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Manganese Uptake and Streptococcal Virulence

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Abstract

Streptococcal solute-binding proteins (SBPs) associated with ATP-binding cassette transporters gained widespread attention first as ostensible adhesins, next as virulence determinants, and finally as metal ion transporters. In this mini-review, we will examine our current understanding of the cellular roles of these proteins, their contribution to metal ion homeostasis, and their crucial involvement in mediating streptococcal virulence. There are now more than 35 studies that have collected structural, biochemical and/or physiological data on the functions of SBPs across a broad range of bacteria. This offers a wealth of data to clarify the formerly puzzling and contentious findings regarding the metal specificity amongst this group of essential bacterial transporters. In particular we will focus on recent findings related to biological roles for manganese in streptococci. These advances will inform efforts aimed at exploiting the importance of manganese and manganese acquisition for the design of new approaches to combat serious streptococcal diseases.

Keywords

ABC transporter; manganese; zinc; cluster A-I SBP; metal binding; solute-binding protein; Streptococcus; virulence

Manganese acquisition pathways

Prokaryotic Mn²⁺ acquisition occurs via two main types of integral membrane transport pathways, the ATP-binding cassette (ABC) permeases and the natural resistance-associated macrophage protein (NRAMP) transporters (Papp-Wallace and Maguire 2006). Although genes homologous to the ABC permeases and the NRAMPs are present in almost all of the available genome sequences of the streptococci, to date there have been no direct reports of NRAMPs contributing to metal ion homeostasis. This is in stark contrast to many other Gram-positive pathogens that have been shown to employ both types of transporters. For example, in *Staphylococcus aureus*, which encodes both a Mn²⁺-ABC permease (*mntABC*)

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Conflicts of Interest

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and NRAMP (*mntH*), loss of one or both Mn^{2+} transporters has been reported to impact virulence of the pathogen, albeit to different extents (Diep et al. 2014; Horsburgh et al. 2002a; Kehl-Fie et al. 2013). By contrast, lack of the Mn^{2+} -specific ABC permease in streptococci invariably comprises their capacity for mediating disease in animal models (Crump et al. 2014; Janulczyk et al. 2003; McAllister et al. 2004; Wichgers Schreur et al. 2011). In addition to the ABC permeases and the NRAMPs, a third class of transporters has also been reported, the Mn^{2+} -transporting P-type ATPases; however, examples have, so far, been restricted to a limited number of bacterial species and the broader significance of this class of Mn^{2+} -transporter remains to be determined (Groot et al. 2005; Hao et al. 1999). Collectively, the streptococci appear to be reliant on ABC permeases as the primary and essential pathway for Mn^{2+} acquisition.

ABC permease solute-binding proteins

ABC permeases or ABC uptake transporters in prokaryotes are typically comprised of an integral transmembrane domain (TMD) through which the solute passes and a nucleotide-binding domain (NBD) that energizes transport via ATP hydrolysis. Together the TMD and NBD form the ABC transporter component of the permease, which also requires an extra-cytoplasmic solute-binding protein (SBP) component. The ligand specificity of the ABC permease complex is defined by the SBP component. Solute-binding proteins, also referred to as periplasmic binding proteins, membrane-bound receptors, substrate-binding proteins, and substrate-binding domains, are a class of proteins associated with membrane proteins involved in transport of compounds or signal transduction processes. Although SBPs are present in all prokaryotes, the terminology, structure, and localization differ between Gram-positive and Gram-negative bacteria. In Gram-positive bacteria the SBPs were often grouped together as the lipoprotein receptor antigen I (LraI) class of cell-surface receptor proteins due to their historical discovery as streptococcal adhesins (Jenkinson 1994). Whereas SBPs move freely through the periplasm of Gram-negative bacteria, SBPs from Gram-positive bacteria are typically attached to the cell membrane via an N-terminal S-diacylglyceryl-cysteine, such that a short tether exists between the membrane surface and the functional domain of the protein. A number of classification systems for the metal ion-specific SBPs have arisen over the last two decades and include the LraI subclass, the Cluster IX (or 9) grouping and, most recently, the Cluster A-F subgroups (Berntsson et al. 2010; Dintilhac and Claverys 1997; Jenkinson 1994). Although no classification completely addresses the breadth of potential biological functions, the structural-based classification of the SBPs into 6 distinct clusters (A–F), as described by Berntsson and co-workers (2010), provides an excellent framework for our current understanding of SBP structural/functional relationships.

Metal ion-recruiting SBPs are widespread throughout prokaryotic genome sequences, but are absent from any known eukaryotes. The overall fold of all SBPs is well conserved and consists of two structurally conserved domains that are connected by an α -helical linker region. Metal ion binding occurs within a cleft formed by the interface between the two domains. Upon ligand binding the SBP undergoes a conformation change, which stabilizes the closed conformation of the protein such that the two domains are tightly packed together with the substrate buried at the interface. Release of the metal ion is then presumed to be

dependent upon interaction of the closed conformation of the SBP with the ABC transporter, but direct *in vitro* evaluation of this inference remains to be performed. Mutational studies of metal ion-specific ABC permeases have shown that the SBPs play a crucial role in metal ion uptake, as the ABC transporter component alone is incapable of acquiring the cognate ligands in the absence of the SBP. This is consistent with studies of other prokaryotic ABC permeases, in which the ABC transporter component appears to lack the capacity to directly import the metal ion in the absence of the SBP. This is often construed as the ABC transporter lacking internal binding sites for the metal ion (Davidson et al. 2008; Locher 2009). However, to date there have been no direct studies to ascertain whether internal metal ion binding sites are present within the transmembrane channel.

The SBPs have a crucial role in metal ion acquisition, as they recruit the required metal ion directly from the extra-cytoplasmic space and, thereby, effectively define the specificity of the ABC permease. For ABC permeases involved in metal ion uptake, SBPs from the cluster A and D subgroups are utilised (Berntsson et al. 2010). The cluster A group is subdivided into two further sub-clusters; the cluster A-I SBPs, which bind metal ions (iron, zinc, or manganese) via direct interactions with the metal ions; and the cluster A-II SBPs, which bind chelated metals by siderophores like enterobactin, catecholate and hydroxamate or heme. The cluster D subgroup is subdivided into four distinct sub-classes (Berntsson et al. 2010). However, only the D-III and D-IV sub-clusters have been associated with metal ion recruitment. The SBPs in cluster D-III bind tetrahedral oxyanions, such as molybdate and tungstate, while cluster D-IV contains iron-binding proteins. The D-IV SBPs bind either ferrous or ferric iron, usually via direct interactions with protein side-chains, but also sometimes with a synergistic anion. The metal ion coordination used by cluster D-IV SBPs is remarkably similar to mammalian transferrins, and this has led to their description as bacterial transferrins, although this similarity is most likely an example of convergent evolution.

