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MN $^{2+}$ AND BACTERIAL PATHOGENESIS

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1. ABSTRACT

Fe^{2+} has traditionally been considered the most important divalent cation involved in host-pathogen interactions. However, recent research indicates a previously unappreciated role for transition metal divalent cations other than Fe^{2+} during infection. Recent studies have identified an absolute requirement for Mn^{2+} in bacterial pathogens that are Fe^{2+}-independent, indicating an important role for Mn^{2+} in pathogenesis. Potential roles for Mn^{2+} in pathogenesis include effects on the detoxification of reactive oxygen intermediates (ROIs), as a cofactor for enzymes involved in intermediary metabolism and signal transduction, and as a stimulus for virulence gene regulation. This review focuses on how these possible roles for Mn^{2+} may affect bacterial pathogenesis and the outcome of an infection.

2. INTRODUCTION

Divalent cations are absolutely required for the survival of all living things. They function in a variety of capacities, from acting as enzymatic co-factors and prosthetic groups to stabilizing macromolecular complexes such as DNA and cell membranes. Until recently Fe^{2+} has been thought to be the most important divalent cation in biological systems. This is due to the numerous cellular functions of Fe^{2+}, including roles as: 1) a transporter of oxygen; 2) a catalyst in electron transport processes; and 3) as a co-factor or prosthetic group for many enzymes of intermediary metabolism (1). The importance of Fe^{2+} in infection was inferred from observations that Fe deprivation is often bacteriostatic, and that extreme Fe deficiency for extended periods is often lethal for bacteria (2, 3). This link was further strengthened by evidence that the incidence of fungal and bacterial infection increases under conditions of Fe overload, while reduced Fe levels are associated with enhanced resistance to infection ((4, 5), and references therein). Additionally, it became apparent that many pathogens had developed sophisticated Fe-acquisition systems to acquire and utilize host Fe, despite extensive efforts by the host to sequester free Fe from microorganisms (5).

Some bacterial pathogens have further subverted the host's strategy of Fe limitation by becoming totally independent of an Fe requirement. *Borrelia burgdorferi*, the etiological agent of Lyme's disease, has no requirement for Fe in its metabolism, apparently having replaced Fe^{2+} as a cofactor with Mn^{2+} (6). In addition, there is evidence that the porcine pathogen *Streptococcus suis*, the probiotic bacterium *Lactobacillus plantarum*, and possibly the human pathogens *Treponema pallidum* and *Mycoplasma pnuemoniae* can grow in the total absence of Fe^{2+} (7-11). Interestingly, when these bacteria no longer require Fe^{2+}, they appear to have acquired an absolute requirement for Mn^{2+} (12). Therefore Mn^{2+} is an essential divalent cation for microorganisms in this most extreme case of Fe^{2+} independence. Further, although information on mammalian Mn-specific binding proteins is lacking, it has been observed that the same proteins responsible for the sequestration of Fe (e.g. transferrin, ferritin, lactoferrin) also bind, and possibly sequester, Mn (13-20). These observations indicate that other divalent cations can also play an important role in infection, and even in Fe^{2+}-dependent pathogens Mn^{2+} probably has a greater role in virulence than previously anticipated.

3. ROLES FOR MN $^{2+}$ IN PATHOGENESIS

There are a number of possible roles that Mn^{2+} may play in pathogenesis. These roles can be split into

three broad classes: 1) the role of Mn^{2+} in the defense against oxidative stress; 2) the role of Mn^{2+} as a cofactor for enzymes involved in intermediary metabolism and cell signaling pathways; and 3) the impact of Mn^{2+} on virulence gene expression. The role of Mn^{2+} in intermediary metabolism (ChapterX) and the defense against reactive oxygen intermediates (ROIs; Chapter Y) is discussed in detail by other authors in this edition. Therefore these subjects are only covered briefly here, and we refer the reader to these chapters for further information on these topics.

3.1. Mn^{2+} and the defense against oxidative stress

3.1.1. MnSOD and KatG (Mn catalase)

The role of inducible oxidative stress responses in the virulence of bacterial pathogens has been the topic of much investigation in the last 20 years. The two enzymes believed to play to largest roles in this response are superoxide dismutase (SOD) and catalase. SODs are responsible for the conversion of superoxide into molecular oxygen and hydrogen peroxide, while catalase converts hydrogen peroxide into water and more molecular oxygen. The role of these bacterial enzymes in pathogenesis has been most extensively studied using the intracellular pathogen *Salmonella enterica* serovar Typhimurium and the systemic murine typhoid model.

