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MECHANISM OF ARSENIC RESISTANCE IN MICROBIAL CELLS

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Abstract: ***The use of microorganisms for the recovery of arsenic from waste streams has achieved growing attention. Microorganisms have evolved complex mechanisms to counter the toxic effects of arsenic. Biotechnological techniques exploit several mechanisms which might be evolved to control the arsenic pollution by microorganisms. Thus, there is a high level of interest in developing methods aimed at cleaning up or detoxifying arsenic contaminated sites with the fewest environmental side effects. To protect themselves against the toxic effects of arsenic, microorganisms generally evolved strategies for detoxification and the best among these is the microbial reduction of arsenate to arsenite by means of the ars system, an enzymatic process in which energy is actually consumed to drive the reduction. Arsenic detoxification has been documented in Escherichia coli, Staphylococcus aureus, and Staphylococcus xylosis, and is controlled by ars operon consist of three or five genes ars RDABC or ars RBC organized on a single transcription unit. The ars R and ars D are the regulators, where as the ars A and ars B forms the oxyion pumps which efflux out the arsenic. Ars C codes for the arsenate reductase convert the arsenate to arsenite, which is then efflux out by the oxyion pump. In some organisms, resistance involves overproduction of intracellular thiols. In many cases, resistance to arsenic salts is the result of removal of the metalloid from the cytosol, usually by extrusion from the cell. Arsenate poisoning generally results from the transport of this ion by the phosphate transport system thereby competitively inhibiting the oxidative phosphorylation pathway. One phosphate transport system (Pit) takes up both, phosphate and arsenate, at similar rates, whereas the other (Pst, phosphate specific transport) is highly specific for phosphate. Four general mechanism operated in the microbial system involves: Keeping the toxic ion out of the cell (reduced uptake); Highly specific efflux pumping (i.e. removing toxic ions that entered the cell by means of transport systems evolved for nutrient cations or anions), Intra or extra-cellular sequestration by specific mineral-ion binding components (e.g.: metallothioneins) and/or segregation into complex compounds; Enzymatic detoxification (oxydoreductions) that converts a more toxic ion to a less toxic one. The present review paper summarizes the work done in this area and provides the reader better understanding of the roles of microorganisms in cycling of arsenic which may lead to improved processes for Bioremediation of contaminated sites.***

Key words: Arsenic toxicity, Microbial system, Resistance mechanism.

Arsenic, the thirty-third element, is synonymous with poison. Discovered in 1250 by Albertus Magnus, it has a colorful history, reputed (but unlikely) to be the cause of death of such notables as Napoleon and the American president Zachary Taylor. It is classified as a metalloid, meaning it has both metallic and nonmetallic properties. Arsenic is situated in Group 15 of the periodic table, below nitrogen and

phosphorous. Arsenic commonly forms complexes with other metals and is readily able to form covalent bonds with carbon, hydrogen, and oxygen. Arsenic is subject to eight electron reductions and can occur in +5, +3, 0 and -3 states. In its inorganic form, arsenic primarily exists in two redox states: the reduced form, arsenite [As (III)], and the oxidized form, arsenate [As (V)]. In soils, the most often encountered arsenic forms are inorganic As (III) (arsenite) and As (V) (arsenate) [1-4].

Methylated species, monomethyl arsonic acid (MMAA), dimethyl arsinic acid (DMAA) and trimethyl arsine oxide (TMAO), dominate in biomass, but have also been detected in soils [5,6] . In addition, As (V) and As (III) can be volatilized to arsine (AsH_3); MMAA to monomethylarsine (CH_3AsH_2; MMA); DMAA to dimethylarsine [$(CH_3)_2AsH$; DMA]; and TMAO to trimethylarsine [$(CH_3)_3As$), TMA] [1]. Arsenic forms organic compounds and is methylated by microorganisms, but neither group is considered as toxic as inorganic As (III) nor As (V) compounds [7]. Arsenic has a natural affinity for sulfur as evident by many natural arsenic containing minerals such as As_2S_3 (orpiment), AsS (realgar), and FeAsS (arsenopyrite).Arsenate and phosphate are similar and may substitute for one another. For example, stereochemical properties of arsenate result in arsenate incorporation instead of phosphate and the uncoupling of oxidative metabolism from ATP biosynthesis [8]. This may account for some toxicity effects of arsenic. Toxicity depends on physical state (gas, solution, or powder-particle size), rate of absorption into cells, rate of elimination, presence of impurities, and the nature of chemical substituents in the toxic compound [9].

Arsenic is found naturally in trace amounts in the earth's soils, waters, and organisms [10]. There is a large range of arsenic concentrations found in natural waters, from less than 0.5 to more than 5000 µg/L [10]. This has become a major concern in areas such as India, Bangladesh, Argentina, and Mexico where high arsenic concentrations in natural aquifers are influencing drinking water and millions of people are exposed to toxic water resources [8,10]. Other areas of naturally occurring high arsenic concentrations are found near geothermal springs such as those surrounding the Greater Yellowstone Ecosystem of Wyoming and Montana, USA. Nimick et al. [11] found that arsenic discharge from a geothermal system (900 to 3560 µg/L) into the Madison River near West Yellowstone generated base flow concentrations of 250 to 370 µg/L.

