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SENSING AND ADAPTING TO ENVIRONMENTAL STRESS: THE ARCHAEAL TACTIC

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1. ABSTRACT

Archaea occupy a considerable diversity of niches encompassing extreme environments with extreme of pH, salinity and temperature that cannot be tolerated by other forms of life. Survival and colonisation requires the capacity to sense, and adapt to environmental change. In this review we consider the issues of adaptation to environmental stresses, in particular the mechanisms that might be employed by different Archaea to respond to the specific challenges of their particular niche. We lay emphasis on the strategies adopted to respond to oxidative and chemical stress. In particular, this paper reviews major key points in the generation of reactive oxygen species in Archaea, defense mechanisms and genetic responses to oxidative stress. Finally, we discuss complex biological response mechanisms to chemical damage with particular attention to detoxification from metals and drugs.

2. INTRODUCTION

The third domain of life, Archaea, encompasses organisms capable of growing under extreme conditions of pH, salinity, temperature (1). During evolution, each organism thriving in hard conditions has acquired genotypes that fit for their adaptation and survival in that particular niche.

Many external agents of biological, physical or chemical nature, like temperature, ion concentration, oxygen availability, osmolarity, metal and pollutant concentrations, can determine a further stress for the cell and hence it appears critical to sense and to adapt to rapid and subtle changes in the environment.

The molecular mechanisms responsible for response to environmental stress have been only partially elucidated in some Archaea by identifying proteins/enzymes or pathways directly involved in counteracting the effect of the stress agents (2) (figure 1). By homology to better established bacterial counterparts, it has emerged that the strategies adopted correlate with the organism's lifestyle. For example, anaerobic euryarchaea, like *Pyrococcus furiosus* or *Archaeoglobus fulgidus*, have developed alternative or redundant pathways with unique and interesting features both at molecular and evolutionary levels to protect from oxidative damage (3, 4). The relevance of such peculiar means is demonstrated by the fact that these systems are so finely regulated.

The recent intense development in genome sequencing and the completion of several sequencing projects of Archaea now available in specific data-bases (5), allowed *in silico* analyses focused in identifying complex regulated biochemical pathways. This new tool, alongside with comparative genomics and functional genomic studies, can help in unravelling how these microbes survive, adapt to their environment and lifestyle,

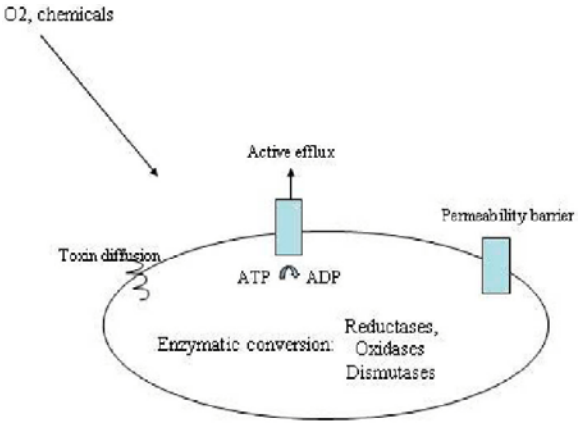

Figure 1. Schematic diagram of environmental stress resistance mechanisms in Archaea.

and recycle disparate organic molecules in the environment. In this review we will focus on the general mechanisms faced by Archaea confronting environmental damage and describe the currently available data on means employed to guarantee their survival; particular attention will be paid on oxidative and chemical stresses.

3. OXIDATIVE STRESS

The evolution of oxygenic photosynthesis about 2.4 bilion years ago led to one of the greatest threats to have challenged the microbial world: the accumulation of ever increasing levels of oxygen gas in the atmosphere. Although this global change allowed the evolution of more efficient forms of respiration using oxygen as a terminal electron acceptor, it also created a strong selective pressure for enzyme that could protect cells against reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide which affect all macromolecules (DNA, lipids and proteins). As a result, organisms either became restricted to niches free from oxygen (anaerobes) or acquired protective mechanisms, including the synthesis of superoxide dismutase (SOD), catalase and alkyl hydroperoxide reductase (Ahp), small proteins like thioredoxin and glutaredoxin, and molecules such as glutathione. Most aerobes have multiple, often overlapping pathways for detoxifying ROS. These multiple enzymes may differ in the time of their expression or regulation. For example, many bacteria induce the expression of catalase or other protective enzymes during the transition from exponential to stationary phase, presumably as an adaptation to protect the genome and other essential cellular components against oxidation.

