Cell

Riboswitch Control of Aminoglycoside Antibiotic Resistance

Xu Jia,[1,2,3,4,5] Jing Zhang,[1,2,3,5] Wenxia Sun,[1,2,3] Weizhi He,[1,2,3] Hengyi Jiang,[1,2,3]
Dongrong Chen,[1,2,3,*] and Alastair I.H. Murchie[1,2,3,*]
[1]Key Laboratory of Molecular Medicine, the Ministry of Education, Department of Biochemistry and Molecular Biology
[2]Institutes of Biomedical Sciences
Fudan University Shanghai Medical College, Shanghai 200032, China
[3]School of Pharmacy, Fudan University, Zhang Heng Road 826, Pudong, 201203, Shanghai, China
[4]Department of Biomedical Science, Chengdu Medical College, Tian Hui Road 601, 610083, Chengdu, China
[5]These authors contributed equally to this work
*Correspondence: aihm@fudan.edu.cn (A.I.H.M.), drchen@fudan.edu.cn (D.C.)
http://dx.doi.org/10.1016/j.cell.2012.12.019

SUMMARY

The majority of riboswitches are regulatory RNAs that regulate gene expression by binding small-molecule metabolites. Here we report the discovery of an aminoglycoside-binding riboswitch that is widely distributed among antibiotic-resistant bacterial pathogens. This riboswitch is present in the leader RNA of the resistance genes that encode the aminoglycoside acetyl transferase (AAC) and aminoglycoside adenyl transferase (AAD) enzymes that confer resistance to aminoglycoside antibiotics through modification of the drugs. We show that expression of the AAC and AAD resistance genes is regulated by aminoglycoside binding to a secondary structure in their 5′ leader RNA. Reporter gene expression, direct measurements of drug RNA binding, chemical probing, and UV crosslinking combined with mutational analysis demonstrate that the leader RNA functions as an aminoglycoside-sensing riboswitch in which drug binding to the leader RNA leads to the induction of aminoglycosides antibiotic resistance.

INTRODUCTION

The aminoglycoside antibiotics have played a historically important role in the treatment of serious bacterial infections. They bind to 16S rRNA in the decoding region of the 30S ribosomal subunit at the A site and cause mistranslation of mRNA and inhibit translocation (Davies and Davis, 1968; Fourmy et al., 1996; Carter et al., 2000). Originally isolated as natural products, they have been progressively developed to combat the spread of antibiotic-resistant infections through first and now second generation semisynthetic approaches (Armstrong and Miller, 2010). The cationic amine groups of the aminoglycosides give them the propensity to bind to negatively charged pockets in structured RNA (Hermann and Westhof, 1999), and additional binding sites have been found in rRNA (Carter et al., 2000; Borovinskaya et al., 2007). Binding sites have also been identified in the HIV *trans*-activating-region and Rev response element (Zapp et al., 1993) and in auto catalytic ribozymes (von Ahsen et al., 1991), and drug binding at such sites can induce conformational changes in the RNA (Davis et al., 2004; Murchie et al., 2004). Their potential to mold RNA structures has been exploited to generate RNA aptamers (Famulok and Hüttenhofer, 1996).

Resistance emerged shortly after their introduction and is associated with the mobile elements on plasmids or integrons responsible for transmissible multidrug resistance (Liebert et al., 1999). Integrons were originally discovered through the proliferation of antibiotic resistance (reviewed in Mazel, 2006). Aminoglycoside resistance is conferred most commonly through enzymatic modification of the drug or of the target rRNA through methylation or by the overexpression of efflux pumps (Nikaido, 2009). Enzymatic inactivation of the drugs is achieved through either N-acetylation (by acetyl transferases), O-adenylylation (by adenyl transferases), or O-phosphorylation (by phospho transferases) of amine or hydroxyl groups by specific enzymes (Figure 1A) (Mingeot-Leclercq et al., 1999). Induction of resistance genes by many classes of antibiotics has been studied for several decades (Lovett and Rogers, 1996) although the molecular details of the mechanism of induction are not yet completely understood. Resistance to the aminoglycosides is known to be inducible (Swiatlo and Kocka, 1987; Mingeot-Leclercq et al., 1999). The best characterized example of induction of ribosomal antibiotic resistance is the induction of the erythromycin resistance methyltransferase ERMC. A key feature of the *ermC* system is ribosomal stalling during the course of translation of a leader peptide through nascent leader peptide-ribosome interactions (Dubnau, 1984; Weisblum, 1995; Vazquez-Laslop et al., 2008).

Over the last decade small-molecule RNA interactions have been identified as a means of regulating gene expression. Riboswitches are regulatory RNAs that bind small-molecule metabolites and cofactors; they exploit specific interactions between low-molecular-weight metabolites and noncoding regions of messenger RNAs to regulate the biosynthetic pathway of the metabolite (Mandal et al., 2003; Nudler and Mironov, 2004;

 ©2013 Elsevier Inc.

Grundy and Henkin, 2006). They utilize a simple feedback mechanism whereby the interplay between two distinct structures in the mRNA controls the level of gene expression, in response to cellular conditions. The equilibrium between competing structures can be controlled by environmental conditions such as fluctuations in metal ion concentrations (Cromie et al., 2006; Dann et al., 2007), changes in pH (Nechooshtan et al., 2009), or temperature (Johansson et al., 2002). They show precise selectivity in controlling the expression of the biosynthetic enzymes of a range of metabolites that represent an assortment of chemical types ranging from relatively large molecules such as coenzyme-B12 (Nahvi et al., 2002) down to small amino acids like Lysine (Grundy et al., 2003; Sudarsan et al., 2003).

Here, we show that the expression of aminoglycoside antibiotic-resistance genes is controlled by a riboswitch. The aminoglycosides bind to the leader region of the aac/aad mRNA and cause a significant conformational change, leading to induction of a reporter gene. Aminoglycoside-RNA crosslinking and mutational analysis of the leader mRNA reveals the structural features that are important for antibiotic binding. We demonstrate a riboswitch mechanism of induction of aminoglycoside resistance genes in which antibiotic binding induces translation of the resistance gene.

RESULTS

A Conserved Sequence in the 5′ Leader RNA of Aminoglycoside Antibiotic-Resistance Genes

Resistance to the aminoglycoside antibiotics is most commonly achieved through modification of the antibiotic by specific enzymes (Figure 1A) (Mingeot-Leclercq et al., 1999) but can also be conferred by modification of the target site in rRNA or through excretion of the drug (Nikaido, 2009). We analyzed the 5′ leader RNA of a representative set of 50 aminoglycoside resistance genes from drug-resistant strains (Table S1A available online). Multiple sequence alignment (Larkin et al., 2007) of the 5′ leader RNA regions revealed that the leader RNA of two aminoglycoside acetyl transferase (AAC) and three aminoglycoside adenyl transferase (AAD) genes within this gene set show significant sequence identity (Figure 1B). The leader RNA has putative short open reading frames (ORFs) that encode leader peptides embedded upstream of the resistance gene, consisting of a ribosome-binding site (SD1) and start and stop codons for the leader peptides and a second ribosome-binding site (SD2) and start codon for the AAC/AAD coding sequences. The leader RNAs are predicted to adopt stable secondary structures. The sequence was found to be widely distributed upstream of aac and aad genes in the antibiotic-resistance (R) plasmids that confer multidrug resistance among a number of clinically important pathogens (Liebert et al., 1999; Hall et al., 2007; Nikaido, 2009). Leader RNA sequences from a range of organisms are shown in Figure 1C and Table S1B (the full sequence alignments are shown in Data S1). Further analysis of this RNA sequence revealed that nucleotides 1–39 were identical throughout the antibiotic-resistant strains, whereas the following nucleotides were more variable (Figure 1C). Significantly, the presence of identical nucleotides (1–39) upstream of two discrete classes of antibiotic-resistance genes, encoding an N-acetyl transferase and an O-adenyl transferase, respectively, for a number of bacterial strains suggests that there might be a common regulatory mechanism for expression of these enzymes.

The Aminoglycosides Induce Reporter Gene Expression through the 5′ Leader RNA of aac/aad

The natural promoter and transcription start site of aac in *Pseudomonas fluorescens* has been identified (Jacquier et al., 2009). There are 126 nucleotides (nt) from the transcription start site to the coding sequence of the resistance protein and 75 nt from the first SD1 to the coding sequence. To investigate whether the 5′ leader RNA of aac/aad has a regulatory role, we constructed reporter plasmids pGEX-leaderRNAaac/aad-lacZα in which the leader RNA (126 or 75 nt) was under the control of the IPTG-inducible tac promoter (Ptac) and positioned upstream of a β-galactosidase (β-gal) reporter gene. This construct does not include the AAC/AAD protein. The controllable Ptac promoter enables careful analysis of leader RNA function (Bailey et al., 2008). The reporter plasmid was transformed into *E. coli* strain JM109 and β-gal activity was examined in the presence of aminoglycoside antibiotics by agar diffusion assays. Strains containing the aac genes are typically resistant to the 4,6 deoxystreptamine aminoglycosides; kanamycin B (KanB), sisomycin, tobramycin, netilmycin, gentamycin, amikacin (Mingeot-Leclercq et al., 1999). We therefore used these drugs for agar diffusion assays. The 4,5 deoxystreptamine derivatives ribostamycin and paromomycin, and neamine, a fragment molecule were used as controls. Initially, we performed agar diffusion assays using the reporter plasmid containing the 126 or 75 nt leader RNA in the presence of IPTG and antibiotics (KanB, sisomycin, ribostamycin, and neamine). For constructs containing the 126 or 75 nt leader RNA, we observe induction of reporter gene expression with KanB and sisomycin but not with ribostamycin or neamine (Figures 2A and 2B). A more detailed investigation with all controls was therefore carried out using the reporter plasmid containing the 75 nt leader RNA. On titration of the 4,6 deoxystreptamine antibiotics a blue-green circular zone of induction is visible around the filter for KanB and sisomycin (Figure 2D), and also for tobramycin, netilmycin, gentamycin, and amikacin (Figure S1A) but not for the control molecules ribostamycin, neamine (Figure 2D), or paromomycin (Figure S1C). No induction by KanB was observed in cells transformed with the reporter plasmid without IPTG (under conditions in which the Ptac promoter is inactive) (Figure 2C) and no induction was observed on plates without KanB (Figure 2C) or the other aminoglycosides (data not shown). To verify that the induction of the reporter is specific for the leader RNA, we performed analogous experiments on a control plasmid pGEX-leader RNA-cat-86-lacZα in which the leader RNA of the cat-86 (encoding chloramphenicol acetyltransferase) (Duvall et al., 1984) gene replaced that of aac/aad gene and found this control construct to be unresponsive to addition of KanB (Figure 2C). To further confirm and quantify the agar diffusion assay, we also measured β-gal activity in solution (Zhang and Bremer, 1995) for KanB, sisomycin, ribostamycin, neamine, and paromomycin. The solution measurements of β-gal activity are in good agreement with the plate based agar diffusion assay (Figures 2E, S1B, and S1D). Significantly, these results show that the 4,6