Structure, function and metal specificity of cluster A-I SBPs

Manganese ions are acquired only by SBPs from the cluster A-I subgroup. However, SBPs belonging to this group are also involved in the recruitment of Fe^{2+} and Zn^{2+} ions. The underlying mechanistic basis for metal ion discrimination by structurally similar, yet functionally distinct SBPs is only now being elucidated. To date, there are 38 characterized cluster A-I SBPs, of which 13 have high-resolution structural data. *In vitro* studies of metal ion interaction with these SBPs has shown 3 broad classes of behaviour; Zn^{2+} -specific SBPs, $\text{Mn}^{2+}/\text{Fe}^{2+}$ -binding SBPs, and SBPs that appear to interact with some combination of Mn^{2+} , Fe^{2+} and/or Zn^{2+} ions. Phylogenetic analyses of these cluster A-I SBPs of known function revealed that the proteins divided into 3 distinct groups with a distribution that was consistent with their reported metal binding properties (Fig. 1). The Zn^{2+} -binding SBPs comprise 1 major sub-group, which is separated into 2 distinct clades depending on the whether the SBPs were from Gram-negative or Gram-positive organisms. The sole exception was ZnuA from *Treponema pallidum*, which clustered separately from the Gram-negative ZnuA-like proteins. All Zn^{2+} binding cluster A-I SBPs from Gram-negative organisms, including ZnuA from *T. pallidum*, were found to possess a histidine-rich loop region, albeit of highly varying lengths. This loop region is believed to facilitate recruitment of Zn^{2+} to the

primary binding site of the protein (Falconi et al. 2011). The primary metal binding site of Zn^{2+} -specific SBPs is, in general, formed by three conserved histidine residues, which contribute $\text{N}\epsilon 2$ atoms, and a carboxylate group, from an aspartic or glutamic acid. Exceptions to this rule, as shown in the structures of *Salmonella enterica* and *Escherichia coli* ZnuA, indicate that variations in Zn^{2+} -binding modalities do occur, but the physiological significance of these differences remains to be elucidated. High-resolution structures of the Zn^{2+} -bound SBPs show that the metal ion typically demonstrates four-coordinate binding with mean bond lengths of 1.9 to 2.3 Å between the metal ion and coordinating side chain residues (Table 1). However, while Zn^{2+} has a preference for four-coordinate binding and “soft” ligands, like the amide group of histidine residues, this is not the case for Mn^{2+} and Fe^{2+} . The composition of the metal ion binding site and near ideal bond lengths for Zn^{2+} coordination is consistent with a preference for Zn^{2+} binding over other metal ions. Indeed, a recent study of AdcA from *Streptococcus pneumoniae* showed that the protein had negligible interaction with first-row transition metal ions other than Zn^{2+} (Plumtre et al. 2014) and, to date, there have been no reports of ligands other than Zn^{2+} interacting with SBPs from this subgroup.

The second major group of SBPs was comprised of proteins with established roles predominantly in Mn^{2+} binding, but also potentially in Fe^{2+} binding (Fig. 1). Although Fe^{2+} can interact with SBPs from the cluster A-I subgroup *in vitro*, the *in vivo* significance of iron recruitment via these SBPs is less clear (Crump et al. 2014; Sun et al. 2009). Unlike Mn, which occurs exclusively as the divalent cation when free in biological systems, iron primarily occurs in the Fe^{3+} form in complex with other molecules, such as heme, and is typically acquired via cluster A-II or D-IV SBPs and their associated permeases (Anderson et al. 2004; Brown et al. 2001; Cheng et al. 2013). Nevertheless, Fe^{2+} acquisition cannot be completely excluded as certain organisms, e.g. *S. sanguinis* and *S. pyogenes*, may encounter Fe^{2+} under anaerobic or microaerobic conditions.

The phylogenetic analyses showed that within this group of proteins, the $\text{Mn}^{2+}/\text{Fe}^{2+}$ -binding cluster A-I SBPs from Gram-negative organisms formed a relatively tight phylogenetic clade of 5 proteins, while their Gram-positive homologs displayed a greater level of sequence variation. Despite this, the $\text{Mn}^{2+}/\text{Fe}^{2+}$ -binding cluster A-I SBPs from the Gram-positive organisms clustered in the same part of the phylogenetic tree. With respect to metal ion interaction, in contrast to Zn^{2+} , the incomplete 3d shells of Mn^{2+} and Fe^{2+} result in these ions showing a tendency to favor “hard” ligands, such as oxygen atoms from the carboxylate groups of aspartic or glutamic acid side chains. Both Mn^{2+} and Fe^{2+} also have, in general, a preference for a ligand coordination number of 6. The amino acid side chains of 2 histidine residues and 2 carboxylate-containing residues, i.e. aspartic and/or glutamic acid, typically form the metal ion binding sites for members of this subgroup. The carboxylate groups can contribute one (monodentate) or two (bidentate) ligands each to metal ion coordination such that coordination numbers between 4 and 6 are possible. High-resolution structures of the metal-bound SBPs from this group show that the bound metal ions typically demonstrate 6-coordinate binding with mean bond lengths, between the metal ion and coordinating side chain residues, of 2.0 to 2.4 Å for the histidine residues and 1.7 to 2.8 Å for the carboxylate residues (Table 2). Importantly, this subgroup of SBPs has also provided significant insights into the underlying basis of metal ion discrimination and selectivity. The archetypal Mn^{2+} -

specific SBP, PsaA from *S. pneumoniae*, was the first SBP resolved by high-resolution structural analysis (Lawrence et al. 1998). However, that structure revealed a Zn^{2+} ion, not Mn^{2+} at the metal-binding site despite the physiological role in only mediating Mn^{2+} uptake. Recent studies have shown that the inherent flexibility within protein structure results in the metal-binding site of PsaA being incapable of steric selection of solely Mn^{2+} ions. As a consequence, PsaA has the potential to interact with any first-row divalent transition metal ion (Couñago et al. 2014). Despite this promiscuity of metal ion binding, at a functional level the Psa permease has been shown to be specific for Mn^{2+} uptake (McAllister et al. 2004; McDevitt et al. 2011). This apparent incongruence between metal ion binding and ABC permease specificity was resolved with the identification of the role of the linking helix in coupling protein conformational change to metal ion coordination at the binding site. In PsaA, the smaller ionic radius of Zn^{2+} and its preference for 4 coordinate binding result in the helical linker undergoing greater distortion to coordinate the metal ion at the binding site. By contrast, the larger ionic radius of Mn^{2+} and its requirement for 6 coordinate binding in PsaA results in less distortion of the linking helix during metal ion binding and a less stable metal-protein complex (Couñago et al. 2014). While transition from the metal-free, open conformation of the SBP to the closed, metal-bound state is an essential step for recognition by the ABC transporter, the stability of the metal-protein complexes appears to be crucial for facilitating release of the metal ions. Although direct transport assays within the field are lacking, the apparent irreversible binding of Zn^{2+} by SBPs from this subgroup, in contrast to Mn^{2+} or Fe^{2+} , strongly implicate this as a major aspect of the mechanism by which the ABC permeases achieve functional specificity for their metal ion cargo, despite the inability to the SBPs to prevent other metal ions from binding. Collectively, this appears to be a novel mechanism by which to prevent mistranslocation of Zn^{2+} ions via the $\text{Mn}^{2+}/\text{Fe}^{2+}$ -specific permeases.