3.1. Thioredoxin/glutaredoxin system

Thioredoxin and glutaredoxin are small soluble proteins capable of catalyzing thiol-disulfide redox reactions. The two cysteines of the conserved active site (CX_1X_2C) form a disulfide bond after reduction of exposed disulfides in a variety of substrate proteins. The reduced form is regenerated by the flavoenzyme thioredoxin/glutathione reductase which in turn is kept reduced by NADPH (6). Thioredoxin can help in the cellular defence against oxidative stresses, such as exposure to hydrogen peroxide or hydroxyl radicals and when upregulated or overexpressed, protects against oxidative stress (7). Reactive oxygen species can oxidize cysteine or methionine sulfoxide. Such reactions often inactivate cellular proteins. Reduction of both of these products may be accomplished in microbial systems by thioredoxin and thioredoxin reductase. Another general and important role in cell signalling and in the defence against oxidative damage is the function of thioredoxin as an electron donor for the ubiquitous family of thioredoxin peroxidases or peroxiredoxins (at least six members in mammalian cells) that catalyze the reduction of H_2O_2. It was demonstrated that at least one of the thioredoxin peroxidases (human TPxII) when overexpressed in cells could inhibit induction of apoptosis by decreasing H_2O_2 levels, thereby constituting a thioredoxin-dependent regulatory step of apoptosis upstream that of Bcl-2 (8). Archaea have proteins with thioredoxin/glutaredoxin motifs suggesting the ubiquity of this system in nature. Most of these organisms are anaerobes, have extraordinarily heat-stable proteins and use ingenious strategies for stabilizing nucleic acids and other macromolecules *in vivo*.

The first protein disulfide oxidoreductase isolated from archaea is the protein from the thermophilic methanogen *Methanothermobacter thermoautotrophicum* (9). This glutaredoxin-like protein may be a component of a ribonucleotide-reducing system that is distinct from the system that uses thioredoxin or glutaredoxin. In *Methanocaldococcus jannaschii*, a thioredoxin-like protein with a Cys-Pro-His-Cys in the active site, which had never been observed in either thioredoxins and glutaredoxins, and a thioredoxin reductase homologue were identified. The thioredoxin-like protein exhibits biochemical activities similar to thioredoxin, although its structure is more similar to glutaredoxin. This protein presents the lowest value so far known among redox potentials of the thioredoxin super family (10). This indicates that the lower redox potential is necessary to keep catalytical disulfide bonds reduced in the cytoplasm. The observation that a single thioredoxin system is present in *M. jannaschii* and in *M. thermoautotrophicum* suggested that a single thioredoxin-like protein with a glutaredoxin-like structure is enough to maintain redox homeostasis in methanogens.

Guagliardi *et al.* purified a protein disulfide oxidoreductase from the hyperthermophilic archaeon *Sulfolobus solfataricus* (11). Given its ability to catalyse the reduction of insulin disulfides in the presence of dithiothreitol, the protein was named thioredoxin. The monomeric form of the enzyme has an unusual molecular mass of about 26 kDa, respect to the molecular weight observed in Trx and Glx (12kDa).

A homologous protein disulfide oxidoreductase was purified from the hyperthermophilic archaeon *P. furiosus* (*Pf*PDO) (11). *Pf*PDO shows close similarity to the *S. solfataricus* protein in molecular weight (25648 Da) and dithiothreitol-dependent insulin reduction activity. In addition, both proteins display thioltransferase activity by catalysing the reduction of disulfide bonds in L-cystine (12, 13). The *Pf*PDO primary structure does not show any

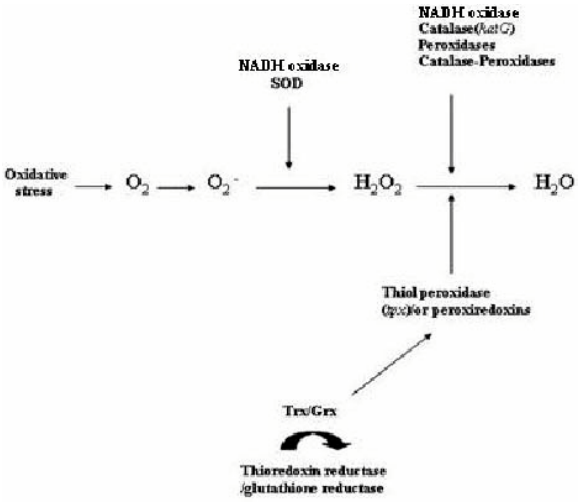

Figure 2. Components of the oxidative stress responsive mechanism (20).

overall sequence similarity to known protein disulfide oxidoreductases. Interestingly, it has two potential active sites with the conserved CXXC sequence motif. A CPYC sequence is located at the C-terminal half of *Pf*PDO, which is the conserved active sequence of the glutaredoxin family, usually located at the N-terminus.

In addition, redox protein homologue genes have been found in the genomes of other hyperthermophilic archaea. The *Pyrococcus horikoshii* genome contains a glutaredoxin-homologue gene (88% identity to the glutaredoxin from *P. furiosus*) (14). However, no gene having a sequence that is homologous to the glutathione synthesis enzyme has been found. The glutaredoxin-homologue protein directly mediates the electron transfer from a thioredoxin reductase-like flavoprotein to protein disulfide. In some obligate anaerobic bacteria systems for oxidative stress response related to some redox proteins have been found. In the case of *P. horikoshii*, the thioredoxin system described may also play a critical role in resistance to oxidative stress against traces of oxygen and for the maintenance of redox homeostasis in the cytoplasm.

