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Viewpoint

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Silencing of Antibiotic Resistance in *E. coli* with Engineered Phage Bearing Small Regulatory RNAs

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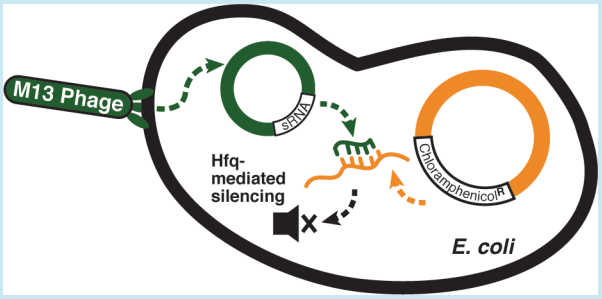
Supporting Information

ABSTRACT: In response to emergent antibiotic resistance, new strategies are needed to enhance the effectiveness of existing antibiotics. Here, we describe a phagemid-delivered, RNA-mediated system capable of directly knocking down antibiotic resistance phenotypes. Small regulatory RNAs (sRNAs) were designed to specifically inhibit translation of chloramphenicol acetyltransferase and kanamycin phosphotransferase. Nonlytic phagemids coding for sRNA expression were able to infect and restore chloramphenicol and kanamycin sensitivity to populations of otherwise resistant *E. coli*. This modular system could easily be extended to other bacteria with resistance profiles that depend on specific transcripts.



The emergence of resistance to conventional antibiotics has motivated interest in techniques to extend or complement their effectiveness.[1] Bacteriophage act to kill bacteria through mechanisms distinct from those of small-molecule antibiotics. Therefore, bacteriophage may serve as complementary therapeutics or bacterial control agents in some contexts.

As with classical antibiotics, the direct application of bacteriophage to cause cell death rapidly selects for resistant strains.[2] Combination therapies that employ multiple phage or drugs can slow the evolution of resistance. Previous work has shown that bacteriophage and antibiotics can act synergistically, with modified bacteriophage altering host physiology to increase drug sensitivity.[3]

In this work, we developed an M13 bacteriophage to knock-down the expression of two genes responsible for antibiotic resistance in *E. coli*: chloramphenicol acetyltransferase and kanamycin phosphotransferase (Figure 1). We used rationally designed, sequence-specific sRNAs to target and silence the transcript for each gene directly.[4] Because such sRNAs can be designed to complement any mRNA, the approach could extend to any antibiotic resistance bestowed by specific transcripts.

The system is RNA-mediated and does not require additional protein expression. Further, the construct is delivered by a nonlytic phagemid system and targets only antibiotic-resistance transcripts. By limiting the disruption of host physiology we have sought to minimize the fitness burden imposed by the construct. In this way, the evolutionary pressures favoring phage-resistance are minimized until a lethal dose of antibiotic can be applied to a formerly resistant population.

RESULTS AND DISCUSSION

Following the design of Na et al.,[4] we created two sRNA expression constructs targeting chloramphenicol acetyltransferase and kanamycin phosphotransferase (Figure 2). These constructs were incorporated into an M13 phagemid, which was then assembled and released by *E. coli* with the help of an M13 phagemid helper plasmid. Because phagemids do not code for viral structural proteins, we reasoned that a phagemid-based delivery would minimize the physiological disruption and fitness cost of our construct to the infected host.[5]

As described in the Methods, populations of chloramphenicol or kanamycin resistant *E. coli* were infected with phagemid bearing the relevant sRNA silencing construct. Antibiotic sensitivity was assayed by plating on chloramphenicol or kanamycin at a range of concentrations.

Our construct restored substantial antibiotic sensitivity to otherwise resistant populations (Figure 3). At high chloramphenicol concentrations (500 μg/mL), we observed >99% killing for infected populations, but no significant killing in control populations (Figure 3a). Lower chloramphenicol concentrations killed less efficiently, but the effect of the

