The Relationship between Aminoglycosides' RNA Binding Proclivity and Their Antiplasmid Effect on an IncB Plasmid†

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Received December 20, 2004; Revised Manuscript Received February 2, 2005

ABSTRACT: Bacteria routinely become resistant to antibiotics through the uptake of plasmids that encode resistance-mediating proteins. Such plasmid-based resistance is seen extensively in clinical settings and has been documented for a wide variety of bacterial infections from both Gram-positive and Gram-negative organisms. We recently reported that a small molecule could be used to mimic a natural process of plasmid elimination, called plasmid incompatibility, and that the addition of this compound causes elimination of an IncB plasmid from *E. coli* and a subsequent resensitization to antibiotics [DeNap, Thomas, Musk, and Hergenrother (2004) *J. Am. Chem. Soc. 126,* 15402−15404]. Described herein is a further substantiation and validation of the notion that plasmid incompatibility can be mimicked with small molecules that bind to important RNA targets controlling plasmid replication. In this study, the dissociation constant and stoichiometry of RNA binding are determined for 12 aminoglycosides with stem−loop I (SLI) of the IncB replication machinery. Importantly, it is found that compounds that do not bind to this RNA replication control element fail to induce plasmid loss in vivo, whereas those that do bind to the RNA typically cause measurable plasmid loss. These results highlight the potential for targeting key RNA regions for induction of plasmid loss and the subsequent resensitization of bacteria to antibiotics.

Bacterial resistance to antibiotics poses a significant threat to public health (*1*, *2*). The seven major classes of antibacterial agents can be grouped into three broad categories according to their mechanism of action: ribosome binders (tetracyclines, aminoglycosides, oxazolidinones, macrolides), cell wall biosynthesis inhibitors (β-lactams/cephlasporins, vancomycin), and inhibitors of DNA gyrase (fluoroquinolones) (*3*). Bacterial resistance to these antibiotics is a constant concern in hospital settings, and notorious bacteria such as vancomycin-resistant enterococci (VRE)[1] and methicllin-resistant *Staphylococcus aureus* (MRSA) are common nosocomial pathogens (*4*, *5*). In fact, of drug-resistant hospital-acquired infections in 2002 almost 60% were resistant to methicillin, and over 20% were resistant to vancomycin, generally regarded as the drug of last resort (*6*). Paradoxically, as drug-resistant bacteria have become more pervasive, antibacterial programs at major pharmaceutical companies have been scaled back dramatically (*6*, *7*).

Much of bacterial resistance to antibiotics has arisen through lateral (sometimes called horizontal) DNA transfer events (*8*). In lateral DNA transfer a bacterium acquires a piece of DNA from another microorganism in a process not associated with bacterial replication and cell division. This is in contrast to the standard "vertical" DNA transfer that takes place in bacterial cell division. Through lateral DNA transfer a bacterium will often obtain a piece of DNA that encodes proteins mediating antibiotic resistance. Typically such proteins either will enable the direct chemical modification of the antibiotic (e.g., aminoglycoside kinase, β-lactamase), (*9*) catalyze the chemical modification of the target of the antibiotic (e.g., erythromycin methylase) (*10*, *11*), or assist in actively exporting the antibiotic out of the cell (e.g., multidrug-resistant protein pumps) (*12*, *13*). In each of these cases, acquisition of the foreign DNA ultimately confers on the host resistance to the antibacterial agent. Although there are a number of mechanisms for lateral DNA transfer, plasmid acquisition is among the most common. (*8*) Plasmids can be easily transferred from bacteria to bacteria and have the capacity to replicate in diverse bacterial hosts, enabling the rapid dissemination of antibiotic resistance genes throughout a mixed bacterial population.

Plasmid-mediated resistance to antibiotics has been directly implicated in a multitude of clinical drug-resistant bacterial infections (*14*, *15*). Plasmid-encoded resistance to β-lactam antibiotics is ubiquitous and has been observed in a variety of bacteria including *Escherichia coli* (*16*), *Enterobacter* sp. (*17*), *Pseudomonas putida* (*18*), *Klebsiella pneumoniae* (*19*), *Salmonella* (*20*), *Vibrio cholerae* (*21*), and many others (*22*, *23*). Plasmid-mediated resistance to aminoglycosides (*24*−*27*), macrolides (*28*, *29*), and tetracyclines (*30*) is also quite common. While resistance to quinolones was once believed to be conferred solely by chromosomal mutation, plasmid-encoded resistance to this class of antibiotics was first reported in 1998 (*31*), and it is now clear that an increasing fraction of strains of quinolone-resistant *K. pneumoniae* isolated from infected patients harbor resistance-mediating plasmids (*32*, *33*). Perhaps most troubling is the recent report

† This work was supported by the National Institutes of Health (R01-GM68385) and the Office of Naval Research (N00014-02-1-0390).

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[1] Abbreviations: IncB, incompatibility group B; MRSA, methicillin-resistant *Staphylococcus aureus*; SLI, stem−loop 1; SLIII, stem−loop III; VRE, vancomycin-resistant enterococci.

