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Zinc Homeostasis in *Streptococcus pyogenes* and *Streptococcus pneumoniae*: A Literature Review

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Abstract

Zinc is an important trace metal that plays an essential role in controlling innate immune responses and regulating bacterial pathogenesis in hosts. To serve as a metalloenzyme cofactor, the concentration of zinc is crucial for maintaining essential catalytic function of enzymes within bacteria. Designated transport proteins contained within the bacterial envelope facilitate the optimum concentration of zinc. Zinc also maintains the transcription and regulates the expression of numerous genes that aid in virulence and bacterial pathogenicity. *Streptococcus pyogenes* and *Streptococcus pneumoniae* are two pathogenic bacteria that causes diseases like strep throat, pharyngitis, pneumoniae, etc. all ranging from mild to severe contagious streptococcal infections. Streptococcal virulence is greatly influenced by the Zn^{2+} content along with other transition metal ions like Fe^{2+}, Cu^{2+}, Mn^{2+} etc. This review, however, primarily focuses on how metal induced protein regulators, transporters and chaperones mediate zinc to optimize its requirement for maintaining streptococcal virulence in host body. The paper begins by discussing the bacterial relationship with metals, the activities of metal-related virulence and a synopsis of both the streptococcal systems that use metals to promote nutrition and pathogenicity. The discussion then delves into a detailed analysis of the metal binding sites of different importers, exporters, chaperones, and their chemical/structural organization that enable necessary zinc binding. This literature review demonstrates a study of how zinc is acquired, transported, and used within *S. pyogenes* and *S. pneumoniae* providing a scope of knowledge on what the potential cellular targets should be for antimicrobial therapies.

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1. Introduction

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1.1 Bacterial Metallostasis

Bacteria requires transition metal ions for survival and as a source of nutrition. Hosts can induce metal limitation and metal intoxication to limit bacterial growth[1]. Due to constant change in extracellular environment, bacterial physiology has evolved to combat and adapt to metal extremity as well as scarcity. While metal limitation can suppress bacterial growth, excess metals can cause metal toxicity that kills the bacteria. To maintain an optimum level of essential metals, bacteria can regulate the ebb and flow of metals within their internal and external environment. This maintenance is conducted by a host of sensor-proteins, metalloregulators, transporters, storage proteins, chaperones, and scavengers that can initiate and/or terminate the sequestration, efflux, storage or capture of necessary metals according to bacterial needs. This diverse array of cellular mechanism is known as metallostasis, a process by which bacteria maintains the availability of transition metals within its environment so as to avoid possible extreme circumstances of metal starvation or toxicity[2]. Metallostasis is a continued and layered process that uses a diverse range of protein structures that perform designated functions in maintaining homeostasis, some exclusively transporting one metal while others handling a variety of transition metal transportation.

Three major categories of proteins that involve in metallostasis are: Metalloregulators, Metallotransporters and Metallochaperones. Although these categories often overlap in functional characteristics within different bacterial systems, their general trends in metal binding and transportation remain the same. Metalloregulators are DNA binding transcriptional regulators that undergo allosteric changes through metal induction. They control the bioavailability of metals by physically inhibiting the transcription of metal transporting genes at

the promoter region or by freeing the promoter region to initiate transcription of those genes[2].

Metallotransporters are a wide range of protein transporters that line the bacterial envelope to

conduct in metal uptake or efflux whose transcription is controlled by metalloregulators. The

proteins that acquire and bind to specific target metals to physically facilitate delivery through

metallotransporters are known as metallochaperones[2]. For example, Zinc Uptake Regulator (Zur)

is a metalloregulator that transcribes ZnuABC transporter system in *E. coli* in which ZnuBC is

the transporter and ZnuA is the chaperone that delivers zinc to the transport complex.

2. Bacteria and Metals

2.1 Relationship of bacteria and metals

Among various reasons, bacterial infection can cause the bioavailability of metals to vary in the

host body. While metals are required for important cellular processes, an over intake of metals

can also adversely affect the cellular pathways of the pathogen[4]. The "tug-of-war' for metal

availability is prominent in the host-pathogen interface. The host body is rich in metal content.

During initiation of bacterial infection, both host and pathogen compete for the use of essential

metals for necessary metabolic activities[2]. Transition metals like Fe, Cu, Zn, Mn etc. play

important roles as nutrients to form structural components of essential proteins complexes that

drive the basic metabolic activities in the bacteria[4]. Approximately, between one-third to one-

half of all cellular proteins require metal interactions in some form many of which require metals

for structural stability and/ or function[6]. Some protein regulators in bacteria evaluate

environmental cues like nutrients availability, chemical stressors, host temperature and immunity

to regulate host-virulence genome expression and coordinate necessary cellular responses to

combat stress[7].

