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REVIEW

Metalloproteomics: Unraveling the Metal Binding Proteins of Diverse Metal-Resistant Bacteria

N. JAMUNASRI, ASWETHA IYER, M. LAKSHMI PRABHA, REYA ISSAC* and S. MURUGAN*

Division of Biotechnology, Karunya Institute of Technology and Sciences, Coimbatore-641114, India

*Corresponding authors: E-mail: reya@karunya.edu; murugan@karunya.edu

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The release of metals into the environment raises serious concerns about their harmful effects on both the wildlife and human health. The biosphere is experiencing with the pervasive presence of heavy metal pollutants such as arsenic (As), cadmium (Cd), mercury (Hg), lead (Pb), chromium (Cr), copper (Cu) and nickel (Ni), which pose significant environmental challenges. While certain metals are essential for regulating fundamental metabolic processes and upholding the overall physiology of microorganisms, excessive exposure to heavy metals can be detrimental to their survival and function. As a result of their remarkable adaptability, microorganisms, particularly bacteria such as *Pseudomonas fluorescens, Escherichia coli*, *Serratia marcescens*, *Bacillus cereus* and *Alcaligenes* sp., have evolved sophisticated defence mechanisms to combat the stress caused by heavy metals. One such process is the creation of metal-binding proteins (MBPs), which may bind and sequester metals, thus significantly lowering their toxicity in bacteria. Metalloproteomics, a subfield of metallomics, focuses on the discovery and characterization of such metal-binding proteins (MBPs) in metal-resistant bacteria, resulting in the opening of the doors for innovative bioremediation techniques and therapeutic treatments against bacterial diseases. This review explores the intriguing world of MBPs in metal-resistant bacteria and emphasizes their significant role in metal resistance, detoxification and homeostasis. Furthermore, metallochaperones in bacteria have been extensively studied using the metalloproteomic methodologies and techniques utilized in metal-binding proteins. This study also provides useful information on the interactions between these metallochaperones and different MBPs, which advances our understanding of how bacteria respond to exposure to such heavy metals.

Keywords: Bacteria, Bioremediation, Metalloproteomics, Metal-binding proteins, Detoxification.

INTRODUCTION

The heavy metals are toxic and persistent in the natural environment, leading to increased contamination throughout the environment. In view of this, a widespread research attention has focused on heavy metal pollution. An inorganic element with an atomic density of more than 5 g cm^{-3} is referred to as a heavy metal [1]. Although heavy metals are found naturally in the environment, anthropogenic activities like extraction, melting and industry process have raised the amount of pollution caused by such metals, making them dangerous to living things [2]. Heavy metals can be divided into two types: essential and non-essential (Fig. 1) [3]. Many biological activities depend on certain heavy metals, including iron (Fe), manganese (Mn), copper (Cu), chromium (Cr), cobalt (Co), magnesium (Mg), zinc (Zn) and nickel (Ni). Because these metals are necessary for 'life' they are characterized as essential elements. However, other heavy metals, such as arsenic (As), beryllium (Be), tin (Sn), cadmium (Cd), lead (Pb), mercury (Hg), selenium (Se), silver (Ag), thallium (Tl), vanadium (V) and zinc (Zn) can be harmful if their exposure exceeds certain levels, leading to a number of health issues, including renal disease, cancer and central nervous system disorders [4].

The recycling of heavy metals in the environment is significantly influenced by bacteria. Certain bacteria can withstand the effects of heavy metals and store them in their cells [5]. The resistance and accumulation of heavy metals by bacteria are also used for a variety of applications, such as bioremediation of contaminated sites and also as biosensors for detecting the presence of such heavy metals in plants [6]. Metal-resistant bacteria (MRB) have been studied extensively for their potential in bioremediation and their capacity to lower the amount of heavy metals present in polluted places [7]. An overview of the intercellular process shown in MRB have shown in Fig. 2

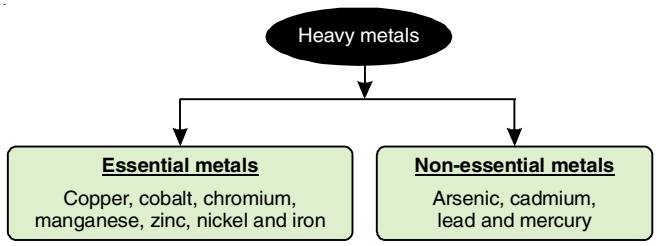

Fig. 1. Heavy metals: essential and non-essential

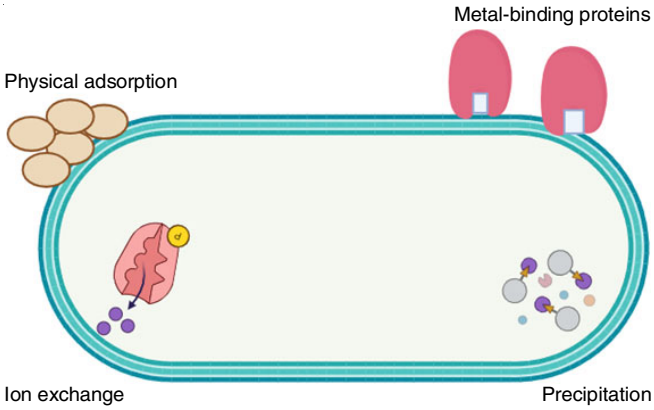

Fig. 2. Metal sequestration in bacteria

about intercellular process. Several bacterial species, including *Pseudomonas fluorescens, Bacillus cereus*, *Escherichia coli*, *Serratia marcescens* and *Alcaligenes* sp. have demonstrated remarkable resistance to a diverse array of heavy metals such has Hg, Cu, Cd, Pb, Cr and Ni. Plasmid-cured *Bacillus* species have also been shown to possess heavy metal resistance, indicating that resistance can be mediated by both plasmid and chromosomal DNA [8]. Other metal-resistant bacteria, such as *P. aeruginosa*, *Paenalcaligenes faecalis*, *Bordetella petrii* and *Paenalcaligenes hominis*, have showed to have high resistance to heavy metals such as Pb, Ni, Cr and Cd. These metal-resistant bacteria have the potential to be used in the biological treatment of wastewater containing heavy metals, offering a promising approach for remediation techniques [9]. In addition, metal-tolerant rhizospheric bacteria, such as *Novosphingobium humi*, *Cupriavidus basilensis*, *Bacillus zanthoxyli*, *Paenibacillus alvei* and *Ralstonia syzygii* have been isolated and shown to enhance plant growth and tolerance to heavy metals like Cu and Fe [10].

