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Environment and Health Impacts of Lithium Environmental Impact Report Needed
I think that alternative energy generation facilities sited by the California Energy Commission should include and analysis of the environmental and health impacts of installation, use and disposal because it has not yet been determined whether lithium is a biologically an important or toxic element to living organisms? references:
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Is lithium biologically an important or toxic element to living organisms? An overview
B Shahzad, MN Mughal, M Tanveer, D GuptaÃ¢â‚¬Â¦ - Ã¢â‚¬Â¦ Science and Pollution Ã¢â‚¬Â¦, 2017 -
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The Industrialized world is exposing living organisms to different chemicals and metals such as lithium (Li). Due to their use in common household items to industrial applications, it is imperative to examine their bioavailability. Lithium belongs to the group IA and also has wider uses such as in batteries, air conditioners to atomic reactors. Lithium occurs naturally in soil and water, mostly at low concentrations, and enters the food chain. It is not one of the essential minerals though various studies indicate that low levels of Li have beneficial effects.
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Lithiumâ€‘sensing riboswitch classes regulate expression of bacterial cation transporter genes

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Lithium is rare in Earthâ€™s crust compared to the biologically relevant alkali metal cations sodium and potassium but can accumulate to toxic levels in some environments. We report the experimental validation of two distinct bacterial riboswitch classes that selectively activate gene expression in response to elevated Li+ concentrations. These RNAs commonly regulate the expression of nhaA genes coding for ion transporters that weakly discriminate between Na+ and Li+. Our findings demonstrated that the primary function of Li+ riboswitches and associated NhaA transporters is to prevent Li+ toxicity, particularly when bacteria are living at high pH. Additional riboswitch-associated genes revealed how some cells defend against the deleterious efects of Li+ in the biosphere, which might become more problematic as its industrial applications increase.

Although most organisms are exposed to only modest amounts of lithium ions from the environment, elevated concentrations of this metal cation are toxic to certain eubacteria and eukaryotic species1. Seawater commonly maintains a Li+ concentration of~25 Î¼M2

but higher concentrations can occur near thermal vents. Concentra□tions in soil average~20 mg kgâˆ’1, but amounts can vary by several orders of magnitude depending on regional
geological characteristics3,4
. Unfortunately, the molecular mechanisms of Li+ toxicity in general, and the reasons for its therapeutic efects on humans5
, are poorly understood6,7
.
Some evidence indicates that Li+ inhibits certain bacterial and eukaryotic phosphatase enzymes, thereby
causing accumulation of the nucleotide 3Í□â€²-phosphoadenosine 5Í□â€²-phosphate (pAp)8â€“11 and the disruption of
sulfur metabolism12,13. In addition, Li+ is known to afect signaling processes by disrupting the function of
enzymes involved in phosphatidylinositol and glycogen metabolic pathways6
. Li+ is also predicted to broadly
afect enzymes that exploit Mg2+-ATP complexes7
. Presumably, other molecular targets of Li+ exist wherein cation
binding disturbs normal cellular functions.
One strategy to investigate the mechanisms of Li+ toxicity resistance is to discover how cells sense elevated
concentrations. Previous discoveries of riboswitches that sense fuoride14â€“16 or the divalent heavy metal cations
nickel and cobalt17 revealed genes whose protein products are expressed to help mitigate the deleterious efects
of these ions. Similarly, biological systems are likely to have evolved mechanisms to overcome the adverse efects
of Li+, and these might be revealed by identifying links between Li+ sensing and toxicity mitigation factors.
Herein we report the experimental validation of two distinct classes of Li+-sensing bacterial riboswitches
that regulate genes whose protein products presumably mitigate Li+ toxicity. Specifcally, we used bioinformatic,
genetic, and biochemical analyses to provide support for the function of these RNAs as direct sensors of Li+
that activate gene expression only when this ion is high in concentration. Our fndings indicate that the primary
strategy used by bacteria to overcome the toxic efects of Li+ is to eject the ion from cells.
Results and discussion
Structured RNA motifs discovered by comparative sequence analysis were riboswitch can□didates for elemental ions. Tree structured, noncoding RNA (ncRNA) classes were previously dis□covered that all commonly associate with genes whose protein products are predicted to be monovalent ion
transporters18. Tese ncRNA classes, called the nhaA-I, nhaA-II, and DUF1646 motifs (Fig. 1Aâ€“C, top), were