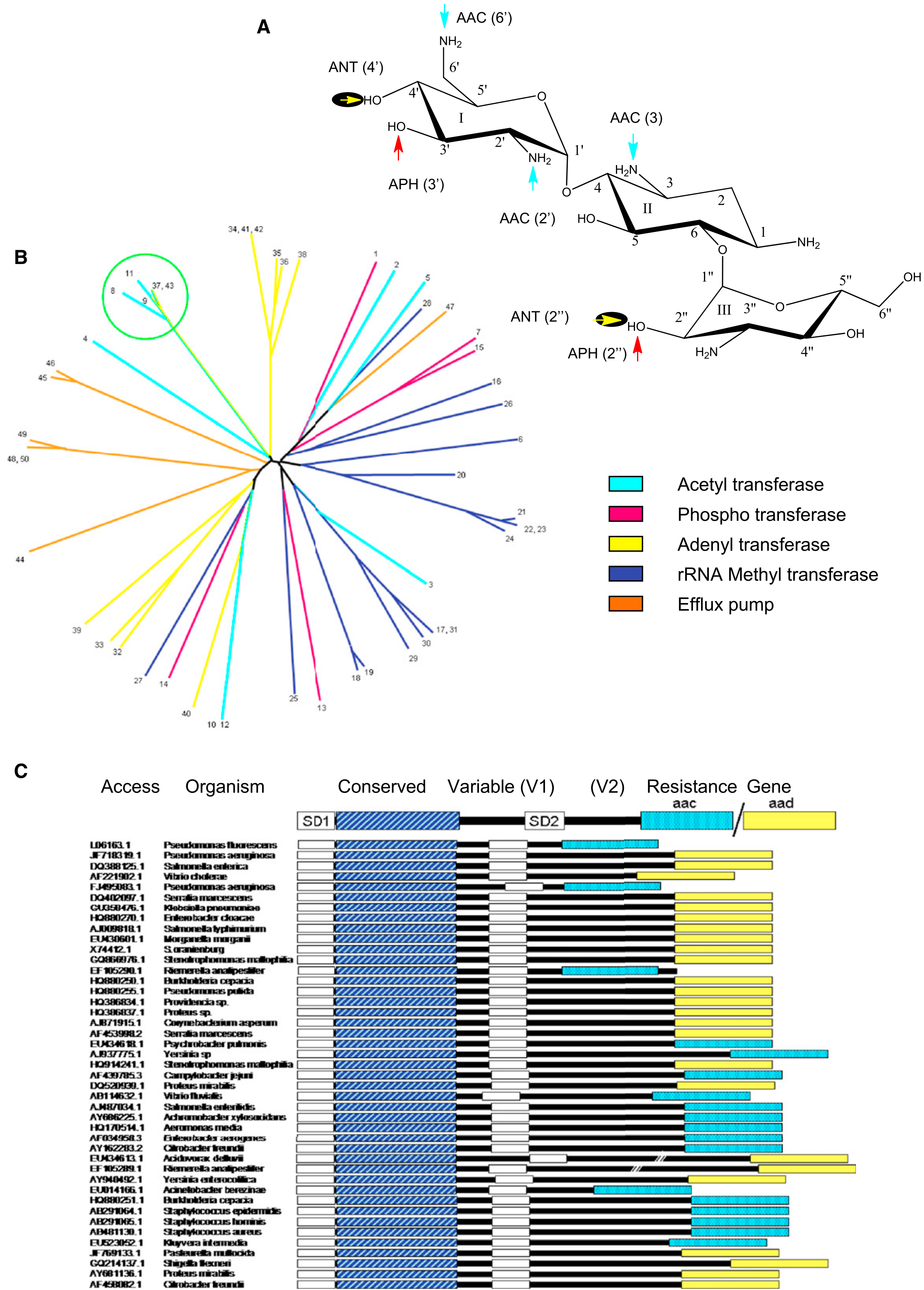


Figure 1. A Highly Conserved Sequence in the 5′ Leader RNA of Aminoglycoside Resistance Genes

(A) Structure of KanB (KanB), arrows indicate the positions that resistance acetyl transferase (AAC) (cyan), phospho transferase (APH) (red) and adenyl transferases (AAD) (yellow) modify.

(legend continued on next page)

deoxystreptamine aminoglycosides (such as KanB or sisomycin) can induce expression of the reporter gene and that the leader RNA is required for induction. However, none of the 4,5 deoxystreptamine aminoglycosides ribostamycin, neamine, or paromomycin induced the reporter gene (Figures 2D, S1C, and S1D). Thus induction of reporter gene expression requires the presence of specific aminoglycosides and the leader mRNA, suggesting that the interaction of the specific aminoglycosides with the leader RNA may have a role in the induction of the resistance protein.

The Aminoglycosides Induce a Change in the Leader RNA Structure

To investigate the secondary structure of the leader RNA, we performed in-line probing, DMS probing and selective 2′-hydroxyl acylation analyzed by primer extension (SHAPE) on the 75 nt RNA in the absence of KanB by capillary electrophoresis with fluorescence detection. The data from the three independent probing methods are shown in Figure 3B and in combination with computational folding a potential RNA secondary structure was predicted (Figure 3A). To investigate the effect of drug binding on the leader RNA structure, in-line probing was further carried out on the 75 nt RNA on titration of KanB (Figure 3C). In response to KanB titration, some nucleotides show progressively increased or decreased cleavage and nucleotide scission is generally increased at the 3′ end (where SD2 and AUG is located) and decreased at the 5′ end (SD1) (Figures 3A and 3D), suggesting that KanB induces a structural transition. In particular, on titration of KanB fragmentation at SD2 is increased, implying that KanB causes changes in the RNA structure such that SD2 may become more accessible (Figures 3C and 3D). Similar results are observed by in-line probing of the 75 nt RNA on titration of the inducing antibiotics such as sisomycin and amikacin (Figures S2B and S2C). In contrast, the pattern of fragmentation remains unchanged with control antibiotics with no increase in cleavage at SD2 (Figures 3C and S2A). DMS probing of the 75 nt RNA with KanB is also consistent with the in-line probing data (Figure S3C). In-line probing of the full-length 126 nt RNA on titration with KanB show that the fragmentation patterns between SD1 and the resistance protein start site (that correspond to the shorter 75 nt RNA) are similar to those of the 75 nt RNA, suggesting that the folding and function of the 75 nt RNA is independent of the upstream sequence (Figures S3A and S3B). In reporter assays, KanB induces reporter gene expression from both the 75 and 126 nt RNAs. The 75 nt RNA can therefore be considered a minimal functional RNA. From the in-line probing data of the 126 nt RNA, we also observed increased accessibility at SD2 (Figures S3A and S3B). These data together suggest that aminoglycoside binding to the leader RNA causes a change in the RNA structure so that SD2 may become more accessible to the ribosome.

The Aminoglycosides Bind to Specific Regions of the 5′ Leader RNA

To examine aminoglycoside-leader RNA binding directly, we used surface plasmon resonance spectroscopy (SPR) (Hendrix et al., 1997). The minimal leader RNA was prepared by in vitro transcription using T7 RNA polymerase. Biotinylated leader RNA was immobilized on an SA-biosensor chip and the binding of each aminoglycoside was measured by flowing them over the immobilized RNA. The same set of molecules was used as before. Titration of the antibiotics led to an increase in the measured response that was consistent with the formation of an aminoglycoside-RNA complex. Figure 4 shows the dissociation constants (k_D) for aminoglycoside- RNA complex formation measured by SPR (Figures 3E and S4). Note that tobramycin, KanB, and sisomycin have the highest affinity for the leader RNA at 2.19, 2.78, and 6.8 μM, respectively, and under these conditions display noncooperative-binding behavior (with Hill constants n $\sim$1) consistent with the formation of a 1:1 complex (Figures 3E and S4). In contrast, ribostamycin, neamine and paromomycin bind with lower affinities at 589 μM, 47 μM, and 12 μM respectively and ribostamycin and paromomycin may exploit a different binding mode (n $\sim$ 0.6) (Figure S4). Thus, we find that the aminoglycosides that induce reporter gene expression in the reporter assays induce a conformational change to the RNA upon binding also display the highest affinity for the leader RNA in SPR measurements. In contrast, the control antibiotics display a different pattern of fragmentation in in-line probing and have the lower affinity for the leader RNA.

Sisomycin is a 4, 6 deoxystreptamine aminoglycoside that has an unsaturated double bond between the 4′ and 5′positions of ring one (Figure 4) and that is suitable for UV crosslinking to RNA. Drug-RNA UV crosslinks can be mapped by primer extension (Porse et al., 1999). Sisomycin induces reporter gene expression (Figures 2A, 2B, 2D, and 2E), binds to the leader RNA in the μM range by SPR (Figures S4 and 4) and induces an altered RNA structure (Figure S2B). To identify the region of the leader RNA that the aminoglycosides bind, we performed UV crosslinking experiments in the presence of 100 μM sisomycin; RNA was reverse transcribed using a fluorescent primer and sequenced directly. The sites of specific crosslinks were detected by the position and incidence of abortive reverse transcripts when compared to UV-treated RNA in the absence of the drug (Porse et al., 1999), and a crosslink was identified between A18 and sisomycin, suggesting that A18 may be involved in aminoglycoside binding (Figures 3F, 3G, and 3A).

