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Review Article

REVIEW ARTICLE

Regulation and distinct physiological roles of manganese in bacteria

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One sentence summary: This review provides an overview of Mn^{2+} transport and the regulation of its homeostasis in bacteria, with a focus on lactobacilli, and describes functions of Mn^{2+} beyond being a co-factor for enzymes, such as oxidative stress and competitive exclusion.

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ABSTRACT

Manganese (Mn^{2+}) is an essential trace element within organisms spanning the entire tree of life. In this review, we provide an overview of Mn^{2+} transport and the regulation of its homeostasis in bacteria, with a focus on its functions beyond being a cofactor for enzymes. Crucial differences in Mn^{2+} homeostasis exist between bacterial species that can be characterized to have an iron- or manganese-centric metabolism. Highly iron-centric species require minimal Mn^{2+} and mostly use it as a mechanism to cope with oxidative stress. As a consequence, tight regulation of Mn^{2+} uptake is required, while organisms that use both Fe^{2+} and Mn^{2+} need other layers of regulation for maintaining homeostasis. We will focus in detail on manganese-centric bacterial species, in particular lactobacilli, that require little to no Fe^{2+} and use Mn^{2+} for a wider variety of functions. These organisms can accumulate extraordinarily high amounts of Mn^{2+} intracellularly, enabling the nonenzymatic use of Mn^{2+} for decomposition of reactive oxygen species while simultaneously functioning as a mechanism of competitive exclusion. We further discuss how Mn^{2+} accumulation can provide both beneficial and pathogenic bacteria with advantages in thriving in their niches.

Keywords: manganese; lactobacilli; bacilli; oxidative stress; competitive exclusion

INTRODUCTION

The divalent cation of manganese (Mn^{2+}) has a variety of important roles in bacterial cells (Fig. 1). Depending on the species and conditions, it can function as a cofactor for diverse enzymes involved in central carbon metabolism (Holland and Pritchard 1975; Chander, Setlow and Setlow 1998), nucleotide metabolism (Martin and Imlay 2011), translation (Stetter and Zillig 1974) and signaling (Missiakas and Raina 1997). Moreover, it is known to associate intracellularly with nucleic acids, proteins and metabolites (Chandrangsu, Rensing and Helmann 2017; Waters 2020) and therefore also plays a role beyond catalytic functions. While the unconventional role of helping in radiation resistance of extremophiles had been known for some time (Daly *et al.* 2004), recently more roles of Mn^{2+} have been discovered, showing the diverse functions of this trace metal in (i) lactobacilli that scavenge it to gain a competitive growth advantage over yeast and mold in dairy (Siedler *et al.* 2020), and (ii) highly specialized newly isolated bacteria in which it serves as an independent energy source (Yu and Leadbetter 2020) (Fig. 1).

The most widespread and essential role of Mn^{2+} across bacterial species relates to oxidative stress resistance (Fig. 1). Under normal growth conditions, most bacteria do not have a high requirement for Mn^{2+}, as most enzymes use iron (Fe^{2+}) and not Mn^{2+} as a cofactor (Jakubovics and Jenkinson 2001; Horsburgh

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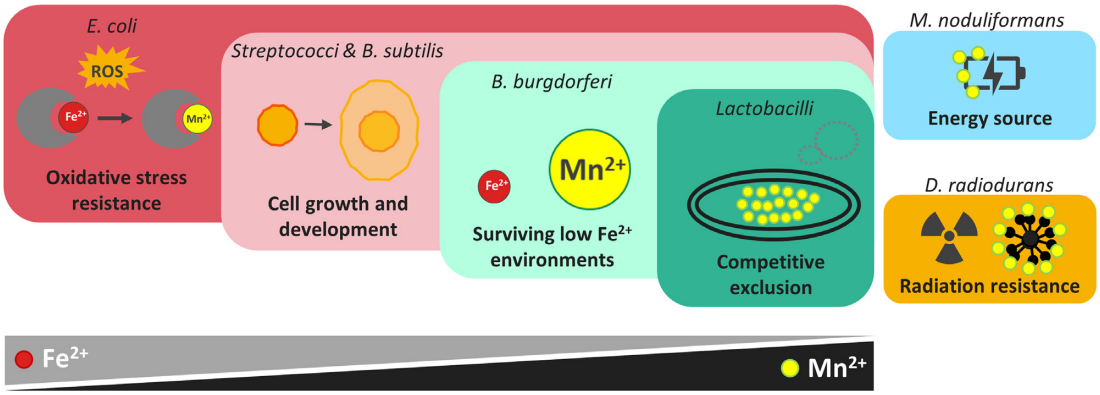

Figure 1. Functions of Mn^{2+} in bacteria. From left to right, the organisms become more manganese centric and use Mn^{2+} for an increasing number of cellular functions of which they share the ones depicted as overlapping boxes; the two depicted separately at the very right have very specialized functions for Mn^{2+} that are not shared by other species. The species given in the top row are the most-studied examples, but the traits are not necessarily exclusive to these species.

et al. 2002; Helmann 2014). Moreover, Fe^{2+} is an essential cofactor for the respiratory chain and hence required for all respiring organisms (Jakubovics and Jenkinson 2001). The requirement for Mn^{2+} and Fe^{2+} differs depending on the species, and bacteria can be divided into three different groups based on their preference and requirement for Fe^{2+} or Mn^{2+}. *Escherichia coli* is the best-studied example of a bacterial species with a highly so-called iron-centric metabolism, as it requires Fe^{2+} for normal growth and Mn^{2+} only under oxidative stress conditions (Anjem, Varghese and Imlay 2009; Paruthiyil *et al.* 2019). In contrast, some bacteria have been found to require little to no Fe^{2+} for normal growth and instead display a high requirement for Mn^{2+}, and hence are called manganese centric. Such bacteria are found in the genus *Borrelia*, the causative agent of Lyme disease, but are especially abundant in species formerly belonging to the recently reclassified *Lactobacillus* genus, particularly those of the current *Lactiplantibacillus* and *Lacticaseibacillus* genera (Helmann 2014; Chandrangsu, Rensing and Helmann 2017). For simplicity, the term lactobacilli will forwardly in this review refer to species of the former broadly defined *Lactobacillus* genus. An intermediate group of bacteria including *Bacillus subtilis* can be considered neither iron- nor manganese centric as they require both Fe^{2+} and Mn^{2+} for normal growth and functioning (Chandrangsu, Rensing and Helmann 2017). In these organisms, most enzymes use Fe^{2+} as a cofactor, but Mn^{2+} is used as well, for example as an essential cofactor for sporulation in *B. subtilis* (Jakubovics and Jenkinson 2001; Que and Helmann 2002).

While lactobacilli can accumulate and tolerate very high amounts of Mn^{2+}, excess accumulation of Mn^{2+} in nonmanganese-centric organisms can easily lead to cytotoxicity - primarily through mismetallation of proteins (Chandrangsu, Rensing and Helmann 2017). Distinct bacteria thus have to tightly regulate Mn^{2+} homeostasis to different extents to ensure sufficient levels for Mn^{2+}-dependent stress responses and other functions, yet low enough to prevent cytotoxicity (Waters 2020). They achieve this mostly through the control of importers and exporters, mediated by transcription factors and riboswitches responsive to Mn^{2+} and/or oxidative stress.

Several recent reviews are available on Mn^{2+} transport and homeostasis (Juttukonda and Skaar 2015; Chandrangsu, Rensing and Helmann 2017; Waters 2020). In the current review, we will first provide a detailed description of the regulation of Mn^{2+} transport and homeostasis regulation, highlighting differences between various classes of bacteria based on whether they are iron- or manganese centric. Second, we will discuss the roles of Mn^{2+} in these distinct bacterial classes in light of homeostasis and functional origin, and elaborate on how Mn^{2+} is involved in not only oxidative stress but also in both beneficial and harmful interactions between bacteria and other organisms. Throughout the topics of this review, we particularly focus on the intriguing manganese-centric physiology of lactobacilli.

Mn^{2+} TRANSPORTERS

Two major types of bacterial Mn^{2+} importers are known: the NRAMP (natural resistance-associated macrophage protein) family type transporter MntH, and the ABC (ATP-binding cassette) transporter type MntABC (Fig. 2). The NRAMPs are a family of secondary active transporters involved in metal ion homeostasis (Shi, Zhao and Kong 2014; Jensen and Jensen 2015). The bacterial NRAMP type was first discovered and characterized in *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) and *E. coli* based on homology with eukaryotic transporters, and named MntH as it is a highly specific Mn^{2+} transporter that symports protons (H^+) (Kehres *et al.* 2000). For the ABC transporter, the nomenclature varies between organisms, e.g. SitABC, SloABC and MtsCBA (see Table S1, Supporting Information). For reasons of clarity, we refer to this transporter as MntABC throughout this review. In *Lactiplantibacillus plantarum*, a potential third type of importer, named MntA (not to be confused with MntABC), has been identified (Hao, Reiske and Wilson 1999), but its role in Mn^{2+} import is not fully confirmed (Groot *et al.* 2005). In this review, it will not be further discussed and, in line with other literature, we are referring to the A-subunit of MntABC whenever MntA/*mntA* is mentioned.