Metal binding proteins (MBPs) have the ability to bind and accumulate heavy metals, such as Cu, Zn and Cd [11]. MBPs consist of histidines $(ghhphg)_2$ (HP), cysteines (gcgcpcgcg) (CP), metallothioneins (MTs), phytochelatins (PCs) and Cd-binding peptides (CdBPs) [12]. The ability of bacteria to bind metals, as well as their accumulation and tolerance to heavy metals, can be increased by the expression of certain genes encoding for such MBPs [13]. In addition to MBPs, bacteria emphasize binding protein production, exclusion, compartmentalization and complex rendering process have been emphasized by bacteria for withstanding the stress of heavy metals. Transcription factors called metalloregulatory proteins control the expression of certain gene groups called regulons and metal sensing regulatory transcription factors control these regulons. Metal-responsive transcription factors, such as Zur and MTF1, in bacteria and eukaryotes, respectively, are essential for maintaining metal homeostasis in them [14,15]. These proteins bind to metal ions and regulate the transcriptional activity of target genes involved in metal uptake, efflux and intracellular trafficking [15]. In bacteria, Zur regulons not only control the expression of metal uptake systems but also biosynthetic clusters, ribosomal proteins, enzymes and virulence factors. The metal sensing transcription factors in *E. coli* form a complex regulatory network that allows the bacteria to adapt and survive in different environments, including inside the host animals. The metal-dependent gene expression is crucial for maintaining cellular metal ion homeostasis and preventing the harmful effects of reactive oxygen species (ROS) [16].

A diverse array of bacterial metalloregulatory proteins, including ArsR-SmtB, MerR, CsoR-RcnR, CopY, DtxR, Fur and NikR, play vital roles in mediating heavy metal resistance and these are involved in detecting and responding to different metal ions [17]. These proteins can regulate gene expression directly associated with metal homeostasis and alter metabolism to reduce the cellular demand for metals. In addition to metalloregulatory proteins, bacteria also utilize metallochaperones, a specialized protein group that shields metal ions from reacting and distributes them to target metalloproteins. Riboswitch RNAs have also been discovered as metalloregulatory elements that selectively bind low-abundance transition metal ions, such as Ni^{2+} and Co^{2+} [18]. These riboswitches bind metals cooperatively and have a high affinity for metal ions. Metal-binding proteins have the ability to interact with heavy metals accumulated in bacteria, which could help in the reduction of heavy metal contaminants in wastewater [19].

Metalloproteomics is a field that focuses on identifying and characterizing MBPs and motifs in bacteria [20]. Several studies have been conducted to investigate metal binding in bacteria that are resistant to metals. In one of the study, metalloproteomic analysis of *Streptococcus pneumoniae* identified putative MBPs and peptides, with a focus on copper and zinc binding [21]. In 2021, studies revealed that these metal-binding proteins are involved in a number of biological processes and might have an impact on bacterial diseases. Another study analyzed metal ions in *Mycobacterium* species and identified metal transporters using *in silico* analysis [22]. Metallothioneins (MTs) are a collection of proteins that have a part in metal binding which possess detoxification mechanisms in microorganisms. These proteins have been extensively studied using various methods such as western blot and high-performance liquid chromatography (HPLC). The study of MBPs in MRB provides a profound understanding of the mechanisms of metal resistance and the interconnections between metals and proteins. The computational and experimental approaches play crucial roles in understanding the structure, function and interactions of MBPs through metalloproteomics. Future research in this field holds the promise of new bioremediation strategies, the creation of innovative MBP-based applications and an improved understanding of metal resistance processes in bacteria.

Origin of heavy metals in the environment: Naturally occurring elements with high atomic mass and concentrations

are known as heavy metals [23]. They can be found in various environmental matrices including air, water, soil and sediments. The origin of heavy metals in the environment can be attributed to both natural and anthropogenic sources [24]. Natural sources include weathering of rocks, volcanic activity, erosion and biological processes. Anthropogenic sources include industrial activities, agricultural practices and urbanization [25]. The heavy metals are transported and distributed and their persistence is determined by these two factors (Fig. 3).