The third group of SBPs consisted of proteins that have the ability to bind Zn^{2+} , Mn^{2+} and potentially Fe^{2+} (Fig. 1). This sub-group of cluster A-I SBPs are primarily from organisms associated with sexually transmissible infections (STI). These proteins formed a separate clade positioned between the groups of Zn^{2+} - and $\text{Mn}^{2+}/\text{Fe}^{2+}$ -binding SBPs. This group includes MntC from *Neisseria gonorrhoeae*, TroA from *T. pallidum* and YtgA from *Chlamydia trachomatis*. In addition to these STI-causing organisms, MntA from *Bacillus subtilis* and TroA from *Streptococcus suis* were found to cluster within this group of $\text{Mn}^{2+}/\text{Fe}^{2+}/\text{Zn}^{2+}$ -binding SBPs. However, the physiological role of proteins clustered within this sub-group are the least well established. Notably, the precise physiological role of *T. pallidum* TroA remains unclear, while it has been implicated in potentially mediating Mn^{2+} , Fe^{2+} and/or Zn^{2+} uptake (Desrosiers et al. 2007; Hazlett et al. 2003). Similarly, YtgA from *Chlamydia trachomatis* has been associated with Fe^{2+} , Fe^{3+} and/or Zn^{2+} transport processes, although a direct physiological role has not been shown (Akers et al. 2011; Miller et al. 2009; Thompson et al. 2012). One of the very few functional roles that have been definitively established within this subgroup is for TroA from *S. suis* and its role in physiological Mn^{2+} acquisition (Wichgers Schreur et al. 2011). However, *S. suis* TroA has also been shown to bind Zn^{2+} , but whether this is an irreversible interaction, as observed for many members of the $\text{Mn}^{2+}/\text{Fe}^{2+}$ subgroup, has not been established. An examination of the two available Zn^{2+} -bound crystal structures of representatives from this subgroup indicates

that proteins of this subgroup employ a different metal ion binding modality (Table 3) in comparison to the Zn^{2+} -specific SBPs and the $\text{Mn}^{2+}/\text{Fe}^{2+}$ -specific SBPs (Lee et al. 1999; Zheng et al. 2011). The significance of these crystal structures and, by extension, the significance of the observed binding mechanism in the absence of other metal-bound forms, remains to be ascertained. Collectively, while SBPs assigned to the other two sub-groups have well-defined functional roles, either Zn^{2+} -specific or primarily Mn^{2+} -specific, members of this last subgroup show a greater heterogeneity of potential metal ion uptake roles. More studies are clearly required to ascertain whether this subgroup is another group of predominantly Mn^{2+} -recruiting SBPs, or whether it reflects a subset of more specialised niche-specific metal ion recruiting SBPs. Defining or clarifying the physiological metal ion specificity of these SBPs will require a combination of *in vitro* protein-metal ion binding studies with a complementary assessment of their role in cellular metal homeostasis. This latter determination has been achieved via a number of different strategies in the literature including assessment of the intracellular metal ion concentrations in SBP gene deletion strains, quantitating mRNA levels of the SBP under defined metal ion restricted conditions, and examination of niche-specific factors that can attenuate the *in vivo* virulence of SBP mutant strains.

Cluster A-I Mn^{2+} -transporting SBPs as streptococcal virulence factors

The $\text{Mn}^{2+}/\text{Fe}^{2+}$ subgroup of cluster A-I SBPs are currently of most interest due to their pivotal contribution to streptococcal virulence (Table 4). It was shown that mutation of *fimA* in *S. parasanguinis* (Burnette-Curley et al. 1995) or *sloC* in *S. mutans* (Kitten et al. 2000; Paik et al. 2003) virtually eliminated infective endocarditis virulence in a rat model. A study performed in *S. sanguinis* examined the effect of mutating each of 52 predicted lipoproteins on endocarditis virulence in a rabbit model (Das et al. 2009). The *ssaB* mutant was the only strain to exhibit normal *in vitro* growth but reduced virulence (Table 4). In subsequent experiments the mutant was recovered in numbers 4 to 5 logs lower than the parent strain (Crump et al. 2014; Das et al. 2009). Deletion of the *mtsABC* operon of *S. pyogenes* completely eliminated virulence at two of three inoculum levels in a mouse skin infection model (Janulczyk et al. 2003). The *mtuA* mutant of *S. uberis* was unable to infect dairy cows after intramammary inoculation whereas the parent strain was uniformly infectious, causing a marked inflammatory response and overt mastitis (Smith et al. 2003). A *troA* mutant of *S. suis* caused no mortality or significant morbidity in a murine intraperitoneal (IP) infection model in contrast to the parent strain, which caused severe infection or death in 60% of the mice. Likewise, mutant bacteria were not recovered from the liver, spleen, or brain of mice infected with the *troA* mutant whereas $\sim 10^8$ CFU/g of the parent strain was recovered from all three tissues (Wichgers Schreur et al. 2011).

PsaA from *S. pneumoniae* has received the most scrutiny of any Mn^{2+} -specific SBP with respect to bacterial virulence. In one report, *psaA* mutants caused no death in 14 d intranasal infection experiments employing BALB/c and QS mice, whereas the D39 parent produced a median survival time of ~ 3 d in both mouse strains (Berry and Paton 1996). IP infection of the QS mice resulted in a median survival time of ~ 1 d with D39, whereas a *psaA* mutant caused no death up to 14 d. IP infection of BALB/c mice produced a much smaller survival difference; however, bacteria recovered from the *psaA* mutant mice were found to have an

intact *psaA* gene, indicating that there had been selection for loss of the integrated plasmid that had disrupted *psaA*. In a later study, a non-revertable deletion mutant of *psaA* killed no BALB/c mice in a 21 d IP infection study whereas the median survival time for D39 was ~3 d (McAllister et al. 2004). Nasopharyngeal colonization of CD-1 mice was also examined by the same group; no *psaA* mutant bacteria were recovered at 96 h compared to $>10^3$ D39 CFU/nasopharynx for D39. The most extensive virulence study was performed by Marra et al. (Marra et al. 2002). In a respiratory tract infection model employing CD-1 mice, D39 recovery was $\sim 10^7$ CFU/lung pair and $\sim 10^6$ CFU/ml of blood whereas a *psaA* deletion mutant was not recovered from either tissue. When the mice were injected IP rather than intranasally, the LD₅₀ for the *psaA* mutant was $>10^4$ -fold higher than that of D39. All D39-infected mice died from infection by day 3, whereas none of the *psaA* mutant mice died or showed any signs of illness one week post-infection. Lastly, both strains were examined in a gerbil middle ear infection model. D39 reached levels of $>10^7$ CFU/ml of middle ear exudate and disseminated into the bloodstream, where counts reached $\sim 10^5$ CFU/ml at 4 d, whereas the *psaA* deletion mutant was not recovered from the middle ear or the blood of any of the animals. Finally, a *psaBC* deletion mutant of TIGR4 was compared to the parent strain in an intravenous infection model employing CBA/N mice. The TIGR4-infected mice exhibited a median time to death or moribundity of ~4 d, with no mouse surviving past day 7, whereas none of 10 mice infected with the *psaBC* mutant died during the 21 d experiment (Johnston et al. 2004). This result suggests that the importance of PsaA for virulence is not specific to strain D39. The combined data indicate SBPs of the Mn²⁺/Fe²⁺ subgroup are critical for virulence in a range of streptococcal species that cause a wide variety of invasive diseases. Indeed, there appears to be no published report in which virulence for invasive disease has not been sharply reduced or eliminated by mutation of a gene encoding a streptococcal Mn²⁺-specific SBP.

