RSC Advances

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

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Microbial Stress Response to Heavy Metal in the Environment

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Abstract: Heavy metal contamination is a global environmental issue as it possess significant threat to public health and exposure to metals above a certain threshold level can cause deleterious effects to all living organisms including microbes. In order to survive in such harsh environments, some microbes evolved a few defence mechanisms to metabolize and transform heavy metal into a less hazardous form and simultaneously induce the formation of heavy metal resistant microbes. Heavy-metal resistant microbes can be used in bioremediation to remediate contaminated areas. Bioremediation uses natural biological activities, is relatively low-cost and has high public acceptance. Here, we summarize interactions and mechanisms that occur between microbes and heavy metal; including stress response and defence mechanisms that involve aggregate and biofilm formations, production of extracellular polymeric substances (EPS), development of resistance genes and signalling pathways against heavy metals.

Keywords: microbes; two-component signalling transduction; defence mechanism; stress response; environment; extracellular polymeric substances (EPS)

1. Introduction

Heavy metals can be defined by various criteria including density, atomic weight, atomic number, chemical properties and lewis acid behaviour[1], however density is the main aspect to be consider as the defining feature .[2] The metals consisted of atomic density exceeding 5 gcm^{-3} and atomic number above 20.[3-5] Accumulation of heavy metals above the threshold level is mainly due to anthropogenic activities including mining, chemical manufacturing, agriculture[6], hospital wastewater[7] and electronic waste[8]. Heavy metals can pose cytotoxic, carcinogenic and mutagenic effects and most heavy metal are hazardous to human even in low concentration.[9] It was proven that the accumulation of heavy metal in the body has caused a severe effect in the heart, brain, kidney, bones and liver.[10]

Heavy metal pollution is considered as most severe environmental issue since the pollutant capable to infiltrate deep into the bed of groundwater sources and surface water, and affect public health.[11-12] These heavy metals will end up in the food chain and form bioaccumulate and transfer from one food chain to another.[5] Metals are able to exert their toxicity because it is non-degradable and are only transformable via methylation, sorption and complexation and alteration in a valence state which influence the bioavailability of metals and mobility.[12] Urban areas with high population density and accelerated anthropogenic activities such as mining are considered as a reservoir of pollution commonly made up of heavy metals.[13-14] Mine water pollution could cause severe impacts to biological systems as species diversity and total biomass composition in aquatic and terrestrial ecosystems can be affected due to acidity and heavy metal contamination.[15]A recent issue on heavy metal contamination, containing mostly iron, zinc and copper, that occurred in Colarado, US was reported in August 2015 where a million gallons of wastewater spilled out from an abandoned mine and caused severe heavy metal pollution in the Animas River.[16] It has been reported that heavy metals contamination due to mining activities involved around 2 million hectares out of 10 million hectares of heavy metals contaminated land in China.[14] Another study that was conducted to evaluate chemical speciation of heavy metal in sediment of former tin mining area at Selangor, Malaysia proved that the sediment were contaminated with chromium, zinc, arsenic , copper, lead and mainly with tin.[15]

Bioremediation process that uses biological agents to effectively remove organic and inorganic toxic wastes from the environment which generally has a major public acceptance could be the key for solution.[17-19] The application of microbial metabolism as an alternative to physio-chemical methods to remediate contamination are considered to be safer, more effective and less expensive.[20] Thus, a further understanding on the mechanisms involved in heavy metal resistance and application of resistant bacteria in bioremediation are crucial to overcome this condition.

2. Interaction between microbes, minerals and metals

In biogeochemical cycling of heavy metals, microbes exhibit an important role in cleaning up the metals.[22] Metals are classified into three major classes according to its biological roles and effects: (i) the essential metals with recognized biological role (Na, Ca, K, Mn, Mg, V, Fe, Cu, Co, Mo, Ni, Zn and W), (ii) the toxic metals (Ag, Sn, Cd, Au, Ti, Hg, Pb, Al and metalloids Ge, Sb, As and Se) and (iii) the non-essential, non-toxic with no biological effects (Rb, Sr, Cs and T).[18] The top most prevalent environmental toxic metals like As, Pb, Cb and Hg are dangerous to public health.[23]

Heavy metal are grouped into five categories according to primary accumulation mechanisms in sediments: (i) adsorptive and exchangeable, (ii) bound to reducible phases (Mn oxides and Fe), (iii) bound to carbonate phase (iv) bound to organic matters and sulphides and (v) detrital or lattice metals.[22] Interaction between microbes, metals and minerals occur in both natural and unnatural conditions with some alteration to their physical and chemical states; at the same time, metals and minerals are also capable of influencing microbial growth, activity and survival by involving directly or indirectly in all phases of microbial metabolism, growth and differentiation.[18]

Metals such as Na, Zn, K, Ca, Cu, Co, Mg, Mn and Fe that go beyond the threshold concentrations will exert toxicity to cells even though it is essential for life.[18] Metals like Cu, Co, Cu, Cr, Ni, Zn, Mg, Fe, Na, K and Mn are micronutrients that are required by cells and are involved in the redox reaction.[24] These micronutrients stabilize molecules via electrostatic interactions, regulate osmotic pressure, act as components of various enzymes and form

concentration gradient and charge across cytoplasmic membranes.[25] Physio-chemical properties of the particular environment and chemical behaviour of the metal species affect metal toxicity. Some metals even cause microorganisms to flourish despite toxicity in sites that are polluted with metals with various mechanisms to develop resistance toward metals.[18] This condition has caused the development of heavy metal resistant bacteria that have been isolated from various environmental sources globally (Table 1).

Table 1: List of selected heavy metal resistant bacteria isolated from various environmental sources globally.

Heavy metal	**Microorganism**	**Location**	**Reference**
As	*Enterobacter agglomeran* *Acinetobacter lwoffii*	Combodia	[26]
Cu, Pb, Cd	*Bacillus megaterium X4*	Korea	[27]
Cu	*Sphingomonas* sp. *Stenotrophomonas* sp. *Arthrobacter* sp.	Chile	[28]
Cu, Co, Ni, Zn, Cr, Cd, Pb	*Pseudomonas aeruginosa ASU 6a*	Egypt	[29]
Pb, Cr, Zn, Cu	*Streptomyces* *Amycolatopsis*	Morocco	[30]
Hg, Cr, Ag	*Bacillus* sp. *Pseudomonas aeruginosa* *Enterobacteriaceae* strain	Brazil	[6]
Cu, Cd, Pb, Cr, Ni	*Pseudomonas putida* *Cupriavidus necator*	China	[31]

	Eiguobacterium sp. *Bacillus aquimaris* *Bacillus cereus* *Alcaligenes* sp.		
As, Pb	*Bacillus* sp.	India	[32]
As, Hg	*Bacillus* sp. *Lysinibacillus* sp.	Iceland French Guiana Spain	[11]
Hg	*Pseudomonas sp.* *Escherichia coli* *Serratia marcescens*	Iran	[33]

Bioremediation are carried out *in-situ* or *ex-situ*. *In-situ* bioremediation is executed in the polluted area, which cost less and discharge less pollutants to the environment whereas in *ex-situ* bioremediation, the contaminated material will be removed to be treated elsewhere and requires shorter treatment time frames. In comparison, the conventional processes that have been used to eliminate heavy metal from industrial wastewaters such as chemical precipitation, oxidoreduction, filtration, electrochemical technique and sophisticated separation processes using membrane are far more expensive.[34] The addition of exogenous microorganisms that are genetically modified or with natural catabolic genes to enhance and expand indigenous population is known as bioaugmentation.[35] Engineered bioremediation may speed up the growth of microbes and optimize the detoxification process.[36] Reducing the bioavailable concentration and interaction of the toxic metal with the cell helps in boosting the organic bioremediation process.[34]

Present of parameters with optimum level such as adequate nutrient, optimum growth, temperature, oxygen level, solute concentrations and pH enable microbes able to flourish at the peak of their growth rate. Any alteration in this parameter is considered as an environmental stress, thus the microbes need to sense and react toward it in order to sustain in that environment. As a matter of fact, majority of bacteria that able to thrive in a constant

state of stress with the optimum growth conditions, are mostly exist only inside the laboratory environment. Bacteria have the potential to sense and react to stress stimuli via coordinated alteration in gene expression.[21] Response mechanisms against alteration in surviving environment are generally available and changes usually lead to the synthesis of specific molecules that respond to the adverse environmental conditions .[21] Microbes that develop resistance toward metals can be utilised as a bioremediation agent. Biochemical evolution in microbes, in order to defend against heavy metal toxicity, can be advantageous in the application of bioremediation.[37]

