The Metal Requirements of *Bacillus subtilis*

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Abstract

Metal ions are essential for many biological processes across all domains of life. In *Bacillus subtilis*, they function as essential cofactors for enzymes, a component of biosensors for oxidative damage, in the assembly of the ribosome, in the folding of mRNA, and a myriad of other processes. In *Bacillus subtilis,* the minimum metal quota per cell to sustain growth has not previously been established. In this study, we establish a metal-limited minimal medium that enables us to probe the interaction of metals at the thresholds for growth and determine how the cell responds to limitation. Here we determine the minimum concentrations of magnesium, iron, and manganese needed for full growth. Furthermore, these requirements are dependent on the levels of all metals within the total metal pool. We were unable to demonstrate any growth limitation in the absence of added zinc in wildtype cells; however, in cells mutant in the zinc importer, encoded by *znuC*, zinc is limiting for growth. Development of this medium allows us to examine how cells adapt to trace metal availability. Under iron-limited conditions, the cell initiates an iron-sparing response. In the absence of the regulator of this response, *fsrA,* the cells require higher iron for growth. Not only does this media give us insight into the complex balance of intracellular metal pools, but it provides a tool for the analysis of metal limitation in *Bacillus subtilis*.

Key words: metal, metal limitation, *Bacillus subtilis,* magnesium, manganese, iron, iron-sparing response, metal homeostasis

Introduction

All life on earth is largely composed of carbon, hydrogen, nitrogen, oxygen, phosphorous, and sulfur. However, other elements, which are far less abundant, can be just as crucial for life. Metal ions, such as magnesium, manganese, iron, zinc, and cobalt, act as cofactors for many essential chemical reactions. These cofactors are crucial for almost all aspects of metabolism and bioenergetic processes on earth, including cellular respiration, photosynthesis, and nitrogen fixation (Chandrangsu et al., 2017). Metal homeostasis depends on coordinating transport, buffering, and storage. Metalloregulators, proteins that sense metal ion levels, coordinate activity of transporters to maintain intracellular concentrations at physiologically compatible levels. These processes are crucial to cell growth, and while much work on elucidating the molecular mechanisms of metal homeostasis maintenance has been done, many questions remain unanswered.

Metal ion homeostasis is critical in bacteria, as in all living organisms. In order to study metal physiology in bacteria, it is useful to explore how metals affect the requirements for other metals in a model organism. *Bacillus subtilis* is an aerobic, Firmicute soil bacterium that produces endospores and secretes a variety of enzymes, making it an essential organism in industry, agriculture, biomaterials, and medicine (Su et al., 2020). Because of its ease of genetic manipulation and its widespread use in industry, *Bacillus subtilis* is one of the most well-studied bacteria in areas of physiology, metabolism, chromosome replication, and cell differentiation.

The roles of certain trace metals, primarily Mg, Mn, and Fe, have been well characterized in *B. subtilis*. Magnesium is a major cation and is universally essential for life in every organism. Hundreds of enzymes require Mg to be catalytically active, including all reactions that use ATP or other nucleotides, which must engage Mg to function. RNA polymerase requires two

magnesium ions coordinated in the active site to proceed with RNA synthesis (Svetlov & Nudler, 2013). Ion pairs of magnesium are also used to stabilize the tertiary equilibrium structure of transfer RNA (Schauss et al., 2021). Because of its essential role as a divalent cation, Mg import and export must be tightly regulated in the cell. In *B. subtilis*, MgtE is the primary Mg importer and is regulated by a magnesium-responsive riboswitch (Wakeman et al., 2014). Since Mg is toxic in excess, regulating the export is also crucial and is controlled by the MpfA efflux system (Pi et al., 2020).

Fe is another major cation that is universally essential for life in nearly every organism. The only known exceptions to this general requirement are certain bacteria that grow in severely Fe-limited environments, including Lactobacilli (Weinberg, 1997) and *Borrelia burgdorferi* (Posey & Gherardini, 2000). Fe has many major functions in cells, including in cytochromes and other heme proteins, Fe/S clusters, mononuclear, di-iron, Fe-oxogluturate, and other non-heme Fe proteins (Merchant & Helmann, 2012). Fe limitation across many organisms has an impact on the primary production of oceanic species, the yields of crops, and the health of humans. Many microbial species have Fe-sparing responses to survive in low Fe conditions, including *Escherichia coli, Saccharomyces cerevisiae,* and *Bacillus subtilis.*

Mn is another major cation that is widely used in most organisms on earth. Mn is used in oxygenic photosynthesis and in mediating resistance to damage by reactive oxygen species, like as a cofactor for mitochondrial SOD in certain organisms (Aguirre & Culotta, 2012). However, despite its essentiality in most organisms, there is little evidence for a sparing response resembling the Fe-sparing response. In *Bacillus subtilis,* the MntR regulator directly senses Mn levels and represses acquisition systems, as well as activating efflux (Paruthiyil et al., 2020; Que & Helmann, 2000).

Despite the large amount of research into certain metals, the precise composition of the metallome is not well understood. One way to measure the concentration of metals in the cell with high sensitivity is inductively coupled plasma mass spectrometry (ICP-MS), which can detect metals in liquid samples at very low concentrations. However, the presence of a certain metal in the cell does not necessarily confirm that it is required for life; for example, non-physiological cofactors like lead or uranium may be found bound to enzymes, even though they are not beneficial for the cell (Cvetkovic et al., 2010).

Understanding the metal requirements of *Bacillus subtilis* and how the cell adapts under metal limitation can lead to advances in our understanding of nutritional immunity, environmental adaptation, and antibiotic resistance. Metals have been used as antimicrobials for thousands of years; for example, copper is used for the treatment of MRSA and *Pseudomomas* infections (Mittapally et al., 2018). Although metals were replaced with organic antibiotics in the twentieth century, there is renewed interest in metals in light of the antibiotic resistance pandemic ravaging the medical field. One strategy that hosts use naturally to defend against pathogens is nutritional immunity, which is when the host sequesters metals and other nutrients to limit pathogenicity (Hennigar & McClung, 2016). In particular, hosts often decrease iron and zinc to starve the pathogen of those essential elements. Thus, it is essential to study the lower threshold of what the cell requires for iron and zinc and how the cell responds when the concentration in the cell is below that threshold. Using our newly optimized metal limited minimal media (MLMM21), we can examine these lower thresholds of metal requirements in *Bacillus subtilis,* which has broad implications for basic science and even the medical field.