Do Mn²⁺-specific SBPs act as adhesins?

Cluster A-I SBPs first came under study when they were identified as potential adhesins in oral streptococci. The FimA protein was originally believed to be the structural subunit of *S. parasanguinis* fimbriae (Oligino and Fives-Taylor 1993). Both anti-fimbrial antiserum and purified FimA blocked adhesion of *S. parasanguinis* to the model tooth surface saliva-coated hydroxyapatite (SHA) (Oligino and Fives-Taylor 1993). Similarly, the SsaB protein from *S. sanguinis* was found to inhibit adhesion of cells to SHA (Ganeshkumar et al. 1988). Independently, a ~38 kDa protein from the related oral species *S. gordonii* was identified as a potential adhesin mediating coaggregation with *Actinomyces neaslundii* (Kolenbrander and Andersen 1990). It was not until six years after the DNA sequence of the first cluster A-I SBP gene (*fimA* of *S. parasanguinis*) was reported (Fenno et al. 1989), that a streptococcal SBP was first associated with virulence (Burnette-Curley et al. 1995). In this study, *fimA* mutants of *S. parasanguinis* were found to be markedly deficient for infective endocarditis virulence in an animal model. The *fimA* mutants also exhibited reduced adherence to fibrin, a major component of heart valve “vegetations” that are colonized in the establishment of infective endocarditis. Given this history, it came as no surprise when mutation of the *psaA* gene encoding the homologous SBP from *S. pneumoniae* resulted not only in reduced

virulence, but also reduced adherence, in this case to A549 human lung carcinoma cells (Berry and Paton 1996).

The first suggestion that the chief function of this SBP family might be Mn^{2+} transport rather than adhesion came from the finding that a homologous cyanobacterial protein was a component of an ABC Mn^{2+} -transport system (Bartsevich and Pakrasi 1995). Further support came from the demonstration that a *psaA* mutant of *S. pneumoniae* exhibited a growth defect that could be complemented by Mn^{2+} (Dintilhac et al. 1997). When *fimA* and *scaA* mutants were found to retain SHA binding (Froeliger and Fives-Taylor 2000) and coaggregation activity (Kolenbrander et al. 1998), respectively, a role for cluster A-I SBPs as oral adhesins became doubtful. A study performed with *S. mutans* established that a strain lacking the SloA NBD component of the transport system (Table 4), yet still expressing the SloC SBP was just as deficient for endocarditis virulence as the *sloC* mutant (Paik et al. 2003). This result indicated that loss of transport activity was sufficient to explain the reduced virulence of the SBP mutant. Recently, an adhesin function for *S. sanguinis* SsaB in endocarditis causation was not supported when an animal study demonstrated that an *ssaB* mutant adhered to cardiac vegetations with the same efficiency as the parent strain (Crump et al. 2014).

Controversy remains as to whether PsaA from *S. pneumoniae* acts as an adhesin. On the one hand, strong support for an adhesin function has been voiced by some investigators, citing studies demonstrating reduced adherence of *psaA* mutants to eukaryotic cells, cell-surface accessibility of PsaA in certain strains, and evidence for specific binding of purified PsaA to a specific eukaryotic cell receptor (Rajam et al. 2008). On the other hand, other investigators have argued against cell-surface accessibility—a prerequisite for adhesin activity—based on the protein's length being less than would be needed to traverse the cell wall and on experiments indicating interference of the cell wall and capsule with binding of anti-PsaA antibodies to whole cells of certain other strains (Johnston et al. 2004). Moreover, as with *S. mutans*, investigators created mutants lacking the PsaB and/or PsaC transport components but still expressing PsaA, and found that these mutants had virulence defects comparable to that of a *psaA* mutant in multiple infection models (Johnston et al. 2004; Marra et al. 2002; McAllister et al. 2004). In addition, Johnston et al. (Johnston et al. 2004) found that a *psaBC* deletion mutant exhibited the same adherence defect to D562 nasopharyngeal cells as an isogenic *psaA* mutant. Lastly, it remains unclear as to how the primary physiological function of the SBP, delivery of extracellular Mn^{2+} to the PsaBC components, could be fulfilled if PsaA were adhered to eukaryotic cells. Thus, while an adhesin function for PsaA cannot be completely excluded, it is clear that the reduced virulence of a *psaA* mutant can be explained by loss of the capacity to acquire Mn^{2+} from the extracellular environment.

Biological functions of Mn^{2+} in streptococci

Apart from adhesion, three functions that have been ascribed to Mn^{2+} -specific cluster A-I SBPs: metal binding (and by extension transport), virulence, and resistance to oxidative stress. The first two functions have been addressed above. Concerning the last, a common finding has been that SBP mutants grow less well in Mn-limited media in the presence of oxygen (Crump et al. 2014; Jakubovics et al. 2002; Janulczyk et al. 2003; Johnston et al.

2004; Kolenbrander et al. 1998; Marra et al. 2002; McAllister et al. 2004; Paik et al. 2003; Tseng et al. 2002) and show increased sensitivity to reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (H_2O_2) (Janulczyk et al. 2003; Johnston et al. 2004; McAllister et al. 2004; Tseng et al. 2002). These defects have been corrected by addition of Mn^{2+} , thus linking Mn^{2+} deficiency to oxygen sensitivity (Table 4). Further, there is good evidence in animal models of streptococcal (McDevitt et al. 2011) and staphylococcal (Damo et al. 2013; Kehl-Fie et al. 2013) invasive diseases that Mn^{2+} limitation sharply reduces virulence. Given these findings, it is reasonable to suggest that it is the reduced resistance to oxidative stress that causes the reduced virulence of SBP mutants. How, then, does Mn^{2+} contribute to oxygen tolerance? This question has not been definitively answered, although numerous possibilities have been suggested (Horsburgh et al. 2002b; Jakubovics and Jenkinson 2001; Kehres and Maguire 2003; Lisher and Giedroc 2013; Papp-Wallace and Maguire 2006). Although it is not possible to cover this topic comprehensively in this review, several mechanisms by which Mn^{2+} may contribute to oxygen tolerance are discussed below.