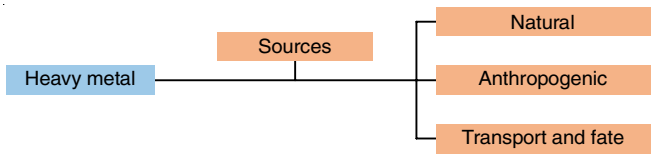

Fig. 3. Sources of heavy metals

Metal resistant bacteria: Metal-resistant bacteria (MRB) are a group of microorganisms that possess the capacity to endure and develop in environments where contamination with heavy metals occurs frequently. These bacteria possess unique genetic and physiological adaptations that allow them to tolerate and detoxify heavy metal ions, which are toxic to most other organisms. Bacteria develop resistance through various mechanisms such as efflux, sequestration and transformation within the cell. Bacteria with metal resistance are advocated for metal removal applications due to their rapid growth rates. Indigenous bacterium from cultivable land such as *Bacillus tropicus* MCCC 1A01406, has shown resistance against multiple heavy metals and have the potential for eco-friendly recovery of heavy metal pollutants [26]. The impact of heavy metal pollution on microbial communities and the evolution of metal resistance determinants are still being studied and there is a need to better understand the microbial metal resistome at the community level [27]. One of the main mechanisms by which the metal resistant bacteria can withstand heavy metal toxicity is through the production of metal binding proteins [28]. These proteins, known as metallothioneins are small, cysteine-rich proteins that can bind to heavy metal ions such as Cd, Hg, Pb and Zn have a high affinity for heavy metals and can sequester them, preventing their harmful effects on cellular processes [29]. They are synthesized in response to heavy metal exposure and play a crucial role in protecting the bacterial cells from metal-induced damage. One such strategy is the efflux of heavy metals from the cell. Bacteria possess efflux pumps that actively transport heavy metal ions out of the cytoplasm, thereby reducing their intracellular concentration [30]. This efflux mechanism helps in maintaining cellular homeostasis and prevents the accumulation of toxic levels of heavy metals. Members of MRB can also undergo genetic adaptations to develop resistance against heavy metals [31]. This includes mutations or acquisition of genes that encode for specific transporters or enzymes involved in heavy metal detoxification. For example, some bacteria have acquired plasmids (small circular DNA molecules) that carry genes encoding proteins that are resistant for specific heavy metals. These plasmids can be transferred between bacterial cells through horizontal gene transfer, allowing the spread of metal resistance genes within the microbial communities [32].

The presence of metal-resistant bacteria has been observed in various environments contaminated with heavy metals, including industrial sites, mining areas and polluted water bodies [33]. These bacteria are often found in close association with metal rich minerals or sediments, where they can form biofilms or aggregates that provide protection against metal toxicity. Biofilms are complex communities of microorganisms embedded in a matrix of extracellular polymeric substances (EPS), which offer physical and chemical protection against heavy metals [34]. Metal-resistant bacteria have attracted significant attention due to their potential applications in bioremediation. Bioremediation is a process in which living organisms are utilized to degrade or remove pollutants from contaminated environments. Metal-resistant bacteria can be used to remediate metal-contaminated sites by either immobilizing heavy metals through biosorption or by actively transforming them into less toxic forms [35].

Biosorption involves the binding of heavy metals to the cell surface or to extracellular polymeric substances of bacteria, effectively removing them from the environment. On the other hand, some metal-resistant bacteria have enzymatic activities that can convert toxic heavy metal ions into less harmful forms through processes such as reduction, oxidation, or methylation. Metal homeostasis and resistance in bacteria are complex and delicate balance. Bacteria have evolved various mechanisms to maintain metal homeostasis, including import and mobilization pathways for metal limitation and efflux and storage pathways for excess metals [36]. Metal homeostasis systems in pathogenic bacteria are crucial for resisting host efforts to manipulate metal availability and toxicity. Metalloregulatory proteins, metallochaperones and related proteins play key roles in regulating metal speciation, buffering intracellular metal concentrations and delivering metals to correct intracellular targets [37]. Bacteria have also developed resistance determinants to enhance tolerance to toxic metals, such as Cd and As [38]. These resistance determinants often co-selected with other types of resistance and vary across different Gram-positive bacteria. The evolution of copper resistance mechanisms in bacteria, such as the *cus* and *pco* systems, has been influenced by human activities and environmental copper deposition [39]. Copper may exist in two different redox states (oxidized as Cu(II) or reduced as Cu(I), which makes it a very useful as a catalytic factor in proteins that do electron transport or redox reactions. It is generally recognized that Cu(I) may have harmful consequences in addition to being beneficial as a cofactor, while the exact chemical pathways behind these effects are unclear [40]. The study of metal-resistant bacteria has provided deep knowledge about the mechanisms of microbial metal resistance and the potential for bioremediation of metal contaminated environments. Studying the adaptation and survival mechanisms of these bacteria in the presence of heavy metals may help in developing strategies for environmental cleanup and enhancing human health in areas impacted by metal contamination.

Mechanisms for heavy metal resistance in bacteria cells: Bacteria have evolved a variety of mechanisms to resist heavy

metals, which are toxic elements that can damage cellular components and disrupt essential biological processes. Numerous processes enable bacterial cells to be resistant to heavy metals. One method involves active or passive bioaccumulation of metal ions within the cell, extracellular precipitation and the outflow of heavy metals from the cell exterior to the surface of the microbial cell [13]. Furthermore, to withstand adverse conditions, metal-tolerant organisms use efflux mechanisms, cellular impermeability, external and internal cellular sequestration and metal-ion reduction [41]. These bacterial strains were capable of condensing metal ions. Strains reducing chromate (CrO_4^{2-}), vanadate (VO_4^{3-}) and molybdate (MoO_4^{2-}) ions were detected in the environment. In order to produce energy, the bacterial isolates used metal ions as electron donors. An example, strain of *S. aureus* that exhibits resistance to arsenic As^{5+}/As^{3+}. Additionally, bacteria have metal resistance genes that control the accumulation of heavy metals either on the genome or in plasmids [42]. These genes are essential for the intake, accumulation, mineralization, oxidation or reduction of heavy metals to less harmful forms by enzymes and their removal from cells. These mechanisms of heavy metal resistance in bacteria are of fundamental importance in microbial ecology and have applications in biogeochemical cycling of heavy metals. In biological systems, metal ions play crucial functions as metallobiomolecules or occasionally as free ions. As parts of metalloproteins and as cofactors or structural components for enzymes, metal ions are essential to several biological processes [43]. An overview of the evolution of heavy metal resistance action in bacterial cells as shown in Fig. 4.