Although arsenic does occur naturally throughout the world, mining and industrial uses of arsenic have substantially increased arsenic availability to humans and other organisms of the earth's surface. This recent (since the industrial revolution) anthropogenic loading of arsenic has created concern for human exposure through consumption of contaminated water and for aquatic biota subjected to increased concentrations [8,10]. Throughout history arsenic has been used in a variety of interesting ways. The history of arsenic use includes many stories of homicide and suicide from the middle Ages. There is evidence of active research by Scheele in 1775 to find a method to counteract arsenic as a homicide agent [8]. Thomas Fowler, a British physician, created Liquor Arsenicalis to treat a plethora of health conditions accepted into London Pharmacopoeia in 1809 and the U.S. Pharmacopoeia in 1820 [8]. Similar forms of this solution were common until the 1960s. Over 8000 arsenic based compounds were used to treat asthma, malaria, tuberculosis, diabetes, and skin diseases, and some arsenic based compounds were used until the mid 1980s for treating narcolepsy (sleeping sickness) [8]. In Syria and Tyrol, healthy people ingested orpiment (As_2S_3) as a luxury food to increase their health and virility. There are also rumors of current arsenic use to lighten skin complexion. Although arsenic has almost exclusively been associated with criminal poisoning for many centuries, the matter of concern today is its contribution to environmental pollution through man's use of arsenic containing insecticides, herbicides, fungicides, pesticides and wood preservatives and through mining and burning of coal, electroplating and paint manufacturers [6]. Thus, anthropogenic use makes arsenic a common inorganic toxicant found at contaminated sites nationwide. Ironically, it is these "sources" are of the most concern to human health on a global basis. Globally, in developed countries, pollution of the aquatic system is controlled by the union under the framework 'Dangerous Substances Directive' which has led to certain environmental protection acts and regulations enforced by environmental agencies. Consequently, all effluents need to be assessed and require integrated pollution documentation before their final discharge. The effect of arsenic on human health is an issue of global concern. A large-scale shift in water resource allocation from surface water to ground water in West Bengal, India, and Bangladesh

(tube well water) and the exposure of local populations to ground water containing arsenic at concentrations of several hundred mg/L have resulted in very extreme environmental health effects. Spurred by increasing concern over exposure to low levels of increased arsenic mobility in natural environments is a major concern in the creation of new wells and water supply systems in areas that are rich in arsenic.

Worldwide, arsenic in soil ranges from 0.1 to 40 mg kg^{-1} with a median concentration of 6 mg kg/L [10]. Arsenic in seawater averages 1.7 µg/L with a relatively homogeneous range from 1.5 to 5 µg/L. In contrast, freshwater from lakes and rivers transport. Freshwater arsenic concentrations range from 1 to 10 µg/L with an average of 1.7 µg/L. The recommended maximum concentration for arsenic in irrigation water is 100 µg/L with the drinking water standard being at 50 pg/L [10]. Arsenate is more predominant in oxygenated water while arsenite is more common under reduced anaerobic conditions. The total arsenic influx into oceans is estimated at 246,110 metric tons/year. Of this total 62,900 metric tons is dissolved arsenic, 178,900 metric tons is sediment suspended arsenic and 4,310 metric tons is from the atmosphere per year.

Biological properties of arsenic and its compounds:

The impacts of arsenic on biological systems are concentration dependent and vary from organism to organism. In general, the toxicity of arsenic is dependent on its oxidation state: Both states are toxic to most organisms. Arsenite (specifically the arsenite ion, AsO_3^{-3}) trivalent arsenic forms are approximately 100 times more toxic than the pentavalent derivatives, which, interferes with sulfhydryl groups in amino acids and can disrupt protein structure. Thus, As (III) inhibits enzyme reactions requiring free sulfhydryl groups, leading to membrane degradation and cell death. As (V), in most environments, arsenite is generally thought to be the more soluble and mobile form, which increase its potential toxicity. However, arsenate is the thermodynamically favorable form in most aerobic systems [1]. In general, methylated species, MMAA and DMAA, are less toxic forms than inorganic As (V) and As (III) because of their low solubility and reduced affinity to tissues [6]. For plants, however, organic arsenic compounds are highly toxic when applied foliarly [12]. For more developed organisms, gaseous arsines are the most toxic forms of arsenic due to their ability to combine with hemoglobin within the red blood cells, causing destruction or severe swelling of the cells and rendering them nonfunctional [5,6,13].

Mechanism of resistance:

Heavy metals are often required by the cell in trace amounts for biochemical reactions, however, at higher concentrations, they can have toxic effects [14]. The cell may use the low concentrations of some heavy metals (such as iron, copper and nickel) in redox reactions, or other heavy-metal ions (such as magnesium or zinc) to stabilize electrostatic forces, and still others (such as Mg^{2+}, in Mg ATP or Zn^{2+}) in often bind to sulfhydryl groups and thereby inhibit the activity of certain enzymes by interacting with ligands present in all enzymes. Some heavy metals also interact with physiological ions and inhibit the activity of that ion. Other heavy metals bind glutathione in Gram-negative bacteria, and the resulting complex reacts with molecular oxygen to form bisglutathione, the metal cation, and hydrogen [15]. Heavy metals, when present, may bind to any or all of these sites and compounds, however, some sites are considered more "sensitive" than others and therefore, binding at these sites causes more damage to the cell than binding at other, less "sensitive" sites [16]. This explanation allows for an understanding of why most cells are protected to some extent against low heavy metal concentrations, and why higher concentrations are often more toxic. Due to the presence of highly concentrated areas of heavy metals in the environment, heavy metal resistance mechanisms are commonly found in bacterial genomes. There is some debate as to when these resistance genes may have been formed. One theory is that the resistance genes were present when bacteria evolved into a world already polluted with heavy metals from volcanic activity and other geological events. Another suggests that these genes arose much later, after humans polluted the world [17,18]. Regardless of when these genes may have arisen, heavy metal resistance is known to be a common phenotypic characteristic encoded by chromosomal and plasmid Uptake will describe the fact that toxic ions enter the cell while efflux will describe the fact that the cell removes these ions.