Whereas primary active transporters such as MntABC require the hydrolysis of ATP for function, secondary active transporters like MntH couple the favorable energy of the passage of one molecule to power the transport of another (Bane 2015) (Fig. 2). It is not yet fully understood what the distinct roles of the two importers MntH and MntABC are. In *Streptococcus* spp., deletion of either *mntH* or *mntABC* decreases virulence, albeit to different extents (Eijkelkamp, McDevitt and Kitten 2015; Kajfasz *et al.* 2020). In several bacteria, including streptococci and *B. subtilis*, the two transporters were shown to have distinct affinities for Mn^{2+}, and to be active at different stages of growth (Que and Helmann 2002; Wang, Tong and Dong 2014). In *S.* Typhimurium, the affinity for Mn^{2+} was found to be 0.1 μM (1.8 ng/L) for both MntH and MntABC (Kehres *et al.* 2000, 2002). The cation inhibition profiles were found to be strikingly similar

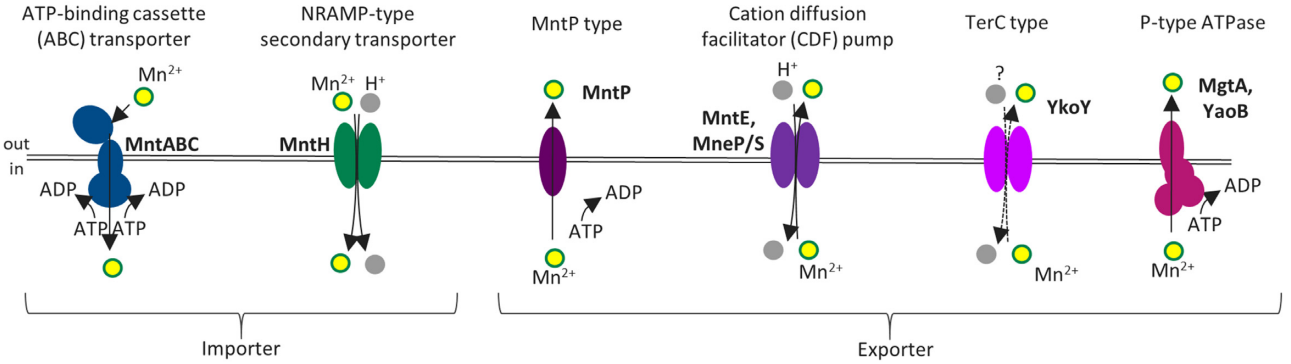

Figure 2. Manganese transporters in bacteria. For presence and absence of the different transporters and their regulation, see Table S1 (Supporting Information) and Figs 3–5. Note that the nomenclature for the ABC transporter differs per species. Throughout this review, we will refer to it as MntABC, but other names are SloABC, MtsABC and SitABC (or CBA). Figure based on Bane (2015).

between these two structurally different transporters, with the exception of Zn^{2+}, which strongly inhibits MntABC but not MntH (Kehres *et al.* 2000, 2002). Moreover, MntH in S. Typhimurium was shown to function best under acidic conditions, while MntABC works optimally at slightly alkaline pH and loses functionality in mildly acidic conditions (Kehres *et al.* 2002). Several lactobacilli contain two or even three MntH transporters as well as the ABC transporter; presumably all these have different affinities and physiological functions (Serata, Yasuda and Sako 2018; Siedler *et al.* 2020), which will be further discussed later in this review.

For Mn^{2+} uptake, no molecular equivalents to the siderophores such as those used for Fe^{2+} chelation have been described in detail. The peptide antibiotic bacitracin produced by bacilli has been reported to bind Mn^{2+} and increase its import and hence could potentially be considered as a 'manganiphore' (Haavik 1979; Archibald 1986), but little follow-up literature is available on this topic. Moreover, bacitracin has been described to bind other metal ions and to have low affinity for Mn^{2+}, with no binding at low pH (Ming and Epperson 2002). Therefore, competition for Mn^{2+} appears to occur through differences in transporter affinity and efficiency. This could explain why several manganese-centric *Lactobacillus* spp. encode for multiple *mntH* importer genes, as high intracellular Mn^{2+} concentrations are essential in these species (Serata, Yasuda and Sako 2018; Siedler *et al.* 2020).

While only two types of Mn^{2+} importers are conserved throughout bacteria, up to five different classes of bacterial Mn^{2+} exporters are described in literature (Fig. 2) (Zeinert *et al.* 2018; Waters 2020). Except for the GTT1-family MneA from *Vibrio cholera*, we will discuss the regulation of these exporter classes in the next sections. Mn^{2+} export takes place whenever intracellular Mn^{2+} levels are too high and intoxicate cells, leading to a rapid release of Mn^{2+} into the environment after a short period of bacteriostasis (Fisher *et al.* 1973; Huang *et al.* 2017). Only recently, the two major Mn^{2+} exporters of *B. subtilis*, MneP and MneS, belonging to the cation diffusion facilitator family, were identified (Huang *et al.* 2017). This was later followed by the discovery of two more putative secondary Mn^{2+} exporters YceF and YkoY, belonging to the TerC family (Paruthiyil *et al.* 2019) (Fig. 2; Table S1, Supporting Information). In *E. coli*, a putative export pump MntP, belonging to its own family, has been described. Deletion of *mntP* resulted in elevated intracellular Mn^{2+} levels and increased manganese sensitivity (Waters, Sandoval and Storz 2011). A detailed review on Mn^{2+} transporters has recently been published by others (Waters 2020).

REGULATION OF Mn^{2+} HOMEOSTASIS

Genetic regulatory elements

Regulation of Mn^{2+} homeostasis mostly takes place on the transport level. Even though functions of Mn^{2+} and the level of manganese centricity of a given bacterial species differ, most regulators of Mn^{2+} transporters are shared between the distinct groups (Patzer and Hantke 2001). First and foremost, the metalloprotein transcriptional regulator MntR, for Mn2+ transport regulator, constitutes the central regulator of Mn^{2+} homeostasis across bacteria, even though MntR homologs display only around 30% similarity between Gram-positive and Gram-negative bacteria (Shi, Zhao and Kong 2014). MntR controls intracellular Mn^{2+} levels by coordinating the transcription of importers, and, depending on the organism, also exporters (Figs 3–5; Table S1, Supporting Information). MntR forms a homodimer that, through binding of one Mn^{2+} ion per subunit, undergoes a conformational change, which increases the affinity for its DNA binding sites. In *B. subtilis*, it has been shown that the dynamic response range of MntR spans from 4 to 20 μM (0.22 to 1.1 μg/mL) (Huang *et al.* 2017). This relatively minor range reflects that tight regulation is required to correctly balance the intracellular concentration of a trace element that is both essential and toxic.

Besides MntR, three other transcription factors are known to influence Mn^{2+} concentrations in bacterial cells, which will be described in later sections in detail. Fur, for Ferric uptake regulator, like MntR also belongs to the metalloprotein transcriptional regulator family and responds directly to Fe^{2+} ions. Fur represses mostly Fe^{2+} uptake genes under high Fe^{2+} concentrations (Bags and Neilands 1987), and plays a role in Mn^{2+} homeostasis. Additionally, the H_2O_2-sensing transcription factors PerR in Gram-positives and OxyR in Gram-negatives (Patzer and Hantke 2001) induce expression of Mn^{2+} uptake mechanisms either directly or indirectly under oxidative stress (Guedon *et al.* 2003). PerR of *B. subtilis* binds Fe^{2+} or Mn^{2+} depending on the respective availability of the two metals, which is a prerequisite for PerR to bind DNA and repress transcription of regulon members. PerR with Fe^{2+} as cofactor is sensitive to metal-catalyzed oxidation by H_2O_2, which causes the release of PerR from its DNA binding boxes enabling expression of downstream genes (Chen, Keramati and Helmann 1995; Herbig and Helmann 2001; Lee and Helmann 2006). OxyR gets activated directly by H_2O_2 through oxidation of a cysteine residue and controls its regulon by direct interaction with RNA polymerase (Imlay 2008; Anjem, Varghese and Imlay 2009; Shi, Zhao and Kong 2014).

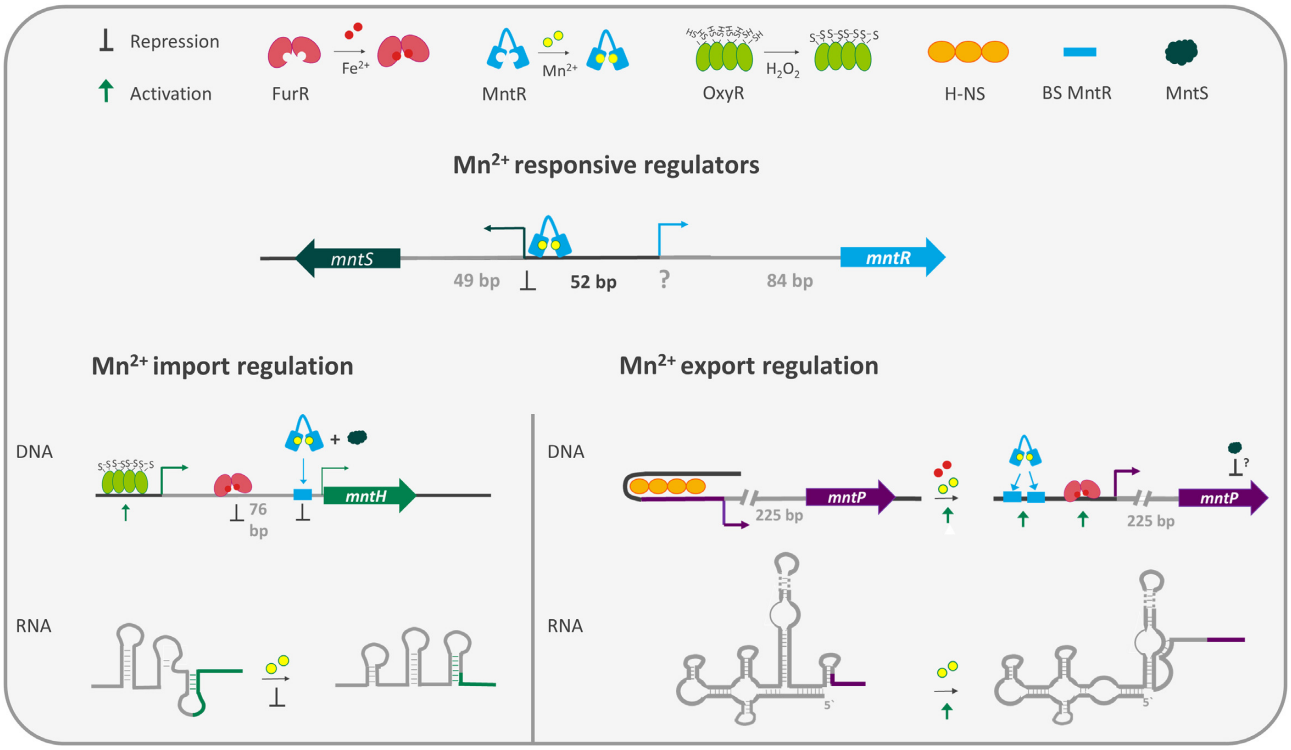

Figure 3. Manganese transport and regulation in the iron-centric *E. coli*. The individual transcriptional regulators are depicted at the top, including their mechanisms, e.g. metal binding, as well as in the figure to indicate their binding sites. For MntR, an additional symbol for the DNA binding site (BS-mntR) in light blue has been chosen for clarity. Repression and activation symbols indicate the influence of the individual transcription factors shown above upon Mn^{2+} addition. Genes are depicted in thick arrows and transcription start sites are shown in thin arrows. The base pair (bp) numbers indicate the distance from the gene to the transcription start site. The 2D structure of folded RNA is depicted with and without Mn^{2+} addition. The coding regions of *mntR* and *mntP* are shown in green and lilac, respectively, while the upstream region is shown in gray.

