Transport of Magnesium by a Bacterial Nramp-Related Gene

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Abstract

Magnesium is an essential divalent metal that serves many cellular functions. While most divalent cations are maintained at relatively low intracellular concentrations, magnesium is maintained at a higher level (~0.5–2.0 mM). Three families of transport proteins were previously identified for magnesium import: CorA, MgtE, and MgtA/MgtB P-type ATPases. In the current study, we find that expression of a bacterial protein unrelated to these transporters can fully restore growth to a bacterial mutant that lacks known magnesium transporters, suggesting it is a new importer for magnesium. We demonstrate that this transport activity is likely to be specific rather than resulting from substrate promiscuity because the proteins are incapable of manganese import. This magnesium transport protein is distantly related to the Nramp family of proteins, which have been shown to transport divalent cations but have never been shown to recognize magnesium. We also find gene expression of the new magnesium transporter to be controlled by a magnesium-sensing riboswitch. Importantly, we find additional examples of riboswitch-regulated homologues, suggesting that they are a frequent occurrence in bacteria. Therefore, our aggregate data discover a new and perhaps broadly important path for magnesium import and highlight how identification of riboswitch RNAs can help shed light on new, and sometimes unexpected, functions of their downstream genes.

Introduction

Metal ions are essential and serve many cellular purposes, including functioning as cofactors for numerous metalloenzymes. The latter are responsible for a diverse array of biochemical reactions and, together, comprise one third of all cellular proteins [1–3]. Conversely, all metals elicit toxic effects when they accrue to excess. Therefore, specific mechanisms are required for maintaining intracellular pools. In many instances, metal-sensing regulatory proteins (metalloregulatory proteins) control expression of transport proteins, which have been shown to transport divalent cations but have never been shown to recognize magnesium. We demonstrate that this transport activity is likely to be specific rather than resulting from substrate promiscuity because the proteins are incapable of manganese import. This magnesium transport protein is distantly related to the Nramp family of proteins, which have been shown to transport divalent cations but have never been shown to recognize magnesium.

Nrpamp genes are members of a large gene family, with numerous representatives in all three domains of life. For example, the sequence identity between bacterial and mammalian Nramps is high, often in excess of 35% [10]. Interestingly, just as mammalian Nramps are involved in microbial resistance, bacterial Nramps may be simultaneously required during infection by intracellular pathogens [11–13], although the importance of these proteins during infection remains a subject of debate [14,15]. Essentially, bacterial and mammalian Nramps may compete for the same metals within the phagosome at the interface of host-pathogen interactions [16,17]. Also, in addition to their important roles during microbial pathogenesis, genes encoding Nramps are required by many bacterial genomes as fundamental transporters of divalent ions.

Nramps share common structural features, including 10–12 transmembrane domains and conserved residues interspersed throughout. High-resolution structural data from X-ray crystallography is still lacking though, restricting knowledge of the structural basis of metal selectivity and transport. It is generally presumed that Nramp family members are employed for transport of manganese or iron, although some family members have been

References

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Author Summary

Magnesium ions are essential for life, and, correspondingly, all organisms must encode for proteins to transport them. Three classes of bacterial proteins (CorA, MgtE and MgtA/B) have previously been identified for transport of the ion. This current study introduces a new route of magnesium import, which, moreover, is unexpectedly provided by proteins distantly related to Natural resistance-associated macrophage proteins (Nramp). Nramp metal transporters are widespread in the three domains of life; however, most are assumed to function as transporters of transition metals such as manganese or iron. None of the previously characterized Nramps have been shown to transport magnesium. In this study, we demonstrate that certain bacterial proteins, distantly related to Nramp homologues, exhibit transport of magnesium. We also find that these new magnesium transporters are genetically controlled by a magnesium-sensing regulatory element. Importantly, we find numerous additional examples of similar genes sharing this regulatory arrangement, suggesting that these genes may be a frequent occurrence in bacteria, and may represent a class of magnesium transporters. Therefore, our aggregate data discover a new and perhaps broadly important path of magnesium import in bacteria.

shown to exhibit broad transport activity for other divalent ions [18–21]. However, magnesium has not been shown to serve as a substrate for Nramps that have been characterized. Indeed, the initial characterization of a bacterial proton-dependent manganese transporter (MntH), which is a member of the Nramp family of proteins, demonstrated that it could import iron or manganese even in the presence of 5 mM magnesium, suggesting that magnesium was unlikely to serve as an MntH substrate [22].

Magnesium is the most abundant divalent metal in living cells and is required for numerous cellular activities, including serving as cofactor for enzymatic reactions and maintaining the structures of membranes and ribosomes [23–25]. Cytoplasmic levels of most transition metals are maintained at relatively low concentrations through action of high affinity metalloproteins [6,26–28]. In contrast, intracellular free magnesium is maintained at a higher level (0.5–2.0 mM), which requires specific magnesium transport proteins [24,29–33].

