

GENETIC BASIS OF HEAVY METAL TOLERANCE IN BACTERIA: A REVIEW

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ABSTRACT

Throughout the world heavy metal pollution is considered a serious issue. Pakistan is also facing problem of industrial, municipal, vehicular and air pollution. While some of the heavy metals play a vital role in biological systems there are others which are purely toxic with no known cellular role. Excessive levels of even the most essential metals can be highly toxic to the organism. Essential heavy metal ions present a dual challenge as these are useful but can also be lethal. Genetic basis of heavy metal tolerance in bacteria has been studied by researchers and interesting facts are found which can lead towards the better use of these naturally occurring mechanisms for the betterment of environment for all the living organisms.

Key-words: Heavy metals, bacterial, resistance/tolerance, genetic, pollution.

HEAVY METALS

Heavy metals are trace metals with a density at least five times that of water (Morris, 1992). Nies (1999) and Lemke (1993) defined heavy metals as the 53 metals with a density above 4.5 g/cm³. Kennish (1992) classified heavy metals as elements having atomic weights between 63.546 and 200.590.

In recent years, ground soil and other materials polluted with heavy metals such as mercury, arsenic and lead have become a serious environmental problem throughout the world due to their use in industrial countries for a variety of applications (Endo *et al.*, 1997). Heavy metals are used in many manufacturing processes, and end up as waste in industrial effluent, through which heavy metals can enter the water cycle, and then the food chain where they are concentrated ultimately reaching the toxic levels (Stillman and Presta, 2000).

Pakistan is also facing environmental pollution which includes: industrial, municipal, vehicular and air pollution, deforestation, desertification, water logging and salinity (Khan, 2000). This country generates over 50,000 tons of solid waste per day; out of which only 20 to 25 percent is collected but not disposed off in the proper manner: causing serious air, water and land pollution and health hazards (Khan, 2000). Only 3 percent of the industry treats their wastes while the rest discharges untreated effluent into river, lakes and sea. Dumping of untreated municipal and industrial wastes have caused contamination of surface and ground water resources and threatened the aquatic life to the endangered level (Blumenthal *et al.*, 2001).

BENEFICIAL AND TOXIC HEAVY METALS

Metals play a vital role in biological systems as a living cell cannot exist without metal ions. Copper, nickel, cobalt and iron are essential to all organisms because of their use as catalytic and structural elements in enzymes and other molecules (Cobine *et al.*, 1999). Trace amounts of some other heavy metals are also required by living organisms, including cobalt, copper, iron, manganese, molybdenum, nickel, vanadium, strontium, and zinc (Gort *et al.*, 1999).

While some of the heavy metals are purely toxic with no known cellular role (Shi *et al.*, 2002), other metals are essential for life at low concentration but become toxic at high concentrations (Badar *et al.*, 2000), high concentration of all the heavy metals inhibits the activity of sensitive enzymes (Koropatnick and Leibbrandt, 1995).

Excessive levels of even the most essential metals can be highly toxic to the organism (Gadd, 1992; Franke *et al.*, 2003). Essential heavy metal ions present a dual challenge to both eukaryotic and prokaryotic cells in that they are useful but can also be lethal. Therefore, a cell must meet its physiological requirement for essential metal ions while preventing their deleterious effects (Adaikkalam and Swarup, 2002).

SIGNIFICANCE OF MICROBIAL TOLERANCE TO HEAVY METALS

Although microbial tolerance to heavy metals has been studied over the past 30 years, the last 15 years have been outstanding with respect to discoveries at molecular level. As a consequence, the amount of work and information is substantial.

Nies (1999) described 3 possible uses of comprehensive metal resistance studies in biotechnology:

1. Metal resistance can be added to a micro-organism in order to facilitate a biotechnological process;
2. Metal resistant bacteria can be used in bio-mining of expensive metals (bioleaching);
3. Metal resistant bacteria can be utilized in bioremediation of metal-contaminated environments.

We need a better understanding of the microbial tolerance mechanisms in order to reduce the overall effect of toxic heavy metals in the environment.

Before focusing on the genetic mechanisms involved, overview shall be given about basic facts concerning heavy metal toxicity and specific characteristics of each important metal. An attempt has been made to discuss all the general issues related to heavy metal tolerance in bacteria.