2.2 Relationship of bacteria and zinc

Zinc is the second most abundant metal after iron. For long, the study of iron on host-pathogen relationship has been widely studied[8]. However, in recent years, the study of zinc in bacterial virulence has become more popular as more and more studies have explored bacterial dependence on transition metals for pathogenic strategies. Studies have shown that metal chelators like calprotectin (CP) and Tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN) induces zinc starvation in the bacteria in the scope of required catalytic activity and pathophysiological processes by zinc[9]. Hosts can employ zinc intoxication by using neutrophils, monocytes and macrophages as a possible way of zinc poisoning antimicrobial to inhibit Group A Streptococcus (GAS) growth[10]. Zinc, like other transition metals also participate in cellular processes, DNA replication, protein binding for structural stability as well as working as a key player in the catalytic cellular functions. The structural role of zinc is widely used to stabilize chromosomes and filaments. The concentration of zinc in organisms usually range from 10^{-11} M in the cytoplasm m to 10^{-3} M within vesicles. Zinc binding sites in proteins rich tend to be within the β-sheet structures where the relative rigidity along with the strength of the fold makes a suitable position for the tetrahedron structure of zinc to bind. In zinc binding extracellular enzymes, zinc acts as a cross-link between histidine and thiolate groups into a four-ligand structure within the β-sheet stabilizing the form of the enzyme[11]. Zinc is tightly bound in proteins and therefore, pathogenic bacteria apply high affinity zinc sequestration protein complexes to optimize needs. For example, the ABC transport system in gram-negative bacteria known as ZnuABC is a high affinity zinc acquiring system were disruption in system results in remarkable loss of pathogenicity[12].

3. Streptococcal Physiology and Attributes

3.1 Key Streptococcal characteristics

Streptococci are clustered, gram-positive, non-motile, nonsporeforming cocci (spherical shaped bacteria) that are found in pairs or chains. Streptococci are ecologically part of the natural microbial flora within humans and animals while their diseases also range from subacute to severe/invasive. Some of the significant streptococcal diseases in humans are scarlet fever, rheumatic heart disease, glomerulonephritis, and pneumococcal pneumonia. Most streptococci are facultative anaerobes meaning that these can grow either in the presence or absence of oxygen and most require to grow on blood rich agar to grow[13, 14]. Due to the lack of an outer periplasmic membrane in gram-positive bacteria, they, unlike gram-negative bacteria consists of a thick layer of peptidoglycan which lies at the outermost part of the cell envelope[38]. Surface proteins in gram-positive bacteria may come attached to the teichoic acid component of the peptidoglycan layers noncovalently, and some covalently to the peptides anchored within the peptidoglycan layers, while many are anchored in the cytoplasmic membrane lying underneath the peptidoglycan layer [38-41].

Streptococcal species are diverse, and their characteristics overlap. They can be classified based on morphology, hemolysis, biochemical reactions and on serologic specificity. Serologic grouping of streptococci divides the species into groups A-V. *S. pneumoniae* and *S. pyogenes* largely separate due to hemolytic differences. Hemolysis refers to the lysis of red blood cells. Based on hemolysis, Streptococci can be divided into the following: α-hemolytic, β-hemolytic, and γ-hemolytic. β-hemolysis refers to the complete lysis/ break down of red blood cells where

the lysis shows up as clear on the blood agar whereas α-hemolysis is partial or "green"

hemolysis. *S. pyogenes* is β-hemolytic and *S. pneumoniae* is α-hemolytic[13, 14].

3.2 Characterization of *S. pyogenes* and *S. pneumoniae*

S. pyogenes is also majorly known as Group A Streptococcus. Newer species are being added

under the category as well. It causes acute diseases in the respiratory tract, bloodstream, and

skin. Acute *S. pyogenes* infections may cause pharyngitis, scarlet fever, impetigo, cellulitis etc.

Some of the invasive forms of infections are include necrotizing fasciitis, myositis and

streptococcal toxic shock syndrome[14]. Virulence determines if the bacteria mostly depend on the

cell envelope where most of colonization and evasion strategies are concerned[13]. Antigenic

virulence factors include M protein, peptidoglycan and lipoteichoic acid, hyaluronic acid

capsule, capsular polysaccharide, pyogenic toxin etc[13, 14]. Currently, there are M protein vaccines

that contain antigens from the C-repeat segment of the proteins. Other vaccines targeting the C5a

peptidase, capsular polysaccharide and fibronectin-binding proteins are also considered to be

highly promising[15].