Three families of magnesium transporters have been discovered in bacteria: CorA, MgtE, and MgtA/MgtB P-type ATPase proteins [33–38]. While many metalloregulatory proteins have been identified as sensors of transition metals, less is known regarding control of magnesium homeostasis. One mechanism is through cytoplasmic gating domains of CorA and MgtE, which help couple intracellular magnesium demand with transport activity. In addition, a few genetic regulatory mechanisms have been discovered for magnesium homeostasis. Best studied in this regard is a two component regulatory system in Salmonella enterica that completes phosphoryl transfer from a sensor kinase (PhoQ) to response regulator (PhoP) in response to fluctuations in extracellular magnesium [39]. PhoP activates genes for magnesium homeostasis, such as mgtA, as well as genes important for growth and replication within a host cell. Interestingly, the mgtA transcript is also subject to a second, post-initiation layer of magnesium-responsive genetic regulation [40]. Changes in magnesium alter the secondary structure within the mgtA 5’ leader RNA; stabilization of one particular configuration is coupled with control of transcription elongation, which has the effect of limiting mgtA transcription to conditions of low magnesium [40].

Signal-responsive RNA elements, akin to S. enterica mgtA, which coordinate chemical cues with regulation of downstream gene expression, are referred to as riboswitches. A second, and mechanistically distinct, magnesium riboswitch, sometimes called the ‘M-box’, has also been discovered in bacteria. Originally discovered upstream of the Bacillus subtilis mgtE gene this riboswitch is also broadly conserved in numerous distantly-related bacteria [41–45]. It is almost always located upstream of one of the three known classes of magnesium transporters: CorA, MgtA, and MgtE. Riboswitches are generally composed of two portions: a signal-responsive aptamer and a downstream region that couples conformational changes of the aptamer with control of transcription, translation, or mRNA stability [44–48]. The structure of the magnesium-bound M-box aptamer domain has been resolved by X-ray crystallography and its mechanism for sensing magnesium has been investigated by various biochemical and biophysical experiments [49,50]. Together, the aggregate data on this riboswitch suggest strongly that it serves as a metalloregulatory RNA for control of magnesium transport genes [42].

Given the close regulatory relationship between the M-box riboswitch and magnesium transporter genes, we were surprised to discover a subset of riboswitches situated upstream of Nramp-related genes. This observation established an intriguing conundrum. While M-box riboswitches respond to magnesium fluctuations in vivo, no Nramp or Nramp-related proteins have been found to transport this divalent ion. Therefore, one of these general assumptions must be incorrect. Either these particular riboswitches have been adapted to sense a divalent ion other than magnesium, or, alternatively, these particular Nramp-related homologs exhibit an unexpected role in magnesium homeostasis. Our combined data support the latter. We find that a Clostridium acetobutylicum ATCC 824 magnesium riboswitch controls expression of an Nramp-related gene and, moreover, that this particular transporter is surprisingly proficient in magnesium transport. These data, therefore, identify this subset of solute carrier proteins as a fourth class of magnesium transporters, designated herein as NrmT (Nramp-related magnesium transporter).

Results

Identification of magnesium riboswitches upstream of Nramp-related genes

M-box magnesium riboswitches [41,42] are widespread in bacteria, and are almost always positioned upstream of putative magnesium transport genes (i.e., corA, mgtE, or mgtA). The riboswitch is presumed to control expression of the transport protein in a magnesium-responsive manner, as it does for Bacillus subtilis mgtE [41]. Most magnesium riboswitches affect gene expression by controlling formation of an intrinsic transcription terminator (Fig. 1A). A three-dimensional structural model of the aptamer (ligand-binding) domain revealed the presence of between 6 and 9 functionally important divalent ion binding sites [41,49,50]. However, this structural model alone cannot rule out the intriguing hypothesis that there might still exist aptamer variants that sense divalent ions other than magnesium. Motivated by this hypothesis, we searched using Infernal [51] for instances where M-box riboswitches were located upstream of genes for transport of metals other than magnesium. This search uncovered multiple instances where it appeared that putative Nramp family genes were located immediately downstream of M-box riboswitch candidates, mostly in Clostridia and Deltaproteobacteria. Since Nramp transporters are generally assumed to mediate transport of manganese and/or iron, and have never been shown to transport magnesium, we chose to examine more closely a few representative
examples of M box-regulated Nramp-related genes. For this, we chose two separate loci within the *Clostridium acetobutylicum* ATCC 824 genome (*Ca_c0685* and *Ca_c3329*) (Fig. 1B).

**Magnesium-specific regulation by the Ca_c0685 riboswitch**

As a biochemical test of magnesium riboswitch function, the aptamer portions of the putative *Ca_c0685* and *Ca_c3329* riboswitches were transcribed in vitro and subjected to analytical ultracentrifugation measurements (Fig. 1C). Previous data using this technique demonstrated a large, and characteristic, change in hydrodynamic radius for *B. subtilis* magnesium riboswitches in response to binding of magnesium [41,42,49]. The sedimentation velocity measurements of the *Ca_c0685* and *Ca_c3329* riboswitches revealed an identical compaction with magnesium, suggesting they are likely to function similar to the previously characterized riboswitch.
by the Ca_c0685 riboswitch as extracellular magnesium was increased. However, only minor repression was observed with Ca_c3329.