10.1021/bi0473298 CCC: $30.25
Published on Web 04/13/2005

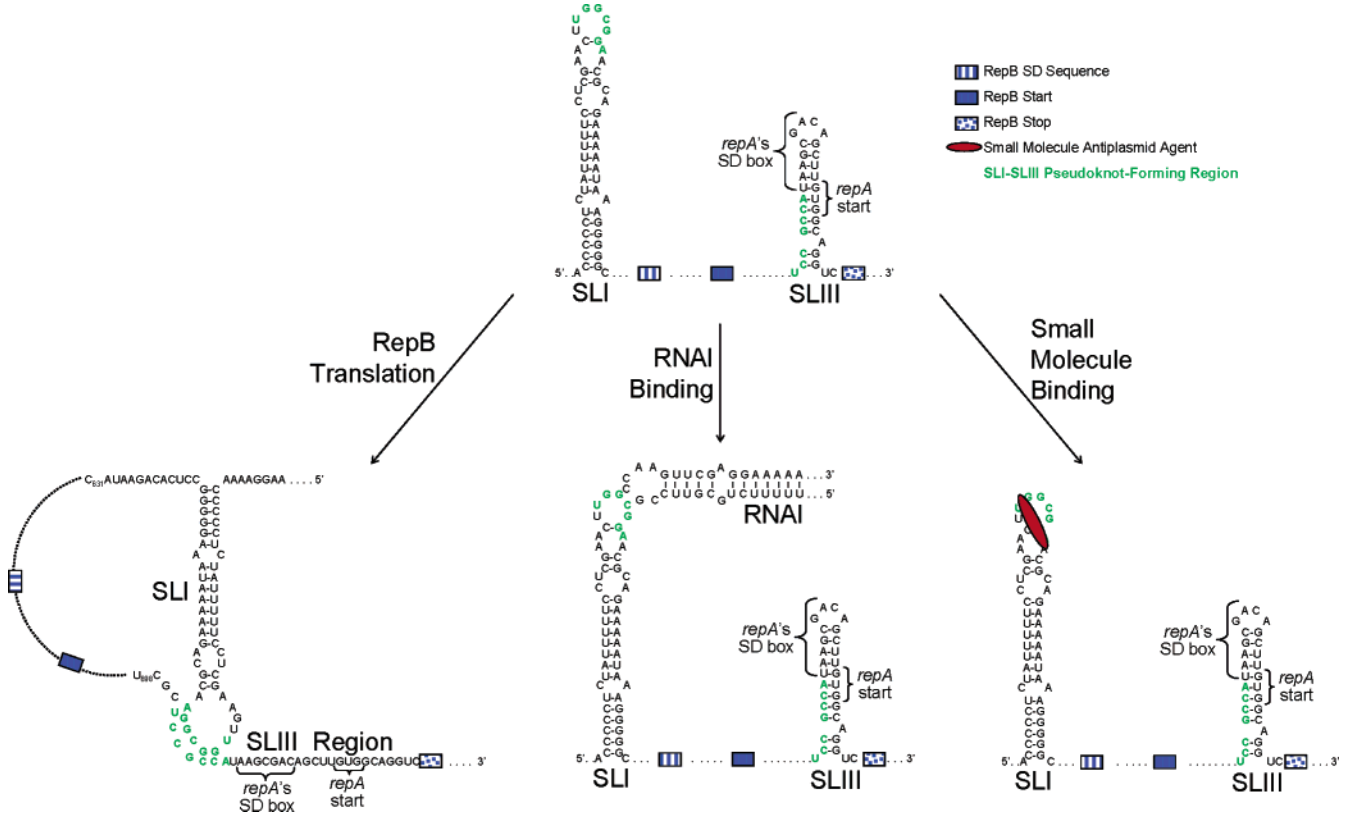

FIGURE 1: The IncB replication machinery (*47*). Translation of RepB opens SLI, allowing its formation of a psuedoknot with SLIII. The countertranscript RNAI blocks the SLI−SLIII interaction (and hence plasmid replication) largely by sequestering critical bases on SLI. A small molecule that binds tightly to SLI could also turn off plasmid replication.

that a plasmid encoding proteins mediating vancomycin resistance had transferred from *Enterococcus faecalis* to *S. aureus* (*14*, *34*). Vancomycin is the antibiotic of last resort for many infections, including methicillin-resistant *S. aureus* (MRSA); widespread resistance of *S. aureus* to vancomycin would be disastrous.

If a systematic method existed to vanquish these plasmids from bacterial cells, then the bacteria could no longer produce the resistance-mediating proteins and would be resensitized to standard antibiotics (*35*). Plasmid incompatibility, a natural mechanism for plasmid elimination, offers clues about how to develop a mechanism-based plasmid elimination agent. Two plasmids are "incompatible" if they will not stably cosegregate to daughter cells after bacterial cell division (*36*, *37*). The mechanisms for plasmid incompatibility are well documented and typically involve the use of a macromolecular "incompatibility determinant" that inhibits the replication of an incompatible plasmid and controls plasmid copy number (*38*, *39*). Incompatible plasmids have similar replication elements and thus compete with one another for the various macromolecules involved in plasmid replication. The net result of the introduction of an incompatible plasmid is that one of the plasmids is not stably replicated and is thus ultimately not passed onto daughter cells. Although incompatibility determinants can take several forms, a common theme in plasmid replication control is the use of a small, highly structured RNA as the incompatibility determinant (*38*, *40*, *41*).