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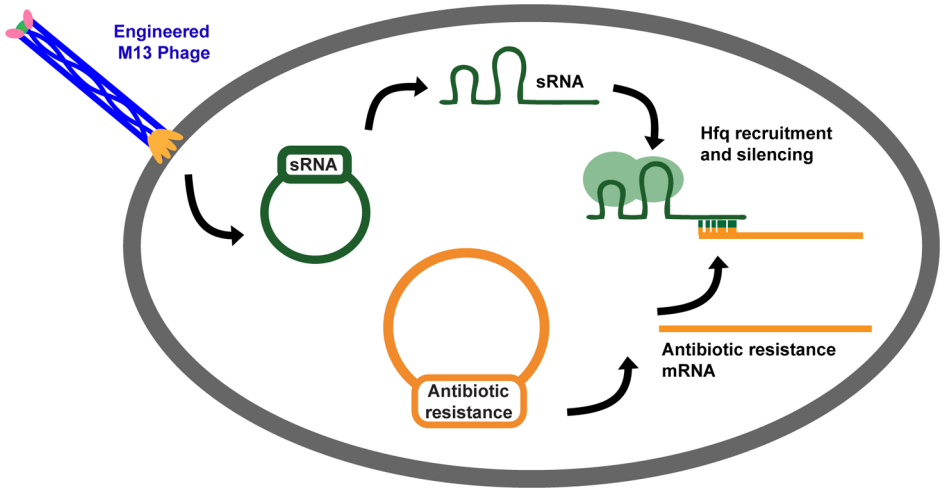

Figure 1. Scheme for antibiotic silencing with phagemid-delivered sRNA. M13 bacteriophage capsules infect the *E. coli* host and deliver a modified nonreplicating phagemid bearing the sRNA expression construct. A 24 bp complementary sequence in the sRNA targets the antibiotic resistance mRNA, while an MicC scaffold sequence recruits the Hfq protein to promote hybridization and degradation.[4]

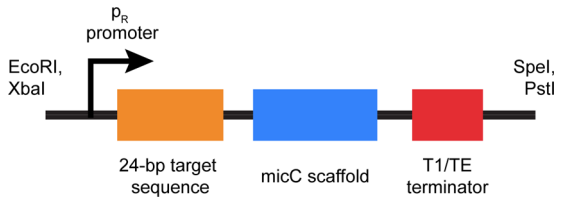

Figure 2. Structure of the sRNA module for the silencing of chloramphenicol acetyltransferase. The entire 419 bp construct was synthesized and cloned into a phagemid vector with BioBrick-compatible restriction sites. The 24 bp target sequence is complementary to the +1 to +24 sequence of the target gene. The moldular design is easily changed to target other resistance-associated mRNAs.

sRNA was still evident at 250 μg/mL. Similar results were obtained for kanamycin (Figure 3b).

We observed significant variability among biological replicates in our plating assay, particularly at intermediate antibiotic concentrations (Supporting Information Table S1). Plating efficiency may have been affected by the stress of high-antibiotic concentrations, even in genetically resistant cells. Other sources of variation may include variable titers or the accumulation of mutations in phage collected from overnight cultures. In the case of the Kanamycin-silencing construct, phagemids were produced using the M13K07 phagemid-helper plasmid. This plasmid itself contains a target for the expressed sRNA. Although the plasmid was maintained at kanamycin concentrations below the levels at which we observed reduced resistance, this interaction may have contributed to the accumulation of mutations.

Because our observed killing efficiencies were less than 100%, we next sought to investigate the ways in which bacteria may escape the killing effect. A cell may escape killing in our system either because it does not become infected with the phagemid or because the phagemid does not effectively silence resistance. Because our phagemid also codes for GFP expression, we were able to estimate the infection rates of the surviving cells.

We scored GFP fluorescence, indicating phagemid infection, in 71% of surviving colonies (Figure 3C). Thus, in the majority of cases, cell survival is due to a failure of the sRNA to adequately silence gene expression. This may be due