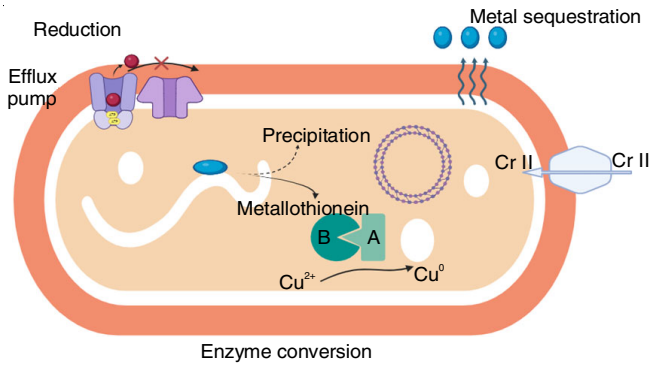

Fig. 4. Entry of heavy metals into the bacterial cell: 1) efflux, 2) sequestration, 3) enzymatic reduction, 4) methylation, 5) precipitation

Nearly half of all metabolic activities in bacteria are catalyzed by metal ion containing enzymes, which are responsible for cell maintenance and are important for metal homeostasis at levels that are both high enough to fulfill cellular demands and low enough to prevent toxicity [44]. Bacterial metal ion resistances are widespread and include genes for toxic metal ion resistances such as Ag^{+}, AsO^{2-}, Cd^{2+}, Co^{2+}, CrO_4^{2-}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, TeO_3^{2-}, Tl^{+} and Zn^{2+} [45]. These resistance systems function through energy-dependent efflux, enzymatic transformations or MBPs. Metal ions, such as Zn(II), are essential for bacterial growth and virulence factors, but their sequestration by the host can limit bacterial infection processes. Additionally, metal ions like Zn(II) are involved in antibiotic resistance mechanisms, such as the expression of metallo-β-lactamases (MβLs) that require Zn(II) ions for their activity [46]. The secretion of siderophores, which are tiny molecular weight molecules that are generated mainly made for ferric iron acquisition, is another way that metal resistance is mediated [47]. As multipurpose metabolites, siderophores can bind other metals outside of the cell and provide protection against metal toxicity. The synthesis of pyridine-2,6-*bis*(thiocarboxylic acid) (PDTC) siderophore by *P. stutzeri* were performed in the environment [48].

Metal-binding proteins for bacterial resistance: Proteins that can bind to metals help bacteria defend against the harmful effects of such metals. By capturing and preventing the interaction of metals, with cellular components these proteins safeguard the integrity of the cells. MBPs are compounds that have the ability to attach to a variety of metals such as Fe, Cr, Zn, As, Cd, Ni and Pb. Naturally occurring proteins, like phytochelatins and metallothioneins contain a number of cysteine residues, which contribute to their ability to bind capability of binding metals [12]. Bacteria are able to live in surroundings with high levels of heavy metals because of this detoxifying process. Depending on the kind of protein and the metal ion involved, different MBPs are involved in executing specific mechanism. Betaproteobacteria is resistant to a wide range of metals, including As, Cd, Cr, Pb, Hg and Zn. This resistance is due to the presence of a number of genes that encode proteins that can detoxify these metals [49]. An example of a MBPS with Betaproteobacteria is shown in Fig. 5.

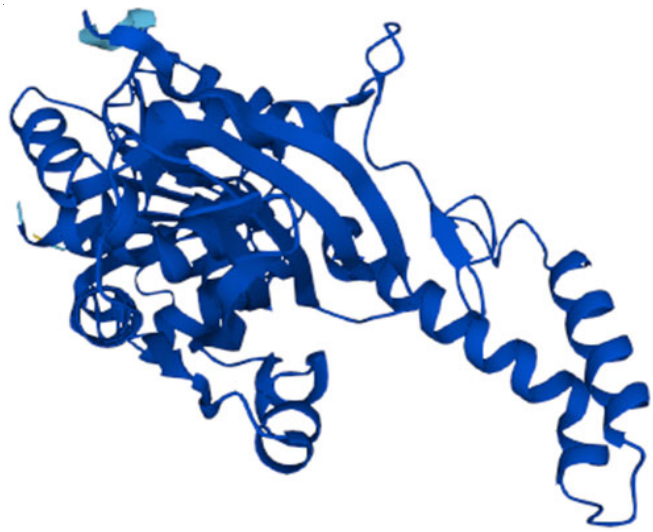

Fig. 5. Aspartate-semialdehyde dehydrogenase protein, encoded by the asd gene, in the betaproteobacteria bacterium *HGW-Betaproteobacteria-16* that contains 376 amino acids and is involved in metal binding (https://www.uniprot.org/uniprotkb/A0A2N2TB33/entry)

Arsenic-binding protein: In the thermophile *Thermus thermophilus* HB27, the adjacent genes TtsmtB and TTC0354 are involved in arsenic resistance and are transcribed from the specific promoters with differential regulation at the transcriptional level [50]. Several types of membrane-bound transporter proteins such as ArsB, ArsY (ACR3) and ArsP. Bacterial arsenic transporters includes the major facilitator protein superfamily

(MFS) and major intrinsic protein (MIP) [51]. These transporters are encoded in arsenic resistance (Ars) operons found in bacterial strains. Additionally, other proteins involved in arsenic resistance, such as ArsK, have been also discovered. ArsK is a suspected major facilitator protein superfamily (MFS) gene that functions as an efflux transporter for trivalent arsenicals and antimonials.