Plasmids of the IncB group have been extensively studied, and their mechanism for copy number control and incompatibility have been well characterized. A detailed picture of their replication has emerged over the last 20 years (Figure 1) (*42*−*51*), and they are representative of the large group of plasmids in which small pieces of RNA dictate incompatibility and control plasmid replication. The replication of IncB plasmids is ultimately controlled by the cellular levels of the RepA protein; the production of this protein is rate-limiting for plasmid replication (*52*, *53*). Accordingly, to tightly regulate plasmid copy number, the amount of RepA produced is intricately controlled at the translational level (*48*). Upon translation of a leader peptide (RepB), stem−loop III (SLIII) of the mRNA for RepA is opened, allowing the binding of SLI and exposing the RepA Shine−Dalgarno sequence (*44*, *50*, *51*). The resulting SLIII−SLI "pseudoknot" enables ribosome binding and translation of the RepA protein (*47*). As a control on plasmid copy number, the small countertranscript RNA (RNA I) binds to SLI (*42*, *48*, *49*). This binding sequesters key pseudoknot bases, thereby inhibiting the SLI−SLIII interaction and blocking plasmid replication (*47*, *48*).

To develop a systematic, mechanism-based approach for plasmid elimination, we chose to mimic plasmid incompatibility by identifying small molecules that (like RNA I) bind to SLI. We have recently shown that the aminoglycoside natural product apramycin will bind tightly to SLI (K_d = 93 nM) in the critical SLIII binding region (*54*). In addition, this compound causes the elimination of an IncB plasmid encoding β-lactamase from *E. coli*, resensitizing these bacteria to ampicillin (*54*). Mutagenesis of SLI confirmed that strong binding of apramycin to SLI was essential for its plasmid elimination effect (*54*). Thus, apramycin mimics an incompatible plasmid and causes the elimination of plasmid from *E. coli*.

Described herein is the relationship between the plasmid-eliminating effect of aminoglycosides and their affinity for SLI. In this study, we have determined the dissociation constants and stoichiometry of binding for a number of different aminoglycosides with SLI and its mutants. In addition, we have evaluated the antiplasmid effect of these compounds on *E. coli* harboring an IncB plasmid. In general, we find a correlation between binding affinity and plasmid-eliminating ability, consistent with the notion that these compounds induce plasmid elimination by mimicking plasmid incompatibility in the bacterial cell.

EXPERIMENTAL PROCEDURES

Materials. All reagents were obtained from Fisher unless otherwise stated. All solutions were made with Milli-Q purified water. Aminoglycosides were purchased from Sigma, and all RNA was purchased with a 5′-fluorescein tag from Dharmacon Research. The Bacto-agar and Bacto-casamino acids were purchased from Becton, Dickinson and Co. The pin transfer device was purchased from V&P Scientific, Inc. The strain of *E. coli* K12 used in this study is JP4821 (*thi-1 thr-1 aroL513 leuB6 gyrA379 gal-351 lacZDM15 lacY1 tonA21 supE44 nalA hsdR*). The plasmid used in this study is pMU2403. Both were obtained from Professor A. J. Pittard (*48*).

Fluorescence Binding Assays. The ligand solutions were prepared as serial dilutions in TM_1 buffer (10 mM Tris, 1 mM $MgCl_2$, pH 7.5) at a concentration four times greater than the desired final concentration to allow for the subsequent dilution during the addition of the RNA solution. The appropriate ligand solution (25 μL) was then added to a well of a black 96-well plate (Nunc 237105), in triplicate. Refolding of the RNA was performed using a thermocycler as follows. The RNA, stored in 10 mM Tris and 0.5 mM EDTA, pH 7.5, was first denatured by heating to 95 °C for 2 min; the temperature was then lowered to 4 °C for 5 min followed by incubation at room temperature for 15 min. After refolding, the RNA was diluted to a working concentration of 37.5 nM through addition of the appropriate amount of TM_1 buffer (<5 μL added into 3000 μL of buffer). The tube was mixed by inversion, and 75 μL of the RNA solution was added to each well containing ligand. This subsequent dilution lowered the final RNA concentration to 28 nM. The fluorescence was measured on a Criterion Analyst AD (Molecular Devices) with an excitation filter of 485 $\pm$ 15 nm, an emission filter of 530 $\pm$ 15 nm, and a 505 nm dichroic cutoff mirror. Binding was allowed to proceed for 30 min to achieve equilibrium. Equilibrium was determined when three identical curve were obtained. All curves were fit to a single site model using TableCurve 2D v5.01 (equation 8108):

$$y = ax/K_d + x$$

where y is the fraction bound, x is the ligand concentration, and a is the asymptotic limit.

Job Plot. The Job plot, or method of continuous variation, requires that the total concentration of ligand and receptor be at least five times that of the K_d (*55*). In an effort to reduce the amount of RNA consumed, each Job plot was performed such that the total concentration of the ligand and RNA was kept at five times the K_d for the particular aminoglycoside−RNA pair. To a well of a black 96-well plate (Nunc 237105), 50 μL of the appropriate ligand concentration was added to 150 μL of the appropriate RNA concentration. In the fluorescence-based assay binding is determined by the change in fluorescence relative to the unbound state. Thus, each well that contains ligand and RNA must have a control well that contains only RNA at the appropriate concentration as a reference point. Each bar on the graph in Figure 4 represents the change in fluorescence observed, which was obtained by subtracting the fluorescence RNA−ligand well from the fluorescence of RNA alone well.