One of the most important functions of Trx is the reduction of ROS, which is performed by the interaction of thioredoxin and thioredoxin peroxidases. Therefore, the study of the thioredoxin system in aerobic hyperthermophilic archaea should be informative. In the genome database of the aerobic hyperthermophilic archaeon *Aeropyrum pernix* K1, a new gene which codes for a 37 kDa protein with a redox-active site motif (CPHC) was found (15). This protein is about three times as large as the normal Trx. To understand the role of the gene and Trx/TR (thioredoxin reductase) system in aerobic hyperthermophilic archaea two genes were cloned from *A. pernix*, the first encoding a protein (APE0641) *Ap*Trx containing a redox active site motif (CPHC) and the second a thioredoxin reductase homologue protein (APE1061) (*Ap*TR). The active site motif, CPHC, which is present in *Ap*Trx is the same as that of *E.coli* DsbA, the most powerful oxidant among thiol-disulfide oxidoreductases (16). The two central residues within the active site motif play a critical role in determining the redox potential (17). Nevertheless, the redox potential of *Ap*Trx is −262 mV, which is very different from that of *E.coli* DsbA (125 mV). This indicates that amino acids other than those within the active site are also important in determining redox potential (18) and that the lower redox potential is necessary to keep catalytic disulfide bonds reduced and to cope with oxidative stress (10). The molecule of *Ap*Trx is larger than the other thioredoxin homologues in size due to an extended region at the N-terminus. This region showed no homology to sequences in the databases and the function is unknown. However, *Ap*Trx protein exhibits biochemical activities similar to classical thioredoxin. *Ap*Trx contains two extra cysteine residues, but its activity is not affected by preincubation with dithiothreitol, indicating that the activity of *Ap*Trx is not dependent on the redox state of the protein.

*Ap*TR is phylogenetically closer to the bacterial than mammalian TRs. The deduced amino acid sequence is most homologous to TR-like protein (54% identity and 73% similarity) from *S. solfataricus* and shows a relatively high homology to the TR of *E. coli* (34% identity and 54% similarity). This protein possesses three conserved motifs responsible for binding of FAD near the N-terminus (GXGXX [G/A]) and the C-terminus (GXFAAGD) and NADPH near the middle of the protein (GGGXXA) in addition to a redox active center (CSVC). Typically, enzymes of this family contain two identical subunits, each subunit containing one redox active disulfide, one mole of FAD per subunit, and conserved FAD and NADPH binding motifs. This is the first example of a functional thioredoxin system in aerobic hyperthermophilic archaea (19) (figure 2).

3.2. Peroxiredoxins

Peroxiredoxins (Prxs) are a ubiquitous family of antioxidant enzymes and are divided into three classes. Typical 2-Cys Prxs; atypical 2-Cys prxs; and 1-Cys Prxs. All Prxs share the same basic catalytic mechanism, in which an active-site cysteine (the peroxidatic cysteine) is oxidized to a sulfenic acid by the peroxide substrate. The recycling of the sulfenic acid back to a thiol is what distinguishes the three enzyme classes. Peroxiredoxins (Prxs) have received considerable attention in recent years as a new and expanding family of thiol-specific antioxidant proteins, also termed the thioredoxin peroxidases (TPx) and alkyl-hydroperoxide-reductase-C22 proteins. Prxs exert their protective antioxidant role in cells through their peroxidase activity (ROOH and $2e^- \rightarrow$ ROH + H_2O), whereby hydrogen peroxide, peroxynitrite and a wide range of organic hydroperoxides (ROOH) are reduced and detoxified. Indeed, these enzymes are truly ubiquitous having been identified in yeast, plant and animal cells, including both protozoan and helminth parasites, and most, if not all, eubacteria and archaea. The peroxidatic functions of Prxs probably overlap to some extent with those of the better known glutathione peroxidases and catalases, although it has been suggested that their moderate catalytic efficiencies ($\sim 10^5 M^{-1}s^{-1}$) compared with those of

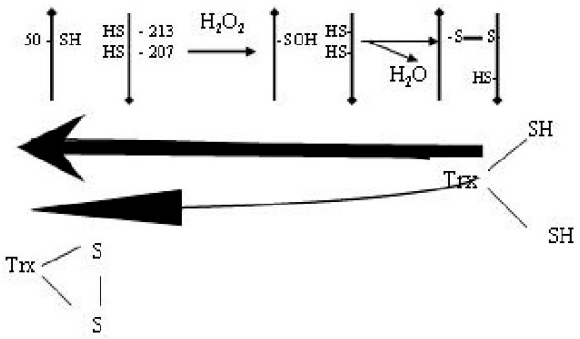

Figure 3. Proposed reaction mechanism for *Ap*TPx.

glutathione peroxidases (~$10^8 M^{-1}s^{-1}$) (21) and catalases (~$10^6 M^{-1}s^{-1}$) (22) makes their importance as peroxidases questionable. Nonetheless, the high abundance of Prxs in a wide range of cells and a recent finding that a bacterial Prx [alkyl hydroperoxide reductase C22 (AhpC)] and not catalase is responsible for reduction of endogenously generated H_2O_2 (23) argue that Prxs are indeed important players in peroxide detoxification in cells. Despite differences in quaternary structure and catalytic cycle, all three classes share the same peroxidatic active-site structure. Some bacterial and mammalian typical 2-Cys Prxs undergo redox-sensitive oligomerization, and this might be a property of typical 2-Cys Prxs in general. Prx peroxidase activity might be regulated *in vivo* by cysteine oxidation, phosphorylation and limited proteolysis (24).

