

# Temperate and lytic bacteriophages programmed to sensitize and kill antibiotic-resistant bacteria

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Edited by Jennifer A. Doudna, University of California, Berkeley, CA, and approved April 28, 2015 (received for review January 25, 2015)

**The increasing threat of pathogen resistance to antibiotics requires the development of novel antimicrobial strategies. Here we present a proof of concept for a genetic strategy that aims to sensitize bacteria to antibiotics and selectively kill antibiotic-resistant bacteria. We use temperate phages to deliver a functional clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated (Cas) system into the genome of antibiotic-resistant bacteria. The delivered CRISPR–Cas system destroys both antibiotic resistance-conferring plasmids and genetically modified lytic phages. This linkage between antibiotic sensitization and protection from lytic phages is a key feature of the strategy. It allows programming of lytic phages to kill only antibiotic-resistant bacteria while protecting antibiotic-sensitized bacteria. Phages designed according to this strategy may be used on hospital surfaces and hand sanitizers to facilitate replacement of antibiotic-resistant pathogens with sensitive ones.**

CRISPR–Cas | positive selection | lysogenization | ex vivo treatment

The clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins have evolved in prokaryotes to protect against phage attack and undesired plasmid replication by targeting foreign DNA or RNA (1–3). These systems target nucleic acids, based on short DNA sequences, called spacers, that exist between repeats in the CRISPR array. Transcribed spacers guide Cas proteins to homologous sequences within the foreign nucleic acid, called protospacers, which are subsequently cleaved. The CRISPR–Cas systems have revolutionized molecular biology by providing efficient tools to precisely engineer genomes and manipulate gene expression in various organisms (4–10). CRISPR–Cas systems have also recently been used to phenotypically correct genetic diseases in live animals (11), and their utility is being explored for various therapeutic approaches in mammals. Nevertheless, only limited studies have shown the use of CRISPR–Cas systems to target antibiotic resistance genes or a specific population of virulent bacterial strains (12–17).

Two recent elegant studies demonstrated that phage-transferable CRISPR–Cas systems are capable of specifically killing pathogens or resensitizing them to antibiotics (16, 17). These studies, and another study (13), also showed that the transferred CRISPR–Cas system is capable of eliminating specific bacterial populations. Furthermore, they demonstrated that the system might be used against pathogens to effectively treat infected animals. Consequently, it was suggested that the system could be used as a potent antimicrobial agent. Nevertheless, although the results of these studies highlight the potential of a phage-transferable CRISPR–Cas system, the concept of using the system as a direct antimicrobial is similar to conventional phage therapy, which currently faces various obstacles (18). One major obstacle is phage administration into infected tissues; this stems from the phages' immunogenicity and relative large size compared with antibiotics. One may argue that it would be more efficient to directly kill a pathogen by a lytic phage if it were possible to deliver the CRISPR–Cas–encoding cassette into this pathogen by a phage. Moreover, using the proposed systems in infected patients to resensitize pathogens to antibiotics while antibiotics counterselect for these

sensitized pathogens would most likely fail due to escape mutants that are selected by the antibiotics.

Here we demonstrate a strategy to counteract the emerging threat of antibiotic-resistant bacteria that evades the above shortcomings. Instead of directly killing the pathogens, we propose to sensitize the pathogens on surfaces or in the human skin flora while concomitantly enriching for these sensitized populations. Patients infected by these antibiotic-sensitive bacteria would thus be treatable by traditional antibiotics. In this strategy, the CRISPR–Cas system is used to destroy specific DNAs that confer antibiotic resistance and to concurrently confer a selective advantage to antibiotic-sensitive bacteria by virtue of resistance to lytic phages. The selective advantage enables to efficiently displace populations of nonsensitized bacteria by killing them with lytic phages. In contrast to conventional phage therapy, this approach does not require administration of phages into the host's tissues. In addition, it does not aim to directly kill treated bacteria but rather to sensitize them to antibiotics and to kill the nonsensitized bacteria. Therefore, there is no counterselection against the sensitization. The strategy relies on CRISPR spacers that can be rationally designed to target any DNA sequence, including those that encode resistance genes and lytic phages. It thus allows genetically linking a trait that is beneficial to the bacteria (i.e., spacers protecting from lytic phage) with a trait that reverses drug resistance (i.e., spacers targeting resistance genes). The genetic linkage enables selecting antibiotic-sensitized bacterial population by using lytic phages. The integrated construct is designed not only to actively eradicate existing resistance genes but also to eliminate horizontal transfer of these genes between bacteria. Extended

