Reversing Bacterial Resistance to Antibiotics by Phage-Mediated Delivery of Dominant Sensitive Genes

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Pathogen resistance to antibiotics is a rapidly growing problem, leading to an urgent need for novel antimicrobial agents. Unfortunately, development of new antibiotics faces numerous obstacles, and a method that will resensitize pathogens to approved antibiotics therefore holds key advantages. We present a proof-of-principle for a system that restores antibiotic efficiency by reversing pathogen resistance. This system uses temperate phages to introduce, by lysogenization, genes \( rpsL \) and \( gyrA \) conferring sensitivity in a dominant fashion to two antibiotics, streptomycin and nalidixic acid, respectively. Unique selective pressure is generated to enrich for bacteria that harbor the phages encoding the sensitizing constructs. This selection pressure is based on a toxic compound, tellurite, and therefore does not forfeit any antibiotic for the sensitization procedure. We further demonstrate a possible way of reducing undesirable recombination events by synthesizing dominant sensitive genes with major barriers to homologous recombination. Such synthesis does not significantly reduce the gene’s sensitization ability. Unlike conventional bacteriophage therapy, the system does not rely on the phage’s ability to kill pathogens in the infected host, but instead, to deliver genetic constructs into the bacteria, and thus render them sensitive to antibiotics prior to host infection. We believe that transfer of the sensitizing cassette by the constructed phages will significantly enrich for antibiotic-treatable pathogens on hospital surfaces. Broad usage of the proposed system, in contrast to antibiotics and phage therapy, will potentially change the nature of nosocomial infections toward being more susceptible to antibiotics rather than more resistant.
INTRODUCTION

Bacteria have evolved to overcome a wide range of antibiotics, and resistance mechanisms against most of the conventional antibiotics have been identified in some bacteria (7). Accelerated development of newer antibiotics is being overrun by the pace of bacterial resistance. In the USA, for example, over 70% of hospital-acquired infections involve bacteria resistant to at least one antibiotic, and in Japan over 50% of the clinical isolates of Staphylococcus aureus are multidrug-resistant (11).

This increasing threat has revived research into phage therapy. For example, a clinical phase I and II control trial was recently completed successfully for the treatment of chronic bacterial ear infections (21). Nevertheless, although phage therapy has been practiced for several decades in some of the former Soviet Union countries and Poland, there are still many doubts as to its ability to replace antibiotics. Major concerns over the use of phage therapy include neutralization of phages by the spleen/liver and by the immune system, their narrow host range, bacterial resistance to the phage, and lack of sufficient pharmacokinetic and efficacy studies in humans and animals (1, 11).

A recent study used phages as a genetic tool to increase bacterial susceptibility to antibiotics. That study used phage M13, of the Gram-negative Escherichia coli, to genetically target several gene networks, thus rendering the bacteria more sensitive to antibiotics (10). It demonstrated that disrupting the SOS response by M13-mediated gene-targeting renders the bacteria several-fold more sensitive to a variety of antibiotics. It also demonstrated that phage-mediated gene transfer combined with antibiotics increases the survival of mice infected with pathogenic E. coli. Overall, the study showed
that transferring genes by phage M13 weakens the bacteria, and render them more susceptible to killing by antibiotics. We believed that some aspects of that study required further modification. First, the transferred genes target a beneficial pathway in bacteria, and therefore significantly reduce the fitness of the bacteria harboring this phage. Consequently, negative selection pressure is constantly being applied against transfer of these genes by the M13 phages. Second, no mechanism to facilitate genetic transfer of the M13 genes was used: high multiplicity of infection in the experimental settings compensated for this shortcoming. Nevertheless, such settings cannot be used in field experiments. Third, the phage was experimentally tested in vivo, in mice, but immune responses against it were not examined. Therefore, despite the novelty of that study in terms of unique genetic-targeting by phages, the end result is very similar to conventional phage-therapy practices, in which phages are used to directly kill the pathogen.

Different approaches make use of phages as "disinfectants" of pathogens present on edible foods, plants, and farm animals. In addition to increasing the shelf life of these products, the treatment is intended to prevent occasional outbreaks of disease. The US Food and Drug Administration recently approved the use of an anti-Listeria phage cocktail for application on meat and poultry as a preventive measure against Listeria (5). Other phage cocktails have been approved as food additives in Europe, and many are currently being developed by phage biotech companies. These applications demonstrate that phages can be dispersed in the environment and efficiently target pathogens in their surroundings.

Here we present a proof-of-principle for genetic delivery of constructs using phages to target pathogens in the environment. In the described system, phages are
genetically engineered to reverse the pathogens' drug resistance, thereby restoring their sensitivity to antibiotics. The phages transfer, by lysogenization into the pathogens, a drug-sensitizing DNA cassette, which was previously shown to render bacteria sensitive to agents to which they had acquired resistance (6). Pathogens that are lysogenized by the designed phages are selected by tellurite because the phage is engineered to contain a DNA element conferring resistance to this bactericidal agent. Rather than being administered to patients, the phages are intended for dispersion on hospital surfaces, thus gradually reversing the occurrence of drug-resistant pathogens and competing with the resistant pathogens residing in hospitals. This method would thus enable the use of well-established antibiotics against which resistance has been acquired.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains used in this study are listed in Table S1 in the supplemental material, as well as in Tables 1 and 2.