Resistance to a given metal can be inducible or constitutive. Many authors found two types of uptake systems for metal

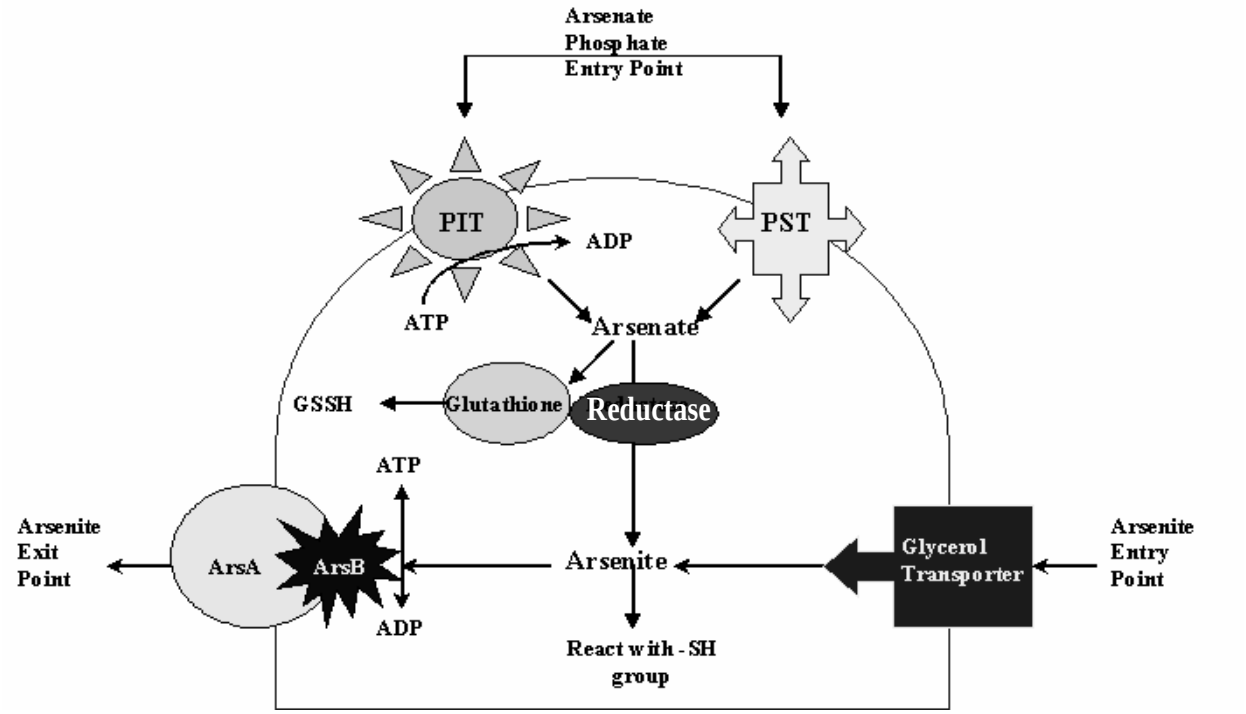


Figure 1 shows a schematic overview of the cellular arsenite and arsenate uptake {After Silver and Phung [22]}

ions whose expressions were either inducible or constitutive. One is fast, unspecific and generally driven by the chemiosmotic gradient (membrane potential gradient, usually 200 mV across the cytoplasmic membrane of bacteria (constitutive) whereas the other one is slower and has high substrate specificity. The latter often uses ATP hydrolysis as the energy source. It is expensive and inducible (in times of need or starvation). Silver [19] distinguishes following 4 mechanisms of bacterial metal resistance:

a). Keeping the toxic ion out of the cell (reduced uptake).

b). 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.

c). Intra or extracellular sequestration by specific mineral-ion binding components (e.g.: metallothioneins) and/or segregation into complex compounds.

d).Enzymatic detoxification (oxydoreductions) that 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. In general, metals become toxic for organisms when their concentration is higher than the demand from the metabolism, at this point the metal can act as inhibitor of metabolic pathways by strongly binding to enzymes or by forming unwanted radicals or less stable reaction products and therefore wasting energy. Cells have developed mechanisms to avoid the toxicity of metals, a selective uptake regulated by the metabolic need for the metal, an efficient excretion mechanism or specific metabolic pathways by which the toxic form of the metal is transformed into a non- or less toxic form.

Uptake and excretion:

Cellular membranes are formed by hydrophobic lipid bilayers, which are nearly impenetrable for charged compounds. In the course of evolution, membrane-spanning transporters were developed for the exchange of ions between the surrounding environment and the intracellular space. These transporters either form simple pores, by which energy-independent diffusion is possible or they transport their freight through membranes by consuming energy.

Passive cellular uptake and excretion by simple diffusion:

Simple diffusion of metals through cell membranes is not a biologically relevant process, simply because of the hydrophobic nature of cell membranes and the hydrophilic nature of the ions. Diffusion through cell membranes is used by gases like oxygen, nitrogen or methane [14]. Diffusion is energy independent since it is driven by a concentration gradient. It is likely that volatile metal compounds for

example Me_3As, Me_2Hg or AsH_3, synthesized by the metabolism in the cell, are also excreted by passive diffusion.