In order to examine metal specificity in vivo for regulation by the Ca_c0685 riboswitch, cells were cultured to mid-logarithmic growth phase, exposed to 2 mM EDTA, and resuspended in medium containing excess iron, manganese, copper, zinc or magnesium (Fig. 3B). Expression of the Ca_c0685-yfp reporter was evaluated alongside control measurements of B. subtilis metal transport genes. This analysis confirmed a three-fold reduction of yfp in response to magnesium by the Ca_c0685 riboswitch. Excess zinc also moderately reduced the yfp transcript (~37%); however, excessive levels of iron, manganese, or copper had no effect on yfp, although the presence of these metals did affect transcripts known to be under their regulatory influence. These data together revealed that the Ca_c0685 riboswitch is a magnesium-sensing regulatory element. In contrast, it remains unclear from these experiments why the Ca_c3329 riboswitch appears to be unresponsive within the B. subtilis host organism.

Expression of Ca_c0685 and Ca_c3329 does not complement a deficiency in manganese transport

Prior experimental evidence has primarily demonstrated that many bacterial Nramp homologues are for transport of manganese or iron [22,25–56]. Although the Ca_c0685 and Ca_c3329 proteins are only distantly related to Nramp family proteins, the genes encoding Ca_c0685 and Ca_c3329 were heterologously expressed in B. subtilis under IPTG-inducible control (Fig. S1). To investigate a potential role in manganese transport, markerless deletion mutants of B. subtilis manganese transport genes, mntH and mntABCd, were introduced into these strains. The ΔmntH/ΔmntABCd double mutant (bCAW2022) containing markerless deletions of mgtE, yloB, and yfjQ exhibited a strong defect in magnesium transport activity (Fig. 4B; Fig. S2), and requires ~50 mM extracellular magnesium to restore growth in rich medium [57]. As a preliminary check of the specificity of this magnesium transport-deficient phenotype, the known B. subtilis manganese transporters, mntH and mntABCd, were ectopically integrated into the genome under inducible control (creating bCAW2073 and bCAW2076). Expression of these manganese transporters was unable to complement the severe magnesium deficiency exhibited by bCAW2022. This supports the hypothesis that the bCAW2022 strain exhibits a specific defect in magnesium transport activity.

To examine the impact of Ca_c0685 and Ca_c3329 expression on magnesium transport, they were integrated single-copy into the bCAW2022 genome under inducible control and growth was assessed under conditions of magnesium limitation. Expression of Ca_c0685 fully restored growth to resemble that of wild-type cells (Fig. 4C; Fig. S3). Moreover, when this strain was inoculated onto solid medium that contained a gradient of magnesium from submicromolar to 3.0 mM, growth was observed on all portions of the plate, in contrast to bCAW2022 (Fig. 4D; Fig. S3). This suggests that Ca_c0685 rescued growth even under conditions of submicromolar magnesium. Also, Ca_c3329 was able to rescue the magnesium transport-deficient phenotype for bCAW2022; however, it rescued growth only when magnesium was included at
Figure 3. Metal specificity of *C. acetobutylicum* riboswitch-yfp reporter fusions. (A) The strains expressing either the Ca_c0685 riboswitch-yfp or Ca_c3329 riboswitch-yfp reporter fusions were cultured in glucose minimal medium supplemented with 50 μM magnesium until reaching an OD_{600} of ~0.5–0.7, at which point 2 mM EDTA was added and cells were incubated for 1 hour. These cells were harvested by centrifugation and the pellet was washed three times and resuspended with an equal volume of chelated glucose minimum medium (chelated with Chelex-100). Either EDTA (2 mM final concentration) or varying magnesium concentrations were added and the cells were incubated for another 1 hour before harvesting. Total RNA was assessed by staining of rRNA bands. Abundance of the yfp gene was monitored by S1 mapping. (B) To assess the specificity of the *C. acetobutylicum* riboswitch-yfp reporter fusion, we cultured cells expressing either the Ca_c0685 riboswitch-yfp or Ca_c3329 riboswitch-yfp reporter fusions in glucose minimal medium supplemented with 50 μM magnesium and appropriate antibiotics until reaching an OD_{600} of ~0.5–0.7, at which point 2 mM EDTA was added for 1 hour. These cells were harvested by centrifugation and the pellet was washed three times and resuspended with an equal volume of chelated glucose minimum medium (chelated with Chelex-100). Either EDTA (2 mM final concentration) or 100 μM various metals were added and the cells were incubated for another 1 hour before harvesting. Radiolabeled DNA probes (Table S2) were used for S1 mapping of the yfp transcript, and for several control transcripts that are known to respond to other metals (e.g., *mgtE* (magnesium), *dhbA* (iron), *mntH* (manganese), *mntA* (manganese), *copZ* (copper), *yciA* (zinc)). These data demonstrate that the Ca_c0685 specifically controls gene expression in response to magnesium, although addition of zinc also resulted in a moderate reduction in gene expression. Shown is a representative gel with quantification derived from experimental triplicates.