S. pneumoniae is a lancet-shaped α-hemolytic streptococci. It is a natural part of the respiratory

tract flora. *S. pneumoniae* has been the primary cause of first community caused pneumonia in

the United States[14]. The bacteria colonize the nasopharynx and causes diseases like otitis media,

bacteremia, and meningitis. The polysaccharide capsule is the most virulent factor of the bacteria

which combats phagocytosis, restricts autolysis and exposure to antibiotics[14, 16]. This organism is

also one of the common causes of sinusitis and conjunctivitis beyond early childhood[14].

4. Zinc Homeostasis in *Streptococcus pyogenes* and *Streptococcus pneumoniae*

4.1 Transcription initiation of metalloregulators: Zinc uptake and efflux

Transcription factors are a group of sequence specific DNA binding proteins that function as either an activator or a repressor for the transcription of other genes. These multi subunit protein complexes generally bind upstream of genes on the DNA on areas known as promoter regions. Stretches of discrete nucleotides known as response elements are available in the promoter region allowing for transcription factor selectivity before transcription initiation[17, 18]. The binding of the transcription factors to the promoter region determines the repression of the transcription of genes. The release of the transcription factor from the promoter region activates the transcription of the target gene by the RNA polymerase.

Metalloregulators are specific kinds of transcription factors that undergo allosteric changes where a cognate metal binds at its binding site to[1, 19]. Together they activate or repress the transcription of metal sequestration, metal effluxion or storage genes by binding at their promoter regions to maintain a steady equilibrium of essential metals[1]. In other words, metalloregulators are sensor-proteins that maintain a balance of the production of metallotransporters that ultimately works directly in the import, export, and flow of metals within its internal and external environment. There are different ways in which metalloregulators sense metals and initiate transcription. One way is by using direct sensing of metals where metals directly bind to the metalloregulators. For example: Zn^{2+} directly binding to protein regulator Zur in *Bacillus subtilis*[20]. Another process is by sensing metal indirectly through a metal-dependent catalytic reaction, specifically related to iron or heme sensing[21]. Functions of zinc metalloregulators are largely dependent on zinc quota, which dictates the total zinc content inside the bacteria[22].

Each individual zinc metalloregulator like Zur, AdcR, etc. fall under a broader family of sensor or regulators. For example, Zur falls under the Ferric Uptake regulator (Fur) family and MtsR falls under the DxtR family of regulators. Across bacterial species, each family has a host of homologous metalloregulators where one homologue plays a dominant role as the main transcriptional activator or repressor in one species and another homologue plays a role in another.

4.1.1 Role of AdcR in Zn acquisition

The role of Adhesion competence repressor (AdcR) is well studied within *S. pneumoniae* and *S. pyogenes* and is known to be the leading metalloregulator for zinc acquisition in both species. The other most highly studied zinc regulator is Zur, which falls under the Fur superfamily of regulators but its zinc uptake metalloregulatory activities are dominant zinc regulators in gram-negative *E. coli*.

4.1.2 Role of AdcR in both bacterial systems: Zn acquisition

The AdcR repressor protein is the member of the Multiple antibiotic resistance repressor (MarR) superfamily[24]. In *S. pneumoniae*, AdcR represses the transcription of an *adcRABC* operon by encoding the transcription of an ABC transporter protein complex and therefore, facilitating the uptake of Zn^{2+} and Mn^{2+} [24, 25]. In a study, it was shown that the cellular growth of *S. pneumoniae* is 5-6 fold lower in conditions of no added Zn^{2+} compared to conditions of 0.2µM added Zn^{2+} [24]. AdcR is also well conserved in *S. pyogenes* and Group A Streptococcus (GAS) family which is a versatile pathogen that uses this metalloregulator to induce ABC transporter genes for zinc influx[23, 24, 26]. In GAS, studies have shown that AdcR is upregulated in the presence of CP mediated Zn^{2+} starvation. AdcR mediatedregulons that are involved in zinc homeostasis, like

adcABC, *rpsN.2*, *adcAII*, *phtD*, are therefore also affected by CP zinc starvation. It was further shown that there are significant differences in the intracellular zinc content of GAS compared to WT mice and individual mutant strains ($\Delta adcA$, $\Delta adcAII$, $\Delta phtY$, $\Delta phtD$, $\Delta phtD\Delta phtY$) suggesting that AdcR regulated genes participate in GAS defense against CP mediated zinc limitation[9].