(B) Multiple sequence alignment of the leader RNA sequence; unrooted dendrogram of 50 aminoglycoside resistance genes including genes encoding AAC (cyan), APH (red), AAD (yellow), rRNA methyl transferases (blue) or efflux pumps (orange) for key see table S1. Two highly conserved AAC and three AAD genes are circled.

(C) Organization of the leader RNA of aac/aad in antibiotic-resistance strains; the highly conserved sequence and relative positions of ribosome-binding sites (SD1 and SD2), variable regions and resistance genes are marked for key see Table S2.

See also Data S1.

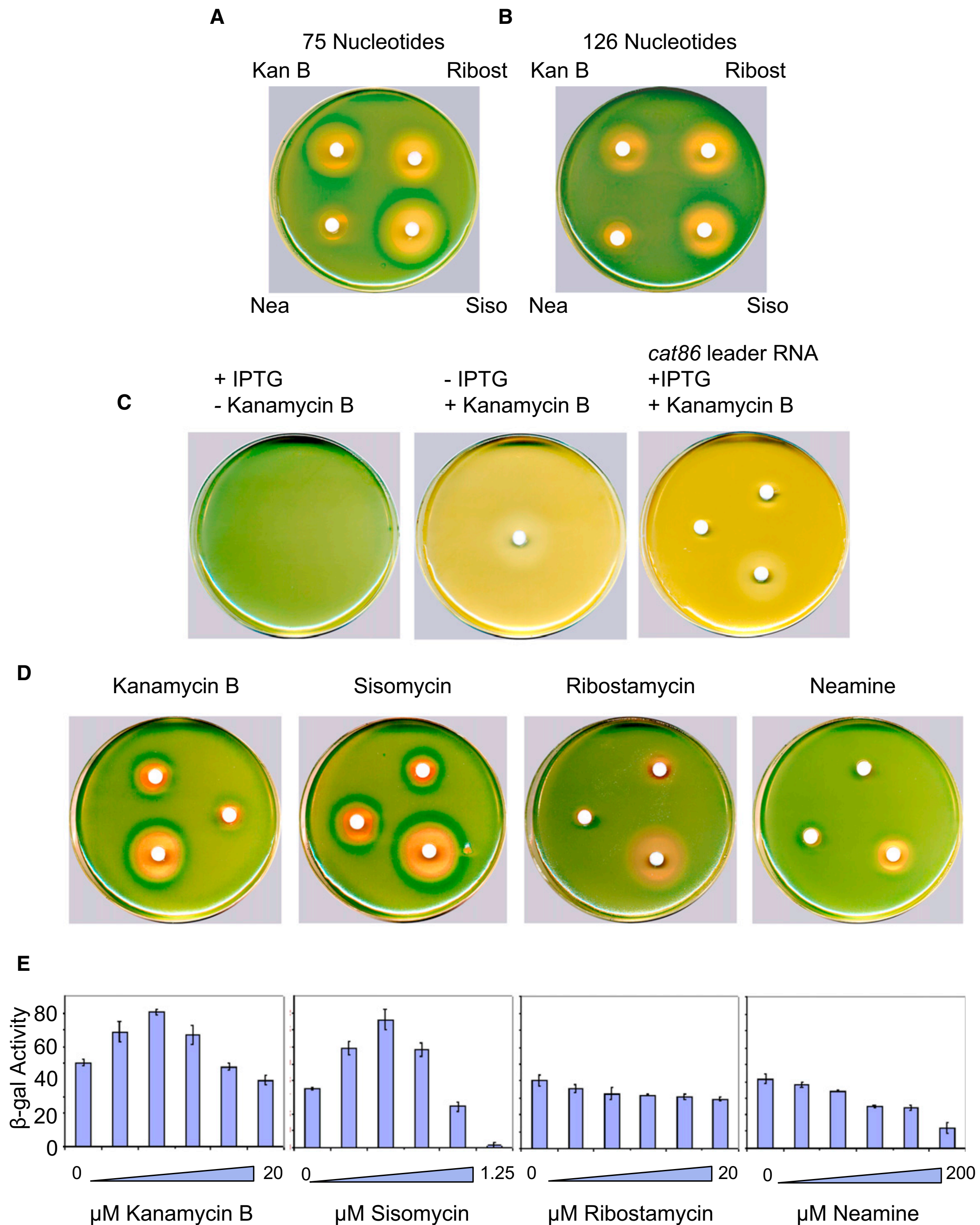


Figure 2. The Aminoglycosides Induce Reporter Gene Expression through the 5′ Leader RNA of aac/aad

(A and B) Agar diffusion assays of cells transformed with the reporter plasmid containing the 75 or 126 nt RNA grown on plates with IPTG; each filter disc was spotted with 1 µl of 100 mM KanB (Kan), sisomycin (Siso), ribostamycin (Ribost), or neamine (Nea).

(C) Control plates for (D); cells transformed with the reporter plasmid containing the 75 nt RNA grown on plate without KanB in the presence of IPTG, cells with the reporter plasmid grown on plate with 1 µl of 100 mM KanB in the absence of IPTG under conditions in which the Ptac promoter is inactive, cells transformed with plasmid pGEX-leaderRNAcat-86-lacZα that have the cat-86 leader RNA in place of aac/aad, with 1 µl of 100 mM (bottom filter [B]), 10 mM (top [T]) and 3 mM KanB (left [L]), and IPTG.

(D) Agar diffusion assay of cells transformed with the reporter plasmid containing the 75 nt RNA grown on plates in the presence of IPTG and titration of aminoglycosides. Filters were spotted with 1 µl of 100 mM (B), 10 mM (T) and 3 mM KanB (Right (R), 1 µl of 100 mM (B), 10 mM (L) and 3 mM (T) sisomycin, 1 µl of 100 mM (B), 10 mM (T) and 3 mM (L) ribostamycin, and 1 µl of 100 mM (B), 10 mM (L) and 3 mM (T) neamine.

(legend continued on next page)

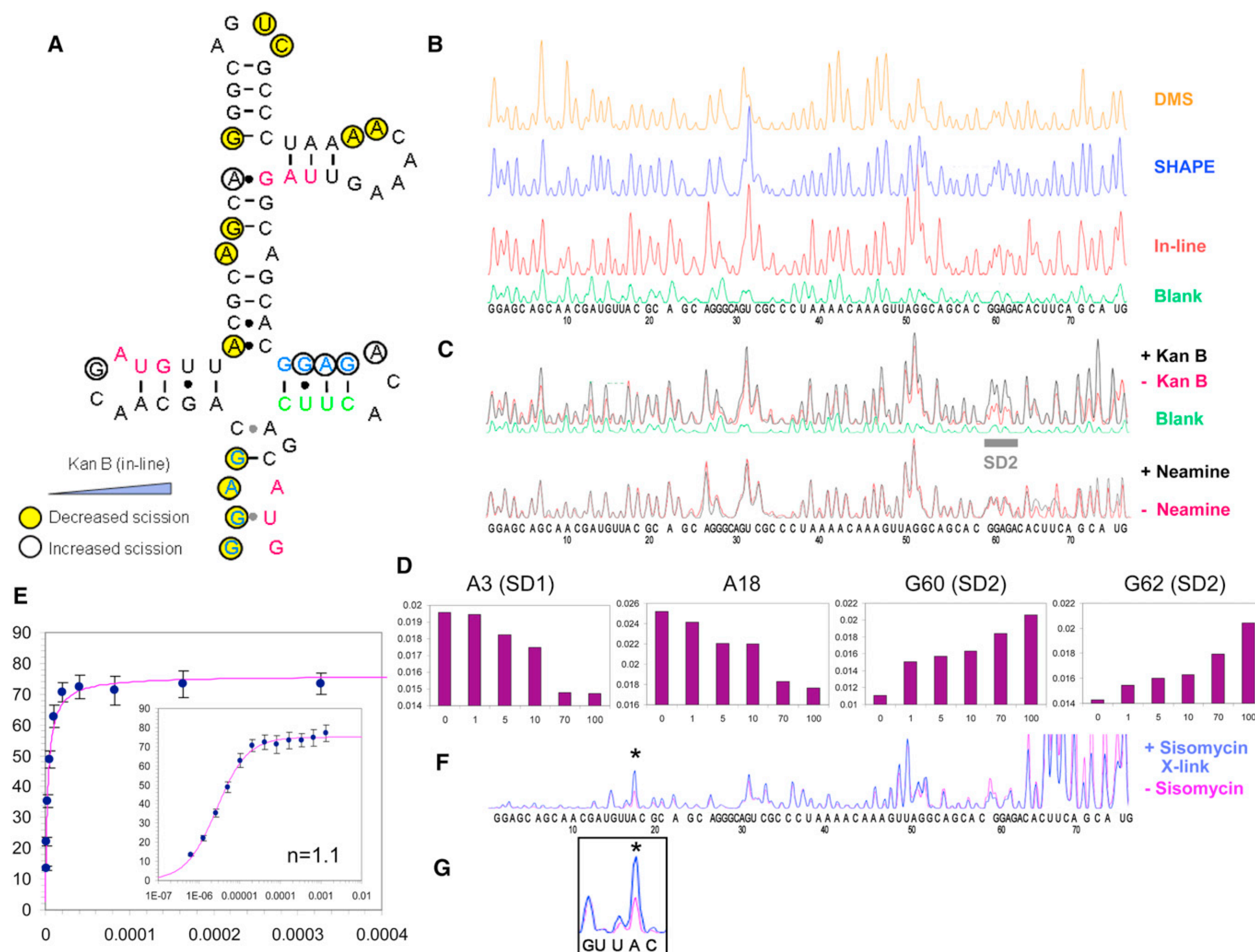


Figure 3. The Aminoglycosides Bind to Specific Regions of the 5′ Leader RNA of aac/aad and Induce a Change in the RNA Structure

(A) Predicted secondary structure of the 75 nt RNA by computational folding and structure probing analysis; the start and stop codon of the peptide and the start codon of the resistant protein are in red, SD1 and SD2 are in blue, the anti-SD is in green.