Active cellular uptake:

The first barrier to penetration is the wall, which provides some protection for the cytoplasmic membrane. Cell walls, especially those of fungi, can be used as biosorbents [20]. Thus, many works have focused on the description of Langmuir or Freundlich isotherms to describe binding [21]. However, walls cannot act as a perfect barrier to entry of some ions that are essential trace elements for microorganisms. So, the metal ion is first transported into the cytoplasm in spite of its high concentration, which is the first reason why metal ions are toxic. Molecules spanning cellular membranes for transporting other molecules are often protein-complexes with hydrophobic and hydrophilic domains. They can form pores suitable for passive transport through the membrane, like members of the permease family. These proteins span the membrane, but they are not able to transport against a concentration gradient. They allow energy independent unhindered diffusion of hydrophilic compounds, like water, through hydrophobic membranes "downhill". Active transport of transporters can either be selective for a specific molecule or molecule-group or non-specific transporters. As an example, the up-to-date knowledge about the uptake of arsenate and arsenite is briefly discussed in the following paragraph.

Arsenate uptake takes place via phosphate-transporters, because of the structural similarity of phosphate and arsenate ions. So far, examination of prokaryotic microbes showed they possess two different phosphate transporters; one with a high affinity for is regulated via a feedback mechanism. Members of the low-affinity Pit-family are expressed predominantly when the phosphate concentration in the environment is high. These transporters belong to the permease transporter channels. Members of the Pst-family are ATP-dependent phosphate transporters. The affinity of the Pit-transporter for arsenate is higher than that of the Pst-transporter.The uptake of arsenite ($As(OH)_3$) or the structurally similar antimonite ($Sb(OH)_3$) is facilitated by transporters, which also transport glycerol, water and other polyols. Transporters of this kind are involved in the osmoregulation of every cell. The ability of glycerol transporters to transport arsenite is probably a result of the similarity of arsenite to other polyols [14].

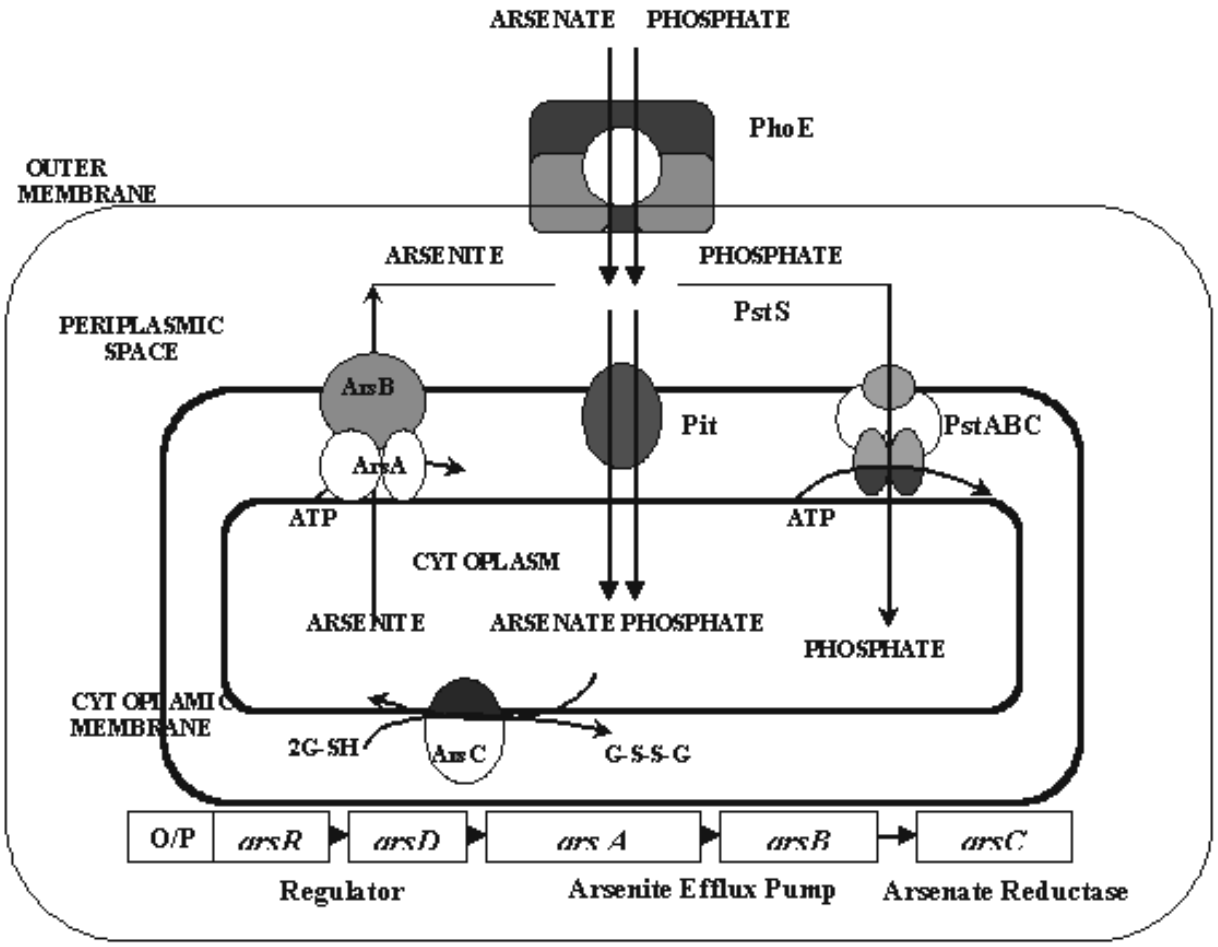
Active cellular excretion:

