

Midbiotics: conjugative plasmids for genetic engineering of natural gut flora

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ABSTRACT

The possibility to modify gut bacterial flora has become an important goal, and various approaches are used to achieve desirable communities. However, the genetic engineering of existing microbes in the gut, which are already compatible with the rest of the community and host immune system, has not received much attention. Here, we discuss and experimentally evaluate the possibility to use modified and mobilizable CRISPR-Cas9-encoding plasmid as a tool to induce changes in bacterial communities. This plasmid system (briefly midbiotic) is delivered from bacterial vector into target bacteria via conjugation. Compared to, for example, bacteriophage-based applications, the benefits of conjugative plasmids include their independence of any particular receptor(s) on host bacteria and their relative immunity to bacterial defense mechanisms (such as restriction-modification systems) due to the synthesis of the complementary strand with host-specific epigenetic modifications. We show that conjugative plasmid in association with a mobilizable antibiotic resistance gene targeting CRISPR-plasmid efficiently causes ESBL-positive transconjugants to lose their resistance, and multiple gene types can be targeted simultaneously by introducing several CRISPR RNA encoding segments into the transferred plasmids. In the rare cases where the midbiotic plasmids failed to resensitize bacteria to antibiotics, the CRISPR spacer(s) and their adjacent repeats or larger regions were found to be lost. Results also revealed potential caveats in the design of conjugative engineering systems as well as workarounds to minimize these risks.

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Introduction

The possibility to engineer gut microbiome has become a notable avenue of research. Restoration of microbial balance in the gut can provide a cure to a multitude of complex diseases. Nonetheless, stable installation of foreign beneficial microbes in the gut is problematic. Studies have shown that dietary supplement bacteria (probiotics) disappear from the community soon after their ingestion ceases.^{1,2} This has led many teams to compile bacterial cocktails that would establish a more stable population within the gut.³ Also, the near-complete replacement of gut flora has been used to revert dysbiosis. This so-called bacterial transplantation is an effective approach to cure especially recurrent diarrhea caused by *Clostridium difficile*,^{4–6} but could also be used to improve various other conditions.⁷ The composition of gut flora is also sensitive to diet, and, for example,

increase of fiber can result in notable shifts in the community composition.⁸ In some circumstances, however, the possibility to modify the genomes of existing bacteria in the gut could provide an alternative to remodel the system.

So far, the genetic engineering of bacterial communities *in situ* has mainly focused on bacteriophage-based applications.^{9,10} Conjugative plasmids offer an alternative route with differing engineering qualities. They are circular antagonistic genetic elements that can mediate their own transfer from one bacterium to another. In addition, these self-transmissible plasmids can co-transfer non-conjugative plasmids with appropriate *oriT* site.¹¹ The relaxosome of the conjugative plasmid recognizes the similar *oriT* site in non-conjugative plasmid and mobilizes it through conjugation.¹² The exact conjugation mechanisms vary between plasmids, but they all form a channel between the cells through which the plasmid is usually transported as

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a single-stranded DNA molecule to the recipient bacterium. Plasmids can be readily modified with various molecular biology methods, thus providing a relatively simple platform for carrying out *in situ* genetic engineering of bacterial cells. Given that the diversity of gut microbiota varies even between genetically identical twins,¹³ the attempts to colonize maladapted (engineered) bacteria within an already established community can be a challenging if not an impossible task. In this respect, the introduction of an engineered mobile element into the existing community instead of relying on the establishment of an entire bacterium provides a potential workaround for deploying desired functionalities within the system. Given the established concepts of probiotics (health-promoting bacteria) and prebiotics (nutrients that promote the growth of beneficial bacteria), "midbiotics" (plasmid-probiotics in a sense) provide yet an alternative form of biotic substances that can be used to acquire beneficial changes in the gut flora. Naturally, such plasmids have only limited use, albeit, in certain instances, they may be even a preferable choice over probiotics such as when only particular genes need to be removed from the community.

