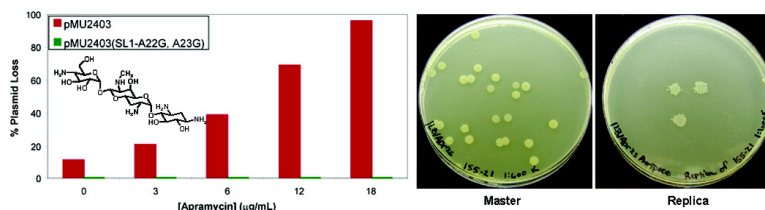


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Combating Drug-Resistant Bacteria: Small Molecule Mimics of Plasmid Incompatibility as Antiplasmid Compounds

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Multidrug resistant bacteria are now ubiquitous in both hospital settings and the larger community.¹ For example, methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), and amikacin- and β -lactam-resistant *Klebsiella pneumoniae* are common pathogens, and it was recently estimated that one-third of enterococci in intensive care units are resistant to vancomycin.² Due to this prevalence of drug-resistant bacteria, there is a pressing need for novel classes of antibacterial agents. Currently, the majority of clinically useful antibacterial drugs target one of three operations in the bacterial cell: cell-wall biosynthesis, protein synthesis, or enzymes involved in bacterial DNA replication.³ Indeed, the problem of antibiotic resistance has been exacerbated by the fact that until recently a major antibacterial agent with a novel mechanism of action had not been introduced since the fluoroquinolones in the mid-1970s. Clearly, new strategies and targets are needed to combat drug-resistant bacteria.

Many bacteria become resistant to antibiotics through the uptake of a plasmid that codes for resistance-mediating proteins.⁴ This lateral DNA transfer confers resistance on the host and accounts for the rapid dissemination of antibiotic resistance genes into diverse bacterial populations.⁵ As a consequence, significant plasmid-encoded resistance is observed clinically for the major classes of antibiotics. For example, bacteria are often resistant to β -lactams, macrolides, tetracyclines, aminoglycosides, and glycopeptides (such as vancomycin) by virtue of plasmid-encoded proteins.⁶ Plasmid-encoded antibiotic resistance also transcends bacterial genus and species, as plasmid-encoded resistance has been observed in the clinic for a variety of Gram-negative and -positive pathogenic bacteria, including both VRE and vancomycin-resistant *Staphylococcus aureus* (VRSA).⁷ If one were able to eliminate these plasmids from the bacteria, then the infections would be readily treatable with antibiotics to which the bacteria had been previously resistant.

There exists a natural mechanism for plasmid elimination from bacterial cells, a phenomenon known as plasmid incompatibility. Two plasmids are said to be incompatible with one another if the plasmids do not co-segregate into daughter cells during cell division.⁸ The net result of the presence of two incompatible plasmids in the same bacterial cell is the elimination of one of the plasmids. Indeed, plasmid incompatibility is a common way to characterize plasmids, and plasmids are often classified according to their incompatibility groups.⁹ In searching for a systematic method to eliminate plasmids from bacterial cells, we have chosen to mimic the natural process of plasmid incompatibility with small molecules. Described herein is the identification of a small molecule that mimics the features of an incompatible plasmid and causes plasmid elimination *in vivo*.

The use of small untranslated RNAs for replication control is a common theme of many replication/incompatibility systems for a variety of plasmids from both Gram-positive and -negative bacteria.¹⁰ These short stretches of RNA (often called RNA I) are

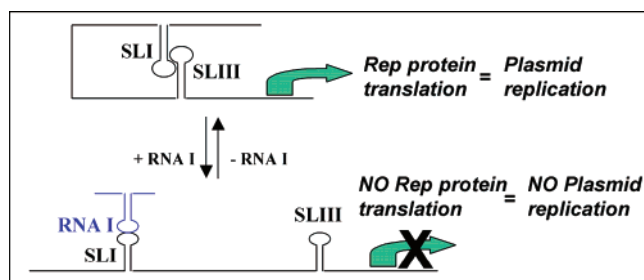


Figure 1. In many plasmid systems, Rep protein translation (hence, plasmid replication) is controlled by the reversible binding of RNA I, a small, untranslated piece of RNA; RNA I is the incompatibility determinant. Small molecules that mimic the function of RNA I should also disrupt plasmid replication and ultimately lead to plasmid elimination.