In this study, we first optimized the components and methodology of the metal limited minimal medium MLMM21 to maximize the purity and efficacy at limiting for trace metals. We

then established the minimum concentrations of Mg, Mn, and Fe required for growth of *Bacillus subtilis*. We demonstrated growth inhibition by metal limitation of Mg, Mn, and Fe, then explored the requirements of other trace metals, such as Zn, of which it has previously been impossible to demonstrate limitation for due to contamination in the media as well as effective import systems to accumulate metals in the cell. Because it's difficult to remove all sources of trace metal contamination, one strategy to demonstrate Zn limitation is to use a mutant in Zn importer *znuC.* Without this importer, cells are more sensitive to low Zn, and we showed that increasing Zn concentration increases growth in *znuC* mutants. After establishing these minimum metal requirements, we then used the MLMM21 to study the mechanisms of the cell's response to limitation. We first studied the Fe-sparing response. When the cell is limited for Fe, the ferric uptake repressor Fur derepresses a small RNA *fsrA*, which prevents expression of proteins that bind Fe but aren't critical for growth (Gaballa et al., 2008). We demonstrate that a mutant in *fsrA* is more sensitive to low Fe conditions. We then explored the mechanisms of functional substitution under metal limitation, in which a replete metal substitutes for a scarce metal. In the future, other trace metals, such as cobalt, can be studied using our optimized MLMM21.

Materials and Methods

Metal-limited minimal medium

Media was prepared from stock solutions of Bacillus salts containing 20 g/L $(NH_4)_2SO_4$ and 10 g/L potassium glutamate (10x), 1 M KPO_4 buffer at pH 7.0 (500x), 50% glucose (25x), 2 mg/mL tryptophan (200x), and 1M potassium morpholinopropane sulfate (MOPS) at pH 7.4 with KOH (25x), 1 mM $MgSO_4$ (20x), 100 µM $Fe(II)SO_4$ in 0.1N HCl (100x), and 100 µM $MnCl_2$ (100x). All stocks were dissolved in milli-Q water or equivalent and were then filter-sterilized and stored at room temperature. All components except Mg, Mn, and Fe were combined and treated with 5% W/V of chelex-100 stirring for 30 minutes. After filter-sterilization, Mg, Mn, and Fe were added and volume was amended with filter-sterilized milli-Q water. The final concentrations of components in the minimal media were Bacillus salts (2 g/L $(NH_4)_2SO_4$ and 1 g/L potassium glutamate), 40 mM MOPS, 5 mM KPO_4, 50 µM tryptophan, 2% glucose, 50 µM $MgSO_4$, 1 µM $MnCl_2$, and 1 µM $FeSO_4$. Media was used immediately.

Bioscreen Growth Assays

Cells were grown overnight in MLMM21, diluted 1:25 in 2.4 mL of media, then immediately subcultured 1:20 into 190 uL fresh MLMM21 with indicated amendments in a 96-well plate. Cell Growth (OD_{600}) was monitored every 15 min for 24 hours using a Bioscreen growth analyzer (Growth Curves USA, Piscataway, NJ) at 37^0C with continuous shaking. Experiments were conducted at least three times with three biological replicates each time.

Quantification of metal content by ICP-MS

Cells were grown in MLMM21 overnight and subcultured 1:200 ratio into fresh MLMM21 to an OD_{600} of about 0.4. Aliquots of 2 mL of cell culture were harvested or 2 mL aliquots of media were taken, and levels of intracellular metals (Mg, Fe, Mn, Zn and Co) were monitored by inductively coupled plasma mass spectrometry (ICP-MS). All cell samples were washed once with buffer 1 (1X PBS buffer, 0.1 M EDTA) then twice with buffer 2 (1X chelex-treated PBS buffer). Cell pellets were resuspended in 400 µL of buffer 3 (1X chelex-treated PBS buffer, 75 mM NaN_3, 1% Triton X-100) and incubated at 37^0C for 90 min to lyse the cells. Lysed samples were spun down by centrifugation and the total protein content was quantified using a Bradford assay. Then, samples were mixed with 600 µL buffer 4 (5% HNO_3, 0.1% (v/v) Triton X-100) and heated in a 95^0C sand bath for 30 min. The debris was removed by centrifugation and the total metal ions in the diluted samples were analyzed by Perkin-Elmer ELAN DRC II ICP-MS. Media samples were diluted into 2% HNO_3 and evaluated the same way. Gallium was used as an internal standard. The total intracellular ion levels are expressed as µg ion per gram of protein content (mean ± SD; n=3). For calculating the metal content of media and cells in µM and in atoms/cell, we assumed an average cell volume of 1 μm^3 (Jeong et al., 1990), a dry weight of a single cell of *Bacillus subtilis* of 2.2 x 10^{-13} grams (Jeong et al., 1990), and an average dry weight protein content of a cell of 55% (Dauner et al., 2001). Using these values, we calculated an average mass of protein per cell of *Bacillus subtilis* of 1.21 x 10^{-13} g.

Results

Metal-limited minimal medium recipe

We first optimized the components and methodology of the MLMM21 to maximize the purity and efficacy at limiting for trace metals. The previous protocol for MLMM in the Helmann Lab contained 822 μM Mg, 0.08 μM of Mn, and 5 uM Fe (Chen et al., 1993), with Mn and Fe levels varied by experiment. We worked to adjust the concentrations of each metal to the minimum sufficient level needed for full growth to an absorbance at OD_{600nm} measured over 1. Based on these preliminary experiments, we developed a metal-limited minimal medium recipe (MLMM21) that contained 50 μM Mg and 1 μM Mn and Fe (Table 1). These concentrations were sufficient for full growth but were not in excess. We noticed that to balance a drastic reduction in the Mg and Fe concentrations, we needed to raise the Mn concentration slightly to achieve full growth. These much lower concentrations of Mg and Fe reduced the likelihood of contaminants of other trace metals in the media, especially from the Mg, which was reduced from 822 μM down to 50 μM. We also tested different durations and frequencies of chelexing the combined media components prior to addition of any Mg, Mn, or Fe; chelex is a resin that binds transition metal ions. We determined that 1 round of chelexing for 30 minutes was the most effective and efficient. The chelating material reduces the likelihood of trace metals in the medium prior to addition of desired metals. However, even though we did not add Zn to the MLMM21, there was still significant Zn measured in the medium. This background level of Zn is reproducible in different preparations and contributes to the difficulty in limiting for Zn. Despite the absence of Co from our media, trace amounts of Co were still detected (Table 1). However, this low concentration of Co is below the level of detection of the ICP-MS.

	Calculated (μM)	Measured (μM)
Mg	50	47.98 ± 5.95
Fe	1	1.22 ± 0.16
Mn	1	1.10 ± 0.38
Zn	0	0.140 ± 0.08
Co	0	0.004 ± 0.002

Table 1: Metal concentrations in metal-limited minimal media (μM) as described in protocol and as measured by ICP-MS, with three independent preparations. Standard deviation is shown.

