A Mn-sensing riboswitch activates expression of a Mn\(^{2+}\)/Ca\(^{2+}\) ATPase transporter in Streptococcus

Julia E. Martin\(^{1,*}^{,1,}\), My T. Le\(^{2,1}\), Nabin Bhattarai\(^{1}\), Daiana A. Capdevila\(^{3}\), Jiangchuan Shen\(^{4}\), Malcolm E. Winkler\(^{5}\) and David P. Giedroc\(^{3}\)

\(^{1}\)Department of Biological Sciences, Idaho State University, Pocatello, ID 83209, USA, \(^{2}\)Department of Cell Biology, Faculty of Biological Sciences, Vietnam National University, Hanoi, Vietnam, \(^{3}\)Department of Chemistry, Indiana University, Bloomington, IN 47405, USA, \(^{4}\)Department of Cellular and Molecular Biochemistry, Indiana University, Bloomington, IN 47405, USA and \(^{5}\)Department of Biology, Indiana University, Bloomington, IN 47405, USA

Received March 21, 2019; Revised May 08, 2019; Editorial Decision May 14, 2019; Accepted May 31, 2019

ABSTRACT

Maintaining manganese (Mn) homeostasis is important for the virulence of numerous bacteria. In the human respiratory pathogen *Streptococcus pneumoniae*, the Mn-specific importer PsaBCA, exporter MntE, and transcriptional regulator PsaR establish Mn homeostasis. In other bacteria, Mn homeostasis is controlled by *yybP-ykoY* family riboswitches. Here, we characterize a *yybP-ykoY* family riboswitch upstream of the *mgtA* gene encoding a PII-type ATPase in *S. pneumoniae*, suggested previously to function in Ca\(^{2+}\) efflux. We show that the *mgtA* riboswitch aptamer domain adopts a canonical *yybP-ykoY* structure containing a three-way junction that is compacted in the presence of Ca\(^{2+}\) or Mn\(^{2+}\) at a physiological Mg\(^{2+}\) concentration. Although Ca\(^{2+}\) binds to the RNA aptamer with higher affinity than Mn\(^{2+}\), in vitro activation of transcription read-through of *mgtA* by Mn\(^{2+}\) is much greater than by Ca\(^{2+}\). Consistent with this result, *mgtA* mRNA and protein levels increase ≈5-fold during cellular Mn stress, but only in genetic backgrounds of *S. pneumoniae* and *Bacillus subtilis* that exhibit Mn\(^{2+}\) sensitivity, revealing that this riboswitch functions as a failsafe ‘on’ signal to prevent Mn\(^{2+}\) toxicity in the presence of high cellular Mn\(^{2+}\). In addition, our results suggest that the *S. pneumoniae* *yybP-ykoY* riboswitch functions to regulate Ca\(^{2+}\) efflux under these conditions.

INTRODUCTION

Small non-coding regulatory RNAs (sRNAs) are ubiquitous and found in all domains of life and play myriad roles in regulating gene expression (1,2). In bacteria, these sRNAs exist as short transcripts with lengths of 30–500 nucleotides (3,4). Bacterial sRNAs are broadly classified into two categories: *cis*-acting (riboswitches) or *trans*-acting (independent transcripts) RNAs. *Trans*-acting sRNAs modulate the stability or translation of mRNA transcript(s) by imperfect base-pairing interactions (5,6) at or near the ribosome binding site (RBS) on the target transcript. Riboswitches, on the other hand, are highly structured regulatory segments located within the 5′ untranslated regions (UTRs) of mRNAs that interact with small molecules, such as metals and other ligands, resulting in a change in transcription or translation mediated directly by environmental changes. sRNAs may also interact with RNA-binding proteins thereby modifying their activities (7,8).

Bacterial sRNAs function in many cellular processes, including the response to environmental stresses, e.g. those mediated by the immune system, and maintaining homeostasis (5,9). Accumulating evidence suggests that sRNAs are differentially expressed in bacteria when transitioning from colonization to an active infection which suggests that sRNAs are key players in mediating invasive disease (10–13). *Streptococcus pneumoniae* is a Gram-positive bacterium that commonly colonizes the nasopharynx of healthy individuals, persisting as a harmless bacterial commensal (14). Despite a largely asymptomatic colonization, *S. pneumoniae* remains a leading causative agent of sinusitis, otitis media (middle ear infection), and the life-threatening invasive diseases pneumonia, bacteremia, and meningitis globally (15,16). Transition from the harmless commensal to pathogen often occurs after a viral respiratory tract infection and is triggered by numerous factors, many of which are unknown (14). Progression into the lungs and invasion into the circulatory system, exposes the pneumococcus to numerous stress conditions and environmental
changes (17), including transition metal fluctuations, an important mediator in the ‘fight or flight’ response during infection (18,19). The capacity for bacteria to quickly adapt to these different environments may derive in part from sRNAs modulating the expression of virulence factors.

Multiple (>100) sRNAs have been identified in S. pneumoniae (20–25), but the functional role for most of these remains unknown. One particular candidate sRNA, SN44, identified in S. pneumoniae TIGR4 using genome-tilling arrays (21) is of interest here. SN44 is predicted on the basis of sequence homology to be a family response element (riboswitch) that is conserved among different Streptococcus species (21) (Supplementary Figure S1). The yybP-ykoY motif defines a ubiquitous class of riboswitches that are upstream of genes that encode uncharacterized or poorly characterized proteins many of which have been linked to Mn$^{2+}$ homeostasis. To date, yybP-ykoY-regulated gene products, MntP of Escherichia coli (26), MntX of Neisseria spp. (27), and YaoB (a predicted P-type II ATPase) of Lactococcus lactis (28), have been independently implicated as Mn$^{2+}$ efflux transporters capable of relieving cellular Mn toxicity. Other studies show that several of these associated yybP-ykoY family riboswitches respond to high Mn$^{2+}$ in vitro and in vivo modulating transcription and translation of downstream gene(s) within the transcript (28–30). Although yybP-ykoY riboswitches have generally been observed to bind Mn$^{2+}$ in vitro with highest affinity among other transition metals examined, none thus far exhibit strict Mn$^{2+}$ specificity (30).

The yybP-ykoY riboswitch aptamer domain adopts a four-helix junction architecture with P2 stacked on P1 and P4 stacked on P3, with the transition metal sensing pocket formed by nucleotides in the L1 loop (in P1) and L3 loop in P3 (Figure 1A). P2 and P4 are not strictly conserved and can be replaced by a short single-stranded connecting region or a longer helical stem, just above the cross-over point (Figure 1). A recent structural analysis of the L. lactis yybP-ykoY riboswitch aptamer domain suggests that the metal site may well be plastic and capable of adopting a range of coordination structures and nuclearity, with bincyclic and trinuclear metal (Cd$^{2+}$) complexes observed in that study (30).

The bincyclic site in a previous structure of the L. lactis yybP-ykoY riboswitch (28) was modeled as Mg$^{2+}$ or Mn$^{2+}$ in the $M_A$ site and Mn$^{2+}$ in the $M_B$ site (Figure 2A). The $M_B$ site is thought to provide specificity for Mn$^{2+}$ given the presence of five inner-sphere coordination bonds, including the N7 of A41 in the L3 loop (Figure 2A–C).

