9 Metallotolerant Bacteria: Insights into Bacteria Thriving in Metal-Contaminated Areas

Dina Barman, Dhruva K. Jha, and Kaushik Bhattacharjee

Abstract

The overall condition of the environment is inevitably linked to nature of life on the Earth. However, due to industrial revolution, the global upsurge of accumulation of toxic metals has increased enormously which is posing a serious problem to human health. In such environment, where survival of indigenous microorganisms is difficult, metallotolerant bacteria are able to thrive by tolerating high levels of heavy metals. To cope with this extreme condition, they employ diverse mechanisms to overcome the toxic effects of metals and metalloids with alteration of different genes and proteins, and these mechanisms also help their possible commercial exploitation. Hence, it is essential to understand their unique metabolic capacity or physical structure which encourages thriving in these metal-rich environments. This chapter also sheds light on evolutionary strategies that facilitate the metallotolerant bacteria to adapt to the environment and associated ecophysiological aspects.

Keywords

Metallotolerant bacteria · Bioaccumulation · Biotransformation

Abbreviations

APX	ascorbate peroxidase
CAT	catalase
CDF	cation diffusion facilitators

D. Barman · D. K. Jha
Microbial Ecology Laboratory, Department of Botany, Gauhati University, Guwahati, India

K. Bhattacharjee (✉)
Division of Life Sciences, Institute of Advanced Study in Science and Technology, Guwahati, India

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EPS	exopolysaccharide
FT-IR	Fourier-transform infrared
GC-MS	gas chromatography-mass spectrometry
GST	glutathione S-transferase
HGT	horizontal gene transfer
IAA	indole-3-acetic acid
LC-MS	liquid chromatography-mass spectrometry
MFP	membrane fusion protein
MIP	major intrinsic protein
MTs	metallothioneins
NMR	nuclear magnetic resonance
NTPs	nucleoside triphosphates
OMF	outer membrane factors
PGPB	plant growth-promoting bacteria
PMF	proton motive force
POD	peroxidase
RND	resistance-nodulation-cell division
SOD	superoxide dismutase
SRB	sulfur-reducing bacteria

9.1 Introduction

In the present era, the consequences of heavy metal accumulation in our planet are increasing day by day due to various natural processes (bioweathering of metal-containing minerals, volcanic emissions, forest fires, deep-sea vents, and geysers) and human anthropogenic activities (mining, surface finishing, energy and fuel producing, fertilizer, pesticide, metallurgy, etc.). Both of these have contributed significantly toward the increase in metal contamination (Ayangbenro and Babalola 2017; Romaniuk et al. 2018). Though most of the metals play an important role in various life processes, they become toxic at their high concentrations (Romaniuk et al. 2018). However, some of the heavy metals including mercury (Hg), lead (Pb), chromium (Cr), arsenic (As), cadmium (Cd), uranium (U), selenium (Se), silver (Ag), gold (Au), and nickel (Ni) have negligible biological role and are toxic even in trace amount (Gupta et al. 2016). Toxic effect of heavy metals in various environmental niches is mainly influenced by change of pH and temperature. At acidic pH, the bioavailability of heavy metal increases due to the presence of more protons (H^+) available to saturate the metal-binding sites, thus decreasing the attraction between adsorbent and metal cations. Under basic conditions, protons form other species by replacing metals ions, such as hydroxo-metal complexes which lead to the formation of metal complexes, of which some are soluble (Cd, Ni, Zn) and some are insoluble (Cr and Fe) (Olaniran et al. 2013).

Metals present in the environment are in nondegradable form and cannot be broken down by chemical or biological processes. Due to this, they become persistent

in the environment for long time and thus adversely influence the microbial community in environment. It causes serious damage to the ecosystem and soil fertility and negatively impact human health by causing diseases like chronic lung disease, cancer, neurodegeneration, and diabetes (Oyetibo et al. 2015; Fashola et al. 2016; Gupta et al. 2016; Ayangbenro and Babalola 2017). To mitigate the higher concentration of heavy metal ions, various physicochemical approaches including chemical precipitation, chemical oxidation and reduction, ion exchange, filtration, and electrochemical treatment have been used. However, due to various harmful impacts of these physicochemical approaches on the environment, the researchers have explored microbial world to ameliorate metal toxicity (Giovanella et al. 2017).

Among the different organisms on the Earth, microbial world is highly diverse, and some of them possess higher adaptability to tolerate a range of extreme environmental conditions (Gupta et al. 2016; Subhashini et al. 2017). Microbes can respond to the fluctuation of environmental conditions by changing their genetic makeup, by transferring genetic elements and by using many other adaptability mechanisms (Ryan et al. 2009). Metallotolerant bacteria are one of the extremotolerants or extremoresistants that can tolerate high levels of heavy metal concentration by means of their intrinsic properties or have unique metabolic capacities and/or physical structures to tolerate and survive in these extreme conditions (Tse and Ma 2016). These bacteria can survive in toxic metal-rich environment by expressing metal-resistant genes which are further used by bacteria to remediate those metal-contaminated areas (Das et al. 2016). They exert different mechanisms to resist the heavy metal toxicity which includes extracellular barrier, efflux of toxic ions from bacterial cells, incorporation of heavy metals into complexes by metal-binding proteins, and enzymatic transformations of metals (Singh et al. 2011; Romaniuk et al. 2018). A variety of genes and proteins are likely to be involved in such mechanisms which can be understood by transcriptomics, proteomics, genomics, and metabolomics studies. Genes responsible for tolerance to the metal toxicity/detoxification are reported by various researchers (Das et al. 2016). The genes responsible for conferring resistance to cadmium toxicity have been identified as *cadB* and *cadD*, which can protect bacterial cell by binding cadmium at cell membrane (Perry and Silver 1982; Robinson et al. 1990; Crupper et al. 1999). Similarly, *cueO* gene in *E.coli* can detoxify copper toxicity by oxidizing Cu(I) to less toxic Cu(II) (Yu et al. 2014). The *pbrD* gene, encoding a Pb(II)-binding protein, can reduce the toxic effect of Pb (Borremans et al. 2001). The bacterial Ni/Co transporter (NiCoT) gene encodes transporter proteins which mediate energy-dependent uptake of Co and Ni ions into the cell facilitating bioaccumulation (Gogada et al. 2015). *merA* and *merB* are some of the genes which are expressed in response to toxicity of mercury (Schaefer et al. 2011; Dash et al. 2014).

In the present chapter, we have summarized various mechanisms employed by metallotolerant bacteria in order to successfully thrive under various metal-rich environments.

9.2 Strategies to Study Metallotolerant Bacteria

Metallotolerant bacteria can be isolated from metal-contaminated sites since bacteria isolated from these sites are more resistance to a range of metals in comparison to other non-contaminated sites (Sarma et al. 2016). They can be isolated in laboratory with or without enrichment technique. Generally, soil, sediment, sewage, or water collected from different metal-contaminated sites should be serially diluted in saline water (0.85%) or low phosphate buffer followed by mixing vigorously at 120 rpm for 2 h at 30 °C. After that, the diluted samples are spread plated on suitable agar medium followed by incubation at appropriate temperature for allocated time (Romaniuk et al. 2018). The heavy metal-resistant bacteria can be also selectively isolated by serial dilution method using appropriate agar medium by incorporating the desired metal ions (Marzan et al. 2017). Enrichment and isolation of metallotolerant bacteria can be performed by suspending the metal-contaminating environmental sample in a low-nutrient broth supplemented with the desired metal and allowed to incubate in an incubator shaker at 120 rpm and at 30 °C for 24 h. After tenfold serial dilutions of this enrichment culture, the desired metal tolerant bacteria can be isolated by plating on standard media (Fig. 9.1) (Sarma et al. 2016).

