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Exploiting CRISPR-Cas nucleases to produce sequence-specific antimicrobials

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Antibiotics target conserved bacterial cellular pathways or growth functions and therefore cannot selectively kill specific members of a complex microbial population. Here, we develop programmable, sequence-specific antimicrobials using the RNA-guided nuclease Cas9 (refs. 1,2) delivered by a bacteriophage. We show that Cas9, reprogrammed to target virulence genes, kills virulent, but not avirulent, *Staphylococcus aureus*. Reprogramming the nuclease to target antibiotic resistance genes destroys staphylococcal plasmids that harbor antibiotic resistance genes[3,4] and immunizes avirulent staphylococci to prevent the spread of plasmid-borne resistance genes. We also show that CRISPR-Cas9 antimicrobials function *in vivo* to kill *S. aureus* in a mouse skin colonization model. This technology creates opportunities to manipulate complex bacterial populations in a sequence-specific manner.

Advances in DNA sequencing technologies are revealing the diversity of complex microbial populations in different environments. They are also providing evidence for the contributions that individual species make to both populations and environments. Perhaps the most striking example of this is the human microbiome and its influence on human health[5,6]. Studying the microbiome has not only shown the importance of certain species for human health, but has also revealed the undesired side effects of traditional antimicrobials (including antibiotics) that lack killing specificity. In addition to important negative effects on human health[7], antibiotic use promotes the emergence of antibiotic resistance. There is a pressing need to develop species-specific, selective antibiotics that can be used to manipulate complex microbial consortia such as the microbiome.

Cas9 (CRISPR-associated protein 9) is a double-stranded (ds)DNA nuclease present in the type II CRISPR (clustered, regularly interspaced, short palindromic repeats) immune system of bacteria that uses a 20-nt small RNA guide (the CRISPR RNA, crRNA) to specify the site of cleavage[8]. We and others recently showed that reprogramming the Cas9 nuclease against bacterial genomic sequences is lethal, most likely due to the introduction of irreparable chromosomal lesions[9,10]. This observation led us to explore the possibility of using this nuclease as a sequence-specific antimicrobial, a tool that would allow selective killing of one or more bacterial species within a heterogeneous population. To achieve this, the type II CRISPR system has to be delivered to as many target cells as possible (if not all), without the need for selection, and in a manner that can easily be used to treat bacterial populations in their natural environment. Bacteriophages naturally package their DNA into capsids, which can then inject their content into host bacteria. Therefore we opted to deliver the *cas9* gene and its RNA guide/s sequences using a phagemid, which is a plasmid that is designed to be packaged in phage capsids[11] (**Fig. 1a**). This strategy was in part inspired by the recent discovery of a phage carrying its own CRISPR system[12].

We tested whether this technology could selectively kill antibiotic-resistant and virulent *S. aureus* strains. Staphylococci are both predominant members of the human skin microbiota[13] and one of the most common causes of nosocomial infections[14]. The recent increase in staphylococcal pathogenicity is largely due to the transfer of antibiotic resistance and virulence genes by means of conjugative plasmids and other mobile genetic elements that has led to the rise of hospital- and community-acquired methicillin- and vancomycin-resistant *S. aureus* (MRSA and VRSA, respectively) strains that are very difficult to treat[3,4]. To investigate whether Cas9 cleavage of chromosomal sequences is sufficient to kill staphylococci, we inserted *Streptococcus pyogenes cas9*, tracrRNA (trans-activating crRNA, a small RNA required for crRNA biogenesis[8]) and a minimal CRISPR array optimized for one-step cloning of crRNA sequences, into the staphylococcal vector pC194 (ref. 15), generating pDB114. This plasmid was programmed to target the *aph-3* kanamycin resistance gene. The resulting construct, pDB114::aph, was used to transform either *S. aureus* RN4220 (ref. 16) or RNK, an isogenic derivative carrying a kanamycin resistance gene in the chromosome. Transformation efficiency of RNK cells was at least two orders of magnitude lower than RN4220 (**Supplementary Fig. 1**). We interpret this as showing sequence-specific, Cas9-mediated killing of staphylococci, similar to the results previously obtained for killing of other bacteria (*Streptococcus pneumoniae*[10,17], *Escherichia coli*[9,10] and *Salmonella enterica*[9]).