Like other annotated yybP-ykoY family riboswitches, the putative SN44 riboswitch overlaps the RBS within the target gene transcript and features a transcription terminator helix just 5’ to the RBS (Supplementary Figure S1). The SN44 RNA in S. pneumoniae D39 serotype 2 is encoded within the 5’ UTR of the mgtA (spaA388: previously caxP or PacL) transcript, encoding a PuI-type ATPase transporter. MgtA was previously suggested to function in Ca$^{2+}$ efflux (25,31), while in a different report was suggested to be implicated in the response against Zn$^{2+}$ and Mn$^{2+}$ toxicity also (32). The idea that MgtA in S. pneumoniae is not restricted to Ca$^{2+}$ and could also transport Mn$^{2+}$ is also supported by the fact that the MgtA protein is encoded from the negative-sense DNA strand immediately upstream of the mntE gene, which encodes the constitutively expressed Mn$^{2+}$-specific cation diffusion facilitator exporter MntE (33,34). Both MgtA and MntE are important for the virulence of S. pneumoniae (31,33). Despite intensive study, the physiological roles of many genes critical for virulence, including those encoding divergent metal transporters like MgtA, remain unclear.

In this study, we examine the metal binding and metal-induced folding of the mgtA aptamer and the regulation of mgtA in S. pneumoniae D39 strain. We find that the S. pneumoniae D39 mgtA aptamer RNA binds stoichiometric Mn$^{2+}$ and Ca$^{2+}$ in a background of physiological Mg$^{2+}$ and each is capable of stabilizing the same compact conformation; however, Mn$^{2+}$ stimulates in vitro read-through transcription to a far greater extent than Ca$^{2+}$. We establish for the first time that the mgtA mRNA is upregulated during Mn-stress in this organism, and that this increased transcription in vivo requires the mgtA (yybP-ykoY family) riboswitch. Further investigation reveals that MgtA may export Mn$^{2+}$ under conditions of extreme Mn-stress thereby protecting cells from Mn$^{2+}$ toxicity. The mgtA riboswitch may also function to regulate Ca$^{2+}$ export under these conditions.

**MATERIALS AND METHODS**

See Supplementary Material detailing methods for bacterial strain and plasmid construction, bacterial growth, disk diffusion assay, β-galactosidase activity assay, RNA isolation and quantitative real-time PCR, western blot, yybP-ykoY RNA synthesis, and inductively coupled plasma-mass spectrometry (ICP-MS) for measurement of total cell-associated divalent cationic metals. Bacterial strains and plasmids used in this study are listed in Supplementary Table S1 and relevant DNA oligonucleotide primers in Supplementary Tables S2 and S3.

**Native polyacrylamide gel electrophoresis (PAGE) analysis of yybP-ykoY RNAs (35)**

Purified RNA (2 µg) was heated at 90°C for 2 min followed by incubation at room temperature for 3 min, then subsequently allowed to refold in 50 mM HEPES [pH 7.5], 50 mM NaCl at room temperature for 10 min. Various divalent metal ion concentrations ranging from 0.01 to 5 mM were added and RNA mixture was left at room temperature for an additional 30 min prior to mixing with loading dye (10% glycerol and 0.01% xylene cyanol). RNA was separated by native PAGE (8% gel prepared with 19:1 acrylamide/bisacrylamide) at 4°C in running buffer containing 34 mM Tris, 66 mM HEPES [pH 7.5], and 3 mM MgCl$_2$, which was recirculated every hour. RNA was stained with ethidium bromide and observed with a Gbox (Biorad).

**SHAPE probing**

SHAPE was performed as previously described using 1-methyl-7-nitrosoatoic anhydride (1M7) derivatization
Figure 1. Secondary structure diagrams of the aptamer domains of (A) *E. coli* alx sequence (30) and (B) *Streptococcus pneumoniae* D39 mgtA riboswitch domains. In panel A, nucleotides in L1 (blue) and in L3 (red) that correspond to those shown in the structural models (Figure 2) are highlighted in bold, and correspond to the numbering convention of the *L. lactis* yybP-ykoY riboswitch (see Figure 2A) (28). In panel B, we used the natural nucleotide residue numbers, and those nucleotides targeted for substitution in this work in L1 (blue) and L3 (red) are highlighted in bold. For example, the loop L1 G8-G9-L.*lactis* system corresponds to the G16-G17-A18 in the *S. pneumoniae* RNA, while the *L. lactis* C40-A41 L3 sequence corresponds to the C49-A50 sequence in *S. pneumoniae*. A41 in the *L. lactis* RNA (A50 in the mgtA RNA) makes an inner sphere coordination bond with the MnB metal (see Figure 2A). The gray nucleotides in panel B are non-native and were added for *in vitro* transcription.

Small angle X-ray scattering (SAXS)

Small angle and wide angle X-ray scattering data were acquired at three different RNA concentrations (0.8, 1.6 and 3.2 mg/ml) of select RNAs folded in buffer containing 50 mM HEPES, pH 7.5, 50 mM NaCl, 3 mM MgCl₂ and 0.5 mM of the metal ion indicated. Data were collected at beamline 12-ID of the Advanced Photon Source at the Argonne National Laboratory. The wavelength (\(\lambda\)) of X-ray radiation was set to 1.033 Å. Procedures for SAXS/WAXS measurement were similar to those previously described (40,41). Thirty images were collected for each RNA concentration and its corresponding background buffer. SAXS data were averaged, and the background was subtracted using the NCI-SAXS program package. The averaged scattering profiles of three RNA concentrations were merged using PRIMUS in the ATSAS program package (http://www.embl-hamburg.de/biosaxs/). A GUINIER plot was generated as \(\ln(I(q))\) versus \(q^2\) to check sample quality and to obtain \(I_0\) and the radius of gyration (\(R_g\)) within the range of \(q_{\text{max}} \times R_g < 1.3\). The data from each RNA concentration were normalized with \(I_0\). Conformation of the RNAs was examined using the Kratky plot for \(q < 0.3\) Å⁻¹. Scattering profiles of RNAs were then Fourier-transformed using GNOM of the ATSAS package to obtain the normalized pair distance distribution graph.

Ab in initio modeling was performed using the program DAMMIN in a slow mode (42). For each RNA, 10 models were analyzed using the program reflexion (43). RNA constructs consisted of the wild-type sequence from the *S. pneumoniae* D39 mgtA 5′ UTR (containing the yybP-ykoY aptamer domain) flanked by SHAPE flanking sequences (37). RNAs were *in vitro* transcribed from PCR templates and purified similarly to that described above for RNA synthesis. Purified RNAs (10 pmol) was refolded in 50 mM HEPES, pH 7.5, 50 mM NaCl buffer before 1M7 derivatization. Reverse transcription of the derivatized RNA was performed with a FAM 6-labeled reverse primer. Unmodified RNA (2 pmol) used as a sequencing ladder was synthesized using reverse transcriptase, NED-labeled reverse primer, and ddCTP or ddGTP. An equal volume of sequencing ladder and reaction mixture with and without 1M7 was precipitated with 0.3 M sodium acetate, pH 5.2 and 100% ethanol, washed with 70% ethanol, air-dried, and re-dissolved in 15 \(\mu\)l water. Fragment analysis was performed using capillary electrophoresis by GENEWIZ (Plainfield, NJ, USA). Data processing was performed using QuShape following a protocol as previously described (39).
were obtained, filtered, and averaged using the DAMPUP, DAMFILT and DAMAVER of the ATSAS package (http://www. embl-hamburg.de/bioSAXS). Normalized spatial discrepancy (NSD) between each pair of the models was computed. The model with the lowest NSD value was selected as the reference model for superimposing onto other models. Outliner models (two models) with an NSD above mean +2* variation were removed before averaging.