considered riboswitch candidates, wherein the conserved domain of each RNA functions as an aptamer that
binds a ligand to regulate expression of the adjacent gene. Given the gene associations common for the three
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motifs (Fig. 1Aâ€“C, bottom), we speculated that they would sense one or more biologically relevant alkali ions
to trigger riboswitch-mediated gene control. Indeed, we have established that DUF1646 motif RNAs function
as highly selective riboswitches for Na+ ions19. Representative DUF1646 motif RNAs (hereafer called Na+-I
riboswitches) were shown to activate gene expression in response to elevated Na+ concentrations, thereby regu□lating the expression of various genes either known or predicted to be relevant to sodium transport or utilization
(Fig. 1C). Tese RNAs bind Na+ with 1-to-1 stoichiometry and strongly reject all other mono- and divalent alkali
and alkaline earth cations tested19.
Te consensus sequence and secondary structure model for Na+-I riboswitch aptamers (Fig. 1C) is similar to
that for nhaA-I motif RNAs (Fig. 1A). Despite their similarities, the consensus models exhibit distinct conserved
features in the lower portion of the frst base-paired region (P1) and in the loop of P2 that served as the basis for
sorting representatives into two groups. Some genes associated with Na+-I riboswitches, such as DUF1646, nhaD,
and oadG, are also occasionally associated with nhaA-I motif RNAs. Tus, we speculated that nhaA-I motif RNAs
might sense a ligand that is chemically similar to Na+, such as a diferent alkali metal cation. Moreover, although
the nhaA-I motif shares no similarity in either sequence or architecture to the nhaA-II motif (Fig. 1B), they both
most frequently associate with nhaA genes. Tis observation strongly suggested that

nhaA-I and nhaA-II motifs
form distinct RNA structures that sense the same ligand.
Members of the nhaAâ€‘I and nhaAâ€‘II motif classes function as Li+
â€‘specifc genetic
switches. NhaA proteins (pfam06965, pfam07399 and COG3004) are known to function as Na+/H+ anti□porters, and these membrane-localized proteins are generally implicated in Na+ homeostasis20,21, along with
another demonstrated Na+/H+ protein class called NhaB22. However, previous biochemical assays21,23,24 reveal
that NhaA proteins robustly transport both Na+ and Li+. In Escherichia coli, it was demonstrated that delet□ing the nhaA gene results in increased Li+ toxicity, whereas deleting the nhaB gene alone has no efect on Li+
sensitivity25. Furthermore, expression of the bacteria NhaA protein confers tolerance for Li+, but not Na+, in Sac□charomyces cerevisiae26. Because the nhaA-I and nhaA-II motif RNAs are associated with nhaA genes and never
nhaB genes, we considered the possibility that these RNAs might function as selective Li+-sensing riboswitches.
To assess the ligand binding and gene regulation functions of nhaA-I motif RNAs, we frst prepared a genetic
fusion (translational) between a Î²-galactosidase (lacZ) reporter gene and the riboswitch candidate based on the
nhaA-I motif representative from Azorhizobium caulinodans (Fig. 2A). Transcription is driven by a heterologous
(Bacillus subtilis lysC) promoter known to be constitutively active27,28. Tis construct, evaluated in surrogate E.
coli cells grown on LBK agar plates at pH 9.1 using agar difusion assays (see Materials and Methods), yielded
low reporter gene expression in the absence of added Li+ and robust gene expression when cells were experienc□ing Li+ toxicity (Fig. 2B).
Furthermore, mutant E. coli cells lacking the gene coding for the native NhaA protein (Î”nhaA) were sensi□tive to lower concentrations of Li+ and exhibited higher reporter gene expression even when Li+ was not sup□plemented in the growth medium. Tis result suggested that the Î”nhaA strain could not efciently expel Li+
Figure 1. Tree distinct classes of riboswitch candidates for alkali metal cations. (Top) Sequence and
secondary structure models for the nhaA-I, nhaA-II and DUF1646 motif RNAs. Consensus models for nhaA-I
and nhaA-II were generated based on updated sequence alignments (see supplemental fles). Te consensus
model for DUF1646 (Na+-I riboswitch aptamer) was prepared as described elsewhere19. (Bottom) Annotated
functions of proteins encoded by genes (parentheses) commonly associated with the three riboswitch candidates
(see also Supplementary Table I in the Supplement). Data are based on the total number of non-redundant
representatives as noted.