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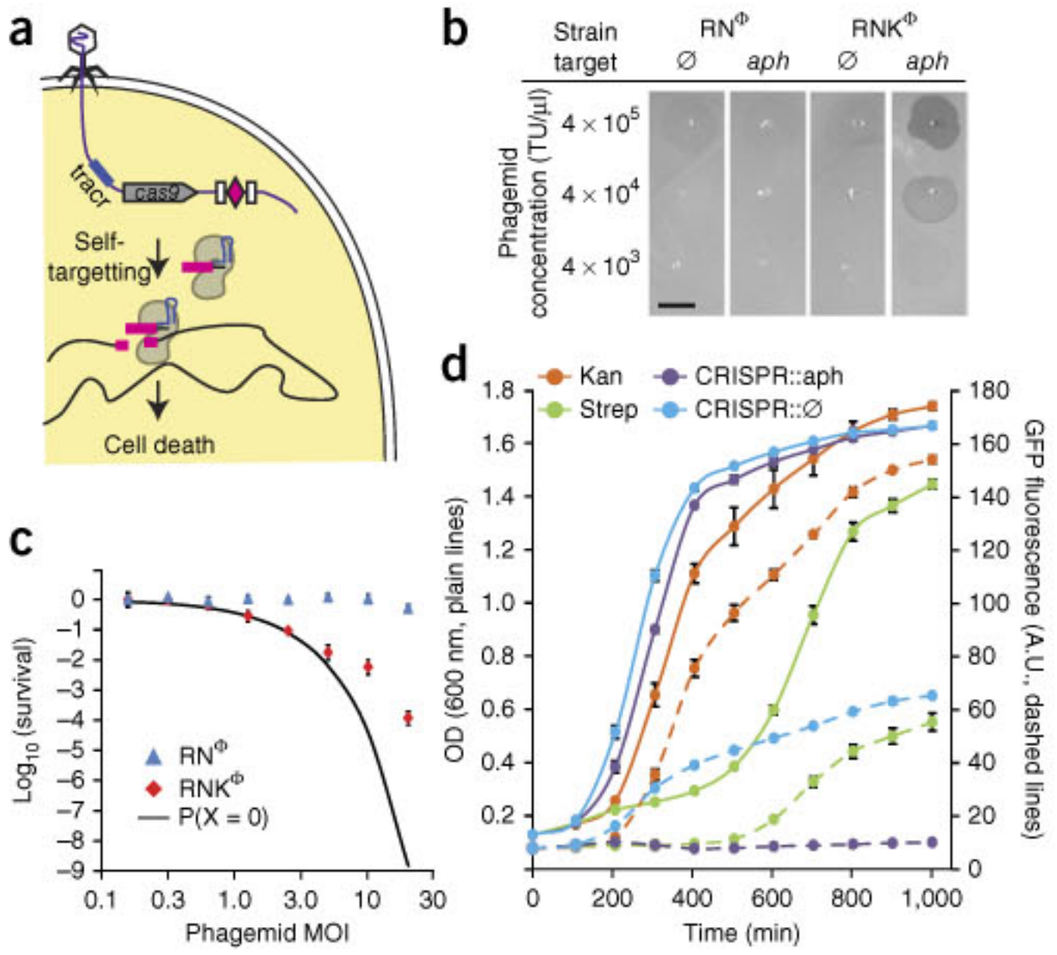

Figure 1 Sequence-specific killing of *S. aureus* by a phagemid-delivered CRISPR system. (**a**) The ΦNM1 phage delivers the pDB121 phagemid to *S. aureus* cells. pDB121 carries the *S. pyogenes* tracrRNA, *cas9* and a programmable CRISPR array sequence. Expression of *cas9* and a self-targeting crRNA leads to chromosome cleavage and cell death. (**b**) Lysates of pDB121 phagemid targeting the *aph-3* kanamycin resistance gene or a nontargeting control are spotted on top-agar lawns of either RN$^{\Phi}$ or RNK$^{\Phi}$ cells. Scale bar, 5 mm. (**c**) Treatment of RN$^{\Phi}$ (blue triangles) or RNK$^{\Phi}$ (red diamonds) with pDB121::aph at various MOI. Survival is calculated as the ratio of CFU recovered after treatment to CFU from an untreated sample of the same culture (mean ± s.d.). The black curve represents the probability that a cell does not receive a phagemid making the assumption that all cells have the same chance of receiving phagemid. (**d**) Time course of treatment of RNK$^{\Phi}$/pCN57 (GFP reporter plasmid) cells in a mixed culture with nontargeted RN$^{\Phi}$ cells. Plain lines show OD and dashed lines GFP (mean ± s.d.). Kanamycin (25 µg/ml) is shown in orange, streptomycin (10 µg/ml) in green, pDB121::aph (MOI ~20) in purple, pDB121::Ø (MOI ~20) in blue.

In order to develop a phagemid system suitable for *S. aureus*, we cloned a ~2-kb fragment containing the *rinA*, *terS*, and *terL* genes and packaging site from the staphylococcal ΦNM1 phage[18] into plasmid pC194, to yield the phagemid pDB91. To assess the efficiency of packaging of pDB91 in ΦNM1 capsids, a transduction assay was performed. RN4220 cells containing pDB91 were infected with ΦNM1, and the lysate was used to transduce RN4220 cells previously lysogenized with ΦNM1, referred to as RN$^{\Phi}$. The lysogenic strain is resistant to superinfection with wild-type phage thereby allowing us to detect only phagemid transduction. We determined that a lysate with a titer of 1.6×10^7 plaque-forming units (PFU)/µl contained 3.8×10^6 transfer units (TU)/µl, showing that our phagemid is packaged in 24% (TU/PFU) of the particles. We cloned the CRISPR sequences of pDB114 and pDB144::aph into the pDB91 phagemid to obtain pDB121 and pDB121::aph, respectively. The proportion of phage particles that contained phagemids was substantially lower (2.9% for pDB121, see **Supplementary Fig. 2**) than that for pDB91, most likely due to the larger size of pDB121 (10.3 kb versus 5.3 kb), but remained sufficiently high to facilitate delivery to a large number of cells (**Supplementary Fig. 2**). When spotted on a lawn of RNK$^{\Phi}$ cells, but not on a RN$^{\Phi}$ lawn, pDB121::aph elicited strong growth inhibition (**Fig. 1b**). Conversely, the nontargeting pDB121 phagemid did not produce inhibition of either strain.