Cadmium-binding proteins: Cadmium-resistant bacteria have been found to possess various mechanisms for binding cadmium. One mechanism involves the efflux of cadmium from bacterial cells through the action of efflux pump proteins encoded by the gene systems cadA and cadB gene synthesis [52]. Another mechanism involves the binding of cadmium to functional groups such as NH_2, COOH, PO_4^{3-} and OH present on the bacterial surface. Enzymes are also involved in detoxifying cadmium and making the bacterial membrane impermeable to the metal. The protein All3255 of *Anabaena* sp. PCC7120 has been identified to have a CadD efflux pump that is involved in cadmium resistance and other heavy metals [53]. Cadmium stress has been shown to result in different protein expression in *Acinetobacter calcoaceticus* strain STP14, exhibiting resistance to cadmium and other metals [54]. In response to cadmium stress, *Lactococcus lactis* has been shown to activate antioxidant capacity and up-regulate cadA, suggesting a role in cadmium resistance [55]. A subset of the EF-hand calcium-binding S100 proteins is released extracellularly and, *via* metal sequestration, contributes significantly to host defense by acting as an antibacterial agent [56]. S100A8 and S100A9 form a heterodimer to form the S100 protein complex calprotectin, which binds and sequesters zinc, manganese, iron and nickel [57].

Copper-binding proteins: Acidophilic organisms like *Acidithiobacillus ferrooxidans* possess high-level resistance to Cu and other metals. Transcriptional expression of copper-binding proteins, such as CopZ like chaperone, rusticyanin and AcoP, increase in the presence of Cu and these proteins confer resistance against copper when expressed in a Cu-sensitive strain [58]. In *Enterococcus faecium* and *Escherichia coli*, copper affects the stability of the bacterial cell envelope and counteracts β-lactam resistance mediated by β-lactam-insensitive LD-transpeptidases (LDTs) [59]. Bacterial Cu storage proteins, such as Csp3, prevent Cu toxicity and allow bacteria to safely accumulate large quantities of copper in their cytosol [60]. Copper resistance in *Streptococcus pneumoniae* develops through the use of differently expressed proteins involved in cell wall [61]. Another set of Cu-resistant proteins like PcoC and CopC exhibit distinct copper chemistry but both have His1 as a bidentate ligand and are involved in intermolecular Cu transfer reactions.

Mercury-binding proteins: MerA is a Hg-binding protein that is produced by the metal-resistant bacteria *Alcaligenes eutrophus*, which is able to detoxify mercury. Mercury-resistant bacteria possess the Mer operon system, which plays a crucial role in Hg biodegradation and bioremediation by converting reactive forms of Hg to inert, volatile forms [62]. The MerA protein in Hg-resistant bacteria involved in the transformation of Hg^{2+} to Hg^0 and its sequences and conserved motifs vary widely in both Gram-positive and Gram-negative bacteria [63]. Another protein called MerT in the Mer operon, is involved in mercuric ion (Hg_2^{2+}) transport. Bacteria resistant to Hg was isolated from a gold mining area were resistant to Hg and exhibited changes in protein synthesis after Hg induction [64].

Lead-binding proteins: PbrD is a Pb(II) binding protein derived from the Pbr lead resistance operon found only in *Cupriavidus metallidurans* CH34. Its ability to sequester Pb(II) presents a great potential for development as a lead biosorbent in wastewater bioremediation [65]. Techniques like attenuated total reflection have been used to confirm the existence of these functional groups such as carboxyl, phosphate and amide are studied using FTIR [66]. In addition, P-O-Pb and C-O-Pb interactions are formed during the binding of lead to bacterial cells. The development of efficient microbial lead remediation solutions can be facilitated by an understanding of the molecular basis of lead binding in bacteria [67]. It has been discovered that the lactic acid bacterium *Lactobacillus plantarum* YW11 has a great capacity to both absorb and withstand lead, with an absorption rate of up to 99.9% [68]. Abundant proteins involved in a variety of biological activities, including as substance transport and cell wall production, were identified by proteomics analysis of YW11.

Zinc-binding proteins: Several studies have investigated the binding properties of these proteins and their impact on bacterial survival. In 2022, Rosen *et al*. [69] demonstrated that the solute-binding proteins (SBPs) called PsaA and MntC in *Streptococcus pneumoniae* and *Staphylococcus aureus*, respectively, bind Zn reversibly and exhibit a preference for Zn ions over other metals. Researchers have uncovered several mechanisms by which bacteria resist zinc toxicity. In 2016, Colaço *et al.* [70] reported that protein ZinT in *E. coli* plays a role in resistance to cobalt, cadmium and mercury. Another study found that knockout mutants of ribosomal proteins in *E. coli*, including RpmJ, RplA, RpmE, RpmI and RpsT, exhibited zinc resistance, suggesting a close connection between ribosomal proteins and zinc resistance [71]. Moreover, Li *et al.* [72] reported that zinc limitation induces ribosome hibernation and aminoglycoside resistance in mycobacteria, highlighting the significance of zinc binding proteins in bacterial survival under zinc-depleted conditions. Furthermore, Neupane *et al*. [73] elucidated the structural and mechanistic details of Zn binding by the Zn-specific SBP AztC, which is crucial for Zn acquisition from the metallochaperone AztD.

Metallothioneins: Structural and functional insights: Metallothioneins (MTs) are low molecular weight protein family that is abundant in cysteine and has a remarkable affinity for various metal ions. Their exceptional ability to chelate a wide range of metals, including both essential and non-essential elements, has allowed them to play a critical role in metal homeostasis [74]. By sequestering excess metal ions, MTs safeguard cells from the deleterious effects of metal toxicity. Their primary function is to store, transport and bind metals, which enables microorganisms to detoxify heavy metals. Pioneering the identification of metallothioneins (MTs), *Cyanobacterium synechococcus* harbors the SmtA protein, which exhibits a robust affinity for Zn and Cd, thereby safeguarding cellular

metal homeostasis [75]. Also, MTs are essential for maintaining Cu homeostasis in yeast because they chelate excess Cu ions to help store copper and prevent copper toxicity. Two MTs from *Saccharomyces cerevisiae*, CUP1 and Crs5, predominantly bind to Cu but can also bind to Zn and Cd ions. A diverse group of proteins called cysteine rich protein (CRPs) are characterized by a high abundance of cysteine residues, which form thiol groups that can bind to metal ions. Recent studies using a functional metatranscriptomic approach have identified new families of eukaryotic CRPs involved in resistance to Cd or Zn [76]. Several of these genes, when introduced into Cd-sensitive yeast mutants, were able to restore Cd resistance, suggesting their potential role in cellular detoxification [77]. An example of a metalloprotein like structure was retrieved from PDB chains [SCOP 1.75] (Fig. 6).