Apart from transcription factors, Mn^{2+} can influence the expression of several genes via Mn^{2+}-responsive riboswitches. One of these is known as the *yybP–ykoY* motif, named after the *yybP* and *ykoY* genes that it precedes in *B. subtilis*. *yybP–ykoY* motifs are widespread in bacteria (Barrick *et al.* 2004) and shown to bind Mn^{2+} in *E. coli*, *B. subtilis* (Dambach *et al.* 2015) and *Lactococcus lactis* (Price *et al.* 2015) in which they mainly regulate Mn^{2+} export. A second Mn^{2+} riboswitch that influences Mn^{2+} import rather than export is found and conserved in several iron-centric Gram-negative enteric bacteria. Both riboswitches will be described in more detail in the respective sections later. The fact that the quantity of Mn^{2+}-related proteins are tuned on both transcriptional and translational levels again highlights the essentiality of tightly regulating intracellular Mn^{2+} levels (Dambach *et al.* 2015). In addition to the regulators introduced earlier, nongenetic factors such as pH and organic acids play a role in regulating Mn^{2+} homeostasis, which will be discussed in a separate section.

The first studies on the function and regulation of Mn^{2+} metabolism focused on *E. coli* and *S.* Typhimurium as model organisms. Many subsequent investigations in *B. subtilis* and to some extent also in streptococci and lactococci have been performed. Remarkably few studies have focused on lactobacilli. This is surprising, as Mn^{2+} plays such a major role in these species that are among the most manganese-centric organisms known, together with *Borrelia* (Lisher and Giedroc 2013). As described later, oxidative stress responses as well as intracellular Mn^{2+} levels differ in bacteria with an iron- or manganese-centric metabolism, which is reflected in the way Mn^{2+} levels are regulated under such and normal conditions. Due to this, and in combination with the fairly low homology between Gram-positive and Gram-negative MntR, we will further discuss the molecular basis of regulation of Mn^{2+} homeostasis separately for each bacterial group (iron centric, iron- and manganese dependent, and manganese centric).

Iron-centric bacteria (most Gram-negatives)

Escherichia coli solely depends on Mn^{2+} under oxidative stress conditions (Anjem, Varghese and Imlay 2009) and harbors one importer (MntH) and one exporter (MntP). Several layers of regulation are involved to fine-tune the expression of both transporters, which ensures the optimal Mn^{2+} concentration for a given condition (Fig. 3). Under Fe^{2+} and/or Mn^{2+}-rich conditions, Mn^{2+} is scarcely imported into the *E. coli* cell because *mntH* transcription is repressed both by Fur and MntR (Patzer and Hantke 2001; Anjem, Varghese and Imlay 2009; Shi, Zhao and Kong 2014). Inactivation of Fur disrupts the Fe^{2+}-based but not the Mn^{2+}-based regulation of *mntH*, indicating that MntR and Fur function independently (Kehres *et al.* 2002). Expression of *mntH* becomes strongly upregulated by the presence of H_2O_2 (Anjem, Varghese and Imlay 2009), but does not respond to paraquat, an inducer of the formation of O_2^- radicals (Kehres *et al.* 2000). Accordingly, the *E. coli mntH* promoter contains separate binding sites for MntR, Fur and OxyR, but not for the main O_2^- sensor SoxS (Fig. 3) (Kehres *et al.* 2000). It was experimentally shown that the H_2O_2-based OxyR-based regulation is dominant over Fur/Fe^{2+} regulation (Kehres *et al.* 2000). We speculate that MntR regulation, in

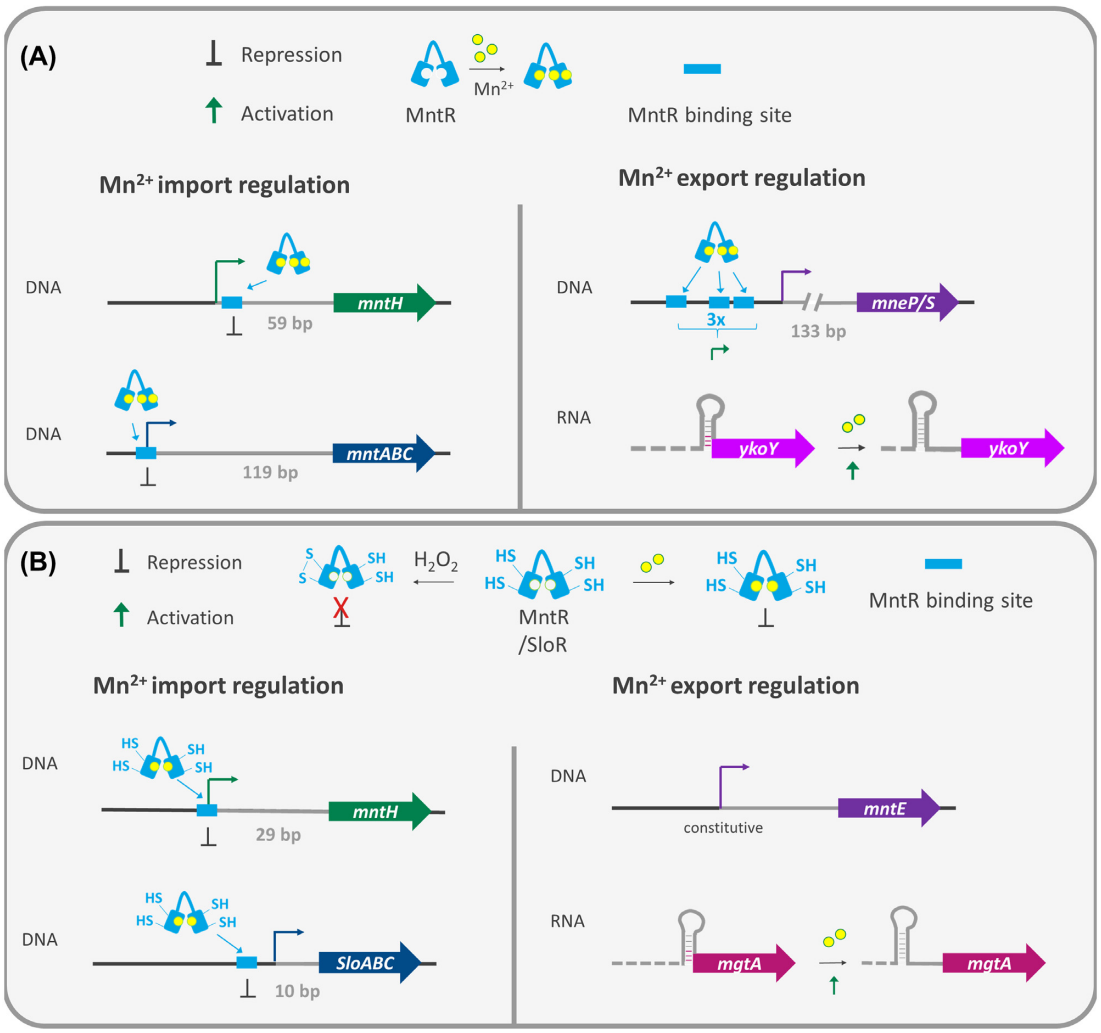

Figure 4. Manganese transport and regulation in *B. subtilis* **(A)** and *Streptococcus oligofermentans* **(B)**, which require both Fe^{2+} and Mn^{2+} and hence are neither iron- nor manganese centric. MntR homodimer is depicted in light blue and its regulation, e.g. metal binding, and H_2O_2 availability, are shown at the top. A thick, light blue line represents the DNA binding site. Genes are depicted in thick arrows and transcription start sites are shown in thin arrows. Repression and activation symbols indicate the influence of the individual transcription factors shown above upon Mn^{2+} addition. The base pair (bp) numbers indicate the distance from the gene to the transcription start site. The 2D structure of folded RNA is depicted with and without Mn^{2+} addition.

its turn, overrules that of OxyR, as MntR binding indicates that a sufficient amount of Mn^{2+} is present in the cells even under oxidative stress conditions.

In *E. coli*, MntH levels are also affected by a small protein named MntS, the expression of which is MntR controlled (Fig. 3) (Waters, Sandoval and Storz 2011). MntS helps MntR to repress *mntH* under moderate to high Mn^{2+} conditions, although it currently remains unknown how MntS exerts this effect (Martin *et al.* 2015).

Another layer of regulation was originally observed in an *S.* Typhimurium mutant lacking *mntR* where addition of Mn^{2+} still resulted in slight repression of the generally increased *mntH* transcription. This led to the discovery of a conserved terminator-like structure in the 5′ Untranslated Region (UTR) of the *mntH* gene, which functions as a Mn^{2+}-responsive riboswitch (Shi, Zhao and Kong 2014). The riboswitch, later on also found in *E. coli* and other enteric bacteria, constitutes a traditional 'OFF' riboswitch with a highly selective affinity for Mn^{2+} (Shi, Zhao and Kong 2014). When Mn^{2+} is bound to the riboswitch, the latter adopts a Rho-independent terminator-like structure that confers premature termination of *mntH* transcription under high Mn^{2+} conditions, thereby fine-tuning *mntH* expression (Shi, Zhao and Kong 2014) (Fig. 3; Table S1, Supporting Information).

