Responding to toxic compounds: a genomic and functional overview of Archaea

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1. ABSTRACT

Archaea occupy a considerable diversity of niches ranging from extreme of pH, salinity to temperature that cannot be tolerated by other forms of life. There is an increasing consciousness that they have a key role both on the biogeochemical cycling of elements and in the bioremediation of polluted habitat. A greater understanding of metal homeostasis and resistance to toxic compounds in this life domain is required to design new strategies for the bioremediation of contaminated sites. This review describes the strategies developed by Archaea to transform xenobiotic compounds and metal ions present in the environment. The adaptation and/or response to such chemicals and the molecular mechanisms of resistance evolved in Archaea are discussed.

2. INTRODUCTION

The origin of toxic compounds can be various: they can be man-made or can be released by natural environment. Microorganisms have colonized so many different environments that can offer the chance not only to understand the mechanism to survive in harsh habitat but also to contribute to the degradation or metabolic utilization of toxic compounds. Survival and colonisation require the capacity to sense and adapt to environmental changes. Microbial cells respond to such stressful conditions mostly by switching global patterns of gene expression to relieve the environmental stress. Different strategies have been developed by microorganisms to remove toxic compounds such as xenobiotic substances or transition metals.
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For many aspects Archaea represent a very interesting model system both for their genetic-molecular characteristics and for their growth conditions. The archaeal microorganisms have colonized habitats mainly characterized by extreme temperature, pH, and salinity; nonetheless the metagenomic analyses have revealed their presence in several types of environments (1). Recent molecular-based studies have shown that Archaea colonize also oil-containing environments, such as petroleum reservoirs, underground crude oil storage cavities and hydrocarbon-polluted aquifers. They are involved in the biodegradation of petroleum hydrocarbons mainly through methanogenesis. Indeed, methanogens are the most abundant Archaea in the contaminated soils. At same time various archaeal microorganisms are capable to grow in environments contaminated by high concentration of heavy metals. It is clear that deep investigations on the mechanisms involved in homeostasis and resistance to toxic compounds and metal ions are required. Fortunately, new and improved techniques of analysis, combined with an increasing number of genome sequences are rapidly advancing the field of metal ions and xenobiotics metabolism in Archaea. We provide an overview of the strategies developed by Archaea to remove toxic compounds present in the environment. This review will be mainly focused both on different pathways that are involved in the detoxification of xenobiotics/multidrugs and on metal ions with different characteristics regarding their levels of toxicity and their link with fundamental metabolic processes.

3. DRUG DETOXIFICATION IN ARCHAEA: THE STATE OF ART

Toxic chemical compounds are either natural products, which are generated as a consequence of the metabolic activities of living organisms, or xenobiotic compounds, which are mainly produced by human activities (2). Most xenobiotic compounds are recalcitrant to degradation because they contain structures or substituents that are not normally present in natural molecules and hence limit their biodegradability. Owing to their human origin, they have been released into the environment very recently, and therefore, only a small number of organisms, mainly microorganisms, have developed various ways to resist to their toxic effects thus adapting to their new natural ecosystems (3). The majority of non pathogen microorganisms are also multi-drug resistant and the genes and proteins responsible for resistance are homologous to those found in pathogens, strongly suggesting horizontal gene transfer (4). Several distinct mechanisms account for drug resistance. For example, drugs may be inactivated before reaching their targets by hydrolysis or by formation of inactive derivatives. Hence, multidrug resistance occurs by the accumulation, on resistance plasmids or transposons, of genes, coding for resistance to a specific agent, and/or by the action of multidrug efflux pumps, each of which can pump out more than one drug type (5). Antibiotic resistance starts as a natural phenomenon, but microorganisms can become more resistant due to the massive selection pressure provided by antibiotics themselves.

Aromatic hydrocarbons are pollutants mainly generated by anthropogenic activities associated with the industrial production of dyes, plastics, explosives, detergents, insecticides, and pharmaceuticals. They also constitute one of the three classes of compounds found in petroleum and the majority of them are toxic for human health being mutagenic and carcinogenic, with extent of toxicity mainly depending on the chemical nature of substituent groups and their position in the benzenic ring.

At molecular level, the stress response and the ability to metabolize recalcitrant chemicals and synthetic compounds generally is due to the expression of specific enzymes and biochemical pathways to degrade or transform these compounds (6). If the transformed compounds are still toxic for the cell, coupled multidrug efflux pumps efficiently extrude such molecules. These latter have been demonstrated to be important also for cell homeostasis and intercellular signal trafficking (7).

Metabolism of pollutants, as well as multidrug resistance, is coordinated by the integration of environmental and physiological signals into regulatory systems able to control the expression of target genes in a way that cells can also respond to subtle changes of environmental conditions (8, 9).

These issues will be dealt with in this part of the review mainly focusing on microorganisms of the archaeal domain and discussing on: i) degradative pathways of aromatic compounds ii) xenobiotic/multidrug transporters and iii) transcriptional regulation mechanisms involved in the adaptation and/or stress response to drugs.

3.1. Degradative pathways of aromatic compounds in Archaea

Knowledge on bio-transformation of toxic compounds in this domain of life, even if still at an infancy stage, comes out mainly from the analysis of biodegradation/biotransformation of organic pollutants as well as by “omics” investigation and functional studies.

Catabolic pathways of aromatic compounds in the Archaea have been basically inferred from genomic data comparisons and characterisation of few enzymes. Whole-genome sequencing of Archaea (115 sequenced and annotated to October 2011, http://archaea.ucsc.edu/) and genome comparisons revealed an increasingly complex picture of archaeal phylogeny, evolution, cellular features and processes (10). Archaeal microorganisms harbour many novel enzymes catalyzing reactions and pathways that are not present in Bacteria and Eukarya (11).

Biodegradation of aromatic compounds by thermophilic Archaea has been reported only in a few cases. The first evidence that hyperthermophilic Archaea can anaerobically oxidize aromatic compounds came from a study demonstrating that Ferroglobus placidus could grow at 85°C in anaerobic medium with a variety of aromatic compounds among which benzoate, phenol, 4-hydroxybenzoate, benzaldehyde and 4-hydroxybenzaldehyde as the sole electron donors, whereas
two close relatives, *Archaeoglobus profundus* and *A. veneficus* could not (12). Some years later, Izzo et al., proved that *Sulfolobus solfataricus* P2 is able to grow aerobically on phenol as the sole carbon source (13). More recently, it was also shown that another *S. solfataricus* strain, 98/2, was able to utilize phenol as carbon and energy sources after adaptation on glucose with a small amount of phenol (14). The authors hypothesised the capability for *Sulfolobus spp.* to metabolize a larger number of aromatic hydrocarbons such as cresols, benzene, toluene, and polycyclic aromatic hydrocarbons (14).

In hyperthermophilic Archaea, the degradation pathways seem to be similar to those found in Bacteria. In the so-called upper pathway, the aromatic ring is first converted by mono- or dioxygenases to dihydroxylated compounds; they undergo ring cleavage reactions producing non-cyclic molecules which are in turn converted, in the lower pathway, into species that can enter in the citric acid cycle. Ring cleavage reactions are catalyzed by extradiol-cleaving dioxygenases (ECDs) and intradiol cleaving dioxygenases (ICDs), which incorporate both atoms of dioxygen into the aromatic substrate and cleave the aromatic ring at positions meta and ortho (15).

A genome analysis of *S. solfataricus* P2 performed in 2005 by Izzo et al., revealed the existence of: (i) a cluster of *orf* coding for the subunits of a hypothetical bacterial multicomponent monooxygenase, (ii) an *orf* coding for a lower pathway protein of the catechol metabolism, and (iii) an *orf* coding for a putative catechol 2,3-dioxygenase (*Sso1223*, EC 1.13.11.2) (13). Two additional *orfs*, *Sso2053* and *Sso2054* have been hypothesised to code for enzymes involved in phenylacetate metabolism. Expression and characterization of the putative catechol 2,3-dioxygenase suggested that it could be the enzyme involved in phenol metabolism (13). A catechol 2,3-dioxygenase (*C230*) was also characterised from *S. solfataricus* strain 98/2. The gene was found in the same genomic environment of a gene cluster encoding for a putative multicomponent monooxygenase, matching with the homologous region of *S. solfataricus* P2 (16). A comparative genomic analysis performed at http://microbes.ucsc.edu indicated that a homologue of the *c230* gene is also present in *S. islandicus* strains LS215, M164 and LD85, but nor in other *Chrenarchaeota* or *Euryarchaeota*, while homologues of *Sso2054* have been found in *S. tokodaiii* (13), in *S. islandicus* strains LS215, M164 and LD85, in *S. acidocaldarius*, Metallosphaera sedula and *M. cuprina*. To date, no functional characterisation has been reported nor for any of them or for *Sso2054*. We inspected the *S. solfataricus* KEGG pathways of xenobiotics (www.genome.jp/kegg/pathway.html), (17) and found that *C230* (*Sso1223* and/or *Sso2054*) could be involved in the catabolism of different aromatic compounds, like toluene, xylene, benzoate, styrene, 1,4-dichlorobenzene.