Determination of MIC Values. The MIC values of the aminoglycosides were determined by growing *E. coli* JP4821 containing plasmid pMU2403 diluted to 10^5 colony forming units (CFU)/mL in the presence of varying concentrations of the aminoglycosides (0, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 μg/mL) for 24 h in the wells of a 96-well plate (in duplicate) and determining the lowest concentration at which no bacterial growth occurred (as determined by OD_{600} reading). The MIC values are as follows: hygromycin B = 128 μg/mL; sisomicin = 2 μg/mL; spectinomycin = 64 μg/mL; neomycin B = 16 μg/mL; tobramycin = 4 μg/mL; kanamycin A = 16 μg/mL; kanamycin B = 8 μg/mL; butirosin B = 16 μg/mL; amikacin = 32 μg/mL; paromomycin I = 32 μg/mL; gentamicin = 4 μg/mL; apramycin = 32 μg/mL.

Growth of Bacteria in 96-Well Plates for Plasmid Elimination Determinations. A single colony from a streak plate of *E. coli* JP4821 containing plasmid pMU2403 was added to 10 mL of minimal media (MM) in a 50 mL conical tube. M63 medium was used, prepared as instructed in ref *89* except that 2× glucose was used. The culture was shaken at 225 rpm overnight at 37 °C, and the overnight culture was used to begin twelve 10 mL cultures, each containing an aminoglycoside at the given concentration (~60% of their MIC) or no aminoglycoside (for control). After growing overnight, these cultures were transferred to the wells of a 96-well plate (16 wells per aminoglycoside, 200 μL per well), and a pin transfer device was used to transfer ~200 nL from this 96-well plate into a 96-well plate containing fresh media/aminoglycoside. Sixteen wells were grown in the absence of aminoglycoside as controls. After being incubated overnight, the OD_{600} was read before pin transferring the cultures to the next plate at a ~10^6 dilution (this is the first passaging). The plasmid-eliminating ability of the aminoglycosides was evaluated at the following concentrations: hygromycin B = 70 μg/mL; sisomicin = 1.6 μg/mL; spectinomycin = 57 μg/mL; neomycin B = 8 μg/mL; tobramycin = 2 μg/mL; kanamycin A = 8 μg/mL; kanamycin B = 6 μg/mL; butirosin B = 10 μg/mL; amikacin = 12 μg/mL; paromomycin I = 18 μg/mL; gentamicin = 2 μg/mL; apramycin = 18 μg/mL.

Determination of Plasmid Loss. After 24 passagings at ~11 generations per passage (as determined by dilution plating) plasmid loss was determined by diluting an aliquot of culture from each of the wells and plating it onto a MM agar plate containing the appropriate aminoglycoside. After the colonies were allowed to grow overnight, the plates were photographed before being replicated onto plates identical to the master except for the presence of ampicillin at 100 μg/mL. After the replicas had grown, the number of colonies that did not replicate was determined visually. By totaling