In a variety of organisms from bacteria, eukarya, and archaea , >40 proteins have been found to have a similar function. The gene, which has some homology to TPx, has been found in archaea such as *Thermoplasma volcanium*, *Thermotoga maritima*, *Thermoplasma acidophilum*, *Sulfolobus tokodaii*, and *A. pernix*. A gene (APE2278) encoding the peroxiredoxin homologous protein of yeast and human was identified in the genome of *A. pernix*. (25). The gene was cloned and the encoded protein produced in *E. coli* cells. The isolated recombinant protein shows peroxidase activity *in vitro* and uses the thioredoxin system of *A. pernix* as an electron donor. These results indicate that the recombinant protein is in fact the thioredoxin peroxidase (*Ap*TPx) of *A. pernix*. *Ap*TPx forms a hexadecamer composed of two identical octamers, and the mechanism of its action appears to be identical to 2-Cys Prx. The novel oligomeric structure may represent a tactic for getting multifunctional antioxidant system and achieving hyperthermostability. The novel structural and catalytic characterization of the Trx-dependent peroxidase in aerobic hyperthermophilic archaea show that *Ap*TPx is dependent on a functional NADPH/TR/Trx system to reduce H_2O_2 *in vitro* (figure 3). Since a themostable superoxide dismutase and a thioredoxin/thioredoxin reductase system were found in the genomic sequence of *A. pernix* (26, 15) then the thioredoxin peroxidase/thioredoxin system may reflect the ancestral features of the antioxidant defense system.

Another example of peroxiredoxins in Archaea seems to be represented by a 25 kDa protein whose production was greatly increased when a sulfur- and ferrous iron-oxidizing species of *Sulfolobus* was switched from growth on tetrathionate to growth on ferrous iron. The gene encoding the protein was cloned and sequenced. The predicted amino sequence shows significant similarity to those of the alkyl hydroperoxide reductase/thiol specific anti-oxidant family of proteins that appear to be involved in responses to certain types of oxidative stress (27).

3.3. Hydroperoxidases

Hydroperoxidases are heme proteins evolved to neutralize potentially lethal ROS. The group consists of two classes of proteins: **catalases** and **peroxidases**. The former is characterized by electron pair transitions in which H_2O_2 is decomposed to O_2 and H_2O, whereas the latter is characterized by single electron transfers resulting in the oxidation of various organic compounds by H_2O_2. Along with superoxide dismutases, catalases play an important role in defending the cell against oxidative stress, and are distributed in almost all aerobic and facultatively anaerobic organisms. Catalases isolated from higher organisms are similar to each other in that they have molecular weights in the range of 225,000 to 270,000, contain four equally sized subunits each containing one ferric heme prosthetic group, show activity in a broad pH range (5 to 10.5) and are specifically inhibited by 3-aminio-1, 2, 4-triazole.

Catalases are assigned to three phylogenetically distinct groups: two groups comprised of **heme catalases** and one group of **nonheme catalases** (28). The monofunctional (or typical) catalases, which are one type of heme catalases, have been found in many bacteria, archaea, plants, fungi, and animals. They display a broad range of subunit sizes (55 to 84 kDa) and are generally tetrameric enzymes. Monofunctional catalases usually do not harbor peroxidase activity. Representative enzymes are bovine liver catalase and *Escherichia coli* HP II .

Peroxidases, like catalases, are heme enzymes, but they are monomeric proteins showing diversity in their molecular weight. Moreover, the heme iron of catalases is not reducible, whereas the heme iron of peroxidases can be reduced by dithionite.

The bifunctional **catalase-peroxidases**, the hydroperoxidases, the other type of **heme catalases**, have been found in bacteria, archaea, and fungi. Besides their difference in size (subunit size, approximately 80 kDa), they exhibit not only catalase activity but also peroxidase activity. The catalase-peroxidases are not phylogenetically related to the monofunctional catalases but rather resemble plant and fungal peroxidases. They are only similar to typical catalases in that they exhibit a tetrameric molecular weight in the range of 240,000 with equally sized subunits. However, these enzymes show properties which are distinct from those of typical catalases in that they possess narrow pH ranges for maximal activity and increased sensitivity to temperature, are inactivated by H_2O_2, and are not inhibited by 3-aminio-1, 2, 4-triazole. Despite the detection of catalase-peroxidase in diverse organisms, we do not know how universally distributed this new enzyme class may be. Both a catalase-peroxidase and a monofunctional catalase were purified from the halophilic archaeon *Halobacterium halobium* (29, 30). In *H. halobium*, it has been investigated