Efflux systems, which essentially pump the toxic ions out of the cell prohibiting them from accumulating to levels high enough to inhibit growth or cause cell death, are the most common mechanism of "heavy metal" resistance [22]. Resistance systems to all metals other than mercury appear to use one of several variations of the energy-dependent efflux mechanism of detoxification. Efflux detoxification may involve both the plasma-membrane-bound ATPases, which are cation pumps that form a phosphorylated intermediate during the catalytic cycle, and the energy required to maintain such a specific pump or gradient far exceeds the "genetic cost of having plasmid genes in the population that can spread when needed" [23]. Therefore, the normal, non-specific uptake systems constitutively expressed and are very energy efficient considering that they transport several types of heavy-metal ions by the same system. However, high concentrations of intracellular metal ions can interfere with the function and transport of other metal ions [24]. Resistance mechanisms, that are often plasmid-encoded, have evolved to allow the induction of metal-ion efflux systems [23,24]. Efflux pumps reduce the intracellular concentration of metals by means of transport systems, without any enzymatic transformation [14]. This mechanism is more widespread than enzymatic detoxification. Uptake and efflux mechanisms can be classified in 8 protein families approximately: the most important are the ABC family (ATP Binding Cassette), the P- and A-type ATPases family, the RND family (Resistance, Nodulation and cell Division) and the MIT family (Metal Inorganic Transport). Excretion of metals from cells is normally an energy demanding process involving selective or non-selective transport molecule complexes. Some metal ions like iron are normally not excreted in free ionic form. Others like arsenic are excreted as free ions as well as in metabolised form. These exporters are, as the importers, transmembrane protein complexes. Most contain binding domains for ATP or other energy-rich molecules, necessary for delivering the energy for the transport, in addition to the substrate-binding site. Not all metals imported into the cell can be excreted as well. For example iron exporters are, in contrast to importers, virtually unknown. Partial responsibility may lie with iron being a limiting factor for microbial growth in most environments, because of its low solubility. The iron export in iron-respiring microbes has not yet been studied. It may be similar

to the only known iron export cellular toxin. Arsenite is able to inhibit every enzymatic activity where sulfhydryl groups are participating. Cells with a defective arsenite export-system are extremely sensitive to the lowest environmental arsenic concentrations. The enzyme systems involved in the excretion of arsenite are known in detail in prokaryotic and eukaryotic cells. These transporters are either plasmid or chromosomal encoded. In all studied species so far these transporters belong all to the ATP-binding-cassette-protein super family, despite their variability.

Genes for arsenic resistance:

Microorganisms have evolved a number of mechanisms to cope with metal toxicity, and some organisms even benefit from the presence of metal ions. In some cases metal toxicity is avoided by minimizing the amount of arsenic that enters the cell. Toxic metals in the environment select and maintain microbes possessing genetic determinants which confer resistance to the toxic compounds. In bacteria heavy metal resistance genes are frequently located on plasmids. Resistance genes are also encoded by the chromosomal or plasmid encoded system. Chromosomally encoded resistance occurs by the activation of a phosphate uptake pump with an increased selectivity for phosphate, two phosphate uptake systems are present, Pit (inorganic Pi transport) under abundant phosphate conditions, the high Vmax but less specific Pit system fulfills the phosphates need of the cell and leads also to arsenate accumulation [25]. Under conditions of phosphate starvation, the more specific Pst (phosphate specific transport) system is induced [26]. Pst discriminates between phosphate and arsenate 100-fold better than Pit [27, 28, 29, 30]. Thus, one way for the cell to adapt to arsenate stress is to inactivate the Pit system by a pit mutation, which leads to moderate arsenate tolerance due to the discrimination between arsenate and phosphate by the Pst system. During periods of phosphate starvation or arsenate toxicity the Pst system is activated and despite having an identical Ki for arsenate, the reduction in cellular arsenic is achieved by the higher affinity for phosphate. The Km for phosphate is 0.25 pM, one hundred times greater affinity than the Pit system [28]. The genetic determinants for there arsenic resistance are studied in *E. coli, Pseudomonas aeruginosa, Bacillus subtilis* and *Mycobacterium tuberculosis*. Thus the activation of the Pst system confers higher levels of arsenate resistance by virtue of reduced uptake of arsenate. As more and more bacterial genomes are sequenced, it has become clear that arsenic resistance operons are ubiquitous. The resistance determinant systems are functional and provide arsenic tolerance; inactivating these *ars* operons leads to 'hypersensititivity' to arsenic compounds. Bacterial *ars* systems confer arsenic resistance primarily by encoding a specific efflux pump that extrudes As (III) from the cytoplasm, thus lowering the intracellular concentration of the toxic arsenic [31]. In some plasmid-determined systems of Gram-negative bacteria, the efflux pump consists of a two-component ATPase complex. The *arsA* gene product is a soluble ATPase subunit, which physically associates with an integral membrane protein, the product of the arsB gene [31,32,33]. *ArsA* and *ArsB* genes product are involved in the export of arsenite and antimonate while *ArsC* gene product is required to confer resistance to arsenate. The ArsA protein encodes two distinct adenylate-binding consensus sequences which have binding affinity for nucleotides and specifically catalyzes the hydrolysis of ATP The oxy-anion pump is composed of only two proteins, a 63 KD hydrophilic ArsA protein and a 45.5 KD ArsB protein [34]. The *ArsA* gene has been sequenced and the deduced amino acid sequence shares homology with several adenylate-binding proteins such as nitrogenase and the 6-subunit of the mitochondrial ATPase. The binding of ATP by ArsA is independent of the presence of oxyanions; however, the rate of ATP hydrolysis is dependent on their presence and is stimulated 5-fold by the addition of arsenite and 50-fold with the addition of antimonite [34]. The ArsA protein is mainly cytosolic but a portion is found sedimented within the cell membrane and is thought to complex with ArsB. ArsB is found in the inner membrane of *E. coli* and has been postulated to be the portion of the pump responsible for the export of anions from the cell. The deduced amino acid sequence of ArsB reveals several regions of the protein are potentially transmembrane regions. Bacterial arsenate reductases can be grouped the 16 KD ArsC polypeptides modify the ArsA-ArsB complex allowing the pumping of arsenate. ArsC is not required for the efflux of arsenite or antimonate [35], it is grouped into two families according to their structure and consequent catalytic mechanisms, but both associated with proteins that promote thiol oxidation/ reduction, such as glutaredoxin (Grx) or thioredoxin (Trx), in order to catalyze disulfide bond reduction [36]. The best-studied group is the *E. coli* resistance plasmid R773 family. Resistance to arsenic is inducible and recently a fourth gene has been identified which regulates the arsenic resistance operon. Plasmid encoded resistance results from the activation of an anion-translocation ATPase with high selectivity for arsenate, arsenite and antimonate [34]. Plasmid-encoded resistance for arsenate/arsenite is widespread among different bacterial species [37]. In *Staphylococcus*, three genes are also involved in conferring resistance to arsenic, however, sequence analysis indicates only ArsB, the gene encoding the transmembrane protein,