Studies on the degradation/transformation of aromatic hydrocarbons in hypersaline environments by halophilic Archaea have shown that *Halolacthaea* are able to degrade aromatic compounds and to use them as carbon sources. For example, the *Halolacthaea* strain EH4 was found to be capable of degrading a wide range of *n*-alkanes and the aromatic hydrocarbons acenaphthene, phenanthrene, anthracene, and 9-methylanthracene (18). *Halolacthaea* D1227 isolated from oil brine-contaminated soil was shown to degrade monoaromatic carboxylic acids, such as benzoate, cinnamate and 3-phenylpropionate (19). Later on, it was shown that this microorganism metabolized these aromatic acids by initial 2-carbon shortening of the side chain to benzoyl-CoA via a mechanism similar to fatty acid beta-oxidation, followed by aromatic degradation using a gentisate pathway. The ring cleavage enzyme is a gentisate 1,2-dioxygenase (EC 1.13.11.4, Figure 1) (20).

Differently from *H. volcanii*, *Haloarcula* sp. D1 was shown to be able to metabolize also p-hydroxybenzoic acid through a pathway very similar to that found in *H. volcanii* involving 2,5-dihydroxybenzoic acid (gentisic acid) as an intermediate (21). Gentisate 1,2-dioxygenases from the two different haloarchaeal genera are closely related belonging to a protein family with members in both Bacteria and Archaea, distinct from other bacterial intra-diol and extra-diol dioxygenases. Adjacent to the *gentisate* 1,2-dioxygenase genes were also found additional genes, which are highly conserved in both microorganisms, and could participate in the aromatic degradative route (22). More recently, Bonfa et al., isolated ten further *Haloferax* strains from five hypersaline sites able to metabolize mixtures of aromatic compounds and suggested that this ability is a common widespread feature among the *Haloferax spp* (23).

The partial genome sequence of *Haloterrigena* sp. H13, an extreme halophilic archaeon, compared to that of other bacterial and archaeal halophiles has revealed genes that may be involved in biodegradation of several aromatic pollutants, like naphthalene, anthracene, 1,2-methylnaphthalene and genes of the benzoate degradation pathway via benzyol-CoA formation. Gene homologs of (S)-2-haloacid dehalogenase (EC 3.8.1.2) and salicylate hydroxylase (EC 1.14.13.1), which might be involved in the degradation of dichloroethane and gamma-hexachlorocyclohexane were also found (24).

Among Archaea, methanogens also can metabolise aromatic and polyaromatic compounds; they use aromatic molecules to produce methane in anaerobiosis. For example, toluene and benzene were partially transformed to carbon dioxide and methane by mixed methanogenic cultures derived from fermentative enrichment (25). The metabolic intermediates detected suggested that benzene and toluene degradation occurred via initial oxidation by ring hydroxylation or methyl oxidation respectively, which would result in the production of phenol, cresols, or aromatic alcohols. Furthermore, Chang et al. demonstrated a direct association between anaerobic biodegradation of naphthalene and phenanthrene and methanogenesis using an inhibitor of methanogenesis, bromoethanesulfonic acid (BES), and monitoring the presence of methanogenic populations with 16S rRNA sequencing of isolated microorganisms before and after the
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Figure 1. Aromatic ring cleavage reaction catalysed by: a) catechol-2,3-dioxygenase (ECD); b) catechol-1,2-dioxygenase (ICD); c) gentisate-1,2-dioxygenase.

addition of BES (26). However, aromatic degradation via methanogenic consortia is likely due to interspecies hydrogen exchange between primary aromatic fermentor, acetogens and methanogens (27)

The potential of proteomics to analyze global protein synthesis has been demonstrated to be a powerful tool to characterize archaeal response to several stresses, especially the response to heat and salt stress, or to analyse protein patterns in cells grown with different substrates, providing a more global view of all of the proteins/enzymes involved.

The proteome of *S. solfataricus* from cells grown on ethanol as the sole carbon sources (inhibitor of cell growth) has been compared with cells grown on glucose; the global translational responses were investigated; the majority of the changed proteins were either annotated as hypothetical or having energy metabolism related functions. Among these, the well characterised Sso2536 alcohol dehydrogenase was found almost ten fold overexpressed (28).

In *H. volcanii* the effect of salt stress on the microorganism determines the up-regulation of a homologue of the phage shock protein *pspA*, also found in other archaeal genomes. In Bacteria this protein has a role in sensing a variety of stresses, including heat shock, osmotic shock and prolonged stationary-phase incubation (29).

*Methanosarcina acetivorans* is an acetate- and methanol-utilizing methane-producing archaeon. A combination of advanced proteomics and DNA microarray analyses of *M. acetivorans* grown with either acetate or methanol, showed gene up-regulation for stress-related proteins in acetate- versus methanol-grown cells, including enzymes specific for polyphosphate accumulation and oxidative stress and allowed the identification of putative regulatory proteins belonging to the Multiple antibiotic
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### Table 1. Number of positive hits obtained by a search for archaeal primary and secondary transporters at the NCBI Protein Database

<table>
<thead>
<tr>
<th>Archaeal classes</th>
<th>ABC-multidrug transporter</th>
<th>MATE</th>
<th>SMR</th>
<th>MFS</th>
<th>RND</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crenarchaeota</td>
<td>25.6 %</td>
<td>7.7 %</td>
<td>12%</td>
<td>35.4%</td>
<td>26.9%</td>
</tr>
<tr>
<td>Euryarchaeota</td>
<td>72.9 %</td>
<td>92%</td>
<td>82%</td>
<td>61.1%</td>
<td>69.3%</td>
</tr>
<tr>
<td>Other</td>
<td>1.4%</td>
<td>0.3 %</td>
<td>4%</td>
<td>3.4%</td>
<td>3.7%</td>
</tr>
</tbody>
</table>

Data have been carefully checked and the duplicate sequence have been removed from the output files. DME members are not included in Table 1 since the number of their predicted sequences is not statistically relevant.

Resistance Regulators (MarR), and Tetracycline Regulators (TetR) families (30).

In response to environmental stresses, many Archaea are capable of forming biofilm. Cells within the biofilm have an increased tolerance to otherwise toxic environmental conditions (31). Proteomic and transcriptomic analyses to describe physiological and regulatory features associated with biofilms in three *Sulfolobus spp.*, showed alteration in the expression of proteins putatively involved in cellular functions like energy production, energy conversion, adaptation to environmental changes and stress, and substrate transport/binding activities. A small heat shock protein (Hsp20) was found to be biofilm-up-regulated, as well as two other stress related proteins, a thioredoxin and a peroxiredoxin. One gene, encoding 3-oxoacyl-(acyl-carrier-protein) reductase (*fabG*) was found to be down-regulated in biofilm-associated cells. In *P. aeruginosa* the enzyme FabG is involved in the production of a quorum sensing autoinducer (32).

Taken together, the reported proteomic/transcriptomic data reveal that adaptation to different lifestyles requires changes in the expression profiles not only of stress related proteins and global regulators, but also of an unpredictable plethora of cellular processes.

### 3.2. Drug transporters

Prokaryotes and eukaryotes are able to expel actively drugs across the cytoplasmic membrane against their concentration gradients in order to prevent their intracellular accumulation (33, 34). This mechanism is catalysed by transmembrane-proteins, the so-called primary or secondary transporters that couple translocation of substrates to the free energy released upon ATP hydrolysis or to the electrochemical (proton or sodium) gradients across the membrane, respectively (35).

Both primary and secondary transporters are in general pleiotropic towards their substrates. Indeed, they are active not only on drugs but also on a variety of different compounds such as sugars, amino acids, peptides, vitamins, ions, xenobiotics and even polypeptides. Thus, the functions of drug-efflux and metabolite-efflux often overlap, linking this class of proteins to various cellular functions that range from energy supply to osmoregulation, detoxification and virulence (34, 36). The efflux systems that accommodate a wide range of structurally dissimilar drugs, constitute the so-called multidrug transporters family (34, 37). The polyspecific nature of multidrug-resistance efflux pumps is responsible for the “multiple resistance” phenomenon to antibiotics and chemotherapeutic agents as well as to natural substances produced by the host (38, 39, 40).

Survey of their genomes revealed that Archaea possess both primary and secondary multidrug transporters and that predicted transporters are more abundant in Euryarchaeal than in Crenarchaeal branch. So far, very few archaeal members have been functionally characterised and no archaea-specific transporter family has been identified. However, a high number of membrane proteins are still categorized as “hypothetical proteins” in the annotated genomes, suggesting that the lack of archaea-specific transporter families might be traced back to their limited functional characterization (2). Indeed, the complexity of the procedures to keep these proteins active has hindered their functional and structural characterisation.