In order to quantify the observed killing, infection experiments were performed at different multiplicities of infection (MOI). Here, we define the MOI as the number of TU per recipient cell. In targeting conditions, cells are killed when the MOI becomes greater than one, while nontargeted cells remain unaffected (**Fig. 1c**). At an MOI of 20, the survival rate is 1.1×10^{-4}. Assuming that all cells have an equal chance of taking up phagemid DNA, we can estimate the number of cells in the population that will not be injected with a CRISPR system and are thus expected to survive. The Poisson distribution with a mean of 20 gives an expected survival rate of only 1.5×10^{-9}, which is substantially lower than the survival rate observed. Four different effects could explain this discrepancy: (i) the injection of the phagemid in the recipient cells might not be completely random, that is, some cells might be more likely to receive phagemids than others; (ii) after it has been received, the phagemid might sometimes be lost through segregation before it kills the cells; (iii) cells might receive phagemids carrying a defective CRISPR system; (iv) cells could survive CRISPR targeting through the introduction of mutations in the target sequence.

In order to investigate the nature of the survivor cells, colonies recovered after treatment were restreaked on chloramphenicol plates (pDB121 carries a chloramphenicol resistance marker). We found that 6/8 colonies were still sensitive to chloramphenicol, supporting scenario (i) or (ii) (indistinguishable in this chloramphenicol sensitivity assay). The remaining 2/8 chloramphenicol-resistant colonies were further analyzed to determine whether the *aph-3* target and/or the CRISPR system were still intact. We found that they contained plasmids incapable of CRISPR targeting in which the *cas9* gene was deleted (**Supplementary Fig. 3a**,**b**). None of the cells (8/8) able to escape phagemid treatment contained target mutations (**Supplementary Fig. 3c**), indicating that such mutations happen at a frequency lower than 1 in 1.3×10^5 cells. This preliminary experiment shows that survivor colonies either did not receive the phagemid, lost the phagemid or received a phagemid containing a defective CRISPR system . In all cases survivors are still sensitive to a second round of treatment, and their numbers were reduced using a higher MOI (data not shown).

Following treatment with conventional antibiotics, which eradicate most members of a bacterial community, the incidence and spread of resistance is fueled by the lack of competitor bacteria in the treated niche. As demonstrated above, sequence-specific killing is not exempt from the generation of cells that escape treatment, however, CRISPR-Cas9 antimicrobials have the benefit of killing only a fraction of the whole population, which could potentially leave other members of the community (including those of the same species) to colonize the niche following treatment and thereby limit the growth of resistant organisms. To investigate the effects of sequence-specific killing on populations, we transformed the pCN57 GFP reporter plasmid[19] into RNK$^{\Phi}$ in order to monitor the growth of RNK$^{\Phi}$ when grown in co-culture with RN$^{\Phi}$ during different treatments (**Fig. 1d**). The mixed RN$^{\Phi}$/RNK$^{\Phi}$ culture was grown in a plate reader that measured OD and GFP, and flow cytometry was performed at the end of the experiment to confirm the results (**Supplementary Fig. 4a**). Treatment with pDB121::aph killed RNK$^{\Phi}$ cells only, stopping the increase of the GFP signal. In contrast, RNK$^{\Phi}$ kept growing when treated with spacerless control (**Fig. 2d**, compare the dashed purple line with the dashed blue line). We also compared our CRISPR-Cas9 antimicrobial with traditional antibiotics. Kanamycin kills the RN$^{\Phi}$ cells leaving only RNK$^{\Phi}$ cells in the culture, resulting in a strong fluorescence signal (**Fig. 1d**; dashed orange line). A near minimum inhibitory concentration of streptomycin temporarily stops the growth of both RN$^{\Phi}$ and RNK$^{\Phi}$ cells, but after 6 h resistant RN$^{\Phi}$ and RNK$^{\Phi}$ cells resume growth (**Fig. 1d**; green lines). Therefore in this specific experiment the CRISPR-Cas9 antimicrobial treatment was