which of the activities of the catalase-peroxidase would function maximally as a consequence of alterations in environment. Under acidic conditions, catalase activity is greatest, whereas peroxidase activity is elevated in a shift to a basic intracellular environment. It should be noted that *H. halobium* contains another hydroperoxidase (31). This protein, like the hydroperoxidase reported before, has both peroxidase and catalase activities and is salt dependent. However, the two enzymes appear to be different in size, salt requirement, and the ability to be reduced by dithionite. This apparent redundancy might be accounted for by unique physiological substrates and therefore specific functions for the two proteins. A catalase-peroxidase was also purified from *Haloarcula marismortui* (32). The enzyme exhibits both catalase and peroxidase activities to remove the harmful peroxide molecule from the living cell and its three-dimensional structure has been solved (33). A gene encoding catalase-peroxidase was cloned from chromosomal DNA from the archaeon, *Halobacterium salinarum* too (34). In methanogenic archaea, monofunctional catalases were purified from *Methanosarcina barkeri* (35) and *Methanobrevibacter arboriphilus* (36). In (hyper)thermophilic archaea, a putative catalase-peroxidase gene was found in the genome of the obligately anaerobic archaeon *A. fulgidus* . Its gene was expressed in *E. coli*, and the recombinant product was characterized (4).

The **nonheme catalases**, which are sometimes referred to as pseudocatalases, constitute one minor catalase group and have a relatively small subunit size (28 to 36 kDa). They utilize manganese ions instead of ferric heme in their active site and are therefore also known as manganese catalases. Until now, only four manganese catalases, all from bacteria, had been purified and characterized (*Lactobacillus plantarum*, *Thermus thermophilus*, *Thermus* sp. YS 8-13, and *Thermoleophilum album*). In archaea only one **manganese catalase** has been identified in *Pyrobaculum calidifontis* VA1 (37). *P. calidifontis* strain VA1 is a facultatively anaerobic hyperthermophilic archaeon which grows optimally at 90 to 95°C and pH 7.0. Oxygen serves as a final electron acceptor under aerobic growth conditions, while oxygen can be replaced by nitrate under anaerobic conditions. Catalase activity was detected in aerobically grown VA1 cells and the catalase gene (kat_{Pc}). was cloned and sequenced. The deduced amino acid sequence shows similarity with that of the manganese catalase from a thermophilic bacterium, *Thermus* sp. YS 8-13. At present, the hyperthermophilic archaeon *P. calidifontis* VA1 is the only archaeal strain that seems to harbor a manganese catalase, and hence can be considered a valuable tool for investigating the physiological roles and evolution of (manganese) catalases. The presence of the gene in *P. calidifontis* VA1 may also represent an interesting example of lateral gene transfer.

No catalase orthologues were found in all other genomes, including those of the thermophilic archaea *M. thermoautotrophicum* (38), *M. jannaschii* (39), *T. acidophilum* (40), and *T. volcanium* (41) and the hyperthermophiles *P. horikoshii* (42) and *Pyrococcus abyssi* (http://www.genoscope.cns.fr/Pab/). Moreover, the complete genomes of the "aerobic" (hyper)thermophilic archaea *A. pernix* (43), *S. solfataricus* (44), and *S. tokodaii* (45) also do not harbor putative catalase genes in spite of having superoxide dismutases which produces hydrogen peroxide. They might withstand hydrogen peroxide stress using other peroxiredoxin systems (e.g., alkyl hydroperoxide reductase) (46).

3.4. NADH oxidase

The H_2O-producing NADH oxidases are members of the flavoprotein disulfide reductase superfamily of enzymes in the subclass usually represented by glutathione reductase (GRX). Within this GRX family, the NADH oxidases are members of the recently discovered subgroup of disulfide reductases that contain a single redox active cysteine at their active site. The three current members of this subgroup are NADH oxidase (NOX; $O_2 + 2NADH + 2H^+ \rightarrow 2H_2O + 2NAD^+$), NADH peroxidase (NPX; $H_2O_2 + NADH + H^+ \rightarrow 2H_2O + NAD^+$), and coenzyme A disulfide reductase (CoADR; CoA-S-S-CoA + NADPH + $H^+ \rightarrow$ 2CoASH + $NADP^+$). All of these enzymes contain an absolutely conserved single redox active cysteine, corresponding to Cys42 of the *Enterococcus faecalis* NPX, rather than the cysteine disulfide usually found at the active site of the disulfide reductases. NPX and NOX are believed to play a role in oxidative stress defense and in the regeneration of oxidized pyridine nucleotides for these organisms.

In several bacterial cells, the NADH formed under aerobic conditions by various dehydrogenases is converted to NAD^+ by NADH oxidase (NOX), which is considered responsible for the maintenance of the intracellular redox balance. The enzyme has been isolated and characterized from various mesophilic and thermophilic bacteria. There are two types of NADH oxidase; one catalyzes the four-electron reduction of O_2 with formation of H_2O, and the other catalyzes the two-electron reduction of O_2 to H_2O_2. The latter is found in many microorganisms including thermophilic bacteria.