that accumulated in cells even when only trace amounts were present in the medium. Tese gene regulation
and toxicity characteristics were specifc for Li+ because no other monovalent ion tested triggered riboswitch
function or inhibited cell growth (Fig. 2B).
Likewise, a reporter-fusion construct integrating a representative of the nhaA-II riboswitch candidate
(Fig. 2C) yielded similar reporter expression results (Fig. 2D). Tese fndings were consistent with the hypothesis
that at least two distinct Li+-responsive riboswitch classes exist among species of bacteria, wherein the RNAs can
sense toxic levels of this alkali metal cation and activate the expression of NhaA proteins. Tus, Li+-responsive
riboswitches and NhaA proteins appeared to be key components of Li+ toxicity defense responses in some bacte□rial species. Hereafer, we refer to these riboswitch classes as Li+-I (nhaA-I motif) and Li+-II (nhaA-II).
Notably, reporter assays for both nhaA-I and nhaA-II constructs yielded patterns of faint blue color nearest
to the flter disk wherein Li+ was applied, and a more intense blue ring farther from the disk. Tis pattern could
be due to a reduction in cell growth caused by Li+ toxicity (faint blue) near the disk, and Li+-triggered induction
of gene expression mediated by the riboswitch at a distance where cells are less stressed. Similar patterns are
observed for other validated riboswitches when evaluated using agar difusion assays29.
Quantitative reporter gene assays were also conducted with the Li+-I and Li+-II riboswitch-reporter fusion
constructs described above. E. coli cells carrying the WT nhaA-I (Li+-I) riboswitch-reporter fusion construct
(Fig. 2A) and cultured in rich medium at pH 9.0 exhibited robust reporter gene expression when exposed to
50 mM Li+, but these cells were largely unresponsive to media supplementation with 50 mM Na+ or K+ (Fig. 3A,
lef). Furthermore, the introduction of mutations that altered two nucleotides of the highly conserved loop
region (Fig. 2A, construct M1: G52A, G53A) substantially diminished reporter gene expression in response to
Li+ addition (Fig. 3A, right). Tis fnding was consistent with the observation that the M1 construct exhibited
poorer afnity for Li+ compared to the WT construct (see below). E. coli cells carrying the WT nhaA-II (Li+-II)
Figure 2. Li+ triggers gene expression mediated by bacterial nhaA-I and nhaA-II motif

RNAs. (A) A nhaA-I
riboswitch-reporter fusion construct was created by fusing a representative associated with the nhaA gene of
Azorhizobium caulinodans to a Î²-galactosidase gene (lacZ). Red nucleotides correspond to the highly conserved
nucleotides characteristic of this RNA motif class (Fig. 1A). Boxed nucleotides identify locations of G-to-A
mutations present in construct M1, which is a mutant riboswitch RNA used in several subsequent experiments.
(B) Agar-difusion assays were conducted with E. coli cells (either wild type [WT] or a nhaA gene deletion
[Î”nhaA] strain) carrying the nhaA-I reporter construct in A. Cells were spread on LBK (pH 9.1) agar media
containing X-gal, and flter disks using 10 Î¼L applications of 5 M chloride salts of various monovalent ions as
indicated, except that reduced concentrations of KCl (3 M) and RbCl (0.5 M) were used due to limited solubility.
(C) A nhaA-II riboswitch-reporter fusion construct was created by fusing a representative associated with the
nhaA gene of Brevundimonas subvibrioides to lacZ. Additional annotations are as described for A. (D) Agar□difusion assays with various cations were conducted with the nhaA-II reporter as described in C. Additional
annotations and details are as described for B.

riboswitch-reporter fusion construct (Fig. 2B) exhibited a similar selective response to Li+ (Fig. 3B). Tese fnd□ings indicated that Li+-I and Li+-II riboswitches most likely use their highly conserved sequence and structural
elements to respond exclusively to Li+, which is also consistent with the fact that cells become more sensitive only
to Li+ when a gene most associated with Li+ riboswitches was deleted (Î”nhaA strain) (Fig. 2).
Biochemical assays confrm ligand responsiveness by Li+
â€˜I and Li+
â€˜II riboswitches. Selective
cation recognition by Li+-I riboswitches was also established using in-line probing30,31, which is a method that
exploits the chemical instability of RNA to monitor folding changes in response to ligand binding. For example,
an RNA construct called 73 nhaA (Fig. 4A), whose design is based on a representative Li+-I aptamer associ□ated with the nhaA gene of the bacterium Pseudomonas monteilii, produced a spontaneous degradation pattern
(Fig. 4B) that was largely consistent with the proposed consensus secondary structure model18. Even though

2 mM Mg2+ and 100 mM K+ were present in the in-line probing reaction mixtures, the RNA underwent changes
in band intensity in several locations upon the addition of Li+, most noticeably in the linker region between
stems P1 and P2 (Supplementary Fig. I in the Supplement).
Tree sites of band intensity changes, chosen because these RNA fragments are produced upon cleavage
within sequence or structural regions typical of this aptamer class, were evaluated to assess the ligand-binding
characteristics of the RNA. Albeit modest, band intensity changes at these sites occurred in a concerted fashion,
yielding a ligand binding curve consistent with a 1-to-1 interaction and an apparent dissociation constant (KD)
of~30 mM (Fig. 4C). Similar results were observed for a Li+-I riboswitch aptamer from the bacterium Ralstonia
eutropha (Supplementary Fig. II in the Supplement), which exhibited a KD of no lower than~20 mM for Li+.
In contrast, Na+ began to induce changes in band intensities for the 73 nhaA RNA construct only at concen□trations~tenfold higher (Supplementary Fig. III in the Supplement), indicating that Li+ is the preferred ligand.
In-line probing reactions were also used to survey the efects of several other alkali metal ions and ammonium
(NH4
+) at 200 mM or Rb+ at 50 mM (Fig. 4D, Supplementary Fig. IV in the Supplement). Te RNA aptamer
strongly rejected K+, Rb+ and Cs+, and exhibited only poor afnity for Na+ and NH4
+. Relatively low cellular
concentrations of ammonium32 and Na+ under non-osmotic stress conditions33 in combination with their poorer
riboswitch afnities likely prevent these ions from triggering gene regulation through Li+ riboswitches. Fur□thermore, mutation of strictly conserved G nucleotides to A nucleotides (construct M1: G56A, G57A) (Fig. 4A)
greatly weakened the response of the riboswitch reporter construct to Li+ (Supplementary Fig. V in the Sup□plement), indicating that these positions are important for ligand binding. Tis latter observation is consistent
with the fact that the M1 construct exhibited substantially reduced responsiveness to Li+ in riboswitch-reporter
fusion assays (Fig. 3B, right).
Figure 3. Quantitation of gene expression mediated by representative Li+-I and Li+-II riboswitches. (A) Lef:
E. coli cells carrying the WT nhaA-I (Li+-I) riboswitch-reporter fusion construct (see Fig. 2A) grown in low
salt LB medium at pH 9 either without or with supplementation with 50 mM LiCl, NaCl, or KCl as indicated.
Either X-gal (top) or ONPG (bottom), respectively, was added afer overnight incubation of cultures to visualize