S. Typhimurium encodes five SODs, one MnSOD (SodA), an FeSOD (SodB), and three Cu, Zn SODs (SodCI, SodCII and SodCIII). For this bacterium to have five functional SODs encoded within its genome suggests that the defense against oxidative stress is very important for the pathogenicity of this microorganism. It is possible that this level of functional redundancy with respect to these numerous SODs may be to maximize the ability of the bacterium to adapt to oxidative stress in multiple environments with varying divalent cation concentrations. However, of these five SODs only SodCI and SodCII have been definitively implicated in virulence and protection of *S.* Typhimurium from the oxidative burst of phagocytic cells (21-23). The more-recently discovered SodCIII has not been tested for a role in virulence, although preliminary evidence suggests it to be redundant to SodCII (24); the role of SodB in virulence has yet to be investigated. In contrast SodA was found to increase the resistance of *S.* Typhimurium to early killing by macrophages, but was not involved in virulence in the murine typhoid model (25). In addition, although *sodA* is positively regulated by the SoxRS oxidative stress regulatory system (26), deletion of SoxS had no effect on survival of *S.* Typhimurium within macrophages nor on virulence (27).

Interestingly, recent microarray analysis of *S.* Typhimurium gene expression upon infection of a monocyte-macrophage cell line indicates that of the three SODs present on the array (*sodA*, *sodB*, and *sodCI*), *sodB* and *sodCI* are upregulated and *sodA* is downregulated in this system (28). This suggests that there will be different patterns of expression of these five SOD genes, and potential functional overlap between these enzymes in any given environment. However, if these five SODs are functionally redundant experiments must be carried out in strains deleted for the other four SODs to truly determine the impact of each individual SOD on virulence. Therefore the role of MnSOD in *S.* Typhimurium's response to oxidative stress *in vivo* remains unclear.

Other human pathogens have been found to encode MnSODs, including the mucosal pathogen *Moraxiella catarrhalis* (29), the emerging opportunistic pathogen *Aeromonas hydrophila* (30), *Streptococcus pneumoniae* (31), *Haemophilus influenzae* type b (32), and *Pseudomonas aeruginosa* (33). The MnSOD in *P. aeruginosa* is inferred not to play a role in pathogenesis (33). In contrast, MnSOD is important for virulence in *Strep. pneumoniae* and *H. influenzae* murine infection models (31, 32). Therefore, further characterization of these enzymes will yield further insights into the role of MnSODs in pathogenesis.

Three catalase enzymes have been identified in *S.* Typhimurium: KatE and KatG are heme-catalases and specifically require Fe^{2+} for function, while KatN is an Mn catalase. KatN is similar to the Mn catalases of *L. plantarum* and *Thermus* spp., and is regulated at the transcriptional level by RpoS (34, 35). Catalase in general has not been identified as being essential for *in vivo* infection, as *S.* Typhimurium lacking both KatE and KatG was not attenuated in the murine typhoid model (36), and the genes encoding these enzymes are downregulated during infection of macrophages (28). Further, deletion of OxyR, member of another oxidative stress response regulatory system and a regulator of *katG*, had no effect on susceptibility to killing by human neutrophils (37). However, although KatN has not been tested for a role in virulence, there is a correlation between expression of *katN* and an increase in peroxide resistance of *S.* Typhimurium (35). The presence of this novel catalase in *S.* Typhimurium as well as regulation of *katN* by RpoS, a defined virulence-associated regulator (38), indicates that KatN may be involved in the oxidative stress response of *S.* Typhimurium during infection, and therefore Mn catalase may play a role in pathogenesis of this microorganism. To our knowledge, no other Mn catalases have been identified in human bacterial pathogens, which makes the study of KatN intriguing and of definite relevance to the role of Mn^{2+} metalloenzymes in the defense of pathogens against ROIs.