Oligonucleotides. Oligonucleotides used in this study are listed in Table S2 in the supplemental material.

Isolation of resistant mutants. Over twenty different overnight cultures of a total of ~10^{11} \textit{E. coli} K-12 cells were inoculated on Luria-Bertani (LB)-agar plates containing 50 \textmu g/ml streptomycin or 50 \textmu g/ml nalidixic acid. Resistant mutants emerged, in both cases, at a median frequency of ~1 in 10^9 CFU, and were picked from different cultures, to reduce the occurrence of sibling mutants. These bacteria were streaked on an agar plate...
containing the appropriate antibiotic. The \textit{rpsL} or \textit{gyrA} genes of resistant mutants emerging on the plate were PCR-amplified followed by DNA sequencing.

**Plasmid construction.** Plasmids were constructed using standard molecular biology techniques. DNA segments were amplified by PCR. Standard digestion of the PCR products and vector by restriction enzymes was carried out according to the manufacturer's instructions. Plasmid sequences are provided in the supplemental material.

**Phages.** Genetic engineering of the different phages was carried out using \textit{\lambda}gt11/\textit{EcoRI}/Gigapack® III Gold Cloning Kit (Stratagene) according to the manufacturer's protocols. Briefly, \textit{EcoRI}-digested arms of phage \textit{\lambda}gt11 were used to construct the lysogenizing phage carrying the different DNA inserts, encoding a chloramphenicol-resistance gene. DNA inserts were PCR amplified from plasmids pRpsL-wt, pRpsL-sil, pRpsL\textit{\Delta}4 (Fig. 1A) using primers 231F/R (Table S2), and digested with \textit{MfeI} restriction enzyme, which produces ends that are compatible with \textit{EcoRI}. Ligation was carried out using T4 DNA ligase (New England Biolabs). The ligated products were transformed into \textit{E. coli} strain Y1088, which supports \textit{\lambda}gt11 growth. Generated plaques were propagated in \textit{E. coli} Y1088 or \textit{E. coli} ymel, which were then used to lysogenize the hosts. In several cases, phages were further manipulated in a host which lacks \textit{supE}, a suppressor gene necessary for phage growth. In such cases, the phage was transferred by P1-mediated transduction to a permissive host and propagated there. Phages carrying tellurite resistance were constructed by homologous recombination-based genetic engineering of the tellurite-resistance marker instead of the chloramphenicol-resistance gene. The tellurite-resistance genes \textit{tehAB} were amplified from the \textit{E. coli} chromosome using primers N1/N2 (Table S2) for \textit{\lambda}-RpsL\textit{\Delta}4-tell, \textit{\lambda}-
RpsL-wt-tell, λ-RpsL-sil-tell, λ-Ctrl-tell, and λ-GyrA-tell (Fig. 1B). Primers RE22/N2 (Table S2) were used for construction of λ-2xRpsL-tell. The obtained PCR products were used for homologous recombination-based genetic engineering as described below. Sequences of the phage inserts are presented in the supplemental material.

**Homologous recombination-based genetic engineering.** Homologous recombination using short-homology flanking ends was performed as described previously (13). Briefly, an overnight culture of lysogens carrying different DNA inserts encoding the chloramphenicol-resistance gene was diluted 75-fold in 25 ml LB medium with appropriate antibiotics and grown at 32°C in a shaking water bath to an OD$_{600}$ of 0.6. Then, half of the culture was heat-induced for recombination function of the prophage at 42°C for exactly 4 min in a shaking water bath. The remaining culture was left at 32°C as the uninduced control. The induced and uninduced samples were immediately cooled on ice slurry and then pelleted at 3600 g at 0°C for 10 min. The pellet was washed twice in ice-cold ddH$_2$O, then resuspended in 200 μl ice-cold ddH$_2$O and kept on ice until electroporation with ~500 ng of a gel-purified PCR product encoding the tellurite-resistance genes. A 25-μl aliquot of electrocompetent cells was used for each electroporation in a 0.1-cm cuvette at 25 μF, 1.75 kV and 200 Ω. After electroporation, the bacteria were recovered in 1 ml LB for 1 h in a 32°C shaking water bath and inoculated on selection plates containing 1 to 4 μg/ml tellurite. The DNA insertion into the resulting phages, λ-RpsLΔ4-tell, λ-RpsL-wt-tell, λ-RpsL-sil-tell, λ-Ctrl-tell, λ-GyrA-tell, and λ-2xRpsL-tell, was confirmed by PCR using primer 233F along with 232F or 232R.
**Lysogenization.** Overnight culture of the resistant mutants was diluted 1:100 in LB with the appropriate antibiotics, 10 mM MgSO$_4$ and 0.2% (W/V) maltose. When the culture reached an OD$_{600}$ of 0.6–0.8, 100 μl was mixed with 10 μl phage λ, carrying a resistance gene, in a 1.5-ml tube and incubated at room temperature for 20 min. Cells were inoculated on appropriate selection plates and incubated overnight at 32°C. Lysogens emerged on selection plates to which the phage carried a resistance gene. Lysogenization was validated by plating the lysogens at 42°C: lysogens cannot grow at this temperature because the prophage is induced to its lytic cycle (see Materials and Methods).