or quantify Î²-galactosidase reporter activity. Right: E. coli cells carrying the WT nhaA-I (Li+-I) or M1 nhaA-I
(Li+-I) riboswitch-reporter fusion construct (see Fig. 2A) cultured in low salt LB medium at pH 9 either without
or with supplementation of 50 mM LiCl. Te mean and standard deviation values are presented for experiments
conducted in triplicate (n=3). (B) E. coli cells carrying the WT nhaA-II (Li+-II) riboswitch-reporter fusion
construct (Fig. 2C) were cultured in low salt LB medium at pH 9.0 without or with supplementation of 50 mM
LiCl, NaCl, or KCl as indicated.

Rare variants of Li+
â€‘I riboswitches exhibit altered ion specifcity and are robustly triggered by
Na+
. Although we speculate that most Li+-I and Li+-II riboswitches regulate gene expression by controlling
ribosome access to the ribosome binding site (RBS) of the adjoining open reading frame, rare variants of Li+-I
riboswitch aptamers carry an intrinsic terminator stem34,35 as an expression platform36. Tus, we speculated that
these unusual variants might bind the ligand and prevent transcription termination from occurring upstream
of the protein coding region of the messenger RNA as a mechanism to regulate gene expression. Indeed, we
observed that a representative of this variant collection associated with a hypothetical gene from the bacte□rium Desulfobulbus propionicus (Fig. 5A) robustly activated transcription upon ligand binding as determined
by in vitro transcription assays, whereas mutants M2 and M3 failed to respond (Fig. 5B). However, surprisingly,
the construct chosen for this analysis was strongly activated upon the addition of Na+ and was only weakly trig□gered by Li+.
To further investigate this unexpected outcome, we hypothesized that some RNAs originally included in the
nhaA-I motif collection carry nucleotide changes that switch the specifcity from Li+ to Na+. Natural riboswitch
specifcity changes are well demonstrated to occur through evolution37â€“39. Consistent with this hypothesis, a
construct carrying 71 nucleotides encompassing the variant D. propionicus (called 71 hypo, Supplementary
Fig. VIA in the Supplement) exhibited structural modulation only with Na+ among the alkali metal cations tested
(Supplementary Fig. VIB in the Supplement). In addition, the estimated KD value for

Na+ binding was in the low
Figure 4. Li+ induces structural modulation of a Li+-I aptamer. (A) Sequence and secondary structure model
for the Li+-I riboswitch aptamer construct 73 nhaA derived from the nhaA gene from P. monteilii. Lowercase
g letters identify non-native nucleotides added to facilitate production by in vitro transcription and red
nucleotides are highly conserved in nhaA-I motif RNAs as depicted in Fig. 1A. Boxed nucleotides at positions
56 and 57 were mutated to A nucleotides in construct M1. Nucleotides circled in red are among those that
undergo reduced spontaneous cleavage during in-line probing assays upon the addition of Li+, as determined
from the autoradiogram depicted in B. (B) PAGE analysis of in-line probing reactions with 5â€² 32P-labeled 73
nhaA RNA in the absence of Li+ (â€“), or in the presence of Li+ concentrations ranging from 2 to 200 mM. NR, T1
and â€“
OH identify RNAs subjected to no reaction, partial digestion with RNase T1 (cleaves afer G nucleotides)
and partial digestion with hydroxide (cleaves afer each nucleotide). Bands corresponding to RNAs carrying a 3â€²
G nucleotide are identifed according to the numbering system in A. A lower contrast version of the gel image
is presented in Supplementary Fig. I in the Supplement. (C) Plot of the estimated fraction of RNAs bound to
Li+ versus the logarithm of the Li+ concentration. Fraction bound values were estimated by quantifying band
intensities at sites 1, 2 and 3 in B, which correspond to the nucleotides annotated with red circles in A. Note that
fraction bound values were set to 1 at the maximum Li+ concentration tested because the band intensities are
near zero (maximal possible suppression). Te solid line depicts a theoretical 1-to-1 binding curve with a KD of
30 mM. (D) PAGE analysis of in-line probing assays as described for B wherein reactions were supplemented
with 200 mM of the ions indicated, except Rb+ was tested at 50 mM. Reduced band intensities at site 3 depicted
here are indicative of ion binding. A lower contrast version of the full gel image and comparisons of sites 1, 2
and 3 are presented in Supplementary Fig. IV in the Supplement.