(B) DMS probing, SHAPE and In-line probing analysis of the 75 nt RNA in the absence of drug.

(C) In-line probing analysis of the 75 nt RNA ± 100 μM KanB and the control antibiotic neamine (100 μM).

(D) In-line probing analysis of position A3, A18, G60, and G62 on titration of KanB.

(E) Change in SPR signal in response units (RU) on KanB binding to immobilized aac/aad 5′ leader RNA, inset is a Hill plot of KanB binding and the Hill coefficient (n). Error bars are standard deviations of at least three independent experiments.

(F) UV crosslinking of sisomycin and the leader RNA * indicates the position of the crosslink.

(G) Enlargement of crosslink site.

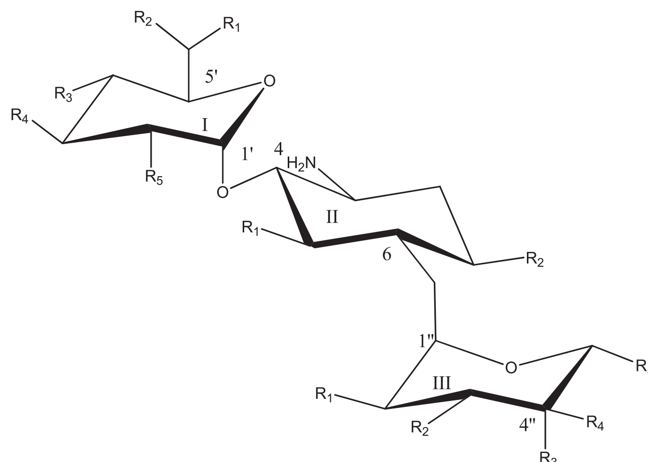
See also Figures S2, S3, S4 and Table S3.

Mutational Analysis of the Leader RNA

To investigate the importance and function of the structural elements of the leader RNA a series of deletions and mutations were introduced into the RNA (Figure 5A and 5B). The effect of these mutations on reporter gene induction in the presence of KanB was determined by reporter assays. The minimal leader RNA comprises three possible loops. For Loop1, a point mutation in Loop1 (M3) and a mutation that disrupts the stem (M5) result in a loss of reporter gene induction by KanB. For Loop2, deletion of Loop2 (M6) eliminates induction of the reporter gene. The formation of stem 2 is confirmed by the disruptive (M7) and restorative (M8) mutations in the reporter assay (Loop 2 and stem 2 are confirmed by the chemical probing data (Figures 3A and 3B). Loop 3 and stem 3 are also consistent with the chemical

(E) β-gal activity (Miller units) of the reporter gene on titration of aminoglycosides; cells were grown in the presence of 0, 2.5, 5, 10, 15, and 20 μM KanB, 0, 0.08, 0.16, 0.31, 0.63, and 1.25 μM sisomycin, 0, 0.31, 1.25, 2.5, 5, and 20 μM ribostamycin, 0, 5, 10, 50, 100, and 200 μM neamine. Error bars are standard deviations of at least three independent experiments.

See also Figures S1 and S7, and Table S2.



Ring I					Ring II			Ring III											
R1	R2	R3	R4	R5	R1	R2	6	R1	R2	R3	R4	R5	Antibiotic	Affinity	(μM) (+/-)	Charge	Induction	In-line
H	NH2	OH	H	NH2	OH	NH2		OH	NH2	H	CH2OH	OH	**Tobramycin**	2.19	(0.12)	+ 5	+	+
H	NH2	OH	OH	NH2	OH	NH2		OH	NH2	H	OH	CH2OH	**Kanamycin B**	2.78	(0.26)	+ 5	+	+
(		¥		)	OH	NH2		OH	NHMe	Me	NH2	OH	**Sisomycin**	6.80	(0.43)	+ 6	+	+
H	OH	OH	OH	NH2	Ŧ	NH2	OH						**Paromomycin**	12.20	(0.50)	+ 5	-	-
MeNH	Me	H	H	NH2	OH	NH2		OH	NHMe	Me	H	OH	**Gentamycin**	12.70	(0.27)	+ 5	+	+
(		¥		)	OH	NHCH2Me		OH	NHMe	Me	OH	H	**Netilmycin**	32.60	(1.99)	+ 5	+	+
H	NH2	OH	OH	OH	OH	#		OH	NH2	H	OH	CH2OH	**Amikacin**	35.20	(4.21)	+ 4	+	+
H	NH2	OH	OH	NH2	OH	NH2	OH						**Neamine**	47.40	(2.93)	+ 4	-	-
H	NH2	OH	OH	NH2	ribose	NH2	OH						**Ribostamycin**	589.00	(82)	+ 4	-	-

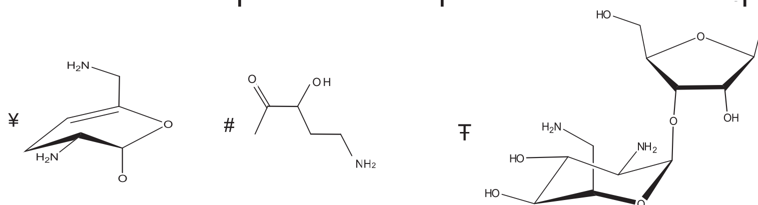
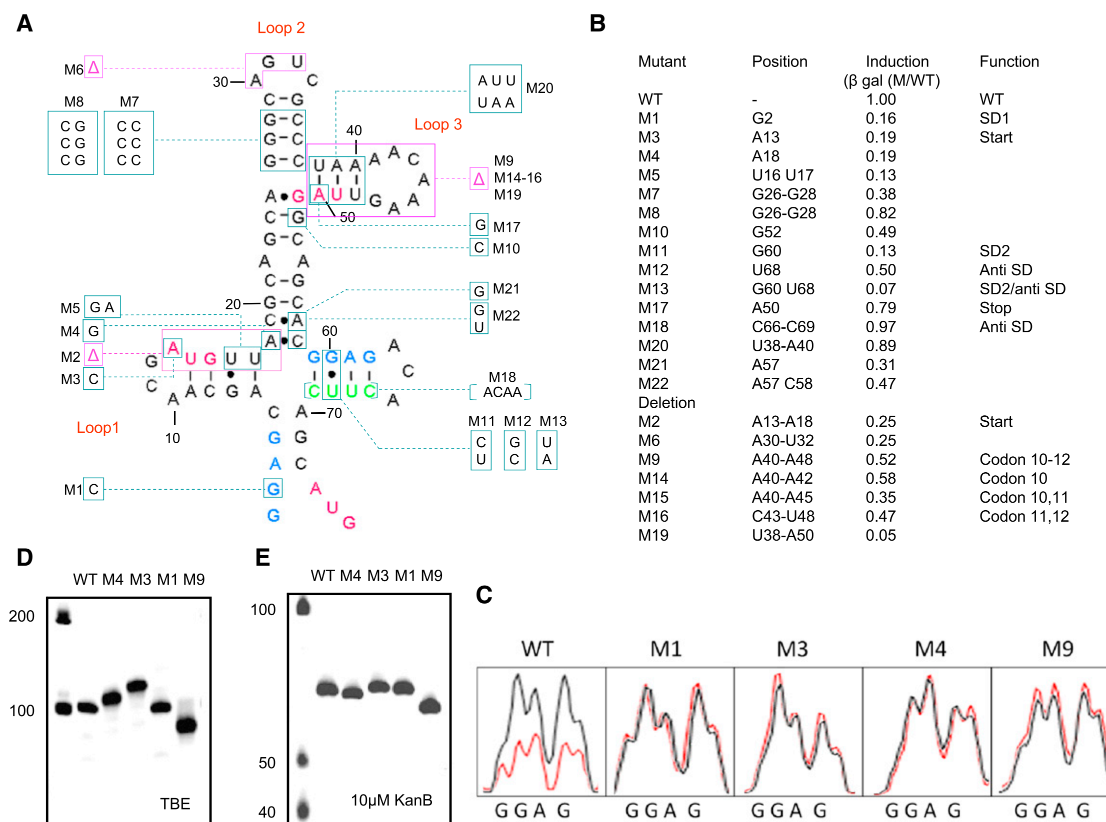


Figure 4. The Relationship between Aminoglycoside Structure, Binding Affinity, Charge, and Reporter Gene Induction
See also Tables S2 and S3.

probing data (Figures 3A and 3B). A compensatory mutation to stem 3 (M20) shows near wild-type levels of reporter gene induction and deletions to Loop 3 and stem 3 (M9, M14–16, M19) show reduced levels of induction of the reporter gene. A18 was identified as a possible binding site for the drug (Figures 3A and 3 F) by UV crosslinking, and the point mutation, M4, at A18 causes a significant reduction in induction of the reporter gene. Consistent with this, the mispaired nucleotides A18:C58 and C19:A57 appear to be critical to the function of the leader RNA; the mutations M21 and M22 that introduce Watson-Crick base pairs at these positions eliminate induction of the reporter gene. These mutational data are supported by the chemical probing data and the crosslinking data. Taken together these data show the structural and functional importance of stem loops 1–3 and specific structural features for antibiotic binding.