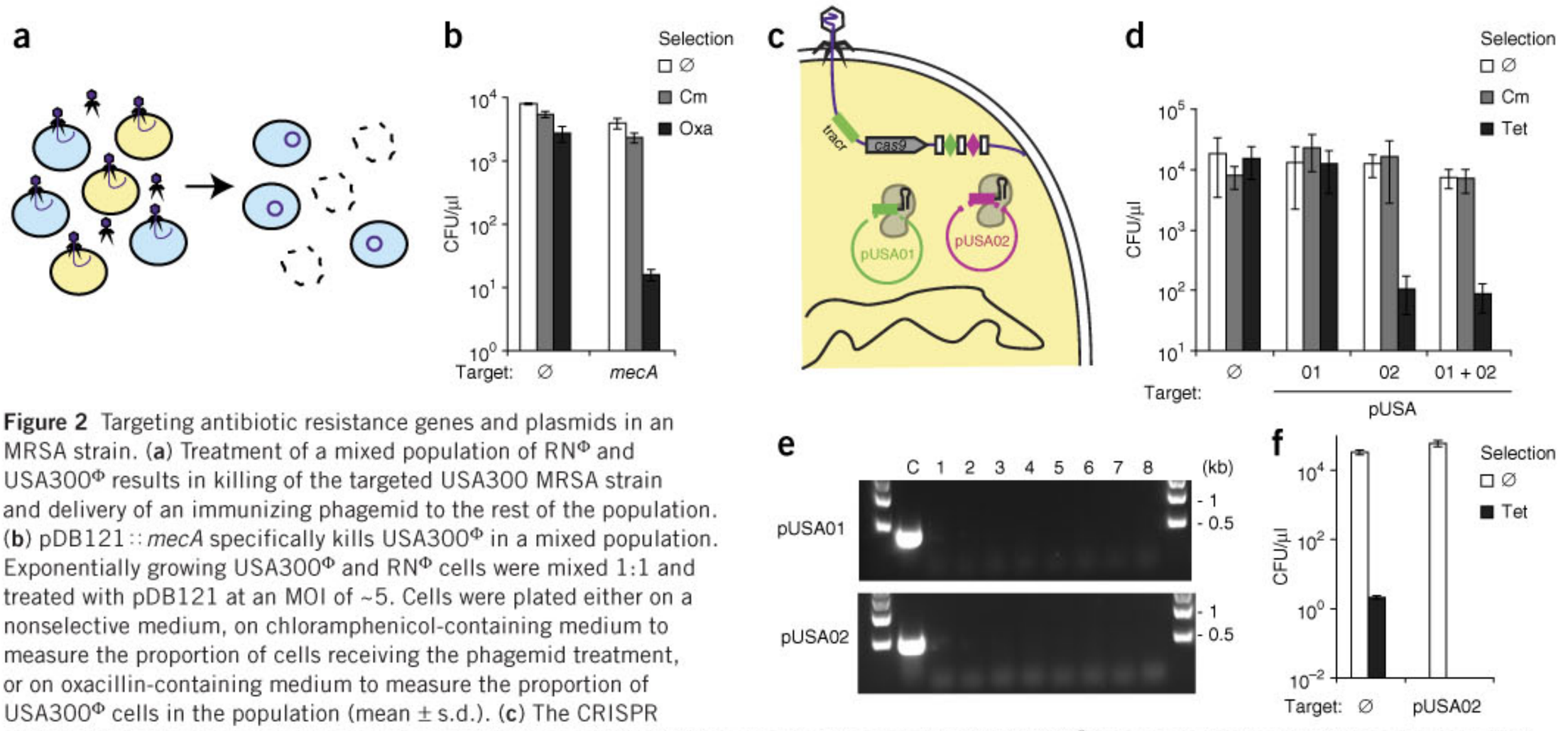

Figure 2 Targeting antibiotic resistance genes and plasmids in an MRSA strain. (**a**) Treatment of a mixed population of RN$^{\Phi}$ and USA300$^{\Phi}$ results in killing of the targeted USA300 MRSA strain and delivery of an immunizing phagemid to the rest of the population. (**b**) pDB121::*mecA* specifically kills USA300$^{\Phi}$ in a mixed population. Exponentially growing USA300$^{\Phi}$ and RN$^{\Phi}$ cells were mixed 1:1 and treated with pDB121 at an MOI of ~5. Cells were plated either on a nonselective medium, on chloramphenicol-containing medium to measure the proportion of cells receiving the phagemid treatment, or on oxacillin-containing medium to measure the proportion of USA300$^{\Phi}$ cells in the population (mean ± s.d.). (**c**) The CRISPR array sequence is programmed to target the pUSA01 and pUSA02 plasmids simultaneously. (**d**) USA300$^{\Phi}$ was treated with pDB121 lysates targeting each plasmid individually or in combination. Cells were plated either on a nonselective medium, on chloramphenicol-containing medium to measure the proportion of cells receiving the phagemid treatment, or on tetracycline-containing medium to measure the proportion of cells cured of pUSA02 (mean ± s.d.). (**e**) Plasmid curing was confirmed by the lack of PCR amplification with plasmid-specific oligonucleotides in eight independent CFUs after treatment with the double targeting construct. (**f**) A population of RN$^{\Phi}$ cells was immunized against plasmid horizontal transfer by treatment with the pUSA02-targeting pDB121 phagemid. 30 min after treatment, the population was transduced with a ΦNM1 stock grown on USA300. Cells were plated either without selection or on tetracycline to measure transduction efficiency of the pUSA02 plasmid (mean ± s.d.).