Metal binding affinity in organism is the measure of cytoplasmic ability to buffer transition metals like zinc from the cytosolic pool within the internal and external environment. It is the reciprocal of the metal association equilibrium or the metal affinity constant[19, 22]. AdcR is the first studied MarR family metalloregulator that binds two Zn^{2+} as a cognate metal with an affinity of $\geq 10^9$ M^{-1} at pH 6.0 and $\geq 10^{12}$ M^{-1} at pH 8.0. AdcR has high pH reliant affinity for zinc which is unique compared to other zinc metalloregulators. AdcR is a homodimer, a protein with two identical polypeptide chains containing histidine residues H108 and H112 at the C-terminal regulatory domain and His42 at the N-terminal DNA binding domain. Studies show that His108 and His112 are important for strong Zn^{2+} binding affinity as metalloregulatory zinc ligands[27]. AdcR is an α-helical protein consisting of 6 α-helices where the dimerization domain is connected through the α2 and α5 helices to the DNA binding domain at the C-terminal through a hydrogen bonding network[27, 28]. This suggests that the hydrogen bonding network in AdcR may play an important role in the allosteric positive change caused by Zn^{2+} [28]. Structure of AdcR is crucial in Zn^{2+} binding. This Zn^{2+} binding is highly specific and causes conformational change in AdcR to slightly bend away from the promoter region to allow for transcription[29]. Zn-bound AdcR binds to target promoter regions and downregulates the expression of the AdcR regulon during higher zinc availability. In both S. pyogenes and S. pneumoniae, AdcR works as

metalloregulators to transcribe ABC transporters and Pht, zinc scavenger proteins for zinc mobilization during zinc stress[23, 24, 26].

4.1.3 Zn efflux metalloregulation

During neutrophil invasion, phagocytosis can take place and as a result, bacteria can experience zinc toxicity[30]. This demands transcription of efflux and export-related zinc transporters. Efflux transport systems are controlled by efflux metalloregulators. The most important bacterial zinc efflux metalloregulators are ZntR from the MerR superfamily and SmtB from the ArsR superfamily[31]. However, ZntR and SmtB are strictly not the efflux sensor-proteins in most Streptococcal species. The way AdcR is exclusive to *S. pneumoniae* and *S. pyogenes* in zinc sequestration transcription initiation, similarly, both species have distinct efflux regulators that regulate the transcription of efflux transporters.

4.1.4 Role of SczA in S. pneumoniae: Zn efflux

SczA is the efflux metalloregulator that promotes zinc effluxion during cytotoxicity and is highly conserved in a few streptococcal species. SczA is the Streptococcal *czcD* activator which is the first metalloregulatory protein under the Tetracycline repressor protein (TetR) family that has been described as a zinc efflux regulator in *S. pneumoniae* that activates *czcD*[32, 33]. But conical TetR regulators generally mediate toxicity through the general paradigm of ligand-mediated transcriptional depression whereas SczA is an exception to it as it allows for the transcription of an efflux pump[34]. During zinc toxicity, SczA is induced by cognate metal ions Zn^{2+}, Co^{2+}, and Ni^{2+} to undergo conformational change and release from the upstream promoter region of zinc efflux gene *czcD*[32, 33]. The study that identified SczA as a transcriptional factor for *czcD* was conducted by creating several *lacZ* fusions and truncations in palindromic sequence of the *czcD*

promoter region. This identified that changes in the conserved bases of the motifs abolished the zinc dependent activation of SczA, blocking *czcD* activation. Two orthologues of *czcD*, known as *spr1671*, a MerR family regulator and *adhB*, a zinc containing alcohol dehydrogenase were also downregulated, the same as *czcD*. SczA binds to the *czcD* promoter at the H_6-SczA region of the metalloregulator[32]. SczA can also act as a repressor for genes but the reason for this switch is yet to be identified[10].

The structure of SczA is essential for the binding of Zn^{2+} to SczA for transcriptional initiation. SczA, like most other TetR regulator contains a highly conserved N-terminal DNA binding domain ($\alpha 1$-$\alpha 3$) helix turn helix structure and a C-terminal regulatory domain that attaches to metal ligands. SczA also has two metal binding sites, the A-site and the B-site that are both located on the opposite ends of the SczA C-terminal regulatory domain, both important for the transcription of *czcD*[34].

In *S. pneumoniae*, Zn^{2+} plays a key role as a cognate metal for the activation of SczA and therefore, upregulation of *czcD*. Cognate Ni^{2+} binds differently to SczA than Zn^{2+}. Two Zn^{2+} ions can bind at each *czcD* promoter region, each with a small difference in metal binding affinity with pH at a high of pH 8.0 and low of 6.0. The dependency on pH level for metal binding is consistent with the Zn-histidine ligation on the metal binding sites[34]. Site B is consisting of residues H67, E114, H117 and H118 from the α and β helices of the protein and is predicted to anchor Zn^{2+} to the site[34].