Manganese-cofactored superoxide dismutase

The biological role most commonly ascribed to first-row transition metal ions including Mn^{2+} is protein binding to impart structure, participate in catalysis, or both (Lisher and Giedroc 2013). If the most important role of Mn^{2+} in oxidative stress is serving as an enzyme cofactor, then the Mn^{2+} -cofactored enzyme must be needed primarily in the presence of oxygen. This would be the case for proteins involved in oxidative stress resistance, exemplified by the Mn-type superoxide dismutase, SodA, which is ubiquitous among streptococci (Bishop et al. 2009). It should be noted that although Mn^{2+} is clearly associated with both *sodA* expression and function in the streptococci, the physiological cofactor remains less well understood. To date, there have been several reports of streptococcal SodA proteins containing cambialistic (mixed Fe/Mn) cofactors (De Vendittis et al. 2010; De Vendittis et al. 2012; Eijkelkamp et al. 2014). SodA eliminates the toxic oxygen product superoxide, which can be generated adventitiously from oxidative metabolism or by host phagocytic cells as a defense mechanism (Imlay 2008). Mutation of *sodA* in *S. gordonii* (Jakubovics et al. 2002), *S. mutans* (Nakayama 1992), *S. pneumoniae* (Eijkelkamp et al. 2014; Yesilkaya et al. 2000), *S. agalactiae* (Poyart et al. 2001), *S. pyogenes* (Gibson and Caparon 1996), and *S. sanguinis* (Crump et al. 2014) has been shown to impede growth in the presence of oxygen. Several of these studies established that *sodA* mutants also have increased sensitivity to paraquat (Crump et al. 2014; Eijkelkamp et al. 2014; Gibson and Caparon 1996; Yesilkaya et al. 2000), which induces intracellular superoxide production. Moreover, SBP mutants of *S. pneumoniae* (Tseng et al. 2002), *S. pyogenes* (Janulczyk et al. 2003), and *S. sanguinis* (Crump et al. 2014) exhibit reduced SodA activity. Finally, *sodA* mutants of *S. pneumoniae* (Yesilkaya et al. 2000), *S. agalactiae* (Poyart et al. 2001), and *S. sanguinis* (Crump et al. 2014) demonstrate reduced systemic virulence. These findings could be interpreted as suggesting that the sole function of Mn^{2+} in oxygen tolerance and virulence is to serve as a cofactor for SodA; however, a study done in *S. gordonii* found that mutation of *sodA* had less effect on ROS resistance than did mutation of the Mn transporter, ScaC (Jakubovics et al. 2002). Also, although the mutants were not examined together or by the same group, the decrease in virulence of the *S.*

pneumoniae *sodA* mutant (Yesilkaya et al. 2000) was less than typically reported for *psaA* mutants (Johnston et al. 2004; Marra et al. 2002; McAllister et al. 2004), and the *sodA* mutant was recovered in high numbers from tissue and blood. Finally, isogenic *S. sanguinis* mutants of *sodA* and *ssaB* were tested for virulence in a rabbit model of endocarditis and for growth in rabbit serum at oxygen levels found in arterial blood, mimicking the environment of the infected heart valve. In both instances, the *ssaB* mutant exhibited a significantly greater defect than the *sodA* mutant, despite the *ssaB* mutant possessing far greater superoxide dismutase activity than the *sodA* mutant (Crump et al. 2014).

The above results suggest that reduced SodA activity is not sufficient to explain the reduced oxygen resistance or virulence of SBP mutants; however, it is important, and may well be a necessary component. Addition of tiron, a superoxide scavenger (Krishna et al. 1992), to *S. pneumoniae* strain TIGR4 cultured in a low-Mn²⁺ medium restored growth to the same extent as added Mn²⁺ (Johnston et al. 2004). Similarly, addition of tiron to an *ssaB* mutant cultured in rabbit serum restored growth to wild-type levels (Crump et al. 2014). One complication of these studies is that tiron also chelates iron (Krishna et al. 1992). In the *S. pneumoniae* study, addition of Fe²⁺ to the low-Mn medium was shown to increase oxygen sensitivity; thus, chelation of Fe may be required in combination with superoxide scavenging for full growth restoration.

Manganese-cofactored ribonucleotide reductase

Streptococci, like many other bacterial species, possess two ribonucleotide reductases (RNRs) that provide the dNTPs essential for DNA replication and repair (Gon and Beckwith 2006). The class III RNR (NrdDG) is inactivated by O₂ and, thus, provides dNTPs only under anaerobic conditions. The class Ib RNR (NrdEF) requires O₂ for cofactor formation and, thus, provides dNTPs only under aerobic conditions. For many years, it was thought that class Ib RNRs possessed an Fe³⁺₂ cofactor. This conclusion was based in part on metal analysis of class Ib RNRs heterologously expressed in *E. coli* and in part on the ability of class Ib enzymes to self-assemble an active enzyme with an Fe³⁺₂-tyrosyl radical (Y•) cofactor *in vitro*. Recently, class Ib RNRs from several bacterial species were shown to contain a Mn³⁺₂-Y• cofactor (Stubbe and Cotruvo Jr 2011). The breakthrough was due to isolation of class Ib RNRs from their native source and to the characterization of NrdI, an unusual flavodoxin that was essential for oxidizing the Mn²⁺₂ form of NrdF to the active Mn³⁺₂-Y• cofactor *in vitro*.

S. sanguinis NrdEF was recently analyzed for *in vitro* activity in the presence of Fe²⁺ and Mn²⁺. NrdEF was shown to function *in vitro* with Fe²⁺, and if NrdI was included, with Mn²⁺ (Makhlynets et al. 2014). The Fe³⁺-cofactored form possessed 30% of the activity of the RNR with the Mn³⁺-cofactor. Deletion of the genes encoding either NrdEF or NrdI completely eliminated aerobic growth. The mutants were also tested in a rabbit model of endocarditis (Rhodes et al. 2014). Neither the two mutants nor their DNA could be recovered from heart valves 20 h post-infection, despite the recovery of ~10⁶ to 10⁷ CFU of the co-inoculated parent and *nrdD* mutant strains from the same rabbits. The finding that the *nrdI* mutant was just as deficient as the *nrdEF* mutant indicates that Fe³⁺ could not substitute for Mn³⁺ as a cofactor to support *in vivo* aerobic growth or virulence. Whether this is

because of a difference in enzyme properties *in vivo* or because concentrations of available Fe^{2+} are limited by cells under aerobic conditions as a defense mechanism (see below) is not clear.

Other manganese-cofactored enzymes

The relatively minor effect of substituting Fe^{2+} for Mn^{2+} on the *in vitro* activity of the NrdEF RNR relative to the sharp dependence of this protein on Mn *in vivo* highlights the difficulty of confidently assessing the metal dependence of enzymes based on *in vitro* activity assays. This is especially problematic with Mn^{2+} , since many of the enzymes for which it has been shown to serve as a cofactor *in vitro* are also active with Fe^{2+} (Anjem et al. 2009) or Mg^{2+} (Kehres and Maguire 2003). Mn^{2+} has been identified as a cofactor or activator of ~90 *E. coli* proteins (<http://www.ecocyc.org>). Further, as illustrated with PsaA, the presence of a metal in a crystallized protein is also not a definitive indication of *in vivo* relevance (Couñago et al. 2014; Lawrence et al. 1998; McDevitt et al. 2011). Nevertheless, there are a number of putative Mn^{2+} -utilizing enzymes that are of particular significance to streptococci, and these are discussed below.