We observe that the ribosome-binding site SD2 (GGAG nucleotides 59–62) becomes more accessible toward chemical probes with the drugs that induce reporter gene expression. In contrast, accessibility of SD2 shows no change on addition of drugs that cannot induce reporter gene expression (Figures 3C and S2A). Furthermore, the nucleotides 66–69 (CUUC) are complementary to both SD2 and SD1 (GGAG nucleotides 1–4). We therefore speculate that in the absence of the antibiotic SD2 is sequestered by the anti-SD2 sequence blocking ribosomal access. Specific antibiotic binding induces a structural transition that allows anti-SD2 to pair with SD1 and consequently frees SD2 for ribosome binding (Figure 6). To assess this proposed model, we made some mutations to SD1 or SD2 and anti-SD2 sequence. Predictably M11 and M13, mutations in the ribosome-binding site SD2, show a significant reduction in the reporter gene expression, suggesting that function of the leader RNA is dependent on ribosome recognition and binding. The mutation M18 to the anti-SD2 that abolishes base-paring between SD2 and anti-SD2 exhibits a similar level of reporter gene expression to wild-type leader RNA with the drug. In contrast, M12, a point mutation to the anti-SD2 sequence that strengthens the base-paring between SD2 and anti-SD2 shows a reduced level of reporter gene. The point mutation M1, in SD1,



Mutant	Position	Induction (β gal (M/WT)	Function
WT	-	1.00	WT
M1	G2	0.16	SD1
M3	A13	0.19	Start
M4	A18	0.19	
M5	U16 U17	0.13	
M7	G26-G28	0.38	
M8	G26-G28	0.82	
M10	G52	0.49	
M11	G60	0.13	SD2
M12	U68	0.50	Anti SD
M13	G60 U68	0.07	SD2/anti SD
M17	A50	0.79	Stop
M18	C66-C69	0.97	Anti SD
M20	U38-A40	0.89	
M21	A57	0.31	
M22	A57 C58	0.47	
Deletion			
M2	A13-A18	0.25	Start
M6	A30-U32	0.25	
M9	A40-A48	0.52	Codon 10-12
M14	A40-A42	0.58	Codon 10
M15	A40-A45	0.35	Codon 10,11
M16	C43-U48	0.47	Codon 11,12
M19	U38-A50	0.05	

Figure 5. Mutational Analysis of the Leader RNA

(A) The positions of mutations in the leader RNA.

(B) β-gal activity of the leader RNA mutations with 10 μM KanB. Mutant RNA activity is expressed as a proportion of wild-type RNA activity. The positions of potential functional sites in the leader RNA are indicated.

(C) In-line probing analysis of the SD2 in the wild-type and mutant (M1, M3, M4, and M9) leader RNA with KanB. See also Figure S5.

(D and E) The wild-type and mutant (M1, M3, M4 and M9) leader RNAs analyzed by 10% native gel electrophoresis in TBE or with 10 μM KanB. Markers are double-stranded RNA.

See also Figures S5 and S6, and Table S3.

would prevent ribosome binding at SD1 and also destabilize the base-paring between SD1 and anti-SD2 in the presence of antibiotics and consequently hinder the structural freedom of SD2. M1 does not induce reporter gene expression. In-line probing of inactive mutants M1, M3, M4, and M9 show that addition of KanB has no effect on the RNA structure (Figure S5). On drug binding, SD2 of wild-type RNA becomes more accessible, but in M1, M3, M4, and M9, it is unchanged (Figure 5C). This is consistent with the proposed model and indicates that antibiotic mediated unmasking of the SD2 sequence is important for the function of the leader RNA.

Mutations to the leader RNA may interfere directly with drug binding or they may interfere with correct RNA folding. RNA structure changes can be detected through differences in their mobility on electrophoretic gels. We performed gel electrophoretic mobility analysis on the wild-type leader RNA, M1, M3, M4, and M9 with or without KanB. KanB induces a conformational change into wild-type and mutant RNAs. In the absence of KanB M3 and M4 are relatively retarded in the gel compared to wild-type RNA, suggesting that point mutations in M3 or M4 affect the conformation of the RNA without the drug (Figures 5D and 5E). Mutational and functional analysis confirms the main features of the secondary structure of the leader RNA and the importance of the structural elements within it for aminoglycoside binding and is consistent with the chemical probing and crosslinking data.

The Mechanism of the Aminoglycoside Dependant Induction of Gene Expression through the 5′ Leader RNA of aac/aad

We next examined how aminoglycoside-leader RNA interactions induce gene expression. Riboswitches can control gene expression at the level of transcription or translation (Breaker, 2011). To test the possibility that induction of aac/aad by KanB occurs at a transcriptional level, we analyzed mRNA abundance of the β-gal reporter on the plasmid pGEX-leaderRNAaac/aad-lacZα in response to increasing amounts of KanB by real-time PCR. Titration of KanB elicits only slight changes in β-gal mRNA abundance relative to the (AmpR) control (Figure 7A) and there is no obvious correlation between β-gal mRNA abundance

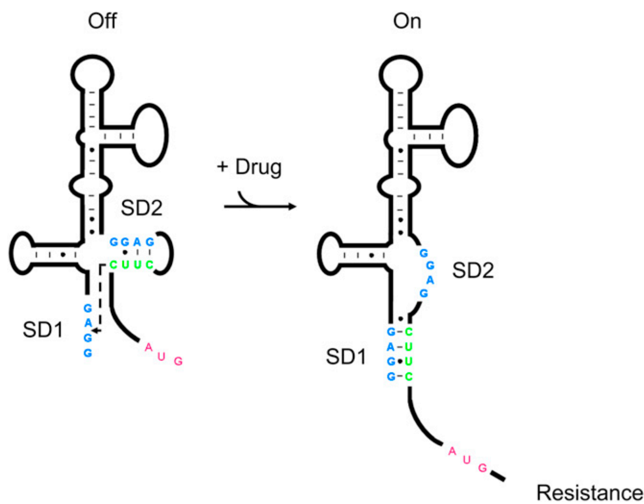


Figure 6. A Schematic Representation of the Proposed Model for the Induction of Aminoglycoside Resistance

Aminoglycoside binding to the leader RNA induces a change in the leader RNA structure such that the Anti-SD2 sequence CUUC base-pairs with SD1 consequently unmasking SD2 for ribosomal binding and translation of the resistance gene. SD1 and SD2 are colored blue, the anti-SD2 sequence is green and the start codon of the resistance gene is red.

and the KanB dependant induction of β-gal that we measure (Figures 2D and 2E). Thus, the main mechanism of regulation of aminoglycoside-dependent gene expression through drug-leader RNA interactions probably occurs at the level of translation and is in agreement with previous observations on the expression of integron encoded genes (Jacquier et al., 2009).

For the induction of the erythromycin-resistance methyltransferase ERMC, by macrolide antibiotics, the nascent leader peptide encoded by its leader RNA plays an important role in ribosomal stalling (Dubnau, 1984; Weisblum, 1995; Vazquez-Laslop et al., 2008). The leader RNA also encodes a putative short leader peptide; to investigate a possible role for the nascent leader peptide in the induction of aminoglycoside antibiotic resistance, we introduced a series of mutations to the RNA codons of the leader peptide. We made three conservative mutations (M23, M24, and M25) in which the leader RNA sequence was altered, but the amino acid sequence of the leader peptide was retained (Figure 7B). The mutants were analyzed by reporter assays (both agar diffusion and solution assays). Although the leader peptide sequence remained unaltered, we found that the mutations to the leader RNA completely abolished or greatly reduced the induction of the reporter gene (Figure 7C and Figure S6B). If the nascent leader peptide played a critical role in controlling the expression of the reporter genes, as it does with *ermC*, we would not expect to lose induction of the reporter gene in these mutants. Conversely, the mutations to the RNA overlap extensively with the region of the leader RNA that have important structural features for its function (Figure 5); thus such mutations would be expected to interfere with induction of the reporter gene. The mutations M23–M25 contain multiple nucleotide mutations; however, we note that the single-point conservative mutation that retains the peptide sequence (M4) also completely abolishes the induction of the reporter gene and displays similar levels of β-gal activity to the mutations that inactivate SD1 and SD2 (M1 and M11) of the leader RNA (Figure 5B). Additionally, a point mutation positioned after the peptide stop codon (M10) that should produce a normal leader peptide shows only intermediate instead of full levels of induction of the reporter gene (Figures 5A and 5B). The deletion mutants M9 and M14–16 also vary the position of the stop codon of the putative peptide and also have intermediate levels of reporter gene induction (Figures 5A, 5B and S6A). These data suggest that leader RNA/drug interactions have an overriding role in the regulation of induction of aac/aad expression by the aminoglycosides compared to nascent peptide stalling.

Although we have shown that the induction of the reporter gene does not depend on nascent leader peptide stalling, a single point mutation (M1) in SD1 (the ribosome-binding site for the peptide) causes a loss of induction of the reporter gene. More predictably, a single point mutation (M11) in SD2 (the ribosome-binding site of the resistance gene) eliminates induction of the reporter gene. It is noteworthy that both SD1 and SD2 are required for induction, suggesting that SD1 may have a role in the recruitment of the ribosomal small subunit that is part of the initiation complex for the translation of the resistance gene. The point mutation A13C (M3) to the putative leader peptide start codon in Loop 1 (Figure 5A) would be predicted to interfere with the initiation of leader peptide translation and is also inactive (Figure 5B). We cannot exclude the possibility that this mutation may interfere with the formation of the translational initiation complex. Overall these mutational data suggest that leader RNA/drug interactions play a dominant role in the regulation of induction by the aminoglycosides compared to nascent peptide stalling.

Antibiotic binding by the ribosome has been shown to be required for the induction of *ermC* resistance (Gryczan et al., 1984) and antibiotic binding to ribosomes can also induce conformational changes that inhibit coordinated interactions between ribosomal sites (Lentzen et al., 2003; Borovinskaya et al., 2007; Harms et al., 2008; Ramu et al., 2011). We have shown a correlation between the induction of translation and a drug dependant structural transition in the aac/aad leader RNA. However, aminoglycoside antibiotic activity is caused by drug binding at the ribosomal A site and leads to a loss in translational fidelity (Davies and Davis, 1968; Fourmy et al., 1996).