Plasmids are divided into incompatibility groups (Inc) based on their potential to stably coexist in a bacterial cell. In other words, two plasmids that share the same Inc-group cannot be maintained in a single cell indefinitely. Conjugative plasmids also often encode entry-exclusion mechanisms that prevent related plasmids from entering the cell. Due to these natural features, the plasmids used for engineering should be uncommon in the targeted flora. Naturally, determining the existence of certain plasmid types routinely from a heterogenous community is a laborious task. Yet, certain antibiotic resistance-conferring plasmids of Enterobacteriaceae, for instance, are relatively rare in patients. Indeed, in a metastudy, Carattoli reviewed the prevalence of different resistance plasmid families in Extended Spectrum Beta-Lactamase (ESBL) strains.¹⁴ Among the rarest were IncP-type plasmids. Despite this, conjugative IncP-plasmids are well-studied, they have a robust conjugation machinery and a broad host range. As such, they provide an example of potential backbones that could be utilized for engineering purposes.

In bacteriophage-based applications, the genetic material within the phage is replaced, and as it infects a cell after the attachment to a specific receptor on the host cell surface, it delivers the genomic cargo into the bacterial host.^{9,10} Phage-based tools have acquired notable attention and are currently under development toward drugs. The advantage of phages is that they have a narrow host range, and thus, they target specifically only the desired fraction of the bacterial community. Yet, bacteria rapidly become resistant to phages, and phages cannot be easily used to exert activity against even all variants of certain species. In this regard, conjugative plasmids provide qualities that could be useful for alternative and more generally applicable engineering purposes. As noted above, conjugative plasmids are usually delivered as a single DNA strand to the recipient cell. The complementing strand is synthesized in the recipient bacterium and thus it contains all the host-specific modifications in the nucleic acids.¹⁵ This way the host does not recognize the incoming plasmid as foreign genetic material, which, in turn, allows the plasmid to establish itself into a natural community without prior knowledge of the features of bacteria therein. Additionally, unlike phages, conjugative plasmids are not dependent on specific receptors on host cells as plasmids require only cell-cell contact. And once plasmid gets into natural bacteria, it can further disseminate itself into the next host. The transfer rate from one bacterium to another is, of course, slower and less-precise than phage-mediated delivery of DNA. This sets certain boundaries for the utilization of plasmids. Nevertheless, conjugative plasmids can provide a broad host range for introducing genetic material into the gut flora.

The advent of CRISPR-Cas9 editing has provoked numerous studies where specific target sequences within various host organisms are modified,¹⁶ even enabling strain-specific elimination of bacteria from heterologous communities.¹⁰ Introduction of CRISPR-Cas9 editing components into conjugative plasmids provides a potential mean to remove unwanted genes such as those conferring antibiotic resistance from diverse bacterial systems. ESBL carriage refers to non-symptomatic colonization of the gut by bacteria

which are resistant to a wide range of different beta-lactam antibiotics.^{17,18} This is of major concern, as beta-lactams are the most commonly used class of antimicrobials, owing to their broad spectrum and minimal side effects. They are abundantly administered to treat and prevent bacterial infections during various medical procedures. Over the past few decades, ESBL carriage has become increasingly more common among long-term hospitalized patients as well as in the community.¹⁹ ESBL carriage serves as a reservoir of resistance genes and significantly increases the risk of clinical infections.²⁰⁻²² As such, we here set to evaluate the possibility to use conjugatively transferred plasmids to induce the loss of ESBL genes (located either in plasmids or in the chromosome) from a bacterial community.

Results and discussion

We constructed a midbiotic system consisting of a conjugative IncP plasmid RP4²³ and a mobilizable pCas9 plasmid containing *Streptococcus pyogenes*-derived CRISPR/Cas9²⁴ that targets conserved sites in two different beta-lactamase genes via plasmid-encoded CRISPR RNA (crRNA). Part of RP4 origin-of-transfer (*oriT*) site was cloned into pCas9 plasmid in order to make it horizontally transferrable by the RP4-encoded relaxosome complex. Further, 543 bp region, including the target site of the CRISPR/Cas9 system, was deleted from the beta-lactamase gene *blaTEM-2* of RP4 to prevent the system from self-targeting. From now on, the RP4^{*blaTEM-2Δ172-714*} plasmid is referred to as delivery plasmid and the modified pCas9 as pCRISPR plasmid, crRNA/multi-crRNA referring to spacer(s) targeting the beta-lactamase gene(s).