Metal content of cells in metal-replete media

After the MLMM21 was optimized by reducing trace metal concentrations to the minimum level needed for full growth, we used it to compare the metal content of the metal-replete media, metal content of spent media after 24 hours of growth, and intracellular metal content of cells grown in measured media for 24 hours (Figure 1). The metal content was measured with an inductively coupled plasma mass spectrometer (ICP-MS). The intracellular metal content was significantly higher than the metal content of the media, which demonstrates the cell's striking capacity for metal concentration through high-affinity importers.

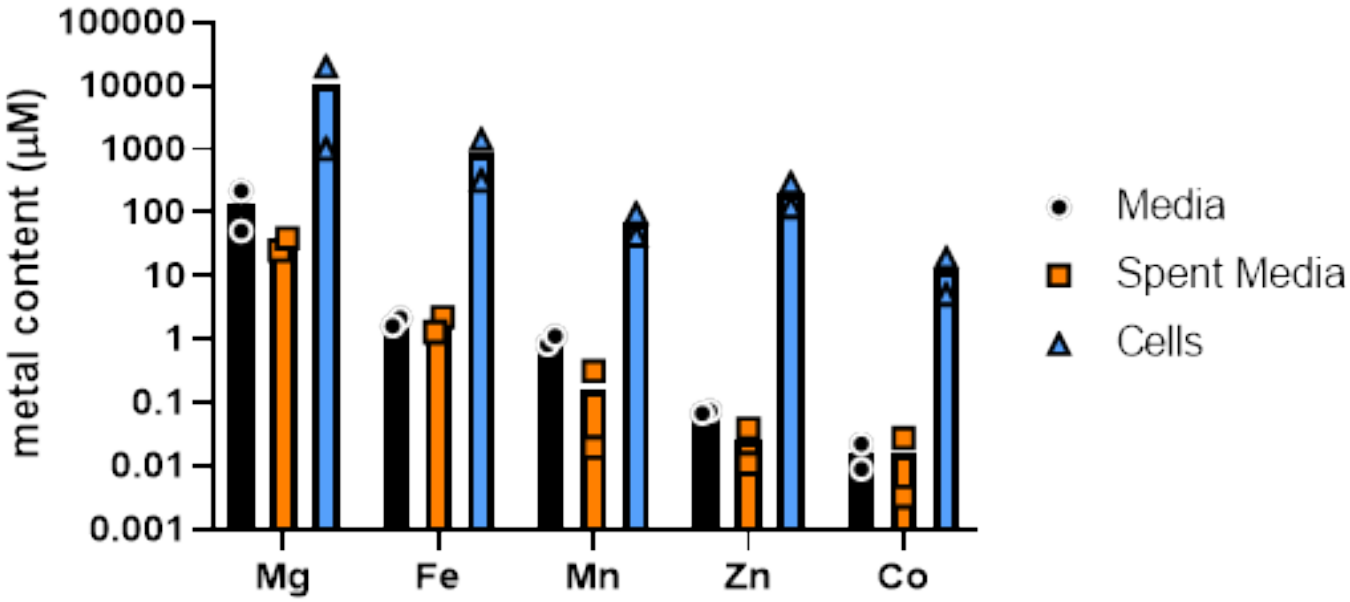

Figure 1: Metal content of cells in metal-replete media- Comparison of the metal content of the metal-replete media, metal content of spent media after 24 hours of growth, and intracellular metal content of cells grown in measured media for 24 hours. Metal content measured with ICP-MS. Average of two biological replicates with individual data points shown.

Growth inhibition by metal limitation

Using the MLMM21, we demonstrated growth inhibition by metal limitation of Mg (Figure 2A), Mn (Figure 2B), and Fe (Figure 2C) in *Bacillus subtilis*. Full growth, as measured by an absorbance at OD_{600nm} of greater than 1, was observed with Mg of 50 µM and Fe and Mn of 1 µM, which are the concentrations used in our MLMM21. Growth yield was affected by varying metal concentration.