3. Bacterial resistance towards heavy metals

Microbial inhibition by heavy metal occurs when heavy metal block essential functional groups or interrupt with essential metal ions incorporation to biological molecules.[34] Heavy metal interrupt binding of essential metal ions to the cellular structure with its high electrostatic attraction and binding affinities to the similar site. This leads to the destabilization of structure and biomolecules (cell wall enzymes, DNA, RNA) which trigger defects in the replication process followed by mutagenesis.[35] Three major mechanisms are involved in the attachment of metals to bacterial cell walls: (i)precipitation via nucleation reactions and (ii) complexation with nitrogen and oxygen ligands, (iii) ion exchange reaction with teichoic acid and peptidoglycan Gram-positive bacteria, especially *Bacillus* sp. possess a high adsorptive capacity as teichoic acid and peptidoglycan contents in the cell wall are high; on the other hand Gram-negative bacteria cell membrane, which has a lower amount of these components are weak metal absorbers.[38]

There are five mechanisms involved in metal toxicity in microorganism: (i) substitutive metal-ligand binding, interruption or destruction in biological function of the targeted molecules when replacement of another metal ions occur at the binding site of specific biomolecules; (ii) covalent and ionic reduction-oxidation (redox), reaction of metals ions with cellular thiols (R-SH), specifically glutathione, reaction between thiols and oxyanions that produce hazardous reactive oxygen species (ROS) as a by-product from reduction. The Pinter-type reaction of thiols with metal oxyanions such as Se and Te oxyanions (SeO_4^{2-}, SeO_3^{2-}, TeO_4^{2-} and TeO_3^{2-}); (iii) Fenton-type reaction, which involves transition metals such as Cu, Ni and Fe that produce ROS. ROS are extremely reactive compounds that could oxidize every biological macromolecules; (iv) inhibition of membrane

transport processes, specific membrane transporter inhibited by toxic metals by engaging to binding sites and/or interrupting with membrane potential that are conserved for essential substrates; (v) electron siphoning by thiol-disulphide oxidoreductase at the respiratory chain caused destruction of cell membrane's protein motive force.[23] Production of oxygen radicals induced by metals affects DNA as well as other cellular composition like polyunsaturated fatty acid residues of phospholipids that are oxidation sensitive.[36]

A rapid and effective process for heavy metal elimination from cells is important to avoid toxicity. Typically, there are two types of mechanisms involved in resistance towards heavy metal ions: (i) intracellular complexation of toxic metal ions mainly in eukaryotes and (ii) reducing the accumulation of cations based on active efflux in prokaryotes.[39] Specifically, heavy metal resistance in bacteria involves five mechanisms: (i) expulsion of metal by a permeability barrier, (ii) extracellular sequestration, (iii) intracellular physical sequestration of metal by binding to protein or other ligands to avoid damage to the metal-sensitive cellular targets, (iv) expulsion by active export of metal from cell and (v) transformation and detoxification.[40-41]

With a strong ionic nature, metals are able to bind to many cellular ligands and dislocate native essential metals from their regular binding site, which is hazardous to cells. Non-enzymatic detoxification may also occur when microbes release inorganic metabolic products including carbonate, sulphide or phosphate ions through their respiratory metabolism and precipitation of toxic metal ions. Cellular sequestration and accumulation or extracellular precipitations are applied by metals to immobilize metals in nature. Metal ions attach to the cell surface through several mechanisms that include van der Waals forces, redox precipitation, covalent bonding, or fusion of these processes. Carboxyl, hydroxyl and phosphoryl are negatively charged group of bacterial cell wall that retain metal cations by mineral nucleation after absorbing the metal cations.[22] Heavy metal toxicity can be reduced by overexpression of metal binding peptides on the microbial cell surface to increase the capacity of adsorption.[42]

Enzyme detoxification is the key mechanism of bacterial resistance toward metals. The presence of resistant genes in bacteria to metals and metalloid is an advantage as observed in *Bacillus spp.* for Hg^{2+} and Cd^{2+} resistance. Synthesis of various metal-binding peptides and proteins such as metallothioneins (MTs) and phytochelatins (PCs) aids in the

regulation of metal ion homeostasis and effect in toxic responses.[18] MTs are low molecular weight protein that encoded by *mt* genes and are expressed in bacteria to boost metal resistance through immobilization while PCs are polypeptides that consist a high number of gamma(PCs), a dipeptide residue. Both MTs and PCs contain high cystein (Cys) level which is an amino acid that contains sulphur (S) atoms to bind metals.[42] In bacteria, altering the fatty acid composition of their lipids is one of the defence and/or repair mechanism used to maintain membrane fluidity. Modification of the lipid acyl chain structure by modifying the ratio of saturation to unsaturation, branched to unbranched formation, *cis* to *trans* unsaturation, acyl chain length and form of branching are executed as a response to toxic agents.[25] Heavy metal stress also causes alteration in fatty acid composition by qualitative and quantitative alteration of lipids, inhibition of biosynthetic pathways and lipid peroxidation.[25]

Due to the fact that concentration of metals above the threshold level is hazardous to microbes in the environment as it poses a deleterious impact on microbial functional activities,microbes that are present in heavy metal contaminated soil have evolved several schemes to exhibit resistance toward the heavy metal.[3,19] Metal-ion-specific Physio-chemical parameters including the Pearson softness index, standard reduction potential (ΔE^0), electron density electronegativity (χ), the solubility product of the metal-sulphide complex (pK_{SP}) and covalent index are related to the susceptibility of microorganisms towards toxic metal species.[23]

Elimination of heavy metals from polluted area are tricky as unlike other pollutant, heavy metals cannot be converted into less hazardous, less mobile and/or less bio-available form via biodegradation process. Basically, microbes could either be resistant or tolerant toward the pollutant. Tolerance is described as the ability of an microorganism to survive in a polluted environment through intrinsic properties of the microorganism while resistance is the ability of microbes to survive in a high concentration of a toxic substance via detoxification mechanisms as direct response toward existence of the similar contaminant.[3,38] Resistance mechanisms in bacteria are encoded typically on the plasmid and transposons. This might due to gene transfer or spontaneous mutations that cause those bacteria to eventually gain resistance to heavy metals as exposure to DNA damaging agents could result in genetic changes.[19,43] Generally, a gene that is responsible for heavy metal resistance is located in the extrachromosomal circular DNA, for example a plasmid that is carried by

metal resistant bacteria.[19] Resistant genes will be induced and expressed in the presence of the specific metals and regulated when certain concentrations of the metals are reached. Promoters and regulatory genes from the bacterial operon that are responsible for resistance used as metal-specific biosensors (promoter-reporter gene fusion), regulate metal resistant genes' expressions in the presence of specific metals in specific concentrations.[3,19]

As some heavy metals are crucial for enzyme function, growth and metabolism, understanding the mechanism of heavy metals uptake in bacterial cells could provide a deeper view of the resistance mechanism. Generally, there are two types of heavy metals uptake mechanisms: (i) by osmotic gradient across cell membrane which doesn't require ATP, and (ii) by specific substrates which are dependent upon ATP released from ATP hydrolysis and is slower when compared to ATP-independent mechanism.[3,19] Some of the mechanisms involved are highly specific biochemical pathways that act as a protective barrier to protect microbes from toxic heavy metals which can be favourable in handling metal contamination. The detoxification process by microbes involves alteration in chemical properties of the metals instead of degrading. Previous studies proved that microbes that belong to a heterotrophic group are capable in the mobilization of metals through the organic acids production whereas autotrophic bacteria like *Thiobacillus* spp. are capable of producing metal-leaching sulphuric acid by oxidizing elemental sulphur.[37]

Biosorption is metabolism-dependent sorption of radionuclide and heavy metal to biomass. The presence of amine, carboxyl, hydroxyl, sufhydryl group and phosphate lead to negatively charged cell surface at neutral pH, thus enable absorption of considerable amount of positively charged cationic metals.[37] Bacterial growth phase, biomass density and living status of the biomass are directly propotional to capacity of biosorption.[44] Gram-positive bacteria's cell wall possesses more affinity than Gram-negative bacteria and is able to attach higher concentration of metals.[3] Microbial detoxification usually requires efflux or exclusion of metal ions from the cell. This phenomenon results in a high local concentration of metals at cell surface which allows reaction with biogenic ligands and precipitates.[37] Biosorption of heavy metal by bacteria depends on non-enzymatic process adsorption which described as the non-specific binding of metal ions to protein and extracellular/cell surface-associated polysaccharides. Microbial biosorbent rely on the microbial species, it either could be active or passive process. Passive uptake of metal ions is a process with rapid, irreversible, independent of cellular metabolism, non-specific to metal species and physical condition and

ionic strength, while in contrast active process is slow and dependent on cellular metabolism.[3]