**Isothermal titration calorimetry**

ITC experiments were performed following standard protocol previously described (30). Briefly, RNAs were heated at 95°C for 2 min, cooled down at room temperature for 3 min, diluted into ITC buffer (30 mM HEPES [pH 6.8], 150 mM NaCl), and incubated at room temperature for an additional 10 min. MgCl₂ was then added to a final concentration of 3 mM and the RNA was subsequently incubated at room temperature for 30 min. RNAs were dialyzed into ITC buffer containing 3 mM MgCl₂, filtered using a 3 kDa MWCO column, and degassed prior to ITC measurement. Divalent metal cations were dissolved in ITC buffer, filtered, and degassed. Experiments were performed with 50–100 µM RNA and 0.5 to 2 mM metal cation at 25°C, depending on affinity and magnitude of enthalpy. Divalent metal cation was injected with 0.5 to 1 µl volume every 300 s. Data were corrected for heats of dilution by subtracting the enthalpies of cations titrated into the ITC buffer from raw data and analyzed using an independent binding model by Nanoanalyse (TA instrument).

**IVT termination assay**

*In vitro* transcription termination assays were performed using standard protocols (28,43). To construct the *in vitro* transcription plasmid, the *S. pneumoniae* mgtA 5′-UTR (containing the aptamer domain, intrinsic terminator, and the first 45-nt of the MgtA protein coding sequence) was fused to a *B. subtilis* glyQS promoter. An ApC dinucleotide sequence was added to the 5′ end of the mgtA aptamer domain with no cytosines present in the next 12 nucleotides. DNA templates were produced by PCR and spin column purified (Qiagen). To assemble stalled RNA polymerase complexes, 20 mM template was mixed with initiation buffer (20 mM Tris–HCl [pH 7.5], 50 mM NaCl, 250 µM MgCl₂, 1 mM DTT and 5% glycerol), 0.15 µCi Curies/µl 32P ATP, 20 µM each of unlabeled ATP/CTP/GTP, and 0.01 U/µl *Escherichia coli* RNA polymerase holoenzyme. Reactions were incubated at 37°C for 15 min for elongation, then transferred to ice for 2 min. For synchronized transcription at each condition, 10 µl of stalled RNA polymerase was mixed with 1.5 µl 10× metal solution, and 1.5 µl 10× elongation buffer (4.5 mg/ml, 650 µM each of unlabeled ATP/CTP/GTP/UTP, 100 mM Tris [pH 7.5], 2 mM DTT, 10% glycerol and 2 mM MgCl₂). Reactions were incubated at 37°C for an additional 15 min, then terminated by addition of 10 µl RNA loading dye (95% formamide, 20 mM EDTA [pH 8.0], supplemented with xylene cyanol). Transcripts (10 µl) were separated by 6% urea denaturing PAGE and analyzed by a phosphorimagor. The sizes of the terminated and full-length RNA products were confirmed by RNA ladder (data not shown). Bands were quantified with ImageJ and each reaction was converted to the fraction of full-length product over total RNA transcribed. The data were fit using Origin 8 data analysis software to the Hill equation (n = 1), where x is the concentration of metal and k is the concentration at which the change in read-through is half maximal. Each assay was performed in triplicate.

**Statistical analysis**

When appropriate, *P*-values were determined relative to non-stressed parent or WT type strains using unpaired *t*-tests or a one-way ANOVA with Dunnett’s post-test determined by GraphPad Prism software.
RESULTS

The *S. pneumoniae* mgtA riboswitch is comprised of a three-way helical junction that is conserved among *Streptococcus* spp.

As described above, the crystallographic structure of the Mn\(^{2+}\) bound *L. lactis* yaoB 5′ UTR (*yybP-ykoY* family) aptamer domain reveals a four-way helical junction (4WJ) comprising of tandem coaxially stacked helices (P2 on P1 and P4 on P3) with the two ‘legs’ of the H-like structure docked at a conserved interface between loop (L) L1 and L3 (Figure 1A) (28). A single Mn\(^{2+}\) ion (M\(_{G}\)) is coordinated by six inner-sphere interactions from nucleotides in loops L1 and L3 including an A41 N7-Mn\(^{2+}\) coordination bond; a Mg\(^{2+}\) ion is also found nearby in M\(_{A}\) (28) (Figure 2). The binding of a single Mn\(^{2+}\) ion influences stability of the *L. lactis* yaoB riboswitch like that of a fluoride-sensing riboswitch (44) but in a way that is distinct from the Mg\(^{2+}\)-sensing riboswitch (or M-box), in which multiple Mg\(^{2+}\) ions cooperatively impact the stability of the riboswitch (45,46).

The *yybP-ykoY* family riboswitch located upstream of the *mgtA* gene in *Streptococcus pneumoniae* D39 reveals, in contrast to the *L. lactis* structure, a three-way helical junction (3WJ) with P4 replaced by an invariant UNAAA (N, can be U or G) sequence (Figure 1B; Supplementary Figure S1C) followed by a transcription termination hairpin with a U-rich sequence (Supplementary Figure S1A). This UNAAA sequence is also found close to a 3WJ in the twister ribozyme (AUAAA), where it induces a sharp turn in the AAA sequence is also found close to a 3WJ in the twister ribozyme (Supplementary Figure S1C) followed by a transcription termination hairpin with a U-rich sequence (Supplementary Figure S1A). This UNAAA sequence is also found close to a 3WJ in the twister ribozyme (AUAAA), where it induces a sharp turn in the backbone at a helical junction (47). The 5′ end of L1 loop is highly conserved, while the 3′ end contains an invariable AGA nucleotide sequence (nucleotides 101–104) preceded by a variable nucleotide at position 101 that is only conserved as a guanosine among *Streptococcus* spp (Figure 1B; Supplementary Figure S1C). These nucleotides are anticipated to contribute to the metal binding pocket of L1 and L3 in the *mgtA* riboswitch. The metal coordinating nucleotides C49 and A50 as defined by the previous structural work as C49 and A41 in the *L. lactis* RNA (Figure 2) (28,30) within the L3 bulge are also invariant. Finally, the C46-G83 base-pair in the P3.1 stem above L3 is anticipated to contribute to hydrogen bond to A18 to form a cross-helix A minor interaction as in other *yybP-ykoY* family riboswitches (Figure 1).

Mn\(^{2+}\) and Ca\(^{2+}\) to the *mgtA* riboswitch alters an RNA conformational equilibrium

A comparison of the *E. coli* mntP riboswitch in the divalent metal-bound and metal-free (Na\(^{+}\) only) apo states suggest that the aptamer domain is pre-organized into a conformation that is quite close to the metal-bound form (Supplementary Figure S2), with major differences occurring in L3, which is largely unstructured in the absence of metal (28). The extent to which this is true in solution, however, and for this particular riboswitch specifically, is unclear. To address this, we monitored the folding equilibrium of the *S. pneumoniae* mgtA riboswitch aptamer domain by native PAGE, evaluating the impact of divalent metal cations Mn\(^{2+}\), Ca\(^{2+}\) and Mg\(^{2+}\) (Figure 3).