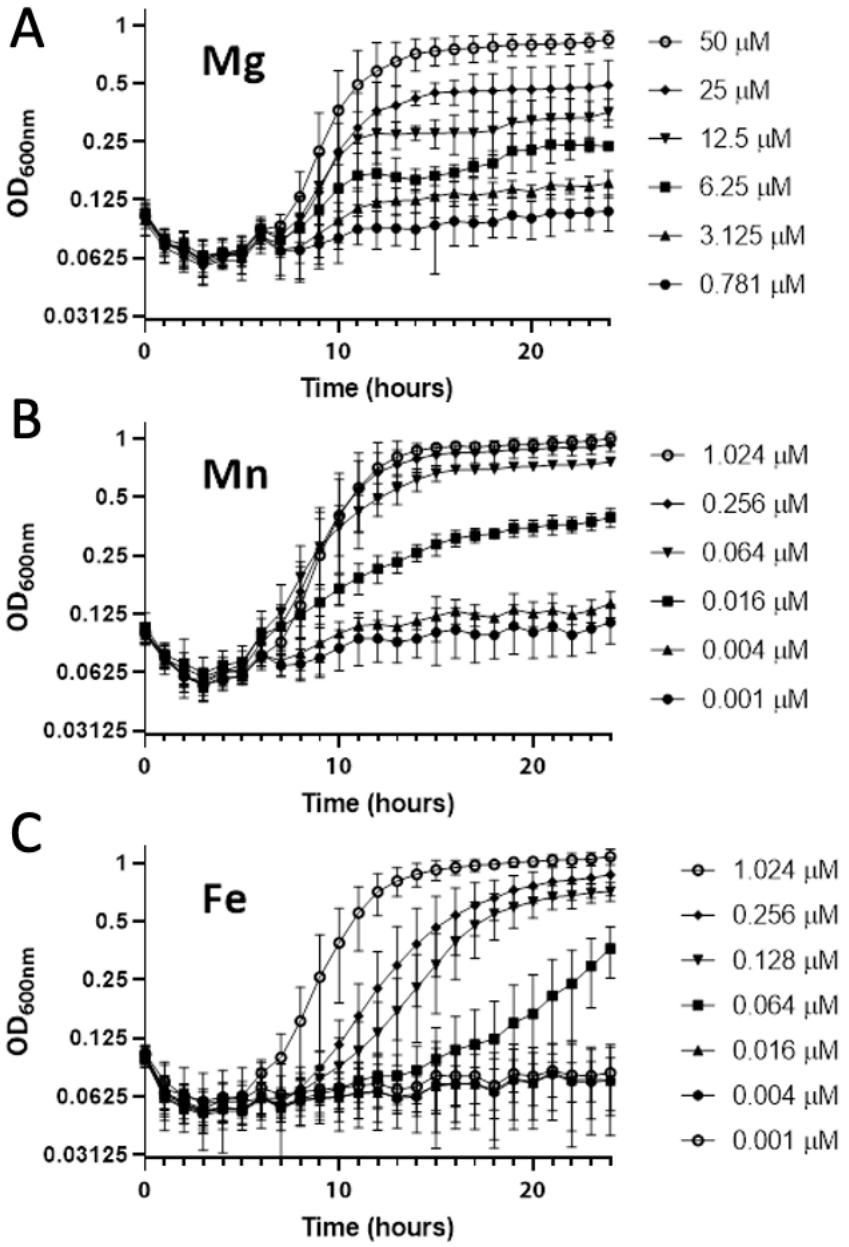

Figure 2: Growth inhibition by limitation of Mg^{2+}, Mn^{2+}, and Fe^{2+}- Using our metal-limited minimal media, we demonstrate limitation of essential metal ions (A) magnesium, (B) manganese, (C) iron by measuring growth hourly at OD_{600nm} at 37 °C for 24 hours under varying metal ion concentrations. Data shown is an average of three biological replicates. Average and standard deviation are depicted.

We observed different thresholds for Fe limitation in different strains of *Bacillus subtilis* (Figure 3). In CU1065, which is a Cornell University isolate of strain 168 that was first published in 1977 (Zahler et al., 1977), there is essentially no observable growth until 0.064 μM Fe. However,

in strain 3610, there is growth to an absorbance of almost 0.5 at the low concentrations of 0.001 μM and 0.004 μM, which are insufficient for any growth in CU1065. The shape of the growth curve of 3610 is also different than the shape of the growth curve of CU1065. The observed difference in growth can be explained by the absence of the bacillibactin synthesis pathway in strain 168. Note that citrate, which promotes iron uptake in bacteria through co-transport, is omitted from the MLMM21, so cotransport is not a factor.

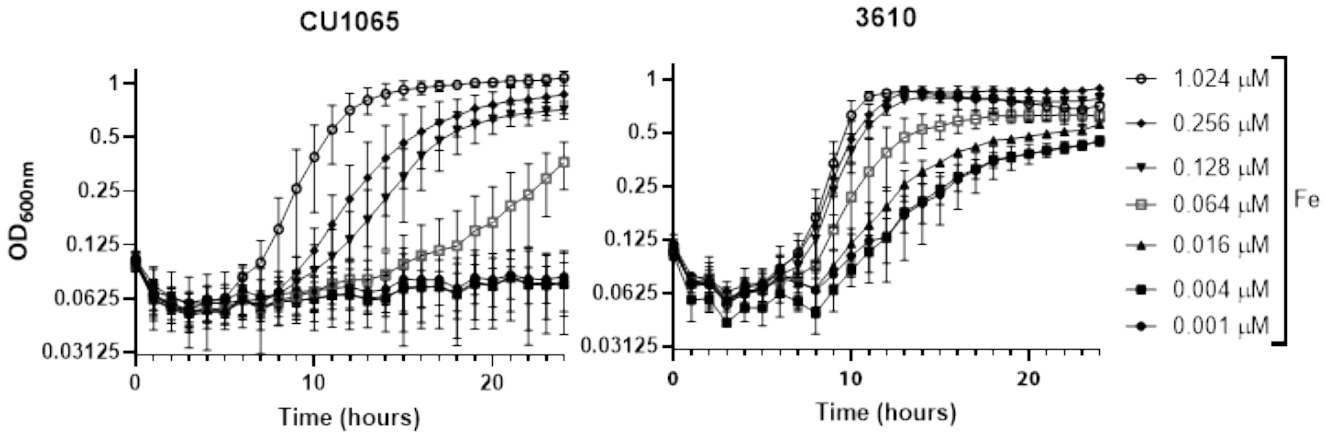

Figure 3: Iron limitation in CU1065 and 3610- We observed different thresholds for iron limitation in CU1065, which does not have siderophores, compared to 3610, which does have siderophores. We measured growth hourly at OD_{600nm} at 37 °C for 24 hours. Data shown is an average of three biological replicates. Average and standard deviation are depicted.

The minimum metal requirements of *Bacillus subtilis*

We established the minimum intracellular concentrations in atoms per cell of Mg, Fe, Mn, Zn, and Co required for growth of *Bacillus subtilis* to an absorbance of 0.5, as measured by the Bioscreen growth analyzer (Figure 4). Potassium was the most abundant ion. Mg and Fe were the next most abundant elements, on the order of 10^7. Mn and Zn were both on the order of around 10^6. Co was the least abundant, on the order of 10^3.