The selection of appropriate media for isolation of metallotolerant bacteria in laboratory condition has significant importance. Different researchers have used

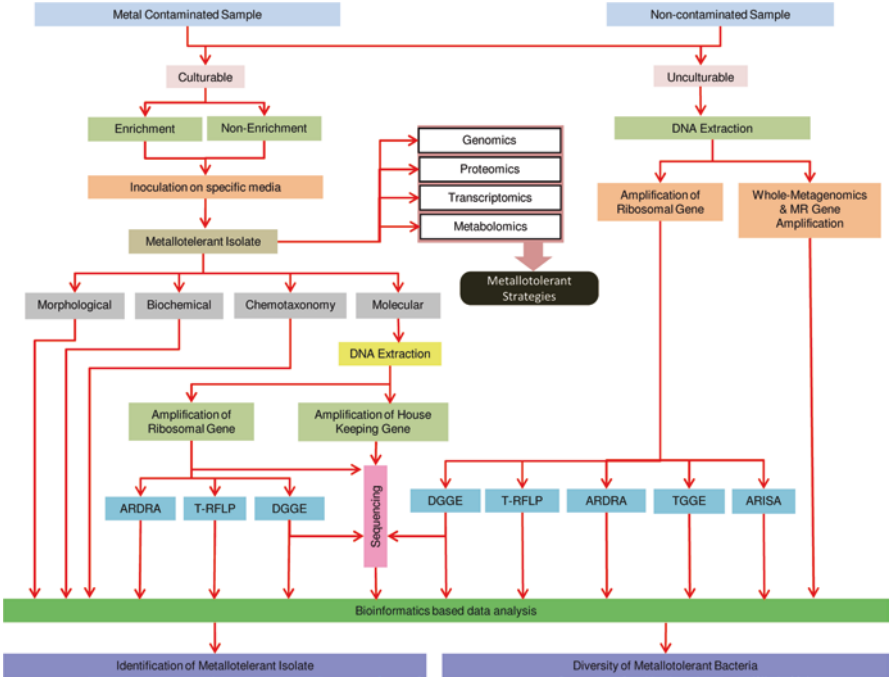

Fig. 9.1 Strategies to study metallotolerant bacteria

different media for isolation of metallotolerant bacteria. A Cr(VI)-tolerant bacterium *Bacillus dabaoshanensis* sp. nov. was isolated from paddy soil altered with sludge compost in Dabaoshan Mine, China (Cui et al. 2015), by inoculating the diluted soil sample with mineral salts medium. Similarly, Abbas et al. (2015) isolated bacteria from tannery effluent (water and sludge sample) collected from Leather Pak Road, Pakistan, on tryptic soy agar amended with various concentrations of heavy metals. Sarma et al. (2016) isolated uranium tolerant bacteria from sediment samples collected from water bodies of three different locations of the uranium rich mining site of Domiasiat in India followed by incubation on tryptone soy agar. Rodriguez-Sanchez et al. (2017) collected soil from lead-contaminated land of Guadalupe, Mexico, and isolated lead tolerant bacteria onto Luria-Bertani (LB) agar plates. Romaniuk et al. (2018) used both R2A and LB media for isolation of metal-resistant bacteria. Oliveira et al. (2009) employed combined carbon medium, a semisolid nitrogen-free medium to isolate arsenic tolerant heterotrophic nitrogen-fixing microorganisms from soil sample collected from both contaminated and non-contaminated soil of Portugal.

Similar to other bacterial populations, metallotolerant bacteria can also be characterized based on morphological, biochemical, and physiological approaches as recommended by Holt et al. (1994). Chemotaxonomic analyses including whole-cell sugar pattern, peptidoglycan type, fatty acid pattern, major menaquinone, and phospholipid type are also useful in characterizing bacteria (Rainey et al. 1996; Maidak et al. 1999). Though morphological, biochemical, physiological, and chemotypic characterization of bacteria can identify genera, sometimes it is not adequate in itself to differentiate between species. The advent of molecular criteria for the characterization of bacteria has provided taxonomists with a set of reliable and reproducible tools for studying the systematics. Molecular characterization of bacteria is performed by 16S rRNA gene amplification of genomic DNA (Shi et al. 2014). Percentage of G + C content of DNA and DNA/DNA-hybridization techniques are also useful tools for the identification of microbes. To characterize taxa at and below the rank of species, the DNA:DNA relatedness, molecular fingerprinting, and phenotypic techniques are methods of choice (Rossello-Mora and Amann 2001).

It is well known that with culture-dependent approach, only 1% of total microbial population can be characterized due to their unknown growth requirements (Vartoukian et al. 2010). In this regard, metagenomics provides a correct scenario regarding the presence of various microorganisms present in different metal-rich environments (Sharma et al. 2008). It also helps to identify the type of microorganism prevalent in metal-rich conditions which enable us to understand the biogeochemical cycles and habits of metal tolerant bacteria (Sharma et al. 2008). For metagenomic analysis, extraction of DNA is the first and foremost requirement followed by cloning DNA into a suitable vector, transforming the clones into a host bacterium, and screening the resulting transformants (Handelsman 2004; Zhang et al. 2017). Metagenomics also provides valuable information about the presence of metal-resistant genes prevalent in microbes thriving in extreme environmental condition (He et al. 2010). One such example is the study of Mina stream sediment, Brazil, which is the world's largest mining regions and is exceptionally rich in iron

and gold ores (Costa et al. 2015). A total of 30,738 operational taxonomic units (OTUs) comprising of 52 bacterial phyla particularly belonging to *Proteobacteria*, *Bacteroidetes*, *Acidobacteria*, *Gemmatimonadetes*, *Cyanobacteria*, and *Spirochaetes* with higher abundance of *Proteobacteria* were found from this mining region. Functional annotations also disclose the presence of higher diversity of several metal resistance genes, which indicate that the bacterial community is able to adapt successfully to metal-contaminated environments. Another good example is the complete metagenomics of the acid mine drainage of the Richmond mine. The bacterial community was dominated by *Leptospirillum*, *Sulfobacillus*, and *Acidimicrobium* which predominantly have metal-resistant genes (Handelsman 2004). Similarly, Tyson et al. (2004) explored the microbial community of acid mine drainage (AMD) biofilm in California and which dominated by the genera *Leptospirillum*, *Ferroplasma*, *Sulfobacillus*, and *Acidimicrobium.*

9.3 Community Structure of Metallotolerant Bacteria in Various Metal-Rich Environments

Among the various heavy metal ions, some of them including iron (Fe), zinc (Zn), copper (Cu), and cobalt (Co) are required for metabolic activities of microorganism at low concentrations, but with increasing concentration, they become lethal to living cells. There are some other categories of metal ions such as Hg, As, Ag, Cd, Cr, and Pb which have no roles in biological activities and become toxic even at low concentration (Rodriguez-Sanchez et al. 2017). The presence of high concentration of heavy metals affects the diversity of microbial communities (Romaniuk et al. 2018). The metal-rich environment is usually found across the world. This type of environment is created due to both natural processes and anthropogenic activities (Romaniuk et al. 2018). Among the natural processes, weathering of metal-containing rocks results in the accumulation of heavy metals and metalloids in soil. Serpentine soil is one type of soil which is originated from serpentine rocks containing silica and high concentrations of heavy metals Fe, Mg, Cr, Ni, and Co. A total of 11 different metal tolerating bacteria isolated from this type of soil of Marmara and Aegean regions of Turkey were found to tolerate Ni, Pb, Cd, and Zn in the range of 50–2000 mgL^{-1} (Turgay et al. 2012). However, it is important to note that extreme polar environments are less influenced by anthropogenic activities. The presence of heavy metals in those areas is mainly due to natural process such as biogeochemical weathering of terrigenous sources and global atmospheric pollution. The physiochemical analysis of soil collected from King George Island of Antarctica revealed the presence of high concentrations of heavy metals mainly copper, mercury, and zinc. About 200 bacterial strains were isolated from these regions and found to have many heavy metal resistance genes including *arsB*, *copA*, *czcA*, and *merA* in 62 different strains (Romaniuk et al. 2018).

The massive accumulation of heavy metal generally results from various anthropogenic activities such as large-scale burning of fossil fuels, mining, and industrial processes. Mexico is among one of the major mining countries and extensive

producer of Ag and Pb in the world. The mining activities generate huge amount of mine spoils rich in high amount of metal content. This type of soil though generally inhibit the establishment of plants, but some of the plants have been found in these areas may be due to the presence of endophytic bacteria which can detoxify the heavy metal content. The endophytic bacteria belonging to Firmicutes, Actinobacteria, and Proteobacteria isolated from *Prosopis laevigata* and *Sphaeralcea angustifolia* collected from Villa de la Paz in the state of San Luis Potosí, Mexico, showed resistance to heavy metals like Pb, Zn, Cu, As(III), and As(V) with minimum inhibitory concentration (MIC) of 1.1, 3.1, 4.3, 11.0, and 94.3 mM, respectively (Román-Ponce et al. 2016). Uranium contamination is another among the major concerns due to its toxicity to the environment and human health. Domiasiat in the state of Meghalaya, India, is reported as uranium rich mining site with an average ore grade of 0.1% U_3O_8 (Sarma et al. 2016). The bacteria isolated from sediment samples collected from Domiasiat belong to *Serratia nematodiphila* and *S. marcescens* subsp. *sakuensis* and are found to tolerate uranium (U), Cd, Cu, Zn, and Pb (Sarma et al. 2016). Similarly, Shreedhar et al. (2014) isolated bacteria belong to Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes having capacity to tolerate uranium from monazite sand of Someshwara beach coast, India. This site is reported to have high radiation because of the deposition of monazite sand containing the actinide element, thorium (Th). Thus, these bacteria provide scope for bioremediation of radionuclide-contaminated sites. Fly ash generated from combustion of coal is also contributing significantly to environmental contamination due to the presence of high content of heavy metals. Bacterial strains isolated from DVC-Mejia Thermal Power Station located at Durlavpur, India, belong to *Bacillus*, *Micrococcus*, *Kytococcus*, and *Staphylococcus* genera and were found to be tolerant to As, with MIC ranging from 14–30 mM for As(III) to 36–72 mM for As(V). Hence, these bacteria are important candidate for As bioremediation in case of fly ash (Roychowdhury et al. 2018).