Biological reduction of some metals cause significant changes to solubility. For instance, U(VI), highly soluble and mobile form of uranium becomes extremely insoluble as U(IV) after undergoing enzymatic reduction by anaerobic bacteria. Anaerobic bacteria use indirect mechanisms to reduce and precipitate some metals. For an example, Fe(III)-respiring bacteria, that capable of catalyzing formation of Fe(II)-bearing minerals to reduce and precipitate high valence metals abiotically[37] and Hg that are more bioavailable to microorganism under anaerobic environment.[45] Another example is *Serratia marinorubra*, a facultative anaerobes marine bacteria that able to transform arsenate to arsenite and methylarsonate under anaerobic condition.[46] Biomethylation that involve formation of volatile and non-volatile methylated compound of metal and metalloids[46] formed in environment by microorganism biotically.[47] For an example, during biotransformation of arsenic, microorganisms such as bacteria, fungi and algae methylated hazardous inorganic arsenic to form monomethylarsonic acid and dimethylarsinic acid.[48]

3.1 Efflux Transporter in Heavy Metal Resistant Bacteria

In bacteria, acquisition of essential metal ions from outside of cells demands consideration. Every Gram-negative bacteria has periplasmic space, an outer membrane and inner cytoplasm which metals ions have to pass through to get to the cytosol. In contrast, Gram-positive bacteria lack of periplasm and the presence of porins on the outer membrane permit metal ions to undergo non-selective passive diffusion across the outer membrane.[49] Heavy metal elimination relies on energy-dependent ion efflux from cell by membrane protein. It acts as an ATPase or chemiosmotic cation/proton antiporters and not by chemical detoxification.[50] High-affinity transport systems in the outer membrane or fixed in the inner membrane aid in the transportation of metal ions into the cytosol. Hydrolysis of ATP on the cytoplasmic side of the membrane drive inner membrane transport systems, for example ATP-binding cassette (ABC) transporters and P-type ATPase or coupled to cation diffusion facilitator (CDF) proteins.[49] Specific and non-specific transporters help in the transportation of essential metal ions into the cytoplasm. In non-specific transporters, it is conducted by chemiosmotic gradient across the bacterial cytoplasmic membrane to transport in metal ions during the presence of excess metals. This situation, also known as ‘open gate’ causes heavy metal ions

to become toxic. For the specific transporter, it requires specific metabolic situation or starvation and is only expressed when needed.[40]

There are three main classes of efflux transporters: (i) P-type ATPase which incorporates in the inner membrane and uses ATP to transport metal ions from the cytoplasm to the periplasm, (ii) CBA transporters which exist in Gram-negative bacteria and are three-component transenvelope pumps that play a role as chemiosmotic antiporters and (iii) Cation diffusion facilitator (CDF) transporters that function as chemiosmotic ion-proton exchangers. P-type ATPase and CDF transporters which export metal ions from the cytoplasm to the periplasm are common in many bacterial species while CBA transporters, a resistance-nodulation-cell divison (RND) protein in Gram-positive bacteria) primarily detoxify periplasmic metal (outer membrane efflux) present in a high-level resistance toward heavy metal. CBA transporter eliminate ions that are transported to the periplasm by ATPase and CDF transporters. P-type ATPase and CDF transporters are functionally identical and can substitute each other but not CBA transporters (Figure 1). Each of these transporters has their own mode of action (Table 2). P-type ATPase transport metal ions from cytoplasm to periplasm in the presence of ATP as the energy source; CBA transporters 'bridge' the whole cell wall (in Gram-negative bacteria) and transport metal ions from periplasms and cytoplasm to the cell exterior by using chemiosmotic gradient; CDF export ions from the cytoplasm to the periplasm and is driven by proton motive force .[39]

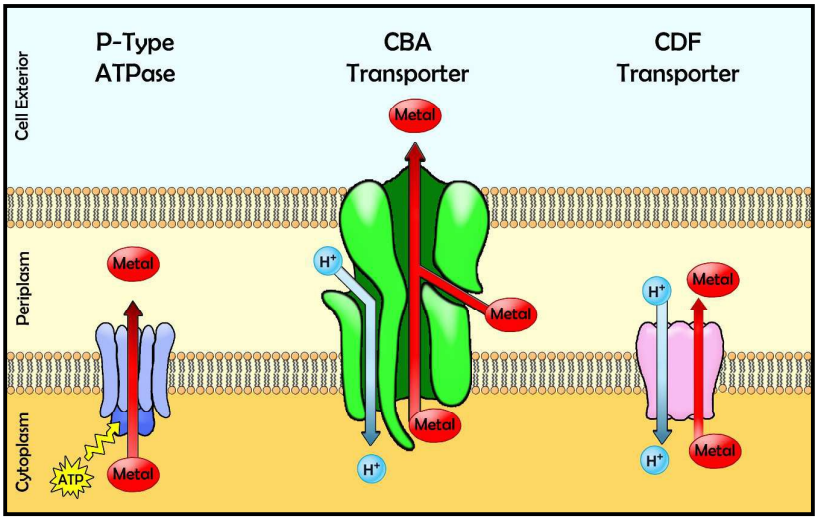

Figure 1: Major transporter families taking part in heavy metal resistance.[39]

Table 2: Types of efflux transporters and their functions

Transporter	Description and functions
P-type ATPase	• Involvement of phosphoenzyme intermediate during reaction cycle contributes to the term P-type. • Driven by energy produced from the removal of γ-phosphate from ATP. Substrates are inorganic substrates like H^+, Na^+, K^+, Mg^+, Ca^+, Cu^+, Ag^+, Zn^+, Cd^+, Co^+ and Pb^+. • ATPase involved in heavy metal translocation are known as CPx-type ATPase because it contains conserved proline residue (P) followed by cysteine residue (C). • Crucial in maintaining homeostasis of vital metals such as Cu^+, Co^{2+} and Zn^{2+} and at the same time pose resistance toward toxic metals Pb^{2+}, Cd^{2+} and Ag^+. • Metal binding domain (MBD) influence specificity of the heavy metal translocating ATPase.
CBA transporter	• RND protein found in the inner membrane is the most essential component which is linked to the bacterial transport protein required in nodulation, cell division and heavy metal resistance. • Known as three-component protein complexes, that made up of: (i) RND protein, (ii) membrane fusion protein (MFP), (iii) outer membrane factor (OMF). Formation of efflux protein complex that functions as a pump that exports substrate from (i) cytoplasm to the periplasm, (ii) periplasm to the outer membrane. • The presence of RND in this export system shows differences between CBA and ABC transport systems. • In many protein complexes, the absence of MFP and RND proteins causes lack of resistance, while the loss of OMF usually only has moderate influence. • RND protein is present in Gram-positive bacteria, but CBA transporter is not functional in the cell walls.

CDF transporter	• CDF can be found in both prokaryotes and eukaryotes. • Mainly involved in Zn^{2+} transportation and also in other metals ($Fe^{2+,}$ $Co^{2+,}$ Ni^{2+} and Cd^{2+}). • Assumed to act as heavy metal buffer when cytoplamic metal concentration is low due to the fact that this system only exhibit extremely low-level resistance.

4. Bacterial Stress Response

Microorganisms in soil are exposed to changes in the environment. To survive these unfavourable conditions, soil microbes developed adaptive defence mechanisms or physiological and structural adaptations which resulted from evolution. The metabolic reaction known as stress response is included in the adaptive mechanism.[51] Microbial stress response induced by the changes in the metabolic activity of cell leads to the repression of synthesis of most proteins that are found in normal physiological conditions and synthesis of specific proteins for cell survival in the new environment.[51] Meanwhile, changes that occur in gene expressions are linked to alteration that involves different sigma protein factors and catalytic core of RNA polymerase. RNA polymerase is needed to identify genes that are required in a particular environmental condition and produce mRNA transcripts that later will be translated into a protein.[21] Table 3 represents the general stress responses in that can be found in bacteria.