A NAD(P)H oxidase (*Ss*NOX38) has been isolated from the archaeon *S. solfataricus* whose primary structure showed no homology with the N-terminal amino acid sequence of a NADH oxidase previously isolated from *S. solfataricus* (*Ss*NOX35) (47). Conversely, it showed 40% sequence identity with a putative thioredoxin reductase from *Bacillus subtilis*, but it did not contain cysteines, which are essential for the activity of the reductase. Another NADH oxidase has been isolated from *S. acidocaldarius* which is a monomer of M_r 27,000 (*Sa*NOX27) (48). *Ss*NOX35 and *Sa*NOX27 also catalyze the transfer of electrons directly from NADH to molecular oxygen to produce hydrogen peroxide. This feature is a distinctive property of thermophilic microorganisms because, up to now, no H_2O-producing NADH oxidase has been isolated from this source. Despite the two other archaeal enzymes, *Ss*NOX38 can also oxidize β-NADPH. This feature is shared by the NADH oxidase isolated from *Thermus thermophilus* (49).

Many NADH oxidases have been reported as scavenging hydrogen peroxide and therefore fuctioning as

peroxidases. They are able to catalyze electron transfer from NADH to several electron acceptors such as methylene blue, cytochrome *c*, 2, 6-dichloroindophenol, and potassium ferricyanide. In addition, other NADH oxidases have a thiol disulfide oxidase activity mediated by the presence of a redox-active disulfide center constituted of two very close cysteine residues. *Ss*NOX38 is different from both types of NADH oxidases because it did not show any electron transfer activity toward 2, 6-dichloroindophenol or DTNB. In fact, as deduced from the sequence of the *Ss*NOX38 gene, no cysteine residues are present in the primary structure of the enzyme. As already suggested for the *Amphibacillus xylanu*s NADH oxidase, the reduction of molecular oxygen to hydrogen peroxide did not require the presence of the two cysteines, even for *Ss*NOX38 the mechanism of the reaction might involve the formation of a flavin C/4a-hydroperoxide adduct followed by the elimination of hydrogen peroxide.

A wealth of H_2O-producing NADH oxidase (NOX) homologues have been discovered in the genomes of the hyperthermophilic Archaea, including two homologues in *P. furiosus* which have been designated as NOX1 and NOX2. In order to investigate the function of NOX1, the structural gene encoding NOX1 was cloned and expressed in *E. coli*, and the resulting recombinant enzyme (rNOX1) was purified to homogeneity (3). The enzyme is a thermostable flavoprotein that can be reconstituted only with FAD. rNOX1 catalyzes the oxidation of NADH, producing both H_2O_2 and H_2O as reduction products of O_2 (O_2 + 1-2NADH + 1-2H^+ → 1-2NAD^+ + H_2O_2 or $2H_2O$). This is the first NADH oxidase found to produce both H_2O_2 and H_2O. Although *P. furiosus* is a strict anaerobe, it may tolerate oxygen to some extent and NOX1 can be involved in the response to oxygen at high temperatures. The most puzzling aspect of the reactivity of rNOX1 is the observation that the enzyme produces both H_2O and H_2O_2 in significant quantities. The production of both products leads to contradictory conclusions regarding the oxidase activity of rNOX1. On the one hand, production of H_2O indicates that rNOX1 functions to combat oxidative stress through the reduction of oxygen to water while at the same time regenerating oxidized pyridine nucleotide for fermentative carbohydrate and amino-acid consumption. On the other hand, the production of H_2O_2 by the NADH oxidase appears not to be physiologically useful. This has been the case with an array of enzymes with H_2O_2-producing NADH oxidase activities. These include the alkyl hydroperoxide reductase of *Salmonella typhimurium* (50), the NADH oxidase of *A. xylanus* (which acts as part of an alkyl hydroperoxide reductase system) (51) and the NADH oxidase of *S. solfataricus* (which appears to be a thioredoxin reductase).

Homologues of NADH oxidase were found in a wide range of archaeal genomes including *P. horikoshii* (42), *A. fulgidus* (52), *M. jannaschii* (39) and *T. maritima* (53) but their function remains to be established.

H_2O_2-producing NAD(P)H oxidases have been characterized from several thermophilic sources (54, 55, 56). Many of these enzymes appear to be members of the thioredoxin reductase subclass of the disulfide reductases (57). Genome sequencing of *A. fulgidus* revealed the presence of eight putative NADH oxidase genes, which were designated *noxA-1* to *noxA-5*, *noxB-1*, *noxB-2* and *noxC*, according to their homology to other NADH oxidase encoding genes (52). The physiological role of the putative NADH oxidases in *A. fulgidus* is still enigmatic. A recently purified membrane associated NADH oxidase from *A. fulgidus* (NoxA-2) was proposed to be involved in electron transfer reactions during sulfate respiration. The high similarity with NoxA-2 of *P. furiosus* suggests a similar role (58). On the other hand, the function of NoxA-1 might be that of a detoxificant enzyme. The most plausible argument against this role however, is the fact that the NoxA-1 and also the enzyme from *P. furiosus* produce predominantly H_2O_2. Thus, instead of preventing oxidative stress through O_2 removal, these Nox enzymes aggravate the problem by producing H_2O_2. Alternatively, H_2O_2 may be converted further by a catalase-peroxidase, which is also active in *A. fulgidus*. But in this case, the amount of oxygen would be reduced by only 50% by the two combined enzyme activities (58). Abreu *et al.* (59) describe a superoxide scavenging system in *A. fulgidus*, and propose that NAD(P)H oxidases may have a role in oxygen detoxification, not by directly reducing oxygen, but via intermediate redox enzymes like rubredoxin and neelaredoxin. This hypothesis certainly deserves further attention, but requires purification of the rubredoxin and neelaredoxin (60).