Industrial processes also have led to the accumulation of metals in the environment which constitutes a major hazard to soil, water, and animal-human health. The soil samples collected from As-polluted areas due to industrial effluents from Estarreja region, Portugal, showed the occurrence of many gram-positive and gram-negative arsenic-tolerant bacteria belonging to Firmicutes, Actinobacteria, and Proteobacteria (Oliveira et al. 2009). Mustapha and Halimoon (2015) also isolated heavy-metal-tolerant bacteria from the outlet of electroplating industry in Klang, Malaysia. In addition to the release of toxic metals from industries, the daily release of sewage from household, agriculture, and health sectors also increases the load of toxic metals in the environment. The banks of Kestopur canal, which runs through the northern fringes of Kolkata, India, is contaminated with heavy metals due to influx of effluents from domestic activities, industries, as well as health sectors. The soil sample collected from this area contained heavy-metal-tolerant bacteria belonging to *Exiguobacterium* and *Bacillus*, which are found to tolerate Cr, Pb, Co, Ni, and Fe with MIC ranging from 7–9 mM, 5–8 mM, 0.6–0.9 mM, 0.6–0.8 mM, to 7–9 mM, respectively (Gupta et al. 2012). Although metallotolerant bacteria are widespread in metal-rich soils of the world, interestingly some of the

metallotolerant bacteria are also present in non-contaminated soil, which supports that metal resistance capacity in those bacteria is present intrinsically (Oliveira et al. 2009).

9.4 Metallotolerance in Bacteria

Metallotolerant bacteria have unique feature to thrive in metal-rich environments due to their small size, high surface area to volume ratio, ability to transfer genetic traits, and adaptability (Das et al. 2016). They can convert heavy metals into non-toxic forms by various mechanisms including exclusion by permeability barrier, effluxing metal ions, oxidizing metals, enzymatic conversion of metals, intracellular and extracellular metal sequestration, and producing metal chelators like metallothioneins and biosurfactants (Igiri et al. 2018). Nonetheless, a complete understanding of the mechanism behind it is yet a major challenge.

9.5 Underlying Mechanisms of Metallotolerance

9.5.1 Extracellular Barrier as a Way for Averting Metal into Cell

Bacterial extracellular membrane, including the plasma membrane, cell wall, and capsule, acts as a barrier for the toxic metal to enter into the cell. Bacterial capsule, mainly carboxyl groups of polysaccharides, acts as a barrier and resists the entry of toxic metal into the cell by adsorbing metal ions (Fig. 9.2). For example, extracellular biopolymers of *Enterobacter chloacae* and *Marinobacter* sp. can accumulate metal ions on the surface of the cell (Iyer et al. 2005; Bhaskar and Bhosle 2006). Similar to the capsule, the plasma membrane also resists the entry of metal ions into cell. In the case of gram-positive bacteria, peptidoglycan layer of cell surface is thick and composed of alanine, glutamic acid, meso-di-aminopimelic acid, polymer of glycerol, and teichoic acid. Contrarily, in gram-negative bacteria, the peptidoglycan layer is composed of enzymes, glycoproteins, lipopolysaccharides, lipoproteins, and phospholipids. All the lipids, proteins, and polysaccharides of the plasma membrane act as active sites for binding of metals on microbial cell surface (Ayangbenro and Babalola 2017). On binding the heavy metals to bacterial cell, bacteria can transform them from one oxidation state to another and thus reduce their toxicity (Ayangbenro and Babalola 2017). The changes in the permeability of the plasma membrane can also inhibit the entry of metal ions into the cell. All the extracellular surfaces of microbial cell are negatively charged containing many ionizable groups (carboxyl, amino, phosphate, and hydroxyl groups) which provide a platform to adsorb the positively charged heavy metals (Diep et al. 2018). Bacteria can trap heavy metal ions and then adsorb metals onto the binding sites of the cell wall by electrostatic interaction, ion exchange, precipitation, redox process, and surface complexation (Ayangbenro and Babalola 2017) which lead to prohibition of transport of metal ions into the cytoplasm.

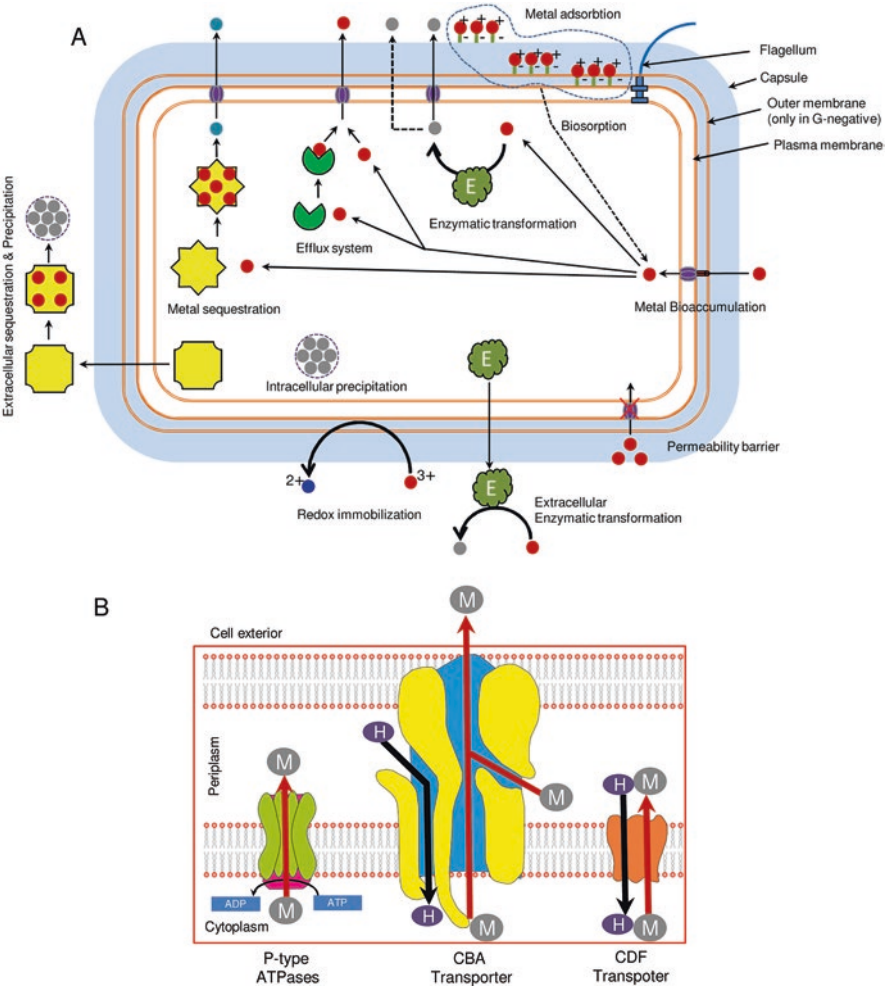

Fig. 9.2 (**a**) A generalized illustration of mechanisms involved in providing tolerance to toxic metals in bacteria. (**b**) Schematic illustration of major types of bacterial efflux system transporter families in heavy metal resistance. (Adapted from Prabhakaran et al. 2016)

9.5.2 Metal Uptake – Biosorption and Bioaccumulation

In bacteria, two different types of heavy metals uptake mechanisms prevail: biosorption and bioaccumulation. Biosorption is the adsorption of metals to the surface layer of bacteria using physicochemical interactions (electrostatic forces, ion exchange), complexation, or chelation and an energy-independent passive metabolic process (Fig. 9.2a) (Diep et al. 2018). On the other hand, bioaccumulation is a metabolically dependent active metal uptake into the living cell across the cell membrane. This process usually involves adsorption of heavy metal ions at the

bacterial cell wall or cell membrane through various physicochemical interactions and then transports into the periplasmic space and translocation through the lipid bilayer into the cytoplasm (i.e., metal import system) (Diep et al. 2018). In this manner, the metals are contained and accumulated within the bacterial cell (Chojnacka 2010). The bioaccumulation of metal ions normally relies upon the translocation of metals from the periplasmic space into the cytoplasm of bacteria through the inner membrane importers which are from three major transporter classes: channels, secondary carriers, and primary active transporters (Diep et al. 2018).

Channels are single component α-helical proteins which facilitate passive diffusion of metal ions in step with their concentration gradient across the inner membrane. They are largely energy-independent, which means that they do not need the proton motive force (PMF) or nucleoside triphosphates (NTPs) like ATP and GTP to translocate their substrates (Saier 2016). Some researchers reported a transporter channel, which belongs to the major intrinsic protein (MIP) superfamily, facilitating diffusion of As and Hg ions in *Escherichia coli*, *Corynebacterium diphtheriae*, and *Streptomyces coelicolor* (Singh et al. 2008; Villadangos et al. 2014). These ion channels appear to be the most effective selection for the uptake of metal ions since there is less energy burden on the cell because of their zero-energy requirement (Diep et al. 2018).