Table 3: General stress responses in bacteria

Type of stress	Response
Chaotropic solutes [52]	• Up-regulating proteins for lipid metabolism protein stabilization, membrane structure, energy metabolism and protein synthesis. Accumulation of compatible solutes.
Osmotic stress	High osmolality

[53]	• Increase in K^+ ion influx i.e. uptake systems: trk, kdp, and kup. • Increased excretion result in drop in intracellular putrescine levels • Synthesis of glutamate (i) glutamate dehydrogenase (gdh) and (ii) glutamate synthase (gs). • Accumulation of disaccharide trehalose.
	Low osmolality • Elongation of the cell envelope and trigger of stretch-activated channels. • Increase the membrane's permeability. • Complex sugar's synthesis; membrane-derived oligosaccharides (mdos).
Nutrition stress [53]	• Inducing the expression of proteins involved in starvation-stress response (SSR). • Collection of cellular nucleotides: (i) cyclic 3, 5-adenosine monophosphate (cAMP) and (ii) guanosine 3, 5-bis(diphosphate). • Major SSR regulators: two alternative σ factors and σE encoded by the *rpoS* and *rpoE* genes. • Activation of nutrient utilization systems which are novel or higher-affinity
Temperature [54]	High temperature • Increased in synthesis of heat shock proteins (hsps). • Protein dnaK and dnaJ, the RNA polymerase σ70 subunit (rpod), groEL, groES, protease and lysU are induced. • Heat shock increases expression of σh target genes.
	Low temperature

	• Involved two signal transduction cascades: the σe and cpx systems. • Increased stability of DNA secondary structure and RNA. Reduced efficiency of transcription, replication and translation.
pH and acid stress [55]	• Induce the acid tolerance response (ATR). • Result in increased expression of synthesized or existing acid shock proteins. • Mg^{+2}-dependent proton translocating ATPase system crucial in some organisms for acid tolerance utilize arginine deiminase (ADI) pathway to produce ATP under acid stress. • Production of Urease (nickel-containing metalloenzyme) to convert urea to carbon dioxide and ammonia.
Oxidative stress [53]	• Controlled by two major transcriptional regulators OxyR and SoxRS (Cabiscol, Tamarit et al. 2010). The OxyR regulon induced by H_2O_2 and the SoxRs induced by superoxide. • In *Escherichia coli,* cytoplasmic Mn-SOD (SodA) and Fe-SOD (SodB) are produce during oxidation to protect protein and DNA. • A periplasmic Cu/Zn-SOD (SodC) defend the periplasmic and membrane constituents from exogenous superoxide. • No molecular oxygen produced during elimination of superoxide via superoxide reductase.
Heavy metal stress [41]	• In *Caulobacter crescentus,* gene regulating against oxidative stress and efflux pumps including metal ion efflux membrane fusion protein and outer membrane efflux protein are up-regulated • Sulphate transporters were down-regulated to reduce

non-specific uptake of the metal

Biochemical changes occur followed by physiological changes such as temporary slowing or stopping of the cell division cycle, morphological changes in cell or development of resistance to stress factors. Activation of defence mechanism becomes impossible when unfavourable stimuli are prolonged and components of cellular structure may be damaged. These severe environment stresses can lead to cell dead and evacuation of susceptible cells. Microbes that have resistance towards these conditions enable themselves to tolerate stress factors without activation of adaptive mechanisms, whereas some microbes require adaptive mechanisms which can delay the synthesis of defence molecules. Microorganisms will enter the stationary phase of growth and cell division will stop when nutrient supply is depleted and the microorganisms are unable to sustain stable growth. Most of the earth's biomass consist of resting microbes and are normally present in a stationary phase due to limited nutrients and harsh conditions that are common in the natural environment.[51]

4.1 Aggregation and Biofilm

Ecological processes such as competition, adaptation, epidemics and succession involve bacterial aggregation. Microbes developed survival skills against harsh conditions such as temporal and spatial changes in stimuli through motility which is an unavoidable part of most microbes' life cycle.[56-57] Aggregates formation resulted in enhanced efficiency in bioremediation.[58] Aggregation leads to formation of biofilm which depends on distinct interactions including synergistic, antagonistic, mutualistic, competitive and commensalism.

Auto-aggregation is defined as the adhesion of the same bacterial species while co-aggregation is the adhesion of two or more different species of bacteria.[59] Co-aggregation is a highly specific adhesion process which happens between two genetically different bacteria via specific molecules, generally mediated by 'adhesin' proteins on one bacteria and a complementary saccharide 'receptor' on the other. Co-aggregation between bacteria from distinct taxonomy is known as intergeneric co-aggregation while interaction between strains that belong to the same species is intraspecies co-aggregation. Molecules associated with surface like proteins and sugars are observed mediating co-aggregation of bacteria and this interaction led to the development of multispecies.[59] Adhesion and capsule with surface

hydrophobicity enable bacteria to adhere to abiotic and biotic surface thus lead to formation of biofilms. Adhesiveness increases with hydrophobicity. Contradictorily, there are studies that show no relationship between the extent of initial binding either to a hydrophobic or hydrophilic substrate and bacteria's surface hydrophobicity.. Auto-aggregation interactions are stronger than co-aggregation which is enhanced by the presence of surface hydrophobicity.[59-61] Physio-chemical properties of surface influence the auto-aggregation phenomena.[62] Cell-to-cell aggregation leads to biogranulation, a self-immobilization of microorganisms and formation of dense aggregates.[61]

Bacterial biofilm involves cell-surface and cell-cell interaction as part of the development process. Bacterial aggregation is the interaction of microbes from cell to cell to form a stable and multi-cellular cluster.[58] Microbial aggregates known as biofilm can consist of single-species or multi-species[51] and is surrounded by self-produced extracellular polymeric substances (EPS).[57] Based on an assay that depends on time and dosage, biofilm consists of subpopulation of cells in it. These cells tend to die at different rates upon exposure of the whole community in biofilm to metal ions.[23]

Ability to synthesize EPS, proteins and nucleic acids that surround the cell surface to form the biofilm matrix are unique characteristic traits of cells living in the form of biofilm.[51] Mechanisms of toxicity for biofilm and planktonic cells are different. Physiological states of microorganisms in biofilm are different even when separated by only 10 μm due to non-uniform distribution in extracellular pH and redox poise. Immature biofilm composed of layers of cells in the early stage of growth show increased of resistance to metal and antibiotics compared to planktonic cells.[23] Compared to planktonic bacteria, formation of biofilm boosts microbial resistance toward hydrogen peroxide, heavy metal, bacteriophage or amoeba[51] and antibiotic up to 1000 times.[57] Biofilm matrix is composed by water (nearly 97%), microbial cells, secreted polymer, nutrients, metabolites, product of cell lysis, particulate materials and detritus from cells's environment.[61] Dead cells in a biofilm community might defend the living cells against toxicity of the metal by precipitating or sequestering the reactive metal species as dead cells are chemically reactive and give biosorption sites that cause formation of metal precipitates and chelates. Dead cells are also able to affect physiological microenvironments and pH discontinuities in biofilm.[23]

Formation of biofilm occurs when suboptimal growth conditions (including lack of easily assimilable nutrients), hazardous stress factors (such as presence of metals or antibiotics) or presence of specific low-molecular weight compounds excreted by plants exists. Resistance of cells towards several environmental stress factors are due to the activation of various stress response mechanisms during formation of biofilm and in mature biofilm.[51] Biofilm alters their physiological characteristics to defend sensitive chemical targets of the reactive metal species to decrease metal toxicity.[23] Carbohydrate and proteins are the major player in the process of metal elimination.[61] Presence of enzymes like peptidase, polysaccharides and phosphatise within the biofilm proved that it helps to boost bioavailability of nutrients in the environment. Physiology properties of cells within biofilm are unlike that of free-floating planktonic cells. Genes that are involved in adhesion, gene clusters and auto-aggregation are highly expressed in biofilm cells or is induced during transition process of biofilm growth phase.[58]

Both natural and engineered microbial biofilm can be applied to handle heavy metal pollution by accumulation of toxic metals ions and/or biochemical modification. Natural processes of phenotypic diversification that occur inside a biofilm population are related to reducing susceptibility of biofilm to toxic metals. An interruption in metabolic processes can be avoided when biosorption of metal ions to components of biofilm (cell membrane, extracellular polymers and cell walls) sequesters these compounds. Metabolic end products produced by microorganisms also react with metals and cause precipitation of bioinorganic metals complexes. For example co-precipitation of heavy metal such as Ni, Cu, U, Zn, Cd and Pb with sulphide (S^{-2}) in biofilm of sulphur reducing bacteria and archaea, and co-precipitation of heavy metal with carbonates (HCO^{-}_{3} and CO^{2-}_{3}) produced during microbial respiration caused the eliminationof toxic metals from the aqueous phase.[23]

Bacteria act as a pool attached to each other (aggregates) and/or on a surface (biofilm) creating sessile communities that are capable of adapting to alteration in the environment or execute extremely specialised task which is same as multi-cellular organisms.[58] Quorum sensing (QS) involved in the information, development and susceptibility to metal toxicity in biofilm by regulates genes that are involved in the different development stages in the biofilm.[23-63] QS is a mechanism of cell-to-cell signalling through excretion of extracellular compounds that recognized as autoinducers. Accumulation of autoinducers in an extracellular medium regulates gene expression and amplification of various types of phenotypes.

Throughout the growth, bacteria produce autoinducers that activate the QS system when it achieves the threshold concentration.