Escherichia coli MntR and Fur not only function as repressors of Mn^{2+} import but also as activators for expression of Mn^{2+} exporters. Under nontoxic Mn^{2+}concentrations, gene expression of the MntP exporter is repressed by H-NS, a general histone-like DNA-binding protein in *E. coli* and related bacteria (Bertin *et al.* 2001). However, when intracellular Mn^{2+} levels become excessively high, both MntR and Mn^{2+}-mismetallated Fur bind the *mntP* promoter, thereby alleviating repression by H-NS (Dambach *et al.* 2015). MntP levels in *E. coli* are also regulated by the Mn^{2+}-responsive *yybP–ykoY*-type riboswitch (which is different from the one mentioned earlier for *mntH*) (Waters, Sandoval and Storz 2011; Dambach *et al.* 2015) (Fig. 3). Moreover, MntS overexpression under high Mn^{2+} conditions resulted in increased Mn^{2+} toxicity, suggesting that MntS could be involved in posttranscriptional regulation of MntP as well (Martin *et al.*

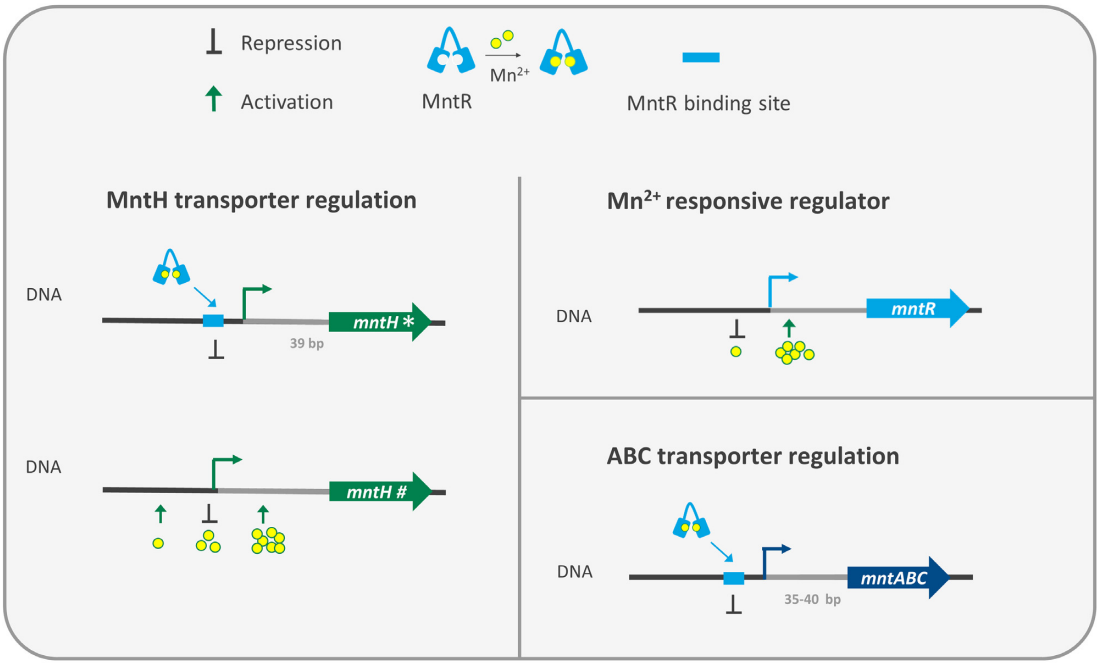

Figure 5. Manganese transport and regulation in the manganese-centric *L. plantarum*. MntR homodimer is depicted in light blue and a thick light blue line represents the DNA binding site. Genes are depicted in thick arrows and transcription start sites are shown in thin arrows. Repression and activation symbols indicate the influence of the individual transcription factors shown above upon manganese access conditions. The base pair (bp) numbers indicate the distance from the gene to the transcription start site. MntH* represents the Mn^{2+} transporter that has been shown to be responsible for competitive exclusion (Siedler *et al.* 2020), while MntH# depicts the regulation of the additional one to two *mntH* genes. No MntR binding site was found in front of *mntH#* or *mntR*, but responsiveness upon Mn^{2+} addition or depletion was shown experimentally. Mn^{2+} concentrations from low to very high are depicted below with the indicated repression or activation symbol to depict the regulation. The responsible regulatory element has not been identified yet. Note that *mntH1* and *mntH2* in the papers of Tong *et al.* (2017a,b) are two subunits of the ABC transporter based on primer sequence and hence depicted and named here as such.

2015). Since these experiments have been performed under nonphysiological conditions—*mntS* is normally not expressed under high Mn^{2+} conditions (Waters, Sandoval and Storz 2011)—further experiments are required to elucidate the physiological role of MntS.

Bacteria utilizing both iron and manganese (most Firmicutes)

Many bacteria, including *B. subtilis* and streptococci among other Firmicutes, require Fe^{2+} along with Mn^{2+} under normal growth conditions. Moreover, in many bacilli, Mn^{2+} is important for sporulation and peptide antibiotic production (Archibald 1986; Jakubovics and Jenkinson 2001). These bacteria have both MntH and MntABC transporters for Mn^{2+} import plus two dedicated exporters MneS and MneP (Fig. 4). *B. subtilis* is believed to have a third uptake system, since an *mntA–mntH* double mutant can still grow when only micromolar levels of Mn^{2+} are provided (Que and Helmann 2002).

Unlike *E. coli*, no Fur-like regulation of Mn^{2+}-related genes takes place in *B. subtilis* (Fig. 4). Rather, Mn^{2+} import and efflux systems are regulated directly by MntR and indirectly by the H_2O_2 response regulator PerR that acts in a complex regulatory circuit involving H_2O_2, Mn^{2+} and Fe^{2+} (Guedon *et al.* 2003). *Bacillus* MntR is among the most extensively studied of all MntR variants. Crystal structures of *B. halodurans* MntR revealed that three instead of the previously assumed two Mn^{2+} molecules are bound in the active dimerized molecule (Lee *et al.* 2019). *B. subtilis mntR* deletion mutants constitutively express both *mntH* and *mntABC*, which increases sensitivity to Mn^{2+} (as well as to Cd^{2+}) and causes Mn^{2+} intoxication (Que and Helmann 2002; Huang *et al.* 2017). Whereas *mntH* and *mntABC* are completely repressed by MntR, *mneP* and *mneS* display constitutive expression, which get 5- to 10-fold enhanced by active (Mn^{2+}-bound) MntR (Huang *et al.* 2017). Binding of MntR to the *mneP/mneS* promoter and subsequent gene activation only occurs at Mn^{2+} levels several folds higher than those required for transcriptional repression of *mntABC* and *mntH* (Huang *et al.* 2017). This is in line with the finding that all three MntR binding boxes in the *mneP* promoter need to be occupied to induce full expression, which entails that more active (Mn^{2+}-bound) MntR molecules are required to activate *mneP* compared with *mntABC* and *mntH* transcription (Huang *et al.* 2017) (Fig. 4).

Streptococcus oligofermentans also utilizes both MntH and MntABC. Mutant generation showed that MntABC rather than MntH is the dominant Mn^{2+} importer in this species (Wang, Tong and Dong 2014). MntABC facilitates protection against H_2O_2 and O_2^-, which are naturally abundant in a common habitat of *S. oligofermentans*, the mammalian oral cavity (Wang, Tong and Dong 2014). MntR, named SloR in *Streptococcus*, binds to and regulates transcription from the *mntABC* promoter (Chen *et al.* 2017). It displays two unique regulatory functions as next to Mn^{2+} it responds to H_2O_2 availability and the redox state of the cell (Chen *et al.* 2017). MntR in *S. oligofermentans* was found to contain three cysteine residues: Cys11 is responsible for DNA binding, Cys123 for metal binding and Cys156 is of unknown function and is less conserved than the other two. H_2O_2 was shown to oxidize all three cysteine residues in MntR, leading to deactivation and even degradation of the regulator. This derepresses *mntABC* transcription and causes an increase in Mn^{2+} uptake, enabling cells to withstand oxidative stress (Chen *et al.* 2017). Note that this form of regulation is comparable to the one seen in *E. coli*,

but instead of having two separate regulators (OxyR and MntR), both roles are now fulfilled by MntR. Contrary to OxyR, the Gram-positive equivalent PerR does not directly bind the *mntH* promoter, and we speculate that this is why MntR took up this function in this Gram-positive organism.

Mn^{2+}-responsive riboswitches present in Gram-positive bacteria differ mechanistically from those in Gram-negatives. In *B. subtilis*, the *yybP–ykoY* riboswitch precedes the genes *yybP* and *ykoY* (Dambach *et al.* 2015). *yybP* and *ykoY* encode a predicted membrane protein of unknown function and encode a TerC family membrane protein, respectively. The role of the Mn^{2+} riboswitch-regulated *yybP* remains elusive, but *ykoY* was recently proposed to constitute a Mn^{2+} exporter, partially overlapping in function with another newly identified exporter YceF (Paruthiyil *et al.* 2019) (Fig. 4; Table S1, Supporting Information). YkoY and YceF might form an extra release valve to back up the two main exporters MneP and MneS, or have a role in metalating secreted or membrane proteins (Paruthiyil *et al.* 2019). Hence, whereas the *yybP–ykoY* riboswitch in *E. coli* regulates the main exporter *mntP*, it seems only of secondary importance in *B. subtilis*.

In *L. lactis* and *Streptococcus pneumoniae*, the *yybP–ykoY* riboswitch controls similar P-type ATPase Mn^{2+} exporters (YoaB in *L. lactis*, MgtA in *S. pneumoniae*) (Price *et al.* 2015; Martin *et al.* 2019). In *S. pneumoniae*, the *yybP–ykoY* riboswitch is located directly upstream of the constitutively expressed Mn^{2+} exporter *mntE* (Martin *et al.* 2019). The constitutive expression of *mntE* might entail that *S. pneumoniae* maintains relatively low intracellular Mn^{2+} levels. Therefore, these species can tolerate high levels of Mn^{2+} unless *mntE* is deleted, resulting in toxic Mn^{2+} accumulation but only when grown under high Mn^{2+} conditions (Rolerson *et al.* 2006; Martin *et al.* 2017). A similar observation was done for *Enterococcus feacalis* (Lam *et al.* 2020)). This effect is worse in an *mntR-mntE* double knockout mutant (Martin *et al.* 2017). Transcription of Mn^{2+} exporters in *S. pneumoniae* and *B. subtilis* only increases under high Mn^{2+} conditions in cells sensitized to Mn^{2+}, for example via deletion of *mntR*. It was therefore suggested that the riboswitch functions as an extra checkpoint to prevent Mn^{2+} toxicity, and, in addition, could be involved in Ca^{2+} efflux (Martin *et al.* 2019). Moreover, magnesium ions (Mg^{2+}) were found to 'pre-fold' the conformation and possibly influence the kinetics of the *yybP–ykoY* riboswitch by priming the affinity of the RNA structure for Mn^{2+} (Sung and Nesbitt 2019).

Altogether, the discovery of different ways of regulating Mn^{2+} transport within Firmicutes hints toward the existence of large interspecies variation in terms of physiological roles that can be attributed to Mn^{2+}.

Manganese-centric bacteria (mostly lactobacilli)

Species within the *Lactobacillus*, *Deinococcus* and *Borrelia* genera have been shown to accumulate remarkably high intracellular levels of Mn^{2+}, which they use for oxidative stress resistance (Archibald and Fridovich 1981), competitive exclusion (Siedler *et al.* 2020), radiation resistance (Daly *et al.* 2004) or circumvention of host Fe^{2+} sequestration (Aguirre *et al.* 2013) (Fig. 1). *Lactiplantibacillus plantarum* does not require Fe^{2+} (Archibald 1983) but instead accumulates as much as 30–35 mM intracellular Mn^{2+} (Archibald and Duong 1984). Some *L. plantarum* strains require at least 2 μM (0.11 mg/L) for growth and show a considerable decrease in biomass and growth rates below this concentration (Hao, Reiske and Wilson 1999; Groot *et al.* 2005). The importance of Mn^{2+} uptake in lactobacilli is indicated by the presence of a notable number of uptake systems, with up to three *mntH* genes as well as *mntABC* (Fig. 5; Table S1, Supporting Information) (Groot *et al.* 2005; Tong *et al.* 2017a,b; Serata, Yasuda and Sako 2018; Siedler *et al.* 2020). Generally, only *mntABC* and one of the *mntH* transporters are mainly expressed under Mn^{2+} starvation conditions in *L. plantarum* (Groot *et al.* 2005). Please note that different studies use different numberings for *mntH* orthologs, depending on genome location.