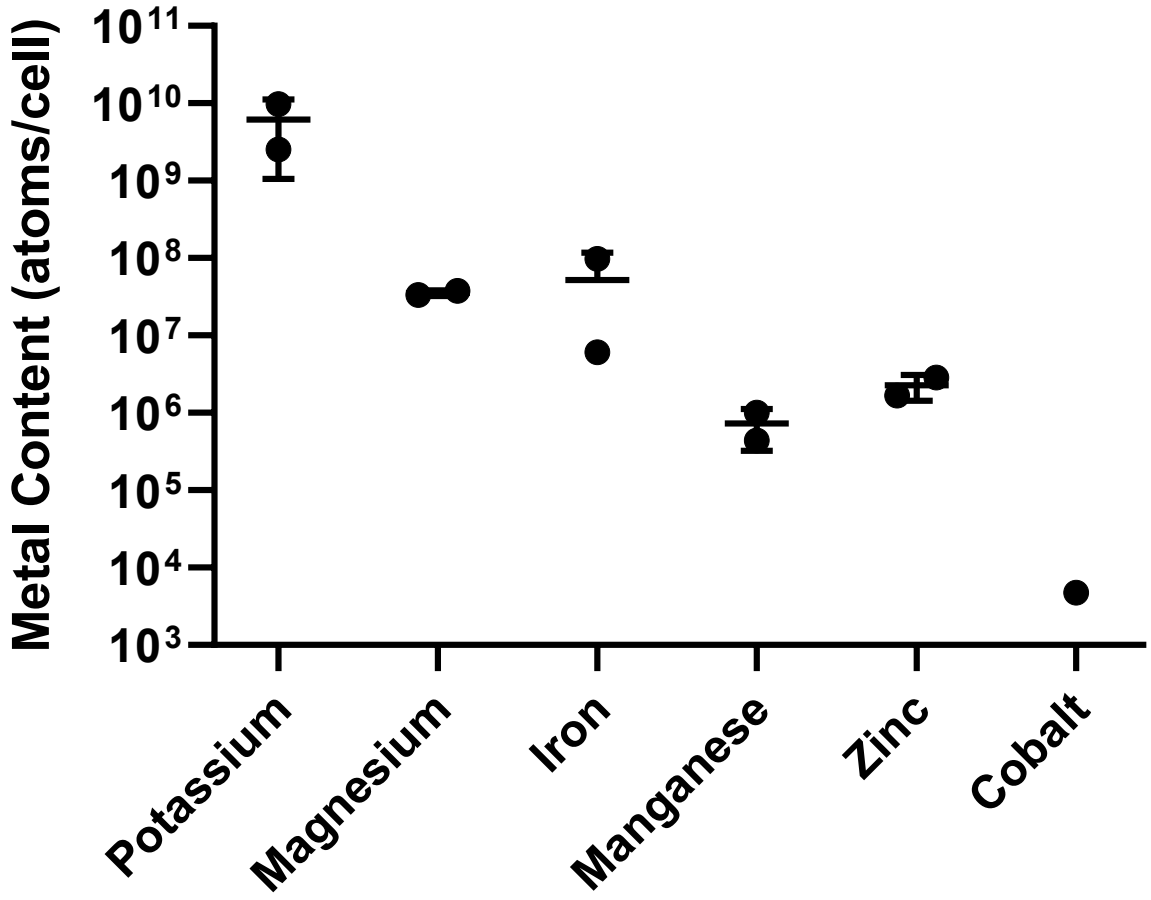

Figure 4: The minimal metal requirements of *Bacillus subtilis*- Minimal metal requirement of *B. subtilis* required for growth to absorbance of 0.5. Data shown is an average of two biological replicates. Average, standard deviation, and individual data points are shown.

Zinc requirement of *Bacillus subtilis*

The Zn requirement of *Bacillus subtilis* is difficult to explore because the cell has such powerful high-affinity import systems for scavenging Zn. In the wildtype strain, there was no demonstrable requirement for added Zn (Figure 5A). However, in a *znuC* mutant, which is deficient in Zn import, we were able to demonstrate growth inhibition from insufficient concentrations of Zn in the media (Figure 5B).

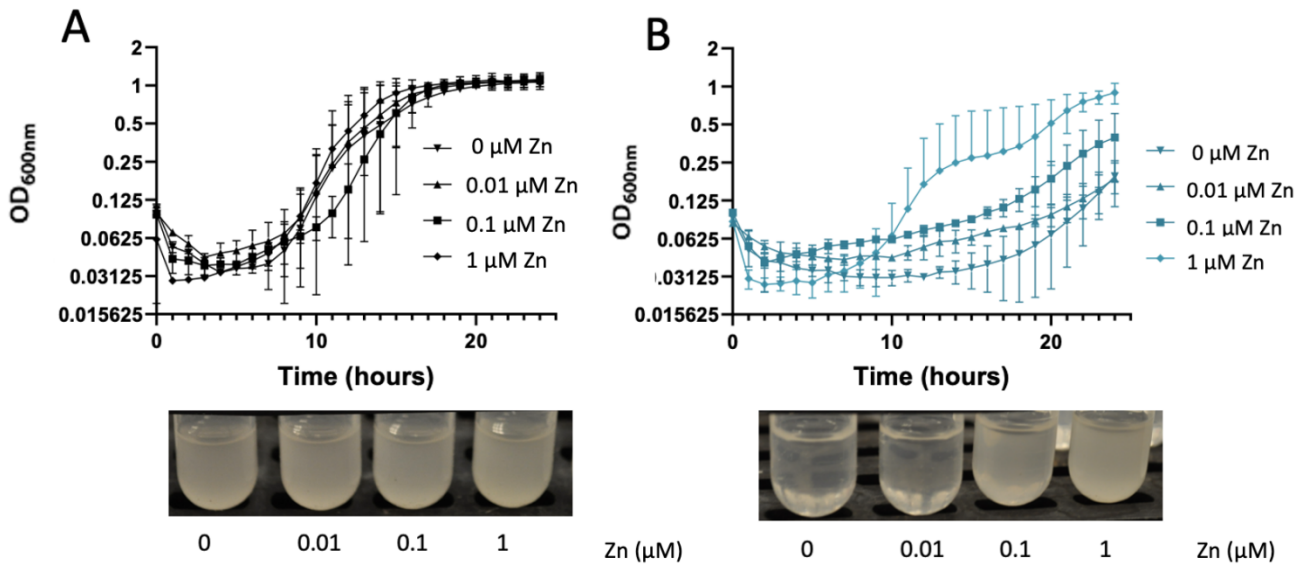

Figure 5: The zinc requirement of *Bacillus subtilis*- (A) In a wildtype cell, there is no demonstrable requirement for Zn addition. (B) In a *znuC* mutant (deficient in zinc import), we were able to titrate for growth, as measured hourly at OD_{600nm} at 37 °C for 24 hours under varying zinc conditions. Data shown is an average of three biological replicates. Average and standard deviation are depicted.

fsrA mutant is more sensitive to low Fe

Since the MLMM21 allows us to limit for Fe in *Bacillus subtilis* 168 strains, we were able to explore the mechanisms of the cell's response to limitation. The *Bacillus subtilis* Fe-sparing response prevents expression of proteins that bind Fe but aren't critical for growth. A key feature of this response is FsrA, a small RNA that represses the translation of non-essential Fe-using proteins. In Fe-limited conditions, cells that were mutant in *fsrA* had lower growth than wildtype cells (Figure 6).