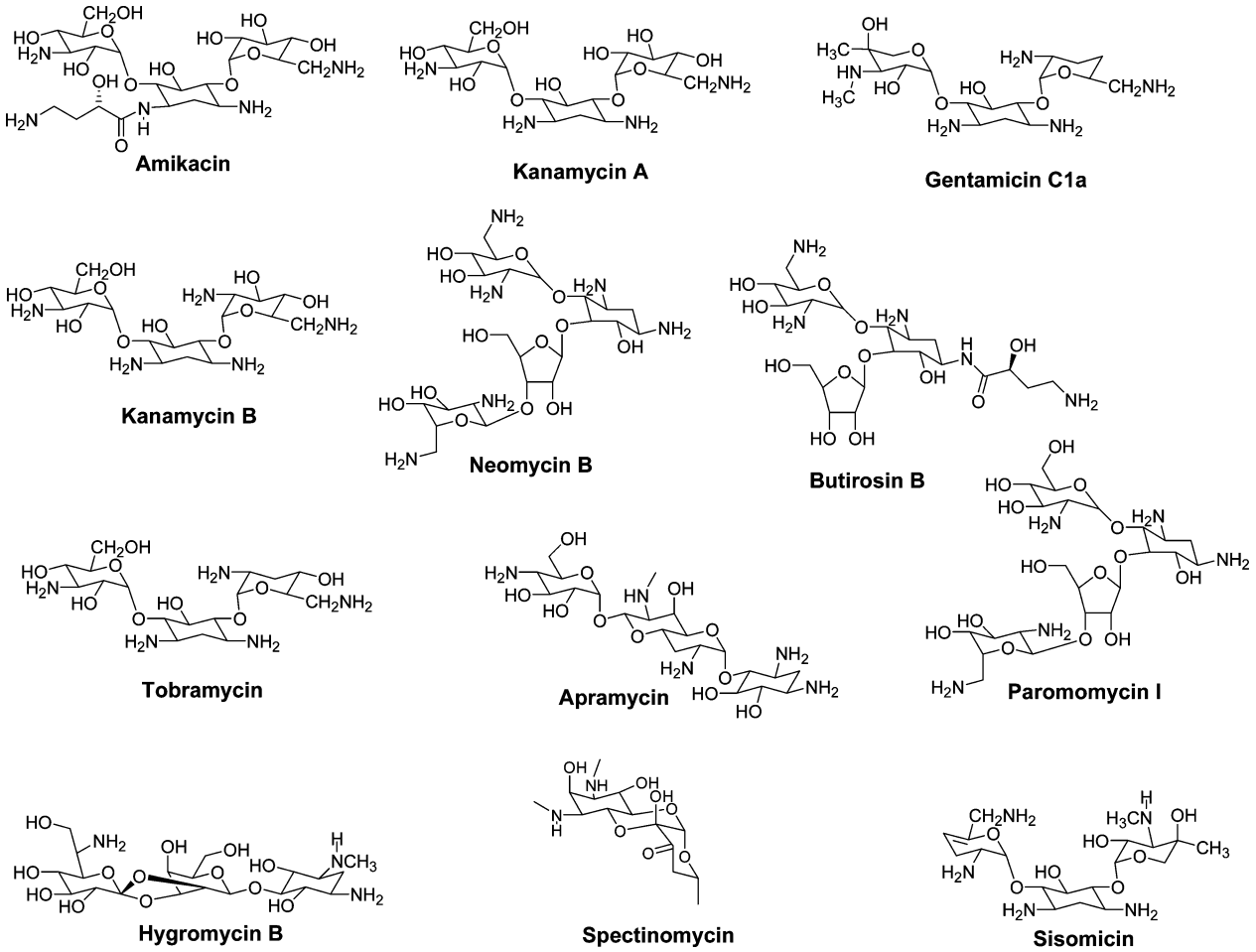

FIGURE 2: Aminoglycosides used in this study.

the number of nonreplicating colonies and dividing that by the total number of colonies, the percent loss is determined. On average, 2400 master plate colonies were counted for each determination in Figure 5.

RESULTS

Binding of Aminoglycosides to SLI. Given the recent success using apramycin to eliminate an IncB plasmid from *E. coli* and the known general proclivity for aminoglycosides to bind RNA (*56*, *57*), we investigated in detail the binding of other aminoglycosides to SLI in vitro and their ability to eliminate plasmids in vivo. Assessment of binding constants for small molecule−RNA interactions poses a considerable challenge, as there is no universal and convenient readout in a binding assay. Although binding determination methods based on the displacement of a fluorescently labeled ligand (*58*−*60*), isothermal titration calorimetry (*61*, *62*), the utilization of surface plasmon resonance (*63*, *64*), or mass spectrometry (*65*) have been described, a more general protocol is the use of a fluorescently labeled RNA. This fluorescent label can be introduced either through an unnatural RNA base or via an end label (*66*−*68*). In this method, the conformational change of the RNA upon binding to the small molecule is read out as either a quench or an increase in fluorescence (*66*). Structural and biochemical data indicate that RNA often experiences a large conformational change upon small molecule binding (*66*, *69*, *70*). In addition, the end-label method for determination of small molecule−RNA dissociation constants has been validated in control experiments (*71*),and is used broadly (*67*, *68*, *72*−*74*). Thus, the binding of aminoglycosides to SLI was assessed through the use of a 5′-fluorescein-labeled version of SLI. This fluorescein-labeled RNA was then incubated with various concentrations of the aminoglycoside, and the change in fluorescence was monitored and a corresponding dissociation constant calculated.

The structures of the aminoglycosides employed in this study are displayed in Figure 2. The structure of SLI is shown in Figure 3A, and the residues that form the pseudoknot with SLIII are shown in bold. Binding curves of SLI with two aminoglycosides are shown in Figure 3B (see Supporting Information for the remaining binding curves). As revealed in Table 1, the aminoglycosides appear to fall into two classes with respect to their affinity for SLI RNA. Two compounds (hygromycin B and spectinomycin) do not bind to the RNA within the concentration range tested and have dissociation constants greater than 4 μM. The second class of aminoglycosides (butirosin B, apramycin, amikacin, neomycin B, kanamycin A, kanamycin B, gentamicin, tobramycin, sisomicin, paromomycin I) bind to SLI with dissociation constants ranging from 0.03 to 0.17 μM. It is notable that such K_d values are quite low, even for an aminoglycoside−RNA interaction, possibly indicative of a conformation of SLI that is favorable for aminoglycoside binding. Dissociation constants for aminoglycosides and various RNAs are typically in the low micromolar range (*58*), although there are some exceptions to this (*75*).

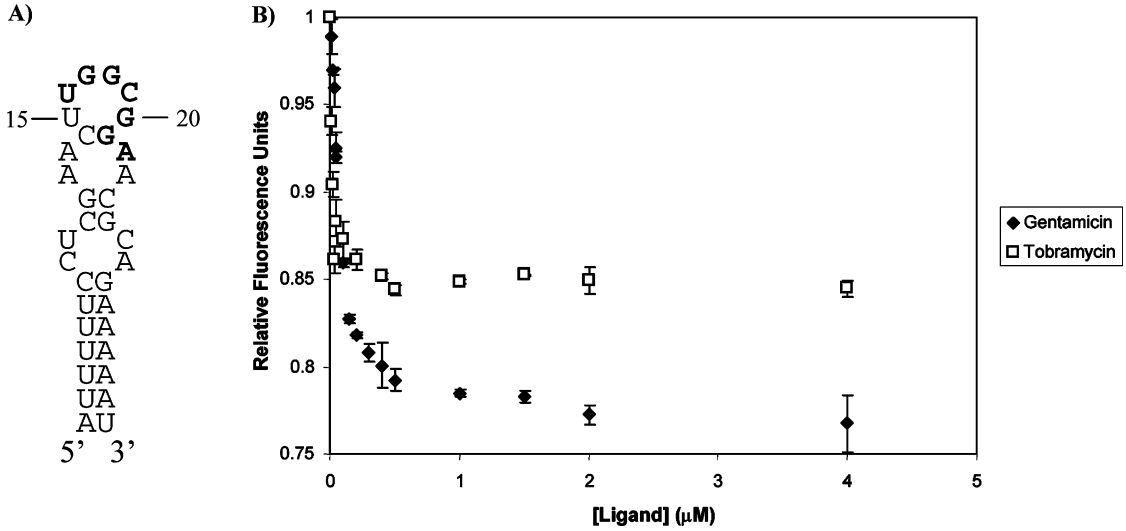

FIGURE 3: (A) Sequence and secondary structure of SLI of the IncB replication system. Residues in bold are those that are complementary to SLIII. (B) Binding of gentamicin and tobramycin to 5′-fluourescein-labeled SLI.