A donor bacterium (*Escherichia coli* HMS174) harboring midbiotic plasmids (delivery and pCRISPR-crRNA plasmids) was cocultured together with recipient *E. coli* strain (HB101) carrying a conjugative ESBL-plasmid pEC15 that encodes *blaTEM-52b* target gene.²⁵ The transfer of these plasmids to ESBL-positive bacteria and the subsequent coexpression of endonuclease Cas9 and crRNA should induce the loss of resistance by guiding the Cas9 complex to ESBL gene and create a double-stranded nick within the target site (Figure 1a). Nicking

linearizes the plasmid and prevents its replication. Indeed, after 24 h, only approximately 1:10 000 transconjugants retained the resistance in comparison to a control treatment lacking the crRNA (Figure 1b). To rule out the possibility that this might result from the unequal conjugation rates between pCRISPR-crRNA and pCRISPR-control plasmid, both were conjugated independently to a recipient HB101 lacking the target plasmid (Figure 2a). Altogether, this suggests that in principle the dispersal of such midbiotics in the bacterial flora would relatively efficiently resensitize the ESBL-harbouring recipients to beta-lactams. Yet, while this approach appears promising in accelerating ESBL loss, there are still potential obstacles to be taken into account when specific genes are targeted with Cas9. These obstacles would be relevant to most *in situ* applications that seek to delete specific functions from the community (and sometimes in applications that attempt to introduce them); hence, we decided to take a closer look at the caveats and the realistic prospects of midbiotic engineering.

In many cases, there can be multiple variants of the genes that encode undesired phenotypes. For example, there is no single guiding crRNA sequence that would direct Cas9 to all possible ESBL variants. However, all classes of beta-lactamase genes share sequences that are usually conserved within the class (Figure 3). Targeting these sites would provide a broad activity against the class regardless of specific knowledge of the variant in any particular case. When various crRNAs are combined into the same plasmid similarly to spacer arrays of natural CRISPR systems, several targets could be abolished with a single pCRISPR plasmid. We tested this by adding two crRNA coding sites separated by a repeat into the pCRISPR plasmid. This pCRISPR-multi-crRNA plasmid was then transferred into two bacterial strains each harboring a different type of an ESBL gene (*blaTEM-52b* and *blaCTX-M-14*). The plasmid exhibited the activity against both ESBL types, leading to a nearly 500-fold decrease in cell density in treated bacteria compared to control, suggesting that combination of crRNA sites could indeed be utilized to achieve broad activity (Figure 1c).