**Transductions.** Transductions were used to transfer antibiotic-resistance markers or complete λ phages between strains (in cases where the strain did not carry suppressor genes required for λ growth). P1 lysate was prepared as follows: overnight cultures of donor strain were diluted 1:100 in 2.5 ml LB + 5 mM CaCl$_2$ + 0.2% (W/V) glucose. After 1 h shaking at 37°C (or 32°C for lysogens), 10$^7$-10$^8$ PFU of phage P1 was added. Cultures were aerated for 1 to 3 h, until lysis occurred. The obtained P1 lysate was used in transduction where 100 μl fresh overnight culture was mixed with 1.25 μl of 1 M CaCl$_2$ and 0 to 100 μl P1 phage lysate. After incubation for 30 min at 30°C without shaking, 100 μl Na-citrate and 500 μl LB were added. Cultures were incubated at 37°C or 32°C for 45 or 60 min, respectively, then 3 ml of warm LB supplemented with 0.7% agar was added and the suspension was poured onto a plate containing the appropriate drug. Transductants obtained on antibiotic plates were streaked several times on selection plates and verified by PCR for the presence of the transduced DNA fragment.
MIC determinations. MIC determination was carried out by following the procedure described by Wiegand et al. (19). Briefly, bacterial cells were grown overnight at 32°C in LB and diluted to $10^7$–$10^9$ CFU/ml. The obtained suspension was serially diluted 10-fold for different spot concentrations, as indicated. Approximately 1 μl of bacterial suspension was then spotted onto selection plates containing different concentrations of either streptomycin or nalidixic acid along with the appropriate selection agent (chloramphenicol or tellurite), as indicated, using a 48-pin replicator. Plates were incubated overnight and photographed using MiniBis Pro (Bio-Imaging Systems). Photographs were digitally manipulated using GIMP2 software to adjust contrast. Liquid-based MIC determination assays were carried out by inoculating serial dilutions of an antibiotic in liquid LB broth with bacterial cultures (OD$_{600}$ ~0.05) in 96-well microtiter plates. Plates were incubated overnight at 32°C and OD$_{600}$ was then measured. The lowest antibiotic concentration at which the relative growth compared to the "no-drug" control was below 10% was determined as the MIC.

RESULTS AND DISCUSSION

Mutations in the target gene, rpsL, constitute a major resistance mechanism to streptomycin. Our overall goal in this study is to provide a proof-of-principle for a genetic system able to restore drug sensitivity to drug-resistant pathogens residing on hospital surfaces. We chose, as a first step, to use streptomycin as the model drug. Streptomycin is highly useful as an effective antibiotic against both Gram-negative and Gram-positive bacteria. For example, streptomycin is a mainstay of tuberculosis therapy.
However, streptomycin-resistant *Mycobacterium tuberculosis* emerge during treatment, and 24 to 85.2% of them have mutations in either *rpsL* or *rrs* (15). The *rpsL* gene product, S12, is an essential, highly conserved protein of the 30S small ribosomal subunit. Most of the acquired resistance to streptomycin is due to specific mutations in *rpsL* that prevent the inhibitory binding of streptomycin to the essential *rpsL* gene product. We wanted to reproduce these findings in a model bacterium, *E. coli*, and then to restore its sensitivity to streptomycin. We therefore inoculated *E. coli* K-12 on LB-agar plates containing 50 μg/ml streptomycin and selected for resistant mutants. This procedure fairly simulates the selection of spontaneous drug-resistant-mutant evolution in hospitals following streptomycin treatment. Resistant colonies emerged with a median frequency of 1 in $10^9$ CFU. Mutations in *rpsL* were found in 21 out of 22 resistant mutants, a frequency that corroborates with that in clinical isolates. As listed in Table 1, 10 mutants harbored a K88R substitution in RpsL, 6 had an R86S substitution, and P42S, K43L, K43N, R54S, K88E substitutions were each identified once. These mutation types also corroborate with previous studies, confirming that a major mechanism for streptomycin resistance relies on mutations in *rpsL* (e.g. (16, 18)). Therefore, targeting this resistance mechanism or reversing its effect should prove highly beneficial in controlling drug-resistant pathogens.