In these experiments, the RNA aptamer domain was denatured and refoldeed at room temperature in a stepwise progression by first adding a monovalent salt (50 mM NaCl), followed by divalent metal cation. Two conformations were resolvable by native PAGE run with Mn\(^{2+}\) in the running buffer: an extended or open form (E, upper band) and a more compact form (C, lower band) (Figure 3A). In the absence of added salt, the majority of the RNA (≥80%) is found in the extended form, while inclusion of the 50 mM monovalent salt drives the RNA towards a compacted form. Addition of 1 mM Mg\(^{2+}\) to 50 mM Na\(^{+}\) shifts the equilibrium further to the compacted form but not completely (Figure 3A). The addition of as little as 0.5 mM Mn\(^{2+}\) is efficient at compacting the RNA with ≈90% of the RNA adopting the compact form (Figure 3A and Supplementary Figure S3A). As anticipated from a cation competition model, the aptamer appears to fold less efficiently when 1 mM Mg\(^{2+}\) and 0.5 mM Mn\(^{2+}\) are simultaneously added into the folding buffer relative to folding in the presence of Mn\(^{2+}\) alone (Figure 3A). Indeed, nucleotides in L1 and L3 of the *L. lactis* yaoB riboswitch can coordinate binding of either Mn\(^{2+}\) or Mg\(^{2+}\) (28). The degree to which increasing concentrations of Mn\(^{2+}\) shifts the conformational equilibrium to a compact conformation can be modelled assuming that the apo form in 3 mM Mg\(^{2+}\) exists as a mixture of extended and compact and Mn\(^{2+}\)-bound form is exclu-
sively compact. This model of the relative concentrations of the two different RNA conformers gives an effective equilibrium dissociation constant ($K_d$) of 0.14 ± 0.02 mM (Supplementary Figure S3A and B) in 3 mM Mg$^{2+}$, or ≈6-fold weaker than that reported for $L$. lactis YaoB riboswitch ($K_d$ of 25 μM for Mn$^{2+}$) using isothermal titration calorimetry (ITC) in a background of 5 mM Mg$^{2+}$ (30). Quantitative ITC data for the binding of the pneumococcal mgtA riboswitch aptamer to divalent cations is presented below.

Although the likely cognate metal ion for the $S$. pneumoniae mgtA riboswitch is Mn$^{2+}$ given its classification as a $yybP$-$ykoy$ family riboswitch, the mgtA riboswitch incorporates the ribosome binding site of the mgtA gene, which encodes $P$. putida-type ATPase transporter (MgtA) that has been implicated in Ca$^{2+}$ efflux (31); furthermore, biophysical experiments show that the analogous transporter from $L$. monocytogenes (LMCA1) transports Ca$^{2+}$ (48). Ca$^{2+}$ binding to this family of RNAs has not yet been investigated. Using native-PAGE, we show that 0.5 mM Ca$^{2+}$ also affects the global hydrodynamics of the RNA aptamer, driving the RNA to the more compact form that is similar in mobility to that obtained upon incubation with Mn$^{2+}$ (Figure 3B).

**Mutant mgtA riboswitch aptamers have altered structures or altered Mn$^{2+}$ sensitivities**

To assess which nucleotides coordinate Mn$^{2+}$ in the $S$. pneumoniae mgtA RNA aptamer, we mutated conserved nucleotides in L1 and L3 that comprise the metal binding site region as detailed in other $yybP$-$ykoy$ structures (Figure 2) and tested the impact of these substitutions on the RNA aptamer folding equilibrium by native-PAGE (Figure 3C). These include G16, G17 and A18 on the 5’ side of L1, C49 and A50 in L3 and A102, G102 and A104 on the 3’ side of L1 (S. pneumoniae numbering) (see Figure 1B). The G16A/G17A and C49A/A50U RNAs show no change in electrophoretic mobility by native-PAGE over the same range of Mn$^{2+}$ concentration that stabilizes the compacted (C) form of the wild-type RNA (Figure 3C and Supplementary Figure S3C and D), suggesting a loss of Mn$^{2+}$ binding ability. We note that the G16A/G17A mutant RNA appears to migrate slightly faster than the extended (E) form of the WT RNA (Figure 3C). Although the C49A/A50U mutant has lost its ability to bind metal (Supplementary Figure S3D), this mutant RNA migrates similarly with the compacted WT RNA. The triple A102G/G103A/A104G (L1) mutant RNA, on the other hand, simultaneously adopts two RNA conformations that migrate closely with that of the compacted WT RNA, but each is formed independent of Mn$^{2+}$ concentration (Supplementary Figure S3E, Figure 3C). Finally, targeting the predicted cross-strand A-minor interaction (nucleotide A18; Figure 1B) with a cytosine substitution (A18C) shifts the E-to-C equilibrium far toward E, as expected, in a background of 3 mM Mg$^{2+}$ (Supplementary Figure S3F and G) while also increasing the apparent $K_d$ for Mn ≈6.5-fold to 0.90 ± 0.01 mM.

Thus, all four mutant RNAs perturb the RNA conformational ensemble, at least as observed by native-PAGE, and only one of four RNAs (A18C) is responsive to the presence of Mn$^{2+}$, albeit at much higher metal concentrations. These data suggest that Mn$^{2+}$ binding may not be required to globally fold the $S$. pneumoniae mgtA riboswitch aptamer into an ‘X-like’ or undocked structure in the presence of physiological (low mM) Mg$^{2+}$, as all mutant RNAs adopt an E- or E-like state, a finding consistent with recent single-molecule FRET experiments (49).

**SHAPE (selective 2'-hydroxyl acylation analyzed by primer extension) probing of the wild-type aptamer**

The impact of divalent metal binding on the structure of the $S$. pneumoniae mgtA RNA aptamer was further investigated by the structural probing method SHAPE. SHAPE experiments measure the relative rates of reactivity of 1-methyl-7-nitroisatoic anhydride (1M7) to 2'-hydroxyls and generally report on local flexibility of an individual nucleotide. In the absence of divalent metal (50 mM Na$^+$ only), nucleotides in L1 (16–21; 102–104) and L3 (residues 48–50) show high to moderate reactivity (Figure 4A), suggesting that no metal-binding pocket is formed. A18 of the A-minor interaction is also moderately flexible in the absence of metal ions. The addition of Mg$^{2+}$ stabilizes base-pairing in the P2 stem (residues 28–29), destabilizing the base of the P3.2 stem, while quenching flexibility of all nucleotides implicated in metal binding, including both sides of L1 and C49 and A50 in L3 (Figure 4B). The modest reduction in flexibility observed for nucleotides A18 in L1 and C49 and A50 in L3 suggests that the A-minor interaction forms in the presence of Mg$^{2+}$ alone. Addition of Mn$^{2+}$ to a background of Mg$^{2+}$ leads only to a small change in the flexibility of nucleotides C49 and A50, which are predicted to directly interact with the Mn$^{2+}$ M8 ion (vide infra), making them more rigid (Figure 4C). The addition of Ca$^{2+}$ results in a SHAPE profile (Figure 4D) that is statistically indistinguishable from that obtained upon addition of Mn$^{2+}$. These data taken together reveal that the RNA conformation obtained in the presence of the 3 mM Mg$^{2+}$ alone, Mg/Mn and Mg/Ca are very similar and that major differences in reactivity relative to the Na$^+$-only structure are localized to the metal-binding region. These findings are consistent with native-PAGE analysis which reveals roughly two distinct conformations with distinguishable, yet rather similar, electrophoretic mobilities.