3.1.2. Mn^{2+} as an antioxidant

Non-enzymatic Mn^{2+} may be important for maintaining bacterial viability in aerobic growth conditions. *L. plantarum* is known to incorporate high levels of intracellular Mn^{2+} (approximately 35 mM; (10)) as a protectant in place of an enzymic SOD (10). This intracellular Mn^{2+} appears to act as a catalytic scavenger of reactive oxygen species by associating with anions such as phosphate, or metabolic intermediates including lactate (39-41). Growth defects generated by aerobic metabolism in MnSOD null mutants of *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus* are rescued by Mn^{2+}-supplementation alone (11, 42-44). Non-enzymic Mn^{2+} also appears to play a significant role in ROI tolerance in the

pathogen *Neisseria gonorrhoeae* (45). Therefore this antioxidant activity of non-enzymic Mn^{2+} may represent an important function for intracellular Mn^{2+}. It may seem redundant to have Mn^{2+} complexes functioning to detoxify ROIs in the presence of SOD and catalase enzymes, especially when Mn^{2+}-dependent isoforms of these enzymes exist in a number of bacterial pathogens (discussed above). However Horsburgh *et al*. (11) hypothesize that this may provide a basal level of protection against ROIs, to increase the fitness of a cell by minimizing energy expenditure on the synthesis of a defense regulon until oxidative stress becomes critical. At this stage the OxyR and SoxRS oxidative stress response regulons, may become important for maintaining bacterial viability.

3.2. Mn^{2+} dependent enzymes in signal transduction and intermediary metabolism

Mn may impact virulence through its role as a cofactor or prosthetic group for various bacterial enzymes. Mn metalloenzymes have many diverse functions within bacterial cells (46, 47). In addition, Mn^{2+} and other cations may be interchangeable in the metal biding sites of many proteins (48), including the substitution of Fe^{2+} for Mn^{2+} in the *Strep. mutans* SOD (49). Indeed Mn^{2+} has been found to be a close, but not exact, surrogate for Mg^{2+}, and substitution of Mn^{2+} for certain enzymes has been noted to result in an increase in enzyme efficiency (50). A number of Mn-dependent enzymes involved in signal transduction systems and intermediary metabolism have been identified to date. A detailed discussion of the mechanism of action of these enzymes is beyond the scope of this work, and we direct the reader to a recent extensive review by Kehres and Maguire (51) for further details. However, the potential roles of these enzymes with relation to virulence are discussed below.

3.2.1. Prokaryotic signal transduction systems: PrpA and PrpB

Originally identified in *E. coli*, the protein phosphatases PrpA and PrpB (52) are homologues of the serine/threonine family of type I eukaryotic phosphatases. Interestingly, *prpA* and *prpB* homologues were identified in *S.* Typhimurium, located within the chromosome at sites associated with pathogenicity and horizontal gene transmission (D.G. Kehres, personal communication). Shi and co-workers (53) cloned the *S.* Typhimurium homologues of *prpA* and *prpB*, and identified that the respective proteins are Mn^{2+}-dependent enzymes. In *E. coli* deletion of *prpA* and *prpB* was found to have an effect on the growth rate of the bacterium at permissive temperatures, and PrpA was further characterized to be a heat shock protein (52). PrpA and PrpB are involved in the regulation of expression of *htrA* in *E. coli* (encoding a periplasmic protease involved in degradation of misfolded proteins), most likely via dephosphorylation of CpxA, one of the known regulators of *htrA* transcription (52). 2D gel analyses suggests that nearly 20 different phosphoproteins are substrates for dephosphorylation by PrpA or PrpB (52), potentially indicating a wide-reaching role in bacterial signal transduction pathways.

Interestingly in *S.* Typhimurium PrpA and PrpB have different specific activities for hydrolyzing phosphorylated serine, threonine or tyrosine, as well as different temperature and pH optimas (53). Preliminary studies indicate that mutation of *prpA* or *prpB* markedly alters the peroxide and temperature sensitivity of *S.* Typhimurium (53), and they are upregulated upon infection of macrophages (28) suggesting possible roles in the heat shock response and in the response to oxidative stress. Their different pH optima also indicates that PrpA and PrpB may have different roles *in vivo* depending on the different environments encountered by *S.* Typhimurium during infection. However, the impact of these protein phosphatases on virulence of *S.* Typhimurium has yet to be studied, and homologues remain to be identified in other bacterial pathogens.