In this study, archaeal sequences encoding for putative multidrug transporters were downloaded from the NCBI Protein database and the relative distribution of primary and secondary transporters in the archaeal domain is showed in Table 1. The Transport Classification Database (TCDB) available at http://www.tcdb.org/ as well as the UniProtKB/Swiss-Prot database was also examined and the output data were cross-checked. The general features of archaeal primary and secondary transporters described below are mainly inferred from sequences comparison with the functionally and/or structurally characterised bacterial counterparts. The analysis of the genomic context of individual genes as well as their comparison across multiple archaeal species were performed at the website http://archaea.ucsc.edu/ (9). It must be pointed out that because of the structural differences between archaeal and bacterial membranes, the export function of archaeal transporters may be accomplished only upon association with different membrane components. The representativeness of these efflux transporters in the landscape of archaeal genomes is also discussed in the context of the evolutionary history of Archaea and of their natural environments.

#### 3.2.1. Primary transporters

The ATP Binding Cassette (ABC)-transporter family includes a variety of members which are widely distributed in all domains of life. ABC transporters are involved in different processes such as substrate, chemotherapeutic drugs and antibiotics uptake or export, osmosensing and osmoregulation and play also a role in multidrug resistance. Mutations in these proteins cause diseases such as cystic fibrosis, hyperinsulinemia, macular degeneration (34, 42, 43).
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Substrate transport is driven by ATP hydrolysis. Generally, these transporters possess two homologous halves, each containing two domains: a transmembrane domain (TMD) arranged into a six-α-helixes surrounding and defining the translocation pore and a cytoplasmic nucleotide binding domain (NBD) bound to the cytosolic face of TMD which drive the translocation of the substrate through the hydrolysis ATP (33). ABC transporters branched off during evolution in importers and exporters that transfer substrates across the membrane in opposite directions. Beside this functional difference, their gene structure is also diverse. Indeed, in exporters TMDs and NBDs are encoded as a single polypeptide, whereas in importers TMDs and NBDs reside on separate subunits. Import systems are so far only found in prokaryotes and require an additional substrate binding protein (SBP) to deliver drugs to the transporter. Exporters recruit their substrate directly from the cytoplasm or lipid bilayer (44). Structural and biochemical data of archaeal ABC transporters are available only for ABC importers (42, 45, 46, 47, 48, 49), but none of them is involved in drug detoxification. For the exporters class, there is an indirect experimental evidence for the presence of an ABC-type multidrug transporter in H. vulcanii. Indeed, an anthracycline-resistant mutant of H. vulcanii was shown to transport rhodamine 123 more efficiently than the wild-type. This efflux activity was reduced by ABC-proteins modulators, such as diltiazem and activated by cytotoxic compounds or metabolites derived from aminoacids (50).

3.2.2. Secondary transporters

Secondary multidrug transporters are categorized according to the number of components that the pump has (single or multiple), the number of trans-membrane segment (TMS), the energy source that the pump uses and the type of substrate exported. Some of these families are members of larger super-families of transporters involved in a variety of other physiological functions.

Multidrug secondary transporters belong to the Multidrug/Oligosaccharide/Poly saccharide (MOP), the drug metabolite transport (DMT), to the major facilitator superfamily (MFS) and to the resistance/nodulation/division (RND) superfamilies.

Among the MOP and DMT families, only those specifically involved in drug/metabolite detoxification will be described, i.e. the Multidrug and Toxic compound extrusion (MATE), the drug metabolite export (DEME) and small multidrug Resistance (SMR) families (36, 40, 51, 52, 53).

Secondary transporters show disparate size of their polypeptide chain(s). Indeed, length ranges vary from 1000 amino acids for the RND transporters down to 100 aminoacids for the SMR transporters. MFS and MATE transporters are instead of an intermedia type transporters as exporters of cationic drugs transporters as as diltiazem and activated by cytotoxic drugs and are widespread in almost all prokaryotes and eukaryotes (55) and are highly represented in the sequence databases. However, being the most recently designated multidrug efflux transport family, only few members (~3% of known MATE-proteins in total) have been characterized so far. They use preferentially a solute/cation antiport mechanism and a Na+-chemiosmotic gradient. Among MATE members, one subgroup is archaeal specific and three others include both bacterial and archaeal proteins that appear loosely related, suggesting that the evolutionary relation between them is to track back to vertical transmission from a common ancestor without horizontal transfer (55, 56). In this study, about 200 putative archaeal MATE sequences were retrieved from the NCBI protein database, but none of them has been functionally characterised. Whereas eukaryotic and bacterial genomes possess multiple MATE family paralogues, in archaeal genomes no more than four MATE paralogues have been found.

3.2.2.2. The DME and SMR families

The DME family includes drugs transporters as well as efflux pumps for aminoacids metabolites and their toxic derivatives. Despite the low sequence conservation, DME members show the same topological organization with ten putative TMSs which clearly exhibit an internal repeat. Few predicted archaeal DME proteins are present in the NCBI and Swiss prot data banks, i.e. Hvo_1714 from H. volcanii, Smar_0735 from Staphylothermus marinus and AF1533 from Archeoglobus fulgidus. Hvo_1714 finds its homologues in the genomes of other euryarchaeal halophiles, as well as in those of several crenarchaeal representatives although they are not annotated as DME members. It has been reported that A. fulgidus has multiple putative DME paralogous on its genome and Pyrococcus horikoshii and P. abyssi contain six homologs in the DME family (57).

SMR members assemble into homo-oligomeric structures (dimers or tetramers), whose subunits are of 100-120 amino acid residues in length and span the membrane as α-helices four times (35, 58, 59, 60). Functionally characterized bacterial members catalyze multidrug efflux driven drug:H+ antiport but the chemical nature of their potential substrates is not well known. SMR members are thought to be the evolutionary building blocks of larger α-helical multidrug efflux proteins. Indeed, the structural arrangements of the TM strands of SMR proteins and of other larger multidrug transporters are similar, despite their low sequence similarity and their remarkable difference in length. Based on this evidences, the bigger multidrug transporters might have arisen from subsequent duplication
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events and/or addition of single TMSs during evolution, i.e. duplication of a 2TMS-encoding genetic element yielded the primordial SMR-type permeases which became first a five-TMSs protein upon addition of one TMS and then a 10 TMS transporter after duplication of the five-TMSs ancestor (61).

Within Archaea, SMR homologues are mainly present in euryarchaeal (Methanogenes and Halophiles) genomes, with only one SMR homologue present on a single genome (61). However, we found that in NCBI protein database, the protein Saci_1019 from *S. acidocaldarius* is annotated as a putative SMR. BLASTP alignment showed identity (ranging from 25 to 35%) with small multidrug members from *Methanomicrobia* and *Archaeoglobi*. Saci_1019 is a 142 aa long protein; secondary structure as well as transmembrane topology prediction suggests that it possesses the typical 4-TMSs topology of SMR members. Furthermore, inspection of its genetic locus revealed that Saci_1019 gene is located next to an aldehyde oxidase, an enzyme characterized by a broad substrate specificity oxidizing aromatic compounds. All together, these observations suggest a functional correlation between detoxification and export of aldehydic drugs at this locus of *S. acidocaldarius* genome. Interestingly, the chromosome of other Sulfolobus species does not contain a Saci_1019 homologue pointing out to a species-specific role of this protein.

In the surveyed archaeal genomes, variants of the typical 4-TMSs SMR are present and predicted to bear only two TMDS. This is the case of MM_0735, identified in the genome of *M. mazeii* and included in the group of Smr-3 (SMR3) in the TCDB. In some sub-members, only homologues of YvaE from *Bacillus subtilis*, are present in the euryarchaeal sub-phyllum of methanogenes. An YvaD gene is commonly located in the same operon of YvaE, but no YvaD homologous are found in Euryarchaeia, thus suggesting that YvaE potentially represents a PSMR progenitor (61).

The first and unique archaeal SMR, functionally and structurally characterized is that from the *H. salinarium* (Hsmr) (62). This exporter shares signature features with the eubacterial counterparts, in terms of the substrates-types transported and distinctive sequence elements. Hsmr finds its homologues in halo-and methano-microbia, with a percentage of identity ranging from 61% to 31% and 39% to 33%, respectively. The oligomeric state of Hsmr and residues involved in the oligomerization has also been investigated (60, 63).

Interestingly, different hosts thriving in similar environmental niches bear SMR homologues suggesting that the selective pressure exerted by environment and/or by lifestyle (aerobic/anaerobic) strongly affect the inheritance and/or maintenance of SMR proteins (61).

3.2.2.3. The MFS superfamily

MFS represent the largest group of secondary active membrane transporters that are ubiquitous in the three domains of living organisms and are constituted of 400-450 residues arranged into a 12-helix structure. Individual members show stringent specificity for their substrates. However, as a family, they transport a huge variety of substrates through uniport, symport and/or antiport mechanisms (64-67).