3.5. Oxygen detoxification without superoxide dismutase

Anaerobic microorganisms possess a quite diverse sensitivity to oxygen that may be explained by the presence of new detoxification pathways not necessarily similar to the ones present in aerobes. In particular, scavenging of O_2^- was recently found to be performed by enzymes distinct from the canonical superoxide dismutases, but their mechanism of action remained to be explained.

Jenny *et al*. (61) found superoxide reductase activity in *P. furiosus*, which reduces superoxide to hydrogen peroxide without forming molecular oxygen. Superoxide dismutase (SOD) or superoxide reductase (SOR) orthologues were found in all the archaeal complete genome sequences. The enzyme was considered a key component of an oxidative stress protection system. SOR reduces superoxide to hydrogen peroxide with reduced rubredoxin and thereby avoids the generation of molecular oxygen. In *P. furiosus*, the regeneration of reduced rubredoxin is made possible by an NAD(P)H: rubredoxin oxidoreductase (62). In this system, the rubredoxin oxidoreductase or neelaredoxin is considered to detoxify superoxide as a superoxide reductase (63). The H_2O_2 product of this reaction, while far less toxic than O_2^-, would still require subsequent removal. Although unclear at present, it has been proposed that the hydrogen peroxide is subsequently reduced to water by a rubrerythrin, which exhibits peroxidase activity (64) and whose gene usually clusters with those of SOR and rubredoxin. It was previously suggested that a NADH peroxidase homologue such as NOX1 might play a role in the removal of H_2O_2.

rNOX1 does not clearly seem to exhibit a significant peroxidase or alkyl hydroperoxide reductase activity. This does not rule out a role for NOX1 in a multienzyme peroxidatic complex, as is seen in the case of the alkyl hydroperoxide reductase activity of the AhpF/AhpC system of *S. typhimurium*. Jenney *et al*. (61) did not detect a catalase activity in crude extracts of *P. furiosus* and no catalase homologues in the *P. furiosus* genome have been identified. This result was not unexpected, as a catalase would simply regenerate oxygen. One or more peroxiredoxin (Prx)-type peroxidase(s) analogous to those characterized for a wide range of other organisms are, however, present in the archaea. Prx proteins, which catalyze the cysteine-dependent reduction of H_2O_2 and alkyl hydroperoxides, are present in both '1-Cys' and '2-Cys' versions, and homologues of both types have been found in the *P. furiosis* genome. Electron donors to Prxs in other organisms include thioredoxin (Trx), bacterial AhpF, or specialized small redox-mediating proteins (3). No homologues of AhpF or Trx appear to be encoded in the *P. furiosis* genome, however, the possibility remains that other redox proteins, as well as the glutaredoxin-like protein purified in this organism (12), might work with one or both Prx homologues in removing peroxides with an AhpF-type activity

A. fulgidus is, so far, the only known anaerobe that contains the genes coding for both a neelaredoxin (Nlr) and a desulfoferrodoxin (Dfx), while all the other known genomes from anaerobes only have genes coding for one of these proteins (59). This suggests that Nlr and Dfx may posses the same function in the cell because their structure and activity are similar. In *A. fulgidus*, the expression level of the two proteins appears to be quite different because Nlr was the main protein found to be reactive towards $O_2^{\cdot -}$. The existence of two proteins with the characteristics of Nlr and Dfx and with similar functions is very interesting from the evolutionary point of view. The mechanism by which Nlr and Dfx detoxify $O_2^{\cdot -}$ is unclear. It was shown that Nlr can both reduce and dismutate $O_2^{\cdot -}$. So, it appears that Nlr and, possibly, Dfx are bifunctional enzymes: in the reduced state and in the presence of its putative electron donor, the enzymes may act as superoxide reductases; however upon oxidation, and in the absence of a reductant, they may still detoxify $O_2^{\cdot -}$ by dismutation. This bifunctionality provides the cells with an efficient way of protection, independent of the cell redox status. The superoxide dismutase activity of Nlr is $10^2 - 10^3$ times lower than that from canonical SODs. But, unlike canonical SODs that are usually present in small concentrations in the cell, Nlr seems to be present in high amounts, which can compensate its lower activity. The bifunctionality of Nlr also explains the assertion of a superoxide reductase activity to Nlr and Dfx, reported previously (64).