Formation of biofilm involves five stages: (i) initial and reversible adhesion, (ii) initial irreversible attachment with production of EPS, (iii) initial maturation and acquisition of biofilm structure, (iv) mature biofilm and (v) dispersion (Figure 2).[63] Primary attachment of a bacterium to a particular surface lead to formation of microcolonies causes maturation of microcolonies into three-dimensional structure enwrapped and braced by EPS. Based on the analysis of biofilm, cell surface structures including fimbriae, pili, EPS, flagella, and outer membrane protein (OMPs) allow primary attachment to a surface that lead to formation of biofilms.[60]

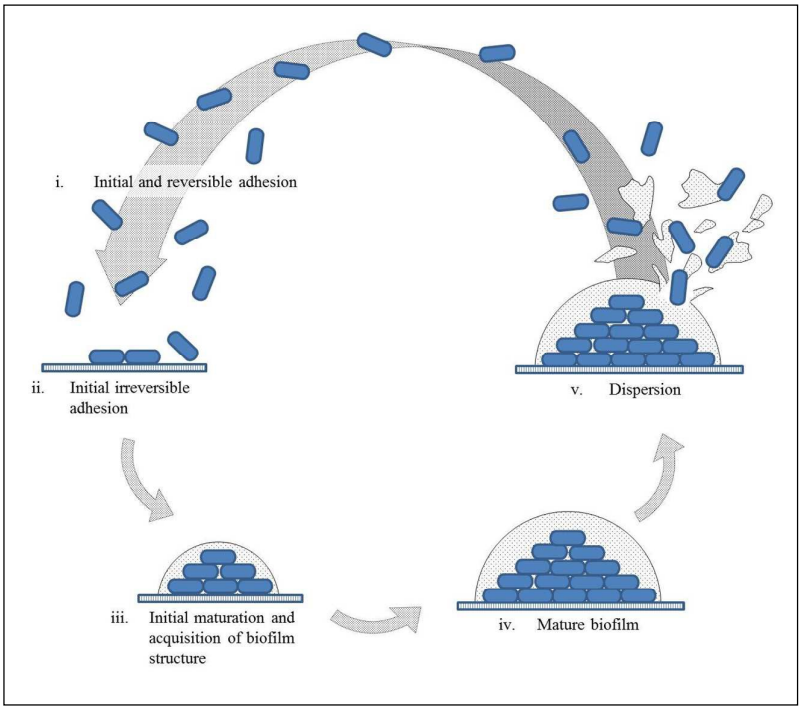

Figure 2: Steps involved in the formation of biofilm.

Rate and extend of attachment of microbial cells are determined by cell surface hydrophobicity, presence of fimbriae and flagella and yield of EPS.[64] Non-motile mutant bacteria showed disability in forming biofilm compared to wild-type cells. Hydrophobicity and ability to co-aggregate and auto-aggregate can increase bacterial adhesiveness. Surface hydrophobicity is usually related to bacterial adhesiveness and is different among organisms and strains and is affected by bacterial age, growth medium and bacterial surface.[60]

Initialization of biofilm involves regulatory processes that indirectly activate genetic and biochemical pathways that are used as a response toward antibiotic and metals exposure by microorganisms. This suggests that microorganisms are able to form biofilm that is multidrug resistant and tolerant when exposed to metals in the environment or in clinical circumstances.[23]

4.2 Extracellular Polymeric Substances (EPS)

EPS are metabolic products with high molecular weight polysaccharides (10–30 kDa) and contain homopolymeric and heteropolymeric compositions[65] and made up of macromolecules including polysaccharides, proteins, nucleic acids and lipids.[58]

EPS have various biological uses such as prevention of dehydration, preserve against environmental stresses including antibiotics and toxins, adherence to surface, symbiosis and pathogenesis under oligotrophic circumstances. EPS play role in microbial survival scheme by separating nutrient materials from the environment and act as a protective layer by restricting diffusion of some antimicrobial agents into the biofilm by being an ion exchanger. Normally, EPS-producing bacteria can be found in environments rich with organic substances, in a capsular material or as dispersed slime without any connection to one particular cell. Factors that affect EPS productions are medium composition (carbon and nitrogen source, pH, temperature), bacterial growth phase[65] and microbial species.[61] EPS production demands lots of activated nucleotide sugars as energy source for building the repeating units, transmembrane translocation and for polymerization; thus production of EPS is predicted to occur under active sugar consumption.[58]

EPS are separated as homopolysaccharides and heteropolysaccharides. Homopolysaccharides possess neutral charge while most of the heteropolysaccharides are polyanionic because of the uronic acids (glucuronic acids, mannuronic acids and galacturonic acids) or ketal-linked pyruvate. Only in few cases EPS can be polycationic. EPS are required in flocculation and binding of metal ions from solutions, thus is relevant to the bioremediation processes.[61] Major categories of macromolecules in biofilm EPS are anionic because of uronic acids or ketal-linked pyruvates and ionisable functional groups that communicate with other molecules, minerals and heavy metal.[61] Uronic acids that influence to anionic characteristic

of the EPS present potential in biotechnology application as they could be use in biodetoxification of heavy metals and waste water considering the heavy metal-binding properties of this polymer.[66-67]

Factors that affect metal binding to biofilm EPS are determined by environmental pH, metal concentration and avaiblity of organic material and biomass. EPS act as a protective layer against heavy metal stress by metal ions binding or by delaying their diffusion within the biofilm.[61] EPS able to sequester heavy metal is mainly due to the presence of ionisable functional groups including carboxyl, amine, phosphoric and hydroxyl groups.[34] Capability of microorganism to catalyse changes in oxidation states of metals that affect their solubility are applicable in bioremediation of heavy metal.[61]

5. Mechanisms of Gene Regulation under Heavy Metal Toxicity Stress

Signalling proteins require two-component signalling (TCS) systems which constitute the major signal transduction system in bacteria.[68] Signal transduction systems which are part of the pathways of intracellular information-processing, act as a bridge between external stimuli and specific adaptive responses. Other proteins like sigma factors, cyclic-di-guanosine monophosphate (c-di-GMP) related proteins and methyl-accepting chemotaxis with flagella proteins are also involved in signal transduction.[69]

5.1 Two-component signalling (TCS) systems

The resistance of bacteria towards heavy metal are linked with cellular signalling pathways. TCS pathway transduction system in bacteria allow them to sense, react and adjust to changes that occur in their environment or in an intracellular state by responding towards signals and stimuli such as nutrient, changes in osmolarity, quorum signals, cellular redox state and antibiotic and are mediated by TCS pathways.[70]

TCS systems react to a various environmental signals and regulate functions, such as sporulation, division, metabolism, motility, virulence, communication and stress adaptation.[71] Simple phosphotransfer are generally used by prokaryotes, while phosphorelays and hybrid kinases are TCS systems in eukaryotes.[72] The typical prokaryotic TCS systems constitute a membrane- bound histidine kinase (HK) and a response regulator (RR). Briefly, HK contains

a variable sensing domain and a conserved kinase domain. When sensing a stimulus, the HK sensor is activated and autophosphorylates at a conserved histidine (His) and effect gene expression by phosphorylating its cognate RR at a conserved aspartate (Asp). The response regulator is usually a DNA-binding transcription factor that undergoes conformational changes due to phosphorylation that controls the expression of the target genes.[73]

TCS begins once stimulus is detected that lead to autophosphorylation of conserved histidine residue on HK protein[74] followed by transfer of phosphoryl group to a RR. Attached output domain will be activated after phosphorylation of the RR on a conserved aspartate residue in its receiver domain. Phosphorylation of RR is linked to changes in the transcription level as DNA-binding domains acts as output domains[70] that give physiological response via repression or activation of genes.[74] Generally, HKs are bifunctional as they are able to catalyse both phosphorylation and dephosphorylation of their related RR. Bifunctional HKs are able to regulate either the kinase or phosphatase activity.[70] One of the properties of the TCS systems is that gene transcription demands both the RR and the signal that triggers its activation, that sensed by the cognate HK. This explains that TCS is controlled by another TCS system transcriptionally, where gene regulated by a system that will be only expressed in a condition when signal that activates both systems is exist.