Bacterial MFS members share low sequence identity and a single signature sequences, DRXXRR, conserved in equivalent position in the two homologues halves constituting the MFS proteins (67). Putative archaeal MFS sequences of transporters involved in drug detoxification retrieved from NCBI are 145, with only 1/3 belonging to the crenarchaeal branch. Only one archaeal MFS member from *H. salinarium*, homologue to the eukaryotic vesicular monoamine transporter (VMAT) has been characterised. VMAT proteins remove neurotransmitters and toxic compounds from the cytoplasm, thereby conferring resistance to their effects. The *H. salinarium* protein, likewise the eukaryotic VMAT, confers resistance by expelling actively fluoroquinolones and chloramphenicol, through a proton motive force-dependent transport (68).

3.2.2.4. RND superfamily

Members of the RND superfamily utilize the proton motive force to catalyze substrate efflux. RND proteins are found ubiquitously in Bacteria, Archaea and Eukaryotes. The interest for this class of proteins stems from their pharmaceutical and medical significance, since the intrinsic drug resistance of Gram-negative bacteria is mainly attributable to RND-type drug exporters (69). Most of the RND transport systems consist of large polypeptide chains (700-1300 amino-acid residues) arranged into a 12-helix structure with two inter-regions between helices 1 and 2 and between helices 7 and 8 extended into large cytoplasmatic domains (70). Generally RND proteins arise from an intragenic tandem duplication event with the exception of few predicted RND proteins from some methanogens species that are of half size.

Bacterial RND representatives, such as AcrB from *E.coli*, work in association with other classes of proteins to exert their drug-efflux function (38, 71, 72). Only few putative AcrB-like members are present in the euryarchaeal genomes and none of them has been functionally characterised.

3.3. Transcriptional regulation in drug detoxification

Microorganisms’ capability to biodegrade a wide variety of natural and man-made toxic compounds is orchestrated by the integration of environmental and physiological signals into regulatory systems that tightly control the expression of genes that are able of metabolizing such molecules (8). In the Archaea, the response to drug exposure is finely regulated by local and/or global regulators belonging to the MarR, Mercury Resistance Regulators (MerR), TetR and Arabinose Catabolism (AraC/XylS) families of transcriptional regulators.

MarR family proteins constitute a diverse group of transcriptional regulators that modulate the expression of
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genes encoding proteins involved in a wide variety of cellular processes including metabolic pathways, stress responses, virulence and degradation or export of harmful chemicals such as phenolic compounds, antibiotics and common household detergents. Adjacent genes located in the genomic locus of marR members are often regulated by the encoded transcription factor (73).

MarR proteins are dimers having a low level of sequence identity and a triangular shape; they bind to their cognate palindromic or pseudopalindromic DNA as homodimers, resulting in either transcriptional repression or activation. The DNA binding domain is a conserved winged helix-turn-helix motif with the two wings located at the corners of the triangle. Another common feature of MarR members is their ability to interact with specific ligands and, upon binding, to modulate DNA recognition. There are more than 12000 MarR-like proteins annotated in bacterial and archaeal genomes to date. In the archaeal domain, the crystal structures of four transcription factors, ST1710 (or StEmrR) from S. tokodaii (74, 75), MTH313 from Methanobacterium thermoautotrophicum (76) PH1061 from P. horikoshii OT3 (77) and BldR from S. solfataricus (78), have been determined.

A coordinate regulatory mechanism to defend against stress by aromatic compounds has been reported for S. solfataricus; this system responds by increasing the expression of a marR operon composed of a permease involved in multidrug efflux and a transcriptional regulator controlling expression of the operon itself and an alcohol dehydrogenase gene (Sso2536adh) responsible for the conversion of the toxic benzaldehyde into the less harmful benzy alcohol (79, 80). Genomic analyses revealed that such an operon is conserved at least in the genus Sulfolobus. A second MarR member, BldR2, has also been characterised in S. solfataricus. It has been proposed that the protein could be involved in the regulation of aromatic catabolic pathways possibly controlling mechanisms different from those regulated by BldR (81).

The MerR family of transcriptional regulators contain a DNA-binding, winged helix-turn-helix domain of about 70 residues. Most MerR-type transcriptional regulators respond to environmental stimuli, like heavy metals, oxidative stress or antibiotics and a subgroup of metalloregulators are bacterial transcription activators that respond to metal ions. Generally, the helix-turn-helix DNA-binding motif is located in the N-terminal part of these transcriptional regulators and is followed by a coiled-coil region. The C-terminal part of MerR-type regulators contains binding regions that are specific to the effectors recognized (82).

MerR type regulators have been found in euryarchaeal (Methanosarcina, Methanococcus, Thermoplasma, Pyrococcus, Archaeoglobus) genomes. Among crenarchaeal representatives, have been found in Sulfolobales.

TetR is a family of transcriptional repressors found in Bacteria and Archaea. The DNA binding domain is composed of a single helix-turn-helix motif. These proteins are involved in the transcriptional control of catabolic pathways, multidrug efflux pumps, differentiation processes, pathogenicity pathways, the control of the biosynthesis of antibiotics, response to osmotic stress and toxic chemicals. The regulator can be released from the operator sequence upon ligand binding or is modulated by another regulator triggering a cell response to react to environmental insults (83). TetR members have been found both in crenarchaeal and euryarchaeal genomes with almost 200 members retrieved from Swiss Prot; however no functional studies have been conducted on archaeal TetR members. A proteomic and microarray analysis of the M. acetivorans grown with acetate or methanol identified several genes differentially expressed encoding regulatory proteins, among which 2 out of 13 are TetR family members (30).

The AraC/XylS family of transcription activator proteins is defined by a 100-amino-acid region of sequence similarity that forms an independent folding domain containing two helix-turn-helix DNA binding motifs. They are involved in the transcriptional regulation of a variety of cellular processes including carbon metabolism, stress responses and virulence (84). In general, these regulators comprise between 200 and 300 amino acids arranged in two domains: a conserved helix-turn-helix DNA-binding domain located at the C terminus whereas a variable N-terminal domain responsible for both protein dimerization and ligand binding (85). Members of the AraC family have been found only in euryarchaeal genomes. An AraC type DNA binding motif has been identified in the transcriptional regulator Bat from Halobacterium sp. NRC-1. It coordinates the synthesis of a structural protein and a chromophore for purple membrane biogenesis in response to both light and oxygen (86).

4. THE DARK SIDE OF METAL IONS: TOXICITY

Some metal ions, many of which are known as heavy metal, have a key role in the physiology of the cell. They can act as cofactors, can be involved in redox reactions or can confer stability to the proteins. At the same time, metal ion concentration has to be strictly controlled; if their homeostasis isn’t preserved, the accumulation can produce toxic effects on cell viability. Metal homeostasis has been studied in detail in Bacteria and Eukarya, while in Archaea the study is still in its infancy. Recently, Cvetcovic A. et al (87) have characterized the metalloproteoma of P. furiosus, and compared it with those of E. coli and S. solfataricus revealing a species-specific assimilation of different metals. The comparison of growth inhibition by different metals showed that S. solfataricus was more sensitive to Ag\(^{+}\), Hg\(^{+}\), and Ni\(^{+}\) with respect to E. coli while for Zn\(^{+}\), Pb\(^{+}\) and Cu\(^{+}\) is true the contrary (88). Furthermore, also inside the Sulfolobales there is a great difference in the metal resistance, e.g. for copper.
The archaeal response to toxic compounds

Table 2. Mechanisms involved in the metal ions detoxification in archaea

<table>
<thead>
<tr>
<th>Ion</th>
<th>Protein</th>
<th>Mechanism</th>
<th>Archaeal microorganism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe²⁺</td>
<td>Dpsl</td>
<td>iron scavenging and DNA shielding against HO²</td>
<td>S. solfataricus, P. furiosus, H. salinarum</td>
<td>98, 99, 100</td>
</tr>
<tr>
<td>Fe³⁺</td>
<td>Ferric reductase</td>
<td>Assimilatory/dissimilatory reduction</td>
<td>A. fulgidus</td>
<td>107, 108, 109</td>
</tr>
<tr>
<td>Ni²⁺</td>
<td>NikR</td>
<td>Repression of nickel transporter genes</td>
<td>Pyrococcus spp., Thermococcus kodakarensis</td>
<td>111, 120, 121</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>CopA</td>
<td>Pₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐ$_{N}^+$ methylation and volatilization</td>
<td>Halobacterium spp.</td>
<td>190</td>
</tr>
<tr>
<td>Hg²⁺</td>
<td>MerA</td>
<td>Reduction/volatilization</td>
<td>S. solfataricus</td>
<td>205</td>
</tr>
</tbody>
</table>

minimal Inhibitory Concentration (MIC) values range from 1 mM for S. acidocaldarias to 200 mM for S. metallicus (89), suggesting that the different sensibility towards metal ions can be bound to very different habitats.