Secondary carriers are single component membrane transport proteins which are involved in the movement of ions across the inner membrane. They are of three different types, i.e., uniporters, symporters, and antiporters (Saier 2016). Uniporter can transport single type of metal ions across a cell membrane based on diffusion gradient. On the contrary, symporters and antiporters are types of cotransporters that transport different types of metal ions across a cell membrane. However, symporters involved in movement of ions in the same direction in relation to each other, and antiporters involved in movement of different ions in both the directions (i.e., in and out) based on electrochemical and concentration gradient. Uniporters translocate positively charged metal ions across the inner membrane which depends on varying the charge difference facilitated by PMF, whereas symporters translocate metal ions by utilizing the protons generated due to the charge difference as a co-substrate during uptake (Diep et al. 2018). For example, symporters are used by *Staphylococcus aureus* and *Helicobacter pylori* to transport Ni, Co, and As^{4+} (Deng et al. 2013).

Furthermore, primary active transporters are multicomponent membrane transport proteins containing transmembrane component for the translocation pathway, a cytoplasmic energy-coupling ATPase component that can hydrolyze phosphoanhydride bond (i.e., in NTPs like ATP and GTP) for translocation of metal ions. Primary active transporters MntA and CdtB in *Lactobacillus plantarum* and *CopA* in *Enterobacter hirae* have been used for Cd uptake and Cu uptake respectively, are belong to the P-type ATPase superfamily (Diep et al. 2018).

Another important class of transporter is porins, which are β-barrel transmembrane protein channels, present across the outer membrane of gram-negative bacteria. These porin channels transport the metal ions from the outer surface to the periplasmic space and later to the cytoplasm by different other transporters present

in inner lipid bilayer (Saier 2016). Schauer et al. (2007) reported about the FrpB4 channels present in *Helicobacter pylori* and their involvement in Ni uptake. The *ropAe* gene encodes a porin-like protein involved in copper transit in *Rhizobium etli* CFN42 (González-Sánchez et al. 2018). A potential role of multiple porins belonging to the OprD family has also been suggested in *P. aeruginosa* in the uptake of Cu (Teitzel et al. 2006).

Microbial exopolysaccharide (EPS) is also one of the significant components that can take part in metal biosorption. They are released by bacterial cells as self-defense against harsh environmental condition. EPS is a negatively charged polymer, and this negative charge is imparted due to the presence of different active and ionizable functional groups such as acetamido group of chitin and hydroxyl groups that can sequester positively charged heavy metals via various mechanisms including ion exchange, precipitation, and complexation and can adsorb heavy metals (Gupta and Diwan 2017). EPS produced by various bacteria including *B. firmus* and *Arthrobacter* ps-5 has been reported which adsorbs various heavy metals (Zhang et al. 2017). Kazy et al. (2002) also studied EPS synthesized by copper-tolerant *P. aeruginosa* strains which showed increase in EPS production in response to higher Cu concentration. EPS can uptake metal ions by different strategies including homogenous consortial EPS, heterogeneous consortial EPS, dead biomass EPS, immobilized EPS, and modified EPS. Metal biosorption by homogenous consortial EPS mainly deals with the EPS from single bacterial culture which is reported by *Methylobacterium organophilum* that can efficiently remove Cu and Pb ions on producing EPS (Gupta and Diwan 2017). Similarly, *Herminiimonas arsenicoxydans* can remove As ions through EPS interaction (Gupta and Diwan 2017). Metal biosorption by heterogeneous consortial EPS means the use of EPS produced by bacterial consortia. For example, activated sludge-mixed cultures can reduce Zn, Cu, and Cr by approximately 85–95% (Gupta and Diwan 2017). Similarly, gram-negative bacterial consortia can reduce the concentration of Zn, Pb, Cr, Cu, Cd, and Co approximately by 75–85% (Gupta and Diwan 2017). Dead biomass EPS also can adsorb metal ions such that EPS of dead biomass of *Ochrobactrum anthropi* could remove cadmium ions. Immobilized EPS is produced by immobilizing bacterial cells to solid surfaces which stimulate EPS production. It was observed that immobilizing of *Paenibacillus polymyxa* in agar beads stimulates the EPS production which can adsorb lead ions. The activity of EPS can also be enhanced by chemical modification including acetylation, phosphorylation, carboxymethylation, methylation, and sulphonylation. This modified EPS is involved in higher sorption of metals in comparison to unmodified ones (Gupta and Diwan 2017).

9.5.3 Efflux of Toxic Ions from Bacterial Cells

Elimination of heavy metals from bacterial cell relies on an energy-dependent ion efflux mechanism. This efflux of toxic ions from the cytoplasm is mainly performed by bacterial cells with three different proteins, which are (i) resistance-nodulation-cell division (RND superfamily) proteins, (ii) cation diffusion facilitators (CDF

family), and (iii) P-type ATPases (Fig. 9.2b). The RND protein family is a group of protein containing RND protein with membrane fusion protein family (MFP) and outer membrane factors (OMF) which form an efflux protein complex and involved in heavy metal resistance by transporting the heavy metals from the cytoplasm, cytoplasmic membrane, or periplasm across the outer membrane directly to the outer surface (Nies 2003). This efflux system is referred to as CBA efflux systems or CBA transporters. *Ralstonia metallidurans* is a gram-negative bacterium that offers resistance to many metal ions including Zn, Co, Cd, and Ni due to the presence of two plasmids, namely, pMOL28 and pMOL30. In pMOL30, the structural gene region contains genes for the OMF CzcC, the MFP CzcB, and the CzcA protein of the RND family which form an operon *czcCBA*. Due to the presence of these genes, *R. metallidurans* offer resistance to Ni and Co. This system can minimize the cytoplasmic and periplasmic concentrations of heavy metal cations; hence, the cations get removed before they enter into the cell. In addition to *czcCBA*, *cnrCBA* and *ncc* are present in *R. metallidurans* which offer resistance to Cd, Co, and Ni. *cnrCBA* encodes for OMF CnrC, the MFP CnrB, and the RND protein CnrA. *ncc* contains regulatory gene region *nccYXH* followed by the structural region *nccCBA*, again encoding a putative outer membrane protein (*NccC*), a MFP (*NccB*), and RND protein (*NccA*) (Nies 2003). Cation diffusion facilitators (CDF family), also a protein family of metal transporter, are involved in providing resistance to Zn and other metal cations. The CDF-encoding gene *czcD* along with TrkA dehydrogenase is present in *B. subtilis* which offers resistance to metals such as Zn^{2+}, Co^{2+}, and Cd^{2+}. Another CDF protein, ZitB, was reported from *E.coli* which makes the strain resistant to Zn on minimizing the gathering of Zn^{2+} by potassium gradient in addition to the proton motive force (Nies 2003). P-type ATPase is another protein family which is driven by ATP hydrolysis. Since this protein family takes part in both import and export of metals, it plays an important role in metal homeostasis and detoxification of heavy metals by effluxing metal ions (Nies 2003).

9.5.4 Biotransformation

The biotransformation of heavy metals is a detoxification process where metals, as a result of biological action, undergo changes in valence and/or conversion into organometallic compounds. It can be achieved either enzymatically or by synthesizing and producing metal-binding proteins such as metallothioneins (MTs) (Fig. 9.2a).

Bacteria can transform toxic effect of metals to harmless one by oxidation, reduction, methylation, and alkylation (Valls and de Lorenzo 2002). Various bacteria can oxidize toxic organic compounds to harmless compounds with oxidoreductase enzymes. They do it by cleaving chemical bonds and catalyzing the transfer of electrons from donor to acceptor (Karigar and Rao 2011). The arsenite-oxidizing bacteria such as *A. faecalis* can tolerate toxic arsenite [As(III)] and oxidized arsenite [As(III)] to arsenate [As(V)] and finally, As(V) forms insoluble sulfides upon exposure to H_2S (Valls and de Lorenzo 2002). Bacteria also reduce and precipitate some

of the metals enzymatically, for example, iron-oxidizing bacteria can reduce Fe(III) to Fe(II) abiotically (Lloyd 2003). Similarly, *Serratia marinorubra* can transform arsenate to arsenite and methylarsonate (Bentley and Chasteen 2002). Bacteria also reduce mercury (Hg^{+}) into less toxic and volatile mercury (Hg^{o}) by mercury reductase and thus released into the atmosphere. Furthermore, highly toxic mercury derivatives such as organomercurials get transformed to mercury (Hg^{+}) and finally to volatile Hg^{o} by enzymatic process of bacteria (Valls and de Lorenzo 2002).

