The role of zinc in the interplay between pathogenic streptococci and their hosts

Sulman Shafeeq, Oscar P. Kuipers* and Tomas G. Kloosterman
Department of Molecular Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 7, 9747 AG, Groningen, the Netherlands.

Summary

Recent studies on pathogenic streptococci have revealed that zinc is a pivotal metal ion in their interaction with the host. In these streptococci, systems exist that ensure optimal use of zinc from the surrounding milieu, as well as export of zinc when concentrations exceed tolerance levels. Zinc uptake is of crucial importance for the virulence of streptococci, whereas elevated zinc levels induced in the host during infection are detrimental for these pathogens. The expression or activity of a number of putative surface proteins and virulence factors depends on zinc. Moreover, several metal sensor proteins that mediate the transcriptional response to zinc in streptococci have recently been characterized. A number of components of zinc- and other metal ion-acquisition systems are suitable as protective antigens and may be future targets for the development of new vaccines, thus providing opportunities for the development of novel therapies. This review will discuss the recent advancements in the important field of metal ion biology in relation to the virulence of pathogenic streptococci, with a central focus on zinc homeostasis in *Streptococcus pneumoniae*.

Why zinc?

Many studies have focused on the importance of iron in the host–pathogen interplay (Brown and Holden, 2002; Schaible and Kaufmann, 2004; Nairz et al., 2010; Hammer and Skaar, 2011). However, other trace metal ions like copper, zinc and manganese have been shown to contribute to the virulence of streptococci as well and recently significant insights into the pivotal role of zinc have been obtained (Fig. 1).

In this review, we have discussed the role of trace metal ions, with an emphasis on zinc, in the lifestyle of an important class of pathogenic bacteria, comprised of the important human pathogen *Streptococcus pneumoniae* and other related streptococci.

Introduction

In order for bacterial pathogens to survive inside their hosts, they need to be able to efficiently exploit and use the available nutrients from their surrounding milieu. Several studies have shown that in pathogenic bacteria there is a complex intertwinement of metabolic (regulatory) networks and the functioning and expression of virulence factors (Brown et al., 2008; Gorke and Stulke, 2008; Hendriksen et al., 2008; Poncet et al., 2009; Somerville and Proctor, 2009; Amon et al., 2010; Eisenreich et al., 2010; Carvalho et al., 2011; Alteredi and Mobley, 2012; Cole, 2012). One of the most highlighted nutritional factors at the intersection of nutrient metabolism and virulence are trace metal ions (Schaible and Kaufmann, 2004; Papp-Wallace and Maguire, 2006; Riccardi et al., 2008; Jakubovics and Valentine, 2009; Dupont et al., 2011; Klein and Lewinson, 2011; Osman and Cavet, 2011; Cassat and Skaar, 2012; Wakeman and Skaar, 2012). All forms of life require trace metal ions for proper functioning and it is estimated that approximately 30% of all proteins and almost 50% of all enzymes contain trace metal ions as cofactors for their faithful activity and/or structure (Tainer et al., 1991; Andreini et al., 2008; Waldron et al., 2009).

In this review, we have discussed the role of trace metal ions, with an emphasis on zinc, in the lifestyle of an important class of pathogenic bacteria, comprised of the important human pathogen *Streptococcus pneumoniae* and other related streptococci.

Zinc can function as a structural and/or as a catalytic component of proteins (Berg and Shi, 1996). Furthermore, it is (like copper) a highly competitive metal ion [high in the Irving-Williams series of the stability of metal complexes: Mn(II) < Fe(II) < Co(II) < Ni(II) < Cu(II) > Zn(II)] that can replace the cognate metal ion of certain metal sensory proteins (Reyes-Caballero et al., 2010; Guerra et al., 2011). Due to a high cellular zinc-chelating capacity, free
Zinc concentrations are kept very low in the cell (Outten and O’Halloran, 2001). Despite the low free zinc concentration in the cell, the total cellular concentration of zinc is in the order of magnitude of 0.1 mM (Finney and O’Halloran, 2003; Jacobsen et al., 2011). Zinc is less redox-sensitive than copper and iron (Outten and O’Halloran, 2001) and might therefore, like manganese, play a role in protection against oxidative stress as for example caused by ROS (reactive oxygen species) produced via Fenton reaction (Gaballa and Helmann, 2002; Faulkner and Helmann, 2011; Ortiz de Orue Lucana et al., 2012).