**Wild-type (wt) rpsL transformed on a plasmid dominantly confers streptomycin sensitivity.** Lederberg first reported in 1951 that wt *rpsL* is a dominant sensitive allele with regard to streptomycin resistance (6). This means that introduction of a sensitive allele of wt *rpsL* into a streptomycin-resistant bacterial cell will result in sensitivity of the bacterium despite the presence of a resistant *rpsL* allele. These findings
were never exploited for clinical practice. To use the dominant sensitivity of \textit{rpsL} as a platform for the next sets of experiments which provide proof-of-principle for use of the constructs in medical settings in the future, we first established the system with our model strains and plasmids. MICs to streptomycin were determined by agar-plate assay (19). In this assay, \textasciitilde10^4 cells are replica-plated on plates with different drug concentrations. The lowest concentration at which there is no visible colony-formation is defined as the MIC. The MICs throughout the study were also measured in a complementary liquid-determination assay, giving a similar readout (not shown). Two representatives of the most common streptomycin-resistant strains obtained above were taken for further study: strains Sm6 and Sm13, harboring mutations in \textit{rpsL} leading to substitutions of R86S and K88R, respectively. Their MICs to streptomycin were 100 \(\mu\)g/ml and 200 \(\mu\)g/ml, respectively, whereas the MIC of the parental strain was 1.56 \(\mu\)g/ml. We transformed these strains with the plasmid pRpsL-wt, encoding the wt \textit{rpsL}, or a control plasmid, pRpsLΔ4, encoding a mock gene (a defective \textit{rpsL} with a 4-bp deletion that disrupts the reading frame after amino acid 26 of the RpsL protein; see Fig. 1 as well as the supplemental material for plasmid sequences and maps) under a modified early \textit{E. coli} promoter from phage T7. Transformed cells were selected on agar plates supplemented with 35 \(\mu\)g/ml chloramphenicol, as the plasmid encodes chloramphenicol acetyl transferase, which confers chloramphenicol resistance. The MICs of the transformed strains to streptomycin were then determined. As shown in Fig. 2A, transformation of the plasmid encoding wt \textit{rpsL}, pRpsL-wt, conferred a dominant sensitive phenotype, restoring the MIC of the resistant mutants Sm6 and Sm13 from 100 \(\mu\)g/ml to 12.5 \(\mu\)g/ml and from 200 \(\mu\)g/ml to 3.125 \(\mu\)g/ml, respectively. A control, streptomycin-sensitive \textit{E. coli}
coli transformed with these plasmids (pRpsLΔ4 or pRpsL-wt) retained similar MICs to streptomycin (not shown). These results demonstrate that a wt rpsL allele delivered on a plasmid into a streptomycin-resistant E. coli renders the cell significantly more sensitive to streptomycin.

*rpsL designed with decreased homology to the wt allele can efficiently restore streptomycin sensitivity.* The system we propose as a proof-of-principle is based on the rpsL-containing construct being transferred horizontally between strains by transformation, conjugation or transduction, as described below. Recombination events between the chromosomal resistant rpsL and the delivered wt rpsL may reduce the efficiency of the construct because it may eventually recombine with an rpsL copy that does not confer sensitivity on the transformed strains (nevertheless, there is no danger that it will confer resistance in sensitive strains as the sensitive allele is dominant). In order to reduce the undesired recombination events between the incoming allele conferring sensitivity and the resistant allele in the transformed cell we designed an allele which cannot undergo homologous recombination with the bacterial copy. Efficient homologous recombination requires identity between recombining genes. Reduction of homology from 100% to 90% decreases the frequency of recombination over 40-fold in E. coli (14). In addition, a minimal efficient processing segment of 23 to 27 bp that is identical to the invading strand is required for efficient homologous recombination (14).

We synthesized an rpsL gene with silent mutations that maximize the incompatibility of recombination with the sequence of wt rpsL. Silent substitutions were made in every possible case, except where codon usage was less than 10% (see the supplemental material for plasmid sequence). Overall, the genes were identical in only 62% of their
sequence, and there was no single minimal efficient process segment between the wt rpsL and the new rpsL allele, thus providing efficient barriers against homologous recombination. We designated this allele rpsL-sil, and the plasmid encoding it, pRpsL-sil. The introduced silent mutations might hamper the folding of the encoded protein or its expression levels (3). We therefore tested whether this allele, like the wt rpsL, can dominantly restore sensitivity. As shown in Fig. 2B, dramatic sensitization to streptomycin was observed, with the MIC values decreasing in Sm6 and Sm13 from 100 μg/ml to 25 μg/ml and from 200 μg/ml to 6.25 μg/ml, respectively. The efficiency of restoration of sensitivity was lower than that observed with the wt rpsL, possibly due to the product's folding efficiency, as already mentioned. Nevertheless, these results indicate that both rpsL and rpsL-sil can efficiently restore sensitivity to streptomycin when expressed from plasmids.