A family II inorganic pyrophosphatase has been identified in multiple streptococcal species as requiring or markedly stimulated by Mn^{2+} *in vitro* (Parfenyev et al. 2001; Rantanen et al. 2007). Binding of Mn^{2+} in place of Mg^{2+} dramatically increases the activity of the enzyme and expands its substrate range (Parfenyev et al. 2001). These enzymes are of great importance for driving many cellular processes that produce inorganic pyrophosphate as a byproduct and, indeed, have been identified as essential in streptococci (Thanassi et al. 2002; Xu et al. 2011). Unlike SodA or the aerobic RNR discussed above, it is not clear why the activity of a pyrophosphatase would become limiting only under aerobic conditions, thus contributing to the oxygen sensitivity of an SBP mutant. This may, nevertheless, occur. Alternatively, this enzyme might be required continuously regardless of oxygen levels; however, as oxygen levels increase, levels of free Mn^{2+} may decrease due to preferential utilization by enzymes whose expression is induced by oxygen, such as NrdEF (Gon and Beckwith 2006) and SodA (Jakubovics et al. 2002). Indeed, SodA levels in aerated *S. gordonii* cells were reported to reach 1.5 – 2% of total cellular protein (Jakubovics et al. 2002). Thus, a significant portion of intracellular Mn^{2+} may be bound to this enzyme, and free Mn^{2+} levels may be sufficient to support full activity of the pyrophosphatase under anaerobic, but not aerobic conditions.

A similar situation may exist with enzymes involved in glycolysis and the pentose-phosphate pathway (Kehres and Maguire 2003). In the related bacterium *Lactobacillus rhamnosus*, it was concluded that five enzymes involved in the conversion of glucose to pyruvate, all of which have been shown to be essential in streptococci (Thanassi et al. 2002; Xu et al. 2011), are most active when bound to both Mn^{2+} and Mg^{2+} , thus explaining the beneficial effect of added Mn^{2+} on lactic acid production. A sixth enzyme, glucokinase, was shown to be more active with Mn^{2+} than Mg^{2+} in *S. mutans* (Porter et al. 1982).

Still another link between Mn^{2+} and oxidative stress resistance may be in relation to (p)ppGpp levels. In streptococci, the (p)ppGpp “alarmone” is synthesized by either of two dedicated synthetases, as well as a bifunctional synthetase/hydrolase called RelA (Kim et al.

2012). Levels of (p)ppGpp can presumably be diminished through either reduced synthesis or RelA-mediated hydrolysis. The hydrolase domain, but not the synthetase domain, has been shown to require Mn^{2+} as a cofactor in streptococci (Mechold et al. 2002) and other bacteria (Kehres and Maguire 2003). Increased levels of (p)ppGpp are thought to protect *S. mutans* from oxidative stress by slowing the growth rate (Kim et al. 2012). Mn^{2+} limitation might therefore reduce streptococcal growth by promoting the accumulation of (p)ppGpp.

Finally, in terms of promoting oxygen resistance by acting as an enzyme cofactor, Mn^{2+} may replace Fe^{2+} in certain mononuclear enzymes that are particularly ROS-sensitive. Studies performed in *E. coli* have suggested that Mn^{2+} protects H_2O_2 -stressed *E. coli* by this mechanism (Anjem and Imlay 2012; Sobota et al. 2014; Sobota and Imlay 2011). Three of these enzymes are widespread in streptococci and important for growth: ribulose-5-phosphate 3-epimerase, involved in the pentose-phosphate pathway; 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase, the first committed enzyme in aromatic biosynthesis; and peptide deformylase, which removes the N-formyl group from initiator methionine residues and is known to be essential in many bacteria including *S. sanguinis* (Xu et al. 2011). H_2O_2 stress is of particular relevance to streptococci, many of which possess the enzymes lactate oxidase (Seki et al. 2004; Taniai et al. 2008) and/or pyruvate oxidase (Carlsson and Edlund 1987; Spellerberg et al. 1996; Taniai et al. 2008), both of which form abundant H_2O_2 from oxygen. Because streptococci lack catalase, H_2O_2 can reach mM levels in some species. Indeed, the green coloration on blood agar plates from which “viridans” group streptococci (including *S. pneumoniae*) derive their name is due to oxidation of hemoglobin by cell-secreted H_2O_2 (Barnard and Stinson 1996). One of the most important mechanisms by which H_2O_2 creates oxidative stress is reaction with Fe^{2+} to yield a hydroxyl radical ($HO\bullet$) via the Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow HO\bullet + OH^- + Fe^{3+}$ (Imlay 2008). Free cytoplasmic Fe^{2+} levels are limited by storage in a non-heme ferritin (Yamamoto et al. 2002); however, Fe^{2+} located at accessible sites within proteins will also react with H_2O_2 . Because Mn^{2+} cannot catalyze the Fenton reaction, replacement of protein-bound Fe_{2+} with Mn_{2+} could protect the enzymes themselves, as well as adjacent biomolecules that might come in contact with H_2O_2 -generated $HO\bullet$.

Manganese-dependent gene regulation

Another possibility is that the primary role of Mn in oxygen tolerance and virulence is to alter gene expression. It has been demonstrated that transcription of genes encoding Mn^{2+} -specific SBPs in multiple streptococcal species is repressed by Mn^{2+} (Crump et al. 2014; Merchant and Spatafora 2014; Ogunniyi et al. 2010). In each case, this repression is mediated by a MntR-like protein often encoded within the ABC transporter operon or nearby. Previous studies have shown that streptococcal MntR-family proteins act as both positive and negative regulators of numerous genes in addition to those contained within Mn^{2+} transport operons (Merchant and Spatafora 2014). One could, therefore, hypothesize that Mn^{2+} contributes to virulence and oxygen tolerance by affecting MntR-mediated gene regulation. With regard to virulence, data are mixed. Two studies have suggested that MtsR of *S. pyogenes* is an important virulence factor (Bates et al. 2005; Beres et al. 2006), whereas a third found that it was not associated with virulence (Hanks et al. 2006). Similarly, in *S. pneumoniae* mutants in the *mntR* homolog, *psaR*, showed reductions in

virulence, no change in virulence, or increased virulence, depending on the study and the strain examined (Hendriksen et al. 2009; Johnston et al. 2006). With regard to oxygen tolerance, there is a clear distinction between MntR and SBP mutants. MntR-family mutants grow at near-normal rates in the presence of oxygen in *S. gordonii* (Jakubovics et al. 2000), *S. mutans* (Paik et al. 2003; Rolerson et al. 2006), and *S. pneumoniae* (Hendriksen et al. 2009; Johnston et al. 2006; Kloosterman et al. 2008) except, apparently, when metals are present at toxic levels. In contrast, when Mn is limited and oxygen is present, SBP mutants of each of these species grow poorly or not at all (Jakubovics et al. 2002; Janulczyk et al. 2003; Johnston et al. 2004; Kolenbrander et al. 1998; Marra et al. 2002; McAllister et al. 2004; Paik et al. 2003; Tseng et al. 2002). Thus, loss of MntR-family activity cannot explain the oxygen sensitivity of SBP mutants, and MntR regulators cannot be essential for Mn-dependent oxygen tolerance.