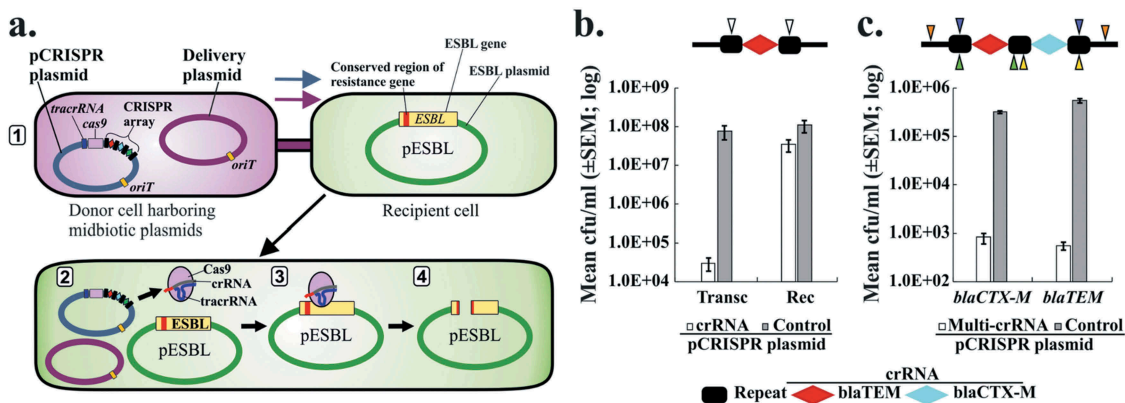


Figure 1. Midbiotic plasmids against ESBL-positive bacteria. (a). 1) Donor cell delivers midbiotic plasmid system (pCRISPR plasmid and delivery plasmid both of which share the same origin of transfer site, *oriT*) via conjugation into recipient target cell that harbors ESBL plasmid (pESBL). 2) After a successful delivery of the plasmids, the new host cell starts producing the components required for CRISPR/Cas9-activity (endonuclease Cas9, crRNA and tracrRNA, encoded by pCRISPR). 3) Cas9 cleaves the ESBL gene based on crRNA that is programmed to target a conserved region within the gene. 4) This results in degradation of ESBL plasmid. (b). Among the transconjugants (Transc) receiving the pCRISPR-crRNA, a difference of nearly four orders of magnitude in ESBL-positive bacteria was observed. Rec denotes the total number of recipient bacteria. Out of the survivors, the deletion of the spacer in the CRISPR locus of pCRISPR-crRNA plasmid (white arrowheads) explained the loss of activity. The mean cell density (cfu/ml) is calculated from a total of six replicates from two different experiments ($n = 6$). The black bars indicate the standard error of mean (SEM). (c). Transformation of pCRISPR-multi-crRNA into target bacteria caused the cell density of HB101(pEC13) (*blaCTX-M*) to decline by two orders of magnitude and HB101(pEC15) (*blaTEM*) by three orders of magnitude. The deletion of either one (green and yellow arrowheads) or both of the spacers (blue arrowheads) resulted in the survival of transformants. Larger deletion in CRISPR locus was most likely the reason for the unsuccessful amplification of some escape mutants, as the primer binding sites were located in the deletion (orange arrowheads). Some survivors contained the intact spacers, suggesting that Cas9 gene or the target sequence might carry mutations. The mean cell density is calculated from three replicates ($n = 3$). The black bars indicate the standard error of mean (SEM).

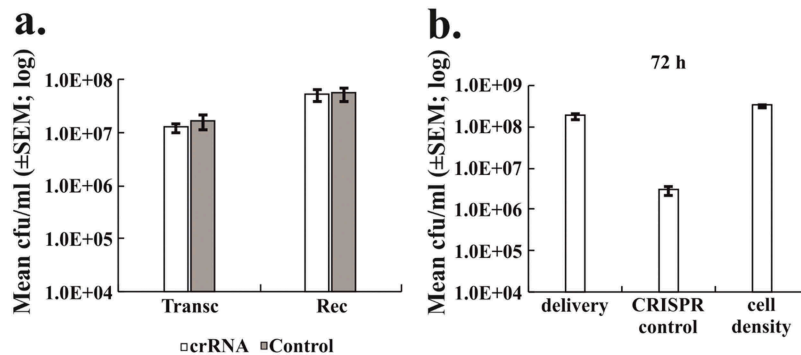


Figure 2. Conjugation of midbiotic system. (a). The conjugation rates of pCRISPR-crRNA and pCRISPR-control plasmid are equal, determined by measuring the mobilization frequencies after 24 h conjugation. Therefore, the presence of spacers does not itself hinder the mobilization rate of the pCRISPR plasmid. The mean cell density was calculated from three replicates ($n = 3$). The black bars indicate the standard error of mean (SEM). (b). After 72 h, the delivery plasmid was observed to conjugate independently without the mobilizable pCRISPR-control plasmid, as the density of cells containing pCRISPR-control plasmid was two orders of magnitude lower than cells with delivery plasmid. Also, when 90 colonies from delivery plasmid selection plate were streaked on plate selecting for pCRISPR-control plasmid, none of them was observed to contain the pCRISPR-control plasmid. On the contrary, all the 90 colonies with pCRISPR-control plasmid also contained the delivery plasmid. The mean cell density was calculated from three replicates ($n = 3$). The black bars indicate the standard error of mean (SEM).

We further studied the individual bacteria that appeared to have avoided the anti-ESBL effect despite having been introduced with the midbiotic system. In other words, some bacteria which had received the pCRISPR-crRNA/multicrRNA

plasmid still retained the resistance to beta-lactams (Figure 1b-c). Sequencing of CRISPR spacer locus of these plasmids (8 escape colonies/replicate/experiment) revealed that the observed tolerance to the midbiotic treatment after 24