better than a nonspecific, traditional antibiotic in limiting the incidence of resistant bacteria. We also carried out the same experiment in a monoculture of RNK$^{\Phi}$ cells (**Supplementary Fig. 4a**). In these conditions, the RNK$^{\Phi}$ cells that survive the treatment resume growth after 7 h, and the CRISPR-Cas9 antimicrobial treatment fares no better than 10 μg/ml of streptomycin. Altogether these experiments highlight the potential benefits of sequence-specific CRISPR-Cas9 antimicrobial killing with respect to the emergence and establishment of resistant strains. When applied to a mixed bacterial population, a sequence-specific antimicrobial allows nontargeted cells to keep growing, which may in turn prevent or reduce the capacity for growth of the small proportion of targeted cells that survive the treatment.

We produced a CRISPR-Cas9 antimicrobial (pDB121::mecA) that targeted the methicillin resistance gene *mecA*[20] to eradicate MRSA strains from a mixed population of bacteria (**Fig. 2a**). This CRISPR-Cas9 antimicrobial phagemid was used to treat the clinical isolate *S. aureus* USA300$^{\Phi}$ (ref. 3) in a mixed culture with RN$^{\Phi}$ cells (both ΦNM1 lysogens). Exponentially growing USA300$^{\Phi}$ and RN$^{\Phi}$ cells were mixed 1:1 and treated with pDB121::mecA at an MOI of ~5. Cells were plated either on a nonselective medium, an oxacillin-containing medium to measure the proportion of USA300$^{\Phi}$ cells in the population or on chloramphenicol-containing medium to measure the proportion of cells receiving the phagemid treatment (**Fig. 2b**). The proportion of USA300$^{\Phi}$ dropped from 50% before treatment to 0.4% after treatment, whereas no significant decrease could be observed in the control experiment using the nontargeting pDB121 phagemid. Plasmids are the main source of antibiotic resistance and virulence genes in pathogenic bacteria. The USA300 strain carries three plasmids, pUSA01-3 (ref. 3), with the pUSA02 plasmid conferring tetracycline resistance. We designed CRISPR-Cas9 antimicrobials that target pUSA01, pUSA02 or both (pUSA03 is unstable, data not shown) and tested them for their ability to eliminate these plasmids from the population (**Fig. 2c**). In all cases, treating USA300$^{\Phi}$ with the phagemid preparation did not result in cell death (**Fig. 2d**, colony-forming unit (CFU) counts without selection similar to the nontargeting control). However, more than 99.99% of the cells became sensitive to tetracycline (**Fig. 2d**), due to the loss of pUSA02 (**Fig. 2e**). In order to investigate the dynamics of plasmid curing, we performed a time course experiment showing a dramatic drop in tetracycline-resistant CFU immediately after the cells and the CRISPR-Cas9 antimicrobial phagemid are mixed (**Supplementary Fig. 5**). This indicates that pUSA02 is rapidly cleaved and degraded, most likely as soon as a CRISPR-Cas9 antimicrobial phagemid enters the cell. Because many bacterial virulence plasmids can transfer horizontally and spread antibiotic resistance[4], we tested whether CRISPR-Cas9 antimicrobial treatment could be used to immunize naive staphylococci against pUSA02 transfer. To achieve this, an exponentially growing culture of RN$^{\Phi}$ cells was treated with a CRISPR-Cas9 antimicrobial targeting the pUSA02 plasmid or a nontargeting control. After 30 min, treated cells were infected with a ΦNM1 phage lysate grown on USA300 cells that can transduce pUSA02. Transduction efficiency was measured by selecting for tetracycline resistance. Although pUSA02 could readily be transferred to cells treated with the control phagemid, no tetracycline-resistant colonies were recovered after cells were treated with the CRISPR-Cas9 antimicrobial (**Fig. 2f**), demonstrating efficient immunization against plasmid transfer.

One advantage of using Cas9-mediated killing is the possibility of programming the nuclease with two or more crRNA guides to target different chromosomal and/or plasmid sequences. This strategy could limit the rise of resistant clones that escape phagemid treatment through the generation of target mutations, and also expand the range of targeted cells. To illustrate the multiplex capabilities of the CRISPR-Cas9 antimicrobial, we expanded the CRISPR array carried by the phagemid to produce a second crRNA targeting either the superantigen