Mn^{2+} uptake and regulation remain to be studied in lactobacilli, despite the importance of this metal ion in the overall physiology of these bacteria. For instance, MntR has not been studied in any *Lactobacillus*, although binding sites have been identified *in silico* upstream of *mntH1* and *mntABC* (Novichkov *et al.* 2013) (Fig. 5). In contrast, PerR and Fur binding sites were not found in the proximity of *mntH* or *mntABC* (Novichkov *et al.* 2013), indicating that the regulation and role of Mn^{2+} in these highly manganese-centric organisms deviate from the previously discussed bacteria. In a *L. plantarum* mutant lacking *mntH2* (*mntH1* in the *L. paracasei* study of Siedler *et al.* 2020), *mntABC* expression increased and, vice versa, *mntH2* expression increased in an *mntABC* mutant. These results suggest the existence of cross-regulation of *mntABC* and *mntH2* (Groot *et al.* 2005), potentially via MntR. Several studies examined gene expression of Mn^{2+} transporters in *L. plantarum* at different Mn^{2+} concentrations. One group reported on the transcription of five individual *mntH* genes of one strain, named *mntH1–5* (Tong *et al.* 2017a,b), but based on primer sequence, *mntH1* and 2 are instead two subunits of the MntABC transporter. The transcription of all these Mn^{2+} transporter genes was upregulated under Mn^{2+} starvation conditions (0 mg/L) compared with standard growth conditions (16 mg/L or 291 μM), while *mntR* expression was mildly downregulated (Tong *et al.* 2017b). When grown with an excess of 960 mg/L (±17.5 mM) Mn^{2+}, all five genes were downregulated, while *mntR* was upregulated; at an even higher concentration of 5760 mg/L (±105 mM) Mn^{2+}, *mntH4* and *mntH5* were upregulated and *mntR* was even more upregulated (Tong *et al.* 2017a) (Fig. 5). This could either indicate a dual role of MntH4 and MntH5 as both importer and exporter, depending on the Mn^{2+} concentration, or indicate that some other molecule is transported. The finding that importers are expressed at lower Mn^{2+} concentrations is in line with another study that showed that *mntH1* and *mntH2* (*mntH2* here likely is *mntH3* in the papers of Tong *et al.*) are not expressed at 100–300 μM (5.5–16.5 mg/L), but their transcripts can be detected below 3 μM (0.165 mg/L) (*mntH1* and *mntH2*) and 10 μM (0.55 mg/L) (*mntH2*) (Groot *et al.* 2005). No expression of *mntH3* was found under the tested conditions, and based on the genetic context of this gene it was suggested to play a role in Fe^{2+} instead of Mn^{2+} transport (Groot *et al.* 2005).

Whereas the K_m in *L. plantarum* for Mn^{2+} uptake (0.2 μM; 0.011 mg/L) is similar to that of other organisms such as *E. coli* and *B. subtilis* (~0.1 μM; 0.0055 mg/L), the V_{max} (rate of uptake) is much higher, namely 23.8 nmol mg^{-1} of protein min^{-1} (Archibald and Duong 1984). In this light, it would be highly relevant to compare the K_m value of *Bacillus* MntR (4–20 μM; 0.22–1.1 mg/L) with that of *Lactobacillus* MntR variants, which presumably have adopted a different dynamic range due to higher Mn^{2+} requirements. Already in early studies on Mn^{2+} homeostasis in *L. plantarum*, scientists speculated about the mechanism behind the extremely high V_{max} of Mn^{2+} uptake. It was suggested that this was either mediated by a specific transporter with extraordinarily high transport activity, or by a high quantity of transporters (Archibald 1986). Recently, it was shown that the main *mntH* gene involved in Mn^{2+} uptake in both *L. paracasei* and *L. rhamnosus* displays extremely high transcription — similar to the level of highly expressed glycolytic genes (Siedler

et al. 2020). This provides an indication that the large quantity of MntH transporters present within the cell membrane is responsible for a high V_{max}, although it cannot be ruled out that the specified *Lactobacillus* MntH also possesses improved Mn^{2+} uptake activity. However, it does imply that organisms encoding an MntH could have the potential to import high levels of Mn^{2+} by increasing transporter copy numbers.

As MntH-mediated Mn^{2+} transport is proton coupled (Fig. 2), it comes at an indirect energetic cost in relation to proton homeostasis. Leakage of only a fraction of the intracellular Mn^{2+} could therefore be detrimental to growth of organisms with high intracellular Mn^{2+} levels. Indeed, this seems to be avoided as no leakage of intracellular Mn^{2+} was observed when *L. plantarum* was transferred from a Mn^{2+}-rich to a Mn^{2+}-depleted medium (Archibald 1986). Interestingly, no reports of Mn^{2+} exporters in lactobacilli are available, so it is currently unknown whether and when they perform active export of Mn^{2+}. Further research is needed to determine how both import and export are regulated.

Taken together, while some facilitators of Mn^{2+} homeostasis have been studied in lactobacilli, the underlying regulation remains largely unknown. The elucidation of such mechanisms that drive high intracellular Mn^{2+} levels will be crucial in generating an understanding of the rather particular and intriguing Mn^{2+} homeostasis found in lactobacilli and other manganese-centric bacteria. Most studies in lactobacilli have looked at expression under fairly or extremely high Mn^{2+} levels. While this is certainly relevant in many environments, it would be of equal interest to investigate the low end of Mn^{2+} concentrations as they exist in milk and fermented dairy (Siedler *et al.* 2020) or the human gut (Juttukonda and Skaar 2015). Also, the importance and level of Mn^{2+} uptake by lactobacilli under oxidative stress conditions remain to be addressed, as all studies so far did not test mutants under oxidative stress conditions.

Nongenetic factors influencing Mn^{2+} transport and intracellular storage

Apart from genetic regulation, other factors are known to affect Mn^{2+} uptake. As stated earlier, the Mn^{2+} importers MntH and MntABC are both influenced by pH. In *S.* Typhimurium, Mn^{2+} uptake by MntABC was highest at pH 8 and was reduced to 50% of its maximum capacity at around pH 6.7 and to nearly 23% at pH 6.0 (Kehres *et al.* 2002). The affinity of MntH for Mn^{2+} was independent of pH (with K_m not impacted), but the maximum uptake rate increased 3-fold as the pH decreased from 8.2 to 5.5 (Kehres *et al.* 2000). It was suggested that the effect of pH is physicochemical and not regulatory. However, more recently, it was found that pH-dependent regulation is exerted through the *yybP–ykoY* Mn^{2+}-responsive riboswitch in *E. coli* and *B. subtilis*. The level of *yybP–ykoY* transcripts increased under alkaline conditions, suggesting that the riboswitch orchestrates regulation by both responding to pH and Mn^{2+} (Dambach *et al.* 2015).

In both *L. plantarum* and *E. coli*, Mn^{2+} uptake is dependent on energy and the proton gradient, as it is inhibited by carbonyl cyanide m-chlorophenyl hydrazine and dinitrophenol, two compounds that uncouple oxidative phosphorylation and dissipate the proton gradient (Silver, Johnseine and King 1970; Archibald and Duong 1984). Since *L. plantarum* lacks a respiratory chain, it generates a proton gradient via ATP hydrolysis by a proton-pumping ATPase. Treatment of cells with an inhibitor of proton-pumping ATPases (*N,N'*-dicyclohexylcarbodiimide) abolished Mn^{2+} uptake (Archibald and Duong 1984). In contrast, treatment with sodium arsenate, an inhibitor of substrate-level phosphorylation, blocked import only partially (Archibald and Duong 1984).

In *E. coli*, uptake improved with increasing temperatures (Silver, Johnseine and King 1970). In *L. plantarum*, Mn^{2+} uptake increases with temperatures up to 37°C, but already at 4°C significant uptake was seen (Archibald and Duong 1984). The effect of temperature has not been further studied or mentioned.

In *Streptococcus*, Co^{2+} and Fe^{2+} were found to inhibit Mn^{2+} uptake but in concentrations 100-fold larger than Mn^{2+} (Kehres *et al.* 2000). The inhibiting effect of Fe^{2+} on both MntABC and MntH was found to be pH dependent, potentially because the transporters obtain an increased affinity for Fe^{2+} at low pH (Kehres *et al.* 2000). While the inhibition profiles were strikingly similar for all other ions, Zn^{2+} strongly inhibited MntABC but not MntH (Kehres *et al.* 2002). The intricate link between Mn^{2+} homeostasis and that of other transition metals is emphasized in a recent study, in which it was unveiled that dysregulation of Mg^{2+} export suppressed Mn^{2+} and Co^{2+} toxicity in a *B. subtilis mntR–mntH* double mutant (Pi, Wendel and Helmann 2020).

As much as 30–35 mM (1.65-1.93 g/L) Mn^{2+} can accumulate intracellularly in *L. plantarum*. In these cells and other hyperscavengers, Mn^{2+} is proposed to be associated with granules of polyphosphate (Archibald and Fridovich 1982). Up to 80% was estimated to be stored in an inactive form in this way, possibly representing a reservoir of Mn^{2+} for times of scarcity while preventing toxicity, whereas the remaining free Mn^{2+} is readily available for metabolic activity (Archibald and Duong 1984). When cells are transferred from Mn^{2+}-rich to Mn^{2+}-limiting conditions, growth rate becomes affected only after several generations, further supporting the idea of Mn^{2+} storage. Rather than changing the total cellular Mn^{2+} content, *L. plantarum* cells are therefore proposed to ensure homeostasis of the freely available Mn^{2+} portion rather than the total cellular content (Archibald and Duong 1984). In line with this hypothesis, the intracellular Mn^{2+} concentration no longer responds to oxidative stress and does not depend on the extracellular concentration when exceeding 200 μM (11 mg/L). In contrast, phosphate limitation leads to a decrease in intracellular Mn^{2+} even if the extracellular concentrations are >200 μM. As an effect of the tight relationship between Mn^{2+} and polyphosphate granules, phosphate-limited cells are unable to accumulate large amounts of Mn^{2+}. Vice versa, polyphosphate granules were not observed in medium with low Mn^{2+} levels (Archibald and Duong 1984). Nevertheless, Mn^{2+} uptake appeared to continue at neutral pH (6.7) regardless of phosphate as long as 20 mM organic acids were present, which seem to make Mn^{2+} more available to the cells (Archibald and Duong 1984). At pH 5.5, cells could only obtain Mn^{2+} efficiently in the presence of citrate or other tricarboxylic acids, while the acids themselves were not taken up (Archibald and Duong 1984).