A variation of the above hypothesis is that Mn^{2+} affects gene expression through regulators other than MntR. There is experimental support for the hypothesis that Mn^{2+} upregulates oxygen defense genes, even if the regulator(s) responsible have not been identified. In *S. gordonii*, it was found that transcription of *sodA* and another oxidative stress response gene, *tpx* (encoding thiol peroxidase and located downstream from the *scaA* SBP gene), was upregulated upon addition of Mn^{2+} to a metal-depleted growth medium (Jakubovics et al. 2002). Upregulation also occurred in a mutant strain lacking the MntR-like regulator, ScaR. Similar findings have been reported for *sodA* expression in *S. pneumoniae* (Eijkelkamp et al. 2014). The combined results suggest that Mn^{2+} may serve as a signal for induction of oxygen tolerance genes.

Non-enzymatic ROS defense

Ionizing radiation damages cell primarily through radiolysis of H_2O to produce $HO\bullet$, and, secondarily, other ROS including H_2O_2 and superoxide (Daly 2009); thus, radiation resistance has much in common with ROS resistance. In *Deinococcus radiodurans* and certain other bacterial species, radiation resistance is associated with high-level uptake of Mn^{2+} and correlates with cellular Mn:Fe ratios (Daly 2009). Recent analyses have suggested that much of the Mn^{2+} in *D. radiodurans* is in the form of low-molecular-weight complexes that have the capacity to scavenge H_2O_2 (Barnese et al. 2012) and superoxide (Daly et al. 2010). This may also occur in streptococci, which have a high Mn:Fe ratio (Crump et al. 2014; Lisher and Giedroc 2013). Some caution in extrapolating results from *D. radiodurans* is needed, however. It is reported that disruption of the *sodA* gene in *D. radiodurans* has very little effect on radiation resistance (Daly 2009) whereas the effect of *sodA* mutation on ROS resistance in streptococci is substantial (Crump et al. 2014; Gibson and Caparon 1996; Jakubovics et al. 2002; Nakayama 1992; Poyart et al. 2001; Yesilkaya et al. 2000), despite there being no reduction in cellular Mn^{2+} levels (Eijkelkamp et al. 2014). Further, given that H_2O_2 levels remain high during aerobic growth for many streptococcal species, it is not clear that H_2O_2 scavenging is important or even operative (Barnard and Stinson 1996; Carlsson and Edlund 1987).

Conclusion

A number of recent advances concerning manganese and Mn^{2+} -specific ABC permeases in streptococci have provided new momentum to the field and important new insights to guide future work in this area. The detailed structural studies outlined above should guide the design and testing of small-molecule inhibitors that could serve as an entirely new class of anti-streptococcal drugs. In this regard, it is interesting to note that unlike ABC transporters for Zn^{2+} and $\text{Fe}^{2+/3+}$, there is no known case in streptococci of multiple ABC Mn^{2+} transporters; thus only one transporter would need to be targeted. The demonstration that a Mn-dependent RNR is ubiquitous in streptococci and is crucial for systemic virulence suggests that the RNR could also serve as a new pan-streptococcal drug target. As with the Mn^{2+} -transporting SBPs, the Mn^{2+} -dependent RNR is not present in humans. We are hopeful that application of recent advances described above will lead to new treatments for some of the most serious bacterial diseases of man, including pneumonia, meningitis, necrotizing fasciitis, and infective endocarditis.

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Figure 1. Phylogeny of the cluster A-I SBPs of known function

The sequences of the 38 SBPs included in this study were obtained from the NCBI and had the following GI numbers: AdcA_Spn, GI:116516060 (Bayle et al. 2011); AdcAII_Spn, GI:116515739 (Bayle et al. 2011; Loisel et al. 2008); FimA_Spa, GI:504525723 (Oetjen et al. 2002); Lbp_Spy, GI:16151821 (Linke et al. 2008; Linke et al. 2009); Lmb_Sag, GI:22537388 (Ragunathan et al. 2009a; Ragunathan et al. 2009b); MntA_Ban, GI:229264967 (Gat et al. 2005); MtsA_Sag, GI:22537676 (Bray et al. 2009); MtsA_Spy, GI:383493478 (Sun et al. 2009; Sun et al. 2008); MntC_Ngo, GI:59800624 (Lim et al. 2008); MntC_Sau, GI:88194402 (Horsburgh et al. 2002a); MntC_Syn, GI:1653002 (Adir et al. 2002); MtsA_Sin, GI:343481042 (Zou et al. 2010); MtuA_Sub, GI:222113460 (Banerjee et al. 2003); PsaA_Spn, GI:116515973 (McDevitt et al. 2011); ScaA_Sgo, GI:310633 (Jakubovics et al. 2000; Kolenbrander et al. 1998); SitA_Eco, GI:84060846 (Sabri et al. 2006); SitA_Sen, GI:16761638 (Zhou et al. 1999); SitA_Sme, GI:15966779 (Chao et al. 2004; Davies and Walker 2007; Platero et al. 2004); SitA_Sps, GI:10135086 (Abate et al. 2014); SitC_Sep, GI:57636840 (Cockayne et al. 1998; Massonet et al. 2006); SloC_Smu, GI:8925939 (Paik et al. 2003); SsaB_San, GI:153826 (Crump et al. 2014); TroA_Ssu, GI:386583456 (Wichgers Schreur et al. 2011; Zheng et al. 2011); TroA_Tpa, GI:1777933 (Deka et al. 1999; Desrosiers et al. 2007; Lee et al. 1999; Lee et al. 2002); YfeA_Ype, GI:1245464 (Bearden and Perry 1999; Bearden et al. 1998); ZnuA_Cje, GI:384442496 (Davis et al. 2009); ZnuA_Eco, GI:298380994 (Patzner and Hantke 1998); ZnuA_Hin, GI:16272089

(Lu et al. 1997); ZnuA_Sen, GI:161353595 (Ammendola et al. 2007; Ilari et al. 2011); ZnuA_Syn, GI:38492833 (Banerjee et al. 2003); ZnuA_Tpa, GI:378974662 (Desrosiers et al. 2007); ZnuA_Vpa, GI:28901162 (Gribenko et al. 2013); ZnuA_Ype, GI:22126135 (Bobrov et al. 2014); ZnuA_Yru, GI:238754865 (Dahiya and Stevenson 2010); YcdH_Bsu, GI:2415736 (Gaballa and Helmann 1998; Gaballa et al. 2002).

The three-letter abbreviation for bacterial species used was: Ban, *Bacillus anthracis*; Cje, *Campylobacter jejuni*; Eco, *Escherichia coli*; Hin, *Haemophilus influenzae*; Ngo, *Neisseria gonorrhoeae*; Nme, *Neisseria meningitidis*; Sag, *Streptococcus agalactiae*; San, *Streptococcus sanguinis*; Sau, *Staphylococcus aureus*; Sen, *Salmonella enterica*; Sep, *Staphylococcus epidermidis*; Sgo, *Streptococcus gordonii*; Sin, *Streptococcus iniae*; Sme, *Sinorhizobium meliloti*; Smu, *Streptococcus mutans*; Spa, *Streptococcus parasanguinis*; Spn, *Streptococcus pneumoniae*; Sps, *Staphylococcus pseudintermedius*; Spy, *Streptococcus pyogenes*; Ssu, *Streptococcus suis*; Sub, *Streptococcus uberis*; Syn, *Synechocystis*; Tpa, *Treponema pallidum*; Vpa, *Vibrio parahaemolyticus*; Ype, *Yersinia pestis*; Yru, *Yersinia ruckeri*.