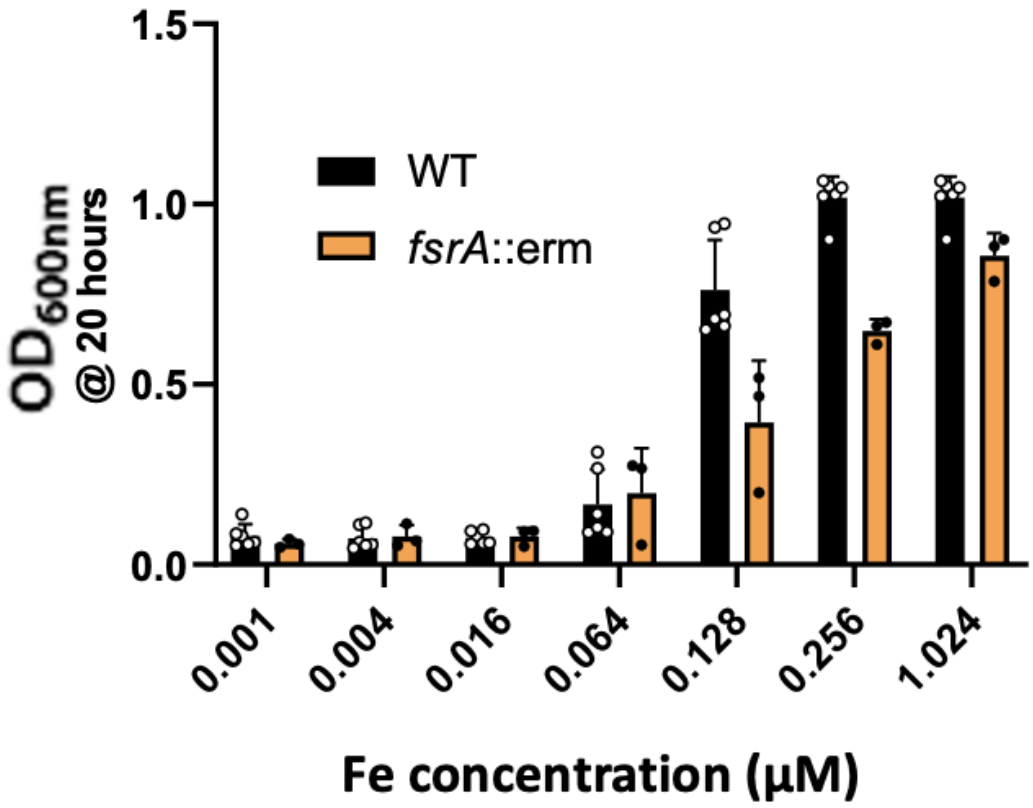

Figure 5: *fsrA* mutant is more sensitive to low Fe- *fsrA* mutant and WT were grown in a gradient of Fe conditions. We measured growth at OD_{600nm} at 37 °C at 20 hours. *fsrA* mutant had lower growth in Fe-limited conditions. Data shown is an average of at least three biological replicates. Average, standard deviation, and individual data points are depicted.

Physiological interactions between metals in limiting conditions

A significant application of the MLMM21 is the exploration of how the limitation threshold of a particular trace metal can be influenced by the levels of other metals in the cell. This can be positive, through functional substitution, or negative, through mismetallation. Functional substitution, in which a scarce metal is replaced by a replete metal, is prevalent in elemental sparing responses (Merchant & Helmann, 2012). Mismetallation, contrastingly, is a result of metal toxicity (Barwinska-Sendra & Waldron, 2017). We found that in Fe-limited conditions, supplemental Zn was able to improve growth, possibly due to functional substitution (Figure 7).

The effect is most prominently seen in the *znuC* mutant when Fe is less than 1 µM. However, the effect can also be observed in WT under 0.256 µM Fe. Cells growing in media with no supplemented Zn had lower growth than cells growing in media with 0.1 µM or 1 µM Zn.

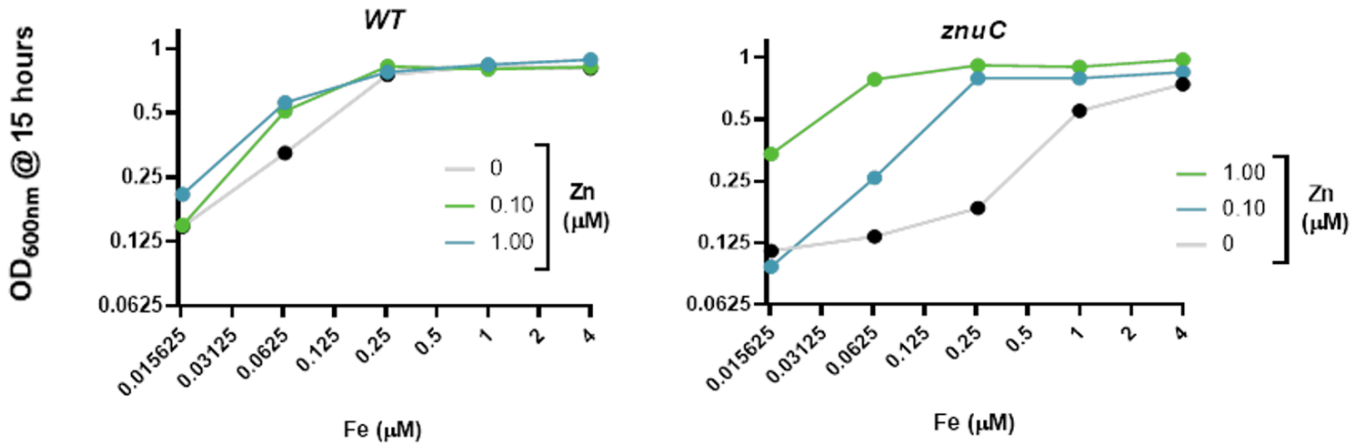

Figure 7: Zn rescues growth in low Fe conditions- WT CU1065 and *znuC* strains were grown in a gradient of Fe and Zn conditions. We measured growth at OD_{600nm} at 37 °C at 15 hours. In Fe-limiting conditions, improved growth was observed with supplemental zinc. Data shown is a representative plot of three independent experiments.

We performed a similar experiment to test the physiological interaction between Fe and Mn. We hypothesized that there may be an ideal ratio of Fe and Mn that maximizes growth in the cell. To test this, we grew *Bacillus subtilis* in the presence of both increasing Fe and Mn concentrations. Increased Mn supported growth in a manner independent of concomitant manipulation of Fe concentrations. Thus, our hypothesis that an ideal ratio of Fe and Mn maximizes growth in the cell was not supported (Figure 8). This lack of interaction is surprising since it has been hypothesized that Fe and Mn serve as alternative cofactors for metabolic enzymes (Imlay, 2014).