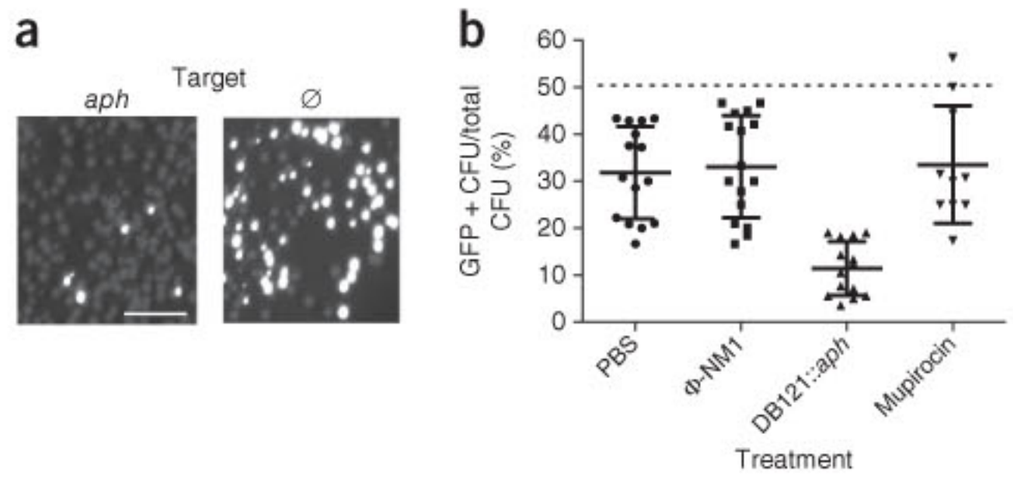

Figure 3 Sequence-specific killing of kanamycin-resistant *S. aureus* in a mouse skin colonization model. Mice skin was colonized with a 1:1 mixture of 10^5 RN$^\Phi$ and RNK$^\Phi$ cells carrying the pCN57 GFP reporter plasmid, followed by treatment at 1 h with PBS ($n = 15$), ΦNM1 ($n = 16$), pDB121::*aph* ($n = 14$) or mupirocin ($n = 10$). After 24 h the skin from the treated area was excised, homogenized and serial dilutions of the homogenate was plated on mannitol salt agar. (**a**) Pictures of two representative plates exposed to a wavelength enabling visualization of GFP. Scale bar, 1 cm. (**b**) The proportion of RNK$^\Phi$ cells in the population was measured as the proportion of green fluorescent CFU on the plates. Data points indicate individual mice; black lines represent the mean ± s.d.

enterotoxin *sek* gene[21] or another region of the *mecA* gene. These arrays were all able to kill USA300 cells with comparable efficiencies. (**Supplementary Fig. 6**). Altogether, these results demonstrate that delivery of the sequence-specific Cas9 nuclease dramatically reduces the plasmid content in a bacterial population without killing the host, can be used to immunize nonpathogenic strains against the transfer of virulence and/or antibiotic-resistant plasmids and can be easily reprogrammed to target multiple sequences.

In order to demonstrate that CRISPR-Cas9 antimicrobials can be used to selectively kill staphylococci *in vivo*, we tested it in a mouse skin colonization model[22,23]. The backs of CD1 mice were shaved and treated with depilatory cream to expose the skin. An area on the back was colonized with 10^5 cells of a 1:1 mixture of RN$^\Phi$ and RNK$^\Phi$ bacteria, the latter harboring the pCN57 plasmid to facilitate detection of targeted cells by measuring green fluorescence. Following colonization, infected areas were topically treated with either the CRISPR-Cas9 antimicrobial pDB121::aph, a control containing phiNM1 phage alone, 2% mupirocin (commonly used in clinical settings to decolonize patients of staphylococci) or streptomycin (200 mg/mouse). After 24 h the treated skin was dissected and homogenized to enumerate staphylococci (**Supplementary Table 1**). The proportion of RNK$^\Phi$ cells in the population was measured as the ratio of GFP CFU to total CFU (**Fig. 3a**). Treatment with the CRISPR-Cas9 antimicrobial pDB121::aph resulted in a decrease in the proportion of RNK$^\Phi$ cells from 50% to 11.4% (±5.7%) that was significantly different from all the other treatment conditions (unpaired Student's *t*-test $P < 0.0001$) (**Fig. 3b**). In comparison, the systemic streptomycin treatment decolonized the mice of all staphylococci (not shown), but the mupirocin left 6/10 mice colonized with RN$^\Phi$ and RNK$^\Phi$ cells in similar proportions. Unexpectedly, a small decrease in the proportion of RNK$^\Phi$ cells (from the expected theoretical 50%) was also observed for mice treated with the control phage, mupirocin or saline, possibly due to a fitness disadvantage of RNK$^\Phi$/pCN57 cells when growing on the mouse skin.