HISTORY OF BACTERIAL TOLERANCE AGAINST HEAVY METALS

Bacteria, being one of the most primitive life forms on earth, naturally developed tolerance to a wide range of toxic heavy metals including As, Cd, Co, Cr, Cu, Hg, Ni, Sb, Te, and Zn (Silver and Walderhaug, 1992; Silver and Ji, 1994) in its genome. Copper, nickel, cobalt and iron are essential to bacteria because of their use as catalytic and structural elements in enzymes and other molecules (Cobine *et al.*, 1999). In many cases, the first response to toxic metal contamination is a large reduction in microbial activity (Pennanen *et al.*, 1996). This is confirmed by the fact that habitats that have had high levels of metal contamination for years still have microbial populations and activities that are smaller than the microbial populations and activities in uncontaminated habitats. Moreover, resistance mechanisms do not offer protection at extremely high levels of free metal ions, and a lethal toxic effect is observed (Malakul *et al.*, 1998; Konopka *et al.*, 1999).

Essential metals for microorganisms as trace nutrients such as Zn^{2+} , Co^{2+} , and Ni^{2+} must be transported into cells against concentration gradient. Highly specific Ni^{2+} uptake systems were found in *R. eutropha* (Lohmeyer and Friedrich, 1987).

Some bacteria have evolved mechanisms to detoxify heavy metals, and some even use them for respiration. Microbial interactions with metals may have several implications for the environment. Microbes may play a large role in the biogeochemical cycling of toxic heavy metals also in cleaning up or remediating metal-contaminated environments.

BASIC MECHANISMS OF TOLERANCE

We are only now beginning to understand heavy metal tolerance mechanisms in bacteria (Cobine *et al.*, 1999). Up to now, four main mechanisms of resistance were identified, i.e., surface binding or reduced uptake (Laddaga *et al.*, 1985), increased efflux (Nies 1992), intracellular sequestration (Diels *et al.*, 1995), and modification to a form which is less toxic (Misra *et al.*, 1992; Summers, 1986). These strategies can occur singly or in combination (Gadd, 1992).

Likewise, Silver (1992) distinguishes 4 mechanisms of bacterial metal resistance:

- 1 Keeping the toxic ion out of the cell (reduced uptake);
- 2 Highly-specific efflux pumping (i.e. removing toxic ions that entered the cell by means of transport systems evolved for nutrient cations or anions). Efflux pumps can be either ATPases or chemiosmotic driven. ATPases are enzymes that use the chemical energy from cleavage of the high-energy phospho-ester bond of ATP to drive the formation of concentration gradients;
- 3 Intra or extracellular sequestration by specific mineral-ion binding components (e.g.: metallothioneins) and/or segregation into complex compounds;
- 4 Enzymatic detoxification (oxydoreductions) which converts a more toxic ion to a less toxic one.

The first two mechanisms can be grouped under the term avoidance, whereas the last two are known as sequestration mechanisms. Quite often, several different resistance mechanisms for a same metal may be found among the same species (Zgurskaya and Nikaido, 2000).

Uptake Mechanisms

In high concentrations, heavy metal ions react to form toxic compounds in cells (Nies, 1999). To have a toxic effect, however, heavy metal ions must first enter the cell. Because some heavy metals are necessary for enzymatic functions and bacterial growth, uptake mechanisms exist that allow for the entrance of metal ions into the cell. There are two general uptake systems -- one is quick and unspecific, driven by a chemiosmotic gradient across the cell membrane and thus requiring no ATP, and the other is slower and more substrate-specific, driven by energy from ATP hydrolysis. While the first mechanism is more energy efficient, it results in an influx of a wider variety of

heavy metals, and when these metals are present in high concentrations, they are more likely to have toxic effects once inside the cell (Nies and Silver, 1995).

Efflux Mechanisms

In bacteria, efflux pumping is the basis of most toxic ion resistance systems, involving transporters such as P-type ATPases or cation/H⁺ antiporters (Silver and Ji, 1994); several efflux pumping systems have been identified for Cu, Cd, Zn, Co, and Ni (Silver, 1996). Efflux pumps reduce the intracellular concentration of metals by means of transport systems, without any enzymatic transformation (Nies, 1999). This mechanism is more widespread than enzymatic detoxification.

Plasmid Mediated Metal Resistance Mechanisms in Bacteria:

Bacterial plasmids contain genes that provide extra functions to the cells, among which resistances to toxic metals is very important. Plasmids are small circular DNA molecule that can move from one bacterial cell to another (Silver, 1997). Thus, the transfer of toxic metal resistance from one cell to another is facilitated. This is why, most of the time, resistance systems are found on these plasmids, but some systems are determined by chromosomal genes in other organisms.