To separate the effects of aminoglycoside binding to the leader RNA from the effects of drug binding to the A site of the ribosome, we developed a system in which the ribosomal A site is protected from drug binding by methylation. The 16S rRNA methyltransferase RmtB confers resistance to 4, 6 deoxystreptamine aminoglycosides through the methylation of N7 of G1405 (Doi et al., 2004; Yu et al., 2010). We cloned RmtB into the reporter plasmid to create plasmid pGEX-RmtBleaderRNAaac/aad-lacZα; cells transformed with this plasmid are significantly more resistant to KanB (Figures 7D, 7E, and 7F) and the 4, 6 deoxystreptamine aminoglycosides (not shown), but not to ribostamycin or neamine. Without IPTG, no induction by KanB was observed and no induction was observed without KanB (Figure 7D). Induction by aminoglycosides of the reporter gene was then examined in the background of resistant ribosomes

by agar diffusion and solution based assays. Under both conditions, we observed induction of reporter gene expression with KanB and sisomycin but not for ribostamycin and neamine (Figures 7E and 7F). A much higher concentration of the drugs was required due to the presence of the RmtB genes, and this leads to a tighter zone of induction in the agar diffusion assays. Hence induction of the reporter gene can also take place in the presence of resistant ribosomes, suggesting that the induction occurs independently of antibiotic-ribosome interactions. The interaction between the aminoglycosides and the 5′ leader RNA therefore controls the induction of the resistance gene.

The aac/aad resistance genes confer resistance by acetylation or adenylation of the antibiotic. To investigate the effect of aminoglycoside modification on induction of the reporter gene, we cloned the aac (6′)-Ib, aminoglycoside acetyl transferase from *Acinetobacter baumannii* into the reporter plasmid to create the plasmid pGEX-AACleaderRNAaac/aad-lacZα. Cells transformed with this plasmid are resistant to KanB and sisomycin (Figure 7G). Agar diffusion and solution-based assays were performed with this resistant strain and the antibiotics (KanB, sisomycin, ribostamycin, and neamine) with controls as described before (data not shown). We observed induction of reporter gene expression on addition of KanB and sisomycin (Figures 7G and S6), but not for ribostamycin and neamine, suggesting that acetylated aminoglycosides may still induce expression of the resistance protein.

DISCUSSION

The data presented here shows riboswitch control of the induction of the AAC and AAD aminoglycoside resistance genes through direct interactions between the RNA and the drug, in which drug binding induces expression of the resistance protein. To support this, we show that the presence of aminoglycoside antibiotics induces a structural transition in the 5′ leader RNA of the aac/aad genes that confer resistance to the antibiotics. The aminoglycosides bind to the RNA with a range of affinities that correlate with the induction of reporter genes under the control of the leader RNA. Mutations to the leader RNA that abolish the induction of reporter genes appear to adopt a different structure. Induction of resistance gene expression is consistent with an aminoglycoside induced conformational change in the leader RNA that unmasks the ribosome-binding site of the resistance gene. Drug binding within the leader RNA is associated with a sequence that is highly conserved across an assortment of antibiotic-resistant pathogens. Taken together, these results are consistent with a riboswitch model for gene regulation whereby small-molecule binding to an aptamer domain modulates expression of the gene leading to the induction of antibiotic resistance.

The aptamer domain of this antibiotic-sensing riboswitch is within the highly conserved nucleotides (1−39) of the leader RNA. Functional analysis of mutant RNAs combined with UV crosslinking and conformation-sensitive nucleotide resolution chemical probing confirms the main features of the secondary structure of the leader RNA and the importance of the structural elements within it for aminoglycoside binding. The drugs may bind directly to A18. Drug binding to the leader RNA causes a significant structural transition in which the ribosome-binding site GGAG (SD2) appears to become more accessible for the ribosome, leading to induction of the reporter gene.

As a riboswitch the aac/aad RNA has a number of features that are common to other well-characterized RNA regulatory mechanisms that control synthesis of the target protein by regulating access to the ribosome-binding site (Grundy and Henkin, 2006). For example the leader RNA of the transcriptional regulator *PrfA* that controls expression of virulence genes in *Listeria moncytogenes* acts as a thermosensor in which the ribosome-binding site is masked at low temperatures. An increase in temperature (on infection) liberates the ribosome-binding site leading to expression of PrfA and consequent virulence (Johansson et al., 2002). The leader RNA of the erythromycin resistance methyltransferase ERMC and the chloramphenicol acetyltransferase (Dubnau, 1984; Weisblum, 1995; Lovett and Rogers, 1996) incorporates an additional ribosome-binding site and encodes a short leader peptide. Induction of resistance to both of these ribosomal antibiotics exploit ribosome-RNA interactions through translational stalling at the nascent leader peptide prior to the initiation of resistance protein synthesis from the second ribosome-binding site (Vazquez-Laslop et al., 2008). Ribosomal attenuation also regulates the *tnaC* cistron of tryptophan catabolizing enzymes (Gong and Yanofsky, 2002). Induction of *ermC* resistance also requires ribosomes that are sensitive to the drug (Gryczan et al., 1984). The untranslated region of the ribosomal protein S15 contains an autoregulatory binding site for S15 that downregulates synthesis of the protein through the stabilization of an alternative fold in the RNA (Ehresmann et al., 2004) that blocks full access to the ribosome-binding site (Serganov et al., 2003) such that the RNA protein complex is entrapped in a blocked preinitiation complex with the ribosome (Marzi et al., 2007).

The aac/aad aminoglycoside-sensing riboswitch may be seen to have appropriated certain features from these systems. There are close parallels with the thermosensor RNA (Johansson et al., 2002), in which conformational changes expose the ribosome-binding site leading to the expression of virulence proteins. Although the aminoglycosides differ from macrolides and phenicols in their ribosomal target sites and mechanisms of antibiotic action, they employ similar mechanisms for the induction of resistance through ribosome binding to the leader RNA (Dubnau, 1984; Weisblum, 1995; Lovett and Rogers, 1996; Vazquez-Laslop et al., 2008). The aac/aad system, however, may have dispensed with the necessity for leader peptide stalling. The requirement for a leader RNA ribosome-binding site (SD1), suggests that ribosome binding to this region is important for antibiotic dependant induction and that SD1 continues to function as an access point into the aac/aad RNA for the assembly of the translational initiation complex. In contrast to *ermC* (Gryczan et al., 1984), ribosomes that are resistant to the aminoglycosides are also capable of inducing resistance through the aac/aad riboswitch. A conformational change in the aac/aad RNA that unmasks SD2 would be similar to that observed for the S15 leader RNA (Serganov et al., 2003) with the corollary that for S15 the structural transition masks SD1. Analogous ligand-dependent screening of ribosome-binding sites is also

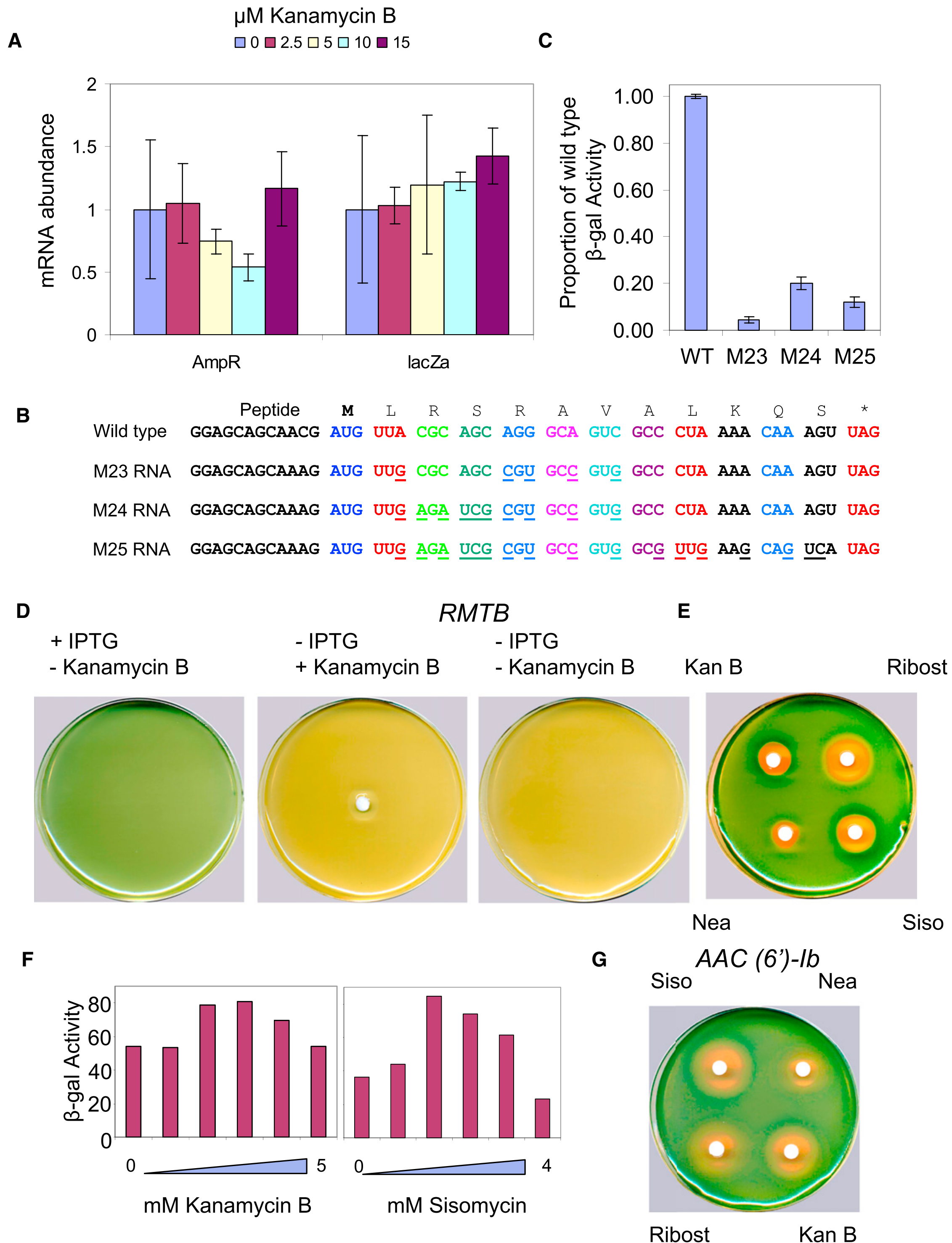


Figure 7. The Mechanism of the Leader RNA Activity

(A) Real-time PCR detection of β-gal mRNA transcripts on addition of the indicated amounts of KanB relative to AmpR as an internal control. Error bars represent the standard deviation of three independent experiments.