3.2.2. Mn^{2+}-metalloenzymes involved in intermediary metabolism

A number of metabolic enzymes appear to either absolutely require Mn^{2+} for function or can tolerate Mn^{2+} as their catalytic divalent cation. Such Mn^{2+}-dependent enzymes in *S.* Typhimurium include 3-phosphoglycerate mutase (involved in glycolysis), aminopeptidase P (peptide cleavage), SpoT (involved in the stringent response) and adenylyl cyclase (involved in the generation of cAMP and the regulation of gene expression primarily associated with carbon source utilization) (53, 12, 54). Enzymes identified to date which can function with Mn^{2+} as the catalytic ion include enzymes involved in nucleic acid degradation, aromatic acid metabolism, amino acid metabolism, sugar metabolism, glycolysis, gluconeogenesis, phospholipid biosynthesis and processing, and central carbon metabolism (12, 51). We refer the reader to Chapter X of this volume, as well as (51) for a detailed description of function of these enzymes and their possible effects on pathogenesis. However, overall their potential role *in vivo* centralizes around their individual roles in intermediary metabolism: if any one of these enzymes was rendered non-functional by the absence of their required Mn^{2+} cation, metabolism of the bacterium would be dramatically impaired. This, in turn, would affect the bacterium's ability to either colonize the host or sustain a productive infection.

3.3. Mn^{2+} and virulence gene regulation

Mn^{2+} may also play a significant role in virulence by affecting the regulation of expression of a number of virulence-associated genes. For example, Mn^{2+} may be involved in the expression of surface proteins involved in colonization and/or virulence of certain microorganisms. In group A *Streptococci*, the glycolytic enzyme enolase is exported by an as-yet-unknown mechanism to the bacterial cell surface (55). Enolase has been found to bind plasminogen with high affinity and thus may be involved in subverting the activity of human plasminogen to their own advantage for tissue invasion (56). It was recently established that enolase expression in *L. plantarum* is repressed by high levels of Mn^{2+} (57), and some have speculated that this is one way in which Mn^{2+} could influence pathogenicity of certain microorganisms (12).

However, a more direct correlation between Mn^{2+} and virulence gene expression lies in the recent identification of a number of Mn^{2+}-responsive regulatory systems in pathogenic microorganisms that appear to regulate virulence genes. The MntR regulatory system has been characterized in both *S.* Typhimurium and *E. coli*, and is involved in expression of the genes encoding the Mn^{2+}-transporters MntH and SitABCD (58, 59). Mn^{2+}-responsive regulatory systems have also been identified in *B. subtilis* (MntR; (60)), *T. pallidum* (TroR; (61)), *Streptococcus gordonii* (ScaR; (9)), and *Staphylococcus aureus* (MntR; (62)). Although the direct impact of deletion of MntR on virulence of these microorganisms has yet to be studied, members of the ScaR and MntR regulons in *Strep. gordonii*, *Staph. aureus*, and *S.* Typhimurium have been found to be virulence-related (9, 62-65). In addition, expression of the *esp* virulence genes of enterohemorrhagic *E. coli* was also found to be dependent on Mn^{2+} concentration, although the regulatory system involved was not defined (66).

The global regulatory protein Fur (ferric uptake regulator) has also been identified to be responsive to Mn^{2+} levels in *E. coli*, *S.* Typhimurium and *Y. pestis* (58, 67, 68). Fur is a regulatory protein that governs expression of approximately 40 genes in *S.* Typhimurium in response to the availability of Fe^{2+}, and to a lesser extent Mn^{2+} (67, 69). Binding of the cation to the Fur apoprotein alters affinity of Fur to bind a "Fur box" in the upstream regulatory region of genes within the Fur regulon, thereby repressing gene expression when levels of Fe^{2+} or Mn^{2+} are high and de-repressing gene expression when levels of Fe^{2+} or Mn^{2+} are low (70). Fur has been implicated in virulence by being involved in the regulation of the *mntH* and *sitABCD* loci in *S.* Typhimurium in response to both Fe^{2+} an Mn^{2+} (58). The role of Fur in virulence is unclear, for although *S.* Typhimurium SL1344 deleted for *fur* is fully virulent (71), other *S.* Typhimurium *fur* strains displayed some level of attenuation (72).

An additional regulatory system, PerR, was originally identified for its role in regulation of genes involved in the inducible peroxide stress response in *B. subtilis* and is the prototype for a group of related peroxide-sensing repressors in a number of bacteria (73). PerR has two metal binding sites per monomer, one which requires a structural Zn^{2+} while the "regulatory" site can contain either Fe^{2+} or Mn^{2+} (74). Expression of *perR* was found to be Mn^{2+}-dependent (75), and in *Staph. aureus* PerR functions as a Mn^{2+}-dependent transcriptional repressor of oxidative stress genes required for full virulence in a skin abscess model of infection (76, 77). Although Fe^{2+} is the metal requirement for PerR in *Strep. pyogenes*, *perR* was found to be required for virulence in a murine air sac model of infection (78). Therefore Mn^{2+}-regulatory systems and their regulons appear to be important for the pathogenesis of a number of different microorganisms.