Across several bacterial species, the *ppk* gene encoding polyphosphate kinase, which catalyzes polyphosphate synthesis from ATP, seems to play a major role in polyphosphate accumulation capacity and oxidative stress resistance (Gray and Jakob 2015). Surprisingly though, only few studies are available on polyphosphate accumulation in general and in lactobacilli specifically, and its exact roles and mechanisms in stress resistance are only partially understood (Gray and Jakob 2015). In some bacteria such as *E. coli* and *L. casei*, polyphosphate has also been shown to play a role in coping with stress induced by salt, heat or low pH, but a similar function could not be identified for *L. plantarum* (Gray and Jakob 2015; Alcántara *et al.* 2018). Besides its role in intracellular stress resistance, polyphosphate is an important immunomodulating signal molecule in bacteria–host

interactions (a process in which reactive oxygen species [ROS] also play a big role) and has been suggested to play a role in the probiotic effects of lactobacilli (Segawa *et al.* 2011; Gray and Jakob 2015).

In general, effective intracellular storage of Mn^{2+} is of prime importance in organisms with high intracellular Mn^{2+} levels. Its association with various low molecular weight (LMW) complexes beyond polyphosphate granules has been reported in several organisms (Archibald and Fridovich 1982; Barnese *et al.* 2012). These include LMW complexes such as inorganic phosphate species, nitrogenous compounds including short peptides, and nucleotides, in addition to carbonate and organic acids (Archibald and Fridovich 1982; Daly *et al.* 2010; Lisher and Giedroc 2013). More research is needed to identify mechanistic details and species-specific variation in these mechanisms, and it also remains to be identified whether dedicated 'chaperones' exist, or unspecific binding is the main mechanism of 'sequestration'.

ROLES, EFFECTS AND FUNCTIONAL ORIGIN OF Mn^{2+} TRANSPORT AND HOMEOSTASIS IN BACTERIA

Understanding how Mn^{2+} transport and regulation is achieved in iron- or manganese-centric bacteria reveals clues about the primary role and evolution of such systems in these bacterial groups, on which we will elaborate in this section. Mn^{2+} is used for diverse functions in the cells, with increasing complexity dependent on the requirements for this trace metal (Fig. 1).

Mn^{2+} in oxidative stress resistance in iron-centric vs manganese-centric bacteria

In aerobic environments, cells are naturally exposed to small amounts of ROS in the form of superoxide (O_2^-) and hydrogen peroxide (H_2O_2). These are formed from molecular oxygen extracellularly by environmental redox reactions and/or intracellularly via enzyme autoxidation (Horsburgh *et al.* 2002; Seaver and Imlay 2004; Imlay 2008). Most aerobic bacteria keep ROS levels within viable limits through the action of superoxide dismutases (SODs), reductases, peroxidases and catalases. SODs scavenge O_2^- and convert it into O_2 and H_2O_2, a redox reaction known as superoxide dismutation. In most organisms, H_2O_2 is then degraded by catalases and peroxidases that convert it into water and oxygen. Some anaerobic bacteria depend on reductases instead of SODs for O_2^- removal (Horsburgh *et al.* 2002; Imlay 2008; Anjem, Varghese and Imlay 2009). The continuous removal of ROS from the intracellular environment is crucial because Fe^{2+}-loaded enzymes, despite their prevalence in bacteria, are inherently susceptible to Fenton reactions, i.e. oxidation reactions of H_2O_2 with Fe^{2+} resulting in highly reactive hydroxyl radicals that cause oxidation of nearby molecules such as proteins and DNA (Imlay 2008; Anjem, Varghese and Imlay 2009).

ROS levels can rise above those in normal aerobic conditions through a variety of causes. For instance, Lactic Acid Bacteria (LAB) and mammalian macrophages can secrete large amounts of H_2O_2 to fight off competitors or pathogenic bacteria (Imlay 2008). Also, plants and some bacteria can produce redox-cycling compounds that generate both H_2O_2 and O_2^- through the oxidation of redox enzymes and concomitant transfer of electrons to O_2 (Imlay 2008). Furthermore, when exposed to light, H_2O_2 can build up in solutions through photochemistry, while an elevation of H_2O_2 might occur whenever anaerobic sediments containing reduced metal and sulfur compounds get in contact with water in which oxygen molecules are dissolved (Imlay 2008). Under conditions with elevated oxidative stress, the regular ROS scavenging enzymes are insufficient to prevent oxidative damage, and additional response systems get activated. Because Mn^{2+}, unlike Fe^{2+}, does not result in Fenton reactions (Jakubovics and Jenkinson 2001; Anjem, Varghese and Imlay 2009), it lies at the basis of both enzymatic and nonenzymatic oxidative stress responses and thus plays a unique and ubiquitous role in coping with high ROS levels.

A bacterium that employs Mn^{2+} in an enzymatic manner during oxidative stress is for example *E. coli*, which possesses two intracellular SODs. One uses Mn^{2+} as a cofactor (Mn-SOD), while the other utilizes Fe^{2+} (Fe-SOD). Under oxidative stress conditions, the latter switches from a Fe^{2+} to a Mn^{2+} isozyme to reduce Fenton reaction-induced self-damage (Imlay 2008). A nonenzymatic use of Mn^{2+} for coping with oxidative stress can be found in many LAB, which are often devoid of either SODs or catalases (Chen *et al.* 2017). For example, the SOD-negative *L. casei* only survives oxidative stress induced by O_2^- generated by paraquat in the presence of Mn^{2+} (Serata, Yasuda and Sako 2018). The exact mechanism by which Mn^{2+} prevents oxidative damage in a non-SOD way is not yet fully understood. The main hypothesis is that Mn^{2+} bound in LMW complexes functions as ROS scavenger, thereby replacing the function of SOD. For example, Mn^{2+} in complex with physiologically relevant anions such as phosphates, carbonates or organic acids is capable of superoxide dismutation (Archibald and Fridovich 1982; Barnese *et al.* 2012). Depending on the anion associated with Mn^{2+}, either catalytic or noncatalytic dismutation can occur. For instance, while Mn-pyrophosphate displays noncatalytic behavior, Mn-orthophosphate was found to act in a catalytic manner (Barnese *et al.* 2012). Moreover, it has been demonstrated *in vitro* that Mn-LMW complexes are capable of decomposing H_2O_2 (Stadtman, Berlett and Chock 1990; Liochev and Fridovich 2004), the product of superoxide dismutation, although it is currently unknown how this translates to *in vivo* activity. Another mechanism has been proposed for *E. coli*, in which under oxidative stress and high Mn^{2+} uptake conditions, Mn^{2+} outcompetes Fe^{2+} in catalytic sites and thereby prevents site-specific Fenton reactions, but this model has not yet been tested (Imlay 2008). Since lactobacilli accumulate little to no Fe^{2+}, it seems likely that such a mechanism is more specific for iron-centric organisms like *E. coli*, and that the mechanisms of nonenzymatic Mn^{2+}-based oxidative stress resistance differ between manganese- and iron-centric organisms.

Since Mn^{2+} does not lead to Fenton reactions, the exchange of Fe^{2+} by Mn^{2+}-mediated enzymatic reactions in itself contributes to oxidative stress resistance. To achieve this, the uptake of Fe^{2+} from the external milieu should be minimized and instead large amounts of Mn^{2+} have to be taken up to outcompete any trace amounts of Fe^{2+}, as Fe^{2+} typically binds enzymes at such high affinities that it easily replaces other metals, causing mismetallation of Mn^{2+}-dependent enzymes (Gerwien *et al.* 2018). Indeed, lactobacilli are known for their Fe^{2+}-independent lifestyle, displaying an absence of a requirement for Fe^{2+} (Bruyneel, vande Woestyne and Verstraete 1989; Weinberg 1997), with Fe^{2+} seemingly completely excluded from some species; down to 2 Fe^{2+} atoms per cell were detected in *L. plantarum*, compared with 1×10^6 for *E. coli* (Archibald 1983). High Mn^{2+} and low Fe^{2+} levels are thus linked, and the latter is likely either an effect or a prerequisite of high intracellular Mn^{2+} levels.

When Fe^{2+} is almost completely expelled from cells, aerotolerance does not depend on the presence of ROS-scavenging enzymes (enzymatic response), which could account for the lack of SODs in many lactobacilli. The depletion of Fe^{2+} further enables the high H_2O_2 production observed for some lactobacilli, as Fe^{2+}-mediated oxygen radical formation is avoided. However, without Fe^{2+}, several cellular functions cannot be carried out. Among those is aerobic respiration that involves Fe^{2+}-dependent cytochromes (Jakubovics and Jenkinson 2001). In line with this, most LAB do not contain heme compounds and use fermentation rather than aerobic respiration for energy generation. In contrast, bacteria that rely largely on respiration for energy storage, such as *E. coli* and *B. subtilis*, cannot avoid the use of Fe^{2+}, and thus have to utilize ROS scavenging and reducing enzymes such as SODs, reductases and peroxidases. Since respiration yields more energy than fermentation, it seems likely that there are additional advantages of the Mn^{2+}-centric metabolism of lactobacilli, which will be discussed next.

Influence of Mn^{2+} on cell growth and development

Besides oxidative stress resistance, Mn^{2+} plays an important role in certain aspects of bacterial growth and development (Fig. 1). While its presence is a prerequisite for cell growth in manganese-centric organisms, its function is multifaceted in the group that is neither iron- nor manganese centric where it additionally influences virulence, sporulation and biofilm formation. Interestingly, this group can be further subdivided into (i) species with a minimal requirement for Mn^{2+}, for which trace amounts of Mn^{2+} present as 'contamination' in Chemically Defined Medium (CDM) are sufficient and (ii) species with a greater demand for this trace metal that require specific addition of Mn^{2+} to CDM, while also still requiring Fe^{2+} (though less than the iron-centric organisms).