TCS systems have unique properties compared to other pathways. The sensor is usually placed at the cytoplasmic membrane and receives periplasmic and/or cytoplasmic signals.[75] Many TCS systems regulate their own expression. Autoregulation allows bacteria to have 'memory' of previous incident with a signal due to abundant amounts of sensors and presence of RR proteins after the signal disappears. Autoregulation is crucial for TCS whereby RR controls target binding sites that are relatively too large to RR made from the constitutive promoter. Furthermore, autoregulation provide a threshold level for gene activation where when a signal remains it will promote adequate levels of phosphorylated RR for gene regulation.[76]

TCS is a functional and accurate system of regulation and is expanded by mutation and gene duplication to play roles from gene regulation to chemotaxis.[77] In *E. coli*, over 40 different TCS systems that respond to different environmental stimuli has been identified.[78] The TCS pathways sense changes in the environment and initiate regulatory factors for formation of biofilm. For example, attachment of *E. coli* cells onto a hydrophobic surface

activates Cpx TCS which is known as the general stress response. Activation of the Cpx system induces genes that code for periplasmic protein folding and protein degradation factors.[63] In *Bacillus subtilis*, transcription of ResD/ResE system that regulates genes needed for anaerobic respiration is manipulated by PhoP/PhoR system that reacts to phosphate starvation.[76] Phosphotransfer network in TCS systems also incorporate components of C-di-GMP signalling pathways. A subfraction of GGDEF/EAL domain proteins are connected to TCS systems. Genes that encoding EAL domain proteins are co-expressed with RR and sensor kinase gene in some bacteria. Many GGDEF/EAL domain proteins consist of N-terminal receiver domains that are phosphorylated by cognate sensor kinase.[79]

Phosphorelay is a common version of a TCS pathway which is started by a hybrid HK that autophsophorylates and transfers its phosphoryl group intermolecularly to a RR-like domain (Figure 3).[70] Phosphorelay is a more complex variant of TCS systems[77] which is applied in complex cellular processes such as development and cell cycle control in bacteria.[80] Phosphorelay was first discovered in *B. subtilis* to initiate sporulation.[81] The phosphoryl group will be moved to a histidine phosphotransferase (HPT) and then to the terminal response regulator which then will arouse related responses.[70] In phosphorelay, first regulatory domain phosphorylated by sensor kinase passes its phosphoryl group to a second phosphotransferase domain that assists as the primary phosphoryl donar to response regulators or transcription factors.[77] Phosophorelay contains sensor kinase, terminal response regulator, intermediate response regulator lacking an output domain and His-containing phosphotransfer protein.[82]

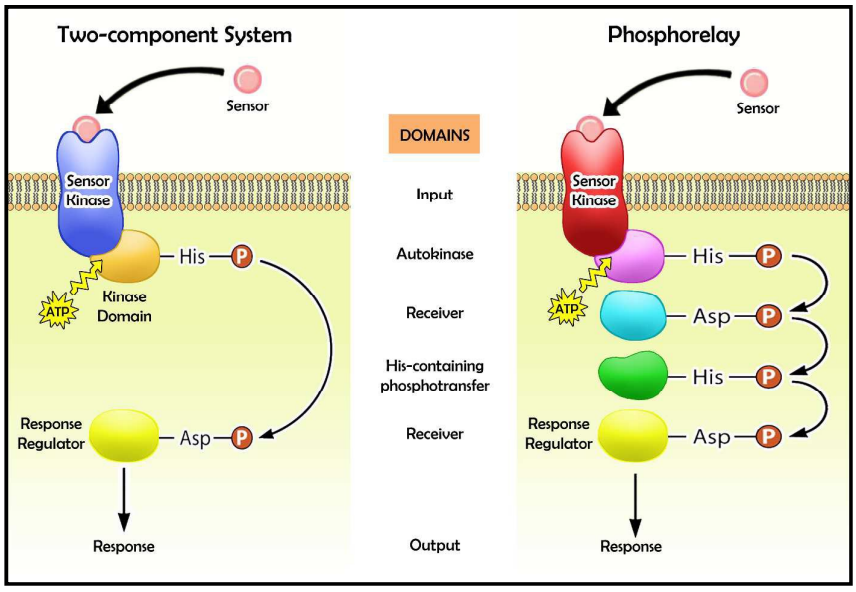

Figure 3: Signal transduction in TCS and phoshorelay.

5.1.2 Examples of TCS in Selected Heavy Metal Resistance

i) Cadmium resistance

Microbial resistance toward Cd^{2+} is generally based on energy-dependent efflux mechanisms. ColRS, which is known for metal resistance or homeostasis ColRS operon, is a type of TCS transduction system. ColR and ColS act as response regulators and HK respectively. Lack of ColRS causes about a fivefold reduction in resistance to Mn^{2+}. This proves that the ColRS signal transduction system is important for regulating resistance or homeostasis of Mn^{2+}.[83]

ii) Zinc resistance

Metal-inducible mechanisms that are based on active efflux of metal ions to avoid hazardous effects to cells took place in the presence of excess Zn^{2+}, Pb^{2+} and Cd^{2+}.[39] P-type ATPase transports only zinc across the cytoplasmic membrane while the resistance-nodulation-cell division (RND) system transports zinc across the complete cell wall of Gram-negative bacteria. Zinc resistance in *Saccharomyces cerevisiae* was mediated via CzcD which are from the cation diffusion facilitators (CDF) family including ZRC-1 protein. *czc* regulatory genes are ordered upstream and downstream of structural genes *czcCBA*. TCS systems are formed between the downstream regulatory regions that contain *czcD*, *czcR* and *czcS* with *czcS* (HK) and *czcR* (RR).[40]

iii) Copper resistance

In copper homeostasis, two copper-responsive regulatory systems involve genes like *cutC*, *cutF* and *ndh*. Sensor-regulator pair formed by *cusRS* triggers the adjacent and at the same time transcribed gene *cusCFBA*. *cusCBA* genes that homologous to a family of proton-cation antiporter complexes are required in the export of metal ions, xenobiotic and drugs while cusF is a putative periplasmic copper-binding protein. CueR, a copper activated homologue to MerR regulates two genes: *cueO* and *copA*. CopA is recognized as Cu(I)-translocating P-type ATPase, while CueO is a putative multi-copper oxidase. In two chromosomal copper-responsive determinants for copper homeostasis, cus determinant are regulated by TCS transduction systems that are encoded by *cusRS* genes.[84]

iv) Silver resistance

In silver resistance, gene *silE* encodes SilE, a periplasmic Ag(I) binding protein. SilE are 47% identical to PcoE in *E. coli* plasmid copper resistance system. SilCBA and Silp, which are two parallel membrane Ag(I) efflux pumps are encoded. Upstream from *silE* is *silRS*, a TCS signal transduction pair, which contains transcriptional regulatory responder, SilR and membrane kinase sensor, SilS that is homologous to other two-component family pairs. *silCBA* genes resemble cadmium, zinc and cobalt resistance system (Czc) of *Ralstonia* sp. and multi-drug resistance system of *E. coli*. SilA forms a cavity of pore for substrates for example, Ag^{+} from the cytoplasmic region straight to outer membrane protein, SilC. This ensures movement across periplasmic space of Gram-negative bacteria and directly to outside of cells without releasing into the periplasmic space. SilB, which is also known as membrane fusion protein, anchors into the inner membrane and connects to the outer membrane protein, SilC. SilP is homologous to membrane P-type ATPase and pump Ag(I) from the cell cytoplasm to the periplasmic space.[50]

5.2 Sigma factors

Changes in gene expression are manipulated transcriptionally via alteration in interaction between different sigma factors and catalytic core RNA polymerase in bacteria. Sigma factors are dissociable subunits of prokaryotic RNA polymerase that manipulate several iron uptake pathways, tolerance to several stresses, alginate biosynthesis, expression of outer-membrane porins and expression of virulence factors.

Sigma factors are classed into two major protein categories, σ^{54} and σ^{70} families (Figure 4) based on literature regarding *P. aeruginosa*.[21,85] Subunits containing the σ^{54} family are typically known as σ^{N}. σ^{N} dependent genes not only regulate nitrogen metabolism in many organisms, but at the same time also contribute to wide range of metabolic processes. *P. aeruginosa* and *P. putida* KT2440 specify 22 σ^{54}dependent transcriptional regulators. Various σ^{54} dependent regulators in KT2440 belong to TCS and exhibit a domain that could be phosphorylated by a sensor-kinase protein in the N-terminal section.