Metal-ion resistance mechanisms mostly revealed on plasmids, some of these Systems and Mechanisms are (Silver, 1996):

mer for mercury (Hg²⁺) and organomercurials in Gram-negative and Gram-positive bacteria through enzymatic detoxification.

ars for Arsenate (AsO₄³⁺ and AsO₂) in Gram - and Gram + bacteria through membrane-associated ATPase (ArsA) and inner-membrane protein (ArsB) for efflux and enzymatic reduction to arsenite through reductase ArsC.

cadA for cadmium (Cd²⁺) and zinc (Zn²⁺) in Gram-positive bacteria through P-type ATPase CadA for efflux.

czc, *cnr*, and *ncc* for cadmium (Cd²⁺), zinc (Zn²⁺), cobalt (Co²⁺), and nickel (Ni²⁺) in Gram – bacteria through inner membrane protein CzcA, outer membrane protein CzcC, and a protein associated with both membranes CzcB.

cop for copper (Cu²⁺) in *Pseudomonas* through periplasmic copper binding proteins (CopA & CopC), outer membrane protein (CopB) and inner membrane protein (CopD). It is also reported in *Enterococcus hirae* in which uptake (CopA) and efflux (CopB) ATPases are reported.

pco for copper in *Escherichia coli* through proteins PcoA, PcoB, and PcoC for copper binding and Pco D for copper efflux.

chr for chromate (CrO₄²⁺) in *R. eutropha* and *Pseudomonas aeruginosa* through membrane proteins which reduce cellular uptake.

pum for Pb²⁺ in Gram + bacteria through accumulation and in Gram-negative bacteria through efflux.

DETAILED STUDIED ORGANISMS FOR METAL TOLERANCE

As far as it is known, there is no general mechanism for resistance to all heavy metal ions, each resistant mechanism is very specific (Silver, 1996).

Many authors reported that microbial communities exhibit variable response to combat heavy metal toxicity at species level (Gelmi *et al.*, 1994; Saier, 2000). Resistant species were found to be *Pseudomonas*, *Enterobacter*, *Citrobacter*, *Alcaligenes*, *Flavobacterium*, *Proteus* and *Bacillus*. There are some very well studied genetic mechanisms of tolerance against heavy metals reported in specific bacterial species.

Escherichia coli:

In *Escherichia coli* at least three systems are involved in copper tolerance. First, the P-type ATPase PcoA pumps excess copper removed from the cell (Fan *et al.*, 2001; Rensing *et al.*, 2000). Second, the multicopper oxidase CueO may protect periplasmic enzymes from copper-mediated damage (Grass *et al.*, 2001). Third, the *cus* determinant confers copper and silver resistance (Franke *et al.*, 2001; Grass and Rensing, 2001; Gupta *et al.*, 2001). *Escherichia coli* survival in copper-rich environments is ensured by the chromosomally encoded copper homeostatic systems (Munson *et al.*, 2000) along with the conjugative plasmid pRJ1004 (Lee *et al.*, 2002). Copper resistance in *Escherichia coli* specified by plasmid is encoded by the *pco* gene cluster, which contains seven genes, *pcoABCDRSE* (Cooksey, 1993), which include, an inner membrane protein (PcoD), an outer membrane protein (PcoB), and two periplasmic Cu²⁺-binding proteins (PcoA and PcoC). Synthesis of this system is governed by two regulatory proteins (the membrane sensor PcoS and the soluble responder PcoR, probably a DNA-binding protein) (Silver and Ji, 1994). The two regulatory genes *pcoR* and *pcoS*, follow the structural genes *pcoABCD* and a fifth structural gene required for copper resistance, *pcoE* follows *pcoABCD* (Tetaz and Luke, 1983; Cooksey, 1993).

Pseudomonas syringae:

One of the best characterized determinants of copper resistance in prokaryotes is reported in *Pseudomonas spp.* is the *copABCD* operon system that reside on a 35 kb plasmid of the copper-resistant strain of *Pseudomonas syringae* (Mellano and Cooksey, 1988; Cooksey, 1994).

Copper resistance by the *cop* determinant, contains six genes, *copABCDRS*, arranged in a single operon and homologous to the equivalent *pco* genes (Cooksey, 1993). In all cases copper resistance has been shown to be inducible (Yoshida *et al.*, 1993; Rensing *et al.*, 2000).