4.1.5 Role of GczA in S. pyogenes: Zn efflux

S. pyogenes has a regulator paralogue of SczA with 50% match in identity that blocks *czcD* transcription at its promoter region[32]. This metalloregulator is known as GczA which falls under

the same TetR family[10, 36, 37]. Research studying *czcD* and *gczA* null mutants of GAS experienced increased effects of zinc toxicity due to neutrophil killing. It showed through transcriptional analyses that GczA, like SczA regulates *czcD* transcription. Results showed that in the absence of zinc, all tested mutants of GAS demonstrated similar growth compared to its wild-type strain. But with increased zinc content ranging from 0.1mM to 1mM, the lag phase of Δ*czcD* and Δ*gczA* mutant strains increased significantly. Further experiments showed that when additional zinc input was stopped, the zinc contents in mutants remained unchanged. However, when zinc content was increased, the mutants accumulated more zinc due to the absence of *czcD* and *gczA* genes that otherwise would have contributed to zinc efflux[36]. Even though GczA is well characterized to be a TetR family regulator and is known to express CzcD, a zinc efflux transporter in *S. pyogenes*, its biochemical and molecular structure is yet to be elucidated. Given that GczA has a paralogous relationship to SczA, it can be hypothesized that both proteins may consist of similar structural organization and construction of Zn (II) binding sites. In *Streptococcus agalactiae* and *S. pneumoniae*, it is found that metal ligand binding for both SczA and GczA are highly conserved[10]. Regardless, GczA is strictly limited to Zn^{2+} binding and is not impacted by the lack or abundance of other metals in GAS.

4.2 Post transcriptional transportation of Zinc

Transporters are import and export proteins that line the cell envelope in both gram-positive and gram-negative bacteria and aid in the influx or efflux of nutrients, metals, ligands etc. Metallotransporters are transport proteins that are expressed highly for the sequestration of metals during metal crisis and release metals during metal toxicity. Metals pass through transporters in ionized form, and therefore, dedicated transporters facilitate the passage of metal

ions using ATP by delivering them either into or out of the cell. Metalloregulators initiate the transcription of genes that translate protein transporters and largely control when transcription should be increased or decreased depending on the metal content within the cell. Zinc transporters can comprise of high affinity and low affinity protein transporters where high affinity refers to zinc uptake from low external zinc content and low affinity refers to scavenging of zinc at non-toxic high external zinc content[41, 42]. For example, ZnuABC transport system transcribed by the Zur metalloregulator acts as high affinity zinc uptake transporter in gram-negative *E. coli*[31, 42].

4.2.1 AdcABC importer: an ABC transport system in Zn uptake

Described earlier, Zn^{2+} dependent metalloregulator AdcR controls the regulation of transporters that aid in zinc uptake in *S. pyogenes* and *S. pneumoniae*[23]. More specifically, AdcR controls the regulation of ATP-binding cassette (ABC) transporters that aid in zinc uptake within these two species[23, 31, 43]. Both in gram-positive and gram-negative bacteria, ABC transporters play an important role in high affinity zinc uptake[41].

ABC transporters are a group of protein complex that transports a wide variety of substrates along cellular membrane[44]. All ABC transporters have some common structural construct[44, 45]. Bacterial ABC transporters have four distinct domains which are two highly conserved variable transmembrane domains (TMDs) composed of two sets of six α-helices and two nucleotide-binding domains (NBDs) located below the TMD in the cytoplasm[31, 44, 45]. In some cases, NBDs and TMDs can be fused into homo- or hetero-dimerizing half-transporters[44, 45]. Since, ABC transporters use ATP to translocate substrates like metals, it conducts three important functions: ATP binding, hydrolysis, or the process of turning ATP to ADP+Pi and phosphate release. A substrate-binding domain (SBD) captures the substrate and docks to the extracellular side of the

TMD[44]. Conformational change in the transmembrane protein shifts the substrate into the NBD where ATP binding, hydrolysis and phosphate release takes place allowing the complete translocation of the substrate[44, 45].

The first ABC transporter described was the ZnuABC transporter in *E. coli* that aids in zinc uptake and has been widely studied. In *S. pneumoniae*, *adcA* gene sequence is 22% match to *znuA*, the *adcB* is 29% match to *znuB* and the *adcC* is 36% match to *znuC*[31, 41]. In *S. pneumoniae*, AdcR regulates the *adcABC* operon to transcribe ABC transporters[31]. Similarly, *S. pyogenes* also uses AdcR to generate adcABC transporters for zinc sequestration[27, 36]. Studies have shown that addition of zinc increased the expression of *adc* in *Streptococcus pneumoniae* and restored the growth of *adc* mutant strains[46]. Here, within the *adcABC* operon, *adcA* is the chaperone for zinc while *adcB* is the transmembrane protein that conducts the permease of the metal and *adcC* is the ATP binding protein that conducts the hydrolysis/ ATPase for the transportation of the metal[47].