Microbial methylation also plays an important role in the transformation of toxic metals, where methyl groups are enzymatically transferred to metals and as a result the metals get transformed into different metalloids with varying toxicity, solubility, and volatility. A range of bacteria including *Clostridia*, methanogens, and sulfate-reducing bacteria can methylate various metals including Pb, Cd, As, tin (Sn), selenium (Se), tellurium (Te), and Hg under anaerobic conditions. For example, on methylation of selenium, it gets transformed into volatile dimethyl selenide. Similarly, arsenic gets transformed into gaseous arsines and lead into dimethyl lead. Some bacteria including *Bacillus* sp., *Escherichia* sp., *Clostridium* sp., and *Pseudomonas* sp. can methylate Hg ion (Hg^{2+}) into more toxic methylmercury [$(CH_3)Hg^{+}$] which in turn is methylated into volatile metallic mercury (Hg^{0}) (Igiri et al. 2018). Furthermore, some bacteria transform methylmercury to volatile dimethylmercury which can be enzymatically reduced to volatile metallic mercury. Another methylation process in bacteria is conversion of phenylmercury to diphenylmercury (Barkay and Wagner-Dobler 2005). Alkylation is another process of biotransformation of metals where an alkyl group other than methyl group is directly bonded to some metals through a carbon atom, for example, $As(C_2H_5)(CH_3)_2$, $As(C_2H_5)_2(CH_3)$, $As(C_2H_5)_3$, and $Sb(C_2H_5)_3$ (Krupp et al. 1996).

Toxicity of metals can also be removed by bacteria on utilizing metallothioneins (MTs). Metallothioneins are small, cysteine-rich metal-binding proteins that can sequester heavy metals intracellularly as complexes (Romaniuk et al. 2018). Biosynthesis of MTs is induced by different factors such as hormones, cytotoxic agents, and metals including Cd, Zn, Hg, Cu, An, Ag, Co, Ni, and bismuth (Bi). Based on cysteine content and structure, MTs are further classified into Cys-Cys, Cys-X-Cys, and Cys-X-X-Cys motifs (in which X denotes any other amino acid). Metallothioneins act as "storehouse" for zinc and can protect the bacterial cells from cadmium toxicity (Das et al. 2016). They can also scavenge free radical and join with harmful molecules like superoxide and hydroxide ions. After cysteine gets oxidized to cystine, the bound metal ions are released into the environment (Das et al. 2016). For example, *Rhizobium leguminosarum* can also sequester cadmium ions by glutathione which makes the strain cadmium resistant (Lima et al. 2006). *P. putida* can tolerate Cd initially by immobilization of cadmium in polyphosphate granules followed by producing cysteine-rich, low-molecular-weight protein, viz., Pseudothioneins (Higham et al. 1986). Similarly, strain of *P. diminuta* having silver-binding proteins can intracellularly reduce the toxic effect of silver (Ibrahim et al. 2001). *P. aeruginosa* strain WI-1 having metallothionein (*BmtA*) can reduce the toxic effect of Pb by intracellular sequestration (Naik et al. 2012).

9.5.5 Precipitation – Intracellular and Extracellular

Bacteria can precipitate metal compounds intracellularly and/or extracellularly, as, for instance, *G. metallireducens* can convert lethal Mn(IV) to Mn(II), poisonous U(VI) to U(IV), and Cr(VI) to less toxic Cr(III) (Igiri et al. 2018). Metal precipitation mainly occurs as a result of dissimilatory metal reduction, sulfide precipitation, and phosphate precipitation (Valls and de Lorenzo 2002). Dissimilatory metal reduction is concerned with the extracellular precipitation of metals which is unrelated to its intake by the action of microbial catalyst, for example, precipitation of uranium, selenium, chromium, technetium, and gold by various bacteria (Valls and de Lorenzo 2002). Sulfide precipitation is another mechanism of metal precipitation where sulfur-reducing bacteria (SRB) produce sulfide to form metal sulfide as precipitate which is followed by entrapment of the sulfide by the exopolymer (Valls and de Lorenzo 2002). For instance, U(VI), Cr(VI), Tc(VI), Pd(II), and As(V) get precipitated by SRBs. *Klebsiella planticola* can precipitate cadmium (Cd) as insoluble sulfides on liberating hydrogen sulfide from thiosulfate (Igiri et al. 2018). Similarly, *Vibrio harveyi* precipitates soluble lead (Pb^{2+}) as complex lead phosphate salt (Igiri et al. 2018). Metal precipitation is also achieved by the release of inorganic phosphate from organic phosphate donor molecules (Valls and de Lorenzo 2002). Metal precipitation by phosphate production is also catalyzed by the liberation of crystallized inorganic phosphate via the action of periplasmic acid phosphatase (PhoN) and also encounters a role for phosphate groups present in the lipopolysaccharide of crystal nucleation (Macaskie et al. 2000; Valls and de Lorenzo 2002). For instance, *Citrobacter* sp. strain isolated from metal-contaminated soil precipitates high levels of uranium, nickel, and zirconium through the formation of highly insoluble metal phosphates. Similarly, *B. thuringiensis* DM55 also precipitates Cd by phosphate production (Valls and de Lorenzo 2002). Iron-reducing bacteria such as *Geobacter* spp. and SRB like *Desulfuromonas* spp. are involved in the precipitation of harmful metals to less or nontoxic metals by an extracellular sequestration.

In addition, bacteria secrete a variety of other metal-complexing metabolites (e.g., siderophores, carboxylic acids, amino acids, surface-active chemical species, and phenolic compounds) to precipitate the heavy metals. Siderophores are low-molecular-weight coordination molecules and highly specific Fe(III) ligands that can bind and transport or shuttle Fe and are secreted by a wide variety of bacteria to aid Fe assimilation. In spite of their preference for iron, they can likewise chelate various other metals with variable affinities (Fig. 9.2a) (Schalk et al. 2011). The production of siderophores in bacteria can also be stimulated by trivalent metals like Al, Ga, and Cr (Schalk et al. 2011). Koedam et al. (1994) reported the production of siderophores in *P. aeruginosa* in the presence of high iron concentrations. The formation of stable complexes between siderophores and other metal cations (other than iron) is prevalent in bacterial biology (Schalk et al. 2011). For example, the complex formation between Ga^{3+}, Al^{3+}, and In^{3+} with hydroxamate siderophore desferrioxamine B (a siderophore) is between 10^{20} and 10^{28} M^{-1}, whereas that with Fe^{3+} is 10^{30} M^{-1} (Schalk et al. 2011). Braud et al. (2009) reported that the pyoverdine and

pyochelin (siderophores) produced by *P. aeruginosa* are able to chelate metals like Ag^{+}, Al^{3+}, Cd^{2+}, Co2+, Cr^{2+}, Cu^{2+}, Eu^{3+}, Ga^{3+}, Hg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Sn^{2+}, Tb^{3+}, Tl^{+}, and Zn^{2+}.

Some bacteria are able to produce surface-active chemical species (i.e., biosurfactants) which help in solubilization and precipitation of metals. For example, rhamnolipids are a class of glycolipid produced by *P. aeruginosa* and several other bacterial strains which act as a biosurfactant (Valls and de Lorenzo 2002). The anionic biosurfactants capture the heavy metal ions through electrostatic or complexation methods. These complexations lead to an expansion in the obvious solvency of metals. In this way, the bioavailability of metals is affected through their decrease by basic metabolic results, which prompts the development of less soluble metal salts including phosphate and sulfide precipitates (Valls and de Lorenzo 2002). There are several other surface-active chemical species produced in the form of polysaccharides, proteins, lipopolysaccharides, lipoproteins, or complex mixtures by a wide range of bacterial strain. For example, *Acinetobacter* spp. produce high-molecular-weight emulsifiers to precipitate metal ions (Mosa et al. 2016).

9.6 Advanced Omics Strategies to Uncover the Truth

Omics strategies provide comprehensive and profound understanding of the underlying mechanism and adaptation strategy in microbial cell in response to metal stress.

9.6.1 Genomics

Genomics refers to the mapping, sequencing, and analysis of the complete set of genes of an organism which provides answer to many unanswered questions (Sharma et al. 2008). It is well known that metallotolerant bacteria can survive in metal-rich environment on expressing different genes such as *cadB*, *chrA*, *copAB*, *pbrA*, *merA*, and *NiCoT* for cadmium, chromium, copper, lead, mercury, and nickel (Das et al. 2016). Genomics provides remarkable information about the various genes involved in resisting metal toxicity which makes us to understand their capacity to grow in metal-rich environment and their interaction with various physical and biological factors (Sharma et al. 2008). In that direction, genomes of many metallotolerant bacteria have already been sequenced (Sharma et al. 2008). During the last few years, the genome of metallotolerant bacteria such as *Sinorhizobium meliloti*, *Mesorhizobium amorphae*, and *Agrobacterium tumefaciens* has been sequenced (Xie et al. 2015). Genome sequencing of *Enterobacter cloacae* B2-DHA isolated from the Hazaribagh tannery areas in Bangladesh provides information on the presence of chromium and other heavy metal resistance genes including *chrR* and *chrA* which make the bacteria to survive in metal-rich environment (Aminur et al. 2017). The genome sequence of hydrocarbon degrading *Geobacillus thermodenitrificans* NG80-2, isolated from a deep oil reservoir in Northern China,