mM range (Supplementary Fig. VIC,D in the Supplement), which is similar to the afnities

measured for Na+-I
riboswitches described previously19.
Te hypothesis of a ligand specifcity change for some nhaA-I motif RNAs was further explored by examin□ing the aptamers grouped into this collection that also associate with intrinsic terminator expression platforms.
Tese variant riboswitches carrying terminator stems were found only in species of Deltaproteobacteria, where
they are located upstream of various genes coding for proteins of unknown function. Tese gene associations
were not found with the remaining RNAs that conform to either Li+-I or Li+-II riboswitch classes, suggesting
that the variant RNAs sense a ligand other than Li+.
Furthermore, most nhaA-I motif RNAs carry a U nucleotide corresponding to nucleotide position 54 of the
RNA constructs derived from D. propionicus (Fig. 5A, Supplementary Fig. VIA in the Supplement), whereas the
natural variant riboswitch carries a C nucleotide at this position. By making a single C54U mutation in the origi□nal riboswitch sequence (M4, Fig. 5A), the ion responsiveness during transcription was broadened to include Li+,
Na+, and NH4
+ (Fig. 5C). Tese results indicate that some RNAs originally grouped with the nhaA-I motif RNAs
carry nucleotide changes that alter their ligand specifcity from Li+ to Na+. Given that these Na+-sensing variant
aptamers described herein carry substantial diferences in the consensus model compared to Na+-I riboswitches
described previously19, we have named these rare variants Na+-II riboswitches.
Concluding remarks
Te discovery of riboswitches for Li+ reveals the only known mechanisms by which bacterial cells sense and
mitigate high concentrations of this toxic alkali metal cation. Te demonstrated selectivity of both Li+ riboswitch
classes in vivo (Figs. 2, 3) highlights the question of how RNAs form binding pockets that favor Li+ binding over
other monovalent ions. Te repetitive negatively charged phosphodiester backbone of RNA clearly creates a
favorable ionic environment for the association of cations. Furthermore, alkali metal cations are well known to
stabilize various structural features of folded RNAs40. Tese characteristics seem to make RNA well suited to form
structures that selectively bind monovalent ions. Indeed, engineered RNA aptamers have been identifed that
bind vitamin B12 only in the presence of Li+, which provides a precedence for the selective binding of this cation41.
Figure 5. Variants of Li+-I riboswitches naturally respond to Na+. (A) Sequence and secondary structure

model for a Na+-sensing riboswitch associated with a gene annotated as â€œhypotheticalâ€ (Supplementary Table I
in the Supplement) from D. propionicus. Orange shading indicates alternative pairing that is predicted to form
an intrinsic terminator stem. Predicted terminated (T) and full-length (FL) RNA transcripts are denoted with
arrowheads. Other annotations are as described for Figs. 2 and 3. (B) Top: PAGE autoradiogram of an example
single-round transcription termination assay series with a DNA template producing the D. propionicus construct
depicted in A. Bands corresponding to FL and T transcripts identifed in A are denoted. Transcription reactions
were supplemented with the ions as indicated, and M identifes the lane loaded with a marker approximating
FL RNA. Bottom: plot of the fraction of FL transcripts for each transcription reaction conducted in triplicate,
where the circle colors matching the panels were derived from the gel shown. Te dashed line represents the
average fraction FL in the absence of additional ion supplementation above the 4 mM Na+ initially present in
the transcription reaction. An uncropped version of the gel image is presented as Supplementary Fig. VIII in
the Supplement. (C) Top: PAGE autoradiogram of an example transcription termination assay series using RNA
construct M4, which carries a C54U mutation that represents the nucleotide at this position most commonly
observed with nhaA-I motif RNAs. Bottom: Plot of the fraction of FL transcripts for each transcription reaction
conducted in triplicate (n=3), where the numbers indicate the mean and standard deviation. Additional
annotations are as described for B. An uncropped version of the gel image is presented as Supplementary Fig. IX
in the Supplement.