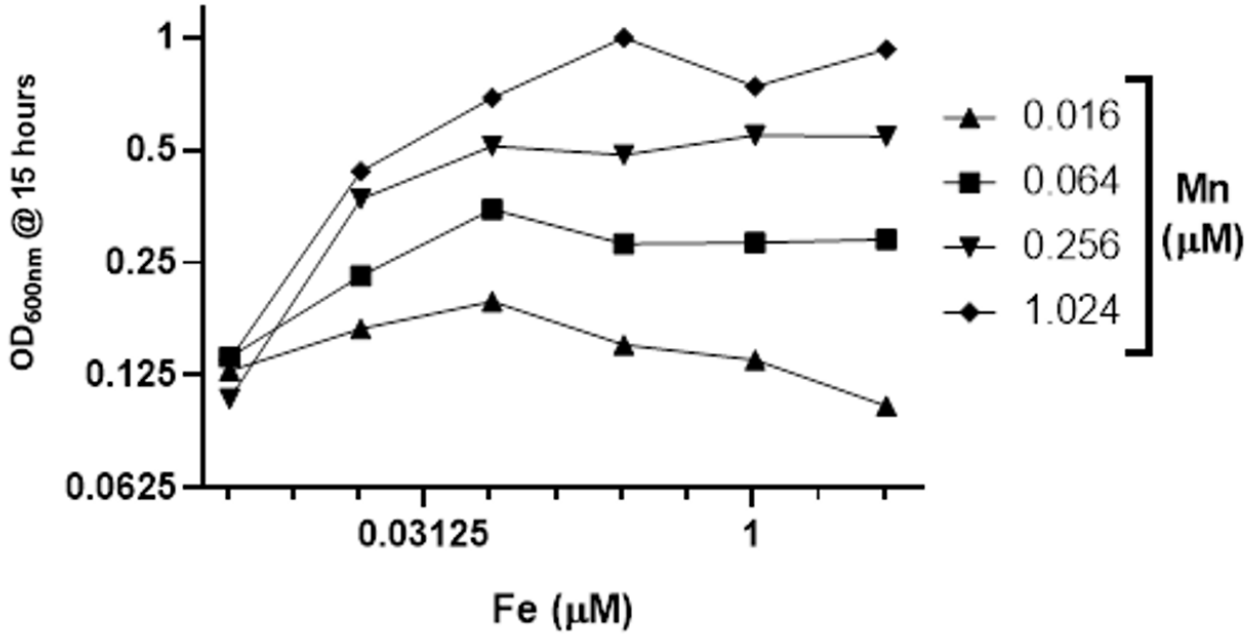

Figure 8: Physiological interaction between Fe and Mn- There was no observable interaction between Fe and Mn. We measured growth hourly at OD_{600nm} at 37 °C for 24 hours. Data shown is an average of three biological replicates.

Discussion

Metal-limited minimal medium recipe

A critical first step in the study of model organisms is the development of a defined medium that allows for the metal ion requirements to be precisely defined. However, it has been difficult to precisely study trace metals because the cell requires these elements in such low amounts, the cell has high affinity uptake systems, and metals are often found as contaminants in other medium components or on tubes (Graham et al., 2009). While metal limited limited media have been developed in other organisms (Kropat et al., 2011), this is not the case for *Bacillus subtilis.* In this work, we were able to create a MLMM where the concentration of Mg was reduced from 800 μM to 50 μM and Fe was reduced from 5 μM to 1 μM. By reducing the Mg concentration so significantly, we may have been able to reduce some of the potential contamination of other metals. However, there was still a significant level of background Zn without any addition of Zn to the media. Further studies could be done to determine which media component was contributing most to this background Zn. By removing one media component at a time and measuring the Zn content of the media, we may be able to determine if there is a single media component that is contributing solely to the background Zn or if it is coming from multiple different sources. We could also measure the Zn content of the media before and after chelexing to determine how effective the chelex is at removing background Zn. Chelex 100 chelating ion exchange resin is known to have a high affinity for divalent ions and transitions metals (Samczyński, 2006), but it is possible that it has a higher affinity for certain transition metals over others. Future studies may explore a pretreatment with another type of chelator to reduce background Zn. For example, Metsorb is patented titanium dioxide that is used to remove a wide variety of heavy metals, including Zn, from drinking water, so studies could be performed to test

whether a combination of Chelex 100 and Metsorb would more effectively remove Zn from the media (Pouran et al., 2013).

Metal content of cells in metal-replete media

The measured metal content in the cells was significantly higher than the metal content of the unused or the spent media. This demonstrates the cell's ability to effectively concentrate metals from the environment using high-affinity importers. In *Bacillus subtilis,* each of the major metals has a dedicated metalloregulator that senses the intracellular concentration of that metal and mediates a response to deficiency or excess. Zn is sensed by Zur (Gaballa & Helmann, 1998) and CzrA (Moore & Helmann, 2005), Fe is sensed by Fur (Bsat et al., 1998), and Mn is sensed by MntR (Que & Helmann, 2000) and Mn-sensing riboswitches (Price et al., 2015). It is significant to note that Co is also concentrated in the cell 1000-fold, even though there is no known role for Co in *Bacillus subtilis.* Future studies may elucidate the mechanisms of Co import and usage in the cell.

Growth inhibition by metal limitation

The Helmann lab has previously shown growth inhibition by limitation of Mg and Mn (Que & Helmann, 2000). The Helmann lab has also shown growth inhibition by limitation of Fe and Zn with soluble chelators, but this poses a problem because the chelators are not always perfectly specific and they are not removable (Gabriel & Helmann, 2009; Ollinger et al., 2006). It is important to demonstrate limitation by absence of media components because then you can add the component back to rescue growth. Our newly optimized MLMM21 allowed us to show growth inhibition by Fe limitation. It is significant to note that the growth curves level off at

certain absorbances for each metal concentration, which demonstrates that the growth is inhibited, not just delayed, by insufficient metal concentration.

It was interesting to observe the difference in the thresholds for Fe limitation in the different strains of *Bacillus subtilis.* Strain 3610 is able to grow to a higher density at lower Fe concentrations because it produces bacillibactin, a catechol-based siderophore with a high affinity for chelating Fe (Miethke et al., 2006). This siderophore allows strain 3610 to scavenge Fe more effectively at low concentrations. Strain CU1065 only makes the bacillibactin precursor DHB(G) but does not synthesize the complete siderophore (Ollinger et al., 2006), so it is more sensitive to low Fe conditions. Future studies with other strains of *Bacillus subtilis* could lend insight into the different mechanisms of scavenging Fe at low concentrations.

The minimum metal requirements of *Bacillus subtilis*

We established the minimum intracellular concentrations of Mg, Fe, Mn, Zn, and Co required for growth of *Bacillus subtilis* to an absorbance of 0.5. Growth to an absorbance of 0.5 is not full growth, but it demonstrates how much of each metal the cell needs simply to survive and replicate. Establishing a baseline metal requirement for the cell can inform further studies of metal utilization and localization. It can also inform understanding of sparing responses and how they relate to nutritional immunity.