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Therefore, these data demonstrated that both Ca_c0685 and Ca_c3329 are capable of magnesium transport activity, with Ca_c0685 potentially showing higher affinity for the magnesium ion.

Magnitude repression of Ca_c0685 and Ca_c3329 in Clostridium acetobutylicum

Our observation that Ca_c3329 acts as a magnesium transporter seems at first glance to be inconsistent with our prior result showing that the PrpsD-Ca_c3329-yfp fusion was unresponsive to magnesium (or any other divalent ions tested). However, heterologous expression of riboswitch-reporter fusions is not always successful. This is, in certain instances, likely to be due to differences in the molecular environment, such as changes in RNase preferences or in RNA recognition by transcription elongation factors. Therefore, it is possible that the Ca_c3329 riboswitch is nonfunctional when expressed in B. subtilis but is still functional in the C. acetobutylicum host for regulation of a transporter. However, it is also possible that the Ca_c3329 riboswitch is nonfunctional in both organisms. As a test of these possibilities, C. acetobutylicum was cultured to OD600 of ~0.8, and then treated with 2 mM EDTA for one hour, at which point the cells were harvested and resuspended in magnesium-free medium. These cells were then aliquoted into media containing varying magnesium concentration and incubated for 2 additional hours before extraction of total RNA. S1 mapping of Ca_c0685 and Ca_c3329 revealed that both genes were subjected to repression of transcription as magnesium was increased (Fig. 5). This indicates that both Ca_c0685 and Ca_c3329 are likely to be repressed by magnesium within the context of their host organism, and that the Ca_c3329 riboswitch is likely to be functionally responsive to magnesium in C. acetobutylicum.

Phylogenetic analysis of Nramp-related transporters associated with magnesium riboswitches

Nramp family transporters are widespread among bacteria and eukaryotes [58] and are hypothesized to have emerged early in evolution. We performed a phylogenetic analysis of putative Nramp-related genes located immediately downstream of M-box RNAs. In addition, we collected representatives from three previously identified groups of bacterial MntH-like proteins (groups A, B, and C, according to a previous classification, [58]),
Nramp homologs from human, Arabidopsis and yeast, and several examples of an Nramp outgroup that was identified previously [59–61]. Members of the branched-chain amino acid transporter family, a part of the APC superfamily containing similar LeuT folds, were used as an outgroup, as in a previous characterization of Nramp phylogeny [60,62]. We constructed the multiple sequence alignment (Fig. S4) and the maximum likelihood phylogenetic tree (Fig. 6) for the 47 selected representatives. All M-box-regulated homologs clustered into a single branch on the phylogenetic tree adjacent to the Nramp outgroup genes, whereas other bacterial Nramp transporters, including known manganese/iron transporters, were distributed in their respective groups. Interestingly, all Nramp-related transporters that appeared to be regulated by the magnesium riboswitch clustered together, suggesting a relationship between magnesium and members of this branch. This analysis indicates that these riboswitch-associated Nramp-related transporters form a distinct clade that is derived from a more distant common ancestor than those of the Nramp family, but that shares a more recent common ancestor with the Nramp outgroup. Given the phylogenetic relatedness of these proteins, we renamed them NrmT, for Nramp-related magnesium transporter.

To search for unique features of NrmT group, we examined the genomic context of representative genes (Fig. S5). Surprisingly, this revealed that the local genomic context of most group members includes a common, additional gene. This latter gene appears by sequence homology to encode for a protein that is specifically homologous to the N-terminal cytoplasmic domain of the MgtE transporter. This protein appears to be encoded by a single gene, except in C. acetobutylicum where the riboswitch-regulated operon Ca_c0685 includes a homolog that is split into two smaller genes (Fig. 1B).

Together, these observations appear to suggest a possible relationship between magnesium homeostasis and the Nramp-like outgroup (NrmT) identified herein. As a preliminary test of this possibility, another member of this grouping, but that lacked a magnesium riboswitch, was arbitrarily chosen for heterologous expression in B. subtilis. Specifically, we identified an nrmT gene from Acidobacterium capsulatum, located downstream of the small gene exhibiting homology to the MgtE cytoplasmic domain. Heterologous expression of the A. capsulatum Nramp-related gene (Fig. S6) in bCAW2022 (i.e., the magnesium transporter-deficient strain) rescued growth, but only in the presence of low millimolar magnesium. The partial MgtE gene was then integrated at a separate locus of the genome and co-expressed with the A. capsulatum putative transporter; expression of both proteins did not further improve rescue of the magnesium transport defect. However, expression of this A. capsulatum transporter gene was fully capable of rescuing the manganese transport defect exhibited by bCAW2105 (ΔmntH/ΔmntABCD) (Fig. S6). These data suggest that the A. capsulatum NrmT protein is likely to function as a manganese transporter, although what role the partial MgtE gene may play in metal transport remains unknown. Therefore, only a subset of the NrmT proteins, sometimes associated with magnesium riboswitches, is likely to exhibit high affinity magnesium transport. These data also illustrate the potential value of using the magnesium riboswitch as an identifying feature of magnesium specificity in associated transporters.