A toxic compound, tellurite, efficiently replaces chloramphenicol as a selection marker. In the above experiments, chloramphenicol, under the constitutive bla promoter, was used as a selection and maintenance marker for the rpsL-encoding plasmids. However, chloramphenicol is not a dispensable antibiotic, and by using it in the proposed system, sensitivity to streptomycin is restored by forfeiting sensitivity to chloramphenicol. This outcome is less desirable than one in which drug sensitivity is restored without forfeiting sensitivities to other drugs. We therefore sought to replace chloramphenicol with a dispensable, yet efficient, selection substance. A resistance gene against tellurite (TeO$_3^{2-}$), a toxic compound, was evaluated. Tellurite is toxic to bacteria as it forms long-lived sulfur complexes, thus disrupting the thiol balance in the bacterial cells. Tellurite was once used as a treatment for microbial infections, especially for
syphilis, prior to the discovery of antibiotics (22). The tellurite-resistance genes, *tehAB*, present naturally in the *E. coli* chromosome, do not confer resistance to *E. coli* under their endogenous promoter due to low transcription (9). Upon expression from an active promoter (e.g. T7), however, the MIC of tellurite against *E. coli* increases 50- to 100-fold. The observed MIC of *E. coli* that either possess or lack the chromosomal *tehAB* genes is equal or less than 2 μg/ml, compared to a MIC of 128 μg/ml in cells harboring extrachromosomal *tehAB* with an active promoter (9). Remarkably, all of the known tellurite-resistance genes, including *tehAB*, are highly specific to tellurite, showing no cross-resistance to other compounds (22), and are therefore a relatively safe choice for our purposes. In addition, the *tehAB* genes are relatively small (~2.5 kb), making them easy to clone and genetically transfer. Linking these genes to the antibiotic-sensitizing DNA cassette would thus constitute an appropriate selectable sensitizing construct.

Plasmids encoding *rpsL*-sil or the mock gene were constructed, carrying the tellurite-resistance genes, *tehAB*, instead of the gene encoding chloramphenicol acetyl transferase. These plasmids were named pRpsL-sil-tell and pRpsLΔ4-tell. The plasmids were transformed into the streptomycin-resistant strains, Sm6 and Sm13, and the MICs of these transformed cells to streptomycin were determined. Restoration of sensitivity by tellurite-based plasmids was comparable to that observed with the chloramphenicol-based plasmids (Fig. 3). pRpsL-sil-tell sensitized Sm6 from a MIC of 100 μg/ml to 1.56 μg/ml, and Sm13 from a MIC of 200 μg/ml to 12.5 μg/ml. These results indicate that tellurite can be used instead of the chloramphenicol-resistance marker. They also demonstrate that tellurite can maintain the plasmids without cross-reactivity with the streptomycin-resistance phenotype.
Streptomycin-resistant bacteria lysogenized with phage \( \lambda \) encoding \( rpsL \) become streptomycin-sensitive. The system is thus shown to restore drug sensitivity using plasmids as a genetic delivery tool without forfeiting other drugs' efficiencies. The system is intended for transfer to resistant pathogens residing on hospital surfaces, thus rendering them treatable by antibiotics. Plasmids are efficiently transferred from host to host mainly via conjugation. However, establishing a conjugation-based system requires dissemination of bacteria harboring a conjugative plasmid, which is not desirable from either regulatory or safety points of view. We therefore evaluated the use of phages as safer delivery vehicles for the designed constructs. We chose \( \lambda \), a model phage which has been extensively studied, as a gene-delivery tool. This phage can infect its \( E. coli \) host and proceed to the lytic or lysogenic cycle. We used a common phage mutant (\( \lambda gt11 \), see Materials and Methods) which is directed to a specific cycle type according to the ambient temperature, and has a deletion (\( nin5 \)) designed to allow stable insertion of up to 5 kb of foreign DNA. This phage mutant was engineered to contain wt \( rpsL \), \( rpsL \)-sil, or a mock-\( rpsL \), each linked to the tellurite-resistance genes and designated, respectively, \( \lambda \)-RpsL-wt-tell, \( \lambda \)-RpsL-sil-tell, and \( \lambda \)-RpsLΔ4-tell (see the supplemental material for maps of the DNA inserts in the phages). One of the streptomycin-resistant strains used above, Sm13, was lysogenized with the recombinant phages and selected on agar plates supplemented with 1.5 \( \mu \)g/ml tellurite at 32°C, a temperature at which it favors the lysogenic cycle. The lysogenized bacteria were propagated and their MICs to streptomycin determined. Lysogenization of Sm13 by the phages resulted in sensitization of the resistant mutants (Fig. 4A). The MIC value for the \( \lambda \)-RpsLΔ4-tell lysogen was 200 \( \mu \)g/ml, compared to 25 \( \mu \)g/ml and 50 \( \mu \)g/ml for \( \lambda \)-RpsL-wt-tell and \( \lambda \)-RpsL-sil-tell,
respectively. Although significant, the sensitization was not as efficient as that observed using plasmid delivery.