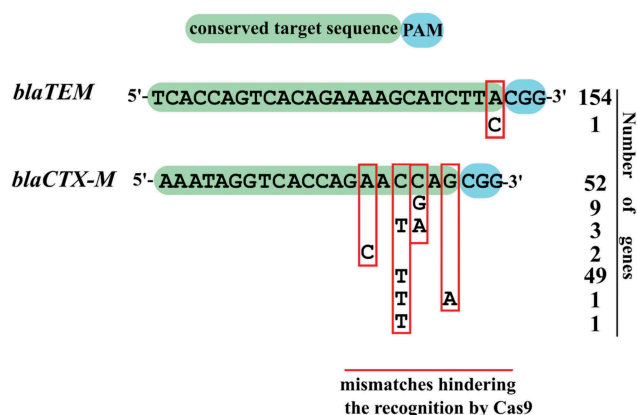


Figure 3. Designing of guide RNA for conserved sites in beta-lactamase genes. A potential obstacle in gene deletion by midbiotic application is the diversity of the genes that need to be targeted. By combining multiple spacers into a single plasmid and selecting conserved sites within target genes, it is possible to increase the coverage. As the beta-lactamase genes belonging to the same class share conserved sites in nucleotide level, these sites can be used to design spacers for CRISPR/Cas9 system in order to target several resistance gene variants with a single spacer. Majority of the genes in class *blaTEM* (154) contain the conserved target sequence (green bar). The target sequence selected for the class of *blaCTX-M* genes (green bar) is not as highly conserved as in the *blaTEM* class, only 52 genes contain the exact sequence. Only one gene in *blaTEM* class has a point mutation (red rectangle) in the first nucleotide next to PAM (blue), whereas genes of *blaCTX-M* class have more variation in these nucleotides. These mismatches in the first seven nucleotides next to PAM might hinder the recognition of the target by Cas9²⁴ and thus the efficiency of the spacer.

h was mainly due to loss of the beta-lactamase-targeting spacer(s) and their adjacent repeat (see the graphic illustration of spacer deletions in Figure 1b-c). In some cases, however, we did not succeed to amplify the crRNA coding region at all, suggesting that a larger deletion might have occurred within the region. On the other hand, sometimes the crRNA site was unaltered, indicating potential changes elsewhere, such as mutations in tracrRNA, Cas9 or PAM sequence.¹⁰ Nevertheless, the emergence of mutants may be difficult to prevent, but in principle several copies of the crRNA regions, for example, could be included in the plasmid, hence allowing it to retain its activity even if one of the sites is lost.

Another potential concern derives from the separation of the midbiotic into two or more plasmids. It is possible that the delivery plasmid mobilizing the pCRISPR plasmid goes ‘rogue’ and

spreads alone in the community, thus attenuating the desired effect. We investigated this possibility by cultivating midbiotic bacteria (harboring pCRISPR control plasmid) together with ESBL-positive strain for 72 h during which the culture was refreshed once a day. All of the studied clones (90 colonies) with the mobilizable pCRISPR plasmid also contained the delivery plasmid. In contrast to this, all bacteria harboring the delivery plasmid had lost the pCRISPR plasmid (Figure 2b). This indicates that the mobilizable pCRISPR plasmid is not always delivered together with the conjugative plasmid, thus requiring countermeasures to minimize the probability of such events. There are at least two possibilities to achieve this: either the pCRISPR plasmid and delivery plasmid could be combined into a single plasmid or the toxin–antitoxin system could be separated so that the pCRISPR plasmid carries the gene for antitoxin and the delivery plasmid encodes the toxin. In the latter case, the dispersal of the delivery plasmid alone would lead to cytotoxic response and death of the recipient cell.

Conjugative plasmids are agents in natural microbial communities, albeit not an inherent part of any particular strain or species. In the recent bloom in microbiota research, they have so far been a seldom utilized tool for inducing genetic changes in existing bacterial communities. Plasmids could be used both to introduce desired genes or remove existing ones. Whether they have applications beyond laboratories is yet to be demonstrated, and the possible spread of malevolent traits via horizontal gene transfer may be a deterrent against using plasmids for engineering purposes. Indeed, the obvious risk in introducing a conjugative plasmid into a bacterial community is that the element may pick up an unwanted gene and disperse it further into other hosts. Before introduction into clinical applications, the resistance genes of delivery plasmid should be deleted to prevent dispersal of new resistance genes. However, it must be noted that the communities aimed to be engineered will nevertheless harbor various types of mobile genetic elements, and, thus, if there is notable selection within the population for acquiring a particular gene, it is likely to disperse anyway. In any event, if the plasmid used for midbiotic-like engineering must be removed

from the community, the plasmid-dependent bacteriophages could provide a way to induce direct selection against the plasmid. However, while *in vitro* experiments suggest that this would result in plasmid loss,^{26,27} it is yet to be determined whether this occurs also *in vivo*.