4. ROLE OF MN^{2+} TRANSPORT SYSTEMS IN PATHOGENESIS

Bacterial pathogens require a number of different divalent cations to maintain normal metabolic processes, as well as their pathogenic nature, *in vivo*. We have discussed above the potential roles for Mn^{2+} during infection. However Mn^{2+} must be able to get into the bacterial cell in order to carry out one or all of its appointed tasks. Further, analysis of the role of Mn^{2+} transport systems can give us further insights into the role of Mn^{2+} in pathogenesis. Studies into bacterial Mn^{2+} transport systems are rapidly expanding, and three main bacterial solute transport systems have been identified to transport Mn^{2+}: the ATP-binding cassette (ABC)-type Mn permeases, the Nramp/MntH family of metal transporters, and the P-type ATPase Mn transporter (57, 79, 80). These transport systems will be discussed briefly here with respect to their potential roles in pathogenesis; as the P-type ATPase has only been identified to date in *L. plantarum* (57) it will not be included in this discussion. For a more detailed description of these transport systems, we refer the reader to Chapter Z of this edition.

The ABC-type family of Mn permeases is the best-defined Mn^{2+} transporter identified in bacterial pathogens to date. Such transporters have been identified in *N. gonorrhoeae, Strep. pneumoniae, Staph. aureus, E. coli,* and *S.* Typhimurium among others, and appear to play a role in the defense against oxidative stress (11, 45, 81, 82). The *S. pneumoniae* transporter, PsaA, was found to be essential for virulence in four different animal models of infection (83). The *S.* Typhimurium Mn permease, SitABCD, was originally identified as a virulence-associated iron transport system encoded within a large pathogenicity island in the *Salmonella* chromosome (65, 84). However, careful study revealed that SitA had a higher affinity for Mn^{2+} than Fe^{2+} , is optimally functional at basic pH (85), and therefore appears to function as a virulence-associated Mn permease in this bacterium (63, 65). ABC-type Mn permeases have also been identified to play a role in the virulence of other pathogenic microorganisms including *Enterococcus faecalis, Strep. mutans* and *Y. pestis* (68, 86, 87).

The second class of bacterial Mn^{2+} transport systems was originally identified based on their homology to the eukaryotic divalent cation transport system, Nramp1 (natural resistance-associated macrophage protein 1). These proteins have been named MntH, for proton (H^+)-dependent Mn transport, and have been identified in many bacteria, including *E. coli, S.* Typhimurium, *P. aeruginosa, Burkholderia cepacia,* and *Mycobacterium tuberculosis* (64, 88, 89). Interestingly, in *S.* Typhimurium the Mn^{2+} transporters MntH and SitABCD have been identified to have markedly different pH optima, as MntH is active at acidic pH, while SitA is most active at a slightly basic pH (85). This suggests that these two transporters are not redundant and may have different roles during infection. However, the role of MntH in virulence of various bacterial pathogens is controversial. To date, no role for *mntH* has been found in the virulence of *M. tuberculosis* (90, 91). Similarly studies in *S.* Typhimurium have suggested either no role, or a minor role of MntH in the murine typhoid model of infection (64, 88), even though *mntH* is expressed in intracellular *S.* Typhimurium (64).

Overall, it is apparent that Mn^{2+} transport systems play a role in bacterial pathogenesis. The role of these transport systems in virulence appears to be intuitive. As divalent cations are necessary for the function of a number of prokaryotic enzymes either involved directly in virulence or in intermediary metabolism, without these cations the bacterium will be compromised for the establishment or maintenance of a progressive infection. This requirement of bacterial Mn^{2+}-transport systems for bacterial pathogens indicates that Mn^{2+} is an important divalent cation for the infectious process.

5. PERSPECTIVE

In summary, there are a number of ways in which Mn could impact the pathogenicity of various microorganisms, including affecting virulence gene expression, affecting the activity of enzymes required for defense against ROIs, and altering the function of enzymes essential for intermediary metabolism. Any and all of these potential roles would impact the ability of a pathogenic microorganism to initiate and sustain a progressive infection. This supports accumulating evidence that divalent cations other than Fe are important *in vivo* for bacterial pathogens, and represents a rapidly expanding and fascinating avenue of research into the field of bacterial pathogenesis.

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