**Small angle X-ray scattering (SAXS) analysis of mgtA riboswitch aptamers**

Small angle X-ray scattering (SAXS) was next used to obtain additional insights into the global fold of the wild-type mgtA RNA as a function of divalent metal ion status, compared to the G16A/G17A L1 mutant which fails to adopt the compact or C-state. Refolding of the aptamer in buffer with NaCl only and no divalent metal ions results in the largest radius of gyration ($R_g$) (Supplementary Figure S4; $\approx 57$ Å) with clear evidence of conformational heterogeneity in this state. Addition of Mg$^{2+}$ results in a significant increase in RNA compaction, with the $R_g$ some 10 Å smaller, to 26.7 ± 0.5 Å. Addition of Mn$^{2+}$ to the Mg$^{2+}$-folded structure results in a similarly compacted structure, that is perhaps slightly more extended ($R_g = 27.6 ± 0.4$ Å) with no significant change observed with the addition of Ca$^{2+}$ to
Figure 4. Mn$^{2+}$ and Ca$^{2+}$ impact nucleotide flexibility in the mgtA RNA aptamer. (A) SHAPE reactivity of the S. pneumoniae mgtA RNA aptamer domain in the presence of various divalent metals. Regions of low reactivity ($<0.4$) are colored gray, moderate reactivity ($0.4$–$0.85$) are yellow, and high reactivity ($>0.85$) are red. Each bar represents a single nucleotide in the sequence as numbered on x-axis. (B) Summary of the flexibility of nucleotides in the RNA aptamer. The Mg$^{2+}$ folded RNA. The dimensionless Kratky plots also reveal that the mgtA aptamer folds upon the addition of Mg$^{2+}$, while inclusion of other transition metals tested only change the overall compaction of the RNA slightly (Figure 5A and B). A significant change in the pair-wise distance distribution is observed with Mg$^{2+}$ showing a larger number of shorter distances (20–40 Å) within the RNA (Figure 5C), consistent with a folded form of the mgtA aptamer RNA. Only a minor change in the pair-wise distance distribution results when Mn$^{2+}$ or Ca$^{2+}$ are present in a background of Mg$^{2+}$ (Figure 5C). Taken together these data are consistent with the native-PAGE and SHAPE results, demonstrating that different divalent metal ions can lead to conformational change and compaction of the mgtA RNA aptamer.

We next used these data to calculate a three-dimensional (3D) envelope (or bead model) of the mgtA RNA aptamer, which could only be determined in the presence of divalent metal ions, a finding consistent with the conformational heterogeneity in the metal-free form of the RNA as evidenced by the non-linear Guinier plot (Supplementary Figure S4A). As expected, the resulting averaged envelopes obtained in the presence of Mg$^{2+}$ plus Mn$^{2+}$ or Mg$^{2+}$ plus Ca$^{2+}$ are similar (Figure 5D and E), but yet distinct from that of Mg$^{2+}$ alone (Figure 5F). A simulated structural model of the aptamer embedded into the SAXS envelope in the presence of Mg$^{2+}$/Mn$^{2+}$ or Mg$^{2+}$/Ca$^{2+}$ fits well (Figure 5D), showing that in the presence of Mn$^{2+}$ or Ca$^{2+}$, the three hairpins likely organize into two coaxially stacked helices (P2 on P1) connected to a third helix (P3) at the three-way junction (3WJ).

In contrast to the WT RNA aptamer, the non-metal binding G16A/G17A mutant RNA that fails to adopt the compact form at any Mn$^{2+}$ concentration by native-PAGE analysis (Supplementary Figure S3C) adopts a more extended average conformation ($R_g = 29.3 \pm 0.4$ Å), independent of Mn$^{2+}$ (Figure 5B and C). The resulting averaged SAXS-derived bead model yields a ‘Y-like’ topology, suggesting that the G16A/G17A mutant RNA is characterized by an
open or extended conformation minimally comprising three
helices connected by a 3WJ (Figure 5G).

The mgtA riboswitch aptamer binds Ca\textsuperscript{2+} more tightly than
Mn\textsuperscript{2+}

The genomic location of the \textit{S. pneumoniae} mgtA riboswitch as well as our own hydrodynamic data (Figures 3–5) sug-
gest that Mn\textsuperscript{2+} and possibly Ca\textsuperscript{2+} may be regulatory. We
next used ITC to monitor the binding of Mn\textsuperscript{2+} and Ca\textsuperscript{2+}
directly, monitored at what we anticipate is near physiologi-
cal conditions of Mg\textsuperscript{2+} (3 mM). The binding of Mn\textsuperscript{2+} to
the mgtA RNA aptamer is exothermic, whereas the binding of
Ca\textsuperscript{2+} is endothermic, where Mn\textsuperscript{2+} binding is more strongly
entropically-driven relative to Ca\textsuperscript{2+} (Figure 6 and Table 1).

Both metals bind to the mgtA riboswitch with an apparent
stoichiometry, \( n \approx 1 \), likely reporting on the displacement
of Mg\textsuperscript{2+} bound to the M\textsubscript{B} site by Ca\textsuperscript{2+} or Mn\textsuperscript{2+} (Figure
2A) (28). The effective \( K_d \) for Ca\textsuperscript{2+} is low micromolar (1.72 ± 0.03 \( \mu \text{M} \)), which is 50-fold higher affinity than for Mn\textsuperscript{2+} (\( K_d = 54 \pm 26 \text{ } \mu\text{M} \)). The \( K_d \) for Mn\textsuperscript{2+} obtained from ITC is
somewhat tighter than that derived from native-PAGE fold-
ing equilibrium analysis, although both are in the 10\textsuperscript{-4} M
Mn\textsuperscript{2+} range (Supplementary Figure S3A and B).

A significant number of the structural models of metal-
bound \textit{yybP-yykO} riboswitches have been reported and
are often used to interpret the sensing mechanism of ri-oswitches (Figure 2B and C). Moreover the recent re-
ported affinities for Cd\textsuperscript{2+}, \( K_d \) in the sub-mM range depend-
ing on the construct (30), suggest that Cd\textsuperscript{2+} could poten-
tially turn on the \textit{yybP-yykO} riboswitches. To test the sig-
nificance of those models in the context of the mgtA ri-
oswitch, we also determined Cd\textsuperscript{2+} binding to the mgtA ri-
oswitch. Although the mgtA RNA aptamer is compacted
in the presence of 500 \( \mu\text{M} \) Cd\textsuperscript{2+} in a background of 3 mM
Mg\textsuperscript{2+} (Supplementary Figure S5A), higher concentrations
of Cd\textsuperscript{2+} drive the RNA aptamer into a more slowly migrat-
ing conformation(s) by native-PAGE. This suggests that at
low concentrations, Cd\textsuperscript{2+} may bind in the same pocket (M\textsubscript{B})
(see Figure 2B) as Mn\textsuperscript{2+} and Ca\textsuperscript{2+}. Both SHAPE (Supple-
mental Figure S5C) and SAXS (data not shown) analy-
eses suggest that an extended or more open conformation
is formed at high Cd\textsuperscript{2+} concentrations. It is also worth not-
ing that low Cd\textsuperscript{2+} concentrations (≤500 \( \mu\text{M} \)) fail to activate

| Parameter\(^{a}\) | Mn\textsuperscript{2+} | Ca\textsuperscript{2+} |
|------------------|----------------|----------------|
| \( K_d \) (\( \mu\text{M} \)) | 54 ± 26 | 1.72 ± 0.03 |
| \( n \) | 1.0 ± 0.0 | 1.07 ± 0.0 |
| \( \Delta H \) (kcal/mol) | 1.1 ± 0.1 | –3.6 ± 0.1 |
| \( T \Delta S \) (kcal/mol) | 7.0 ± 0.2 | 4.3 ± 0.1 |
| \( \Delta G \) (kcal/mol) | –5.9 ± 0.02 | –7.9 ± 0.2 |