The sigma 70 family has two subcategories: (i) the primary sigma factor RpoD (σ70), that involved in the transcription of housekeeping genes and coordinate transcription of genes that essential for bacterial metabolism and growth and (ii) the alternative sigma factors, that play

important roles in the transcription of stress-related genes, which, based on conservation of their primary structures and sequences, can be grouped into four different classes;

- RpoS (σ^{32}) activates expression of multiple genes that needed to sustain cell viability as the cell exit the exponential growth conditions and proceed into stationary phases,
- FliA (σ^{28}) controls flagellin synthesis in *P. aeruginosa.* The mechanism of *fliA* transcription is still unclear but is suggested to be constitutive,[86]
- RpoH (σ^{32}) manipulate the heat shock regulation in *E. coli*. The role of RpoH in *P. putida* is not completely understood
- Extracytoplasmic function (ECF) involved in sensing and responding to conditions in periplasm, the membrane or extracellular environment. *P. putida* KT2440 is reported to have 19 ECF sigma factors.[21,87]

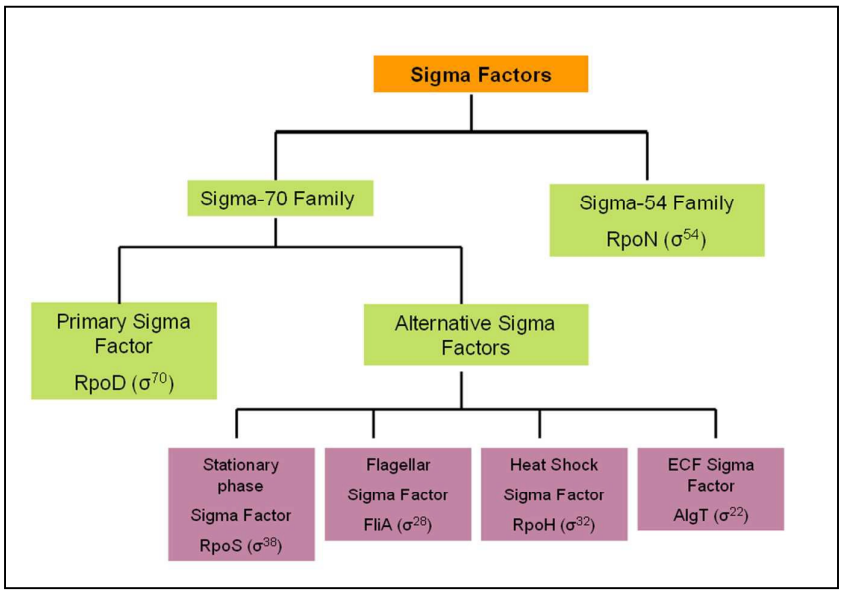

Figure 4: Sigma factors in *Pseudomonas aeruginosa*

Bacteria have different single stress-induced responses to aid in adaptation to specific stress situations by removing the hazardous substances. General stress response is usually regulated by a single master regulator. For example, the master regulator in *E. coli* is σ^S (RpoS).[88] Sigma regulatory proteins are crucial in transition to stationary phase in both Gram negative and Gram positive bacteria.[51] Sigma factors link up with RNA polymerase to create a RNA polymerase holoenzyme that allows the holoenzyme to identify the promoter site in DNA. σ^B from Group III sigma factors that are found in *B. subtilis* regulate σ^B-dependent general stress regulators that are expressed upon exposure of bacterial cell to ethanol, heat,

salt stress, acid, moving to stationary phase or starvation for oxygen, glucose or phosphate. While σ^E from Group IV that can be found in *E. coli*, is an extracytoplasmic function sigma protein responsible for heat-shock stress.[21] In *Caulobacter crescentus*, ECF sigma factor σ^F is one of the regulatory proteins that are required in the regulation of transcriptional response to chromium and cadmium and controls eight genes under chromium stress.[89]

In harsh environments, reductive division and dwarfing cause bacterial cells to shrink into smaller size and acquire spherical shape compared to their log phase counterparts. Reductive division enhances surface-area-to-volume ratio, producing spherical shape while dwarfing is a type of self-digestion caused by degradation of endogenous cell materials especially cytoplasm and outer membrane.[51]

Reorganization causes cell envelope (outer membrane, periplasm, peptidoglycan and inner membrane) to become stiff and resistant to chemical and physical agents. Nucleoid undergoes condensation in which DNA-binding protein from starved cells (Dsp) defend DNA from several damaging agents. Dsp are triggered by OxyR in oxidative stress conditions as a result of an expression dependent on housekeeping transcription factor σ70 whereas in starvation conditions it is by the σS transcription factor. Dimerization of ribosome into an inactive form occurs as a form of preservation as some ribosomes are degraded which explains the low translation levels observed in this conditions. Modifications at the metabolic level require inhibition of transcription of genes coding for rRNA, tRNA and ribosomal proteins which cause a decrease in cellular protein synthesis. Synthesis of cell wall components and lipids also are reduced. Protease and peptidase synthesis increases in the early stages of starvation with increase in protein turnover (as much as five fold in *E. coli*).[51]

5.3 C-di-GMP

C-di-GMP influences complex biological processes such as virulence and biofilm formation in many bacteria.[90] It has been proven that C-di-GMP signaling involved in cell aggregated and biofilm formation in *P. aeruginosa* and other bacteria[56] while in *C. crescentus*, GGDEF domain protein PleD manipulate flagellum ejection and cell morphology.[79] C-di-GMP-metabolizing proteins, phosphodiesterase and di-guanylate cyclase each possess one GGDEF and EAL domain as common domains. GGDEF domains are involved in synthesis and EAL domains are involved in hydrolysis of C-di-GMP. The amino acid sequences and protein

structures in both domains share high similarity, even if the proteins catalyze opposite biochemical reactions.[56] Inactivation of gene encoding GGDEF and EAL domain proteins regulates amplitude of a phenotype or retreive of function is accomplished under unsimiliar environmental conditions, but rarely causes major phenotype changes.[79] C-di-GMP affect biofilm formation and virulence in *Staphylococcus aureus* and fimbrial expression on *P. aeruginosa* despite the fact that it is an intracellular second messenger.[90]

Levels of C-di-GMP are involved in many cellular processes including the conversion between the motile and sessile lifestyle in bacteria (Figure 5). The saturation of C-di-GMP directed processes was attained by the expression of diguanylate cyclase leading to a high C-di-GMP production triggering the sessile lifestyle, favoring phenotypes including extended biofilm formation that are linked with the fimbriae, adhesive matrix components and exopolysaccharides. While C-di-GMP depletion was accomplished by the overexpression of a cytoplasmic phosphodiesterase, which led to motility activities like swimming, swarming and twitching motility.[79] In *P. putida*, EAL and GGDEF domain proteins suppressed the biosynthesis of flagella in the early growth phase.[90] In *hns* mutant *E. coli* which loses swimming motility due to loss of flagella motion was recovered by the production of an EAL domain protein. This proves that down-regulation of C-di-GMP concentration lead to functional activation of structural components that decoupled from synthesis of respective structures. The structure of EAL and/or GGDEF domain proteins (sensor output domain) is same to the sensor HKs and methyl-carrier chemotaxis proteins. It is due to existence of an amino acid that are able to regulate the turnover of C-di-GMP in the similar pattern as they regulate HKs.[79]

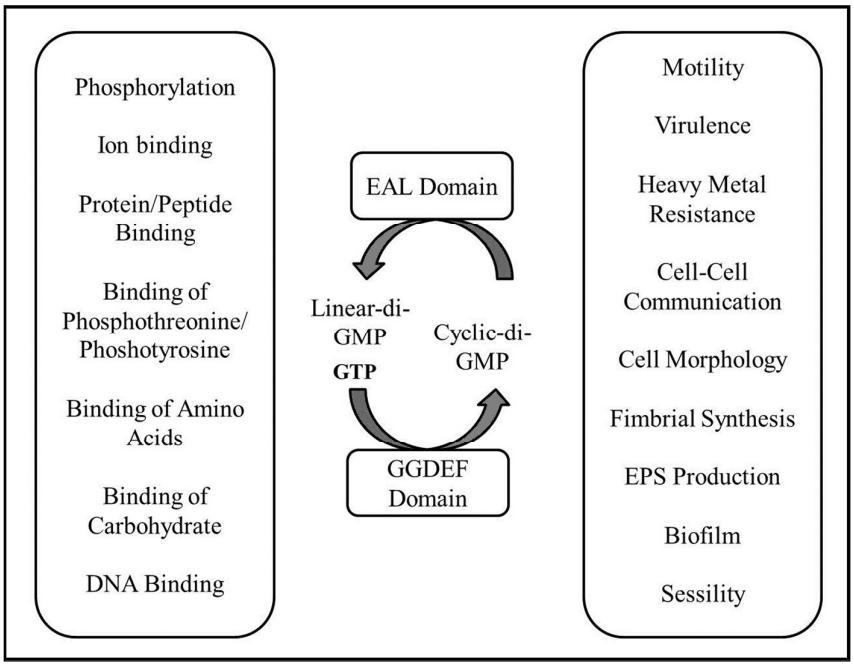

Figure 5: Known input and outputs signals of C-di-GMP metabolism

High concentrations of C-di-GMP in *Salmonella typhimurium* triggered the formation of biofilm, production of adhesive surface organelles including curli fimbriae and cellulose, and suppressed motility. In low concentrations of C-di-GMP, production of adhesive surface organelle and biofilm formation are inhibited with inhibit biofilm formation and production of adhesive surface organelles and induction in swarming and swimming motility. Adhesion of cells to a surface exhibits several C-di-GMP concentrations relying upon whether the cells show twitching motility or produce adhesive extracellular matrix.[90]

5.4 Chemotaxis and Flagellae

Chemotaxis is a process whereby motile unicellular organisms coordinate its movement away or towards from gradients of specific substances, which are either attractants or repellents.[91] Bacterial chemotaxis that involves chemosensory pathway is part of TCS superfamily of receptor-regulated phosphorylation pathways. When a cell swims through different regions of concentration, chemosensory pathway monitors local concentrations of chemical species that vary with time as the cell swims. When a cell swims up an attractant gradient, the chemosensory pathways detects increasing attractant concentration with time and deliver out signal to the propulsion motor, that will lower the chances of a tumble event, thus prolong the average run up the gradient and vice versa.[92] Chemotaxis process requires two separate systems including the chemo-receptors situated in the bacterial cell membrane that crucial for detecting the binding compounds and the transduction proteins that needed in downstream signal transduction in response to the stimuli.[91] Molecular mechanisms of bacterial chemotaxis consist of cytoplasmic chemotaxis proteins (Che proteins), methyl-accepting chemotaxis proteins (MCPs) and flagellae (Figure 6).