typically the incompatibility determinants meaning plasmids that carry identical (or nearly identical) RNA Is are incompatible with one another. The IncB plasmids are representative of this large group of plasmids in which a small untranslated RNA dictates incompatibility. In the IncB system, plasmid replication is ultimately controlled by the amount of the phosphodiesterase RepA that is present in the cell. RepA is a plasmid-encoded enzyme whose synthesis is tightly regulated by RNA I at the translational level (Figure 1).¹¹ When the mRNA for RepA forms an intramolecular pseudoknot between SLI and SLIII (Figure 1), translation of the RepA protein and plasmid replication occur; the exact residues involved in this pseudoknot formation are well documented.¹² When this RNA pseudoknot is disrupted by a small counter-transcript RNA (RNA I, via complementarity to SLI), then RepA translation and plasmid replication are shut down.¹³ This RNA I incompatibility determinant of plasmids in the IncB group has been well-characterized biochemically and genetically.^{11a,14}

To mimic plasmid incompatibility with a small molecule, compounds that imitate the function of RNA I (binding to the SLI portion of the RepA mRNA) were sought. Aminoglycosides and their derivatives have long been known to bind tightly to RNAs from a variety of sources.¹⁵ Furthermore, it has been well documented that aminoglycosides bind to regions of distorted RNA secondary structure.¹⁶ We therefore tested a series of aminoglycosides for their ability to bind tightly to SLI.

The direct binding of aminoglycosides to SLI was assessed *in vitro* using fluorescently labeled RNAs, a common method of assessing small molecule–RNA interactions.¹⁷ Incubation of fluorescein-labeled SLI with apramycin gave a significant decrease in the fluorescent signal (Figure 2), and a corresponding K_d of 93 nM was calculated for the apramycin–SLI interaction. In contrast, when apramycin was incubated with fluorescein-labeled SLIII, no significant change in fluorescence was observed (Figure 2). The binding of apramycin to SLI was further confirmed through RNA footprinting experiments (see below).

The site of the SLI–SLIII interaction has been previously inferred through mutagenesis studies and examination of comple-

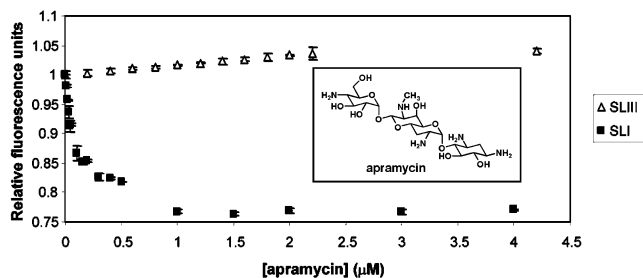


Figure 2. In vitro binding of apramycin to 5'-fluorescein-labeled SLI and SLIII. Apramycin binds to SLI with a dissociation constant of 93 nM, whereas apramycin shows no detectable binding to SLIII. Error bars represent standard deviations from the mean. The strength of the apramycin–SLI interaction was also confirmed with RNA footprinting.

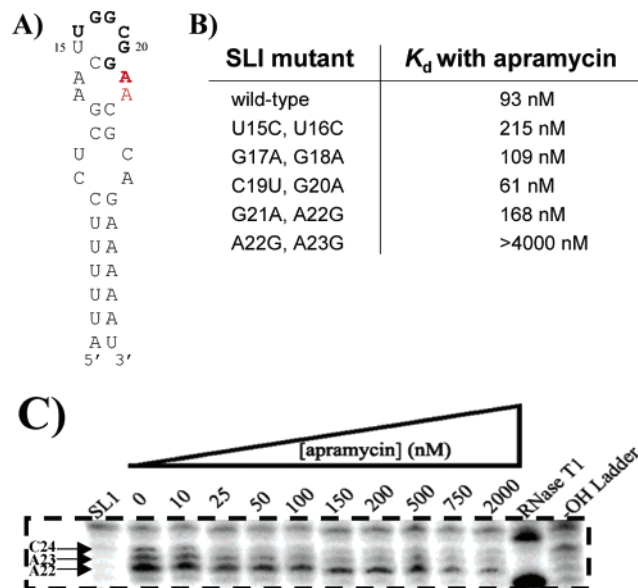


Figure 3. (A) SLI of the IncB plasmid system. Residues in bold are those that are known to bind SLIII, enabling pseudoknot formation and Rep protein translation. (B) The in vitro binding of SLI and its mutants to apramycin. 5'-Fluorescein-labeled mutants of SLI with base changes in and around the SLIII binding site were created, and their binding to apramycin was assessed. The A22G,A23G double mutant has severely diminished affinity for apramycin; these bases are shown in red in A. (C) RNase I footprint of SLI in the presence of various concentrations of apramycin. A22 and A23 are protected from RNase I digested at very low apramycin concentrations (see Supporting Information for entire gel), consistent with the mutagenesis data in B.

mentarity; those residues of SLI that bind to SLIII are shown in bold in Figure 3A.¹⁸ Thus, to assess if apramycin was binding to SLI in this important regulatory region, binding assays with a series of fluorescently labeled RNA SLI loop mutants were conducted. These assays confirmed that certain residues in and around the SLIII binding site are important for a strong SLI–apramycin interaction (Figure 3B; see Supporting Information for graphs). Specifically, residues A22 and A23 are the most critical as the A22G,A23G double mutant has a greatly diminished affinity for apramycin. In addition, an RNase I footprinting experiment confirmed the binding of apramycin to A22 and A23. In the presence of increasing concentrations of apramycin, strong protection of SLI from RNase I digestion was observed for these two residues (Figure 3C). In addition, modest protection was observed for other residues in the loop region.