\(^{a}\) Mean and standard deviations are obtained from duplicated independent experiments. All experiments were performed in a background of 3 mM MgCl\textsubscript{2} at 25 °C. Data reported here are calculated from a one-site random binding model.

Figure 5. Global structure analysis of the \textit{mgtA} RNA aptamer with divalent metal ions. (A) Scattering profiles collected at separate RNA concentrations 0.8, 1.6 and 3.2 mg/ml with indicated metal, then merged. (B) Dimensionless Kratky plots. (C) Pair-wise distance distribution plots for all RNAs. (D) Simulated 3D structure of the RNA aptamer by SimRNA embedded in the envelope obtained from SAXS in the presence of Mn\textsuperscript{2+}. SAXS simulated envelope models for WT RNA with Ca\textsuperscript{2+} (E), Mg\textsuperscript{2+} (F) and the G16A/G17A mutant RNA that is unable to bind metal (G).
Figure 6. Thermograms of the interaction of the mgtA aptamer with Mn\(^{2+}\) (A) or Ca\(^{2+}\) (B) by isothermal titration calorimetry. For each panel, the upper panel shows the raw ITC data plotted as the change in power (μcal s\(^{-1}\)) required to maintain equal temperature between the sample and reference cells as a function of time; lower panel shows integrated heat normalized for mol of injectant added. The thermodynamic parameters for metal binding defined by the continuous lines drawn through the data are summarized in Table 1.

transcription read-through and that higher Cd\(^{2+}\) concentrations are inhibitory (data not shown). Collectively, these data suggest that Cd\(^{2+}\) can bind and affect global change of the mgtA riboswitch, however the functional outcome is not likely physiologically relevant.

The mgtA riboswitch responds more robustly to Mn\(^{2+}\) in vitro

Previously, it was demonstrated by *in vitro* transcription (IVT) termination experiments that the *L. lactis* yaoB riboswitch is activated by 0.5 mM Mn\(^{2+}\) but not by other metal ions tested (Fe\(^{2+}\), Co\(^{2+}\), Ni\(^{2+}\) and Ca\(^{2+}\)); Mg\(^{2+}\) also induced transcription read-through, but only when provided at high concentrations (10 mM), suggesting that Mg\(^{2+}\) is not a preferred metal (28). Likewise, an *L. lactis* yaoB riboswitch chimera harboring L3 mutations to match the *E. coli* mntP and *alx* riboswitch L3 nucleotides, respond to Mn\(^{2+}\) as do other transition metals tested (Cd\(^{2+}\), Co\(^{2+}\), Cu\(^{2+}\), Fe\(^{2+}\) and Ni\(^{2+}\)); however, transcription read-through was not induced by Ca\(^{2+}\), Mg\(^{2+}\) and Zn\(^{2+}\) (30). Other experiments demonstrate that these *yybP-yykO* family riboswitches can also mediate Mn\(^{2+}\)-dependent regulation in *vivo* (28,29). Thus, it is not possible to predict which metal induce transcription for the *mgtA* RNA aptamer based solely on it being part of the *yybP-yykO* family riboswitches, nor on the selectivity in terms of binding for different metal ions. This observation motivated a series of *in vitro* transcription termination assays to determine which metal induces transcription for the *mgtA* RNA.

The binding of Mn\(^{2+}\) to the *S. pneumoniae* RNA aptamer is hypothesized to destabilize the terminator hairpin and permit read-through transcription of full-length *mgtA* mRNA transcript (Supplementary Figure S1C). Indeed, titration of increasing Mn\(^{2+}\) concentrations into the reaction led to the synthesis of full-length transcripts by *E. coli* RNA polymerase (Figure 7A) in a background of 250 μM Mg\(^{2+}\), revealing that Mn\(^{2+}\) binding to the aptamer activates transcription read-through. Ca\(^{2+}\) is also capable of activating transcription read-through (Figure 7B), but to a much lower extent compared to Mn\(^{2+}\) (Figure 7C). The half-maximal transcriptional activation by Mn\(^{2+}\) (0.23 ±
could also mediate Mn2+- and Ca2+-dependent regulation of the MgtA protein. The mean of at least three independent cultures revealed that Mn2+ is the most effective activator of the S. pneumoniae mgtA riboswitch functions as a Mn2+-specific ‘on’ switch in vivo, inducing expression of MgtA.

To further connect aptamer folding with function and confirm metal specificity in vivo, key nucleotides within the S. pneumoniae mgtA RNA aptamer were mutated to disrupt folding of the aptamer were mutated in the context of the 5’ UTR and tested for functionality in vivo in a heterologous B. subtilis expression system. Both L1 (G16A/G17A) and L3 (C49A/A50U) substitutions reduced expression of β-galactosidase for all metals tested except for Zn2+ and Cu2+, which inhibit growth under these conditions, to a level consistent with unregulated expression, i.e. that obtained with aptamer alone (Figure 9C). This mutant 5’ UTR RNA may mis-fold in the cell and lead to a loss or destabilization of the terminator hairpin (see Supplementary Figure S1B). Taken collectively, these data correlate well with our global conformational analyses (Figures 3–5).

The mgtA riboswitch regulates a PII-type ATPase that protects cells against Mn toxicity

Our results so far indicate that the S. pneumoniae mgtA riboswitch functions as a Mn2+-specific sensor that turns on a predicted PII-type ATPase metal effluxer, MgtA, that we reasoned might play a role in cellular Mn homeostasis. To investigate this, we constructed S. pneumoniae D39...
The *S. pneumoniae* mgtA riboswitch senses and responds specifically to intracellular Mn in *B. subtilis*. Exponentially growing *B. subtilis* cells growing in LB treated with indicated metal for 2 h, then harvested. (A) Relative transcription of Pr_{mgtA-lacZ} fusion in ΔmntR cells grown with increasing concentrations of Mn. (B) β-Galactosidase activity in ΔmntR carrying various promoter-lacZ fusions in response to increasing concentrations of Mn. (C) β-galactosidase activity in WT cells containing WT-{mgtA} leader sequence in response to Mn and Ca. (D) β-galactosidase activity in ΔmntR carrying Pr_{Full} (WT) and mutant forms the mgtA riboswitch in response to various metal cations. The mean of at least three independent cultures ± SEM are shown. Pr_{Full}, promoter plus intact 5′ UTR (185 nt; see Supplementary Figure S1); Pr_{Apt}, promoter plus aptamer (nucleotides 1–124); and Pr_{Term}, promoter plus terminator hairpin (nucleotides 101–185).