We considered the possibility that bacterial species living in high Li+ concentration environments might be
more likely to carry these riboswitches. Te nhaA-I RNA motif is present in diverse species of Alpha-, Beta-,
Gamma-, and Deltaproteobacteria, whereas the nhaA-II RNA motif appears to be present only in some species of Gammaproteobacteria. At present, we have not found a notable bias in favor of these riboswitches in organisms that are known to live in environments high in lithium. Tus, it seems likely that bacteria living in
many ordinary environments must have mechanisms to sense and respond to lithium

toxicity, suggesting that
additional sensors for Li+ await discovery.
Most genes associated with Li+ riboswitches appear to be cation transporters (Fig. 1, Supplementary Table I
in the Supplement), and our fndings strongly indicated that the primary function of some NhaA proteins is
to export Li+ as concentrations approach toxic levels. It is notable that cells become more sensitive to high Li+
concentrations under alkaline conditions (Supplementary Fig. VII in the Supplement), and we speculate that this
is a result of the use of Na+ gradients to power solute transport. At high pH, cells can harness the energy present in ion gradients to import various solutes42. Excess cellular Na+ can then be used by Na+-H+ antiporters to adjust internal pH. If this import system is imperfect, then Li+ might also enter cells predominantly at high pH because it mimics the function of Na+. Under these circumstances, Li+ riboswitches and NhaA proteins could selectively sense and preferentially expel excess Li+. Given that NhaA proteins are also ion-proton antiporters, this action would both alleviate Li+ toxicity and contribute to reducing cellular pH.
Intriguingly, among bacterial sequences generated from an environmental bacterial sample, we identifed
several instances wherein riboswitches conforming to the Li+-I class reside in tandem. Such tandem arrangements of riboswitches for the same ligand have been observed previously, and they usually function to reduce the ligand concentration range needed to maximally trigger changes in gene expression43. However, tandem riboswitch arrangements wherein the aptamers sense diferent ligands yield genetic versions of Boolean logic gates44,45, wherein gene expression depends on the concentrations of two diferent chemical inputs. Te genes associated with these tandem Li+-I riboswitches are generally related to cation transport and osmotic stress, and it was not determined if the aptamers are selective for Li+ or Na+. In addition, one example of a triple riboswitch arrangement was observed preceding a nhaD gene, wherein each aptamer is followed by an intrinsic terminator stem. Again, the ligand specifcity of each riboswitch is unknown, but this appears likely to activate the expression of an NhaD cation/H+ antiporter protein in response to very small changes in ligand concentration.
It is known that fluoride riboswitches commonly upregulate the expression of genes for enzymes that are
inhibited by this toxic anion. This action should help maintain the metabolic fux through pathways where
key enzymes have reduced activity due to Fâ˜ inhibition. However, most genes coding for proteins previously
implicated7â€“13 in the biological/toxic efects of Li+ are not listed among those associated with the two Li+-sensing
riboswitch classes. Intriguingly, a gene coding for trehalose-6-phosphate synthase is occasionally associated
with Li+-I riboswitches19 (Supplementary Table I in the Supplement). It has been established46,47 that trehalose
biosynthesis in some eukaryotes is altered by exposure to Li+, and thus some bacteria

might use Li+ riboswitches
to regulate trehalose biosynthesis when concentrations of the cation are high. Alternatively, these riboswitches
might be variants that sense Na+, and regulation might be part of an osmotic stress process involving trehalose.
Other genes associated with Li+ riboswitches might provide clues regarding the molecular targets relevant
to Li+ toxicity. However, the rarity of gene associations beyond nhaA suggests that the deleterious efects of Li+
cannot be easily overcome by upregulating its molecular targets. This conclusion is consistent with the possible
action of Li+ in eukaryotes, which has been proposed to induce its efects by binding to many targets7
. If true,
the most practical way for most bacteria to overcome Li+ toxicity might be to lower its concentration by ejection.
from the cell.

Methods

Chemicals and biochemical. Lithium chloride was purchased from Acros Organics and ammonium chlo□ride was purchased from Macron Fine Chemicals. [Î±-32P]UTP and [Î³-32P]ATP were purchased from Perki□nElmer. All other chemicals and synthetic DNA oligonucleotides were purchased from Sigma-Aldrich. RNase
T1 was purchased from Roche and all other enzymes were purchased from New England Biolabs. All salts were
of 99% or greater purity.
Bioinformatics analyses. Consensus models for the nhaA-I (Fig. 1A) and nhaA-II (Fig. 1B) motifs
were updated by frst conducting searches for additional representatives using CMFinder and Infernal 1.1
algorithms48,49 as described previously18. Te consensus model for DUF1646 (Na+-I riboswitch aptamers) is
depicted (Fig. 1C) as published previously19. Searches were conducted against the RefSeq 80 genomic sequence database and a collection of microbial environmental sequences (env12). Consensus sequence and secondary structure models were created with the computer program R2R50, and covariation annotations were defned using R-scape51.
Gene associations, defned as the frst gene located immediately downstream of each RNA motif representa□tive, were established by manual examination of each representative. In some instances, gene identities were
established by using the NCBI Basic Local Alignment Search Tool (BLAST)52 to identify annotated protein
homologs with known functional annotations. Pie charts were then generated to present the distributions of
genes associated with each motif class.
RNA constructs. Synthetic DNA oligonucleotides (Supplementary Table II in the Supplement) were used

to prepare RNA transcription templates by overlap extension using SuperScript II reverse transcriptase follow□ing the manufacturerâ€™s protocol (Termo Fisher Scientifc). Te resulting double-stranded DNA templates were
transcribed using T7 RNA polymerase in 50 Î¼L reactions (80 mM HEPES [pH 7.5 at~20 Â°C], 24 mM MgCl2,