To assess the antiplasmid effect of apramycin, *Escherichia coli* harboring the IncB plasmid pMU2403 (containing a nontransposable β -lactamase gene)¹³ were grown in the presence of a range of apramycin concentrations (0, 3, 6, 12, and 18 $\mu\text{g}/\text{mL}$). To obtain

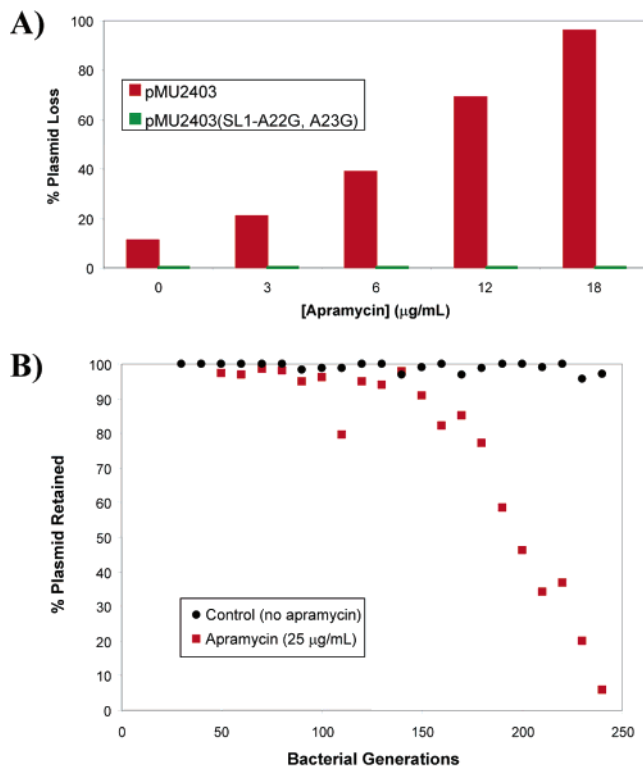


Figure 4. Apramycin causes plasmid elimination from *E. coli*. (A) At each apramycin concentration, cultures of *E. coli* JP4821 harboring the plasmid (encoding β -lactamase) were grown for 250 generations and the percent plasmid loss was determined by replica plating. The value of each point on the graph was determined from counting at least 3500 master plate colonies. The red bars show the effect of a range of apramycin concentrations on retention of the IncB plasmid pMU2403, and the green bars represent apramycin's effect on the retention of pMU2403(SL1-A22G,A23G). This plasmid contains mutations in the critical apramycin binding site on SL1. (B) The rate of plasmid loss versus bacterial generation. *E. coli* harboring the IncB plasmid pMU2403 were incubated in the presence of 25 $\mu\text{g}/\text{mL}$ apramycin, and the amount of plasmid retained was determined by replica plating.

an accurate assessment of the antiplasmid capabilities of apramycin, this experiment was performed simultaneously on 32 separate cultures for each apramycin concentration. Each culture was allowed to grow for approximately 250 generations in the presence of apramycin. Plasmid loss was assessed for each culture by first plating onto agar plates containing apramycin, followed by replica plating onto agar plates containing apramycin and ampicillin (100 $\mu\text{g}/\text{mL}$); these assessments were typically made from an average of 265 colonies for each culture; thus, for each apramycin concentration at least 8500 colonies were evaluated for the presence or absence of plasmid. Apramycin was included in the replica and master plates to ensure that the plasmid elimination pressure exerted by apramycin was maintained during analysis. The data are summarized in Figure 4A (red bars). As indicated by this graph, apramycin causes almost complete elimination of plasmid at 18 $\mu\text{g}/\text{mL}$, and a clear dose dependence of this effect is observed. Controls in the absence of apramycin showed approximately 10% loss, indicating that the plasmid is stable over time. An assessment was also made of the rate of plasmid loss. As shown in Figure 4B, the plasmid is almost completely lost within 250 bacterial generations in the presence of 25 $\mu\text{g}/\text{mL}$ apramycin; experiments conducted at an apramycin concentration of 18 $\mu\text{g}/\text{mL}$ gave similar results. Representative examples of master and replica plates are pictured in the Supporting Information.