Enterococcus hirae:

In the Gram-positive bacterium *Enterococcus hirae*, copper metabolism seems to be much clearer than in the Gram-negative bacteria. *Enterococcus hirae* contains a *cop* operon with two structural genes, both encoding a P-type ATPase. Studies with whole cells had suggested that CopA serves in the uptake and copper nutrition, the 35% identical CopB is responsible for copper efflux and detoxification (Oderematt *et al.*, 1994).

Ralstonia eutrophus:

The best-studied metal cation efflux system in Gram negative bacteria is of *Ralstonia eutrophus* CH34 (Mergeay *et al.*, 1985). This bacterial strain harbors two megaplasmids (pMOL28 and pMOL30) governing multiple resistance to heavy metals (Kohler *et al.*, 2000), totaling more than 400 kb DNA and contains at least seven metal resistance determinants, including the *czc* system (determining resistances to cadmium, zinc and cobalt) (Mergeay *et al.*, 1985); a system that confers nickel and cobalt resistances (*cnr* determinant) (Nies and Silver, 1989), three mercury resistance operons (Dressler *et al.*, 1991); and additional systems for resistances to chromate (*chr*) and to copper (*cop*) (Dressler *et al.*, 1991, Nies and Silver, 1989).

The *czc* determinant contains three structural genes coding for the three subunit of the membrane-bound efflux complex CzcCBA (Nies *et al.*, 1990; Rensing *et al.*, 1997). Driving force for the export of the heavy metal cations is not ATP, but the proton motif force (Nies, 1995). The determinant consists of the three "structural" genes *czcC*, *B* and *A*, plus two regulatory genes *czcR* and *czcD*. More recently, the two additional systems *cnr*, for cobalt and nickel resistances (Liesegang *et al.*, 1993; Siddiqui *et al.*, 1989) and *ncc* (for nickel, cadmium and cobalt resistances) (Schmidt and Schlegel, 1994) also have been cloned and sequenced from *Alcaligenes*.

Chromate resistance in *R. eutrophus* mediated by the *chr* determinant located on plasmid pMOL28 (Nies *et al.*, 1989). This determinant encodes two proteins, ChrB and ChrA, with the *chrB* gene preceding the *chrA* gene in the same transcriptional direction (Nies *et al.*, 1990).

BIOTECHNOLOGICAL USE OF HEAVY METAL RESISTANCE: AN OPINION

Theoretically, there are three fields for heavy metal resistance biotechnology: first, adding metal resistance to a microorganism may facilitate a biotechnological process which has otherwise nothing to do with heavy metal toxicity or resistance. Secondly, heavy metal resistant bacteria may be used for any kind of bio-mining of expensive metals, directly on the ores or by taking back metals from effluents of any industrial process. Thirdly, heavy metal resistant bacteria may be used for bioremediation of metal-contaminated environments. Genetic engineering allows the introduction of desired traits into cells to help meet some of these criteria, and this approach has already been used to construct cells for the bioremediation of mercury (Chen and Wilson, 1997). How a metal resistance should be added to a microorganism of biotechnological use depends on the amount of control over the process, which depends on the in depth knowledge about these processes. In a highly controlled fermentor reaction, the insertion of a heavy metal resistance determinant into the chromosome of the respective bacterium for the purpose of improvement is readily done, if heavy metals pose a threat. On the other hand, a sewage plant with a limited control over the cleaning process probably does not allow the use of a highly modified organism. However, in these cases, heavy metal resistant natural bacteria may be established in the plasmids with a broad host range of replication and metal resistance expression could easily be introduced into the bacterial community. If heavy metals are a problem for the bacteria, the plasmids should remain within the bacterial population. In all cases, efflux systems should be supplied to the bacteria, since detoxification by efflux is more economical for the bacteria than binding, with the exception of mercury (Sahlmen and Skaerfstad, 1993).

For biomining of ores, either the bacteria must be able to solubilize the respective metal directly; e.g. by reduction or oxidation, or the biotechnological transformation of another element or metal is used in an indirect process. Few metals may be reduced or oxidized by bacteria, e. g. copper and iron. For recycling of any metal in an industrial effluent, the value of the metal obtained must be higher than the value of the bacteria used. In most cases,

the high costs for growing the bacteria and the low specificity of the bacterial accumulation process makes such a cleaning procedure unattractive.

In the end it could be evaluated that knowledge of genetic basis of heavy metal tolerance in bacteria can lead towards the better use of these naturally occurring mechanisms for the betterment of environment for all the living organisms.

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