Figure 9. The *S. pneumoniae* mgtA riboswitch senses and responds specifically to intracellular Mn in *B. subtilis*. Exponentially growing *B. subtilis* cells growing in LB treated with indicated metal for 2 h, then harvested. (A) Relative transcription of Pr_{mgtA-lacZ} fusion in ΔmntR cells grown with increasing concentrations of Mn. (B) β-Galactosidase activity in ΔmntR carrying various promoter-lacZ fusions in response to increasing concentrations of Mn. (C) β-galactosidase activity in WT cells containing WT-{mgtA} leader sequence in response to Mn and Ca. (D) β-galactosidase activity in ΔmntR carrying Pr_{Full} (WT) and mutant forms the mgtA riboswitch in response to various metal cations. The mean of at least three independent cultures ± SEM are shown. Pr_{Full}, promoter plus intact 5′ UTR (185 nt; see Supplementary Figure S1); Pr_{Apt}, promoter plus aptamer (nucleotides 1–124); and Pr_{Term}, promoter plus terminator hairpin (nucleotides 101–185).

DISCUSSION

Metal cation homeostasis is essential for colonization and pathogenesis of bacteria. As such, the mammalian host has evolved mechanisms to both restrict or intoxicate invading bacteria with specific metals, depending on the microenvironmental niche. In response, bacteria have evolved complex regulatory strategies to adapt in an effort to maintain homeostasis of essential metal ions. Recent work provides support for the idea that individual metal ions in a bac-
Portance here is that Mg\textsuperscript{2+} levels are thought to be main-

tained in the low mM range, primarily controlled by the Mg\textsuperscript{2+}-sensing ‘M-box’ riboswitch in B. subtilis, for example (45), while Mn\textsuperscript{2+} concentrations are transcriptionally controlled by Mn\textsuperscript{2+}-sensing protein repressors MntR in B. subtilis (56) and a related repressor PsaR, in S. pneumoniae (57). The M-box riboswitch is tuned to \(~\text{mM}\) affinity (55). The \(K_{d,\text{Mn}}\) for the regulatory ‘sensing’ site in PsaR is in the low \(\mu\text{M}\) range (1.3 \(\mu\text{M}\)), while the Salmonella MntR \(K_{d,\text{Mn}}\) is about 10-fold lower affinity (13 \(\mu\text{M}\) at pH 8.0) (55). This ‘set-point’ model suggests that Mn\textsuperscript{2+} may be buffered in the low \(\mu\text{M}\) range, which in S. pneumoniae may be further re-

stricted by the constitutive expression of MntE, to which effuxes Mn\textsuperscript{2+} from the pneumococcal cell (50). In contrast to what is known about Mn\textsuperscript{2+} homeostasis, intracellular bac-

terial Ca\textsuperscript{2+} homeostasis remains largely unexplored, and to our knowledge a bacterial Ca\textsuperscript{2+}-specific metallosensor or two-component response regulatory system has yet to be described.

In this report, we describe the physical and functional characteriza-
tion of a Mn\textsuperscript{2+}-sensing riboswitch that is found upstream of the mgtA gene. Using a combination of native gel mobility experiments, SHAPE probing, SAXS analysis and in vitro transcription experiments, we show that this ri-

boswitch aptamer domain is capable of binding Mn\textsuperscript{2+} and Ca\textsuperscript{2+} in the presence of mM Mg\textsuperscript{2+}, and that all three metals, including Mg\textsuperscript{2+} alone stabilize a compact state, relative to an extended or ‘open’ conformation. All three metals stimu-

late transcription read-through in vitro using E. coli RNA polymerase, but Mn\textsuperscript{2+} is clearly the most potent activating metal in these experiments, both in terms of maximal read-

through activity and metal sensitivity (\(<100 \mu\text{M}\) metal).

The half-maximal Mn\textsuperscript{2+} concentration required to shift the folding equilibrium of the RNA to the compact form and stimuate in vitro transcription is within a factor of four of the \(K_{d,\text{Mn}}\) measured by calorimetry (50–200 \(\mu\text{M}\)); furthermore, the response in all three experiments conducted in 3 mM Mg\textsuperscript{2+} fits well to a rectangular hyperbolic function, consistent with a single Mn\textsuperscript{2+}-sensing site on the riboswitch, which we speculate is the MnB site (see Figure 2A). As anticipated from previous work, mutations in the metal-site pocket abrogate or greatly weaken (A18C) Mn\textsuperscript{2+}-dependent folding, and when incorporated into the mgtA 5’ UTR in cells, render them functionally inactive.

These physical characteristics are largely recapitulated in pneumococcal cells and in a heterologous B. subtilis host, with Mn\textsuperscript{2+} the only divalent metal ion capable of read-

through transcription in either cellular background, which in S. pneumoniae results in a corresponding increase in the cellular amount of MgtA protein. Ca\textsuperscript{2+} is not a strong mgtA inducer in vivo. However, significant Mn\textsuperscript{2+}-dependent mRNA or protein expression in either bacterial host is only observed in a strain that is extremely sensitive to Mn\textsuperscript{2+} toxicity, i.e. in \(\Delta mntR\) B. subtilis and in \(\Delta mntE\) S. pneumoniae. Under these conditions where mgtA mRNA and MgtA pro-

tein levels are higher, MgtA is capable of lowering total cell-

associated Mn\textsuperscript{2+}, consistent with a role in Mn\textsuperscript{2+} efflux by MgtA. These physiological findings are entirely consistent with the observed in vitro metal sensitivity of the riboswitch itself, which is in the 50–200 \(\mu\text{M}\) Mn\textsuperscript{2+} range, or set \(\geq 5–10\)-fold higher than the metalloregulator proteins MntR and PsaR; unfortunately, the metal binding affinity of the MntE
Figure 11. Threshold model for Mn\textsuperscript{2+} sensing and detoxification in \textit{S. pneumoniae}. The Mn\textsuperscript{2+} binding affinity of the \textit{S. pneumoniae} mgtA riboswitch is such that there is sufficient free intracellular Mn\textsuperscript{2+}, governed by the relative affinities of the transcriptional Mn uptake repressor PsaR and the constitutively expressed Mn-specific efflux pump MntE (34,57), to ensure MgtA is not induced and that key intracellular Mn-dependent enzymes are active. At concentrations >1 \muM Mn\textsuperscript{2+}, PsaR will bind Mn\textsuperscript{2+}, increasing its affinity to DNA and repress \textit{psaBCA} transcription, thereby reducing Mn import; excess Mn\textsuperscript{2+} will continue to be effluxed by MntE. Our data suggest that as free Mn\textsuperscript{2+} rises to \geq 100 \muM, the \textit{mgtA} riboswitch functions as a failsafe ‘on’ signal inducing MgtA expression to prevent Mn\textsuperscript{2+} toxicity. In addition, the riboswitch also functions to regulate Ca\textsuperscript{2+} efflux under this condition.

has not yet been determined but might be expected to be in the low \muM range. Thus, if these systems are present and functioning, they would prevent intracellular Mn\textsuperscript{2+} from reaching the \approx 100 \muM range, and thus this riboswitch and subsequent MgtA protein production would simply not fire. Thus, our data support the hypothesis that the MgtA production functions as a ‘fail-safe’ or back-up system to allow \textit{S. pneumoniae} to adapt to acute phases of Mn\textsuperscript{2+} toxicity in the host when Mn\textsuperscript{2+} uptake regulated by PsaR and Mn\textsuperscript{2+} efflux by MntE fail to avoid cellular toxicity (Figure 11) (50).