Overall, the fraction of the community that can be engineered with conjugative plasmids is equal to the fraction of the flora that receives them. Studies suggest that plasmid dynamics and persistence in a community is a complicated matter where trophic levels and various characteristics of plasmids, their hosts and the environment play an indispensable role.^{28,29} Without extensive selection for the midbiotic plasmid, it is unlikely to spread to even all possible hosts. Therefore, as in the case of ESBL carriage, the midbiotic system could be considered as a booster which accelerates ESBL curing rather than an outright treatment. Sometimes, however, even a small fraction of engineered bacteria may be enough, such as in the case of making the midbiotics encode externally secreted bacteriocins against unwanted bacterial species. Yet, the overall improved understanding of the survival conditions of plasmids can help us find ways both to get rid of conjugative plasmids and, if necessary, to facilitate their dispersal. Nevertheless, while caution is necessary, the ability to introduce or remove genes within natural bacterial communities is a real possibility that could be considered as a potential tool for genetic engineering of existing bacterial systems or, for example, modification of gut microbe transplants prior to their implementation.

Materials and methods

Plasmids, bacterial strains and culture conditions

In this study, the so-called midbiotic system consists of the conjugative RP4^{*bla*TEM-2Δ172-714} plasmid (delivery plasmid) and mobilizable pCas9 plasmid (pCRISPR plasmid, a gift from Luciano Marraffini, Addgene plasmid # 42876) encoding the *S. pyogenes* CRISPR/Cas9 system²⁴ with crRNA(s) targeting conservative sites of different beta-lactamase resistance genes in ESBL plasmids (Table 1). pCas9 was made mobilizable by cloning RP4 *oriT* site^{12,30} (50980–51793 bps, amplified with primers

RP4oriT-F and RP4oriT-R, Supplementary Table 1) into pCas9 digested with Sall (ThermoScientific; Waltham, Massachusetts, United States) into region spanning 7377–7486 bps. The phosphorylated ESBL-gene-targeting crRNA oligonucleotides (2 μM each) were first annealed together in 50 μl reaction with 1x of T4 ligase buffer (New England Biolabs; Ipswich, Massachusetts, United States) and 0.05 M NaCl by heating first at 95°C for 5 min and then cooling it down gradually (1°C/35 sec) to 20°C. Then, crRNA insert was ligated into BsaI (ThermoScientific) digested pCas9 plasmid by T4 ligase in T4 ligase buffer (New England Biolabs; Ipswich, Massachusetts, United States). In order to prepare the pCRISPR-multi-crRNA plasmid, the multi-crRNA insert was multiplied by PCR from a synthetic plasmid (GenScript; Nanjing, China) with primers spacer-multi-crRNA-F and spacer-multi-crRNA-R (Supplementary Table 1). PCR product was purified according to instructions of Qiagen PCR purification kit before being ligated (similarly as above) into the plasmid. The pCRISPR-control plasmid was otherwise similar but lacked the crRNA (Table 1). If not mentioned otherwise, all the PCRs were done according to instructions of Phusion Hot Start II High-Fidelity PCR mastermix (ThermoScientific), except for an extended initial denaturation (from 5 min to 7 min 30 s), using C1000 Thermal Cycler (Bio-Rad Laboratories Inc.; Hercules, California, United States). Both ESBL plasmids, pEC13 and pEC15, in recipient strains, originate from nosocomial isolates,²⁵ and the conserved sites of their respective beta-lactamase genes (Table 1) were selected as targets for the CRISPR/Cas9 system of pCRISPR plasmids.

All the bacterial cultures were grown at +37°C in Luria Bertani Lennox-broth (LB)³¹ and, as necessary, plated on LB-agar (1%) plates. When appropriate, the following antibiotic concentrations were used: rifampicin (50 μg/ml), streptomycin (25 μg/ml), kanamycin (25 μg/ml), chloramphenicol (25 μg/ml) and ampicillin (150 μg/ml). Liquid cultures were shaken at 220 rpm.