In this report we present the use of the programmable Cas9 nuclease as a sequence-specific antimicrobial to manipulate heterogeneous bacterial populations. Such CRISPR-Cas9 antimicrobials could be used to decolonize patients of antibiotic-resistant bacteria such as beta-lactam- or vancomycin-resistant staphylococci, enterococci, enterobacteria and toxigenic clostridia. Our strategy has several advantages over both small-molecule antibiotics and phage therapy. We have shown that it can be more efficient than an antibiotic treatment when nontargeted bacteria are free to occupy the niche. Although more specific than traditional antibiotics, phage therapy could also kill both pathogenic and commensal bacteria indiscriminately, potentially disturbing the microbiota and selecting for resistant organisms. Another drawback of phage therapy is that phages used are often poorly characterized. In contrast, the composition of our CRISPR-Cas9 antimicrobial and the function of all its genes are well understood. Undoubtedly the main obstacle to translation of this technology into a viable therapeutic is the efficient delivery of the Cas9 and its RNA guide/s into bacterial cells. As opposed to bacteriophages, which can produce hundreds of copies of themselves when they kill a cell, our phagemid system does not produce more particles after infection. This means that the amount of phagemid used in the treatment needs to be much larger than the size of the target population. Furthermore, delivery of the phagemid in an environment more complex than the mouse skin remains to be investigated. Although phagemids provide a suitable delivery for some applications, difficulties associated with their purity, large-scale production and narrow host range could preclude their extensive use. Other potential delivery methods for programmable Cas9 nucleases, such as polymeric nanoparticles[24] will need to be explored in the future. Also, although necessary for the characterization of the technology in this study, antibiotic resistance genes need to be removed from phagemids to avoid their spread.

Despite these caveats, CRISPR-Cas9 antimicrobials offer numerous potential advantages over traditional antimicrobials. Besides selective killing, provided with a suitable delivery system, the built-in multiplex feature of CRISPR-Cas systems could be exploited to target several different species at the same time and/or several sequences of the same bacterium to prevent the rise of resistant mutants. Our approach can also be used to cure plasmids and other mobile genetic elements from a population without killing the host. Moreover, the technology could be easily adapted to reduce or abolish the expression of antibiotic resistance, virulence and other genes of interest without causing the death of the host using dCas9, the nuclease-defective version of Cas9 (refs. 25,26). These unique features create opportunities for the application of this technology in medical, environmental and industrial settings, offering the possibility to shape complex bacterial populations.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

D.B. and L.A.M. designed the experiments. D.B. and W.J. performed the *in vitro* experiments. D.B., P.M.N. and C.W.E. performed the animal experiments. G.W.G. isolated phage phiNM1 and constructed strain RNK. V.A.F. and X.D. participated in the conception of the project.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Strains and culture conditions. *S. aureus* strain RN4220 (ref. 16) was grown at 37 °C in tryptic soy broth (TSB), when appropriate, with the following antibiotics: kanamycin (Kan, 25 μg/ml), chloramphenicol (Cm, 10 μg/ml) and tetracycline (Tet, 5 μg/ml). *S. aureus* USA300 (ref. 27) was provided by the Fischetti laboratory. Phage ΦNM1 was isolated from the *S. aureus* Newman (ref. 28) strain. The supernatant of an overnight culture was used to infect RN4220 in a top-agar layer. Single plaques were isolated and passaged three times to ensure purity. ΦNM1 lysogens of RN4220 (RN^{Φ}) and USA300 (USA^{Φ}) were isolated by restreaking cells from the middle of a turbid plaque twice. Chromosomal integration of ΦNM1 was checked by both PCR and by ensuring resistance to ΦNM1 superinfection. A kanamycin resistance gene was introduced in RN4220 by using a derivative of the pCL55-itet integrative vector where the chloramphenicol resistance gene was replaced with the *aphA-3* kanamycin resistance gene to produce pKL55-itet. Briefly, *aphA-3* was amplified from strain crR6 (ref. 17) using primers L484/L485 and pCL55-itet was amplified with primers L482/L483, followed by digestion with XhoI and ligation. Integration in the RN4220 chromosome was achieved by transformation in electrocompetent cells and selection on TSA + Kan.

Plasmid construction. To assemble the pDB91 phagemid, the rinA-terS-terL region of ΦNM1 was amplified with oligos B234/B235, and pC194 with oligos B233/B127. PCR products were digested with KpnI and SphI followed by ligation and transformation in RN4220-competent cells. The pDB114 plasmid was constructed in two steps. First the full M1GAS *S. pyogenes* CRISPR02 system was cloned on pC194 by amplifying *S. pyogenes* genomic DNA with oligos L362/W278 and pC194 with oligos W270/W282, followed by digestion with BglII and BssSI and ligation, giving pWJ40 (ref. 29). The pWJ40 plasmid was then amplified with oligos B334/L410 and the BsaI CRISPR array from pCas9 with oligos L409/B333, followed by Gibson assembly[30]. To construct pDB121, pDB114 was amplified with oligos B351/W278 and pDB91 with oligos L316/L318, followed by Gibson assembly of the two fragments. Spacers were cloned by digestion with BsaI, and ligation of annealed oligonucleotides designed as follows: 5′-aaac+(target sequence)+g-3′ and 5′-aaaac+(reverse complement of the target sequence)-3′, where the target sequence is 30 nt and is followed by a functional protospacer-adjacent motif (PAM) NGG. Alternatively, two spacers were cloned in a single reaction in the BsaI-digested pDB121 vector. Two pairs of oligonucleotides were annealed and ligated with the vector. The pair carrying the first spacer was designed as follows: 5′-aaac+(target sequence)+GTTTTAGAGCTATG-3′ and 5′-AACAG CATAGCTCTAAAAC+(reverse complement of the target sequence)-3′, and the pair carrying the second spacer as follows: 5′-CTGTTTTGAATGGTCC CAAAAC+(target sequence)+g-3′ and 5′-aaaac+(reverse complement of the target sequence)+ GTTTTGGGACCATTCAA-3′. A list of all spacers tested in this study is provided in **Supplementary Table 2**, and a list of oligonucleotides in **Supplementary Table 3**.