## Significance

**Antibiotic resistance of pathogens is a growing concern to human health, reviving interest in phage therapy. This therapy uses phages (natural bacterial enemies) to kill pathogens. However, it encounters many obstacles such as delivery barriers into the tissues and bacterial resistance to phages. Here, we use phages for delivering a programmable DNA nuclease, clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated (Cas), to reverse antibiotic resistance and eliminate the transfer of resistance between strains. This approach combines CRISPR–Cas delivery with lytic phage selection of antibiotic-sensitized bacteria. The strategy may reduce the prevalence of antibiotic-resistant bacteria in treated surfaces and on skin of medical personnel, as it uses phages in a unique way that overcomes many of the hurdles encountered by phage therapy.**

Author contributions: I.Y., M.M., and U.Q. designed research; I.Y. and M.M. performed research; I.Y., M.M., and U.Q. analyzed data; R.K. contributed new reagents/analytic tools; and U.Q. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1500107112/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1500107112/-DCSupplemental).

use of this technology should thus reduce drug-resistant populations of pathogens on major sources of contamination. Consequently, well-established antibiotics for which resistance currently exists could once again be effective.

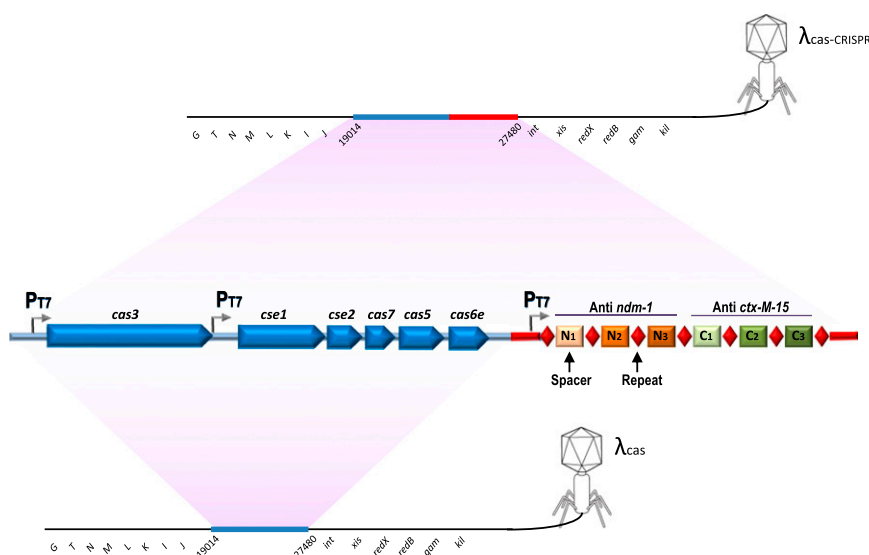
## Results

**CRISPR-Cas System Delivery by a  $\lambda$  Phage.** To sensitize bacteria carrying antibiotic resistance genes, we first constructed a transferable CRISPR-Cas system. We used PCR to amplify the CRISPR cascade genes (*cse1*, *cse2*, *cas7*, *cas5*, and *cas6e*) and *cas3* of the *Escherichia coli* type I-E CRISPR system. These genes encode proteins that are sufficient to eliminate DNA molecules encoding targeted protospacers (19). The PCR product was introduced by homologous recombination into a  $\lambda$  prophage (Fig. 1), in place of genes that were shown to be dispensable for phage growth and lysogenization (20, 21). A CRISPR array, encoding spacers that target conserved sequences of the resistance genes *ndm-1* and *ctx-M-15*, was also introduced into the same lysogen, immediately downstream of the *cas* genes (Fig. 1). These genes encode  $\beta$  lactamases that confer resistance to carbapenems, which are  $\beta$  lactam antibiotics that are often the last line of effective antibiotics against resistant pathogens (22). The prophage was then induced, and its progeny was used to lysogenize naïve *E. coli* bacteria. The engineered CRISPR-Cas system, designed to target and destroy plasmids encoding genes *ndm-1* and *ctx-M-15*, was thus made transferable to bacteria by lysogenization. Lysogenized bacteria could outcompete bacteria harboring resistance plasmids, indicating that the genetic fitness cost of the transferred prophage is smaller than that of the tested plasmids (Fig. S1).