Heavy metal ions as nickel, cobalt, mercury, copper, arsenic play their toxic role inside the cell in different ways. They can bind to thiol groups of enzymes inhibiting their catalytic activity or they can interact with other divalent cations sequestering the ion and hence inhibiting its physiological role, or they can cause oxidative stress indirectly through uncoupling of electron transport chain, depletion of glutathione (GSH), and accumulation of Reactive Oxygen Species (ROS).

To establish if a metal has a toxic effect, it has to enter inside the cell. Generally the ions transport can be primary or secondary. The main primary metal ions transport systems in Prokaryotes are ABC and Energy-coupling factor (ECF). In addition some ions can enter utilizing specific or unspecific channels (90).

The strategies utilized by microorganisms to defend themselves from toxic ions are essentially based on ATP driven efflux by membrane transporters, detoxification enzymes, and metal sequestration. Here we describe how in Archaea different toxic ions are transported inside the cell and how they respond to metal attack (Table 2). The levels of toxicity of diverse metal ions and their link with fundamental metabolic processes are also analysed. In particular: 1) iron (Fe²⁺/Fe³⁺) that is an important trace element with low toxicity; 2) nickel (Ni²⁺), cobalt (Co²⁺) and copper (Cu²⁺/Cu³⁺), that are important cofactors but at same time are toxic elements; 3) arsenic [As(III) /As(V)] and mercury (Hg²⁺) that have limited beneficial effects, but are highly toxic.

4.1. Iron

The development of oxygenic photosynthesis 2.5 billions of years ago generated a drastic change of life on the Earth. Among the effects produced by oxygen accumulation one regards the reactivity with iron. Iron is the second most abundant element in Earth’s crust and is required for the life of almost all organisms because it is involved in the major cellular functions as photosynthesis, respiration, N₂ fixation. Iron can be bound in Fe-S cluster or in heme groups to allow the proteins to play their biological functions, or it can modulate the function of transcriptional regulators (91, 92). Iron mainly exists in two different redox forms: the reduced Fe²⁺ and the oxidised Fe³⁺.

The oxidation of Fe²⁺ to Fe³⁺ makes the iron insoluble and not available for cellular biological processes; on the other hand Fe³⁺ can be extremely toxic in presence of ROS generating double damage e.g. iron release and protein inactivation (93). Through Fenton reactions (a), Fe⁵⁺ generates highly reactive and very dangerous hydroxyl radicals (OH⁻) that damage DNA, proteins and lipids.

\[
\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^+ \quad (a)
\]

Furthermore, proteins containing a cluster 4Fe-4S can be damaged during oxidative stress (93). Therefore, it is fundamental to provide the iron necessary for the growth but also to keep it in non toxic form. For these reasons iron homeostasis must be strictly controlled and different strategies have been adopted. They comprise: uptake of free or sequestered iron; intracellular iron storage by proteins such as ferritins; iron-dependent gene regulation; scavenging of free-radicals. Generally, iron transport utilizes siderophores, low-molecular-weight compounds that show a high affinity and selectivity for Fe³⁺. Siderophore-Fe³⁺-complexes enter in the cell using specific membrane bound receptors and then they are delivered in the cytosol by ABC transporters and released as Fe²⁺ upon reduction (94). Various uptake processes have been described in Bacteria and Eukaryotes, while a puzzling picture regarding Fe³⁺ uptake and reduction has been achieved in Archaea (Figure 2).

Inside the cell iron can be stored in different ways that involve mainly iron storage proteins as ferritins and haem-containing bacterioferritins, and DNA binding protein from starved cells (Dps). Ferritins and haem
containing bacterioferritins are composed by 24 identical subunits assembled in a spherical protein with a central cage that acts as iron reservoir; it can host at least 2000-3000 iron atoms in oxidized ferric form. These proteins take the iron in soluble form and store it in the protein cage as oxidized ferric form. The oxidation requires a ferroxidase centre in each subunit.

Dps, a dodecameric protein, can accommodate about 500 iron atoms. Twelve ferroxidase sites are present in the dodecamer, two between each dimer. In several Dps iron has been found bound in these sites characterized by conserved histidine and carboxylate residues. Dps monomers are small proteins with a MW about 22 kDa, with a folding of compact four-helix bundle. The fold is essentially similar to that of ferritin and bacterioferritin, with a common ancestor (95, 96). Despite the fold conservation with ferritin and bacterioferritin, Dps-like (Dpsl) proteins exhibit also a variety of activities: they protect from oxidative stress and binds DNA aspecifically to protect from HO• damage caused by reox stress (97). Furthermore, it was shown its preference to oxidize Fe2+ in presence of H2O2; indicating a preferential role as iron scavenging and DNA shielding against OH•. To date, over a thousand of putative Dps-like proteins have been identified and about 3% have been found in Archaea.

Recently, Dpsl proteins were characterized from *S. solfataricus*, *P. furiosus* and *H. salinarum* (98-100). Dpsl from *S. solfataricus* maintains the 12-mer organization and a N-terminal extension that mediates the interaction with DNA; Dpsl possesses a different ferroxidase center, located in the four helix bundle monomer, composed by two iron ions coordinated with two histidine and four acidic residues (95, 101). In addition, a pair of cysteine residues (Cys101 and Cys126), juxtaposed between the exterior surface and the channel of the ferroxidase center, can form a disulfide bond. It is possible that such a disulfide bond plays a structural role but a possibility that cysteine residues could be involved in peroxidase activity through the redox cycle of reduced and oxidized forms must be investigated. Like peroxiredoxin (102), SsDpsl could be oxidized by H2O2 forming a disulfide bond, that is reconverted in thiolic form by an electrons redox cascade. As hypothesized by Maaty et al. (103) from “omics” results for *S. solfataricus*, Dpsl together with superoxide dismutase (Sod) and peroxiredoxin could constitute a stressosome complex that would act in a coordinated way to remove ROS.

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The iron metabolism has been investigated in extreme acidophiles *Ferroplasma acidiphilium* spp revealing proteins with a higher iron content (104). This feature could be linked to a role of iron in protein stabilization in a moderately acidic cytoplasm (105). However, the high iron concentration inside the cell makes it highly exposed to oxidative damage. As a consequence, these microorganisms should be equipped with an array of putative antioxidant enzymes, proteins for iron storage and putative proteins involved in iron transport (105). In *H. salinarum* under low-iron growth conditions, no siderophores were determined in culture supernatants, nonetheless various xenosiderophores can be utilized to transport iron across the membrane; however which is the transport system involved and how the iron is reduced must be still clarified (106). In *A. fulgidus* a ferric reductase was identified and its structure solved. Unfortunately, it is still unclear, if the enzyme is involved in the assimilation of iron or in the dissimilatory reduction (107-109).

4.2. Nickel and Cobalt

Nickel and cobalt cationic forms (Ni2+ and Co2+) are essential nutrients for microorganisms playing a key role in the metabolism. These metal ions are cofactors of numerous proteins: nickel is fundamental for various metalloenzymes as, NiFe-hydrogenase, carbon monoxide dehydrogenase (Ni-CODH) and NiSod, involved in detoxification of superoxide radical (O2•-). On the other hand, cobalt is mainly found as component of Vitamin B12 (110). To date, the mechanisms of nickel toxicity are poorly understood and possible mechanisms have been suggested to explain the damage of this ion: i) the essential metal of metalloproteins is replaced by nickel, ii) enzyme inactivation or indirect inhibition of oxidative stress response. (111). Cobalt toxicity in E. coli derives from completion with iron on Fe-S clusters, or on protoporphyrin IX of cytochrome and from sulfur assimilation (112). The two ions can be transported both by active ATP driven
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Figure 3. Nickel and cobalt uptake and detoxification systems. (●) Ni, (○) Co, (□) NiCoT transporter, (●) ECF transporter, (□) ABC transporter, (○) P₁bATPase.

system and by chemiosmotic gradient. In Archaea three families of nickel and cobalt transporters are identified: NiKABCDE, Nik/CbiMNQO belonging to ABC and ECF transporters family, respectively (113), and NiCoT belonging to a family of secondary metal transporters (114) (Figure 3).

NikABCDE system, likely to ABC cassette system, couples ATP hydrolysis to its uptake. The system is composed by two TMDs that constitute a channel across the membrane and two ATP-hydrolyzing subunits that supply energy for the transport; in addition, a soluble cytoplasmic protein with high affinity to substrate binds and delivers the ion to a transmembrane protein. Putative transporters belonging to the NikA BCDE family were found in the genomes of M. acetivorans, M. barkeri and M. mazei (1, 115).