**Discussion**

The three major classes of bacterial magnesium transporters (CorA, MgtE, MgtA) were discovered using complementation strategies similar to that described herein [35,63–65]. While other, minor routes of magnesium import may be possible, organisms...
from all three domains of life are generally expected to encode at least a subset of these three protein families, as magnesium acquisition is essential. In this study, we employed a similar complementation approach to suggest that \textit{C. acetobutylicum} proteins, unrelated to the known classes of transporters, are capable of magnesium transport. As these proteins are distantly related to the Nramp family of proteins, which have not been found to transport magnesium, this was an unexpected discovery that suggests either the substrate range for Nramp transporters must be expanded to include this divalent ion, or, more likely, that a new class of Nramp-related divalent metal transporters has been introduced. Therefore, these observations together suggest that a subset of Nramp-related transport proteins constitutes a fourth class of dedicated magnesium transporters in bacteria, designated herein as NrmT.

Our data also revealed that a riboswitch upstream of \textit{Ca_c0685} is proficient within the confines of a heterologous host in coupling intracellular magnesium fluctuation with control of downstream gene expression. This observation strengthens the overall body of evidence showing that magnesium is the central signal perceived by the M-box riboswitch. It also suggests that identification of these riboswitches can, in certain instances, be used to help predict which Nramp-related homologues are likely to function as dedicated magnesium transporters, rather than transporters of other divalent cations such as manganese. This is also bolstered by our observations that \textit{Ca_c3329} was both repressed by magnesium in \textit{C. acetobutylicum} and provided magnesium transport activity in \textit{B. subtilis}, albeit at higher concentrations of the ion. Therefore, in total, we speculate that the \textit{C. acetobutylicum} magnesium riboswitches control expression of two magnesium-transporting Nramp-related genes, which may be functionally specialized for different ranges of extracellular magnesium. There are two MntH homologues that are also encoded by this organism (\textit{Ca_p0063} and \textit{Ca_c0628}, representing a MntH-A and MntH-B protein, respectively).

Figure 6. Bayesian phylogenetic tree of 45 Nramp family transporters, Nramp outgroup proteins, riboswitch-associated outgroup members, and branched-chain amino acid transporters. The sequences were aligned using MAFFT v7 using the L-INS-I algorithm [75]. This tree is the consensus of four replicate trees constructed using MrBayes 3.2 [76,77]. Each replicate tree was constructed using four total chains for Metropolis coupling, and 1,000,000 generations with 25% relative burn-in. Priors used were the defaults for amino-acid models with an equal mixture of amino-acid substitution models. Transporter genes preceded by putative magnesium riboswitches are denoted by stem-loops. Branch support values are indicated in red by each internal node. Branch lengths represent expected number of substitutions per position. Similar trees were obtained with different approaches including maximum likelihood (MetaPIGA), minimum evolution, and maximum parsimony (MEGA6), not shown [78,79].

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of manganese or iron. We demonstrate herein that an A. capsulatum outgroup protein, which lacks a magnesium riboswitch, revealed that it is likely to primarily transport manganese, rather than magnesium, despite the presence of an adjacent MgtE-like fragment. Therefore, the MgtE-like fragment that is associated with the A. capsulatum protein would be expected to serve a function other than magnesium regulation, perhaps instead responding to manganese ions. Subsequent biochemical analysis of transport activity will be required to test these predictions, and to reveal the function of the small ORF.

Nramps are believed to be important in most organisms for transport of manganese or iron. We demonstrate herein that an outgroup of Nramp-related proteins are likely to function as dedicated magnesium transporters. Therefore, when considering the potential routes of magnesium transport activity for a target organism, this family of proteins must be considered as potential suspects, along with previously identified magnesium transport classes. Future studies will be required to compare these newly discovered magnesium transporters with CorA, MgtE and MgtA/B proteins, and to determine whether they are also important to infection by bacterial pathogens.