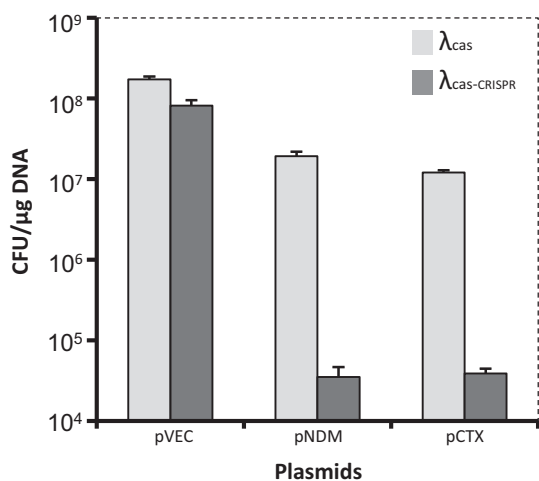
**Lysogenized Bacteria Block Transformation.** Naïve *E. coli* lysogenized with the  $\lambda$  phage encoding the CRISPR-Cas system ( $\lambda_{cas-CRISPR}$ ) or with a control phage lacking the CRISPR array ( $\lambda_{cas}$ ) were transformed with a control plasmid or plasmids encoding *ndm-1* or *ctx-M-15*, all conferring streptomycin resistance. Transformation efficiency was determined by counting colonies that acquired streptomycin resistance. Lysogens of the  $\lambda_{cas-CRISPR}$  were transformed equally well with the control plasmid compared with lysogens of the  $\lambda_{cas}$ . In contrast, these lysogens were transformed with the targeted plasmids approximately three orders of magnitude

less efficiently than lysogens of the  $\lambda_{cas}$  (Fig. 2). To demonstrate that lysogenization can also cure established resistance plasmids, we lysogenized resistant bacteria and determined plasmid loss. Plasmids were cured specifically from bacteria lysogenized with  $\lambda_{cas-CRISPR}$  but not with  $\lambda_{cas}$  (Fig. S2). Together, these results indicate that the CRISPR-Cas system can be transferred by temperate phages into bacteria to specifically prevent horizontal gene transfer of antibiotic resistance elements.

**Protection from Lytic Bacteriophages.** A desired feature of a sensitizing CRISPR-Cas system is the ability to concomitantly confer a selective advantage to the pathogens harboring it, such as resistance to lytic phages. To this end, we engineered lytic T7 phages encoding protospacers that are identical to the *ndm-1* and *ctx-M-15* protospacers targeted by the transferred CRISPR-Cas system. These engineered phages would thus be targeted concomitantly with the resistance genes. We intentionally cloned identical protospacers to ensure that the lysogens could not lose the sensitizing element without also losing phage resistance. In addition, targeting a synthetic protospacer of the phage rather than a naturally occurring sequence does not provide protection against the WT phage and thus does not interfere with the natural ecological balance. Naïve *E. coli* were lysogenized with  $\lambda_{cas-CRISPR}$  or  $\lambda_{cas}$ , and the bacteria were then infected with the engineered T7 phages. Bacteria lysogenized with  $\lambda_{cas-CRISPR}$  and bacteria lysogenized with the control  $\lambda_{cas}$  phage were both sensitive to infection by a control T7-gp8 phage, as expected. In contrast,  $\lambda_{cas-CRISPR}$  lysogens resisted growth of the T7 phages encoding either two protospacers from *ndm-1* (T7-N<sub>1</sub>-N<sub>2</sub>) or two protospacers from *ctx-M-15* (T7-C<sub>2</sub>C<sub>1</sub>) or one from each gene (T7-N<sub>1</sub>C<sub>1</sub> or T7-C<sub>2</sub>N<sub>2</sub>) by at least four orders of magnitude compared with  $\lambda_{cas}$  lysogens (Fig. 3). These results indicate that the transferred CRISPR-Cas system protects bacteria from a specifically programmed T7 bacteriophage, thus linking pathogen sensitization to antibiotics with resistance to lytic phage. Moreover, the system confers resistance only to phages encoding artificial matching protospacers, demonstrating that the system does not interfere with natural ecological interactions.



**Fig. 1.** Schematics of the lysogenizing phages. The CRISPR-associated genes *cas3*, *cse1*, *cse2*, *cas7*, *cas5*, and *cas6e* (blue) were inserted in place of nucleotides at position 19,014–27,480 of the  $\lambda$  chromosome (National Center for Biotechnology Information Reference Sequence: NC\_001416.1) yielding the control lysogenizing phage  $\lambda_{cas}$  (Lower). The  $\lambda_{cas-CRISPR}$  phage (Upper) encodes, in addition to the *cas* genes, a CRISPR array with spacers targeting the genes *ndm-1* (N<sub>1</sub>, N<sub>2</sub>, N<sub>3</sub>) and *ctx-M-15* (C<sub>1</sub>, C<sub>2</sub>, C<sub>3</sub>).  $P_{T7}$ , T7 promoter.