4.2.2 Substrate (Zn) binding lipoprotein/ chaperones

AdcA in *S. pneumoniae*: AdcR regulation of ABC transporters is the most studied in *S. pneumoniae*. SBDs in gram-positive bacteria specifically for zinc and manganese falls under the cluster 9 family of lipoproteins[48, 49]. AdcA and AdcAII are also cluster 9 lipoproteins. It is found that Δ*adcCBA* mutant strains of *S. pneumoniae* faces reduced growth in absence of zinc but growth increases after zinc is added[47]. Interestingly, the mutant can restore growth presumably through the PsaBCA ABC transporter that predominantly transports Mn in the bacteria[47, 50]. In *S. pneumoniae*, Zn^{2+}-specific chaperones AdcA and AdcAII are two metal binding receptors (MBRs) or SBDs of the ABC transport system that plays overlapping roles in zinc recruitment[26, 31, 46, 51]. AdcA and AdcAII share 43% of their genetic identity and is comprised of a specific common structure[51]. The common structure of A-I Zn^{2+}-binding complexes in gram-positive and

gram-negative bacteria is that it consists of two $(\beta/\alpha)_4$ domains that are connected by a rigid α-helix structure. The metal binding site is available at the interface that connects the two domains[51, 52]. But there are a few notable differences in their structure and metal binding processes. The Zn^{2+} binding AdcA, like ZnuA has an extended flexible loop within N-terminal rich in amino acids, histidine and glutamate residues that are essential for metal binding[41, 51-53].

AdcA uses a "trap-door" mechanism to recruit zinc in which the zinc gets trapped/enclosed within histidine and glutamine residues of the C and N terminals of AdcA. In AdcA there are two Zn^{2+} binding sites; the C-terminal and the N-terminal[51]. The zinc binding site of ZnuA in *E. coli* is high in histidine and glutamine residues and happens to have a fully enclosed conformation due to the presence of this outer hydrophobic shell[53, 54]. Contrastingly, AdcA consists of a partial opening in its conformation due to the rotational movement of an off-hanging segment of an α helix, $\alpha7$ perpendicular to the interlobe of another α helix, $\alpha5$ within $AdcA_N$[51]. The $AdcA_N$ site contains His63, His140, His 204 and Glu279[51, 52]. These histidine and glutamine residues form a double lobed binding site to trap Zn^{2+} ion where each form a bond distance ranging from 1.98 to 2.08 Å[51]. The $AdcA_c$ site contains His452, His461 and His463 connecting another single Zn^{2+} ion with bond distances ranging from 1.97 to 2.11 Å[51, 52]. When Zn^{2+} binds to AdcA, the α helical sections interreacting are $\alpha7$, $\alpha12$ and $\alpha15$ of the $AdcA_c$ domain as well as the histidine rich residues of $AdcA_N$ domain. Studies have shown that $AdcA_N$ domain and the zinc acquiring histidine residues are required in *S. pneumoniae* because without the $AdcA_N$ domain, the Zn^{2+} binding capacity is completely abolished. The Zn^{2+} binding specificity, however, is enabled due to the presence of a mobile surface loop in AdcA known as the $\alpha2\beta2$ site. The His63 inside the $\alpha2\beta2$ loop acquires zinc and retracts into the binding site thereby decreasing metal accessibility

of the site. Therefore, zinc acquisition in AdcA is described as a trap door model due to the retraction and conformational change of the α2β2 site after zinc sequestration[51].

AdcAII in *S. pneumoniae*: AdcAII shares 26% sequence identity with AdcA and 30% with PsaA and 67% with proteins titled as Llp in other streptococcal species. Upstream to *adcAII*, the gene *phtD* gene suggests that *phtD* might be for transcriptional coregulation of *adcAII*. AdcAII binds to Zn^{2+} in a 1:1 ratio where the Zn attaches within its two domains and stabilizes AdcAII[48]. The basic structure of AdcAII has been described along with AdcA[48, 51, 52]. The α-helix linkage between the $(β/α)_4$ contains that binds Zn^{2+} consists of histidine and glutamine residues which are His71, His147, His211 and Glu286 each with a bond distance of 1.99 Å, 2.04 Å, 2.08 Å and 2.08 Å respectively. The N-terminal and C-terminal in AdcAII, unlike AdcA, does not contain the histidine rich loops and thereby does not conform to the general zinc MBR characteristics[48].