Phage and phagemid production. Phage and phagemid stocks were produced by growing cells from an overnight culture diluted 1:50 in TSB + Cm + $CaCl_2$ 5 mM until an OD_{600} of 0.6 was reached. The cultures were then inoculated with 10 μl of a concentrated ΦNM1 phage stock and incubated for 3 h. Cell debris was eliminated by centrifugation and filtering of the supernatant through 0.45 μm filters. Phage titers were determined by serial dilution and spotting on a top-agar layer of RN4220 cells on HIA plates supplemented with 5 mM $CaCl_2$. To determine the transducing titer, serial dilutions of the phage stock were produced and used to infect a culture of RN^{Φ} cells grown to OD_{600} ~ 1. After 1 h of incubation at 37 °C, cells were plated on TSA + Cm, and transducing units (TU) were measured from the number of CFU obtained.

Killing and plasmid curing assays. Phage stocks were produced on RN4220 cells carrying a phagemid with the desired CRISPR spacer. Recipient cells were grown in TSB to an OD_{600} of 0.6, diluted 10× in TSB + 5 mM $CaCl_2$ and 100 μl of the culture was mixed with 100 μl of the appropriate phage stock dilution. After 1 h of incubation, cells were plated on TSA. Survival rates were measured as the ratio of CFUs obtained with treatment over CFUs obtained without treatment. When appropriate, cells were also plated on TSA + Cm to measure phagemid transduction efficiency, TSA + Tet to measure pUSA02 curing and TSA + Oxa to measure the proportion of MRSA cells in the population.

Immunization assay. RN^{Φ} cells were diluted 1:100 in TSB and grown to OD_{600} of 0.2. Phagemid was added to an MOI of 10, and cells were incubated 30 min to allow for establishment of the CRISPR system. The pUSA02 plasmid was transduced by infecting with a phiNM1 stock grown on USA300 cells. Cells were plated on TSB, TSB + Cm or TSB + Tet to measure transduction efficiency.

Growth curves and fluorescence measurements. Growth curves and GFP fluorescence were measured in a Tecan microplate reader. Cultures were started by diluting an overnight culture 1:100 in 200 μl of TSB. Phagemid was added to an MOI of ~10 after 80 min of growth.

Flow cytometry. A 1-μl aliquot of cells growing in the Tecan microplate reader were diluted into 3 ml of PBS and were used to acquire flow cytometry data using an LSR II flow cytometer (Becton Dickinson). Data were analyzed with FlowJo software (TreeStar).

Mouse skin colonization. The Rockefeller University's Institutional Animal Care and Use Committee approved all *in vivo* protocols. All experiments were conducted at The Rockefeller University's Animal housing facility, an AAALAC accredited research facility, with all efforts to minimize suffering. An adapted approach from Kugelberg *et al.*[22] and Pastagia *et al.*[23] was used to induce topical skin colonization with *S. aureus* on 6- to 8-week-old female CD1 mice (Charles River Laboratories, Wilmington, MA). Briefly, mice were anesthetized by intraperitoneal injection of ketamine (1.5 mg/animal; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (0.3 mg/animal; Miles Inc., Shawnee Mission, KS). A 2 cm^2 area of the dorsum of each mouse was shaved with an electric razor; Nair depilatory cream was then applied to the shaved area for 1 min and wiped away with 70% ethanol pads. The area was then tape stripped, with autoclave tape, approximately 10 times in succession, using a fresh piece of tape each time to irritate and remove the upper layers of the epidermis. The mice were topically colonized with a 2 μl mixture of cultures of *S. aureus* RN^{Φ} and RNK^{Φ}/pCN57 containing 1×10^5 cells in logarithmic growth phases in PBS. Animals were then immobilized under isoflurane anesthesia. After 1 h, 10 μl of concentrated phagemid lysate containing 2×10^7 TU/μl was applied onto the infected skin area. To obtain this concentration, crude lysates were concentrated using 100 kD Amicon Ultra centrifugal filters and washed once with PBS. Additional mice were treated with either streptomycin 200 mg/mouse or 2% mupirocin. After 24 h, mice were euthanized with CO_2, and tissue from the infected skin area was excised and homogenized in 0.5 ml of PBS using the Stomacher 80. Bacterial dilutions were plated on mannitol salt agar (an *S. aureus*-selective medium) and TSA + Cm.

The experiments were not randomized.

The investigators were not blinded to allocation during experiments and outcome assessment.

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