Materials and Methods

Strains and culture conditions

All B. subtilis strains used in this study were isogenic with common laboratory strains listed in Table S1. Depending on the experiment, they were cultured in liquid rich medium [2xYT; (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl)], solid rich medium [Tryptone Blood Agar Base (TBA)], and glucose minimal medium [20 g/L (NH₄)₂SO₄, 183 g/L K₂HPO₄·3H₂O, 60 g/L KH₂PO₄, 10 g/L sodium citrate, 0.5% glucose, 0.5 mM CaCl₂, 5 µM MnCl₂, and 2 g/L MgSO₄·7H₂O when appropriate] at 37°C. When appropriate, antibiotics were included at: 100 µg/mL spectinomycin, 5 µg/mL chloramphenicol and 1 µg/mL erythromycin plus 25 µg/mL lincomycin. To chelate divalent cations from media, 5 g/100 mL of Chelex-100 resin (Bio-Rad) was added to dissolved medium and equilibrated with stirring for 2 hours, followed by removal of resin by filtration. DNA was transformed into B. subtilis using a modified version of a previously published protocol [60].

Construction of riboswitch-yfp reporter strains

For construction of reporter fusions between C. acetobutylicum riboswitches and yellow fluorescent protein gene (yfp), the putative magnesium riboswitches were amplified by PCR and subcloned into pDG1662 (Table S2). The constitutive promoter from B. subtilis rpoD was subcloned upstream while the yfp gene was placed downstream of the putative riboswitches, respectively. These plasmids were transformed into B. subtilis PY79 for integration into amyE.

Construction of strains for complementation experiments

The wild-type strain for all complementation experiments was derived from B. subtilis 168 by integration of empty pHyperspank vector at the amyE locus [69]. Manganese and magnesium
transporter knockout strains were created by in-frame markerless deletion [57,70]. Complementation strains were created by subcloning of the transporter genes into pHyperspank under the control of an IPTG-inducible promoter and double homologous recombination of the resulting construct into the amyE locus of the appropriate transporter knockout strain. Correct strains were verified by diagnostic PCR and Sanger sequencing. All strains used in this experiment are listed in Table S1.

Growth measurements

*B. subtilis* strains were cultured on TBAB plates, supplemented when necessary with MgCl₂ concentrations indicated in the text, and the appropriate antibiotics. These cells were used to inoculate 5 mL of either 2xYT or glucose minimal media (GMM) [45], that was cultured overnight while shaking at 37°C. An aliquot was then diluted 1:100 in 25 mL 2xYT or GMM supplemented as necessary with antibiotics. These cells were incubated shaking at 37°C until reaching an OD₆₀₀ of ~0.5, whereupon they were pelleted, washed twice with 10 mL 2xYT or GMM, and resuspended to an OD₆₀₀ of ~0.05 in 25 mL containing the indicated amounts of MgCl₂ and/or 0.5 mM IPTG. Klett readings were recorded at regular time intervals using 250 mL flasks. Stationary phase OD₆₀₀ measurements were taken at the second time point after the intersection of exponential and stationary growth phases.

Gradient plate assays

For preparation of agar plates containing a gradient of magnesium, we utilized a procedure described previously [71,72]. Briefly, a slanted 2% agar medium base containing the maximum desired concentration of magnesium was prepared in a standard petri dish, upon which a magnesium-free, 0.8% top agar medium was poured, thus allowing a gradient of magnesium to be established by diffusion from the slanted bottom layer. Approximately 3 µl culture (at ~1×10⁸ cells/µl) were spotted onto magnesium gradient plates (from 0 mM magnesium to either 2.5 or 5.0 mM magnesium), with or without 0.5 mM IPTG and incubated for exactly 10 hours at 37°C at which point the plates were imaged by photography. In a related experiment, a serially diluted culture (from 6.25×10⁵ to 100 cells) was spotted onto glucose minimal medium plates with and without 10 µM manganese chloride, and with and without 0.5 IPTG, and incubated for exactly 10 hours at 37°C at which point they were photographed.

S1 mapping analysis

Total RNA was harvested from cells that were grown to mid-logarithmic phase in 2xYT or in glucose minimal medium [41]. When appropriate, the glucose minimal medium was first subjected to chelation of divalent metals by incubation with various amounts of EDTA, as described in the manuscript. Total RNA was extracted by hot phenol after fixation of cell pellet with RNAProtect reagent (Qiagen), according to the manufacturer instructions and as described previously [28,73]. The quality and quantity of RNA was measured by absorbance spectroscopy and confirmed by resolution on 1.3% formaldehyde-agarose gels. Gene-specific oligonucleotide probes (Table S2) for *Ca_c0685*, *Ca_c3329*, *mntA*, *mntH*, *yph*, *mglE*, *dbhA*, *copZ*, and *yclA* transcripts were used for PCR amplification using *Clostridium acetobutylicum* and *B. subtilis* genomic DNA as template. Each specific DNA probe was radiolabeled with [γ-³²P] ATP and T4 polynucleotide kinase and 30,000–40,000 cpm of labeled probe was used in each reaction. 100 µg of total RNA was pelleted andhypophosphitated; this pellet was then carefully resuspended in 20 µl hybridization buffer [40 mM PIPES (pH 6.4), 400 mM NaCl, 1 mM EDTA, 80% (v/v) formamide]. Individual samples were incubated at 80°C for 25 min and slowly cooled to 42°C. 300 µl of S1 nuclease mix (~100 units in 1 unit S1 nuclease buffer [280 mM NaCl, 30 mM NaOAc (pH 4.4), 4.3 mM ZnOAc]) was added and incubated at 37°C for 45 min. The reaction was terminated by addition of 75 µl of S1 nuclease termination solution (2.5 M NH₄OAc, 0.05 M EDTA). The DNA-RNA hybrid was precipitated by adding 400 µl of isopropanol and the pellet was washed with 70% (v/v) ethanol, vacuum dried, and resuspended in 10 µl alkaline loading dye. The protected DNA fragments were then resolved by 6% (wt/vol) polyacrylamide gels containing 7 M urea. The dried gels were exposed to a phosphor imaging screen (FLA-2000; Fuji) and bands were quantified using Multi Gauge V3.0 or ImageJ.