Studies have shown that Δ*adcAII* mutant has great loss of intracellular zinc concentrations. Mutants that lack both *adcA* and *adcAII* demonstrated morphological defect in zinc deficient conditions. Using Cryo-EM the morphological changes were studied, and it was observed that *S. pneumoniae* in zinc depleted conditions were severely deformed consisting of asymmetrical septa, which then resulted in unequal divisions of bacteria[47, 55]. The single mutants showed a 47-51% reduction in zinc uptake while double deletions of genes resulted in a reduction of 65%-76%. Even in the presence of PhtD in the mutants, which is a zinc scavenger protein in *Streptococcus pneumoniae*, both AdcA and AdcAII account for most of zinc acquisition. In the study, it showed that even though the functions of *adcA* and *adcAII* are redundant, they use the same transmembrane permease protein AdcB to transport zinc. It also shows that with double mutation, even though the growth is substantially low, addition of zinc can reaffirm the growth of the mutant strain[47]. In another study however, chelators were used to test the expression of

adcA and *adcAII* under Zn^{2+} limiting conditions. It was shown that in an Δ*adcAII* mutant, the upregulation of *adcA* was 6-fold in the presence of TPEN while the upregulation of *adcAII* was 80-fold in an Δ*adcA* mutant. This demonstrates that the transcription of *adcAII* is more sensitive to Zn^{2+} concentrations in the environment[55].

AdcA in *S. pyogenes*: The structure and zinc binding specificity of AdcA in *S. pyogenes* is yet to be exclusively characterized but is understood to be a close 61% homologue to the zinc binding AdcA of *S. pneumoniae*. AdcA in *S. pyogenes* like its pneumococcal counterpart has a histidine rich N-terminal that plays important role in zinc binding[57]. N-terminal of AdcA in *S. pyogenes* shares a 43.9% homology with the N-terminal of Bsu-YcdH in *Bacillus subtilis*[56]. The C-terminal extensions of AdcA in *S. pneumoniae* and Spy9 in *S. pyogenes* are shown to have homology with a cadmium induced zinc binding chaperone called YodA, also known as ZinT[48, 58]. YodA is related to the lipocalin/calycin family. It consists of a common up-down β-barrel structure which is the common feature of lipocalins and a helical domain which consists of histidine side chains. The metal binding site of YodA is available at the interface of the helical domain and calycin domain[59]. Since, the structure of AdcA has been established to be highly conserved among all C-terminal domain and AdcA in *S. pyogenes* is homologous to its pneumococcal C-terminal domain, we can hypothesize that the structure of YodA is crucial to understanding AdcA in *S. pyogenes* in the future[48, 56, 59]. In GAS, Δ*acdA* mutants exhibit downregulation in growth during zinc starvation in the presence of CP[60]. The paper shows that Δ*adcA* mutants are hypersensitive to zinc starvation compared to the WT strain. Studies also show that the levels of Lmb/Lsp and HtpA/ PhtD (zinc storage and scavenger protein) increases in Δ*adcA* mutants as an adaptive measure for zinc sequestration[61].

Lsp in *S. pyogenes*: Compared to *S. pneumoniae*, the α-hemolytic counterpart of *S. pyogenes*, its import and export mechanisms has not been as similarly studied. However, in GAS, we know that AdcR aids in the transcription of an extracellular solute-binding lipoprotein known as Lipoprotein signal peptidase (Lsp) that binds to zinc and aids in the uptake of divalent cations[24, 25]. Lsp is also known as Laminin binding protein (Lmb) or Lipopolysaccharide binding protein (Lbp)[9]. It is the homologue of AdcAII from *S. pneumoniae*[57]. Lsp, like AdcA and AdcRII falls under the cluster 9 family of lipoproteins that conducts zinc homeostasis in *S. pyogenes*[25]. Within the cluster 9 family, Lsp falls under the sub-class of LraI family which stands for Lipoprotein receptor antigen I[62, 63]. LraI is found at the bacterial surface anchored to the membrane like most other cluster 9 SBDs[25, 64]. The anchoring of Lsp to the membrane occurs due to the binding characteristic of lapidated peptidase II sequence containing cysteine at residue 20 which is an important common feature in LraI family of proteins[25, 63]. Some other common features of LraI proteins are: i) β1-α-β2, three domain structure, ii) amino acid size of 300-330 and iii) N-terminal consisting of cysteine that anchors the protein to the membrane[63]. ABC transporter system takes the assistance of LraI lipoproteins or SBDs that is transcribed in various streptococcal species and is known to bind different trace metals including Zn^{2+} to transport through the ABC protein complex[64]. Through an X-ray emission analysis and by checking the metal content, it was suggested that Fe^{2+}, Cu^{2+} and Zn^{2+} uptake were associated with the lipoprotein in GAS[65]. Mutation of *mstA*, which is ABC transporter gene under LraI proteins obstructs iron and zinc uptake[65, 66]. However, *S. pneumoniae* demonstrates two LraI operons are *psaBCAD* and *adcCBA* where mutations in *adcC* and *psaA* resulted in increased requirement for Zn and Mn respectively[46, 66].