In bacteria, zinc is present in approximately 5% of all proteins (Andreini et al., 2006b; 2008; Decaria et al., 2010). A recent study suggests that more than 200 copper/zinc-containing proteins (9% of the proteome) are encoded by the genome of *S. pneumoniae* (Sun et al., 2011). Indeed, several important enzymes in streptococci require zinc for their proper activity. Notable examples include pneumococcal phosphorylcholine esterase that affects decoration of the cell surface with choline, thereby influencing the complement of surface-exposed choline-binding proteins, which in turn might be important virulence factors (Lagartera et al., 2005; Hammerschmidt, 2009), and also peptidoglycan deacetylase, which protects against host lysozymes and is a virulence factor (Blair et al., 2005). Other groups of proteins that (likely) bind zinc are zinc-containing alcohol dehydrogenases (Lanie et al., 2007; Stroher et al., 2007; Potter et al., 2010), Pht (Polyhistidine triad) family proteins (Panina et al., 2003; Rioux et al., 2010; Loisel et al., 2011; Plumptre et al., 2012), ribosomal proteins (Panina et al., 2003), several zinc metalloproteases (Zmp) that are implicated in virulence (Blue et al., 2003; Chiavolini et al., 2003; Oggioni et al., 2003; Camilli et al., 2006; Bek-Thomsen et al., 2012), laminin-binding protein LmB/AdcAll (Loisel et al., 2008; Linke et al., 2009), and the major antigen and exotoxin SpeA1 of *Streptococcus pyogenes* (Baker et al., 2001; 2004; Galeotti et al., 2012). Thus, it is evident that in streptococci zinc plays a vital role in protein structure and function.

**Zinc in the human host**

In the human body, zinc is estimated to interact with up to 10% of all proteins and after iron, it is the second most abundant trace element (Whittaker, 1998; Zalewski et al., 2009).

![Diagram](image-url)
2005). Appropriate levels of zinc are important for the functioning of the immune system (Rink and Gabriel, 2000; Andreini et al., 2006a,b). The importance of zinc for immunity is further illustrated by observations of an elevated incidence of several infectious diseases in developing countries that are associated with a poor zinc status, like acute respiratory infections, otitis media and diarrhoea. Dietary supplementation with zinc may have a positive impact on the prevalence of these infectious diseases (Sazawal et al., 1998; Black and Sazawal, 2001; Fischer and Black, 2004; Coles et al., 2008); however in case of pneumonia there is conflicting data available (Basnet et al., 2012).

The zinc concentration in the human body varies among different tissues. For example, plasma zinc concentrations are between 12 and 16 μM (Rink and Gabriel, 2000; Ibs and Rink, 2003; Jayaram et al., 2011), while lung alveolar lavages contain as low as 1–2 μM zinc (Harlyk et al., 1997) and sputum contains around 1 μM (50 μg l⁻¹) zinc (Jayaram et al., 2011). The concentration of freely accessible zinc in the airway epithelium and other mucosal surfaces is thought to be relatively high (Zalewski et al., 2005), whereas in plasma, virtually all zinc is bound to plasma proteins, primarily albumin (Magneson et al., 1987; Wang et al., 2003; Jayaram et al., 2011).