2 mM spermidine, 40 mM DTT) incubated overnight at 37 Â°C. RNA products were separated using denatur□ing (8 M urea) polyacrylamide gel electrophoresis (PAGE), and the gel portions containing the desired RNAs
were excised, crushed, and incubated in 350 Î¼L crush-soak solution (200 mM NH4Cl, 10 mM Trisâ€“HCl [pH 7.5
at~20 Â°C], 1 mM EDTA) for 30 min at~20 Â°C. RNAs were recovered from gel extracts by precipitation with cold
ethanol followed by centrifugation. Te resulting pellet was dried under vacuum and resuspended in deionized
water (dH2O). RNA solutions were quantifed by measuring the absorbance at 260 nm and calculating molarity
using extinction coefcients estimated for each product.
RNAs (75 pmol) were dephosphorylated using rAPid Alkaline Phosphatase (Roche) following the manu□facturerâ€™s protocol. 10 pmol of each resulting RNA was 5â€² 32P-labeled using T4 polynucleotide kinase in a 20 Î¼L reaction containing 5 mM MgCl2, 25 mM CHES (pH 9.0), 3 mM DTT, and 20 Î¼Ci [Î³-32P]-ATP. Radiolabeled RNAs were purifed by PAGE, and salts were removed by performing three dH2O washes through an Amicon Ultra-0.5 centrifugal flter unit (3 KDa molecular weight cut-of).
Inâ€˜line probing assays. In-line probing assays were conducted as described previously30,31, except that the
concentration of MgCl2 used was 2 mM. 32P-labeled RNAs were incubated with candidate ligands for 1 min at
75 Â°C before the addition of room-temperature in-line probing buffer (50 mM Trisâ€“HCl [pH 8.3 at~20 Â°C],
2 mM MgCl2, 100 mM KCl). Te resulting mixtures were incubated at room temperature for between 40 and
70 h. Denaturing 10% PAGE was used to separate the resulting RNA degradation products, which were visualized using a Typhoon FLA 9500 Molecular Scanner (GE Healthcare). Product band intensities were quantified using ImageQuant software and bands whose intensities are modulated by ligand introduction were used for estimating KD values for RNA-ligand interactions. The resulting intensity values were scaled to a fraction between 0 and 1 (greatest change), then plotted against the logarithm of the ligand concentration. Apparent KD values were calculated using a sigmoidal-dose response equation in GraphPad Prism 8.
Riboswitch reporter assays. Riboswitch reporter fusion constructs for both nhaA-I and nhaA-II motifs

were prepared by PCR amplification of synthetic DNA constructs (Supplementary Table II in the Supplement)
with the lysC promoter from B. subtilis directly preceding the riboswitch sequence of interest. Te resulting
DNAs were digested with the appropriate restriction enzyme and ligated into the pRS414 reporter vector (gif
from R.W. Simons, UCLA). Plasmids were used to transform the WT E. coli BW25113 strain and its isogenic
derivative BW25113 (Î”nhaA::kan). E. coli cells were obtained from the Coli Genetics Stock Center at Yale Uni□versity.
For agar-difusion assays, cells with the desired reporter plasmid were grown overnight in LBK media (stand□ard LB media with sodium replaced with 100 mM KCl). LBK agar plates were bufered with AMPSO (100 mM, pH adjusted with KOH) and contained X-gal (5-bromo-4-chloro-3-indolyl Î²-d-galactopyranoside; 100 Âµg mLâˆ’1) and carbenicillin (100 Âµg mLâˆ’1). Te choice of LBK and buffer was based on established methods53. Assays were conducted at pH 9.0 or 9.1 (100 mM K+, 100 mM AMPSO) as noted for each experiment. 10 Î¼L of 5 M LiCl, 5 M NaCl, 3 M KCl, 0.5 M RbCl, 5 M CsCl, or 5 M NH4Cl was added to flter disks as indicated. Te concentrations of stock salt solutions were chosen based on solubility. Plates were incubated overnight at 37 Â°C and were then photographed to record growth and Î²-galactosidase activity.
Liquid cultures inoculated with E. coli strains carrying riboswitch reporter fusion constructs were used to
conduct visual (X-gal) or quantitative (ONPG) Î²-galactosidase assays. Cells with the indicated reporter plasmid
were grown overnight in low sodium LB (yeast extract and tryptone). Ten 100 Î¼L of the overnight culture was
subcultured in low sodium LB bufered at pH 9.0 with 100 mM AMPSO (pH adjusted with KOH) containing
carbenicillin (100 Âµg mLâˆ’1) plus the 50 mM of the supplemented ion as indicated. Higher concentrations of Li+
were found to inhibit cell growth. Cultures were incubated overnight, X-gal (100 Âµg mLâˆ’1) was subsequently
added, and cells were photographed afer development of blue color indicating high Î²-galactosidase activity.
Alternatively, ONPG was added to the cultures to quantify Î²-galactosidase activity by adapting the method
previously described by Miller54. Tree individual replicates of each assay condition were performed with an
additional fourth replicate performed of the WT nhaA-I reporter for comparison to the three replicates of the
M1 nhaA-I reporter.
In vitro transcription assays. Te transcription termination assays were conducted by adapting a previ□ously established method for single-round transcription55. Te DNA templates (Supplementary Table II in the
Supplement) for the RNA transcripts (Fig. 5A) include the native promotor from D. propionicus. Transcription