To test zinc sensitivity of Lsp, several growth assays were conducted under different concentrations of TPEN, that is known to cause zinc starvation. It was noted that *lsp* lacking mutants demonstrated hypersensitivity to zinc compared to its WT counterpart. Studies have shown that when GAS mutants with a *lsp* deletion were injected in mice, 30% of mice exhibited lesions related to murine soft tissue infection which suggested that *lsp* depletion results in lower uptake of zinc from the host. When mice were infected with wild type (WT) strain, all mice exhibited lesions. This suggests that *lsp* upregulation correlates to GAS pathogenesis in soft tissue infection. The association of lack of Lsp to hypersensitivity was further solidified when the addition of a plasmid containing WT *lsp* copy improved conditions of hypersensitivity. When changing the position of Lsp with an alanine allele, the new *lsp* mutant showed more sensitivity than its cysteine positioned WT suggesting that the placement of Lsp on the membrane signifies its effective function[25].

The *lsp* operon is located upstream to fibronectin, *ORFX/fba* and downstream to dipeptide permease, *dppE*[63]. The three-dimensional structure of Lsp contains a protruding α-helix that comprises of the solute binding surface of the protein while two globular domains connected by the α-helix is comprised of four β sheets surrounded by four α-helices[57, 62]. Lsp captures Zn^{2+} in a 1:1 ratio in the interface between the two globular domains where the ion is surrounded by histidine and glutamate residues. The histidine residues His66, His142, His206 are connected to Zn^{2+} by a bond distance of 2.1 Å. The glutamate residue E281 binds within a range of 2.0- 3.1 Å. The residues also form a weak hydrogen bond with each other ranging from 2.6-3.2 Å. Contrastingly, the metal binding sites of AdcAII from *S. pneumoniae*, homologous to Lsp is not histidine rich and do not display highly charged residues. It is demonstrated that their structures are almost identical and binding sites are superimposable[57]. Lsp has also been identified as an

adhesion protein that mediates the adhesion of GAS to host epithelial cells causing diseases like impetigo, scarlet fever, pharyngitis etc[67].

4.2.3 CzcD: Zn exporter in S. pneumoniae and S. pyogenes

CzcA Zn Efflux: As described earlier, SczA regulates *czcD* transcription to facilitate zinc efflux mechanisms in *S.pneumoniae*. Similarly, paralogous to SczA, protein sensor GczA regulates *czcD* transcription in *S. pyogenes*, which also facilitates zinc efflux in the system. CzcD falls under the cation diffusion facilitator (CDF) family that involves zinc resistance and can be induced by Zn^{2+}, Co^{2+}, Cu^{2+} and Ni^{2+} for transcription[8, 32]. In both gram-negative and gram-positive bacteria, CzcD is a heavy-metal-ion-resistance determinant where it not only resists Zn^{2+} but also metals like Co^{2+} and Cd^{2+} using the *czcDCBA* operon[32]. Studies have shown that Δ*czcD* mutant exhibits heavily decreased resistance to zinc compared to the WT strain[32]. Deletion of *czcD* exhibited decreased transcription of the CzcDCBA transport system most likely due to the increase of heavy divalent cations[31, 32, 68].

Conclusion

Streptococcal infection is diverse and continued research on metal sensors, protein transporters, chaperones, etc. can build the foundation towards a well characterized physiology. So, far the studies on proteins have been emerging but there is still more to unravel. Zinc with its high binding specificity and critical involvement in a series of metabolic and virulence pathways seems to be highly conserved in its import and export systems. While metal like iron has a huge panel of uptake systems, zinc only has one high affinity importer system. This makes zinc specific proteins important targets and ideal candidates for the development of novel antimicrobial therapies. AdcR, zinc mediated protein regulator is a conserved protein sensor for

uptake of zinc throughout all streptococcal species. While the uptake systems are well characterized, not enough studies have been done on zinc efflux regulators have been conducted in *S. pneumoniae* and *S. pyogenes*. PerR regulated P_{1B-4}-Type ATPase (PmtA), initially thought to be a zinc efflux an efflux regulator was then characterized for iron regulation in *S. pyogenes* or GAS[10, 35]. Even though the homology of AdcA is widely analyzed, the exclusive study of the structure of AdcA in *S. pyogenes* is yet to be characterized as well. The Pht proteins have been a recent source of interest for potential pneumococcal vaccines with their defining feature, the HxxHxH triad motif where PhtD is considered to be the most promising target due to it being the most conserved throughout all pneumococcal strains[69]. Different studies have also emphasized targeting individualized ABC transporter systems in streptococcal species as potential target for antimicrobial drugs and vaccines. Understanding host nutritional immunity in correspondence to bacterial pathogenicity and studying target protein complexes can open exciting options to combat and target emerging challenges in drug and vaccine discovery in streptococcal infections and chronic diseases.

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