Partial deletion of *bla*TEM-2 in RP4

The part of *bla*TEM-2 gene (172–714 bp) containing the crRNA target site was deleted from RP4 to prevent the midbiotic system from self-targeting the

delivery plasmid. The deletion was first created in the RP4 *bla*TEM-2 gene cloned in pET24 plasmid by polymerase chain reaction (PCR) using 0.2 μ M of primers deletion-F and deletion-R (Supplementary Table 1) with elongation time (5 min 15 s) adjusted so that the plasmid without the unwanted sequence was amplified with extension rate 0.5 kb/min.³² As the deletion was confirmed with agarose gel electrophoresis, pET24-*bla*TEM-2 Δ 172-714 PCR-product was recombined back to circular plasmid by Red/ET recombination in recombineering-proficient *E. coli* strain GB08-red^{Rif^R} (Gene Bridges; Heidelberg, Germany) according to the manufacturer's instructions, with the exception of using 10 ng of DNA for transformation. Briefly, the truncated *bla*TEM-2 Δ 172-714 gene was amplified by PCR with primers del*bla*TEM2-F and del*bla*TEM2-R (Supplementary Table 1). Template of the PCR was removed by DpnI treatment (ThermoScientific), and *bla*TEM-2 Δ 172-714 PCR product was purified from the gel according to instructions of Qiagen's Gel purification kit (Hilden, Germany). RP4 plasmid containing the deletion was obtained by recombining *bla*TEM-2 Δ 172-714 PCR product into RP4 in GB08-red^{Rif^R} strain. This RP4^{*bla*TEM-2 Δ 172-714} was then conjugated from GB08-red^{Rif^R} to BL21 Gold(pCRISPR-crRNA) with a donor to recipient ratio of 2:1 in 3 ml and then cultivated at +37°C, 220 rpm, for 16 h. Transconjugant bacteria were selected on LB agar plates with chloramphenicol-kanamycin selection. The colonies were picked and transferred into LB medium with same antibiotic selection as above and cultivated overnight without shaking. The colonies containing the deletion (RP4^{*bla*TEM-2 Δ 172-714}) were identified by negative selection by plating on LB agar plates with and without ampicillin selection. This RP4^{*bla*TEM-2 Δ 172-714} plasmid was conjugated to HMS174 by incubating donor and recipient in ratio 1:1 in 5 ml cultivation for 2 h at +37°C, 220 rpm. Transconjugants were selected by plating on LB agar plates with rifampicin-kanamycin selection.

Midbiotic conjugation

The efficiency of the midbiotic plasmids in inducing ESBL loss from the transconjugants was investigated with the following setup. Before the experiments, donors HMS174(RP4^{*bla*TEM-2 Δ 172-714})(pCRISPR-

crRNA) and control HMS174(RP4^{*bla*TEM-2 Δ 172-714}) (pCRISPR-control) and the recipient strain HB101 (pEC15) were cultivated overnight with antibiotic selection. To mix the equal number of cells for conjugation experiments, the cell density (colony forming units; cfu/ml) of all the cultures were determined either by plating or by measuring the optical density at OD₅₉₅ (Multiskan FC, ThermoScientific; Waltham, Massachusetts, United States). The experiments were performed in two sets with slightly different concentrations of bacteria (see below), and each conjugation setup was replicated six times in total. The recipient strain ($\sim 3.0 \times 10^5$ cfu/ml in the first experiment and $\sim 7.0 \times 10^5$ cfu/ml in the second) was mixed with the donor ($\sim 1.0 \times 10^5$ cfu/ml in the first and $\sim 2.0 \times 10^6$ cfu/ml in the second) and cultivated 24 h in 5 ml LB without antibiotics. After the experiment, plating was used to measure the cell density of transconjugants (streptomycin-ampicillin-chloramphenicol), recipients (streptomycin-ampicillin), donors (rifampicin-kanamycin-chloramphenicol in the first experiment and rifampicin-chloramphenicol in the second) and of the community (no antibiotics). Donor and recipient strains were distinguished by differing resistance for rifampicin and streptomycin, respectively. The presence of RP4^{*bla*TEM-2 Δ 172-714} was controlled by kanamycin and the pCRISPR by chloramphenicol selection. In order to observe the potential for different midbiotic plasmids to disperse separately, RP4^{*bla*TEM-2 Δ 172-714} and pCRISPR-control plasmid were cultivated for 72 h during which the culture was renewed daily by transferring 50 μ l of culture into fresh 5 ml LB medium. After 72 h, the density of bacteria carrying either RP4^{*bla*TEM-2 Δ 172-714} or pCRISPR-control plasmid and the total cell density was determined by plating with appropriate antibiotics. From the total of 90 colonies (30 colonies/replicate), we determined whether RP4^{*bla*TEM-2 Δ 172-714} or pCRISPR-control plasmid containing colonies also accommodated the other midbiotic plasmid.