Two copies of the \textit{rpsL} gene are significantly more efficient than a single copy in reversing resistance. We suspected that the decreased sensitization observed by lysogenization relative to plasmid transformation is due to a lower number of \textit{rpsL} gene copies introduced by the \textit{\lambda} phage. To test this and improve the sensitization, we cloned the two different \textit{rpsL} alleles (wt \textit{rpsL} and \textit{rpsL}-sil) into the \textit{\lambda} phage, designated \textit{\lambda}-2xRpsL-tell, and used it to lysogenize resistant strain Sm13 as above. Introduction of two gene copies dramatically enhanced the sensitization efficiency of the lysogenized strains, resulting in a significant decrease of the MIC from 200 $\mu$g/ml to 1.56 $\mu$g/ml, comparable to the MIC observed for the sensitive strain (Fig. 4B). As a whole, these results constitute a proof-of-principle for restoration of sensitivity to streptomycin using a phage that carries sufficient copies of \textit{rpsL}, at the "genetic cost" of a resistance marker to a toxic compound.

Nalidixic acid-resistant bacteria lysogenized with phage \textit{\lambda} encoding \textit{gyrA} show restored nalidixic acid sensitivity. The above results demonstrate that streptomycin resistance can be reversed by the proposed system. We wished to expand the proof-of-principle to other antibiotics as well, to demonstrate that a "multidrug-sensitivity cassette" can theoretically be used. We therefore chose to target quinolone resistance, which also manifests dominant sensitivity by the wt allele (12). The quinolone drug family targets the enzyme gyrase, encoded by \textit{gyrA}, resulting in DNA-replication arrest. Mutations in \textit{gyrA} are observed in a specific region termed "quinolone-resistance-determining region" (QRDR). The wt \textit{gyrA} allele is dominant sensitive and may therefore
reverse resistance (12). Nalidixic acid, the first of the synthetic quinolone family
antibiotics, was used here as a representative of the quinolone family. To test whether the
system can restore sensitivity to quinolone, we isolated spontaneous nalidixic acid-
resistant mutants by plating sensitive *E. coli* on 50 μg/ml nalidixic acid. Similar to the
isolation of the streptomycin mutants, we obtained mutants with previously reported
substitutions in the target gene, *gyrA* (Table 2). We identified five D87G substitutions
and three S83L substitutions in the *gyrA* gene product. These results corroborate another
study on pathogenic *E. coli*, which showed that 37 out of 38 isolated quinolone-resistant
*gyrA* mutants have substitutions at either S83 or D87 or both. Out of 36 pathogenic *E.
coli* resistant to high levels of nalidixic acid (MIC ≥ 256 μg/ml), 35 had at least one
mutation in *gyrA* (4). Here, as with the isolation of streptomycin-resistant mutants, the
fact that most of the spontaneous mutants are located in the target gene highlights the
potential benefit of reversing the effect of these mutations. We next introduced the wt
*gyrA* expressed from its endogenous promoter, or a control construct, both linked to
tellurite-resistance genes, into λ phages, designated λ-GyrA-tell and λ-Ctrl-tell
respectively. We used these phages to lysogenize a nalidixic acid-resistant strain, Nal2,
harboring a S83L substitution in GyrA. The lysogens were selected on 4 μg/ml tellurite
and tested for sensitization by measuring MICs as described above, using nalidixic acid
instead of streptomycin. As shown in Fig. 5, the *gyrA* construct significantly reversed the
mutant's resistance. The MIC of the resistant mutants decreased twofold when
lysogenized by a *gyrA*-encoding phage compared to the control phage. The significance
of this sensitization was corroborated by experiments in which we transformed *gyrA-
encoding plasmids into nalidixic acid-resistant mutants and observed a three orders of
magnitude decrease in the number of CFU on 50 μg/ml nalidixic acid compared to resistant cells transformed with a mock plasmid (Fig. S1). Overall, these results indicate that the proposed system can be used to target nalidixic acid resistance as well as streptomycin resistance.