Table 1: K_d Values for Binding of Aminoglycosides to Fluorescently Labeled SLI and Several of Its Mutants[a]

	Fl-SLI (μM)	U15C, U16C (μM)	G17A, G18A (μM)	C19U, G20A (μM)	G21A, A22G (μM)	A22G, A23G (μM)
butirosin B	0.20	0.20	0.20	0.15	0.26	>4.00
amikacin	0.08	0.07	0.13	0.07	0.12	>4.00
neomycin B	0.08	0.07	0.05	0.05	0.03	>4.00
kanamycin B	0.11	0.11	0.10	0.10	0.13	>4.00
kanamycin A	0.17	0.29	0.12	0.30	0.24	>4.00
gentamicin	0.08	0.06	0.10	0.07	0.09	>4.00
tobramycin	0.04	0.09	0.03	0.06	0.07	>4.00
hygromycin B	>4.00	ND	ND	ND	ND	ND
sisomicin	0.03	0.04	0.04	0.03	0.06	>4.00
spectinomycin	>4.00	ND	ND	ND	ND	ND
paromomycin I	0.04	0.04	05	0.05	0.05	>4.00

[a] For the structure of SLI, see Figure 3A. ND = not determined.

Binding of Aminoglycosides to SLI Mutants. To decipher which individual bases of SLI are important for binding the aminoglycosides, mutations at positions in and around the SLIII binding site were created, and their binding to the aminoglycosides was evaluated (Table 1). We chose to create double mutations with transitions of the base pairs under evaluation (purine to purine, pyrimidine to pyrimidine). In general, the majority of these mutations had little or no effect on aminoglycoside binding; the double mutations at positions 15 and 16, 17 and 18, 19 and 20, and 21 and 22 did not significantly alter the dissociation constants of the various aminoglycosides. However, in all cases the double mutant of positions 22 and 23 essentially abolished the binding of the aminoglycosides to this stem−loop. This result is consistent with that previously observed with apramycin (*54*) and suggests that the aminoglycosides share a common binding site on SLI.

Stoichiometry of Aminoglycoside−RNA Binding. To determine the precise stoichiometry of the aminoglycoside−SLI interactions, binding was evaluated at a variety of molar ratios, and Job plots were constructed. In the Job plot, the ratios of ligand and receptor are varied while the combined concentration of the two remains constant. The observed binding is then plotted as a function of the varying ratio. The maximum occurs at the stoichiometric ratio of the ligand and receptor, that is, the maximum defines the stoichiometry of binding (*55*, *76*, *77*).

Analysis of the aminoglycoside−SLI interactions via this method revealed a 1:1 stoichiometry of binding for all compounds evaluated. The Job plots for gentamicin and tobramycin are displayed in Figure 4, and the graphs for butirosin B, amikacin, neomycin B, kanamycin A, kanamycin B, sisomicin, paromomycin I, and apramycin are in the Supporting Information. Again, analysis of the Job plot data is consistent with a common binding site for the aminoglycosides that bind to SLI.

Antiplasmid Effect of Aminoglycosides. Previously, we have shown that apramycin causes significant loss of the IncB plasmid pMU2403 from *E. coli* (*54*). In addition, a plasmid containing the A22G, A23G mutations on SLI that abolish the apramycin binding site was not eliminated with apramycin. To further define the relationship between the antiplasmid effect and aminoglycoside−SLI binding, aminoglycosides with a range of affinities for SLI were sought. As described above, we now have determined that several aminoglycosides will bind tightly to SLI, and mutagenic data indicate that they share a common binding site. As importantly, we have also identified two aminoglycosides, hygromycin B and spectinomycin, which do not bind to SLI with any appreciable affinity. Therefore, these compounds offer an excellent opportunity as controls to confirm the hypothesis that the plasmid elimination effect is linked to SLI binding propensity.

To evaluate the antiplasmid effect of the various aminoglycosides, *E. coli* harboring plasmid pMU2403 was grown for 250 generations in the presence of the compound being tested. To reach 250 generations, bacteria were grown for 10−12 generations (in the presence of the putative antiplasmid agent), at which point a small portion was transferred to fresh media. This cycle was repeated 24 times to give a total of approximately 250 elapsed bacterial generations over the course of the experiment. Plasmid loss was determined by plating bacteria onto nonselective media (containing the putative antiplasmid agent but no antibiotic) followed by replica plating onto media containing ampicillin (100 μg/mL) and the compound under evaluation. As apramycin was found to be the most effective at plasmid