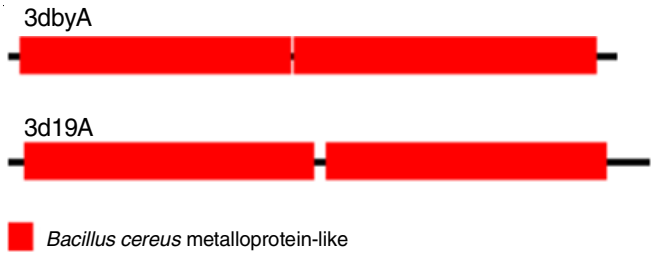

Fig. 6. Domain assignments for the *Bacillus cereus* metalloprotein-like superfamily in PDB chains (SCOP 1.75)

Metallochaperons: A family of metalloproteins known as metallochaperones are responsible for binding metal ions and transporting them along the absorption, functional, storage, or detoxifying routes. Through certain protein–protein interactions, they introduce metal ions into other proteins [78]. One significant class of metallochaperones that is broadly dispersed throughout all domains of life are nucleoside triphosphate hydrolase (NTPases). These proteins perform extremely particular and controlled roles in the metalloenzyme maturation process by binding and hydrolyzing nucleoside triphosphates, which can be either adenosine triphosphate (ATP) and guanosine triphosphate [79]. Bacterial metallochaperones are involved in the control of intracellular metal availability, metallocofactor active site assembly in metalloenzymes and the systems-level response to metal limitation and intoxication [80]. Zinc import by *E. coli* and other bacteria at low ion concentrations is facilitated by metallochaperones of the TroA superfamily, including ZnuA. The zinc transporter ZnuABC, which has been thoroughly characterized, includes ZnuA as a critical component [81]. ZnuA selects free zinc and transfers it to ZnuB, a transmembrane protein that transports Zn^{2+} into the cytoplasm; ZnuC mediates this process by providing the energy needed to complete the process.

Metalloproteomics: Metalloproteomics is the study of the identification and characterization of proteins in organisms that bind to metals, such as metal-resistant bacteria. The distribution of genes linked to heavy metal tolerance in bacterial genomes specifically in proteobacteria and terrabacteria have been the subject of several investigations [82]. These genes are often involved in metabolism, including ionic transport, amino acid biosynthesis and energy production [83]. Additionally, research has shown that metal-resistant bacteria have the ability to accumulate and remove metals from contaminated environments, such as dumpsite leachate and spent engine oil [84,85]. These bacteria have demonstrated high resistance to heavy metals, such as Pb, Cr, Cd and Ni and have been found to be effective in reducing the concentration of these metals in wastewater and contaminated soils [85]. Metalloproteomics also plays a crucial role in the structural and functional characterization of metalloproteins on a genome-wide scale [86]. Additionally metalloproteomics also brings together researchers from various disciplines and utilizes strategies and instrumentation such as mass spectrometry, Fourier transformed ion cyclotron resonance mass spectrometry (FTICR) and X-ray absorption spectroscopy (XAS) [86]. Therefore, metalloproteomics can be a valuable tool for studying the mechanisms of metal resistance in bacteria and for developing bioremediation strategies for metal-contaminated environments.

Approaches for studying metalloproteomics: Metalloproteomics encompasses two distinct approaches: experimental and computational-based methods. Standard genomic DNA, recombinantly produced proteins and subcellular fractions are analyzed using metalloproteomic methods [87]. Top-down and bottom-up methods are further separated. Top-down metalloproteomics is the process of first identifying metal-binding or metal containing proteins by experimentation and then identifying the coding genes. Bottom-up metalloproteomics is a bioinformatic technique to predict metal-binding or metal containing proteins from genomic databases and these methods are subsequently validated through experiments [20]. Inductively coupled plasma mass spectrometry (ICP-MS) has also been widely used in metalloproteomic techniques [88], The experimental methods include nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), X-ray crystallography and immobilized metal affinity chromatography (IMAC).

Experimental approaches

Inductively coupled plasma mass spectrometry (ICP-MS): A flexible analytical method used in the field of metallomics is inductively coupled plasma mass spectrometry (ICP-MS). It has been used in a number of fields, including metalloproteomics, spatial metallomics, single particle analysis and single cell analysis. ICP-MS can quantify many elements with low detection limits, which makes it appropriate for quantifying metalloproteins. Quantification of protein-bound, intrinsic or labelled metal/metalloid elements is possible through the use of this approach, which measures the concentration of certain elements in proteins. Furthermore, in the area of metallomics, ICP-MS may help provide established sources of reference for metalloproteins [89].

Immobilized metal affinity chromatography (IMAC): Immobilized metal affinity chromatography (IMAC) is widely used method in metalloproteomics [90]. This involves the use of metal-chelating agents to selectively bind and separate metalloproteins from complex mixtures [91]. Metal ions such as Ti^{4+}, Zr^{4+} and Ni^{2+} are commonly used in IMAC to form stable complexes with the target proteins [92]. The immobilized

metal ions provide a specific affinity for metalloproteins, allowing for their purification and enrichment. IMAC has been successfully applied in various applications, including the enrichment and identification of phosphopeptides, the purification of biomolecules such as lysozyme and the separation of metal chelating peptides from protein hydrolysates. The development of new crosslinking reagents, such as PhoX, has further expanded the capabilities of IMAC in proteome-wide characterization of protein structures and interactions [93].