**Fig. 2.** Lysoigenization effect on transformation of antibiotic resistance plasmids. *E. coli* K-12 were lysogenized with  $\lambda_{cas}$  (light gray bars) or  $\lambda_{cas-CRISPR}$  (dark gray bars). These lysogens were transformed with a control (pVEC), *ndm-1* (pNDM), or *ctx-M-15* (pCTX) encoding plasmids and plated on agar plates supplemented with streptomycin. Bars represent average and SD of the number of CFUs per microgram plasmid counted after plating serial dilutions of the cultures in three independent experiments.

**Lytic Phage Selection of Sensitized Bacteria.** The transferred CRISPR-Cas system prevented plasmid transformation and protected the lysogenized bacteria from lytic phages. These results indicate that lysoigenization can be used to sensitize antibiotic-resistant bacteria and that the population of sensitized bacteria may be enriched by lytic phages. To simulate treatments that could be applied on hospital surfaces or skin flora, we cultured bacteria harboring control-, *ctx-M-15*-, or *ndm-1*-encoding plasmids. We then treated these cultures by adding either the lysogenizing phage  $\lambda_{cas-CRISPR}$  or  $\lambda_{cas}$ . The cultures were then overlaid on agar plates containing the T7-N<sub>1</sub>C<sub>1</sub> lytic phage, against which the lysogenized bacteria have CRISPR-Cas-mediated protection. Surviving colonies were counted after overnight incubation (Fig. 4A). In all cultures, more than 20-fold more colonies treated with the targeting  $\lambda_{cas-CRISPR}$  phage were resistant to the engineered T7-N<sub>1</sub>C<sub>1</sub> phage compared with those treated with the control  $\lambda_{cas}$  phage (Fig. 4B). Phage-resistant colonies treated with either  $\lambda_{cas-CRISPR}$  or  $\lambda_{cas}$  were inoculated on plates having or lacking streptomycin to test for loss of the antibiotic resistance conferring plasmid. As expected, cultures harboring the nontargeted plasmid (pVEC) remained streptomycin resistant in both types of lysogenizations. However, all of the bacteria lysogenized with  $\lambda_{cas-CRISPR}$  and harboring targeted plasmids (pNDM or pCTX) concomitantly became sensitive to streptomycin, whereas all of the bacteria treated with  $\lambda_{cas}$  maintained this resistance (Fig. 4C).

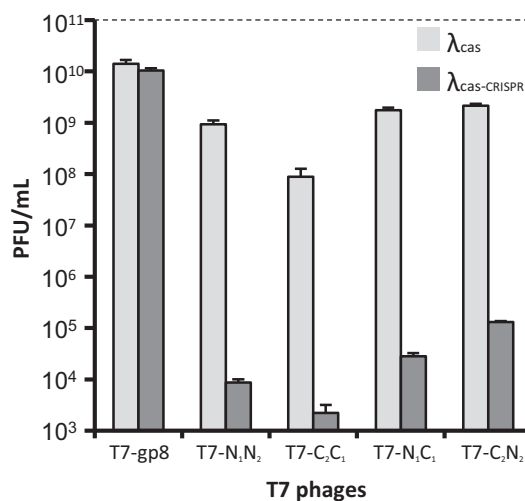
Finally, to demonstrate that multiple resistances in the same bacterium can also be eliminated, we repeated the above described procedure using bacteria harboring two different antibiotic resistance plasmids (pNDM\*+pCTX). As expected, in this case also, bacterial cultures treated with the  $\lambda_{cas-CRISPR}$  resisted the lytic phages, as they carry antiphage spacers (Fig. 4B). Bacteria surviving the lytic phage infection and treated with  $\lambda_{cas-CRISPR}$  were cured from both resistance plasmids, whereas survivors treated with  $\lambda_{cas}$  maintained the resistance plasmids (Fig. 4C). Altogether, these experiments provide a proof of principle that an engineered temperate phage delivering the CRISPR-Cas system can be used along with an engineered lytic phage to facilitate the simultaneous loss of multiple resistance determinants, reduce their horizontal transfer, and enrich for bacterial populations that exhibit both features.