The ovoid-shaped lactic acid bacteria such as enterococci, streptococci and lactococci fall into the first subgroup, and Mn^{2+} has a limited effect on their physiology under normal growth conditions: transcriptomic studies in *S. pneumoniae* grown in Mn^{2+}-limiting conditions revealed changes in only five genes, encoding two subunits of MntABC, a cation efflux system protein and virulence-associated proteins PcpA and PrtA (Ogunniyi *et al.* 2010). Recent studies identified more genes that were differentially regulated in the absence of Mn^{2+} in *S. mutans* and *S. sanguinis* including upregulation of Mn^{2+} uptake systems and changes in genes important for Mn^{2+}-specific cellular functions by Mn^{2+}-dependent enzymes (Kajfasz *et al.* 2020; Puccio *et al.* 2020). To date, eight enzymes are known that employ Mn^{2+} as a cofactor in streptococci (Kuipers *et al.* 2016; Martin *et al.* 2017), including Mn-SOD discussed earlier, and a Mn^{2+}-dependent ribonucleotide reductase RNR NrdEF, required for synthesis of deoxynucleotides from ribonucleotides under aerobic conditions (Makhlynets *et al.* 2014; Rhodes *et al.* 2014). Interestingly, the Mn^{2+}-dependent group of enzymes comprises a set of Mn^{2+}-requiring phosphatases that are crucial for normal cell physiology and development, as well as for virulence. These phosphatases are strongly affected by perturbations in Mn^{2+} homeostasis, which can be achieved by addition of the highly competitive Zn^{2+} transition metal or the metal chelator EDTA, employing mutants devoid of Mn^{2+} import or export, or changing the oxygen availability (Geno *et al.* 2014; Ong, Walker and McEwan 2015; Martin *et al.* 2017; Puccio *et al.* 2020). For instance, a balanced phosphatase activity of PhpP, mediated by Mn^{2+} availability, is crucial for maintaining the phosphorylation status of key cell division proteins in *S. pneumoniae* (Martin *et al.* 2017). In *mntR/mntE* mutant strains, too high Mn^{2+} levels result in hyperactive PhpP phosphatase and aberrant cell division (Martin *et al.* 2017). On the other hand, a shortage of Mn^{2+} was found to render Mn^{2+}-dependent phosphatases inactive. This is likely because the Mn:Zn ratio was altered, leading to the displacement of Mn^{2+} with Zn^{2+} in Mn^{2+}-dependent phosphatases, rendering the latter inactive (Martin *et al.* 2017; Puccio *et al.* 2020). Zn^{2+} is also known to bind the Mn^{2+} solute-binding protein MntC of MntABC with high affinity, essentially blocking uptake and resulting in depletion of Mn^{2+} and altered Mn:Zn ratios (McDevitt *et al.* 2011; Eijkelkamp *et al.* 2014).

Two Mn^{2+}-dependent phosphatases have a major role in streptococcal capsular polysaccharide (CPS) biosynthesis, which is crucial for virulence and biofilm formation (Hardy *et al.* 2001) — namely CpsB and phosphoglucomutase Pgm. Pgm is inhibited by Zn^{2+}, resulting in decreased hyaluronic acid capsule production in *S. pyogenes* (Ray 1967; Ong, Walker and McEwan 2015). CpsB activity increases under increased oxygen availability, i.e. when metabolism switches to a more manganese-centric state (Geno *et al.* 2014). In *S. sanguinis*, expression of *pgm* and *cpsB*, as well as *deoB*, encoding a Mn^{2+}-dependent phosphopentomutase, was found to be downregulated upon Mn^{2+} depletion, while that of *papP*, coding for a nucleotide phosphatase that uses Mn^{2+} as a cofactor and is involved in membrane lipid homeostasis, was upregulated (Puccio *et al.* 2020). CPS production, mediated by CpsB and Pgm, constitutes one of the core virulence factors of streptococci (Hardy *et al.* 2001; Geno *et al.* 2014; Ong, Walker and McEwan 2015; Wu *et al.* 2016), and deletion of *papP* is known to attenuate virulence (Cron *et al.* 2011; Bai *et al.* 2013). Interestingly, *S. aureus* was recently found to express a second metal-independent Pgm under Mn^{2+} limitation, required for growth under such conditions but also necessary for host infection (Radin *et al.* 2019). A clear link exists between Mn^{2+} homeostasis and virulence, which will be further discussed in the next section. Interestingly, also the inorganic pyrophosphatase PpaC relies on Mn^{2+} for optimal function, which has been proposed to have a putative role in the formation of LMW Mn^{2+}-phosphate complexes involved in oxidative stress tolerance (Martin *et al.* 2017). Hence, similar to what is observed in the manganese-centric lactobacilli, phosphate and phosphatases are closely linked to Mn^{2+} homeostasis.

Species such as bacilli and staphylococci belong to the second subgroup. Generally, this group of bacteria is more sensitive to Mn^{2+} restriction, despite possessing high-affinity Mn^{2+} uptake systems. Additionally, Mn^{2+} efflux is under control of MntR in *B. subtilis*, resulting in a highly Mn^{2+}-sensitive *mntR* deletion mutant strain, whereas this is not the case for *mntR* deletion mutants in streptococci, where the exporters are constitutively expressed (Martin *et al.* 2019) (Fig. 4). Moreover, in contrast to the relatively few changes that occur in streptococci, microarray data showed that, in *B. subtilis*, 10% of all of genes were differentially expressed when Mn^{2+} was omitted from the medium (Mhatre *et al.* 2016).

The dependency of *B. subtilis* and *S. aureus* on the Mn^{2+}-dependent isoform of Pgm likely explains why these species require more Mn^{2+}, as this renders Mn^{2+} essential for growth on glucose (Oh and Freese 1976; Radin *et al.* 2019). Recent investigations in our laboratory have shown that *Listeria* spp. have a similar requirement for Mn^{2+} and contain a highly homologous Pgm (van Gijtenbeek *et al.* manuscript in preparation). The role of the Mn^{2+}-dependent Pgm reaches further than its essential roles in glycolysis and CPS synthesis/virulence. Besides being required

for vegetative growth of *B. subtilis*, Mn^{2+} is essential during the early phases of sporulation. This is likely due to Pgm activity, which is also required for sporulation (Oh and Freese 1976; Vasantha and Freese 1979). In addition, it has been suggested that the phosphorylation state of Spo0A, a major regulator during the initiation phase of sporulation, is possibly dependent on Mn^{2+} as well (Mhatre *et al.* 2016). This seems plausible when looking at the strong link between phosphatases and Mn^{2+} as described in the previous paragraph. Also Spo0A-regulated antimicrobial peptide production was reduced in *B. subtilis* grown in medium not supplemented with Mn^{2+} (Mhatre *et al.* 2016). While Mn^{2+} is essential for growth and sporulation in *B. subtilis*, too high concentrations (>15 μM) resulted in reduced growth rate and sporulation in *Bacillus* (Vasantha and Freese 1979; Cheung, Vitkovic and Brown 1982). This might be due to displacement of Fe^{2+} by Mn^{2+} when levels of the latter are too high, evidenced by the upregulation of Fur (Guedon *et al.* 2003) and emphasizing the importance of a strict regulation of Fe^{2+} and Mn^{2+} balance in these organisms.

More recently, it was shown that Mn^{2+} is also required for biofilm formation by *B. subtilis* (Shemesh and Chaia 2013; Mhatre *et al.* 2016), as well as *L. plantarum* (Nozaka *et al.* 2014). Low Mn^{2+} concentrations result in decreased biofilm formation in both species. This seems another way in which Mn^{2+} is involved in stress responses, as biofilm formation and sporulation occur under stress conditions (i.e. stationary phase, which involves nutrient depletion, oxygen stress and toxin production) (Mhatre *et al.* 2016). Interestingly, Mn^{2+} seems to have opposing effects on biofilm formation by iron-centric or manganese- and manganese/iron-centric bacteria. In contrast to the more or fully manganese-centric organisms, biofilm formation in *E. coli* is negatively influenced by the presence of Mn^{2+} (Guo and Lu 2020). Moreover, in uropathogenic *E. coli* low concentrations of Fe^{2+} and Mn^{2+} lead to the formation of biofilm aggregates, which disperse upon an increase in these metal ions, leading to recurrence of the infection (Rowe, Withers and Swift 2010). This is likely related to the success or failure of mammalian hosts to deplete essential ions as a defense mechanism and subsequent virulence development (Rowe, Withers and Swift 2010). Such competition and virulence mechanisms based on Mn^{2+} (and Fe^{2+}) are discussed in the next section.

Nutritional immunity and competitive exclusion: Mn^{2+} in pathogenic and beneficial bacteria as two sides of the same coin

Mn^{2+} and its transport has long been recognized to be involved in streptococcal and enterococcal development toward virulence (Loo *et al.* 2003; McAllister *et al.* 2004; Rolerson *et al.* 2006; Abrantes *et al.* 2013; Kajfasz *et al.* 2020). Although the existence of a clear relationship between Mn^{2+} transport and virulence development by pathogenic bacteria is acknowledged, the role of Mn^{2+} in beneficial interspecies interactions has remained more elusive (Waters 2020). Recently, it is becoming increasingly clear that Mn^{2+} also plays a crucial role in beneficial interactions that occur within bacterial communities, at the bacteria–eukaryotic host interface or in bacteria–plant symbioses. In this section, we briefly explore both harmful and beneficial relationships that exist, sometimes simultaneously, in various niches between two or more species that have been reported to be affected by Mn^{2+} availability. Within this scope, we will also discuss the beneficial role of the hyper-scavenging of Mn^{2+} exerted by highly manganese-centric lactobacilli.

The oral cavity forms an example of a niche where two neighboring organisms use the same Mn^{2+} uptake systems to survive oxidative stress and drive either pathogenesis or prevention thereof. Here, the beneficial commensal *S. oligofermentans* produces large amounts of H_2O_2 to fight off other microorganisms that compete for nutrients, including pathogens. Since *S. oligofermentans* cells do not have a catalase, they rely on high Mn^{2+} uptake to survive oxidative stress imposed by their own H_2O_2 production (Wang, Tong and Dong 2014; Chen *et al.* 2017). Mutant cells devoid of a high-affinity Mn^{2+} transporter showed a 5.7-fold decrease in survival rate, while overexpression of the transporter resulted in a 12-fold increase when challenged with H_2O_2 (Wang, Tong and Dong 2014). Hence, Mn^{2+} import by *S. oligofermentans* is self-beneficiary but at the same time delivers an advantage to the host. In order to overcome the stress induced by H_2O_2 production by *S. oligofermentans*, the cariogenic pathogen *S. mutans*, in its turn, utilizes and requires the same Mn^{2+} importers for its survival, growth and virulence (Paik *et al.* 2003; Rolerson *et al.* 2006; Kajfasz *et al.* 2020). In the same niche, competition for Mn^{2+} to fend off pathogenic microorganisms is also employed by the host itself. The general concept of restricting access to Mn^{2+} and other transition metals such as Zn^{2+} and Fe^{2+} is known as nutritional immunity (Kehl-Fie and Skaar 2010). The specific depletion of Mn^{2+} is realized, among other mechanisms, by the Mn^{2+}-chelating protein calprotectin expressed by healthy epithelial cells in the oral cavity (Gómez *et al.* 1995; Corbin *et al.* 2008).