Mass spectrometry (MS): In metalloproteomics, mass spectrometry (MS) is also an effective method for identifying and measuring low-concentration of metal containing compounds. It is particularly useful when combined with stable isotope tracers, which can be easily distinguished from their natural counterparts by mass spectrometry (MS). This technique has been applied in various metallomic studies, such as the preparation of isotopically enriched metalloproteins and the exploration of biological pathways associated with selenium species [94]. When coupled with inductively coupled plasma mass spectrometry (ICP-MS), it is useful to find the overall amounts of elements in biological samples. However recognition of compounds that bind to metals is not possible with ICP-MS. To overcome this limitation, ICP-MS has been combined with 2D-electrophoresis to determine which components are complexed with which proteins [95].

X-ray crystallography: Another effective technique for studying metalloproteins is X-ray crystallography, which enables high-resolution structural determination. X-ray crystallography has been used to investigate the binding of Pb^{2+} in metalloregulatory proteins such as PbrR, which have a high affinity for Pb^{2+} and selectivity over other heavy metals [96]. It can be used with spectroscopic methods to correlate the structure and function of metal-bound sites [97]. The X-ray absorption spectroscopy (XAS) has been applied in the field of metalloproteomics to characterize the structure and function of metal binding proteins [98]. The initial stage in X-ray crystallography is to obtain a suitable single crystal of the substance being studied. The crystal should be large enough (with all dimensions greater than 0.1 mm) to produce a measurable diffraction pattern. It should also be of high quality, with a pure composition, a regular structure and no significant internal defects such as twinning or cracks [99]. These defects can distort the diffraction pattern and make it difficult to determine the crystal structure.

Nuclear magnetic resonance (NMR): NMR spectroscopy has long been used as an important tool to study the structure and dynamics of macromolecules. Characterization of metalloproteins, identification of metal binding properties including number and type of ligands and metal-ligand geometry and mapping of structure and dynamics of metal binding are important to understand the biological functions of metalloproteins [100]. Paramagnetic nuclear magnetic resonance (NMR) has emerged as a vibrant and dynamic field within biomolecular NMR over the past two decades, proving to be an invaluable tool for characterizing metalloproteins. Metalloproteins constitute a substantial portion of the entire proteome and a significant fraction of these metalloproteins exhibit paramagnetic properties [101].

Computational approaches: Computational approaches have been developed to study metal-binding proteins in metalloproteomics. These approaches use a combination of theoretical and experimental methods to study the chemical reactivity, structure and function of metalloproteins. Scientists can better understand the intricate structure of metalloproteins and their function in biological processes by utilizing homologous proteins and Density Functional Theory (DFT) [102]. The computational approaches for metalloprotein research include methods for prediction and characterization of metal ion binding sites, as well as de novo design and remodeling of metalloproteins. These methods are crucial for understanding the diverse roles of metal binding proteins in cellular function and disease, as well as for developing therapeutic strategies targeting metalloproteins. In addition, deep learning architectures like convolutional neural networks have been used to predict protein metal binding sites, providing valuable information for understanding protein function and facilitating drug development. Numerous databases, including MINAS18, MESPEUS, BioLiP, MetalPDB and MDB, have been created to provide information on metal-binding proteins. The two most notable metalloprotein data sources used are as BioLiP and MetalPDB [103].

Metalloproteomics in metal binding proteins: Using the protein homology/analogY recognition engine v 2.0 (Phyre2) computer programme, the 3D structural modelling of a subset of proteins was completed [104]. The protein tertiary structure is shaped by Phyre2 using distant homology techniques based on HMM-HMM alignment. The modelled proteins with high throughput that satisfied the requirements of query coverage of 50% and confidence of at least 90% were chosen to be used in the structural metal ion binding pattern prediction. Using a fragment transformation strategy based server metal ion binding (MIB) site, the structural motifs were scanned for binding with metal ions [105].

Role of metalloproteomics in environmental bioremediation: Bioremediation is the process of using living or dead biomass to break down organic materials and eventually mineralize them into carbon dioxide, water, nitrogen gas and other substances. It can also be used to remove toxic elements from contaminated environments and remove harmful substances like heavy metals. Both *in situ* and *ex situ* methods can be used to apply the bioremediation process to soil and water environments [106]. Metalloproteomics plays a significant role in environmental bioremediation [107]. It makes it possible to investigate the way metals change the pathways of metabolism, essential for comprehending how organisms react to environmental challenges. Metallomics and metabolomics are two powerful omics techniques used to identify biomarkers that indicate hazardous environmental conditions [108]. These techniques involve the use of organisms as bioindicator to evaluate the biological response to contaminants. Additionally, the study of biomolecules containing metals and the detection of metabolites changes due to contamination are made achievable by the combination of chromatography with ICP-MS and organic MS. Metalloproteomic studies have also allowed for the characterization of metalloproteins and their application in environmental monitoring studies. Overall, metalloproteomics provides essen-

tial into metal uptake, trafficking, accumulation and metabolism in biological systems occurs contributing to the understanding and remediation of environmental pollution.

Applications of metalloproteomics in combating metal resistance and promoting

Bacterial bioremediation: Metalloproteomics has emerged as a indispensable tool in understanding metal resistance and promoting bacterial bioattenuation. It has been discovered that metal resistant plant growth-promoting bacteria (PGPB) increase the effectiveness of phytoremediation by encouraging plant growth, changing the bioavailability of metals in soils and lowering the toxicity of metals in plants [109]. Moreover, metal resistant PGPB can impact the formation of siderophores, extracellular polysaccharides (EPS), metallothionein and efflux systems, all of which support their adaptation and resistance mechanisms against the toxicity of heavy metals [110]. Furthermore, metalloproteomic methodologies have been developed to investigate metal-protein interactions at a proteome-wide scale, providing insights into metal homeostasis and the molecular mechanisms of metallodrugs in biological systems [109]. These advances in metalloproteomics can contribute to the development of more efficient bioremediation technologies, including the use of genetically modified microbes for metal removal [111]. By applying this knowledge into the fundamental mechanisms and raising up the possibilities for new approaches, metalloproteomics is an essential tool for reducing metal resistance and improving bacterial biological remediation.