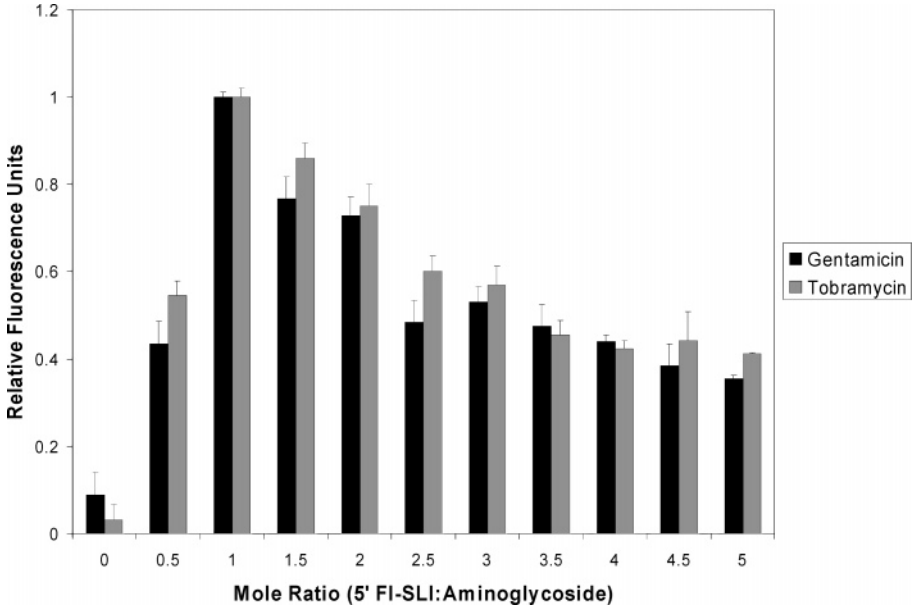

FIGURE 4: Stoichiometry of small molecule−RNA binding as revealed by Job plots.

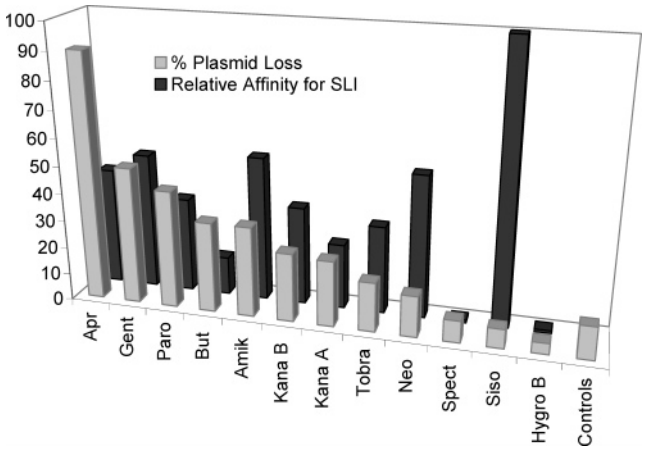

FIGURE 5: SLI-binding aminoglycosides generally cause plasmid elimination from *E. coli* harboring pMU2403. Gray bars indicate the percentage of plasmid loss after incubation with the given compound for 250 bacterial generations; percentages were determined by replica plating. Black bars indicate relative affinities of the aminoglycosides for the SLI RNA, as determined by the fluorescence assay described in the Results section. Sisomicin binds tightest, with a dissociation constant of 0.03 μM. Compounds that are unable to bind to SLI (such as spectinomycin and hygromycin B) show no appreciable antiplasmid effect. See Experimental Procedures for exact concentrations of aminoglycosides used in plasmid elimination experiments.

elimination when used at a concentration that equaled 60% of its MIC (*54*), all compounds were assessed at approximately 60% of their MIC. The MIC values for each compound are listed in the Experimental Procedures.

The data on the antiplasmid effect of the aminoglycosides are displayed in Figure 5; for reference, their relative affinities for SLI are also displayed on this graph. In general, compounds that showed affinity for SLI and could be tested at relatively high concentrations (such as paromomycin I, butriosin B, amikacin, kanamycin A, and kanamycin B) showed a significant degree of plasmid loss after 250 bacterial generations.

More striking is the correlation between the in vitro binding data and the in vivo antiplasmid effect for hygromycin B and spectinomycin. Neither of these compounds binds to SLI to an appreciable extent, and they were both unable to effect any elimination of plasmids from the bacterial cell, even at fairly high concentrations (70 and 57 μg/mL for hygromycin B and spectinomycin, respectively). These data are consistent with the notion that the strong antiplasmid effect observed for apramycin is due to its SLI-binding properties and not to some other general stress effect. Sisomicin is an outlier as it binds strongly to SLI but does not induce an appreciable plasmid loss after 250 generations of bacterial growth. This compound is also quite toxic to *E. coli*, with a MIC of 2 μg/mL, and likely does not effect plasmid elimination due to off-target effects (see Discussion below).

DISCUSSION

Mimicking the natural process of plasmid incompatibility with a small molecule is a potentially general strategy for effecting plasmid elimination from bacterial cells. The IncB group of plasmids have been meticulously characterized both biochemically and genetically and thus provide an ideal proving ground for this approach. Furthermore, there is considerable homology in the key regulatory region of SLI of IncB with plasmids from a number of other systems (*78*, *79*). Gerdes and co-workers have recently reported a systematic comparison of loops in RNA-regulated gene systems from a variety of plasmids (*78*, *79*). From this analysis the YUNR motif was found to be common in the loop region, and SLI of an IncB plasmid was one of the RNAs analyzed; the YUNR in SLI correspond to residues U15, U16, G17, and G18 of Figure 3A. Thus, compounds that are effective against one incompatibility type have potential to be more broadly applicable.