MCPs are reversibly methylated transmembrane chemosensory proteins for environmental stimuli and function as homodimers. A cluster of chemotaxis genes has been located that is *cheB*, *cheJ*, *cheA*, *cheY* and *cheZ*. MCPs together with CheW regulate the

autophosphorylation activity of CheA as a response to temporal changes in stimulus intensity. Methyltransferase CheB and methyltransferase CheR that receives the phosphoryl group from phosphorylated CheA reversibly methylated MCPs at several glutamate residues. Methylesterase activity will increase as phosphorylation of CheB occurs and the level of methylation of MCPs is regulated in response to environmental stimuli. This occurrence, which known as reversible methylation of MCPs, is important for chemical gradients sensing.

Alteration in repellent or attractants concentrations are detected by a protein complex comprising of transmembrane receptors (Tar, Tap, Tsr, Aer and Trg), an adaptor protein CheW and a HK CheA. Autophosphorylation activity of CheA is manipulated by attractant binding (inhibited) and repellent binding (raised) to receptors. The phosphoryl group is immediately transferred from CheA to the response regulator CheY. Phosphorylated CheY (CheYp) alter the direction of motor rotation from counterclockwise (CCW) to clockwise (CW) to allow tumbles by diffusing to flagellar motors. CheZ phosphatase, localized to sensory complexes through binding to CheA, assure a rapid turnover of CheYp, that crucial to rapidly readjust bacterial behavior. Receptor modification boosts CheA activity and reduce sensitivity to attractants. Response is provided by CheB phosphorylation through CheA that raises CheB activity.[91]

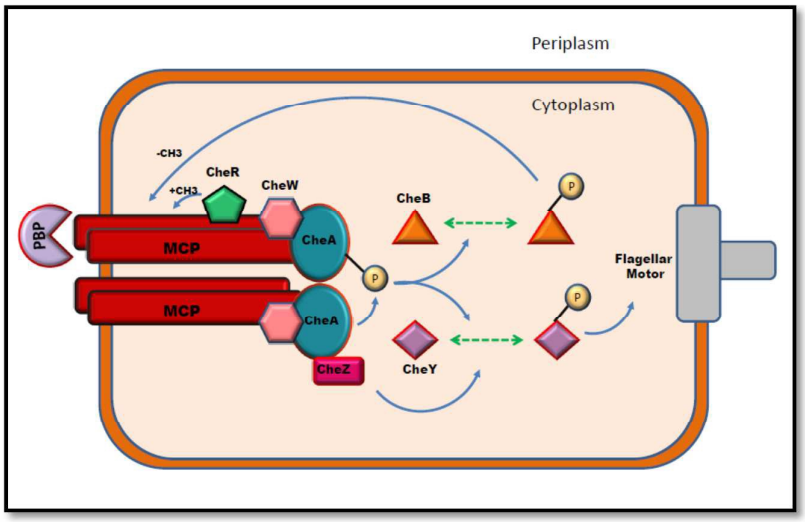

Figure 6: Chemotaxis pathway in bacteria

Flagella are complex organelles generating motility that enable bacteria to propel through liquids (swimming) and through highly viscous environments or along surfaces

(swarming).[93] Six different types of bacterial surface motility are involved in bacteria including swarming, swimming, twitching, darting, gliding and sliding. Among these, swimming and swarming are flagella-dependent.[94] Flagellar rotation and the number of flagella may differ depending on the species. For example *E. coli* and *S. typhimurium* can have up to 10 peritrichous flagellae[94] while *P. aeruginosa* has a single polar flagellum[86] and an exception is *Burkholderia mallei* which are permanently immotile.[95] *P. putida* has been proved to have multiple polar flagellae and typically has between five and seven flagellae inserted at one end to form a tuft. Flagellar filaments are typically 2 to 3 wavelengths long and able to changes the direction in 20 to 30 milliseconds.[96]

Flagellar rotations are in a CCW or CW direction. When flagellae rotate in CCW, the cell moves forward and the cell shows unidirectional swim that recognized as run. Meantime, when some of the flagellae rotate CW and others rotate CCW, cells start to tumble. Cells coordinate the movement by alternating between run and tumble and the alternation is believed to be random. In a situation to exhibit chemotactic behaviour, i.e., sensing the gradients of an attractant or repellent substrate, they change the frequency of tumble and run. When the cells sense increasing concentrations of attractants they tumble less frequently and swim longer times, whereas when they sense decreasing concentrations of attractants they tumble more and decrease run times.[97]

In *C. crescentus* strain CB15N (ATCC 19089), chemotaxis protein McpJ, cell motility proteins and other additional chemotaxis proteins were down-regulated under all metal stresses. Exposure to uranium most significantly down-regulated the protein involved in cell motility and chemotaxis proteins such as flagellin protein FljM. Down-regulation in transcriptional and/or translational of chemotaxis and cell motility proteins also can be observed in *Shewanella oneidensis* MR-1 under Cr exposure and in *Camplylobacter jejuni* and *P. putida* under Cd exposure. This indicates that reduction in cell motility and chemotaxis are a common mechanism in bacterial heavy metal stress response. Interference of heavy metal with chemoreceptors and ability of cells to sense a non-conducive environment may reduce chemotactic activities and cause down-regulation of these proteins.[98]

According to a study about chromium (VI) exposure on *S. oneidensis* MR-1, abundance levels of 7 proteins including 2 chemotaxis proteins (SO1144 and SO3207)

reduced upon exposure of Cr (VI) for 24 hours compared to control conditions. Prevalence of non-motile cells upon prolonged exposure of Cr(VI) causing down-regulation of proteins involved in motility and chemotaxis. This is proved by confocal laser scanning microscopy observation. Chemotaxis genes *cheY1*, *cheA*, *cheW* and *cheB1* experienced transcriptional repression 0.4-fold, 0.5-fold, 0.3-fold and 0.5-fold respectively.[99] Transcriptomic analysis of *B. cereus* ATCC 14579 showed that most of the hook-associated genes (*flgE, flgE* and *fliL*), chemotaxis-related genes (*cheV*, *cheY* and *cheA*), flagellar biosynthesis genes (*fliO* and *flip*), motor switch genes (*fliN* and *fliG*) and basal body rod genes (*flgG* and *flgB*) were down-regulated after exposure to silver nitrate. No changes in flagellar motor switch (*fliR*) expression indicate that it may not be influenced by ionic stress response. A prolonged introduction of silver nitrate has slowed cell motility based on study in *B. cereus* that are related to chemotactic behaviour of silver stress.[100]

Conclusion

Heavy metal contamination is not only hazardous to humans but also to microbes that are present in the environment. Anthropogenic contamination of heavy metal exerts perniciousness when it exceeds certain threshold levels. Non-degradable properties of metals contribute to its toxicity. In order to survive, defence mechanisms were developed by some microbes to adapt to the conditions. Adaptations of microbes in such rough conditions cause not only physiological but also genetical changes. Presence of ion-selective ATPase pumps enhances efflux transport of heavy metal to reduce the toxicity in microbes. Formation of aggregations, biofilm and EPS contribute to application of heavy metal resistant bacteria in bioremediation. Genetic manipulation has been approached to create engineered microbes to be used in bioremediation. Research over the past decade has provided better understanding of mechanisms and signalling pathways required in heavy metal stress in microbes. However, further understanding of these mechanisms and signalling pathways are crucial in coping with heavy metal contaminations that are increasing alarmingly.

Acknowledgement

This study was supported by Ministry of Higher Education of Malaysia, MOHE (FRGS/2/2014/SG05/UKM/03/1). The authors would like to thank Mohd Faiz Mohd Yusoff for his assistance in illustration editing.

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