During inflammation and infections, zinc levels are elevated in several body compartments (Milanino et al., 1993), although levels in the plasma may drop due to redistribution to the liver, which produces elevated levels of the zinc-binding metallothioneins and displays upregulation of zinc transporters (Peretz et al., 1991; Milanino et al., 1993; Sturniolo et al., 1998; Liuzzi et al., 2005; King, 2011). In addition, upon bacterial infection, zinc levels may be depleted locally by the action of human calprotectin released from neutrophils (Kehl-Fie and Skaar, 2010), which chelates these metal ions and potentially inhibits bacterial growth at the sites of inflammation, as shown for abscesses caused by Staphylococcus aureus (Soehne and Hahn, 2000; Lusitani et al., 2003; Corbin et al., 2008; Kehl-Fie et al., 2011). However, this might be counteracted from the side of pathogenic bacteria by upregulation/engagement of high-affinity zinc uptake systems, as has been shown in an animal model for gut infection by Salmonella typhimurium (Liu et al., 2012) and lung infection by the nosocomial pathogen Acinetobacter baumannii (Hood et al., 2012). Moreover, comparison of zinc concentrations in infected and uninfected tissues in mice indicated that the zinc to manganese ratio in infected tissues has increased 2.4-fold in lungs, 2.8-fold in the brain, 5.3-fold in the nasopharynx and 24.4-fold in the blood serum (McDevitt et al., 2011).

Thus, it seems plausible that human pathogens like S. pneumoniae and its relatives have to cope with both conditions of zinc limitation and zinc excess in their hosts.

Systems involved in zinc homeostasis in streptococci

In pathogenic streptococci, several systems involved in zinc homeostasis have been studied. In S. pneumoniae, a high-affinity zinc transport unit is present that consists of an ABC transporter AdeBC with two extracellular zinc-binding proteins, AdeA and AdeAI (also called laminin-binding protein LmB). These are partially redundant in binding zinc on the pneumococcal surface (Loisel et al., 2008; Bayle et al., 2011) and are homologous to the Escherichia coli major zinc-binding protein ZnuA (Li and Jogli, 2007). In addition, S. pneumoniae contains a manganese ABC transporter, the PsaBCA complex, which can bind zinc as well, although it is not involved in high-affinity zinc transport (Lawrence et al., 1998; McDevitt et al., 2011). Similar systems have been studied in other streptococci like Streptococcus suis (Aranda et al., 2009; Zheng et al., 2011), S. pyogenes (Janulczyk et al., 2003; Brenot et al., 2007), Streptococcus gordoni (Jakubovics et al., 2000; Loo et al., 2003) and Streptococcus agalactiae (Bray et al., 2009). The homologues of AdeAI in S. agalactiae and S. pyogenes, which both also have been shown to bind zinc, are involved in adhesion to the extracellular matrix protein laminin, in invasion of HMVE (human brain microvascular endothelial) cells and adhesion to epithelial cells (Sommer et al., 1992; Spellerberg et al., 1999; Elsner et al., 2002; Terao et al., 2002; Tenenbaum et al., 2007; Linke et al., 2009; Ragunathan et al., 2009). The adcA (lmB) gene in all of these streptococci is in an operon with a gene encoding a histidine triad protein Phd that also binds zinc (Panina et al., 2003; Kunitomo et al., 2008; Loisel et al., 2011). Streptococci contain several more proteins belonging to the zinc-binding Phd protein family, in which they are unique compared with non-streptococcal species (Adamou et al., 2001; Plumptre et al., 2012). These Phd family proteins are thought to scavenge manganese and zinc on the bacterial cell surface, and may serve as a reservoir for these metal ions during their starvation (Rioux et al., 2010).

A zinc export system is encoded by czcD in S. pneumo-

niae, belonging to the cation diffusion facilitator (CDF) family, which is present in other streptococci as well (Kloosterman et al., 2007). This gene provides resistance against zinc stress, and to a lesser extent cobalt stress (Kloosterman et al., 2007). In addition to this system, in S. pyogenes a gene called pmtA has been implicated in zinc export (Brenot et al., 2007). This gene is repressed by PerR, but gets expressed upon peroxide stress, leading to an intracellular zinc starvation response (Brenot et al., 2007).