Growth of *Clostridium acetobutylicum*

*C. acetobutylicum* 824 was cultured in 400 mL *Clostridial* growth medium (CGM) [74] until it reached an OD₆₀₀ of ~0.8. EDTA (pH 8.0) was added to a final concentration of 2 mM. After one-hour incubation, the cells were harvested by centrifugation and resuspended in magnesium-free CGM. 40 µl of this cell suspension was then aliquoted into separate containers containing designated concentrations of magnesium. After a two-hour incubation, cells were harvested by centrifugation and stored at ~30°C until lysis and RNA extraction.

Supporting Information

**Figure S1** Expression of *Ca_c0685* and *Ca_c3329* in a manganese-deficient strain. (A) Genotype of strains (Table S1). (B) Expression of *Ca_c0685* and *Ca_c3329* in a manganese-deficient strain. Strains containing inducible control of *Ca_c0685* and *Ca_c3329* were created as described in the text and analyzed alongside control strains. 0.5 mM IPTG was added to exponentially growing cultures for 1 hr, whereupon 100 µg of total RNA was hybridized with the appropriate radiolabeled S1 probe DNA. DNA oligonucleotides used for S1 mapping are listed in Table S2. “+” indicates addition of IPTG, whereas “−” indicates the absence of IPTG. Following S1 mapping, the protected DNA probes were analyzed by phosphor imaging. Representative results are presented in this figure. These data indicate that the *Ca_c0685* and *Ca_c3329* genes are transcribed under these conditions. (C) Growth curves are shown for *B. subtilis* control strains, including wild-type and a ΔmntH/ΔmntABCΔD manganese-deficient double mutant, and transport-deficient strains containing IPTG-inducible copy of *mntH*, *Ca_c0685*, or *Ca_c3329* integrated into the amyE locus. These strains were cultured in minimal medium without added manganese in the presence of 0.5 mM IPTG. (D) They were also serially diluted onto solid growth medium that either contained or lacked 10 µM manganese, and that either contained or lacked 0.5 mM IPTG for induction of Nramp-related genes. Only bCAW2109, containing ectopic expression of MntH, was capable of rescuing growth on the manganese-limiting medium. (PNG)

**Figure S2** Heterologous expression of *B. subtilis* MntH and MntABCD do not rescue a magnesium-deficient phenotype. (A) Genotype legend (Table S1). (B) Expression of manganese transport genes, *mntH*, and *mntA*, within the context of a manganese deficient strain. The transcripts for *mntA* and *mntH* were examined by S1 mapping analysis for the strains mentioned in this figure and described in the text. Total RNA was extracted from exponentially growing cells after one hour of treatment with 0.5 mM IPTG (“+”) or in the absence of IPTG (“−”). Ethidium bromide-stained rRNA is included as a loading control in these
analyses. DNA oligonucleotides used for S1 mapping are listed in Table S2. Following S1 mapping, the protected DNA probes were analyzed by phosphor imaging. Representative results are presented in this figure. These data indicate that the mntH and mntI genes are transcribed under these conditions. (C) Growth curves are shown for B. subtilis control strains, including wild-type and a ΔmgtE/ΔyloB/ΔyfjQ triple mutant that is deficient in magnesium transport activity, and transport-deficient strains containing an IPTG-inducible copy of the mntH or mntABCD genes. These strains were cultured in rich medium in the presence of 0.5 mM IPTG. (D) They were also serially diluted onto solid growth medium that either contained or lacked 50 mM magnesium, and that either contained or lacked 0.5 mM IPTG for induction of either MntH or MntABCD. The petri plates were incubated for 32 hrs at 37°C, to 2.5 mM magnesium. These plates were incubated for 10 hours at 37°C before they were photographed. Only the wild-type strain grew in the absence of 50 mM magnesium. As further evidence, 3 μL of these strains (1×10^4/μL) were spotted onto rich medium plates containing a gradient of magnesium ranging from 0 to 5 mM. Again, only wild-type grew under these conditions.