reactions were performed with 100 nM DNA template in 40 mM Trisâ€“HCl (pH 7.5 at 23 Â°C), 100 mM KCl,
4 mM MgCl2, 0.01 mg mLâ¯1 BSA, 1% (v/v) glycerol, and 0.04 U ÂµLâ¯1 E. coli RNA Polymerase, Holoenzyme
(New England Biolabs). Te E. coli RNA polymerase was supplied in a bufer containing NaCl, thus adding
4 mM NaCl to the fnal reaction. Te transcription reaction was initiated in the presence of ApA dinucleotide
(0.135 mM), GTP and ATP (2.5 ÂµM each), UTP (1.0 ÂµM), and [Î±-32P]-UTP (2 ÂµCi). Tis initial reaction mixture
was incubated for 10 min at 37 Â°C to allow RNA polymerase to stall at the frst C residue of the RNA transcript,
which occurs 16 nucleotides from the transcription start site. Te halted complexes resumed transcription with
the addition of an elongation mixture containing 150 ÂµM each of GTP, ATP, and CTP and 30 ÂµM UTP. Also,
0.1 mg mLâ¯1 heparin was added at that time to prevent further RNA polymerase initiation, and the mixture was
incubated for 30 min at 37 Â°C. It was necessary to add the additional monovalent ions (candidate riboswitch
ligands) with the elongation mixture after the initiation complex was formed, otherwise there was a decrease in
overall yield of transcription products.
All annotations for monovalent ion supplementation do not include 100 mM KCl present in the initial transcription reaction mixture in the buffer. For example, the reaction annotated as supplemented with 200 mM K+

has a total of 300 mM KCl. Transcription products were separated by denaturing 10% PAGE, imaged using a
Typhoon FLA 9500 Molecular Scanner, and quantifed using ImageQuant sofware. Tree replicate transcription termination experiments were performed on three separate days, and a repre□sentative of the resulting PAGE autoradiogram is presented (Fig. 5B, top). Using band intensities, the amounts of full-length (FL) and terminated (T) transcripts were estimated by accounting for the diferent number of U residues in each transcript, and the resulting values for all three experimental replicates are depicted in the
plot (Fig. 5B, bottom). Te number of U nucleotides in the initiation region for all constructs is 5. Te number
of U nucleotides in the elongation region is 45 for the WT and M2 constructs, 47 for the M3 construct, and
46 for the M4 construct. Te percent of [Î±-32P]-UTP relative to total UTP concentration in the initiation and
elongation reactions (7% and 0.2%, respectively) was established, and the relative

amount of radioactivity per
terminated (RT) and full length (RFL) transcripts was calculated for each transcript size using the following
equation: [(Number of U residues in initiation region)(7%)]+ [(Number of U residues in elongation region)
(0.2%)]=R. RT/RFL is equal to the correction factor (X%) that accounts for the increased number of radiolabeled
U residues in the full-length transcript. Te equation used to establish the percent of transcription termination
was: 100{T/[T+(FL)(X%)]}=percent termination. Te resulting values were used to establish the fraction of
RNA transcripts that are full length, which was plotted (Fig. 5B). Tese methods were also used to establish the
data for the M4 construct (Fig. 5C).

Data availability

Te datasets generated and/or analysed during the current study are available in the Rfam repository, [https://
rfam.xfam.org/family/RF03057#tabview=tab1 and
https://rfam.xfam.org/family/RF03038#tabview=tab1]. All
other data needed to evaluate the authorâ€™s conclusions are presented in the main or Supplementary Materials
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Author contributions

N.W., H.S., N.S., and R.R.B. planned experiments. N.W., H.S., N.S., and A.S. performed experiments and all
authors evaluated the data. R.R.B. wrote the manuscript with input and edits from all authors. Project management and funding acquisition was provided by R.R.B.

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