Another protein that might play a role in zinc homeostasis is Dpr, which is a ferritin-like protein that can bind and oxidize iron with the concomitant consumption of hydrogen peroxide, thereby preventing the Fenton reaction and thus

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free radical stress. This protein has been shown to bind zinc in *S. pyogenes*, offering protection against zinc stress (Haikarainen and Papageorgiou, 2010; Tsou et al., 2010; Haikarainen et al., 2011). In *S. pneumoniae*, dpr is regulated by the RitR orphan response regulator that regulates iron metalloprotein genes (Ulijasz et al., 2004), and its expression is iron-dependent (Gupta et al., 2009).

**Zinc-dependent transcriptional regulators in streptococci**

In many bacteria, like *E. coli* and *Bacillus subtilis*, Zur (Zinc uptake regulator) regulates zinc uptake genes (Panina et al., 2003). However, in streptococci the MarR family regulator AdcR is involved in regulation of zinc uptake genes (Panina et al., 2003). During zinc starvation, AdcR binding to its target promoters is relieved, thereby activating the expression of zinc acquisition genes and ensuring the maintenance of intracellular zinc levels (Aranda et al., 2008; Oggunyi et al., 2009; Shafeeq et al., 2011a). Interestingly, the AdcR regulon includes genes encoding the Pht family of zinc-binding proteins (Shafeeq et al., 2011a). In addition, in some streptococci AdcR regulates alternative ribosomal genes, which might be induced upon zinc starvation leading to lower consumption of zinc by ribosomes (Panina et al., 2003; Brenot et al., 2007; Aranda et al., 2009).

A recently identified novel zinc-stress regulator, SczA, which is conserved in streptococci, activates the czcD zinc-eflux gene upon encountering high levels of zinc (Kloosterman et al., 2007). This occurs via an interesting mechanism involving both a repressor site that functions during low zinc levels and an activator site needed for induction of transcription during high zinc levels (Kloosterman et al., 2007). Besides czcD, SczA also induces expression of two downstream genes, nmlR and adhC. NmlR activates adhC expression, which has been proposed to encode a GSNO (S-nitrosoglutathione) or GS (glutathione)-aldehyde reductase, providing resistance against NO (Stroehrer et al., 2007; Potter et al., 2010). Induction of expression of this gene would be consistent with the prediction that AdhC contains zinc, and moreover with a model wherein AdhC is necessary to prevent accumulation of nitrosylated zinc-binding cysteine-containing sites in proteins.

Microarray experiments suggest that AdcR tunes expression of zinc-acquisition systems encoded by the adc and pht genes when zinc in the medium is scarce (lower than a few μM) (Shafeeq et al., 2011a), and SczA takes over control at higher levels of zinc in the growth medium (>10–20 μM; Jacobsen et al., 2011) by proper adjustment of expression of the CzcD zinc-eflux pump (Kloosterman et al., 2007; Jacobsen et al., 2011). This plausible mechanism of the regulation of zinc homeostasis whereby AdcR and SczA act at distinct cytoplasmic concentrations of zinc is consistent with the metal-binding affinities of both zinc sensors (Reyes-Caballero et al., 2010; Jacobsen et al., 2011).

Streptococci also contain a manganese-responsive regulator. In *S. pneumoniae*, this regulator, PsaR, has been shown to be responsible for manganese-dependent repression of the manganese uptake system PsaBCA, as well as the choline-binding protein PcpA and the surface-associated protein PrtA (Kloosterman et al., 2008). In *S. pyogenes*, *S. agalactiae* and *S. mutans*, this regulator is also involved in the response to iron and seems to have an impact on other important physiological processes as well, such as biofilm formation, genetic competence, oxidative stress tolerance and adherence (Berry and Paton, 1996; Dintilhac et al., 1997; Novak et al., 1998; 2000; Marra et al., 2002; Tseng et al., 2002; McAllister et al., 2004; Johnston et al., 2006; Anderton et al., 2007; Bray et al., 2009). Interestingly, the regulon of PsaR was upregulated under conditions with high zinc concentrations (Kloosterman et al., 2008; Jacobsen et al., 2011), likely by preventing manganese-induced interaction of PsaR with its cognate promoters (Kloosterman et al., 2007), and by competing with manganese uptake through PsaA (McDevitt et al., 2011).