Nik/CbiMNQO belongs to the ECF family of transporters. In particular it is constituted by three functional components: S, T and A units (113). S unit is constituted by a hetero-oligomer that binds the substrate and it is encoded by nik/cbiM and nik/cbiN. NickM and CbiM have seven TMDs with a conserved N-terminus containing a His residue in position 2, essential for the transport (113). Despite the large variations in primary structure, NikN and CbiN have similar three dimensional structures. In the Co²⁺ transport system CbiM is larger than CbiN and both units are essential for the transport (116). NikM and NikN are often found fused in a single protein or can be replaced by NikK and NikL (113).

The T component is constituted by a conserved transmembrane protein encoded by nik/cbiQ. Nik/CbiQ shows the classical Walker A and B motifs for ATP binding (117). Finally A unit is formed by a couple of ABC ATPases encoded by nik/cbiO. Nik/CbiO is a cytoplasmic ATP-binding protein that interacts with the cytoplasmic loop of Nik/CbiQ.

Comparative and phylogenetic analyses were performed on 400 microbial genomes. Using SEED comparative genomics platform various ECF transporter families were identified and in 39 archaeal genomes nik/cbiMNQO homologues were found (113, 117, 118). The major differences with the ABC system are represented by the lack of an extracytoplasmic solute binding protein and a different subunit assembly.

NiCoT is a secondary transporter of nickel and cobalt also diffused in eukaryotic organisms; it is characterized by eight TMSs and supports high-affinity
The archaeal response to toxic compounds

uptake (114); homologues are present in the thermoacidophilic Archaea Sulfolobus solfataricus and Thermoplasma acidophilum (1, 115).

In Archaea, the control of nickel and cobalt homeostasis occurs through two different mechanisms: the first is based on the regulation of genes that encode the nickel transporters by the transcriptional repressor NikR and the second regards the active efflux driven by members of the subfamily of P-ATPase named Pnt-type-ATPase.

In E. coli the nikABCDE operon is tightly regulated: in anaerobic conditions it is regulated by fumarate and the nitrate reduction regulator (FNR), whereas in presence of excess of nickel its transcription is repressed by NikR (119). NikR has been crystallized and characterized in the anaerobic archaeon Pyrococcus horikoshii (120, 121). The overall structure is a homotetramer with a central Metal Binding Domain (MBD) and two flanking dimeric ribbon-helix-helix (RHH) domains that bind the DNA. Each MBD contains low and high-affinity nickel binding sites allowing a fine sensing of metal concentration from pico- to nanomolar ranges of concentration (120-122).

Orthologs of E. coli NikR were found in the genomes of different prokaryotes (115). In particular, genome analyses of methanogenic Archaea showed more than four copies of nikR and multiple copies of putative nickel transporters, suggesting the importance to safety nickel homeostasis. Phylogenetical analysis showed that NikR repressors are clustered both in a large group including proteobacteria and Archaea and a smaller group comprising Pyrococcus spp. and Thermococcus kodakarense. NikR binding sites have been also identified in prokaryotic genomes; they have been grouped in four consensus sequence, two of which are present in Archaea. All sequences contain the same palindromic structure and a distance of 13-14 base pairs, between the two half sites. Using the identified consensus sequence, 28 bacterial and 14 archaeal transporters belonging to NiCoT, NiABCDE and NikMNQ transporters have been identified and repression by NikR has been predicted (115).

The second defence system utilizes a subgroup of P-ATPases: the Pnt-type-ATPase. This pump is involved in the efflux across membrane. Pnt-type-ATPases are present in all kingdoms of life and have been identified in most archaeal genomes. The structure and function of Pnt-type-ATPases for the export of different metals ions were elucidated in different bacteria, plants and fungi (122).

4.3. Copper

Differently by nickel and cobalt that have a medium toxicity, copper is highly toxic for the cell when outside physiological range. Copper catalyses, in Fenton reaction, ROS production (123, 124), it can bind with high affinity to His, Cys and Met residues inactivating the proteins (125, 126) or can damage the iron-sulfur clusters (127). At the same time copper is an important transition element used by cells as cofactor of proteins/enzymes involved in a wide range of biological processes e.g. oxidative phosphorylation and antioxidant defence. Likewise for the other ions described above, every organism also possesses systems to maintain and to regulate the homeostasis of this metal. The different level of copper resistance depends on the environments in which the microorganisms live. For example, E. coli can grow in the presence of 1 mM copper while the acidophiles as F. acidarmanus or S. metallicus colonizing habitats with extreme metal contaminations can grow at copper concentration >200 mM (125).

In Archaea the homeostasis of copper involves mainly two systems: copper efflux and metal sequestration (128) (Figure 4).

Copper efflux is driven by Pnt-type ATPases. Those characterized showed high structural stability and different selectivity with respect to Cu⁺ and Cu²⁺, the two different oxidation states, (122). Archaeal genomes analyses have allowed the classification in two different subgroups: Cu⁺-ATPase and Cu²⁺-ATPase (122).

In the archaeon A. fulgidus the two energetic pumps are named CopA and CopB, able to transport Cu⁺ and Cu²⁺ respectively (122, 129). A. fulgidus CopA has been extensively characterized: it consists of eight TMD, an A-domain between helices four and five, an ATP-Binding Domain (BD) between helices six and seven, and a soluble MBD at the N-terminus (N-MBDs). Cu⁺ isn’t transported in its hydrated form to the ATPase subunit but the metal ion is previously bound to the metallochaperone CopZ and then transferred to the N-MBD. The N-MBD binds copper through a conserved CXXC motif determining an allosteric effect on protein conformation that affects the turnover rate of the enzyme (130). The metal ion is then transferred to TM-MBS required for enzyme phosphorylation and for consequent metal translocation (131-134). The residues responsible of ion translocation are two Cys residues in helix six, Asn, Tyr in helix seven and Met, Ser residues in helix eight. In addition A. fulgidus CopA has a MBD at C-terminus, which binds with high affinity Cu⁺, and characterized by a CHHC motif that was only found in one metallochaperone homolog from Thermosipho melanesiensis BI429. Furthermore C-MDB interacts both with ATP-BD and A domain suggesting a more complex role in vivo of CopA (130).

CopB is rich in His residues that could determine the enzyme selectivity versus Cu²⁺. The two putative MBSs, in particular the Cys-Pro-His sequence in TM-MBS of helix 6 and the His 17 residue in the N-MBD, could influence the enzyme specificity and be involved in the turnover, respectively (129).

Copper regulation was studied in various bacteria as cyanobacteria (135, 136), E. coli ( 137) and in the Gram-positiver bacterium Enterococcus hirae (138, 139). The operon for copper resistance comprises copY, copZ, copA and copB. copA and copB encode the copper ATPases involved in the uptake and export of Cu⁺, respectively (138); CopY is a transcriptional regulator and CopZ is a metallochaperone that acts as intracellular copper driver. Inside the cell, Cu⁺ is transferred from CopA to CopZ that delivers Cu⁺ to the dimeric CopY repressor.
CopY is a Zn containing homodimeric repressor that binds to the copYZAB repressing the transcription (140). In presence of Cu⁺ CopY releases the zinc, bounds copper ions, looses affinity for its own promoter, and derepresses the operon transcription (138, 141).

Using comparative genomics the copper resistance gene cluster was identified in various Archaea: in *F. acidarmanus* Fer1 strain, in P2 and 98/2 strains of *S. solfataricus* and in *S. metallicus*.

*F. acidarmanus* Fer1 can grow at high level of copper concentration (~300 mM) indicating high metal-resistance. In this microorganism, copper homeostasis is controlled by a putative transcriptional factor CopY that regulates the expression of a putative metallochaperone (*copZ*) and P-type ATPase (*copB*) that are co-transcribed. CopB shows high similarity with both copper uptake and copper export ATPases (125). Upon metallic stress, five of twenty-one proteins associated to protein stability are up-regulated in *F. acidarmanus* Fer1, suggesting that they could contribute to limit cellular damage (125).

In *S. solfataricus* two P-type ATPases responsible for copper transport have been identified: CopB (Sso2896) and CopA (Sso2651). CopB catalytic domain is characterized by a phosphatase domain (CopB-A), an ATP-binding and phosphorylation domain (CopB-B) and a heavy metal binding domain (CopB-C). The partial alignment of CopB with *E. coli* and *E. hirae* CopA showed the conservation of identical residues in these domains. Furthermore, the capability of CopB-B to hydrolyze ATP, suggests its belonging to the P-type ATPase family (142).

Similarly, a gene copper resistance cluster was also identified in *S. solfataricus* P2 and a molecular characterization was obtained (143). *copR* (Sso2652) encodes the transcriptional regulator, *copT* (Sso10823) encodes the metallochaperone and *copA* (Sso2651) encodes the P-type ATPase. All these proteins have the signature of TRASH domains characterized by the cysteine motif C-Xaa19-22-Caa3-C predicted to be involved in copper sensing, trafficking and resistance (143-145).