Figures 2 summarize the current knowledge about resistance to arsenate in E. coli.

shares homology with sequences of R773 [38]. In most chromosomal arsenic resistance systems of Gram negative bacteria and the plasmids and chromosomes of Gram-positive bacteria, contiguous *arsB* and *arsC* genes are found, but there is no *arsA* gene. Little is known about this subject in algae and fungi. An arsenic resistance gene cluster similar to that of bacteria is found in the yeast *Saccharomyces cerevisiae* [39].There are three contiguous genes in the cluster, *ARR1, ARR2* and *ARR3* (previously called *ACR1*, *ACR2* and *ACR3*). The *arsR* encodes a trans-acting repressor of the ArsR/SmtB family involved in transcriptional regulation. The arsR under non inducing conditions prevents RNA polymerase binding and therefore transcription. The *arsR* is a transcriptional repressor that responds to As (III) and Sb (III) *E. coli* ArsR proteins are dimers in solution and that dimerization is required for DNA binding [40,41,42]. Here the general mechanism of arsenate resistance explains in details. Once inside the cell, there is a problem involving arsenate detoxification: due to the structural similarity, it would be difficult to export arsenate with high affectivity with the high phosphate concentration in the background [23]. Thus, arsenate detoxification has to involve an initial step, which is not possible for phosphate, and this step is reduction of arsenate to arsenite [43, 44]. More specific arsenate efflux resistance systems have been intensively studied in the Gram-negative bacterium *Escherichia coli* (Fig. 1) and in two species of the Gram-positive genus *Staphylococcus*. The chromosomal and plasmid-harbored functions encoded by ars determinants in all resistant organisms mediate efflux of arsenic in an energy-dependent process, driven in *E. coli* by ATP hydrolysis and in *Staphylococcus* by the membrane potential [34,45-48]. The ars determinants also govern resistance to antimonite (which appears to be an alternative substrate for the transport system) and to tellurite [49,50]. Because of the electrochemical gradient across the cytoplasmic membrane, export of any anion has a negative free energy for the bacterial cell. Thus, the cell needs only an arsenic-specific tunnel through the membrane to get rid of the toxic anion (see Fig. 2). These arsenic-specific tunnels are the products of the arsB genes in *E. coli* and *Staphylococcus* [35,44,51].

Resistance to arsenic is inducible and recently a fifth gene *arsD* has been identified which regulates the arsenic resistance operon. The lack Bacterial arsenate reductases can be grouped of *arsD* and *arsA* genes gives the Bacterial arsenate reductases can be grouped more similar to structures of the *ar*s

operons from plasmids of gram-positive bacteria, even though the gene products exhibit only moderate (57% for *ArsB*) to poor (19% for *ArsC*) similarity.

The distinct gene named arsH was also found in both copies of the genomic *ars* operons in *P. pituda*. The *arsH* was originally identified in the ars cluster of a Tn2502 transposon (belonging to the virulent plasmid pYV of *Yersinia enterocolitica* and in *Thiobacillus ferrooxidans* [52,53]. In *Y. enterocolitica*, *arsH* appears to be necessary for arsenic resistance [52]. In *P. putida* both ArsH1 and ArsH2 were highly similar to their counterparts in *Y. enterocolitica* and *T. ferrooxidans* (over 74%). Although *P.fluorescens* strain MSP3 lacks an *arsH* gene [54], *P. aeruginosa* has an *arsH* homologue (87% amino acid similarity) located downstream of *arsC*. The *arsH* gene, which is required for arsenic resistance, although the function of the *arsH* product has not yet been, elucidated chromosomal arsenic-resistance (ars) operon of *A.ferrooxidans* is atypical in that it is divergent, with its *arsCR* and *arsBH* genes transcribed in opposite directions [52]. Furthermore, the amino-acid sequence of the putative *ArsR*-like regulator of the *ars* operon is not conserved in regions that have been shown to be responsible for binding to arsenic.