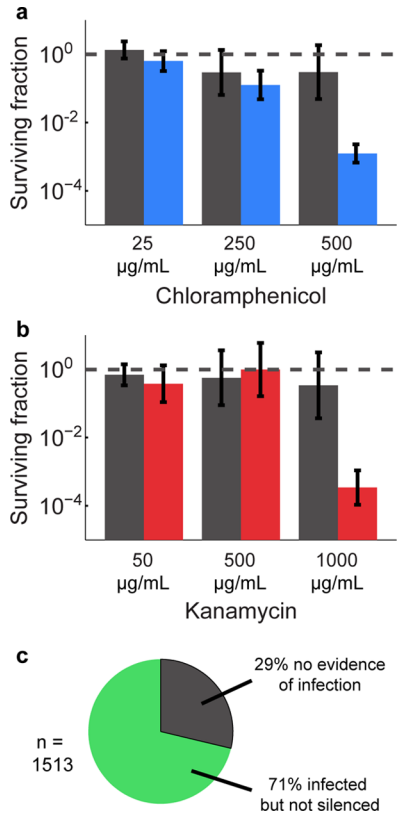

Figure 3. Engineered phagemids restore antibiotic sensitivity to a genetically resistant population. Bacterial populations carrying plasmid-borne resistance cassettes for chloramphenicol (a) and kanamycin (b) were infected with sRNA-expressing phagemids. In the infected populations, significant killing was observed especially at high antibiotic concentrations. In control populations with no sRNA-silencing construct, no significant killing was observed. Dotted lines indicate 100% survival. Error bars represent 95% confidence intevals. (c) Failure modes for sRNA-mediated silencing. Phagemid-encoded GFP was used to score surviving colonies for the presence of the phagemid. The expression of GFP in 71% of surviving colonies (68–74 at 95% confidence) indicates that phagemid infection was successful but sRNA-mediated silencing did not act to restore antibiotic sensitivity.

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insufficient production of the sRNA. The pACYCDuet-1 and pCOLADuet-1 vectors that were used to convey antibiotic resistance are estimated by the manufacturer (Novagen) to be present at copy numbers of 10−12 and 20−40, respectively. The sRNA-targeted transcripts may be in excess of sRNA production. Alternately, the sRNA-mRNA complex may be too slow to form or too weakly bound to completely silence transcription. Finally, mutations in the antibiotic resistance genes could disrupt sRNA targeting.

Future work could address in greater detail the mechanism by which resistant *E. coli* escape silencing. Some routes of escape are easily addressed with simple and modular sRNA design. For example, point mutations in the antibiotic resistance gene could be easily matched with a redesigned sRNA. Other escape mechanisms, such as overexpression of the target mRNA or resistance to phage infection, would be more problematic. In particular, mutations in the pili are known to confer resistance to M13 phage.[2]

The counter-selection of our construct in a bacterial population will be in proportion to the fitness burden it imposes on the host. We have taken steps to reduce the burden of our construct by avoiding protein expression, by minimizing the size of the coding sequence and by targeting only antibiotic resistance transcripts. The targeted genes are nonfunctional in any antibiotic-free medium and costly to translate.[6] A low-burden construct should be more easily maintained in bacterial population prior to the administration of lethal antibiotic doses. However, further population genetic modeling will be required to minimize counterselection and account for the specific dynamics of a particular host−phage system.

Phage therapy and phage-based biocontrol predate the development of conventional antibiotics.[7] Currently, broad-spectrum small molecule drugs remain the antibacterial weapon of choice in most cases, despite recent challenges in the form of evolved resistance. Integral to the general success of antibiotics was the application of synthetic chemistry to improve the stability, specificity, and activity of naturally occurring antibiotic compounds. Increasingly, Synthetic Biology provides the tools to enhance wild phage isolates.[8] Combination therapies featuring semisynthetic antibiotics and designer phage may synergistically increase the value of both approaches.

METHODS

Design of an sRNA to Target Antibiotic Resistance. The method of Na et al.[4] was used to design sRNA transcripts to silence chloramphenicol acetyltransferase and kanamycin phosphotransferase. A 24 bp sequence complementary to the +1 to +24 area of each target gene was inserted within the MicC scaffold sequence, which promotes RNA hybridization through recruitment of the Hfq protein. The P_R promoter and T1/TE terminators were included to control the transcription of the regulatory RNA. With the addition of BioBrick-compatible restriction cloning sites, the complete 419 bp module was synthesized by IDT. Restriction cloning was used to integrate the sRNA-expression module into Litmus28i_J23115-B0032-GFP, an M13-derived phagemid carrying GFP.[9,10]

Construction of Phage and Antibiotic Resistant Strains. Phage-producing cells were created by cotransforming *E. coli* MG1655 with a phagemid and phagemid-helper plasmid M13K07 (NEB). After overnight culture of the phage-producers in selective LB, phage were isolated by centrifugation and filtration at 0.2 μm.