Differently from *S. solfataricus* P2 strain, 98/2 shows a higher resistance to copper. Like the P2 strain, *S. solfataricus* 98/2 copRTA operon encodes a transcriptional regulator, a copper binding protein and a P-type ATPase. In order to preserve internal copper homeostasis CopT and CopA levels are maintained through *copRTA* constitutive expression from the same promoter upstream *copR*; in the presence of high copper concentration, transcription from a second promoter upstream *copTA* is induced to remove the copper excess by CopA mediated efflux (143, 145). Sequence analysis in both *S. solfataricus* strains showed the identity of *copR*, *copT* and promoter sequence upstream *copTA*. It has been suggested that additional trans-acting factors could influence the different copper sensitivity of the two strains.
Copper resistance in *Sulfolobus* spp has also been related to copper sequestration mediated by polyphosphate (polyP). PolyP is a polymer of hundreds of orthophosphate linked by phosphoanhydride bonds whose biosynthesis is catalysed by polyphosphate kinase (PPK) (146) and degradation by an exopolyphosphatase (PPX) (147). In Bacteria a model was proposed in which intracellular cations regulate the activity of PPX releasing Pi from PolyP; the Pi-metal complex is then transported through Phosphate inorganic transport (Pit) outside the cell. In Archaea, polyP accumulation has been described only in *Methanosarcina* spp and in *S. acidocaldarius* (148, 149); pit–like genes have not been found in archaeal genomes (151). Recently, in *S. metallicus*, Remonssellez et al. (128) correlated the increase in copper concentration not only to a decrease in the level of polyP, but also to an increase of both PPX enzymatic activity and phosphate efflux, suggesting the existence of a new system of copper detoxification through metal sequestration.

4.4. Arsenic

The arsenic can be found as arsenite, As(III), or as arsenate, As(V). At physiological levels As(III) and As(V) can be involved in the respiratory chain playing a completely different role, indeed while As(III) could function as an electron donor at the start of a membrane respiratory chain, As(V) functions as a terminal electron acceptor for an anaerobic respiratory chain (152-158). As(V) is a structural analog of phosphate and can inhibit phosphorylation processes by producing unstable arsenylated derivatives. Both in Archaea and Bacteria As(V) can enter cells via two phosphate transport systems: phosphate specific transport (Pst) and Pit (1). Pit system uptakes phosphate and As(V), at similar rates, whereas the Pst is highly specific for phosphate. Considering that As(V) is the thermodynamically favourable form of arsenic under aerobic conditions (159-161), it is likely to be the most common form of arsenic in many environments and in addition it is less labile and toxic than As(III) (159). As(III) results toxic to cells because of its ability to bind to essential sulphydryl groups of proteins and diithiols such as glutaredoxin. Differently from As(V), As(III), due to its unionized form at neutral pH, can passively move across the membrane bilayer or be transported by a carrier protein similar to those that transport unionized organic compounds.

The detoxification system involves the reduction of As(V) in As(III) by the cytoplasmic ArsC-type reductase (EC 1.20.4.1) and then it is pumped directly out of the cell by the ArsB protein (Figure 5).

The genes involved in the detoxification are generally clustered in the operon *arsRCB*: *arsR* coding the arsenite-responsive transcriptional repressor controlling basal levels of *ars* expression (162), *arsC* encoding the arsenate reductase (a) and *arsB* coding an arsenite-specific transmembrane pump (159, 163, 164) (EC 3.6.3.16) (b)

\[
\text{As(V)} + \text{glutaredoxin/thioredoxin} \rightarrow \text{As(III)} + \text{glutaredoxin/thioredoxin disulfide} + \text{H}_2\text{O} \quad (a)
\]

\[
\text{ATP} + \text{H}_2\text{O} + \text{As(III)}_{\text{in}} \rightarrow \text{ADP} + \text{phosphate} + \text{As(III)}_{\text{out}} \quad (b)
\]

ArsR belongs to a metalloregulatory transcriptional repressors family (SmtB/ArsR), and acts as metal-sensitive DNA binding repressor that controls expression of various metal detoxification genes (165, 166). To define the DNA sequence recognized by this family multiple sequence alignments of putative operator sites and corresponding winged helix-turn-helix (HTH)

![Figure 5. Arsenate reduction detoxification pathways.](image)

- (A) As(V), (B) Pit, (C) Pst, (D) ArsC, (E) As(III), (F) ArsM, (G) TMA, (H) ArsB.
motifs were used. Using a web application, the Prokaryotic Inter-Genic Exploration Database (PIGED; http://bioinformatics.uwp.edu/~PIGED/home.htm), 60 SmtB/ArsR DNA binding sites or operators, linked to metal detoxification genes, have been predicted among 230 annotated prokaryotic genome sequences (165). Nine archaeal SmtB/ArsR and associated regulons have been predicted by the computational analysis and the binding activity of one of these proteins from *M. acetivorans* C2A MA4344 was confirmed by biochemical studies (165). In addition, the crystal structure of the product of the *ph1932* from *P. horikoshii* OT3 was solved and it resulted to be an archaeal ArsR (167). In detail, the C-terminal domain is responsible for dimerization and forms a unique hat-shaped helix-bundle; the inside of the hat is a possible effector-binding site of this protein.

Two different bacterial classes of ArsC have been found (152, 168). The first of them is ArsC discovered in *Staphylococcus aureus* pl258 plasmid and in other Gram-positive bacteria. In pl258 ArsC, the reduction of As(V) involves three Cys residues (158) in a disulfide cascade coupled with Trx, Trx reductase and NADPH as recycling system (169). It is different from the enzyme discovered in enteric bacteria such as *E. coli* (i.e. from plasmid R773) which has a single active site Cys residue and requires reduced glutathione (GSH) and Grx during the catalytic cycle (170). Both these classes show a low molecular weight, 131 amino acid residues for *S. aureus* and 141 for *E. coli*, respectively and are monomeric cytoplasmic enzymes (158).

A detailed study of the phylogenetic distribution of *arsC* has been performed showing a widespread presence in all three kingdom of life, suggesting that arsenate reductase is an evolutionarily old enzyme that could be present either in the last universal common ancestor or after the Bacteria/Archaea divergence through early HGT events (159).

Recently the structure of ArsC from *A. fulgidus* Dsm4304 (PDB code 1Y1L) was solved within a structural genomic project (158), showing a Trx fold as other ArsC (171-173). Although the enzyme presents low sequence identity with pl258 ArsC (about 15 %) their structures result to be similar conserving the three catalytic Cys residues. Structurally, it seems evident that the *A. fulgidus* ArsC, and likely the other archaeal members, could be linked to the Trx-coupled system, but this functional prediction needs to be confirmed by biochemical analyses.

ArsB can function alone or with another As(III)-stimulated ATPase pump. Indeed some microbial strains possess additional genetic determinants, often found on plasmids that confer arsenic resistance. *arsA*, encoding As(III)-stimulated ATPase, is allosterically activated by As(III) and it functions as catalytic subunit of ArsB (174) increasing As(III) extrusion (175). It is characterized by the signature sequence DTAPTGHTIRLL (176) and it consists of two homologous modules designated as the A1 and A2 loops, a clear result of an ancestral gene duplication and fusion (163, 177, 178); *arsD* encodes an arsenic chaperone that transfers As(III) to ArsA regulating *ars* expression (178-180). *arsH* has been identified but has an uncertain function (181).

In other Archaea like *F. acidarmanus*, *Metallophaga sedula*, *S. acidocaldarius* and *S. solfataricus* the lack of some genes involved in the arsenic resistance has been highlighted (182). In particular Gihring et al. investigated the arsenic resistance in arsenic-hypertolerant *F. acidarmanus* Fer1 (183). In detail, genes encoding putative ArsR and ArsB homologues were found located on a single operon. A gene encoding putative ArsA, located apart from the *arsRB* operon, was also identified. Arsenate-resistance genes encoding proteins homologous to the ArsC were not found, if indicating that additional unknown arsenic-resistance genes exist for arsenate tolerance (183). Later Austin et al., performing transcriptional analyses of the putative *arsR* and *arsB*, demonstrated that these genes are co-transcribed, and expressed in response to As(III), but not As(V) (184).

Furthermore in silico studies were performed using motif-based searches for identification of genes involved in As(III) resistance (182) suggesting the presence of *arsB* homologues in other two Archaea *T. acidophilum* and *T. volcanium*, but no homologues were found for *S. solfataricus*, *T. kodakii* and in *S. acidocaldarius* strain BC (155).

These Archaea may possess an unpredicted As(V) reductase that does not have significant sequence homology to other ArsC proteins or, finally, completely novel resistance mechanisms may be adopted (182).