provides information that the bacteria have a number of genes which make this organism capable in tolerating a number of contaminated environments including oil reservoirs (Feng et al. 2007). *Lysinibacillus sphaericus* OT4b.31, a native Colombian strain, is generally applied in bioremediation of heavy-metal-polluted environment. The genome sequencing of this bacterium was found to have sphaericolysin B354, the coleopteran toxin Sip1A, and heavy metal resistance clusters of *nik*, *ars*, *czc*, *cop*, *chr*, *czr*, and *cad* operons which support the bacteria to tolerate the metal toxicity (Pena-Montenegro and Dussan 2013). Furthermore, the genome sequencing and annotation of *Halomonas zincidurans* strain $B6^T$ isolated from a deep-sea heavy metal-rich sediment from the South Atlantic Mid-Ocean Ridge provide information that the bacteria encode 31 different genes in relation to heavy metal resistance especially Zn which makes it a candidate for bioremediation of heavy metal-contaminated environments (Huo et al. 2014). The bacterial species *P. putida* is well known for biodegradation of organic compounds (Wu et al. 2011; Yang et al. 2019). Nevertheless, *P. putida* ATH-4 isolated from soil sediments at the "Prat" Chilean military base located in Greenwich Island, Antarctica, is found to be resistant to mercury/tellurite. Interestingly, it showed tellurite resistance only when it was allowed to grow in the presence of mercury, suggesting a cross-resistance mechanism (Rodriguez-Rojas et al. 2016). Further, it is also revealed that the bacterium can resist the toxicity of Cd^{2+}, Cu^{2+}, CrO_4^{2-}, and SeO_3^{2-}. The genome sequencing of *P. putida* ATH-4 provides information that the bacterium possesses more tRNA gene sequences than other known *P. putida* genomes which reflect the adaptation of the bacterium to extreme environmental conditions. On the other hand, in the ATH-43 genome sequence on using IS finder tool, 13 IS elements along with 21 transposases and 17 integrases were found which increase our understanding of its capability to horizontal gene transfer of metal resistance among others (Rodriguez-Rojas et al. 2016). All these information gathered from genomics provide holistic information about the interaction of metallotolerant bacteria with the environment (Sharma et al. 2008).

9.6.2 Transcriptomics

Though genomics provides information about the genes involved in resistance to metal stress, there are different stress response systems which get activated in response to metal stress which can be understood by transcriptome analysis (Peng et al. 2018). Transcriptome analysis of *E. coli* and *B. subtilis* conferred that there are three membrane stress-related regulons, i.e., *cpxRA*, *rpoE*, and *basRS* which get activated in response to metal stress (Hobman et al. 2007). Gene *cpxRA* can enhance the production of membrane chaperons and protease which mitigate periplasmic stress, whereas gene *basRS* controls the biogenesis of capsular- and lipopolysaccharides. Gene *rpoE* gets activated on the introduction of defect of the outer membrane protein assembly. It can restore the protein assembly of the outer membrane by activating the production of chaperon and by upregulating the expression of β-barrel assembly machinery (Peng et al. 2018). The transcriptome analysis of *P.*

putida KT2440 in response to different dose of Zn revealed that with increasing stress, genes responsible to metal homeostasis, cell envelope structure, antioxidant enzyme, and basic cellular metabolism get affected. At lowest dose, genes associated with transportation of metal and membrane homeostasis were influenced. And at an intermediate level, both the above mentioned genes are highly expressed along with the expression of genes associated with oxidative stress and genes for amino acid metabolism. At the higher level of zinc stress, zinc ions can induce the generation of reactive oxidative stress with induction of alkylhydroperoxide reductase and ferredoxin-NADPH reductase which become essential for the maintenance of optimum levels of NADPH. Moreover, at the highest dose, a gene responsible for Fe-S cluster biogenesis gets induced with induction of glyoxylate cycle (Peng et al. 2018). In the case of genus *Sphingobium*, on exposure to high Ni concentration, about 118 genes are differentially expressed. Out of them, 90 were upregulated genes, and a cluster including genes coding for nickel and other metal ion efflux systems (similar to either *cnrCBA*, *nccCBA*, or *cznABC*) and for a NreB-like permease is also found (Volpicella et al. 2017).

9.6.3 Proteomics

Proteomics is suitable to reveal useful physiological profiles of bacteria at protein level (Zhai et al. 2017). The two-dimensional gel electrophoresis (2-DE) and two-dimensional difference gel electrophoresis (2-D DIGE) are the commonly used techniques of proteomics profiling. However, due to the presence of certain limitations, isobaric tags for relative and absolute quantitation (iTRAQ) are more competent which can allow the reliable quantitative description of differentially regulated proteins in complex systems (Zhai et al. 2017). Comparative proteomics easily identify the changes in expression of protein in response to metal stress and also provide information related to molecular mechanisms of tolerance to metal ions (Zivkovic et al. 2018). *P. aeruginosa* is one of the promising candidates for bioremediation which can tolerate high level of Cd by extracellular biosorption, bioaccumulation, biofilm formation, controlled production of siderophore, enhanced respiration, and modified protein profile. The mechanism behind it can easily be understood by proteome profiling of these bacteria (Zivkovic et al. 2018). The resistance of Cd in *P. aeruginosa* is mainly attributed to upregulation of metalloproteins in particular interest to denitrification proteins which are mainly located in the periplasm. These denitrification proteins are overexpressed but not active in exposure to Cd toxicity which suggests their protective role. They also observed the downregulation of siderophore which is regulated by ferric uptake regulation protein (FUR) which showed the effect of Cd on the iron homeostasis (Singh et al. 2011; Zivkovic et al. 2018). It was observed that *Klebsiella pneumonia* isolated from contaminated water sample from river Mula, Pune, is resistant to cobalt (Co^{2+}) and lead (Pb^{2+}). Proteome profiling showed the overexpression of two important proteins, viz., DNA gyrase A and L-isoaspartate protein carboxymethyltransferase type II in response to metal stress (Bar et al. 2007). DNA gyrase A plays an important role in replication,

transcription, recombination, and DNA repair. The overexpression of this protein help us to understand that the organism tried to survive in the presence of metal toxicity by expressing many genes on modifying the cellular processes including transcription and replication. Nonetheless, L-isoaspartate protein carboxymethyltransferase type II is mainly responsible to repair and/or degradation of damaged proteins. The overexpression of this protein revealed that bacterial cells have mechanism to allow repair of protein in response to cobalt stress (Bar et al. 2007). Proteomics analysis of *Acidithiobacillus ferrooxidans* also revealed up- and downregulation of four different proteins in response to high potassium concentration. The upregulation of proteins results in the thickening of glycocalyx layer which may be involved in survival of this bacterium in response to metal stress. However, downregulation of ATP synthase F1 delta subunit and ATP synthase F1 beta subunit proteins is associated with the decreased transportation of metal into the cell (Ouyang et al. 2013).

The iTRAQ analyses of *Lactobacillus plantarum* CCFM8610 which is strongly resistant to Cd and *L. plantarum* CCFM191 which is sensitive to Cd provide useful information about the underlying mechanism in response to Cd toxicity. Both the strains displayed physiological alterations in response to Cd. It was observed that 27 proteins were differently regulated in *L. plantarum* CCFM8610 on exposure to Cd, and 111 proteins were changed in *L. plantarum* CCFM191 in response to Cd stress (Zhai et al. 2017). The strong resistant of *L. plantarum* CCFM8610 to Cd is mainly attributed to specific energy-conservation survival mode, mild induction of its cellular defense and repair system, an enhanced biosynthesis of hydrophobic amino acids in response to Cd, inherent superior Cd binding ability and effective cell wall biosynthesis ability, a tight regulation on ion transport, and several key proteins, including prophage P2b protein 18, CadA, mntA, and lp_3327 (Zhai et al. 2017). Proteomics can also provide useful information of the impact of plant growth-promoting bacterial (PGPB) inoculation in plant for microbe-assisted phytoremediation. The total protein extract of maize plant, grown in normal and peripheral soils with or without PGPB, showed variation in protein profiling and protein responsible for various activities. In normal soil, 85 different maize proteins showed notable variation in response to inoculation of PGPB. The major proteins altered in maize with inoculation of PGPB were up-regulation of photosynthetic proteins which sustain the enhancement of chlorophyll a and total chlorophyll content of leaves, proteins in regulation-signal transduction, cellular metabolism, folding and degradation of protein. However, in peripheral soil, protein regulating the DNA repair, methionine biosynthesis, malate metabolic process, photosynthesis, and carbon fixation were upregulated in maize inoculated with PGPB and decreasing the activity of major antioxidant enzymes such as glutathione S-transferase (GST), catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), and ascorbate peroxidase (APX) (Li et al. 2014).

9.6.4 Metabolomics

Metabolomics is a technology to determine and quantify metabolites involved in different life processes. Since bacteria can synthesize a wide range of metabolites to adopt different stress condition, identification and quantification of these metabolites provide a better understanding of stress biology in bacteria. The metabolic fingerprinting can be performed by different techniques such as nuclear magnetic resonance (NMR), MS, Fourier-transform ion cyclotron resonance mass spectrometry, or Fourier-transform infrared (FT-IR) spectroscopy. The identification and quantification of the metabolites can be done by NMR, GC-MS, liquid chromatography-mass spectrometry (LC-MS), capillary electrophoresis-mass spectrometry (CE-MS), gas chromatography-mass spectrometry (GC-MS), NMR, and FT-IR spectroscopy.