Antibiotic resistant strains were created by transforming *E. coli* MG1655 with pACYCDuet-1 or pCOLADuet-1 plasmids (Novagen) to convey resistance to chloramphenicol or kanamycin, respectively. An F plasmid was also introduced to both strains by conjugation, as F pilus expression is required for sensitivity to M13.

Antibiotic Sensitivity Assay. Resistant *E. coli* were grown to log phase (OD_{600} = 0.7) in selective LB. One milliliter of culture was mixed with 100 μL of harvested phage or plain LB as a negative control. Phage infection was allowed to proceed for 30 min at 37 °C with agitation. Serial dilutions were then plated on selective LB agar with defined antibiotic concentrations. Sensitivity to a given antibiotic was assayed by comparing CFU counts at a specific antibiotic concentration to counts on antibiotic-free LB agar.

Phage Infection Assay. Following infection and plating, the number of phagemid-carrying *E. coli* was estimated by scoring GFP fluorescence of single colonies by eye using an Illumatool model LT-9900 (Lightools Research) with appropriate filters for GFP.

Statistical Analysis. For the antibiotic sensitivity assay, the survival fraction was calculated as a CFU count on antibiotic plates compared to a matched antibiotic-free control. Confidence intervals were estimated using the standard error of the mean for the log-transformed survival fraction. For the phage infection assay, confidence was estimated as a binomial proportion confidence interval.

ASSOCIATED CONTENT

Supporting Information

Exact DNA sequences for the sRNA-expressing phagemids created for this study; raw survival data for the antibiotic sensitivity assay depicted in Figure 3a, b. This material is available free of charge via the Internet at http://pubs.acs.org. Further discussion of this project and its context within the larger work of the 2013 Paris Bettencourt iGEM team is available via our team Web site at http://2013.igem.org/Team:Paris_Bettencourt.

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Author Contributions

[§]V.K.L. and A.G.B. contributed equally to this work. V.K.L., A.G.B., and E.H.W. designed the experiments. A.G.B., V.K.L., and C.B. conducted the experiments. All authors contributed to the analysis and interpretation of results. V.K.L., A.G.B., and E.H.W. prepared the manuscript.

Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Donadio, S., Maffioli, S., Monciardini, P., Sosio, M., and Jabes, D. (2010) Antibiotic discovery in the twenty-first century: Current trends and future perspectives. *J. Antibiotics 63*, 423−430.
(2) Hagens, S., and Bläsi, U. (2003) Genetically modified filamentous phage as bactericidal agents: A pilot study. *Lett. Appl. Microbiol. 37*, 318−323.
(3) Lu, T. K., and Collins, J. J. (2009) Engineered bacteriophage targeting gene networks as adjuvants for antibiotic therapy. *Proc. Natl. Acad. Sci. U.S.A. 106*, 4629−4634.
(4) Na, D., Yoo, S. M., Chung, H., Park, H., Park, J. H., and Lee, S. Y. (2013) Metabolic engineering of *Escherichia coli* using synthetic small regulatory RNAs. *Nat. Biotechnol. 31*, 170−174.
(5) Qi, H., Lu, H., Qiu, H.-J., Petrenko, V., and Liu, A. (2012) Phagemid vectors for phage display: Properties, characteristics, and construction. *J. Mol. Biol. 417*, 129−143.
(6) Andersson, D. I., and Hughes, D. (2010) Antibiotic resistance and its cost: Is it possible to reverse resistance? *Nat. Rev. Microbiol. 8*, 260−271.
(7) Hérelle, F. and Smith, G. (1926) *Bacteriophage and Its Behavior*, The Williams & Wilkins Company, Baltimore, MD.
(8) Lu, T. K., and Collins, J. J. (2007) Dispersing biofilms with engineered enzymatic bacteriophage. *Proc. Natl. Acad. Sci. U.S.A. 104*, 11197−11202.
(9) Ortiz, M. E., and Endy, D. (2012) Engineered cell−cell communication via DNA messaging. *J. Biol. Eng. 6*, 16 DOI: 10.1186/1754-1611-6-16.
(10) Evans, P. D., Cook, S. N., Riggs, P. D., and Noren, C. J. (1995) LITMUS: Multipurpose cloning vectors with a novel system for bidirectional in vitro transcription. *BioTechniques 19*, 130−135.