**Proposed application, safety measures, and advantages of the system.** The proof-of-principle presented here is a step toward solving the major threat of emerging drug-resistant pathogens, against which we have limited new emerging antibiotic weapons. It demonstrates that with simple genetic engineering, bacteria can be resensitized to approved and useful antibiotics. It is suggested that the system be applied in a simple treatment of hospital surfaces to reverse the resistance of nosocomial pathogens. Phages against *Listeria monocytogenes* (ListShield™) and *E. coli* (EcoShield™) are used to spray ready-to-eat food and effectively target the contaminating pathogen (5). Phages are also used in the USA as effective pesticides on edible crops, among others (5). Although the above applications used non-genetically-altered phages, they support the safety and efficacy of our proposed spraying of phages on hospital surfaces. The proposed uses and advantages of the system are presented in Table 3. Extended transfer of the sensitizing cassette by specifically constructed lysogenizing phages might enrich for antibiotic-treatable pathogens on hospital surfaces. This enriched, sensitive population might then interfere with the establishment of newly introduced resistant pathogens by overtaking their ecological niche. Our approach differs from conventional phage therapy in the sense that it does not use phages to kill the pathogens directly. Consequently, there is no selection against the used phage, but rather selection for pathogens harboring the phage because it contains tellurite resistance.
Moreover, the approach avoids the use of phages inside the patient's body, thus overcoming toxicity issues and other drawbacks of phage therapy, such as phage neutralization by the spleen and the immune system (11). The presented system makes use of a temperate phage whose lytic cycle is induced at elevated temperatures (8). This feature carries added value because in the environment, as long as the temperature is below 32°C, there is only selective pressure for being lysogenized and taking up the sensitizing genes linked to the tellurite-resistance marker. However, once the pathogens are lysogenized, they are less bound to infect humans or other warm-blooded animals, because such an infection would induce the lytic cycle of the prophage and consequently kill the bacteria. This added benefit is not essential for a future product, but is an optional addition. If successful, this approach will render most of the nosocomial infections treatable by antibiotics, unlike the current situation in which most nosocomial infections are caused by antibiotic-resistant pathogens. The sensitizing cassette may be expanded to include other sensitizing genes such as thyA, conferring sensitivity in a dominant way to trimethoprim (20). Designing several antibiotic-sensitizing genes on a single construct reduces the probability of spontaneous regaining of resistance against all of these antibiotics simultaneously. We believe that the provided proof-of-principle can be applied to different pathogen-phage systems as the extensive co-evolution of phages and bacteria suggests that it is possible to find lysogenizing phages specific to any bacterium. For example, the described system could quite simply be modified to target pathogenic *E. coli* (e.g. *E. coli* O104:H4) by carrying out several selection cycles of the described λ phage on the desired host, until the phage becomes fully adapted to it. In addition, the dominant sensitivity of both rpsL and gyrA alleles has been shown to be broad across bacterial
species (e.g. (2, 12, 17). Along with the proof-of-principle, we also demonstrate a simple procedure for creating an efficient barrier against homologous recombination. The procedure includes replacement of most of the wobble bases, thus reducing the identity between genes as well as eliminating the minimal efficient processing segment for homologous recombination, without significantly affecting the translated product. This procedure can be carried out with currently available technology for basically any desired gene, as synthetic gene production has become common practice. Further manipulations to reduce the possibility of tellurite resistance being horizontally transferred without the sensitization cassette can be designed in a future product. For example, the sensitization genes can be constructed so that their presence is essential for acquisition of tellurite resistance: the sensitizing genes would be preferentially positioned before a promoterless tellurite-resistance gene, making tellurite resistance dependent on expression of the sensitizing genes. In addition to the system's benefits, which can be improved by implementing the above safety measures, these phages are simple to prepare and to apply, constituting a great advantage. Broad usage of the proposed system, in contrast to antibiotics and phage therapy, will potentially change the nature of nosocomial infections by making the bacteria more susceptible to antibiotics rather than more resistant.

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We thank Nir Osherov for critical reading of the manuscript and Camille Vainstein for professional language editing.
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REFERENCES


5. Lang, L. H. 2006. FDA approves use of bacteriophages to be added to meat and poultry products. Gastroenterology 131:1370.


TABLE 1. *E. coli* K-12 streptomycin-resistant mutants, Sm1–22, isolated on 50 μg/ml streptomycin. Substitutions in the RpsL protein are indicated, as well as the MIC to streptomycin.

<table>
<thead>
<tr>
<th>Streptomycin-resistant mutant</th>
<th>Substitution in RpsL</th>
<th>MIC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sm1</td>
<td>P42S</td>
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<tr>
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<td>800</td>
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<td>Sm22</td>
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TABLE 2. *E. coli* K-12 nalidixic acid-resistant mutants, Nal1–8, isolated on 50 μg/ml nalidixic acid. Substitutions in the GyrA protein are indicated, as well as the MIC to nalidixic acid.

<table>
<thead>
<tr>
<th>Nalidixic acid-resistant mutant</th>
<th>Substitution in GyrA</th>
<th>MIC (μg/ml)</th>
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<tbody>
<tr>
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<td>Nal8</td>
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TABLE 3. Proposed procedure for using the lysogenizing phages and the selection agent (tellurite) compared to the current cleaning and disinfecting procedures in hospitals. Advantages of this procedure are indicated.

<table>
<thead>
<tr>
<th>Surface treatment/frequency</th>
<th>Current procedure</th>
<th>Proposed procedure</th>
<th>Advantage of proposed procedure</th>
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<tr>
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FIGURE LEGENDS

FIG. 1. Plasmid (A) and phage (B) maps. The inserts are drawn to scale, with the relevant genes and genetic elements indicated. CAT - chloramphenicol acetyl transferase, conferring chloramphenicol resistance; Pamp - bla promoter, P A1* - mutated T7-A1 promoter of T7 phage. The promoter could not be cloned without a mutation, and therefore we proceeded with the following 1 bp deletion in the promoter sequence (bolded):

T7-A1: AAAAGAGTATTGACT TAAAGTCTAA CTATAGGATACT TACAGCCAT,
T7-A1*: AAAAGAGTATTGACT TAAAGTCTAA CTATAGGATACT TACAGCCAT;

rpsL Δ4 - encodes a truncated RpsL protein, rpsL-wt - encodes the RpsL protein; rpsL-sil - encodes an RpsL protein, harboring numerous silent mutations, with only 62% identity to the wt sequence; tehA/tehB - encode proteins which confer tellurite resistance; gyrA-wt - encodes the Gyrase A protein.