Future directions and challenges in metalloproteomics: The increasing prevalence of antibiotic resistance, fueled by widespread misuse and mismanagement, necessitates the exploration of novel approaches to combat bacterial infections. Metal and metal oxide nanomaterials have emerged as promising candidates in this domain, exhibiting potent antibacterial and antibiofilm properties. Their antibacterial mechanisms encompass a range of cellular disruptions, including membrane damage, protein destabilization and nucleic acid interference [51]. However, the emergence of metal-resistant bacteria, such as *E. coli*, *Pseudomonas* sp. and *Serratia marcescens*, poses a significant to the widespread adoption of these nanomaterials. Metalloproteomics, the study of metal-containing proteins, offers a great lens to dissect the intricate mechanisms underlying bacterial heavy metal resistance [112]. By elucidating the roles of metalloproteins in metal detoxification, transport and homeostasis, researchers can identify novel targets for therapeutic intervention. This approach holds the potential to circumvent the limitations of conventional antibiotics and mitigate the threat of antibiotic-resistant bacterial infections. Effective strategies for remediating heavy metal-contaminated sites necessitate a comprehensive understanding of the physico-chemical parameters of the environment, the structure and diversity of microbial communities and the nature and concentration of heavy metals. This knowledge is crucial for tailoring remediation strategies to specific characteristics of each contaminated site. By harnessing the insights gained from metalloproteomic studies, researchers can design targeted remediation approaches that effectively neutralize heavy metal contaminants and restore ecological balance. Additional investigations is required to examine the redox reactions and cross-reactivity processes among the synthesis of metallothionein, formation of extracellular polysaccharides, production of siderophores and efflux systems of bacteria resistant to metals. The patterns of resistance that bacterial isolates have against heavy metals are summarized in Table-1, and the different mechanisms that are involved in this resistance are described in Table-2.

TABLE-1
RESISTANCE PROFILES OF BACTERIAL ISOLATES AGAINST HEAVY METALS

Metal	Metal resistance bacteria	Ref.
Arsenic	*Lysinibacillus* sp., *Bacillus safensis*	[18]
	Pseudomonas gessardii, Brevundimonas intermedia	[109]
	Bacillus licheniformis	[113]
	Enterobacter sp., *Klebsiella pneumoniae*	[114]
Cadmium	*Brevundimonas diminuta*	[115]
	Atriplex lentiformis	[116]
	Bacillus megaterium	[117]
	Salmonella enterica, Sedum alfredii	[118]
Copper	*Stenotrophomonas maltophilia*	[119]
	Shigella flexneri	[120]
	Pseudomonas stutzeri LA3	[121]
	Sphingomonas, *Stenotrophomonas*, *Arthrobacter*	[122]
Mercury	*Pseudomonas aeruginosa*	[123]
	Pseudomonas idrijaensis	[124]
	Stenotrophomonas sp	[125]
	Acinetobacter junii, Pseudomonas stutzeri	[126]
Lead	*Sedum alfredii*	[127]
	Bacillus cereus NWUAB01	[128]
	Bacillus xiamenensis PbRPSD202	[129]
	Leclercia adecarboxylata and *Pseudomonas putida*	[130]

Conclusion

Metal-binding proteins are indispensable for bacterial metal resistance, enabling their survival and even growth in metal-contaminated environments. Various bacterial species,

TABLE-2
TYPES OF MECHANISM [Ref. 131]

Mechanism	Description	Example
Efflux	Actively pumps heavy metals out of the cell	P-type ATPases, RND efflux pumps
Sequestration	Binds heavy metals to proteins or other molecules	Metallothioneins (MTs), phytochelatins (PCs)
Reduction	Enzymatically reduces heavy metals to less toxic forms	Mercuric reductase, chromate reductase
Methylation	Methylates heavy metals to volatile forms	Arsenic methyltransferase, mercury methyltransferase
Permeability barriers	Modifies cell walls or membranes to reduce heavy metal permeability	EPS production, biofilm formation

such as *Psuedomonas gessardii*, *Brevundimonas intermedia*, *Enterobacter* sp., *Klebsiella pneumoniae*, *Salmonella enterica*, *Arthrobacter*, *Sphingomonas*, *Stenotrophomonas* and *Sedum alfredii* have resistance to metals such as As, Cu, Zn, Cd and Pb. These bacteria have evolved mechanisms, often involving metal-binding proteins, to selectively bind and sequester toxic metal ions, thereby maintaining cellular homeostasis. Significant understanding of these metal-binding proteins (MBPs) mode of action has been made possible by their structural and functional characterization. Modern techniques in metalloproteomics are highly beneficial for identifying and measuring the metal ions attached to these proteins. Metalloproteomics gives to study the structurally characterize of proteins, focusing on their binding sites for metal ions. This multidisciplinary approach integrates techniques from proteomics, metallomics and structural biology to unravel the intricate details of metal-protein interactions. The metalloproteomics approach has been instrumental in advancing our understanding of how metal-resistant bacteria employ specific proteins to cope with metal stress. By elucidating the structural and functional aspects of these metal-binding proteins, researchers can gain understanding into the adaptation mechanisms of bacteria to metal contaminated environments. This knowledge not only contributes to the fundamental understanding of microbial metal resistance but also holds potential applications in bioremediation strategies and environmental challenges.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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