In all cases (37 out of 37, 100%), attempts to isolate plasmid from the colonies on master plates that did not replicate failed,

confirming that the antibiotic sensitivity of these colonies was due to plasmid loss. In addition, plasmid preparations performed on a large number of colonies that did appear on the apramycin/ampicillin replica plates showed that virtually all (39 out of 40, >97%) of these colonies still contained the plasmid, indicating that resistance had not been transferred to the chromosome. Thus, apramycin causes plasmid elimination from bacterial cells; because they no longer harbor the plasmid that contains the β -lactamase gene, the bacteria are now sensitive to ampicillin.

To explore the possibility that nonspecific stress by subinhibitory concentrations of an antibiotic (apramycin) was causing the observed plasmid loss, an experiment was performed to track the stability of the IncB plasmid in the presence of erythromycin A. Like apramycin, erythromycin A is an antibacterial agent by virtue of its ability to bind to bacterial ribosomes and disrupt protein synthesis. Unlike apramycin, erythromycin A does not bind SLI of the IncB plasmid system ($K_d > 250 \mu\text{M}$, see Supporting Information). We have determined the MIC of both erythromycin A and apramycin to be $\sim 30 \mu\text{g/mL}$ with *E. coli* JP4821. When *E. coli* harboring the IncB plasmid were grown in erythromycin A (25 $\mu\text{g/mL}$) for 500 generations, virtually no plasmid loss (<5%) was observed.

A final key experiment was conducted to probe the potential general stress effect of apramycin and to connect the in vitro RNA binding data with the in vivo plasmid loss. As shown in Figure 3B, the A22G,A23G SLI mutant binds apramycin with greatly reduced affinity as compared to the wild-type SLI ($K_d > 4000 \text{ nM}$ vs $K_d = 93 \text{ nM}$). Therefore, a plasmid containing these mutations should be resistant to the plasmid elimination effects of apramycin. This double mutant was thus created in the pMU2403 plasmid to generate plasmid pMU2403(SLI-A22G,A23G). Incubation of *E. coli* harboring this plasmid with 0 to 18 $\mu\text{g/mL}$ apramycin followed by plating and replica plating revealed virtually no plasmid loss (<1%) after 250 bacterial generations (Figure 4A, green bars). This is in stark contrast to the wild-type SLI, in which almost 100% plasmid loss after 250 generations is observed in the presence of 18 $\mu\text{g/mL}$ apramycin. Therefore, the plasmid elimination effect of apramycin appears to be specific for the given plasmid sequence and not simply a result of nonspecific bacterial stress.

We have demonstrated herein that apramycin causes the loss of an IncB plasmid from *E. coli*. RNA binding experiments indicate that apramycin binds to SLI, and mutagenesis data and RNase I footprinting places the apramycin binding site in the critical regulatory region of SLI. In vivo experiments demonstrate that apramycin causes significant plasmid loss from bacterial cells. In addition, the A22G,A23G double mutant of SLI that results in a greatly diminished affinity for apramycin in vitro is also resistant to the effects of apramycin in vivo. From combined analysis of these experiments, we conclude that apramycin mimics the natural incompatibility determinant RNA I by binding to SLI, disrupting the SLI–SLIII loop–loop interaction, inhibiting plasmid replication, and ultimately leading to plasmid loss.

It is worth noting that although there are many sizes and varieties of plasmids, the mechanism used for their replication control is often similar. A small piece of RNA (such as RNA I) is commonly employed in plasmid replication control, and it typically interacts with another stretch of RNA to form a loop–loop “kissing” complex.^{10c} In addition, it has recently been recognized that the critical RNA loop–loop interactions that control plasmid replication are often mediated by the consensus sequence YUNR (Y = pyrimidine, U = uridine, N = any base, R = purine).¹⁹ In all cases this homologous region consists of the first four bases on the 5' side of the loop sequence, and RNA loops from 45 different

prokaryotic replication control elements were found to contain this consensus YUNR sequence. This similarity is present in plasmids from an array of incompatibility groups from a variety of bacterial hosts,¹⁹ and SLI from the IncB group is among the multitude of RNA loops involved in plasmid replication control that contain this YUNR consensus sequence (the YUNR sequence is from U15 to G18 on the SLI structure in Figure 3A).¹⁹ The presence of this consensus sequence suggests that such plasmids may be prone to small molecule-induced plasmid elimination through a similar mechanism. Of course, there are also non-YUNR-mediated plasmid replication systems and plasmid-encoded aminoglycoside-modifying enzymes. Strategies parallel to the one described herein would be required to effect elimination of such plasmids.

Plasmid-mediated resistance to antibacterial agents is widespread and a significant problem clinically. Incompatibility is a general phenomenon among plasmids. The demonstration herein that plasmid incompatibility can be mimicked by a small molecule and that such a molecule can resensitize bacteria to antibiotics offers a new target for combating plasmid-encoded drug resistance.

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Supporting Information Available: Full experimental protocols and characterization data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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