Zinc also interferes with the regulation of copper homeostasis genes in *S. pneumoniae* via the copper-responsive regulator CopY, as, like in *Enterococcus hirae*, zinc is necessary for CopY-dependent repression (Solioz and Stoyanov, 2003; Solioz et al., 2010; Shafeeq et al., 2011b). The physiological relevance of this is unknown, but it could be a way for the cell to ensure a proper balance between copper and zinc.

The regulators and their regulons discussed above are all highly conserved in different strains of *S. pneumoniae* (Table S1). Especially the adcCBA and psaBCA genes are also conserved in other streptococcal species, underscoring their important functions in the lifestyle of these bacterial species. However, some of the pht genes are specific to *S. pneumoniae* and also the sczA regulon is absent in several streptococci, implying differences in zinc homeostasis.

**Zinc in the interplay of the pathogen and the host**

The importance of zinc for pathogenic streptococci as well as for their hosts implies a role for zinc during colonization and infection. AdcB was found to be important for full virulence of *S. pneumoniae* in a STM lung infection study (Hava and Camilli, 2002). Combined mutation of the genes encoding the zinc-binding proteins AdcA and AdcAII completely abolished virulence in models of nasopharyngeal carriage, pneumonia and sepsis, suggesting that efficient zinc uptake is needed to survive in all of the corresponding host compartments, and that zinc is preva-
lently limiting there (Bayle et al., 2011). Despite the abolished virulence, the adcA/adcAll double mutant was still able to grow, albeit worse than wild-type, in diluted BALF (bronchoalveolar lavage fluid) and human serum. It could be that specific and local sequestration of zinc, by for example, metallothioneins or calprotectin renders zinc concentrations in certain microniches (see also above) in the host tissues low enough to prevent growth of the adcA/adcAll mutant. AdcA and AdcAll could as well have other roles during infection in the host. Indeed, the adcA-adcAll double mutant showed a lag phase even in zinc-replete conditions, suggesting a role besides zinc acquisition. In line with a function that is seemingly unrelated to zinc, the permease component AdcB, was found to be important for adhesion to human epithelial cells (Song et al., 2008). Moreover, in S. gordonii the adc operon contributed to biofilm formation (Loo et al., 2003) and zinc uptake by the proteins encoded in the adc operon in S. pneumoniae is important for competence (Dintilhac et al., 1997). The individual contributions of AdcA and AdcAll to in vivo fitness remain unclear and it is tempting to speculate that each protein has a unique way of contributing to the infection processes. In S. pyogenes, deletion of adcAll alone already confers a strongly debilitated virulence and a defective growth phenotype during zinc depletion (Weston et al., 2009).

Given the fact that there is a strict zinc-dependent control of expression of genes involved in zinc homeostasis by AdcR, it is to be expected that at least at some stages during infection there is no need for these systems, namely when zinc levels are high. Indeed, a recent study shows that during infection, S. pneumoniae might encounter elevated concentrations of zinc and that upon infection of mice the ratio of zinc to manganese increases dramatically, especially in the plasma and in the tissue zinc levels have been observed during pneumococcal infection (McDevitt et al., 2011), showing a significant importance of metal ion homeostasis. Moreover, certain proteins having a role of the zinc efflux gene cccD in streptococcal virulence has not been studied so far, given the fact that increases in tissue zinc levels have been observed during pneumococcal infection (McDevitt et al., 2011), this system might contribute significantly. Another example of a link between zinc-resistance and virulence is S. pyogenes PmtA, which likely functions as a zinc exporter and enhances oxidative stress resistance (Brenot et al., 2007). Derepression of metal homeostasis as a result of perR deletion is thought to render S. pyogenes avirulent. So, while general effects of zinc homeostasis on virulence genes are evident in several pathogenic bacteria including S. pneumoniae, it remains to be resolved at which stages of the infection process the different zinc-regulated and zinc transport genes are required. Streptococci could as well encode hitherto unknown zinc transporters that help in fine-tuning of zinc homeostasis. Moreover, certain proteins having a role in the infection process require zinc. For example, in S. pyogenes, SpeI interacts strongly with AdcAll/LmB and AdcA. Since SpeI, a potent virulence factor in this bacterium, needs zinc for activity, this interaction makes sense (Galeotti et al., 2012). Also, the Phf family proteins, which have zinc-binding capacity, may play roles in protection against the immune system, for example by neutralizing complement, and they might also contribute to adherence (Rioux et al., 2010; Plumptre et al., 2012). In addition, the functionality of numerous other proteins could be affected by zinc during colonization and infection, such as the zinc metalloproteinases, which are involved in virulence as well, and zinc-containing alcohol dehydrogenases (Potter et al., 2010; Bek-Thomsen et al., 2012).