## Discussion

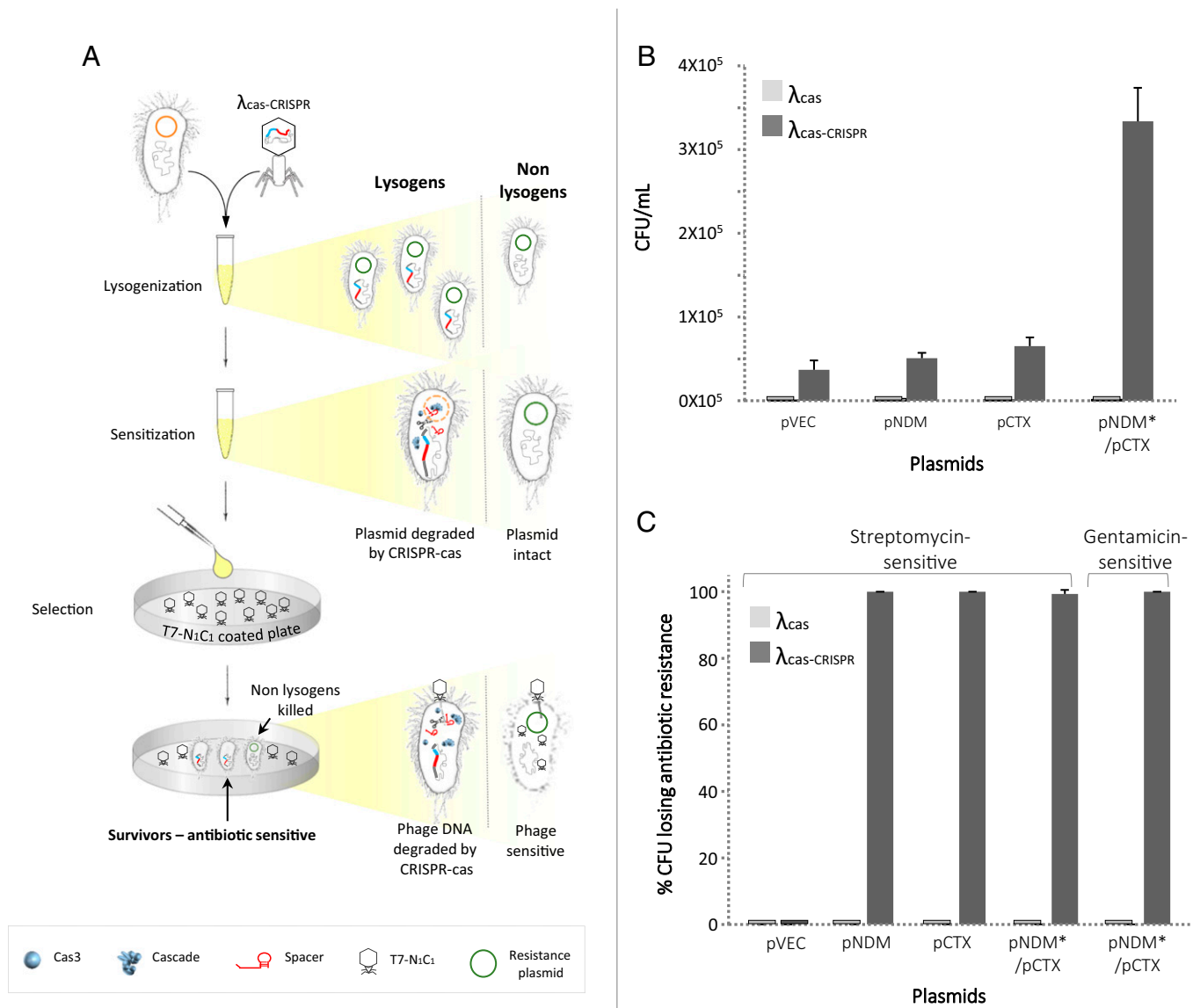
The strategy presented here is a step toward decreasing the threat of emerging drug-resistant pathogens, against which new weapons are desired. It demonstrates that bacteria can be sensitized to approved and useful antibiotics following delivery of a rationally designed CRISPR-Cas system. This strategy may be applied for treating hospital surfaces and hand sanitizers for targeting the skin flora of medical personnel. In contrast to antibiotics and disinfectants that select for resistant pathogens, the proposed treatment enriches and selects for sensitive pathogens. Moreover, the system enriches for pathogens that cannot receive or transfer resistance determinants horizontally and may thus further reduce the spread of antibiotic resistance. The enriched sensitive population could prevent newly introduced resistant pathogens from becoming established by overtaking their ecological niche.