In this study we set out to determine if there was a correlation between the ability of a small molecule to bind to SLI of the mRNA for the RepA protein in the IncB system and its influence on plasmid stability in vivo. Thus, multiple aminoglycosides (compounds that have a proclivity for binding RNA) were evaluated for their ability to bind SLI in vitro. While many compounds were found to bind to SLI

quite tightly (dissociation constants of approximately 0.1 μM), two aminoglycosides (spectinomycin and hygromycin) showed no detectable binding. This discovery of aminoglycosides that do *not* bind SLI was important, as it allowed for testing the general stress of aminoglycosides on *E. coli*, in the absence of SLI binding. If aminoglycosides exerted their antiplasmid effect by induction of a simple bacterial stress response, then any aminoglycoside would be expected to have this effect. However, if the antiplasmid effect was truly a mechanism- and target-based phenomenon, then aminoglycosides that do not bind to the antiplasmid target (in this case SLI) should no longer be effective antiplasmid agents.

As detailed herein, compounds that bind tightly to SLI cause plasmid elimination to various extents; the only exception to this is sisomicin (discussed further below). Importantly, the two compounds that show no detectable affinity for SLI in vitro also cause no plasmid loss in vivo (Figure 5). These data are consistent with the notion that the aminoglycosides that do bind to SLI and cause plasmid elimination do so by binding to SLI in vivo. In this vein, we have previously shown that mutagenic elimination of the critical apramycin binding site on SLI results in a plasmid that can no longer be eliminated by apramycin (*54*); analogous experiments have shown that gentamicin also fails to effect plasmid elimination of this mutant plasmid (data not shown). The combined analysis of these experiments indicates that apramycin and certain other aminoglycosides cause elimination of the IncB plasmid by binding to SLI in a fashion that mimics the plasmid incompatibility process.

There is a rich history of testing aminoglycosides for binding to various RNA targets in vitro (*56*, *57*, *80*). Thus, there is an opportunity to compare aminoglycoside−SLI binding to that of other known aminoglycoside−RNA interactions. Most relevant to the work discussed herein are experiments in which the affinity of aminoglycosides for bacterial targets (such as the A-site of the 16S ribosomal RNA) has been assessed. In general, the aminoglycosides depicted in Figure 2 bind to the A-site of the 16S bacterial ribosomal RNA with dissociation constants ranging from 0.2 to 2.0 μM (*56*, *81*, *82*), with some variation in the values depending on the precise conditions and the method of analysis. Therefore, the affinities observed for aminoglycosides with SLI ($\sim$0.1 μM) may indeed allow for specificity in vivo and likely account for the antiplasmid, but not antibiotic, properties of the aminoglycosides at the concentrations tested.

However, aminoglycosides are known to have some promiscuity in their RNA binding (*63*). It is thus quite possible that the lack of a precise correlation between SLI binding and plasmid elimination is due to some off-target effects of the aminoglycosides. For example, sisomicin binds strongly to SLI but induces virtually no plasmid loss (Figure 5). However, sisomicin is a potent antimicrobial agent and has been reported to bind to the formylmethionyl-transfer RNA (fMet-tRNA^{fMet}) of bacteria in an extremely tight manner, with a IC_{50} of 0.7 nM (*83*); this is one of the strongest aminoglycoside−RNA interactions ever reported. Thus, for a compound like sisomicin, which has another high affinity in vivo target, even a relatively strong affinity for SLI is overridden, and no plasmid elimination is observed. As with any medicinal approach that aims to target a single macromolecule within the cell, the success of a given compound as an antiplasmid agent will be dictated both by its affinity and its specificity for SLI. Although such specificity challenges also exist for protein-based targets, the small molecule targeting of a single RNA in the cell has proven quite difficult and remains an active area of investigation (*80*, *84*, *85*). In fact, other than apramycin binding to SLI of the mRNA of RepA and exerting the antiplasmid effect, there are precious few examples of exogenously added small molecules that regulate mRNA function in vivo. On the other hand, substantial precedent for mRNA targeting exists with endogenously produced small molecules, as exemplified by riboswitches (*86*, *87*).

This mechanism-based approach to inhibiting plasmid replication and inducing plasmid loss from a bacterial population likely has benefits over other systems where simple bacterial stress causes plasmid loss (*88*). However, as even the most potent compound identified at this point requires approximately 200 elapsed bacterial generations before significant plasmid loss is observed (*54*), a validation of this approach in an animal model may require the development of more potent small molecule binders for SLI.

This study further demonstrates and substantiates the notion that plasmid incompatibility can be mimicked with small drug-like organic compounds. As plasmids are commonly classified according to their incompatibility groups, in principle, this approach could be applied to any plasmid system. Such extensions of small molecule mimics of plasmid incompatibility to other plasmids and other organisms are in progress and will be reported in due course.

ACKNOWLEDGMENT

We thank Prof. A. J. Pittard (University of Melbourne) for the gift of the IncB plasmid and *E. coli* bacterial strain. We also thank Dinty J. Musk (University of Illinois) for helpful discussions.

SUPPORTING INFORMATION AVAILABLE

Binding curves for aminoglycosides with SLI and Job plots for all aminoglycoside−SLI interactions. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI0473298