**Figure S3** Heterologous expression of Ca_c0685 and Ca_c3329 in a magnesium transport-deficient strain. (A) Genotype legend (Table S1). (B) Strains containing inducible Ca_c0685 and Ca_c3329 analyzed alongside control strains. 0.5 mM IPTG was added to exponentially growing cultures for 1 hr, whereupon 100 μg of total RNA was hybridized with radiolabeled S1 probe DNA respectively. DNA oligonucleotides used for S1 mapping are listed in Table S2. “+” indicates addition of IPTG, whereas “-” indicates the absence of IPTG. Following S1 mapping, the protected DNA probes were analyzed by phosphor imaging. Representative results are presented in this figure. These data indicate that the Ca_c0685 and Ca_c3329 genes are transcribed under these conditions. (C) Growth curves are shown for B. subtilis control strains, including wild-type and a ΔmgtE/ΔyloB/ΔyfjQ triple mutant that is deficient in magnesium transport activity, and transport-deficient strains containing an IPTG-inducible copy of Ca_c0685 or Ca_c3329 integrated into the amyE locus. The resulting strains were cultured in rich medium in the presence of 0.5 mM IPTG and 2.5 mM magnesium. Expression of Ca_c0685 and Ca_c3329 both fully rescued growth in this medium. (D) Also, 3 μL of each of these strains (~1×10^4/μL) were spotted onto solid medium containing a gradient of magnesium from 0 to 5 mM magnesium. These plates were incubated for 10 hours at 37°C before they were photographed. These results revealed that the Nramp homologue, Acp2977, could partially rescue growth of the magnesium-deficient strain whereas Acp2976 alone was unable to rescue growth under these conditions. (E) Strains that included IPTG-inducible copies of either Acp2976 or Acp2976 and that included deletion of manganese transport genes were serially diluted onto solid growth medium that either contained or lacked 10 μM manganese. Several control strains are included in this analysis and are described in the figure. Under these conditions induction of B. subtilis MntH was sufficient for rescue of growth in the absence of added manganese. Similarly, induction of Acp2977 fully rescued growth in the absence of added manganese; therefore, the Acp2977 Nramp homologue is likely to function as a manganese transport protein.

**Figure S6** Expression of A. capsulata ACP2976 and ACP2977. (A) Schematic representation of the gene arrangement of a Nramp-related gene from Acidobacterium capsulatum. This particular Nramp relative (Acp2977) was chosen as it is related to the magnesium-transporting Ca_c0685 gene but lacks an observable magnesium riboswitch. However, it, like the majority of magnesium associated Nramp homologues is located immediately downstream of an open reading frame that appears to encode for a protein that is homologous to the cytoplasmic domain of the magnesium transporter, MgtE (Acp2976). (B) The Acp2977 gene was integrated into the B. subtilis amyE gene while the Acp2976 gene was integrated into the sacA locus under IPTG- and xylitol-inducible control, respectively. The background B. subtilis strain also included deletions of three putative magnesium transporters, mgtE, yloB, and yfjQ. (C) Analysis of this and other control strains by S1 mapping showed that the Acp2976 and Acp2977 genes were indeed transcribed when induced by xylitol and IPTG, respectively. (D) 3 μL of these strains (~1×10^4/μL) were spotted onto solid medium containing a gradient of magnesium from 0 to 5 mM magnesium. These plates were incubated for 10 hours at 37°C before they were photographed. These results revealed that the Nramp homologue, Acp2977, could partially rescue growth of the magnesium-deficient strain whereas Acp2976 alone was unable to rescue growth under these conditions. (E) Strains that included IPTG-inducible copies of either Acp2976 or Acp2976 and that included deletion of manganese transport genes were serially diluted onto solid growth medium that either contained or lacked 10 μM manganese. Several control strains are included in this analysis and are described in the figure. Under these conditions induction of B. subtilis MntH was sufficient for rescue of growth in the absence of added manganese. Similarly, induction of Acp2977 fully rescued growth in the absence of added manganese; therefore, the Acp2977 Nramp homologue is likely to function as a manganese transport protein.

**Table S1** Bacillus subtilis strains and plasmids used in this study. Shown in this table are all of the strains and relevant plasmids for the experimentation described herein. The construction and characterization of the magnesium transporter deletion strains, including the marker-less AmgtE, AyloB, and AyfjQ deletion strains, are described in detail in a separate publication [57]. Similarly, construction and characterization of markerless deletions of mntABCD, and mntH are also described in this publication [57].

**Table S2** The DNA oligonucleotides that were used in this study are listed and briefly described herein.

**Author Contributions**

Conceived and designed the experiments: JHS CAW WCW DAR. Performed the experiments: JHS CAW DAR JRG. Analyzed the data: JHS CAW DAR JRG. Contributed reagents/materials/analysis tools: BGF RSS. Wrote the paper: JHS CAW DAR JRG RSS WCW.
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