Table 1

Zinc-specific SBP metal ion coordination

Protein	Residue 1 ^a	Residue 2	Residue 3	Residue 4	PDB	Resolution
AdcAII (<i>S. pneumoniae</i>)	His71:Ne2 (1.95 ± 0.1)	His147:Ne2 (2.40 ± 0.4)	His211:Ne2 (2.25 ± 0.2)	Glu286:Oe2 (1.90 ± 0.2)	3CX3	2.40
Lmb (<i>S. pyogenes</i>)	His66:Ne2 (2.05 ± 0.1)	His142:Ne2 (2.10 ± 0.0)	His206:Ne2 (2.10 ± 0.0)	Glu281:Oe2 (2.05 ± 0.1)	3GII	2.45
Lmb (<i>S. agalactiae</i>)	His66:Ne2 (2.10 ± 0.0)	His142:Ne2 (2.30 ± 0.0)	His206:Ne2 (2.20 ± 0.0)	Glu281:Oe2 (2.20 ± 0.0)	3HJT	2.50
ZnuA (<i>E. coli</i>)	Glu77:Oe2 (1.90 ± 0.0)	His78:Ne2 (1.95 ± 0.1)	His161:Ne2 (2.05 ± 0.1)	His225:Ne2 (2.10 ± 0.0)	2OSV	1.75
ZnuA (<i>Synechocystis 6803</i>)	His83:Ne2 (1.90 ± 0.0)	His179:Ne2 (2.05 ± 0.1)	His243:Ne2 (2.10 ± 0.0)	H ₂ O (2.30)	1PQ4	1.90
ZnuA (<i>Salmonella enterica</i>)	Glu59:Oe1 (1.70)	His140:Ne2 (2.10)	His147:Ne2 (2.20)	His211:Ne2 (2.00)	2XQV	2.08

^aMean metal-protein distances for coordinating atoms in the metal-binding site of the indicated proteins

Table 2

Manganese/Iron-specific SBP metal ion coordination

Protein	Residue 1 ^a	Residue 2	Residue 3	Residue 4	PDB	Resolution
PsaA (<i>S. pneumoniae</i>)	His67:Ne2 (2.18 ± 0.1)	His139:Ne2 (2.0 ± 0.0)	Glu205:Oe1 (2.1 ± 0.0)	Asp280:Oδ1 (2.38 ± 0.1)	3ZTT	2.70
			Glu205:Oe2 (2.40 ± 0.0)	Asp280:Oδ2 (2.20 ± 0.07)		
MtsA ^b (<i>S. pyogenes</i>)	His68:Ne2 (2.10)	His140:Ne2 (2.0)	Glu206:Oe1 (2.1)	Asp281:Oδ1 (2.80)	3HH8	1.87
			Glu206:Oe2 (2.40)	Asp281:Oδ2 (2.00)		
MntC (<i>Staphylococcus aureus</i>)	His50:Ne2 (2.10 ± 0.1)	His123:Ne2 (2.05 ± 0.1)	Glu189:Oe1 (2.30 ± 0.1)	Asp264:Oδ1 (2.35 ± 0.1)	4K3V	2.20
			Glu189:Oe2 (2.75 ± 0.1)	Asp264:Oδ2 (2.20 ± 0.0)		
MntC (<i>Synechocystis 6803</i>)	His89:Ne2 (2.10 ± 0.1)	His154:Ne2 (2.38 ± 0.1)	Glu220:Oe1 (2.20 ± 0.4)	Asp295:Oδ1 (2.77 ± 0.2)	1XVL	2.90
			Glu220:Oe2 (2.27 ± 0.3)	Asp295:Oδ2 (1.70 ± 0.1)		

^aMean metal-protein distances for coordinating atoms in the metal-binding site of the indicated proteins^bCrystal structure contains Fe²⁺ bound with bicarbonate ion

Table 3

Manganese/Iron/Zinc-specific SBP metal ion coordination

Protein	Residue 1 ^a	Residue 2	Residue 3	Residue 4	PDB	Resolution
TroA (<i>T. palladium</i>)	His68:Ne2 (2.25 ± 0.1)	His133:Ne2 (2.25 ± 0.1)	His199:Ne2 (2.30 ± 0.0)	Asp279:O61 (2.25 ± 0.1) Asp279:O62 (2.30 ± 0.0)	1TOA	1.80
TroA (<i>S. suis</i>)	His68:Ne2 (2.47 ± 0.0)	His131:Ne2 (2.23 ± 0.0)	His197:Ne2 (2.23 ± 0.0)	Asp281:O61 (2.37 ± 0.1) Asp281:O62 (2.60 ± 0.2)	3MFQ	2.60

^aMean metal-protein distances for coordinating atoms in the metal-binding site of the indicated proteins

Table 4

Characterized streptococcal manganese ABC transport systems

Species	Components				Metal(s) associated with		Virulence ^{a,b}	Reference
	SBP	NBD	TMD	Uptake	Complementation ^d			
<i>S. gordonii</i>	ScaA	ScaC	ScaB	Mn ²⁺	Mn ²⁺	– ^c	– ^c	(Kolenbrander et al. 1998)
<i>S. mutans</i>	SloC	SloA	SloB	Mn ²⁺ , Fe ²⁺	Mn ²⁺	–	Yes	(Paik et al. 2003)
<i>S. parasanguinis</i>	FimA	FimC	FimB	–	–	–	Yes	(Burnette-Curley et al. 1995)
				Mn ²⁺ , Fe ²⁺	Mn ²⁺	–	–	(Oetjen et al. 2002)
<i>S. pneumoniae</i>	PsaA	PsaB	PsaC	–	–	–	Yes	(Berry and Paton 1996)
				–	Mn ²⁺	–	–	(Dintilhac et al. 1997)
				Mn ²⁺	Mn ²⁺	–	Yes	(McAllister et al. 2004)
<i>S. pyogenes</i>	MtsA	MtsB	MtsC	Fe ²⁺ , Zn ²⁺	–	–	–	(Janulezyk et al. 1999)
				Mn ²⁺	Mn ²⁺	–	Yes	(Janulezyk et al. 2003)
<i>S. sanguinis</i>	SsaB	SsaA	SsaC	Mn ²⁺ , Fe ²⁺	Mn ²⁺	–	Yes	(Crump et al. 2014)
<i>S. suis</i>	TroA	TroB	TroC	–	Mn ²⁺	–	Yes	(Wichgers Schreur et al. 2011)
<i>S. uberis</i>	MtuA	MtsB	MtsC	–	Mn ²⁺	–	Yes	(Smith et al. 2003)

^aComplementation of *in vitro* growth or oxygen sensitivity phenotype^bReduced virulence of SBP mutant demonstrated^c–, Not determined