Response of micro-organisms to metal stress-metallothionein:

Metallothioneins (MTs) are ubiquitous proteins of extremely high metal and sulfur content. They are thought to play roles both in the intracellular fixation of the essential trace elements zinc and copper, in controlling the concentrations of the free ions of these elements, in influences of exposure to toxic elements such as cadmium and mercury and in the protection from of a variety of stress conditions. This is one of the most important mechanisms by which bacteria combat heavy metal exposure and subsequent accumulation. Bacterial metallothionein (MT) are commonly grouped in 3 classes. Class I and II are gene-encoded, whereas class III is not. Proteins within the cells may also provide a mechanism for isolating metal ions and conferring a degree of resistance. These groups of proteins are characterized by their resistance to thermocoagulation and acid precipitation, by the presence of ca. 60 non-aromatic amino acids, and by the absence of disulfide bonding [55,56]. Metallothioneins are a particularly well-characterized family of metal binding proteins. The metallothioneins are simple, cysteine rich, small molecule proteins that are found in many organisms, from, and even in some cyanobacteria [57]. These proteins have a high affinity for metal ions in groups 11 and 12 (most commonly Cd^{2+}, Cu^{+}, Hg2+, and Zn^{2+}) and complex 7 to 18 metal ions forming one or two metal-thiolate clusters [56]. Given their unusual structure, labiality, and induction by a variety of agents, role in metal homeostasis, detoxification, and transfer [57,58]. In the prokaryotic cyanobacteria, metal ion sequestration within the cell is performed by the class II metallothioneins. Class II metallothioneins are sulfhydryl-containing, cysteine-rich, metal-binding proteins that sequester metal, thus preventing accumulation of potentially toxic forms of metal ions within the cell metals by limiting their cellular availability [59]. Metal ion binding occurs through the interactions of the ions with the sulfhydryl groups of cysteine residues [60]. The smt locus contains a metal-regulated gene; smtA [61].This operon encodes a class II metallothionein and a divergently transcribed repressor of *smtA* transcription, *smtB*. SB is a trans-acting repressor of expression from the *smtA* operator-promoter region. Metallothionein protein expression is dependent upon the loss of the repressor gene, *smtB*, and subsequent unregulated transcription of *smtA*, has been shown to be advantageous to organisms constantly stressed with changing levels of cadmium, copper, lead, nickel, zinc, or arsenate [62]. These mutant strains, devoid of the functional repressor, show elevated levels of smtA messenger RNA even in the absence of a metal inducer. One such protein, identified in a *P. putida* strain isolated from a metal-polluted site, displays a high degree of homology to the *Synechococcus SmtA* [63,64]. The three-dimensional structure of the *Synechococcus* MT has been determined, and all the cysteine residues present in the peptide have been assigned to the chelation of metals. Alignment of the *Synechococcus* MT with the *pseudomonad* (*P. putida* KT2440 and *P. aeruginosa*) MTs showed the latter to have one or two cysteine residues more (one conserved at position 33) that could play a role in binding the metal [64]. All the cysteine and histidine residues in SmtA involved in metal binding are conserved in the pseudomonad MTs, except His-49. Actually, this residue is different in both *pseudomonad* MTs, being replaced by either methionine or aspartate. *Pseudomonad* MTs are longer than those of other bacteria, although the C-terminal tail lacks cysteine and histidine molecules has been reported for *Synechococcus SmtA*, in which

both cysteine and histidine residues are involved in Zn co-ordination. Metallothionein contains several sulfhydryl groups, which arsenite might bind indicated that arsenite can bind to metallothionein, but the binding is pH dependent, with greater binding occurring at pH < 7. At pH 7, zinc can displace arsenite from binding to metallothionein.

In eukaryotic system, sodium arsenite, and less potently sodium arsenate, induce hepatic metallothionein in mice and rats in a dose-dependent manner after oral, intraperitoneal and subcutaneous administration [65-67]. Both isoforms of metallothionein as well their corresponding mRNA are induced by arsenic [66,67]. Arsenite induced metallothionein levels in other organs of the mouse including kidney, heart, lung, spleen, stomach and small intestine. However, the level of induction in these organs is less than the induction of metallothionein in the liver [66]. Arsenite was found not to be associated with hepatic metallothionein after rats were treated with zinc and arsenite [67]. Yeast cells defend themselves against heavy metals by production of specific proteins, metallothioneins (MTs) by binding to heavy metals [69-72]. Over expression of metal-binding proteins such as MTs in bacterial cells resulted in enhanced Hg^{2+} accumulation and thus offers a promising strategy for the development of microbe-based biosorbents [73,74].

Stress induced proteins:

When microbial species are subjected to abnormal environmental circumstances, the organisms rapidly redirect gene expression and produce a distinct range of newly synthesized proteins which assist their survival and adaptation to the new conditions. Living organisms respond to stressful environmental conditions by increasing the production of specific proteins which alleviate or reduce damage incurred by the cell. Several resistances one of the environmental stresses that activate a specific set of genes called the heat shock genes, which are associated with newly synthesized mRNA [75,76]. The most thoroughly studied stress proteins include the heat shock proteins (HSP), the induction of which is the most highly conserved response across genera. This group of proteins interacts with other cellular proteins to facilitate appropriate folding, assembly into protein complexes, or their translocation into organelles [77,78]. Many environmental stresses are known to induce the production of HSPs that can help protect an organism from damage until stress is removed. One of the most important of the heat shock proteins is GroEL. GroEL is a 58-kDa protein that assembles into two stacked rings of seven subunits each with an additional ring of seven 10-kDa GroES subunits. This complex has been shown to renature proteins, making them again functional [79]. Since their major role is in assisting protein folding with the consumption of ATP, GroE termed chaperonins. Chaperonins provide kinetic assistance to the process of folding of newly translated proteins or proteins disrupted as a result of cellular stress to regain a biologically active conformation [80]. In the bacteria, the genes for GroES and GroEL proteins are arranged into an operon (groESL) and transcription is coordinately expressed by the use of specific stress sigma factors, essential component for maintaining viability with changes in temperature [81], essential proteins for cellular growth and are always transcribed at baseline levels, only under conditions of stress does the transcription rate increase.