(B) Sequence of the wild-type leader RNA and the three mutants M23-25 (mutations underlined). The leader RNA was altered, whereas the leader peptide sequence remained unchanged.

(C) β-gal activity of M23-25 with 5 μM KanB. Mutant RNA activity is expressed as a proportion of wild-type (WT) RNA activity. The error bars correspond to the standard deviation of three independent experiments. See also Figure S6B.

(legend continued on next page)

employed by flavin mononucleotide, S-adenosyl methionine (S [MK]) and the thymine pyrophosphate dependant regulatory riboswitches (Mironov et al., 2002; Winkler et al., 2002; Fuchs et al., 2006). Thus, the aac/aad riboswitch appears to have assimilated a number of regulatory RNA features to fit the particular requirements for the induction of resistance to aminoglycoside antibiotics.

An antibiotic-resistant riboswitch must be able to detect low levels of antibiotic and activate the resistance mechanism before the cells are killed. The aac/aad riboswitch demonstrates progressive induction of reporter genes in response to sublethal doses of the antibiotic. Expression of the resistance gene is delicately balanced; both of the aac/aad reporter constructs display elevated background levels of reporter gene expression in the absence of KanB. Intriguingly, although the acetylation of the drugs blocks their interaction with the ribosome and confers antibiotic resistance, the modified drugs can still induce reporter gene expression. This suggests that, to restrict the build up of unmodified drugs in equilibrium with modified drugs in the cell, this riboswitch acts to maintain high levels of the resistance protein as the modified drugs accumulate. The aminoglycosides are highly efficient inhibitors of translation and cells would be sensitive to even low levels of unmodified drugs. This mechanism allows the cells to respond rapidly to an antibiotic threat and minimizes the commitment of cellular resources to the production of resistance protein. We speculate that the simplicity of this mechanism of activation of antibiotic resistance might be exploited by other RNA-binding antibiotics.

The conservation and wide distribution of the RNA aptamer domain sequence of this riboswitch is noteworthy. They are a constituent of the integron cassette system that accumulates resistance genes for the resistance (R) plasmids that confer multidrug resistance (Liebert et al., 1999; Hall et al., 2007; Nikaido, 2009). The R plasmids are stably maintained within host bacterial strains from which they can be efficiently transferred to other drug-sensitive cells. The accumulation of multidrug resistance on R plasmids is a significant clinical threat (Taubes, 2008). Riboswitches have previously been characterized as chromosomal regulatory elements (Mandal et al., 2003; Nudler and Mironov, 2004). The presence and stability of an antibiotic-sensing riboswitch found as an integral part of a multiple copy, transmissible plasmid suggests that they have an important role in antibiotic resistance. Riboswitches have been regarded as relics from an RNA world (Joyce, 2002). The propagation of this novel riboswitch throughout pathogenic bacteria over the relatively short time-scale of the last 60 or so years, coupled with the recently established role of riboswitches in fundamental cellular processes, suggest that novel riboswitch functions will continue to emerge.

EXPERIMENTAL PROCEDURES

Detailed protocols for all sections are described in Extended Experimental Procedures.

5′ Leader RNA Sequence Analysis

Sequences of five representative sets of bacterial genes that confer resistance to the aminoglycoside antibiotics were obtained from NCBI. These included acetyl transferase, phospho transferase, adenyl transferase, rRNA methyl transferase, and efflux pump genes. The 5′ leader RNA sequences were used for multiple sequence alignment. This analysis identified a series of homologous sequences within the gene sets and further sequence alignments identified them to be present within antibiotic-resistant pathogens.

Generation of Reporter Constructs

The reporter plasmid pGEX-leaderRNAaac/aad-lacZα (75 or 126 nt RNA) was constructed, with an IPTG-inducible Ptac promoter and the aac/aad leader RNA upstream of a lacZα gene and transcription terminator; the derivative pGEX-leaderRNAaac/aad-RmtB-lacZα contains the RmtB gene (Yu et al., 2010) and pGEX-AAC aac-lacZα contains the AAC gene (Figure S7).

Agar Diffusion and β-Galactosidase Assays

Discs of 3MM paper spotted with different amounts of antibiotic were placed onto agar plates inoculated with pGEX-leaderRNAaac/aad-lacZα (75 or 126 nt RNA) transformed cells and incubated at 37°C for at least 18 hr (Bailey et al., 2008). β-Galactosidase assays were performed as previously described (Zhang and Bremer, 1995).

Surface Plasmon Resonance Spectroscopy

In vitro transcripts of aac/aad RNA (75 nt) were 3′ end labeled with biotin (Wu et al., 1996). Aminoglycoside-RNA binding was measured by SPR (Hendrix et al., 1997).

Chemical Probing of RNA

The wild-type leader RNA or mutant transcript RNA with 3′ and 5′ linkers was subjected to in-line probing, DMS probing, and SHAPE analysis in the presence or absence of different amounts of the aminoglycosides, modified RNA was detected by primer extension; fluorescent reverse transcripts were analyzed by capillary electrophoresis with fluorescence detection.

Native Gel Electrophoresis

Transcribed RNA samples were electrophoresed in 10% polyacrylamide gels in TBE with buffer circulation in the presence or absence of 10 μM KanB.

Crosslinking Analysis of Sisomycin with RNA

Sisomycin and leader RNA with 3′ and 5′ linkers were mixed in HBS buffer and UV irradiated at 254 nm for 15 min. An RNA sample with no added sisomycin was prepared in parallel as a control. The presence of crosslinks between RNA and sisomycin was measured by the position of abortive reverse transcripts compared to the UV-treated control RNA in the absence of sisomycin and reverse transcripts were analyzed by capillary electrophoresis with fluorescence detection.

(D) Control plates for (E); cells transformed with the plasmid pGEX-RmtB leaderRNAaac/aad-lacZα grown on plate without KanB in the presence of IPTG, in the absence of IPTG (when the Ptac promoter is not active) with KanB and without both IPTG and KanB.

(E) Agar diffusion assay of cells transformed with plasmid pGEX-RmtB leaderRNAaac/aad-lacZα grown in the presence of IPTG and the aminoglycosides. The drugs used on the plates are KanB (10 μl 1 M), sisomycin (10 μl 1 M), neamine (5 μl 100 mM) and ribostamycin (5 μl 100 mM).

(F) β-gal (Miller units) activity of pGEX-RmtB leaderRNAaac/aad-lacZα in the presence of 0, 1.0, 3.5, 4, 4.5 and 5 mM KanB and 0, 0.13, 0.25, 0.5, 1, and 4 mM sisomycin.

(G) Agar diffusion assay of cells transformed with plasmid pGEX-AAC-aac-lacZα grown with IPTG and the aminoglycosides. Filters were spotted with: KanB (10 μl 1 M), sisomycin (10 μl 1 M), neamine (5 μl 100 mM) and ribostamycin (5 μl 100 mM).

See also Figures S6 and S7, and Tables S2 and S3.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, three tables and one data set and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.12.019.

ACKNOWLEDGMENTS

We thank Professor Eric Westhof (University of Strasbourg) for constructive and insightful discussions. We thank Drs Di Qu, Ming Gui Wang and Jin Wei Huang (Fudan University) for the gift of the RMTB plasmid and *Acinetobacter baumannii* strain. This work was supported by National Key 973 grant (2010CB912602). City of Shanghai Key project grant (09DJ1400601). Natural Science Foundation grant 31170749 to A.M and 31100543 to W.X.S.

Received: February 5, 2012
Revised: June 4, 2012
Accepted: December 13, 2012
Published: January 17, 2013

REFERENCES

Armstrong, E.S., and Miller, G.H. (2010). Combating evolution with intelligent design: the neoglycoside ACHN-490. Curr. Opin. Microbiol. *13*, 565–573.

Bailey, M., Chettiath, T., and Mankin, A.S. (2008). Induction of erm(C) expression by noninducing antibiotics. Antimicrob. Agents Chemother. *52*, 866–874.

Borovinskaya, M.A., Pai, R.D., Zhang, W., Schuwirth, B.S., Holton, J.M., Hirokawa, G., Kaji, H., Kaji, A., and Cate, J.H.D. (2007). Structural basis for aminoglycoside inhibition of bacterial ribosome recycling. Nat. Struct. Mol. Biol. *14*, 727–732.

Breaker, R.R. (2011). Prospects for riboswitch discovery and analysis. Mol. Cell *43*, 867–879.

Carter, A.P., Clemons, W.M., Brodersen, D.E., Morgan-Warren, R.J., Wimberly, B.T., and Ramakrishnan, V. (2000). Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. Nature *407*, 340–348.

Cromie, M.J., Shi, Y., Latifi, T., and Groisman, E.A. (2006). An RNA sensor for intracellular Mg(2+). Cell *125*, 71–84.

Dann, C.E., 3rd, Wakeman, C.A., Sieling, C.L., Baker, S.C., Irnov, I., and Winkler, W.C. (2007). Structure and mechanism of a metal-sensing regulatory RNA. Cell *130*, 878–892.

Davies, J., and Davis, B.D. (1968). Misreading of ribonucleic acid code words induced by aminoglycoside antibiotics. The effect of drug concentration. J. Biol. Chem. *243*, 3312–3316.

Davis, B., Afshar, M., Varani, G., Murchie, A.I.H., Karn, J., Lentzen, G., Drysdale, M., Bower, J., Potter, A.J., Starkey, I.D., et al. (2004). Rational design of inhibitors of HIV-1 TAR RNA through the stabilisation of electrostatic "hot spots". J. Mol. Biol. *336*, 343–356.