Availability of micronutrients such as zinc could also be influenced the presence of other bacterial species, as may the case in the nasopharynx, colonization of which by S. pneumoniae is a prerequisite for subsequent infection. So far, this is an unexplored area of study.

Use in therapies

Since the colonization and virulence of pathogenic streptococci displays a clear dependence on trace metal ion
homeostasis systems (Brown and Holden, 2002; Schaible and Kaufmann, 2004; Riccardi et al., 2008; Jakubovics and Valentine, 2009; Klein and Lewinson, 2011; Cassat and Skaar, 2012) there could be possibilities to use these systems as targets for anti-infective therapies. Either it could be possible to generate compounds that inhibit such systems, or vaccines based on these systems could be developed (Klein and Lewinson, 2011). One class of vaccine targets could be the Pht family proteins, which are highly conserved in pneumococcal strains (see Table S1), especially PhtD which shows very little sequence variability (Riouxf et al., 2010; Plumptre et al., 2012). All Pht family proteins were able to elicit a high level of protection in virulence settings, although in a study by Ogguniyi et al. (2007) the level of protection observed for PhtE and PhtB was not very strong. PhnD and E, as well as PcpA were found to raise antibody responses in humans (Khan et al., 2012; Pichichero et al., 2012), which might compromise pneumococcal adhesion to epithelial cells as this is mediated by these proteins (Khan et al., 2002; Hendriksen et al., 2009). Moreover, PhnE, PhnD and ZmpB were found in an antigen screen using human and mice sera (Beghetto et al., 2006). PcpA also protects against sepsis and lung infection, but despite the fact that it has been reported as an adhesion factor, not against colonization of the nose (Glover et al., 2008). Lmb, which binds zinc and presumably has a similar role as AdcAll in S. pneumoniae, is conserved in all strains of S. agalactiae and might be a good vaccine candidate as well (Ragunathan et al., 2009). S. pneumoniae contains up to four zinc metalloproteases: IgA1, ZmpB, C and D (Bek-Thomsen et al., 2012). Especially ZmpB, highly conserved among serotypes and involved in pneumococcal virulence, is an interesting vaccine candidate, which offers protection against pneumococcal invasive disease in mice, in particular when combined with other antigens (Gong et al., 2011).

Adequate levels of zinc in the host might reduce the incidence of infectious diseases, including those caused by S. pneumoniae (Sazawal et al., 1998; Black and Sazawal, 2001; Strand et al., 2001; 2003; Fischer and Black, 2004; Coles et al., 2008; Basnet et al., 2012). Zinc also reduces the toxicity of pneumolysin to cochlear hair cells in vitro due to an inhibition of the toxin’s incorporation and aggregation into the plasma membrane, a phenomenon which could be a basis for treatment of pneumococcal otitis media (Franco-Vidal et al., 2008).

So far, inhibitory molecules have not been generated based on metal ion homeostasis systems, but such compounds could be well targeted at one of the zinc uptake or efflux systems as described above (Klein and Lewinson, 2011). Thus, a proper understanding of zinc homeostasis in relation to the colonization by and virulence of streptococci might open up new avenues for protection against infections.

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