An example of a different mechanism of arsenic resistance is represented through methylation and subsequent volatilization of As(III) involving the methyltransferase ArsM (EC 2.1.1.137) (c) and (d)

\[
\text{S-adenosyl-L-methionine} + \text{arsenite} \rightarrow \text{S-adenosyl-L-homocysteine} + \text{methylarsonate}
\]

\[
\text{S-adenosyl-L-methionine} + \text{methylarsonate} \rightarrow \text{S-adenosyl-L-homocysteine} + \text{dimethylarsinate}
\]

ArsM catalyzes the formation of a number of methylated intermediates from As(III) with a consequent loss of arsenic, from both the medium and the cells. Because ArsM homologues are widespread in nature, this microbial-mediated transformation is proposed to have an important impact on the global arsenic cycle (185). In 1971 McBride and Wolfe already observed in *Methanobacterium* the production of dimethylarsine (186). Other methanogenic Archaea *M. bryantii*, *M. formicicum*, and *M. Barkeri* were reported to produce volatile forms of methylated arsenic (1, 187).

Also in the genome sequence of *Halobacterium spp*. strain NRC-1 genes homologous to those responsible
for conferring resistance to arsenic have been annotated (188). These genes occur on both the large extrachromosomal replicon pNRC100 (arsADRC, arsR2 and arsM), and on the chromosome (arsB). In particular on pNRC100 were identified: two additional ars genes located near arsADRC, a second arsR gene (arsR2) and another gene, named arsM encoding for a putative methyltransferase homologous to a recently identified mammalian As(III)-methyltransferase (188). The role of all of these ars genes in arsenic resistance has been studied genetically by gene knockout producing sensitivity to As(III). The authors hypothesize that ArsM could methylate intracellular arsenite, creating a concentration gradient outward representing an alternative resistance mechanism by lowering the intracellular arsenic concentration (188).

4.5. Mercury

Mercury is the heavy metal with the highest toxicity. It can be found in different forms such as elemental (Hg⁰), ionized (inorganic salts Hg²⁺ and Hg⁺), organic (typically as methylmercury) or sulfidic (cinnabar) (189). Mercuric ions (Hg²⁺) and methylmercury are major, human-generated, toxic contaminants present on the earth. Bacteria can adopt different strategies to react to mercury exposure, providing a real mean of bioremediation by taking up these compounds and reducing them to volatile, non-toxic, elemental mercury (Hg⁰) (190) (Figure 6).

Three types of mercury/ methylmercury transporters have been previously identified in Bacteria and described in detail in the review by Yamaguchi et al.: MerC, MerT and MerF (190). Each of these has distinct topologies in particular MerC is characterized by a four TMS, while most MerT and MerF proteins have three and two TMSs, respectively. MerC has been shown to bind Hg²⁺ ions, but the mode of transport remains unknown while MerT transports Hg²⁺ into the cytoplasm directly interacting with the periplasmic protein MerP (191). MerP has MBDs homologous to enzymes, chaperones and heavy metal-transporting P-Type ATPases indeed it shares the so named metal binding loop GMTCX and the typical ferredoxin-like fold (190, 192). In addition, it has been observed that the binding loop undergoes a conformational change in response to metal binding (190). Regarding the last known transporter MerF, its NMR structure was determined and it showed a helix-turn-helix core hairpin loop with the two vicinal pairs of Cys residues, involved in the transport of Hg²⁺ across the membrane, exposed to the cytoplasm (193). Differently from MerTs, MerF proteins do not function coupled with MerP (190, 191). MerP homologues are absent in eukaryotes but few examples were observed in Archaea, while homologues of merT, merF and merC genes were not found in Archaea. This observation conducted Yamaguchi et al. to hypothesize that these kinds of mercuric resistance systems appeared late in the evolution, at least after the divergence of Archaea and Eukaryotes from Bacteria (190). Nonetheless, they utilized the ubiquitous MBD to derive the MerP homologues.

Once entered in the cells, Hg²⁺, deriving also by the protonolysis of the organomercurials catalyzed by the organomercurial lyase (MerB) (194), must be converted into the volatile Hg⁰ (1, 189) to be detoxified. The strategy of detoxification better characterized is mediated by the products of mer operon. In detail merTPCAD operon encodes a group of proteins involved in the binding, transport, and reduction of mercury. As previously cited MerP binds Hg²⁺ and transfers it to MerT. MerT transports mercuric ion into the cytoplasm where the NADPH-dependent flavoprotein mercuric reductase (MerA) (EC 1.16.1.1) reduces Hg²⁺ to Hg⁰ that is released from the cell (a)

\[
\text{Hg}^{2+} + \text{NADPH} \rightarrow \text{Hg}^0 + \text{NADP}^+ + \text{H}^+ \quad (a)
\]
The archaeal response to toxic compounds

MerA belongs to the pyridine nucleotide disulfide oxidoreductase family and its core domains share significant sequence identity in the redox active disulfide/dithiol active site, NAD(P)H and FAD binding sites with glutathione reductase, trypantothione reductase, and lipoamide dehydrogenase (LPD). In particular, Pullikuth and Gill (195) showed by means of phylogenetic reconstructions a common ancestor between LPD and MerA. A peculiar characteristic of MerA is the presence of a short C-terminus extension containing a Cys pair which is involved in delivery of Hg^{2+} to the inner redox cysteine active site (196, 197).

Phylogenetic reconstructions of MerA indicated its origin in a thermophilic bacterium following the divergence of the Archaea and Bacteria with a subsequent acquisition in Archaea via HGT (197). The majority of the microorganisms possessing MerA homologues live in oxygenic environments suggesting that the presence of MerA is correlated with oxygenation of the biosphere (197).

In Bacteria the expression of the mer operon is regulated by MerD and MerR (189, 198). Both merR and merD gene products bind to the same operator DNA. In the absence of mercury, MerR binds and sends the DNA interacting with the RNA polymerase keeping in an inactive state and consequentially repressing transcription (189, 199-201). In the presence of mercury, MerR undergoes a conformational change favouring mer operon transcription. The coupled action of MerD and MerR was hypothesized suggesting a displacement of MerD on Hg-bound MerR from the mer operator to allow new synthesis of metal-free MerR able to switch off the induction of the mer genes when the external mercury is exhausted. A protein phylogenetic analysis identified two orthologs of merA and merR in the genome of S. solfataricus. The studies conducted by Schelert et al. (189) represented the first report of an archaeal heavy metal resistance system. The archaeal system proved to employ a MerR protein that acts as a negative transcriptional regulator of merA expression, indeed a merR disruption mutant exhibited elevated Hg(II) resistance and at the same time a constitutive synthesis of the merA transcript. Further findings indicated that the toxicity of mercuric ion in S. solfataricus is in part the consequence of transcription inhibition due to transcription factor B inactivation (202). It has been observed that Hg^{2+} interacts with the regulator MerR (203). Two additional Hg^{2+} inducible S. solfataricus genes, merH and merI located on either side of merA, were identified by Schelert et al. (203) and constituted the merHAL operon. While a chaperone function for mercury immobilization has been suggested for MerH, the role of MerI is still unknown. Different experiments demonstrated that MerR remained bound to DNA, thus exerting a protective effect over the preinitiation transcription complex. In addition MerR contains a motif resembling a distant ArsR homolog (203). Indeed both ArsR and MerR present the wHTH DNA binding domain (203, 204), but constitute two distinct families. The significant difference between these two families resides in their response to metal ligands; ArsR dissociates from DNA while MerR remains bound. The ArsR motif in MerR, however, is slightly different because it lacks the conserved Cys residue required for ligand-induced DNA release. Despite this divergent feature, the S. solfataricus MerR protein could be the first example of Hg^{2+}-responsive ArsR family member.

Another form in which mercuric is found is represented by the methylmercury, a very potent neurotoxin; in Bacteria, the transformation occurs anaerobically, is directly coupled to sulfate respiration and depends on the presence of constitutive and induced methyl transferase pathways (1, 205). Very little is known concerning mercuric methylation in Archaea, making exceptions for some methanogens such as M. maripadulis able to methylate Hg^{2+} if grown together with Desulfovibrio desulfuricans (1, 206). Bacteria are capable of demethylating methylmercury. This process is regulated by an inducible mer operon and serves as a detoxification mechanism in polluted environments. In Archaea, nothing is known on detoxification by demethylation.

5. CONCLUSIONS

All ecosystems on Earth are submitted to pollution. In particular, a wide range of pollutants and heavy metals are actively biodegraded (mineralized or transformed) in extreme environments characterized by low or elevated temperatures, acidic or alkaline pH, high salinity or high pressure.

Since Archaea often represent the majority of the microbial community in these environments, it is expected that these microorganisms play a leading role in the biogeochemistry of the elements as well as in the degradation of xenobiotics. Nevertheless, the overall degradation mechanisms and the enzymes involved are not completely elucidated so far.

To better understand these processes global approaches such as whole genome-transcriptome-proteome analyses and metagenomics are needed. This could reveal enzymes that can direct novel chemical reactions and/or new catabolic aptitudes as well as new archaean species or synthropic communities responsible for bioremediation. From a more applied point of view, this knowledge will be critical for developing strategies aimed to remediate polluted habitats.

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