Doi, Y., Yokoyama, K., Yamane, K., Wachino, J., Shibata, N., Yagi, T., Shibayama, K., Kato, H., and Arakawa, Y. (2004). Plasmid-mediated 16S rRNA methylase in Serratia marcescens conferring high-level resistance to aminoglycosides. Antimicrob. Agents Chemother. *48*, 491–496.

Dubnau, D. (1984). Translational attenuation: the regulation of bacterial resistance to the macrolide-lincosamide-streptogramin B antibiotics. CRC Crit. Rev. Biochem. *16*, 103–132.

Duvall, E.J., Williams, D.M., Mongkolsuk, S., and Lovett, P.S. (1984). Regulatory regions that control expression of two chloramphenicol-inducible cat genes cloned in Bacillus subtilis. J. Bacteriol. *158*, 784–790.

Ehresmann, C., Ehresmann, B., Ennifar, E., Dumas, P., Garber, M., Mathy, N., Nikulin, A., Portier, C., Patel, D., and Serganov, A. (2004). Molecular mimicry in translational regulation: the case of ribosomal protein S15. RNA Biol. *1*, 66–73.

Famulok, M., and Hüttenhofer, A. (1996). In vitro selection analysis of neomycin binding RNAs with a mutagenized pool of variants of the 16S rRNA decoding region. Biochemistry *35*, 4265–4270.

Fourmy, D., Recht, M.I., Blanchard, S.C., and Puglisi, J.D. (1996). Structure of the A site of Escherichia coli 16S ribosomal RNA complexed with an aminoglycoside antibiotic. Science *274*, 1367–1371.

Fuchs, R.T., Grundy, F.J., and Henkin, T.M. (2006). The S(MK) box is a new SAM-binding RNA for translational regulation of SAM synthetase. Nat. Struct. Mol. Biol. *13*, 226–233.

Gong, F., and Yanofsky, C. (2002). Instruction of translating ribosome by nascent peptide. Science *297*, 1864–1867.

Grundy, F.J., and Henkin, T.M. (2006). From ribosome to riboswitch: control of gene expression in bacteria by RNA structural rearrangements. Crit. Rev. Biochem. Mol. Biol. *41*, 329–338.

Grundy, F.J., Lehman, S.C., and Henkin, T.M. (2003). The L box regulon: lysine sensing by leader RNAs of bacterial lysine biosynthesis genes. Proc. Natl. Acad. Sci. USA *100*, 12057–12062.

Gryczan, T.J., Israeli-Reches, M., and Dubnau, D. (1984). Induction of macrolide-lincosamide-streptogramin B resistance requires ribosomes able to bind inducer. Mol. Gen. Genet. *194*, 357–361.

Hall, R.M., Holmes, A.J., Roy, P.H., and Stokes, H.W. (2007). What are superintegrons? Nat. Rev. Microbiol. *5*, C1, author reply C2.

Harms, J.M., Wilson, D.N., Schluenzen, F., Connell, S.R., Stachelhaus, T., Zaborowska, Z., Spahn, C.M.T., and Fucini, P. (2008). Translational regulation via L11: molecular switches on the ribosome turned on and off by thiostrepton and micrococcin. Mol. Cell *30*, 26–38.

Hendrix, M., Priestley, E.S., Joyce, G.F., and Wong, C.H. (1997). Direct observation of aminoglycoside-RNA interactions by surface plasmon resonance. J. Am. Chem. Soc. *119*, 3641–3648.

Hermann, T., and Westhof, E. (1999). Docking of cationic antibiotics to negatively charged pockets in RNA folds. J. Med. Chem. *42*, 1250–1261.

Jacquier, H., Zaoui, C., Sanson-le Pors, M.J., Mazel, D., and Berçot, B. (2009). Translation regulation of integrons gene cassette expression by the attC sites. Mol. Microbiol. *72*, 1475–1486.

Johansson, J., Mandin, P., Renzoni, A., Chiaruttini, C., Springer, M., and Cossart, P. (2002). An RNA thermosensor controls expression of virulence genes in Listeria monocytogenes. Cell *110*, 551–561.

Joyce, G.F. (2002). The antiquity of RNA-based evolution. Nature *418*, 214–221.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., et al. (2007). Clustal W and Clustal X version 2.0. Bioinformatics *23*, 2947–2948.

Lentzen, G., Klinck, R., Matassova, N., Aboul-ela, F., and Murchie, A.I.H. (2003). Structural basis for contrasting activities of ribosome binding thiazole antibiotics. Chem. Biol. *10*, 769–778.

Liebert, C.A., Hall, R.M., and Summers, A.O. (1999). Transposon Tn21, flagship of the floating genome. Microbiol. Mol. Biol. Rev. *63*, 507–522.

Lovett, P.S., and Rogers, E.J. (1996). Ribosome regulation by the nascent peptide. Microbiol. Rev. *60*, 366–385.

Mandal, M., Boese, B., Barrick, J.E., Winkler, W.C., and Breaker, R.R. (2003). Riboswitches control fundamental biochemical pathways in Bacillus subtilis and other bacteria. Cell *113*, 577–586.

Marzi, S., Myasnikov, A.G., Serganov, A., Ehresmann, C., Romby, P., Yusupov, M., and Klaholz, B.P. (2007). Structured mRNAs regulate translation initiation by binding to the platform of the ribosome. Cell *130*, 1019–1031.

Mazel, D. (2006). Integrons: agents of bacterial evolution. Nat. Rev. Microbiol. *4*, 608–620.

Mingeot-Leclercq, M.P., Glupczynski, Y., and Tulkens, P.M. (1999). Aminoglycosides: activity and resistance. Antimicrob. Agents Chemother. *43*, 727–737.

Mironov, A.S., Gusarov, I., Rafikov, R., Lopez, L.E., Shatalin, K., Kreneva, R.A., Perumov, D.A., and Nudler, E. (2002). Sensing small molecules by nascent RNA: a mechanism to control transcription in bacteria. Cell *111*, 747–756.

Murchie, A.I.H., Davis, B., Isel, C., Afshar, M., Drysdale, M.J., Bower, J., Potter, A.J., Starkey, I.D., Swarbrick, T.M., Mirza, S., et al. (2004). Structure-based

drug design targeting an inactive RNA conformation: exploiting the flexibility of HIV-1 TAR RNA. J. Mol. Biol. *336*, 625–638.

Nahvi, A., Sudarsan, N., Ebert, M.S., Zou, X., Brown, K.L., and Breaker, R.R. (2002). Genetic control by a metabolite binding mRNA. Chem. Biol. *9*, 1043.

Nechooshtan, G., Elgrably-Weiss, M., Sheaffer, A., Westhof, E., and Altuvia, S. (2009). A pH-responsive riboregulator. Genes Dev. *23*, 2650–2662.

Nikaido, H. (2009). Multidrug resistance in bacteria. Annu. Rev. Biochem. *78*, 119–146.

Nudler, E., and Mironov, A.S. (2004). The riboswitch control of bacterial metabolism. Trends Biochem. Sci. *29*, 11–17.

Porse, B.T., Kirillov, S.V., Awayez, M.J., Ottenheijm, H.C., and Garrett, R.A. (1999). Direct crosslinking of the antitumor antibiotic sparsomycin, and its derivatives, to A2602 in the peptidyl transferase center of 23S-like rRNA within ribosome-tRNA complexes. Proc. Natl. Acad. Sci. USA *96*, 9003–9008.

Ramu, H., Vázquez-Laslop, N., Klepacki, D., Dai, Q., Piccirilli, J., Micura, R., and Mankin, A.S. (2011). Nascent peptide in the ribosome exit tunnel affects functional properties of the A-site of the peptidyl transferase center. Mol. Cell *41*, 321–330.

Serganov, A., Polonskaia, A., Ehresmann, B., Ehresmann, C., and Patel, D.J. (2003). Ribosomal protein S15 represses its own translation via adaptation of an rRNA-like fold within its mRNA. EMBO J. *22*, 1898–1908.

Sudarsan, N., Wickiser, J.K., Nakamura, S., Ebert, M.S., and Breaker, R.R. (2003). An mRNA structure in bacteria that controls gene expression by binding lysine. Genes Dev. *17*, 2688–2697.

Swiatlo, E., and Kocka, F.E. (1987). Inducible expression of an aminoglycoside-acetylating enzyme in Providencia stuartii. J. Antimicrob. Chemother. *19*, 27–30.

Taubes, G. (2008). The bacteria fight back. Science *321*, 356–361.

Vazquez-Laslop, N., Thum, C., and Mankin, A.S. (2008). Molecular mechanism of drug-dependent ribosome stalling. Mol. Cell *30*, 190–202.

von Ahsen, U., Davies, J., and Schroeder, R. (1991). Antibiotic inhibition of group I ribozyme function. Nature *353*, 368–370.

Weisblum, B. (1995). Insights into erythromycin action from studies of its activity as inducer of resistance. Antimicrob. Agents Chemother. *39*, 797–805.

Winkler, W., Nahvi, A., and Breaker, R.R. (2002). Thiamine derivatives bind messenger RNAs directly to regulate bacterial gene expression. Nature *419*, 952–956.

Yu, F.Y., Yao, D., Pan, J.Y., Chen, C., Qin, Z.Q., Parsons, C., Yang, L.H., Li, Q.Q., Zhang, X.Q., Qu, D., and Wang, L.X. (2010). High prevalence of plasmid-mediated 16S rRNA methylase gene rmtB among Escherichia coli clinical isolates from a Chinese teaching hospital. BMC Infect. Dis. *10*, 184.

Zapp, M.L., Stern, S., and Green, M.R. (1993). Small molecules that selectively block RNA binding of HIV-1 Rev protein inhibit Rev function and viral production. Cell *74*, 969–978.

Zhang, X., and Bremer, H. (1995). Control of the Escherichia coli rrnB P1 promoter strength by ppGpp. J. Biol. Chem. *270*, 11181–11189.