Metabolomics provides unprecedented access to the variations in cellular metabolic architecture in response to metal stress (Booth et al. 2015). It is well known that tellurium (Te) is one of the toxic metals which is harmful to both prokaryotes and eukaryotes. Tremaroli et al. (2009) isolated *P. pseudoalcaligenes* KF707 from soil which was found to be resistant to tellurite and also KF707 mutant (T5) with hyperresistance to tellurite. Metabolomics profiling showed a remarkable variation of metabolites of *P. pseudoalcaligenes* KF707 and T5 in response to tellurite. *P. pseudoalcaligenes* KF707 displayed variation in levels of several metabolites, i.e., increase in threonine, leucine, tyrosine, betaine, serine, lysine, isoleucine, alanine, arginine, valine, glutathione, and adenosine whereas decrease in glutamate, aspartate, glycine, histidine, tryptophan, and tyrosine in T5 with and without tellurite. This variation can be correlated with oxidative stress response, resistance to membrane perturbation, and extensive reconfiguration of cellular metabolism. Metabolomics can also provide useful information about the impact of plant growth-promoting bacterial (PGPB) inoculation in plant for microbe-assisted phytoremediation. The metabolite profiling of maize inoculated with PGPB revealed the upregulation of photosynthesis, hormone biosynthesis, and tricarboxylic acid cycle metabolites of maize which makes the plant to remediate metal-contaminated land as well as better growth and development of the plant in metal-contaminated land (Li et al. 2014). Similarly, in *E. coli* and *B. subtilis*, the synthesis of cysteine and histidine is upregulated on exposure to Zn (Hobman et al. 2007).

9.7 Evolution of Strategies

It is notable that most of the heavy metals are required in low level for smooth functioning of bacterial cell, but at high level, it becomes toxic to cell (Coombs and Barkay 2005). In this aspect, metal homeostasis genes play an important role to regulate the transportation of metals into and out of the cell. On increasing the exposure of heavy metals to bacterial cell, the metal homeostasis genes are gradually evolved to survive in metal-rich environment (Coombs and Barkay 2005). Bacteria also develop their genetic system and

produce specific enzymes to sequester, remove, or transform the harmful effects of metal toxicity and adapt to that environment. Furthermore, it was observed that bacteria can adapt to changing environmental condition but it is poorly understood in relation to evolution (Hemme et al. 2016). The horizontal transfer of genes (HGT) for metal resistance present in plasmid and gene duplication provides a major contribution to understanding the evolution of bacterial genome in response to metal stress (Kandeler et al. 2000). However, identifying and quantifying such events remain elusive. To delineate the problem, the whole genomes sequencing of isolated bacteria from the environment can provide useful information on comparing to the reference. However, most of the metagenomes of environmental samples are found to be dominated by few reference genomes which also can provide information about the evolution of genome to adapt to contaminated environmental conditions.

The role of HGT has been well recognized for the transfer of antibiotic resistance genes in bacteria, but it is less explored in transfer of metal transporting genes. On performing cultivation-independent analyses of community genomic DNA and RNA from groundwater of contaminated sites, Hemme et al. (2016) observed the abundance of metal-resistant *Rhodanobacter.* The amplicon sequence analysis indicated that the genes coding for Fe^{2+}/Pb^{2+}permeases, most denitrification enzymes, and cytochrome c_{553} are not subjected to horizontal gene transfer (HGT). However, the numerous metal resistance genes, particularly Co^{2+}/Zn^{2+}/Cd^{2+} efflux and mercuric resistance operon genes, are found to be mobile within *Rhodanobacter* populations which aid in understanding the dominance of *Rhodanobacter* populations in contaminated sites. Similarly, the conjugal transfer of *czc* genes of plasmid pDN705 found in *A.eutrophus* CH34 to *E. coli* confers resistance against cobalt, cadmium, and zinc in metal-contaminated sites (De Rore et al. 1994). Nongkhlaw et al. (2012) reported the evolution of ecologically important phenotype where HGT of P_{IB}-type ATPase genes among Firmicutes, Bacteroidetes and Proteobacteria in U rich soil from Domiasiat of India.

9.8 Ecophysiology and Application of Metallotolerant Bacteria

9.8.1 Bioremediation

Bioremediation of contaminated sites with bacteria or bacterial consortia has emerged as most probable alternative. The bioremediation potential of microorganisms mainly depends upon the response of microorganisms toward toxic heavy metals to remediate polluted environment (Bestawy et al. 2013). They can perform bioremediation both in aerobic and anaerobic conditions (Azad et al. 2014). The metallotolerant bacteria used various mechanisms for bioremediation, including biosorption, bioaccumulation, biomineralization, and biotransformation (Dixit et al. 2015).

In biosorption, bacteria can eliminate the metal ions on attracting them to cell membranes (Azad et al. 2014). Sorption of metals by bacterial cell is generally mediated by metallothioneins (a family of 0.5–14 kDa proteins) which are rich in cysteine residues as well as histidine residue also. These metallothioneins act as a scavenger of free radical and combine with harmful molecules like superoxide and hydroxide ions. After this, cysteine gets converted into cystine on oxidation, and thus the bound metals are released into the environment (Das et al. 2016). The process of biosorption is often coupled with enzymatic conversion of metals where on being adsorption of metals on bacterial cell, it gets acted upon by enzymes which can precipitate metals as salt (Das et al. 2016). Since the process of biosorption depends on the basic principle of adsorption, it also encounters difficulties in regard to change of pH and ionic strength (Diep et al. 2018). Again, this process of biosorption has limited life spans since it is not influenced by metabolic contribution and often uses dead biomass (Diep et al. 2018). Various bacterial strains such as *B. subtilis* and *Magnetospirillum gryphiswaldense* were tested as probable biosorbents. During the process of bioaccumulation, heavy metal ions pass across the cell membrane into the intracellular space by translocation pathway. On entering the intracellular space, the heavy metals are sequestered by proteins and peptide ligands. This type of metal uptake is referred to as active uptake (Al-Gheethi et al. 2015). In biotransformation process, the toxic heavy metals are converted to nontoxic form by redox conversions of inorganic forms and conversions from inorganic to organic form, and vice versa. Microbes can oxidize certain metals to obtain energy and/or can reduce some metals where they can utilize metals as a terminal electron acceptor (Niggemyer et al. 2001). In biomineralization, bacteria can remove toxic metals from solution by precipitating insoluble metal sulfide and phosphate.

Bioremediation potential of bacteria can also be upgraded basically by two methods. One of the techniques is to improve or redesign microorganisms to enhance the metal-accumulating properties of the cells, and another one is concerned with modifying the binding sites of the cell by developing commercial biosorbents using immobilization technologies or chemical modifications. According to many researchers, metallotolerant bacteria isolated from heavy metal-contaminated sites are more potent candidates of bioremediation due to their better adaptation and heavy metal resistance mechanism (Al-Gheethi et al. 2015). The bacteria *Lysinibacillus* sp., *Staphylococcus sciuri*, *B. fastidiosus*, *B. niacini*, *Clostridium* sp., and *Bacillus* sp. were reported to be tolerant to As, Cd, and Hg, which were also applied in bioremediation (Bhakta et al. 2018). Similarly, various gram-negative bacteria like *Enterobacter* sp., *Stenotrophomonas* sp., *Providencia* sp., *Chryseobacterium* sp., *Comamonas* sp., *Ochrobactrum* sp., and *Delftia* sp. were isolated from activated sludge, which appear to tolerate Cu, Cd, and Co, and can be efficiently utilized for bioaugmentation of activated sludge to treat the industrial effluents efficiently (Bestawy et al. 2013). Wastewater released from industries and domestic, commercial, or agricultural activities should be treated to detoxify the toxic effect of metals prior to release in water bodies or land surfaces. The heavy metal-resistant bacteria of the genus *Micrococcus* isolated from wastewater may be potentially used for bioremediation of sewage sludge, industrial wastes, and

industrial effluent (Benmalek and Fardeau 2017). Marzan et al. (2017) isolated *Gemella* sp., *Micrococcus* sp., and *Hafnia* sp. from tannery effluent where *Gemella* sp. and *Micrococcus* sp. showed resistance to Pb, Cr, and Cd. These bacterial strains can be used as bioremediation agents in toxic tannery effluent treatment technology.