Sensitizing bacteria to various antibiotic resistance genes may easily be accomplished by the CRISPR-Cas system, as it can be programmed to eliminate any gene of interest. In fact, dozens of spacers could be engineered in a single array due to their short sequence, thus re-enabling the use of a vast number of antibiotics against which resistance has developed. In theory, a mixture of a few antibiotics should suffice to treat bacterial infections with minimal probability of simultaneous spontaneous resistance formation by the pathogen. Evolution of resistance to lytic phages can likewise be minimized by using a mixture of lytic phages. The CRISPR array can be designed with multiple spacers to simultaneously protect against several lytic phages that will be used for selection, thus reducing the occurrence of nonsensitized bacterial mutants that escaped these lytic phages. Last, multiple spacers could be used to target each resistance gene and each lytic phage, thus reducing the probability of CRISPR-escape mutants by these targets.

With few adaptations, the use of the strategy may be further broadened. For example, the system may be designed to specifically eliminate phage lysogenizations and transductions by targeting specific phages, thus reducing a significant source of virulence genes transfer (23). Another alteration of this strategy



**Fig. 3.** Lysoigenization effect on protection against lytic phages. *E. coli* K-12 were lysogenized with  $\lambda_{cas}$  (light gray bars) or  $\lambda_{cas-CRISPR}$  (dark gray bars). These lysogens were infected with a control T7-gp8 lacking targeted protospacers, or with T7 phages encoding two protospacers from *ndm-1* (T7-N<sub>1</sub>N<sub>2</sub>) or two protospacers from *ctx-M-15* (T7-C<sub>2</sub>C<sub>1</sub>) or one spacer from each gene (T7-N<sub>1</sub>C<sub>1</sub> and T7-C<sub>2</sub>N<sub>2</sub>). Bars represent average and SD of the number of plaque-forming units (PFUs) per milliliter counted after plating serial dilutions of the phages in three independent experiments.



**Fig. 4.** Enrichment of antibiotic-sensitized bacteria by lytic phages. (A) Schematics of the procedure to enrich for antibiotic-sensitized bacteria. A bacterial culture is mixed with lysogenizing phages, resulting in both lysogens and nonlysogens in the culture. Lysogens are both antibiotic sensitized and phage resistant as the CRISPR-Cas system degrades the antibiotic resistance-conferring plasmid and the lytic-phage chromosome. The treated culture is inoculated on agar-containing lytic phages that selectively kill the nonlysogens and thus enrich for antibiotic-sensitized bacteria. (B) Enrichment of phage-resistant *E. coli*. *E. coli* K-12 harboring a control (pVEC), *ndm-1* (pNDM), *ctx-M-15* (pCTX), or *ndm-1 + ctx-M-15* (pNDM\*/pCTX) encoding plasmids were treated with  $\lambda_{cas}$  (light gray bars) or  $\lambda_{cas-CRISPR}$  (dark gray bars) and plated on T7-N<sub>1</sub>C<sub>1</sub>-coated plates as shown in the scheme presented in A. Bars represent average and SD of the number of surviving CFUs per milliliter counted in three independent experiments. (C) Enrichment of antibiotic-sensitive *E. coli*. Surviving colonies (20–48 CFUs) from each culture described in B were inoculated on plates having or lacking streptomycin or gentamicin. Bars represent percentage and SD from three independent experiments of streptomycin- or gentamicin-sensitive bacteria scored as CFU unable to grow on plates with streptomycin or gentamicin out of the total number of CFU able to grow on plates lacking these antibiotics.

may deal with resistance genes encoded by chromosomal elements rather than those transferred on mobile elements. In such cases, targeting the DNA would counterselect against the transferred CRISPR-Cas as it will kill the host. However, elimination of the resistance element can still be achieved using CRISPR-Cas system that target RNA (12, 24, 25). Although targeting the RNA will eliminate the resistance conferred by the encoded gene, it will not kill the pathogen and would thus avoid counterselection against the delivering temperate phage. The flexibility and ease of genetically engineering spacers combined with the availability of various types of CRISPR-Cas systems may thus allow many useful variations of the strategy. In this respect, the fact that we used the CRISPR-Cas subtype I-E, rather than the more frequently

used subtype IIA, demonstrates that desired outcomes may be obtained with different subtypes.