In other locations of the mammalian body, calprotectin is believed to be secreted by neutrophils that undergo apoptosis in response to microbial infections, for instance in the inflamed gut and at wound sites (Urban *et al.* 2009; Diaz-Ochoa *et al.* 2014). It has been shown to be a critical host element in the defense against a broad range of bacteria (Juttukonda and Skaar 2015). As discussed earlier, nutritional immunity via Mn^{2+} scavenging by mammalian hosts is often paired with production of ROS. It is evident that Mn^{2+} restriction in the presence of ROS leads to a highly toxic environment for bacteria that depend on increasing intracellular Mn^{2+} for oxidative stress survival.

Since manganese-centric lactobacilli are strong competitors for Mn^{2+} and sometimes also produce antibacterial peptides and/or H_2O_2, it is tempting to speculate that these mechanisms, alone or in combination, enable lactobacilli to thrive as beneficial microbiota in the human host. On top of that, the LAB-based beneficial microbiota might help to capture Mn^{2+} in the human gut otherwise available for more harmful bacteria (Knaus *et al.* 2017). Indeed, the high Mn^{2+} uptake by *L. plantarum* has been proposed to be an underlying mechanism for its probiotic properties (Tong *et al.* 2017a). Mn^{2+} restriction and ROS production by the mammalian host can be counteracted by the overexpression of Mn^{2+} importers triggered under Mn^{2+}-limiting conditions. Like *S. mutans*, many pathogens, including various virulent streptococci (Paik *et al.* 2003; Johnston *et al.* 2006; Wichgers Schreur *et al.* 2011) as well as *E. coli* (Sabri *et al.* 2008), *S.* Typhimurium (Diaz-Ochoa *et al.* 2016), *E. faecalis* (Colomer-Winter *et al.* 2018) and *S. aureus* (Kehl-Fie *et al.* 2013), rely on MntABC and/or MntH for survival in the host. Another path to cope with detrimental low Mn^{2+} concentrations has recently been postulated for *S. aureus* and *S. pneumoniae* that have at least one strictly Mn^{2+}-dependent enzyme essential for glycolysis. Under Mn^{2+} limitation, both species shift from glucose utilization to amino acids for energy generation, presumably to reduce the level of Mn^{2+} they require for growth (Ogunniyi *et al.* 2010; Radin *et al.* 2016).

Similar to Mn^{2+} scavenging, mammals have developed a variety of mechanisms to scavenge Fe^{2+} (Johnson and Wessling-Resnick 2012). *Borrelia burgdorferi*, the causative agent of Lyme disease, circumvents this by being fully manganese centric with no requirement for Fe^{2+}, with highly effective Mn^{2+} uptake systems (Posey and Gherardini 2000). Also, *Helicobacter pylori* uses Mn^{2+} instead of Fe^{2+} as a cofactor for several key enzymes to survive in low Fe^{2+} environments (Haley and Gaddy 2015).

Bacterial Mn^{2+} uptake is not only relevant in mammals but has recently also been shown to exert a key function in certain plant symbioses. In young nodules of galegoid-clade legumes, *mntH* and *mntABC* were found to be highly expressed in the plant symbiont *Rhizobium leguminosarum* (Hood *et al.* 2017). Interestingly, both genes appeared essential for nitrogen fixation, and their expression was only upregulated in galegoid-clade and not in phaseloid-clade legumes. It was suggested that this host effect is related to a difference in Mn^{2+} availability and/or ROS concentration in the different plant species (Hood *et al.* 2017).

Another niche where Mn^{2+} scavenging provides a growth advantage for beneficial organisms is found in dairy products, where lactobacilli inhibit growth of yeast and mold during milk-to-yogurt fermentations through Mn^{2+} scavenging—a process known as competitive exclusion (Siedler *et al.* 2020). The analogy between the mechanisms used by lactobacilli for competitive exclusion and mammalian hosts for nutritional immunity is intriguing, as both use homologs of the NRAMP type Mn^{2+} transporter MntH for their defensive Mn^{2+} scavenging.

Functional origin of Mn^{2+} scavenging in manganese-centric lactobacilli

The finding that Mn^{2+} accumulation in lactobacilli has functions beyond basal Mn^{2+} homeostasis and oxidative stress resistance poses an intriguing question with regard to the order at which these functions evolved. One clue might be taken from the analogy between the use of MntH/NRAMP transporters by both lactobacilli and mammals for strategies to outcompete other organisms. It is tempting to speculate that MntH1 in lactobacilli evolved to have a major function in this, as deletion of the *L. paracasei mntH1* only abolished inhibition of yeast and mold related to Mn^{2+} starvation but did not affect normal milk acidification, a proxy for growth of *L. paracasei* (Siedler *et al.* 2020). Contrary to *L. paracasei*, deletion of *mntH* in *B. subtilis* did result in a clear growth defect, as cells did not reach maximum cell densities when provided with <3 μM (0.165 mg/L) Mn^{2+} (Que and Helmann 2002). Since *L. paracasei* has three *mntH* genes, the two other MntH variants, of which at least one is likely to transport Mn^{2+} as evidenced by studies performed in the closely related *L. casei*, (Serata, Yasuda and Sako 2018), could take over a possible growth-supporting role of MntH1. However, neither compensated for the uptake level required to substantially deplete the environment from Mn^{2+}, indicating that MntH1 alone is required and sufficient for competitive exclusion (Siedler *et al.* 2020). This also further indicates a different role and regulation of Mn^{2+} in highly manganese-centric organisms like LAB and iron/manganese-centric organisms like *B. subtilis*, and possibly a different functional evolution of the different systems.

Another hint that MntH1 transporters in manganese-centric lactobacilli might have evolved to serve a distinct role is provided by the conditions under which MntR responds to Mn^{2+} in various bacteria. In *S. oligofermentans*, transcription of Mn^{2+} scavenging systems only becomes derepressed under oxidative stress after three cysteine residues within MntR get oxidized, thereby releasing MntR inhibition (Chen *et al.* 2017). However, MntR in lactobacilli has only one cysteine residue, indicating a difference in the effect of oxidative stress on MntR and hence Mn^{2+} import.

Why many LAB, unlike most other aerotolerant organisms, use nonenzymatic instead of enzymatic mechanisms to cope with oxidative stress and developed a fully manganese-centric metabolism remains a topic of debate. One hypothesis is that bacteria obtained distinct mechanisms to endure the transformation of the world from anaerobic to aerobic after the first photosystems evolved around 2.4–3.8 billion years ago (Khademian and Imlay 2020). Before that, most enzymatic reactions were optimized to function under anaerobic conditions. Iron was the ion of choice for this, since its ferric form is highly soluble and bioavailable under low oxygen conditions and has a highly biologically relevant reduction potential, enabling a multitude of reactions (Khademian and Imlay 2020). One of the results of the rise in atmospheric oxygen was the drop in solubility and hence bioavailability of ferric iron, resulting in a low-iron environment. Moreover, the iron-dependent enzymes became more prone to oxidative damage (Khademian and Imlay 2020). As recently stated by Khademian and Imlay, Fe^{2+} was so important in the metabolism of bacteria that they generally did not evolve in the direction of an iron-free metabolism, but instead developed mechanisms to cope with this new scenario. These include the use of Mn-SOD and Mn^{2+} import for oxidative stress protection, scavenging of Fe^{2+} through the use of siderophores and substituting Fe^{2+} for Mn^{2+} where possible (Khademian and Imlay 2020). Lactobacillaceae are believed to have diverged from staphylococci and bacilli around 1.8 billion years ago (Battistuzzi, Feijao and Hedges 2004; Duar *et al.* 2017) and thus after the rise in atmospheric oxygen. Possibly, they evolved away from Fe^{2+} usage and toward a manganese-centric ion homeostasis to resist oxidative stress, which in turn also provided some of them with an additional growth advantage through competitive exclusion. As such, they constitute one of the few examples of bacteria that have been managed to shift their metabolism to a fully Mn^{2+}-dependent, Fe^{2+}-free one. The intriguing question why apparently only lactobacilli took this solution remains to be answered.

Overall, it is not yet clear in what order the different functions of accumulating extremely high Mn^{2+} levels by certain lactobacilli evolved. Did the accumulation of high Mn^{2+} concentrations result in a competitive advantage after which SOD genes were lost as it became redundant? Or did it originally evolve as a means of dealing with oxidative stress while lacking SOD and did this give the added advantage of competitive exclusion?

CONCLUDING REMARKS AND OUTLOOK

From the current literature, it is clear that Mn^{2+} conveys a variety of long-underestimated roles in bacteria that go beyond being a mere cofactor of enzymes. Overall, the physiological role of Mn^{2+} and the regulation of its homeostasis appear different in bacterial species depending on their level of iron- or manganese centricity. To date, most knowledge on the regulation of its homeostasis and involvement in protein function is derived from iron-centric organisms that require Mn^{2+} in either small amounts or only under certain conditions, such as oxidative stress. However, not much is known about the transporters and the regulation involved in Mn^{2+} homeostasis in manganese-centric bacteria. The high intracellular Mn^{2+} levels in lactobacilli seem to have multifaceted roles beyond oxidative stress resistance, such as competitive exclusion, and enabling both polyphosphate granules and the low Fe^{2+} levels that in turn facilitate H_2O_2 production toward inhibition of other organisms. The negative correlation between Mn^{2+} and Fe^{2+} levels observed

in certain lactobacilli could represent an alternative evolutionary strategy for coping with oxidative stress. Other advantages of high intracellular Mn^{2+} levels such as radiation resistance have been identified and further studies could reveal additional beneficial effects in other organisms, while additional species with high Mn^{2+} concentrations also remain to be identified. Moreover, most studies on Mn^{2+} uptake in lactobacilli have evaluated mutant strains only under standard growth conditions. Characterizing mutant strains of highly manganese-centric organisms under various stress conditions is likely to result in the discovery of yet unidentified functions and regulatory mechanisms of Mn^{2+} homeostasis in both beneficial and pathogenic bacteria.

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SUPPLEMENTARY DATA

Supplementary data are available at *FEMSRE* online.

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