Phytoremediation is another type of bioremediation where hyperaccumulating plants are used to remediate contaminated sites. Plants can perform phytoremediation by different mechanisms, viz., phytoextraction, phytostabilization, phytovolatilization, and rhizofiltration (Rathore et al. 2017). However, the process of phytoremediation generally restricts the growth and biomass of the hyperaccumulating plant when the concentration of metal is very high in the contaminated soil. It can be easily overcome by introducing metallotolerant and plant growth-promoting microorganism to the plant (Tirry et al. 2018). These bacteria can alter the bioavailability of heavy metals to plant by acidification, by releasing chelating substances, and by changing the redox potentials (Whiting et al. 2001). The inoculation of endophytic bacteria in hyperaccumulating plants receives comparable importance due to their capacity to assist the plant growth and development in metal-contaminated sites by producing plant growth regulators, increasing the uptake of mineral nutrients and water, nitrogen fixation, and systemic resistance of plants against pathogens. Endophytic bacteria help the plants to survive in metal-rich sites by converting toxic metal ions to nontoxic forms. They can also degrade contaminants by producing various enzymes (Sharma et al. 2018). The endophytic bacteria belonging to the genera *Acinetobacter*, *Bacillus*, *Arthrobacter*, *Burkholderia*, *Clostridium*, *Enterobacter*, *Micrococcus*, *Paracoccus*, *Rhodococcus*, *Pseudomonas*, *Streptomyces*, *Staphylococcus*, etc. are reported as heavy metal-resistant bacteria which may be used to rehabilitate metal-contaminated sites (Sharma et al. 2018). Similarly, the rhizospheric bacteria in combination with plants play a significant role in phytoremediation of metal-contaminated soils by acidification, phosphate solubilization, releasing chelating agents, and redox changes. The heavy metal-resistant and plant growth-promoting bacterium *Cellulosimicrobium* sp., isolated from the rhizosphere of a contaminated region in the Plain of Sais, Fez (Morocco), enhances the growth of alfalfa plants in heavy metal-contaminated sites (Tirry et al. 2018). Similarly, *B. subtilis* SJ-101 was found to stimulate indole-3-acetic acid (IAA) production which enhances the growth of *Brassica juncea* in Ni-contaminated soil (Mishra et al. 2017). The As tolerant *B. licheniformis*, *Micrococcus luteus*, and *P. fluorescens* were reported to enhance the biomass of grapevines in contaminated sites (Mishra et al. 2017).

9.8.2 Bioleaching

Bioleaching is a process where the insoluble metal sulfides get converted to metal sulfate. The metals are released from metal sulfide mainly by two different mechanisms including direct and indirect bacterial leaching. During direct leaching, bacterial cell directly comes in contact with specific sites of crystal imperfection mineral

sulfide surface, and sulfates get oxidized by different enzymatic pathways (Bosecker 1997). Microbes can solubilize metals by (i) acidolysis, where microbes produce acids by which it can leach metals; (ii) complexolysis, where microbes excrete biogenic agents which solubilize metal ions by ligand formation; and lastly (iii) redoxolysis, where microbes used oxidation and reduction reactions to solubilize metals (Monballiu et al. 2015). This method is widely used by bacteria for removal of heavy metals from metal-rich sites (Jeremic et al. 2016). The process of bioleaching is affected by various physicochemical and microbiological factors. Hence, it is important to maintain optimum growth of microorganisms so that they can leach the metals more efficiently (Monballiu et al. 2015). However, the growth and metabolic processes of microorganisms involved in bioleaching are adversely affected by the occurrence of heavy metals in bioleached materials. The metals can inhibit the enzyme activities, disrupt the membrane transport processes, and ultimately lead to inhibit the growth of microorganisms. Hence, it is better to utilize metallotolerant bacteria for the efficient bioleaching process (Monballiu et al. 2015). Metallotolerant bacteria can sequester metal ions extra- and/or intracellularly and carry metal resistance genes. They can adapt to metal-rich sites by active transportation of metal ions, interaction with extracellular polymeric substances (EPS), formation of cell surface complexes, and metal reduction to a less toxic state (Monballiu et al. 2015).

Most of the microorganisms used in bioleaching process are found to be belonging to the genus of *Thiobacillus* (*T. ferrooxidans, T. thiooxidans*, and *T. cuprinus*). They can oxidize sulfides, elemental sulfur, and thiosulfate to sulfate on utilizing sulfur as an energy source (Roy and Roy 2015). Hence, researchers are progressively focused on this group of bacteria isolated from metal-contaminated sites (mines and mine tailings) for their bioleaching potentials. Except these, many heterotrophic bacteria are also reported to be used for bioleaching. Pyrolusite is a mineral containing manganese dioxide and is important ore of manganese. This ore can be degraded by *Bacillus*, *Micrococcus*, *Pseudomonas*, *Achromobacter*, and *Enterobacter* by enzymatic reduction under both aerobic and micro-aerobic growth conditions (Roy and Roy 2015). Most of the metallotolerant bacteria belonging to the genus *Bacillus* are widely used for bioleaching. For example, *B. mucilaginosus* is one of the bacteria which is resistant to Cr, Ni, and As and can be used for its bioleaching (Monballiu et al. 2015). Furthermore, *T. ferrooxidans* and *P. Aeruginosa*, isolated from waste dump of magnesite and bauxite mines of Salem district in Tamil Nadu, are resistant to heavy metals Mn, Fe, Cu, Cr, and Hg and these two bacteria effective in bioleaching process (Mathiyazhagan and Natarajan 2011).

9.8.3 Biomining

Biomining is a process where microorganisms are used to oxidize iron and sulfur to recover metals from minerals containing copper, gold, and uranium (Valenzuela et al. 2006). In this process, metal tolerant acidophilic bacteria have a special advantage due to their metal resistance mechanisms (Jeremic et al. 2016). Mostly bacteria from the genus of *Acidithiobacillus*, *Leptospirillum*, *Acidimicrobium*,

Ferromicrobium, *Sulfobacillus*, and *Thiomonas* are reported as most potent for biomining (Valenzuela et al. 2006). They have the capacity to withstand extremely acidic environment and can tolerate high metal concentrations. For example, *A. ferrooxidans*, which is most studied biomining bacterium, can tolerate Cu, As, Zn, Cd, and Ni in the concentration of 800 mM, 84 mM, 1071 mM, 500 mM, and 1000 mM, respectively. Similarly, *A. caldus*, *Cupriavidus metallidurans*, *Thiomonas cuprina*, *Thiomonas arsenitoxydans*, *Metallosphaera sedula*, and *Sulfolobus solftaricus* can tolerate some of the heavy metals (Navarro et al. 2013). For that, they have genes related to metal tolerance. It was reported that *A. ferrooxidans* ATCC 23270 cells have at least ten genes relating to Cu homeostasis. These genes are upregulated on introducing this bacterium to a high level of Cu concentration (5–25 mM or higher) (Orell et al. 2009).

9.8.4 Metabolic Engineering

Metabolic engineering is a tool to develop or redesign bacteria to detoxify specific metal more specifically. It is performed by recombinant DNA/RNA technology and can be successfully used to decontaminant heavy metals from contaminated sites, e.g., GE *E. coli* strain JM109 containing *merA* gene removes mercury from contaminated site by expressing metallothioneins and polyphosphate kinase. By the same token, GE *E. coli* containing *arsR* gene removes arsenic from the contaminated site by bioaccumulation of As, and GE *Ralstonia metallidurans* and GE *Caulobacter* sp. strain JS4022/p723-6H removes Cr and Cd respectively from industrial wastewater, respectively (Azad et al. 2014). *Deinococcus geothermalis*, a radiation-resistant thermophilic bacterium, has been genetically engineered for bioremediation purposes on introducing Hg(II) resistant *mer* operon of *E. coli* (Dixit et al. 2015). The metal resistance capacity of metallotolerant bacteria can also be enhanced by inducing mutation with different mutagenic agents. It is observed that *Pseudomonas* sp., a bacterium isolated from soil and factory effluents, tolerate Cu and Zn more specifically when induced by mutagenic agents such as acriflavine, acridine orange, and ethidium bromide (Shakibaie et al. 2008). The Cd^{2+}tolerance is enhanced in *P. aeruginosa* on mutation of *cad* operon by acridine orange and acriflavine (Kermani et al. 2010).

9.9 Conclusion and Future Perspectives

Environmental niches have become more prone to heavy metal pollution due to their long persistent and nondegradable nature. It may be due to the growing industrialization and human activities related to mining, disposal or leakage of industrial wastes, the use of pesticide, and sewage sludge which become hazardous to both ecological and human health. In such condition, where growth of most of the life forms including microorganisms is difficult, metallotolerant bacteria have the capacity to adapt and survive in various metalliferous environments. They develop

various cope-up mechanisms and metal-resistant genotypes to adapt to this unfavorable condition. Different omics approaches shed light on the detail molecular mechanism behind it. In addition, due to their metal tolerance capacity, these bacteria can be exploited for the remediation of heavy metal polluted sites, biomining, bioleaching, and various biotechnological approaches. Since a vast diversity of metallotolerant bacteria are prevalent in metalliferous environment, on exploring the diversity and genetic makeup of these bacteria, there is a possibility to discover novel metal-resistant genes and proteins which make them to survive and adapt more efficiently to these extreme environmental conditions. On understanding the mechanism of metal tolerance potentials of metallotolerant bacteria, it becomes also possible to transform bacteria and/or transfer the gene to other potent bacterial strains for enhanced bioremediation and other areas of biotechnological approaches.

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