The functional significance of Ca\textsuperscript{2+} binding by this riboswitch and Ca\textsuperscript{2+} efflux by MgtA under conditions of extreme Mn\textsuperscript{2+} stress (Figure 10B) are not yet known. MgtA is clearly capable of efluxing Ca\textsuperscript{2+} from cells and clotrimazole and extracellular Ca\textsuperscript{2+} interfere with this process (Supplementary Figure S8), but under what conditions this might occur during the course of a bacterial infection are not known. \textit{mgtA} is the first gene in a co-transcribed two-gene operon with a downstream, non-essential gene (\textit{spd}1382) encoding a glutathione S-transferase (25). Consequently, the defective growth of \textit{\Delta mgtA} mutants, irrespective of Mn\textsuperscript{2+} status, could be due to a polarity on \textit{spd}_{1382} expression and/or a second role of MgtA in cellular metabolism possibly in maintaining Ca\textsuperscript{2+} homeostasis. The former is less likely as the replacement of the native \textit{mgtA} gene with a construct encoding MgtA triple-FLAG-tagged protein linked to an antibiotic resistance cassette did not show defective growth (data not shown). The putative essentiality of \textit{mgtA} was not studied further here, but an implication of this operon arrangement is that expression of the \textit{spd}_{1382} glutathione S-transferase is also controlled by the \textit{mgtA} riboswitch aptamer in response to Mn\textsuperscript{2+} and Ca\textsuperscript{2+}. One possibility is that under metal-replete conditions, small amounts of MgtA produced via leaky expression function in Ca\textsuperscript{2+} efflux, perhaps required to efficiently metallate an obligatory Ca\textsuperscript{2+}-requiring enzyme on the outside of the pneumococcal cell; indeed, such a role for P\textsubscript{1B}-type ATPases in metallating periplasmic, extracellular or membrane-anchored client proteins is not without precedent for Cu\textsuperscript{2+}-specific P\textsubscript{1B}-ATPases in other bacteria (58–60). Alternatively, MgtA might play an important role in maintaining Ca\textsuperscript{2+} homeostasis, but total levels of Ca\textsuperscript{2+} simply do not change much under transition metal-replete conditions. This would be consistent with the classification and biochemical characterization of the bacterial MgtA-like transporters as primarily Ca\textsuperscript{2+} transporters (48). In this model, only under conditions of acute Mn\textsuperscript{2+} toxicity does MgtA function as a Mn\textsuperscript{2+} transporter, a condition bolstered by increased accumulation of the MgtA in the membrane to effect efflux of this transition metal.

As a classic P\textsubscript{1B}-type ATPase, MgtA is predicted to transport two metal cations per reaction cycle, which could in theory involve any combination of Mn\textsuperscript{2+} or Ca\textsuperscript{2+} (48). This observation is not intuitive in terms of the basis of metal ion recognition by the transporter based on the significant chemical differences between Mn\textsuperscript{2+} and Ca\textsuperscript{2+}. However, it is not unprecedented. The dual metal specificity of MgtA offers striking functional parallels to the secretory pathway Ca\textsuperscript{2+}-ATPases (SPCAs) previously identified in yeast and vertebrates and known to transport both Mn\textsuperscript{2+} and Ca\textsuperscript{2+} into the Golgi from the cytosol. Here, the metal is used to metallate key client enzymes in this compartment or is simply secreted via this route (61–63). MgtA in fact shows significant sequence similarity to SPCAs and may well harbor the same Q747A substitution that enhances Mn\textsuperscript{2+} transport relative to Ca\textsuperscript{2+} (Supplementary Figure S12). Biochemical studies of \textit{S. pneumoniae} MgtA coupled with more extensive physiological characterization of the functional role of MgtA are clearly required to further elucidate the metal-
dependence and functional role of the MgtA transport cycle.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS
We thank J.D. Helmann (Cornell University, NY) for providing the necessary plasmids and B. subtilis strains to make the lacZ reporter fusions and IPTG inducible constructs, H. Niu (Indiana University, IN) for providing all necessary reagents for the IVT assays, and the Y.X. Wang group (National Cancer Institute) for help collecting the SAXS data. We appreciate the support of the A.E. Simon’s group (University of Maryland, College Park), and a special thanks to J. May for providing the necessary training for us to perform SHAPE profiling.

FUNDING
Idaho State University start up fund (to J.E.M.); Institutional Development Award from the National Institutes of General Medical Sciences [P20GM103408 to Idaho State University]; National Institutes of General Medical Sciences [R35 GM118157 to D.P.G., R01 GM042569-25S1 to J.E.M., R01 GM127715 to M.E.W.]. Funding for open access charge: Idaho State University start up funds (to J.E.M.).

Conflict of interest statement. The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interests or non-financial interests in the materials discussed in this manuscript.

REFERENCES
1. Barrick, J.E., Corbino, K.A., Winkler, W.C., Nahvi, A., Mandal, M., Collins, J., Lee, M., Roth, A., Sudarsan, N., Jona, I. et al. (2004) New RNA motifs suggest an expanded scope for riboswitches in bacterial genetic control. Proc. Natl. Acad. Sci. U.S.A., 101, 6421–6426.
2. Meyer, M.M., Hammond, M.C., Salinas, Y., Roth, A., Sudarsan, N. and Breaker, R.R. (2011) Challenges of ligand identification for riboswitch candidates. RNA Biol., 8, 5–10.
3. Gottesman, S. (2004) The small RNA regulators of Escherichia coli: roles and mechanisms. Annu. Rev. Microbiol., 58, 303–328.
4. Storz, G., Vogel, J. and Wassarman, K. M. (2011) Regulation by small RNAs in bacteria: expanding frontiers. Mol. Cell, 43, 880–891.
5. Waters, L.S. and Storz, G. (2009) Regulatory RNAs in bacteria. Cell, 136, 615–628.
6. Wagner, E.G.H. and Rompy, P. (2015) Small RNAs in bacteria and archaea: who they are, what they do, and how they do it. Adv. Genet., 90, 133–208.
7. Brantl, S. (2009) Bacterial chromosome-encoded small regulatory RNAs. Future Microbiol., 4, 85–103.
8. Repola, E. and Darfeuille, F. (2009) Small regulatory non-coding RNAs in bacteria: physiology and mechanistic aspects. Biol. Cell, 101, 117–131.
9. Majdalani, N., Vanderpool, C.K. and Gottesman, S. (2005) Bacterial small RNA regulators. Crit. Rev. Biochem. Mol. Biol., 40, 93–113.
10. Song, J., Lays, C., Vandenbosch, F., Benito, Y., Bes, M., Chu, Y., Lina, G., Rompy, P., Geissmann, T. and Boisset, S. (2012) The expression of small regulatory RNAs in clinical samples reflects the different life styles of Staphylococcus aureus in colonization vs. infection. PLoS One, 7, e37294.