FIG. 2. rpsL encoded by plasmids efficiently sensitizes streptomycin-resistant mutants. Streptomycin-resistant mutants Sm6 and Sm13, transformed with a plasmid encoding wt rpsL, pRpsL-wt (A) or rpsL having multiple silent mutations, pRpsL-sil (B), become more sensitive to streptomycin as compared to mutants transformed with a plasmid encoding a mock gene, pRpsLΔ4. Serial 10-fold dilutions starting at 10^5 CFU/spot (from top to bottom) of the different mutants were spotted on plates with the indicated streptomycin concentrations. Chloramphenicol was supplemented at 35 μg/ml in all plates to maintain the plasmid. Plates were incubated overnight and photographed.
FIG. 3. Tellurite-resistance genes efficiently replace chloramphenicol acetyl transferase as a selection marker. Streptomyacin-resistant mutants transformed with a plasmid encoding wt rpsL as well as a tellurite-resistance gene, pRpsL-sil-tell, become more sensitive to streptomyacin as compared to mutants transformed with a plasmid encoding a mock gene, pRpsLΔ4-tell. Serial 10-fold dilutions starting at $10^5$ CFU/spot (from top to bottom) of the different mutants were spotted on plates with the indicated streptomyacin concentrations. Tellurite was supplemented at 1.5 μg/ml in all plates to maintain the plasmid. Plates were incubated overnight and photographed using MiniBis Pro (Bio-Imaging Systems). A representative experiment out of three is presented.

FIG. 4. rpsL genes introduced by phage λ sensitize a streptomyacin-resistant mutant. Phage λ encoding a single copy of either wt rpsL (λ-RpsL-wt-tell) or rpsL-sil (λ-RpsL-sil-tell) sensitizes a streptomyacin-resistant mutant, Sm13, compared to phage λ encoding a mock gene (λ-RpsLΔ4-tell) (A). Sensitization is significantly enhanced when the phage carries both copies of rpsL (λ-2xRpsL-tell) (B). Serial 10-fold dilutions starting at $10^5$ CFU/spot (from top to bottom) of the different lysogens were spotted on plates with the indicated streptomyacin concentrations. Tellurite was supplemented at 1.5 μg/ml in all plates to maintain the prophage. Plates were incubated overnight and photographed using MiniBis Pro (Bio-Imaging Systems). A representative experiment out of three is presented.
FIG. 5. *gyrA* introduced by phage λ sensitizes a nalidixic acid-resistant mutant. 

Phage λ encoding a single copy of wt *gyrA* (λ-*GyrA-tell*) sensitizes a nalidixic acid-resistant mutant, Nal2, compared to phage λ encoding a mock gene (λ-*Ctrl-tell*). Triplicates of the different lysogens, at $10^4$ CFU/spot, were spotted on plates with the indicated streptomycin concentrations. Tellurite was supplemented at 4 μg/ml in all plates. Plates were incubated overnight and photographed using MiniBis Pro (Bio-Imaging Systems). A representative experiment out of three is presented.
Figure 1

A

CAG

pRpsLΔ4

pRpsL-wt

pRpsL-sil

P amp

pRpsLΔ4-tell

pRpsL-wt-tell

pRpsL-sil-tell

P A1*

P A1*
Figure 2

A

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Legend:
1 - pRpsLΔ4
2 - pRpsL-wt

B

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Legend:
1 - pRpsLΔ4
3 - pRpsL-sil
Figure 3

Legend:
1 - pHpl54-tell
3 - pHpl54-sil-tell

0 1.56 3.125 6.25 12.5 25 50 100 200 streptomycin (μg/ml)

Sm6

Sm13
Figure 4

A

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Legend: 1 - λ-RpsLΔ4-tell, 2 - λ-RpsL-wt-tell, 3 - λ-RpsL-sil-tell

B

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<td>4</td>
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</tbody>
</table>

Legend: 1 - λ-RpsLΔ4-tell, 4 - λ-2XRpsL-tell
Figure 5

Legend:
1 - \( \lambda \)-Ctrl-tell
2 - \( \lambda \)-GyrA-tell

Nalidixic acid (\( \mu \)g/ml)

\[ \begin{array}{cccccccc}
0 & 8 & 16 & 32 & 64 & 128 \\
1 & 2 & 1 & 2 & 1 & 2 & 1 & 2 \\
\end{array} \]