The conjugation efficiencies of the pCRISPR-crRNA and pCRISPR-control plasmid were determined to be equal by conjugating the plasmids into HB101 without target ESBL plasmid. The donors were mixed with the recipient in ratio ~ 1 – 1.65 :100 in 5 ml LB and cultivated overnight in the absence of antibiotics. The cell density (cfu/ml) of transconjugants with pCRISPR plasmids (streptomycin-chloramphenicol), recipients (streptomycin) and

donors (rifampicin-chloramphenicol) as well as the total cell density (no antibiotics) were determined by plating.

pCRISPR plasmid with multiple ESBL targets

To test the activity of multi-crRNA, electroporation was used to transform the pCRISPR-multi-crRNA plasmid to ESBL-plasmid harboring strains. Electroporation was performed according to the protocol in manual of recombineering-proficient *E. coli* strain GB08-red (Gene Bridges; Heidelberg, Germany). The optimal density was measured with UV-mini-1240 UV-VIS Spectrophotometer (Shimadzu; Kyoto, Japan) using 1.5 ml semimicro cuvettes (Brand; Germany). A 6.5×10^5 cfu/ml of HB101(pEC13) and 4.0×10^6 cfu/ml of HB101 (pEC15) strain were used for each plasmid transformation. Every plasmid transformation (pCRISPR-multi-crRNA and pCRISPR-control) was conducted in triplicates by using 20 ng of plasmid DNA. One negative control, transformed with 1 μ l of water, per bacterial strain was done. The DNA concentration of plasmids was measured according to the protocol of QubitTM dsDNA HS Assay Kit (Invitrogen; Carlsbad, California, United States) by using Qubit[®] 2.0 Fluorometer (Invitrogen; Carlsbad, California, United States). Transformants were plated on LB agar plates without antibiotics and with the combination of chloramphenicol and ampicillin. Negative control was plated with and without chloramphenicol selection. The activity of different crRNA sites was determined by counting the colonies on each plate.

Target site selection

The conserved regions of the *bla*TEM and *bla*CTX-M beta-lactamase genes for crRNA targets were determined by aligning sequence samples of these classes (obtained from the ResFinder 3.0 database)³³ separately with MUSCLE algorithm with default settings by Geneious 8.1.9 (Biomatters Ltd; Auckland, New Zealand). The most conserved sites with the appropriate PAM sequence were selected for the crRNA spacer sequences.

Escape mutants

The survived escape mutant colonies from the conjugation and transformation were re-isolated by plating them on chloramphenicol-ampicillin. Eight colonies/replicate, except two colonies per control replicate (altogether 32 colonies/experiment), were grown in LB media with chloramphenicol-ampicillin at +37°C without shaking. CRISPR locus of pCRISPR-crRNA, pCRISPR-multi-crRNA and pCRISPR-control plasmid were amplified with PCR using one bacterial colony as a template with primers spacerseqF and spacerseqR (Supplementary Table 1). PCR product was purified from primers and nucleotides with 0.4 U of Exonuclease I (20 U/ μ l, ThermoScientific; Waltham, Massachusetts, United States) and 0.4 U of FastAP Thermosensitive Alkaline phosphatase (1U/ μ l, ThermoScientific; Waltham, Massachusetts, United States). These reactions were incubated at +37°C for 20 min and then at +80°C for 15 min in order to inactivate the enzymes. Sequencing-PCR of ExoSAP-treated DNA was performed with BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems; Foster City, California, United States) according to the manufacturer's protocol. The sequencing reactions were purified using the protocol of BigDye Terminator v3.1 Cycle Sequencing kit except centrifugation was performed with 1109 \times g and 100 \times g and, before adding formamide, samples were dried at +37°C for 10 min. Sequencing was carried out with 3130xl Genetic Analyzer (Applied Biosystems/HITACHI; Foster City, California, United States). The basecalling was performed with Sequencing Analysis Software v6.0 (Applied Biosystems; Foster City, California, United States), and the sequences were analyzed for deletions or mutations in CRISPR locus by mapping them against the original sequence by using Geneious 8.1.9 (Biomatters Ltd; Auckland, New Zealand).

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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