The effectiveness of the CRISPR-Cas system in eliminating plasmid DNA and lytic phages is well established (1, 2, 15, 19, 26). Nevertheless, its utility in clinical settings as a tool to render pathogens sensitive to antibiotics and to reduce horizontal gene transfer of resistance determinants has only recently been demonstrated (16, 17). We believe that the strategy provided here can be applied to different pathogen–phage systems as phages can be found for most of the pathogens, and a compatible CRISPR-Cas system should work in many pathogens. However, implementation of this strategy must overcome several barriers. One characteristic of most phage infections is



their narrow host range. Most phages infect only one species of bacteria and some are limited to certain strains within a species (27). This feature can be advantageous, on the one hand, as it allows targeting specific pathogens without disrupting other bacterial populations (27). On the other hand, this narrow host range may constitute a significant shortcoming, as uninfected pathogens would remain untreated. One way to expand the host range of phages is to select for phage mutants that infect new hosts. In many cases, the selected mutants that adapted to new hosts also maintain their infectivity to the original host, and thus the range is extended (28). An additional way to partially overcome this issue is to use a mixture of phages to target an extended range of the same bacterial species. Successful examples of such approach is the use of mixtures of phages against *Listeria monocytogenes*, *E. coli*, and *Salmonella enterica* in the respective products ListShield, EcoShield, and SalmoFresh, all approved by the US Food and Drug Administration (FDA) (29). These phage mixtures were shown to effectively eradicate targeted pathogens on food and surfaces [[www.intralytix.com/Intral\\_products.htm](http://www.intralytix.com/Intral_products.htm)] (30)]. Moreover, all of these products were given the “ready-to-eat” approval from the US FDA, demonstrating the safety of spreading phages on consumed products and on surfaces.

Spraying surfaces may be an effective method to treat them, as demonstrated by the US FDA-approved phage sprays mentioned above. The spray should contain both the temperate CRISPR-Cas-encoding phages and the lytic phages. Ideally, this surface treatment regime will not replace disinfection and cleansing procedures but rather serve as an additional step. While disinfection reduces the overall amount of bacteria, the proposed treatment changes the ratio of sensitized vs. resistant bacteria. In addition, some pathogens often escape cleansing and disinfection procedures (31); thus, treating these pathogens would gradually increase the relative abundance of antibiotic-sensitized pathogens. Delivery may also be carried out in the form of liquid added to hand sanitizers in hospitals. These delivery methods avoid the use of phages inside the patient’s tissues, thus overcoming toxicity issues and other drawbacks of phage therapy, such as phage neutralization by the spleen and the immune system (27). Optimization of the delivery methods should be specifically determined for every phage type used.

Hospital surfaces contain complex mixtures of bacterial populations: some of them are resistant pathogens belonging to different species, whereas most are harmless bacteria or antibiotic-sensitive pathogens. Importantly, only a few resistant pathogens are responsible for the lion’s share of nosocomial infections in both the developed and developing countries. *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species, generally known as the “ESKAPE” pathogens, are those pathogens that cause the majority of nosocomial infections and effectively escape the effects of antibiotics (32, 33). It follows that efficient and effective treatment against these six species, or even only a few of them, could significantly reduce fatalities and financial burden caused by resistant pathogen infections. This point highlights the potential applicability of the proposed system, as only few such systems would need to be adjusted against selected pathogens to significantly reduce the antibiotic resistance problem. Due to the high specificity of phages to their hosts, in combination with the specificity of the CRISPR-Cas construct that targets resistance-conferring plasmids, selected mixtures of phages can discriminate between antibiotic-resistant and -sensitive pathogens within the same strain in complex bacterial populations. This feature may enable the targeting of the pathogens posing the most relevant threats, with minimal disturbance of the ecological niche of other microbes.

We believe that specific clinical adaptations following further research would produce a system that specifically and efficiently

reduces the occurrence of infection from antibiotic-resistant pathogens. Broad use of this strategy, in contrast to antibiotics and phage therapy, will potentially change the nature of nosocomial infections by making the bacteria more susceptible rather than more resistant to antibiotics.

## Materials and Methods

Reagents, bacterial and phage strains, plasmids, plasmid construction, and genetic engineering procedures are all described in *SI Materials and Methods* and *Tables S1* and *S2*.