Zinc requirement of *Bacillus subtilis*

Zinc is a universally required element that is used as an enzyme cofactor and in protein folding. Zinc is known to be a necessary cofactor for several proteins in *Bacillus subtilis*, most notably for ribosomes, which contain about 20% of the cellular Zn (Chandrangsu et al., 2019). However,

it has previously been difficult to demonstrate growth inhibition by Zn limitation because Zn is required is such small concentrations in the cell, there are high affinity import systems, and Zn is often found as a contaminant in other media components. Experiments with *E. coli* have demonstrated the difficulty of limiting for Zn in chemostats, where cells obtained more Zn than was added to the culture due to leaching from the vessel (Graham et al., 2009). Even using the MLMM21, we were unable to demonstrate Zn limitation in the wildtype cell. To ameliorate this issue, we used a *znuC* mutant that is deficient in Zn import. *znuC* encodes a subunit of the ABC transporter that is the primary mechanism of importing Zn in *Bacillus subtilis*. This mutant is more sensitive to low Zn conditions because it is not as effective at importing Zn from the media. In this mutant strain, we were able to show Zn limitation at concentrations of Zn less than 1 μM. 1 μM appears to be sufficient for full growth in the *znuC* mutant. However, we found that a concentration of 10 μM Zn poisoned the cell, leading to very low growth. Zn homeostasis in the cell must thus be tightly regulated to prevent limitation or toxicity.

During this experiment, the cell's ability to scavenge Zn at very low concentrations was highlighted very clearly when we switched culture tubes from glass to plastic. When we used glass culture tubes, we were unable to get any Zn limitation even in the *znuC* mutant because the glass had traces of Zn on it that the cell was able to effectively scavenge and survive on. Once we switched to plastic, we were able to show Zn limitation because the plastic had fewer traces of contaminating metal.

fsrA mutant is more sensitive to low Fe

Iron in *Bacillus subtilis* is regulated by the ferric uptake repressor Fur, which, under low Fe conditions, becomes inactive and derepresses several different redundant Fe uptake systems.

Under extreme Fe limitation, *Bacillus subtilis* expresses a fur-controlled small RNA FsrA and the chaperone proteins fbpABC, which make up the Fe-sparing response (Gaballa et al., 2008). The *Bacillus subtilis* Fe-sparing response prevents expression of proteins that bind Fe but aren't critical for growth. Recent studies examining the Fe-sparing response have determined that many of the genes turned off by FsrA are involved in metabolism and the TCA cycle (Mars et al., 2016). Previous studies in the Helmann lab determined that the Fe-sparing response is maladaptive in iron-sufficient media (Gaballa et al., 2008). In a *fur* mutant, in which FsrA is produced constitutively because Fur is unable to repress it, there was low growth in Fe-sufficient conditions. While at first it was assumed that this low growth was because cells are taking in too much Fe, a large part of the growth defect is due to the constitutive expression of the Fe-sparing response. This 2008 study also demonstrated that the Fe-sparing response is adaptive under Fe-starved conditions. In that experiment, FsrA was constitutively turned on in the *fur* mutant, but we had never tested if FsrA is important in wildtype cells. My data demonstrate that FsrA helps cells adapt to low Fe conditions because there is lower growth in the *fsrA* mutant than the wildtype cells at low Fe conditions.

Physiological interactions between metals in limiting conditions

Functional substitution, a process in which a replete metal replaces a scarce metal, is common in elemental sparing responses (Merchant & Helmann, 2012). For example, the electron carrier protein ferredoxin is replaced with an Fe-free alternative, flavodoxin, in several organisms. In *Clostridium pasteurianum*, the substitution of ferredoxin with flavodoxin allows the former to be actively degraded in order to release and recycle Fe for maintenance of pyruvate synthase, an Fe/S protein (Schönheit et al., 1979). In Fe-deficient *E. coli* cells, a Fur-regulated substitution

pathway is used in which the *nrdEF* operon encodes an alternative, Mn-utilizing ribonucleotide reductase (RNR) to replace the Fe-dependent RNR that requires an Fe_2S_2 ferredoxin (Andrews, 2011). In this study, we demonstrate that under low Fe conditions, supplemental Zn is able to improve growth of *Bacillus subtilis*, which may suggest that Zn is functionally substituting for some Fe-dependent enzymes. Further studies are needed to explore the mechanisms of this substitution and which Fe cofactors can be replaced by Zn.

Limitations

Although the MLMM21 allowed us to demonstrate metal limitation for Mg, Mn, and Fe, the components were still not 100% pure because we were unable to show Zn limitation in the wildtype strain. Some sources of Zn contamination could be from a low level of contamination in the other metal preparations that we added or from other media components. Although we used 99.99% pure components, the cell is able to scavenge enough Zn to survive from the contaminants due to its efficient and high affinity import systems. This could also be a problem with Co. Even though we don't add Co to the media, the cell still concentrates Co 1000-fold in the cell.

Another limitation of this experiment is the inherent variability in the media and in the cells. Even though we used the exact same concentrations of each media component and supplemental media in every experiment, there were still differences in the absorbance measurements between experiments from different days, or even among biological replicates from the same day. The variation could stem from a variety of different factors, including slight differences in measuring the components, different growth rates or phases of growth among different colonies, different temperatures, and many more unforeseen variables in running an experiment.

Next steps

Now that we have optimized this MLMM21, it can be used by the community to explore the cellular metal quota and the mechanisms of response to metal limitation of other trace metals, such as Co. While many elements are known to be necessary for growth in all organisms, Co is not universally required as a micronutrient. In *Bacillus subtilis,* there is no known role for Co. Recent studies in the Helmann lab have identified a putative B_{12}-coupled cobalt permease *ybaF* (Pi et al., 2020). *Bacillus subtilis* also expresses a putative B_{12}-importer *yvrC*; *yvrC* is controlled by a cobalamin riboswitch that shuts down transcription by enhancing termination (Chan & Mondragón, 2020). I have also shown that Co is concentrated in the cell 1000-fold compared to the media. Thus, even though it is not known to be required for cell survival, it is unknown whether Co could have a beneficial role under certain conditions, such as Zn-limitation. Co and Zn are both transition metals and share many of the same chemical and physical properties, potentially allowing metallation of certain Zn-requiring enzymes. Future studies using the optimized MLMM21 may be able to shed light on the role of Co under Zn-limited conditions, potentially implicating a novel function for cobalt in *Bacillus subtilis* metabolism.

Conclusion

We established MLMM21 that allows us to limit for Mg, Mn, and Fe and address several significant research questions regarding cellular requirements for certain metals and responses to metal limitation. We demonstrated that cells are effectively able to concentrate metals from the media intracellularly. We explored the Zn requirement of *Bacillus subtilis* and showed Zn limitation in a *znuC* mutant. We studied the Fe-sparing response and provided evidence that the *fsrA* mutant is more sensitive to low Fe conditions. We showed the Zn is able to improve growth of an Fe-starved cell, potentially through functional substitution. Based on this work, we can now continue to explore trace metals and their interactions in the cell, as well as mechanisms of the cell's response to metal limitation, using the MLMM21.

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