Stress responses have been noted to occur in *Pseudomonas* species, as initially reported in *P. aeruginosa* [82]. Allan et al. [82] reported an exposure to environmental pollutants and a rapid temperature shift caused elevated synthesis of 17 proteins. Heat, NaCl, and hydrogen peroxide shock exposure of *P. putida* also caused production of clusters of new proteins, where some were similar to starvation-stress proteins and others were condition specific or similar to the HSPs. Despite the universality of the heat shock response, the mechanism and production of HSPs varies greatly and also among different bacterial species, and even differs in one species depending on the stimulation [83,84].

Inductions of heat shock proteins both by several sulfhydryl reagent arsenite have recently been reported. These induced heat shock proteins protect membranes and proteins in a similar way as under heat stress [85]. The induction of mRNA for heat shocks these heat shock or other stressors induces several proteins. In the rabbit, intravenous administration of sodium arsenite (0.8 mg/kg) results in induction of a 74-kDa protein in the kidney, liver and heart [86].This indicates there are organ-specific differences in the ability of sodium arsenite to induce this protein. The differences may be due to the disposition of arsenic, which generally accumulates

in low amounts in the brain [87]. A protein that is induced by arsenite or heat shock in human renal carcinoma cells is P-glycoprotein, which is an expression of the multidrug resistance gene (MDR1) [88].

Microorganisms play an important role in the environmental fate of arsenic with a multiplicity of mechanisms affecting transformations between soluble and insoluble arsenic forms and toxic and nontoxic arsenic forms. Inorganic arsenic forms, As (V) and As (III), are subjected to microbiologically mediated oxidation-reduction reactions. For example, a Pseudomonas strain oxidizes As (III) to As (V), but they were not able to grow with As (III) as the sole energy source, thus suggesting that the ecological role of As (III) oxidation was detoxification of arsenic. Microbial reduction of arsenate is important because arsenite (the reduced form) is more toxic and more soluble (and thus, more mobile) than arsenate. To date, dissimilatory reduction has been observed in several bacteria [89-93]. In addition, microorganisms may possess As (V) reduction mechanisms that are not coupled to respiration but instead are thought to impart arsenic resistance. For e.g. *E.coli* and *S. aureus* reduces As (V) to As (III) for its rapid extrusion from the cell [43,94]. In another study of aerobic contaminated mine tailings, it was found that members of the *Caulobacter*, *Sphingomonas*, and *Rhizobium* families may be responsible for the reduction and mobilization of arsenic [93]. In addition, Banfield [95] isolated a new Thermus strain from an arsenic-rich terrestrial geothermal environment, which was capable of both As (III) oxidation and As (V) dissimilatory reduction.

Potential application for bioremediation of arsenic contaminated sites:

The discharge of heavy metals due to industrial, agricultural and military operations has serious adverse effects on the environment [17,96]. In recent years, we have seen dynamic growth in understanding arsenic as a result of the teamwork of a worldwide community of researchers working on arsenic speciation, transformations, transport kinetics, seasonal cycling, accumulation, biochemistry, molecular biology, geochemistry, and toxicology. New developments in arsenic biological and geochemical behaviour will engender better understanding in developing new, safer and cheaper technology to clean up the arsenic contaminated sites and polluted drinking water.

A wide variety of fungi, algae, and bacteria are now under study or are already in use as biosorbents for arsenic remediation [97,98]. Metal binding by biomolecules manipulated by cultivation conditions (e.g., stress-inducible fungal melanin to improve its metal binding properties [98].

During the last few decades extensive attention has been paid to the hazards arising from contamination of the environment by arsenic [96]. Decontamination of heavy metals in the soil and water around industrial plants has been a challenge for a long time. The use of microorganisms for the recovery of metals from waste streams, as conventional processes used for removal of heavy metals from industrial wastewaters include chemical precipitation, oxidation-reduction, filtration, electrochemical techniques and sophisticated separation processes using membranes [97-100]. These processes are usually expensive when heavy metals are present in moderate concentrations, such as 1 to 100 mg/L. This characteristic stimulates the use of alternative biotechnologies, due to their application. Moreover, the possibility of altering the properties of living species used in heavy metal remediation or constructing chimeric organisms possessing desirable features using genetic engineering is now under study in many laboratories. Many scientists have sought microbial community members responsible for arsenate reduction. Hoeft et al. [101] found that in the anoxic water of Mono Lake (California), two subgroups (*Sulfurospirillium* and *Desulfovibrio*) of the any arsenate that had been produced. Thus, in some environments, both oxidation and reduction of arsenic may occur. In another study of aerobic contaminated mine tailings, it was found that members of the *Caulobacter*, *Sphingomonas*, and *Rhizobium* families may be responsible for the reduction and mobilization of arsenic [93]. Although several studies have attempted to show essentiality of arsenic, a biological role for it has not been demonstrated [8].The studies of interaction of living organisms and their constituents with the arsenic, will prove to be useful for development of alternative technology for the bioremediation of polluted sites.

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