**Transformation Efficiency Assays.** Overnight cultures of *E. coli* IYB5670 and IYB5671 were diluted 1:50 and aerated at 32 °C in 10 mL Luria-Bertani (LB) medium supplemented with 25 µg/mL kanamycin and 10 µg/mL chloramphenicol. When the culture reached an OD<sub>600</sub> of 0.2, 0.2% L-arabinose was added, and the cultures were incubated at 32 °C until an OD<sub>600</sub> of 0.5–0.6 was reached. Bacteria were then centrifuged at 4,600 × g at 4 °C, the supernatant was disposed, and the bacteria were resuspended in 1 mL ice-cold double-distilled water (ddH<sub>2</sub>O) and transferred to a 1.5-mL tube. The cells were spun down for 1 min at 13,000 × g at 4 °C. After an additional washing step, the cells were suspended in 250 µL ice-cold ddH<sub>2</sub>O. Bacterial cells (50 µL) were then mixed in an ice-cold 0.2-mm electroporation cuvette (Bio-Rad) with 12 ng pVEC, pNDM, or pCTX plasmids. The mixture was pulsed in a Bio-Rad micropulser at 200 Ω, 25 µF, and 1.8 kV. Immediately after the pulse, 0.1 mL of yeast extract-tryptone (2YT) broth containing 0.2% L-arabinose was added, and the cells were aerated for 1 h at 32 °C. Various dilutions of the reaction were plated on LB agar plates supplemented with 50 µg/mL streptomycin and 0.2% L-arabinose. Plates were incubated overnight at 32 °C. Colonies emerging on the selection plates were counted, and the colony-forming unit (CFU) number per microgram plasmid was calculated accordingly.

**Assays of Lytic Phage Growth Efficiency.** Overnight cultures of *E. coli* IYB5670 and IYB5671 were diluted 1:50 and aerated at 32 °C in 10 mL LB medium supplemented with 25 µg/mL kanamycin. When the culture reached an OD<sub>600</sub> of 0.2, 0.2% L-arabinose was added, and the cultures were incubated at 32 °C until an OD<sub>600</sub> of 0.5–0.6 was reached. The bacteria were harvested by centrifugation and concentrated to an OD<sub>600</sub> of ~3. One milliliter from the concentrated cultures IYB5670 and IYB5671 was mixed with 10 mL of soft agar supplemented with 0.2% L-arabinose and spread onto LB agar plates supplemented with 25 µg of kanamycin and 0.2% L-arabinose. After the agar solidified, the plates were incubated at 32 °C for 40 min. Fifteen microliters of phage dilutions was plated onto the soft agar, allowed to dry, and then incubated at 32 °C for 15 h. Plaque-forming units were counted on several dilutions, and their number per milliliter was calculated accordingly.

**Lysogenization and Resistance Loss Determination.** Overnight culture of IYB5666 harboring pNDM, pCTX, pNDM\*+pCTX, or control plasmid (pVEC) was diluted 1:50 in LB medium supplemented with 50 µg/mL streptomycin, 10 µg/mL gentamicin (only for the culture harboring pNDM\*), 10 mM MgSO<sub>4</sub>, and 0.2% maltose. Cultures were grown to an OD<sub>600</sub> of 0.5 and then centrifuged at 13,000 × g for 1 min. The supernatant was discarded, and the pellet was resuspended in LB medium supplemented with 10 mM MgSO<sub>4</sub> and 0.2% maltose. Ten microliters of the treated culture was mixed with 10 µL of phage λ<sub>cas</sub> or λ<sub>cas</sub>-CRISPR at a multiplicity of infection of ~10 in a 1.5-mL tube and incubated at room temperature for 30 min. Sixty microliters of LB medium supplemented with 0.2% L-arabinose was then added, and the cultures were aerated at 32 °C for an additional 2.5 h. Cultures were then diluted 1:10, and 84 µL was spread onto LB plates containing 5 µg/mL of tetracycline and 0.2% L-arabinose overlaid by 3 mL of 0.8% LB agar containing 5.5 × 10<sup>5</sup> T7-C1N<sub>1</sub> phage. The plates were incubated 36 h at 32 °C, and surviving colonies were then counted. To determine plasmid loss, 20–48 of the surviving colonies were resuspended in 0.1 mL of LB, and using a plate replicator, the suspension was plated on LB agar plates supplemented with 5 µg/mL tetracycline and 0.2% L-arabinose either with or without 50 µg/mL streptomycin or 10 µg/mL gentamicin. CFUs sensitive to streptomycin or gentamicin were determined as those unable to grow on medium having these antibiotics out of the total number of CFUs.

**ACKNOWLEDGMENTS.** We thank Eyal Molchansky for graphic assistance. This work was funded by European Research Council Grant 336079, Marie Curie International Reintegration Grant GA-2010-266717, Israeli Ministry of Health Grant 9988-3, and Israel Science Foundation Grant 268/14.

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