbank, NC002483) oriT (coordinates 66118 to 66407) and inc (coordinates 48254 to 48349, 49204 to 49451, 35982 to 36642, 37816 to 37878, and 3747 to 3839). DoS 3.0 was constructed from DoS 2.0 by introducing Cas9, gRNA cassette, and AraC + pBAD promoter downstream of the pTet promoter via Gibson assembly.

For all experiments, single clones were grown separately overnight at 37°C for 16 hours with shaking (250 rpm) in LB broth containing appropriate antibiotics [Cm (35 μg/ml), carbenicillin (100 μg/ml; Carb), kanamycin (50 μg/ml; Kan), or tetracycline (10 μg/ml; Tet)]. Unless otherwise stated, all experiments were performed using M9 medium [M9CA medium broth powder from Amresco, lot #2055C146 containing casamino acid (2 mg/ml) supplemented with thiamine (0.1 mg/ml), 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.4% (w/v) glucose]. Solid-phase experiments and all colony-forming unit (CFU) quantification were performed using LB agar plates.

Long-term selection and conjugation dynamics of DoS in liquid culture

Sixteen-hour overnight cultures (3ml of LB media with appropriate selecting agents, density ~ 1×10^9 CFU/ml) were resuspended in M9 medium. For all experiments containing one conjugative plasmid, resident population (MG1655 conjugative plasmid) were diluted to an initial starting density of ~10^7 CFU/ml, while treatment population (Top10 DoS) were diluted to an initial starting density of ~10^4 CFU/ml to achieve a starting ratio of 1000:1. This cell mixture was distributed among three to six well replicates in 96-well plate to a

final volume of 200 µl per well. Plates were covered with an AeraSeal film sealant (Sigma-Aldrich, SKU A9224) followed by a Breath-Easy sealing membrane (Sigma-Aldrich, SKU Z380059). Plates were shaken at 250 rpm for 23.5 hours at 37°C. Plates were then passaged daily at a 10,000× (DoS 1.0) or 1000× (DoS 2.0 and 3.0) dilution into a new 96-well plate containing freshly mixed media (~30 min). For DoS 1.0 and 2.0 experiments, medium was supplemented with Cm (2 or 10 µg/ml, respectively) during the initial treatment phase. In the recovery phase, medium was supplemented with aTc (100 ng/ml) to induce I-SceI. Periodically, an additional 20 µl of the culture was added to a separate plate, serially diluted 10-fold, and plated out on selective plates for subpopulation quantification. A plasmid was considered fully eliminated once the CFU/ml fell under our daily dilution factor. At that point, it was statistically unlikely for at least one cell to be passed into the next day's culture. Experiment duration varied depending on the DoS plasmid being tested. For DoS 3.0 experiments, we used 17-ml round bottom culture tubes (Genesee Scientific, SKU 21-129) to daily passage the cultures (3 ml per tube) and used Cm (25 µg/ml), aTc (100 ng/ml), and 0.2% arabinose when appropriate.

The same passaging protocol was used for the DoS selective experiment with multiple conjugative plasmids. However, for the initial setup, both MG1655 RP4 and MG1655 pOX38 are added to the initial mixed culture at ~10^7 CFU/ml, while Top10 DoS 2.0 is added at ~10^4 CFU/ml. Thus, the initial starting density of this experiment is twofold higher than any other experiment.

Quantification of retrotransfer, plasmid competition, and incompatibility

To quantify retrotransfer, MG1655 containing the F plasmid and Top10 containing G^+ from Lopatkin *et al.* (*60*) were grown for 16 hours (3 ml of LB media with appropriate selecting antibiotics) and resuspended in fresh LB containing no antibiotics. Both cultures were mixed at 1:1 ratio (final density ~ 10^9 CFU/ml) and incubated at 25°C for 1 hour before plated out on LB agar plates containing Xgal (100 µg/ml), 1 mM IPTG, Tet (10 µg/ml), and Kan (50 µg/ml) for CFU counting. Blue colonies corresponded to retrotransfer transconjugants, while white colonies corresponded to regular conjugation transconjugants. The mixture was also plated out on agar plates supplemented with Tet + Xgal + IPTG and Kan + Xgal + IPTG separately to measure the parent population densities. Transfer efficiencies were calculated using the equation from Lopatkin *et al.* (*60*).

To quantify plasmid competition, three different MG1655 cells were grown—one containing F plasmid, one containing F and G^+ plasmids, and one containing F and G^- plasmid from Lopatkin *et al.* (*60*)—along with Top10 for 16 hours (3 ml of LB media with appropriate selecting antibiotics) and resuspended in fresh LB containing no antibiotics. Each MG1655 strain was mixed at 1:1 ratio (final density ~ 10^9 CFU/ml) with Top10 and incubated at 25°C for 1 hour. The mixtures were plated on LB agar plates containing streptomycin (Str; 50 µg/ml) + Tet (10 µg/ml) to select for transconjugants containing the F plasmid. The mixture was also plated out in the same manner as the retrotransfer experiments to measure parent population densities. The mixture of MG1655 F^+ G^+ and Top10 was further plated on LB agar plates containing Str (50 µg/ml) + Kan (50 µg/ml) and Str (50 µg/ml) + Kan (50 µg/ml) + Tet (10 µg/ml) to measure all possible transconjugants in the experiment.

To quantify incompatibility, DoS 1.0 plasmid was transformed into competent MG1655 cells containing RP4 and plated on LB agar containing Kan (50 µg/ml) + Cm (35 µg/ml) for transformants containing both plasmids. Separately, DoS 1.0Δinc plasmid was also transformed into MG1655 $RP4^+$ and plated on the same plates for transformants containing both plasmids.

Supplementary Materials

This PDF file includes:

Supplementary Information
Tables S1 to S4
Figs. S1 to S6

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