3.6. Superoxide Dismutase

The predominant ubiquitous defense system against ROS is mediated by the superoxide dismutases (SODs), which catalyze the disproportionation of superoxide into hydrogen peroxide and oxygen (65, 66). In the majority of archaea, along with some species of anaerobic bacteria, distinct systems have evolved to protect the cell from reactive oxygen. In the aerobic or microaerobic (hyper)thermophilic archaea, SOD seems to functionally replace SOR. In general, SODs are assigned to four groups on the basis of their metal cofactors: Cu/Zn-SOD, Mn-SOD, Fe-SOD, and Ni-SOD. Mn- and Fe-SODs are closely related in amino acid sequence and three-dimensional structure. Among (hyper) thermophilic archaea, Fe-SODs have been reported from *S. solfataricus* (67), *S. acidocaldarius* (68), *Acidianus ambivalens* (69), and *T. acidophilum* (70). Although Mn- and Fe-SODs generally exhibit a strict metal specificity for SOD activity (71), several cambialistic SODs, which are active with either Mn or Fe, have been reported. This is the case in the enzymes from *P. aerophilum* (72, 73) and *A. pernix* (74). At present, Cu/Zn-SODs have not been found in archaea. Interestingly, the archaea that harbor SOD do not necessarily utilize a catalase. In fact, the only catalases reported from hyperthermophilic archaea are the Mn catalase from *P. calidifontis* (37) and the heme catalase from *A. fulgidus*, which does not harbor a SOD (36). Recently, a SOD has been also identified and characterized (75) making *P. calidifontis* the only hyperthermophilic archaeon known to utilize an SOD-catalase system to detoxify reactive oxygen species. Both genes were found to be regulated at transcriptional levels by the presence or absence of oxygen. Furthermore, the fact that *P. calidifontis* is a facultative aerobe allows to study how hyperthermophiles respond to the presence or absence of oxygen at high temperatures. Subsequent genome sequence data from aerobic (hyper) thermophilic archaea have revealed in *P. aerophilum* an orthologue of the Mn catalase gene from *P. calidifontis*. Although aerobic, the genomes of *S. solfataricus* (44), *S. tokodaii* (45), *A. pernix* (43), *T. acidophilum* (40), and *T. volcanium* (41) do not harbor catalase orthologues. Furthermore, rubrerythrin orthologues are not found in these organisms, suggesting that the majority of aerobic (hyper) thermophilic archaea may utilize an SOD-Prx system. When strictly anaerobic microorganisms come in contact with O_2, generally O_2^- and H_2O_2 are generated by autoxidation of reduced iron-sulfur proteins and/or flavoproteins which, if not immediately removed by the enzymes superoxide dismutase and catalase, will damage the cells. The presence or absence of one or both of these enzymes determines whether an anaerobe is more or less aerotolerant (76). Instead of a superoxide dismutase, *P. furiosus* contains a superoxide reductase that catalyzes the reduction of O_2^- to H_2O_2 with reduced rubredoxin (61).

Initially, methanogenic archaea were thought to contain neither superoxide dismutase nor catalase. In 1981, Kirby's group reported the presence of an iron superoxide dismutase in *Methanobacterium bryantii* (77). Later, Takao *et al*. (78) cloned and sequenced the *sod* gene from *M. thermoautotrophicum* strain $^{\delta}$H and heterologously overexpressed it in *E. coli*. *sod* and *kat* genes were, however, not found in *M. jannaschii* (39). In the genome of *A. fulgidus,* a sulfate-reducing archaeon most closely related to the Methanosarcinacaea, no *sod* gene but a bifunctional catalase-peroxidase was found (79). *M.*

barkeri, besides a catalase, also contains an iron superoxide dismutase (80).

4. CHEMICAL STRESS

4.1. Metal detoxification

Microorganisms require the presence of a number of transition metals, heavy metals and metalloids playing essential role as cofactors, in redox processes and in stabilising protein structures (81). Metals may accumulate above physiological concentrations and become toxic by blocking enzyme activities, inhibiting transport systems, and disrupting cellular membrane integrity.

Therefore, detoxification and resistance systems to rid the cell of these potentially toxins have evolved (82) and in all cells there are mechanisms for metal ion homeostasis that frequently involve a balance between uptake and efflux systems (83). Knowledge of metal toxicity and how archaea survive in metal rich environments, may provide insights in bioremediation of contaminated sites and in the development of novel biotechnological processes like for example the transfer of metal resistance gene operons creating multi metal resistant strains.

In most cases, the expression of such resistance systems is transcriptionally controlled by metal sensor proteins that "sense" specific metal ions. Resistance operons to heavy metal ions have been stably integrated into bacterial chromosome or can be located on endogenous plasmids or transposons containing multiple resistance or detoxification operons. *In silico* studies have indicated the presence of homologues of metal resistance operones in a number of archaeal genomes by searching for analogues of known systems.

Usually, these operons encode resistance proteins such as metal-specific efflux pumps, membrane-bound transporters, metal reductases, cytoplasmic or periplasmic metal transport proteins, or metal-sequestering proteins. They also encode at least one trans-acting-metal-responsive transcriptional regulator for the operon (84), which can act as repressor or activator. In some cases, the extent of amino acid sequence identity among resistance proteins is so high to suggest horizontal gene transfer between bacteria and archaea.

The basic mechanism responsible for cellular resistance to metals generally involves an enzyme-catalysed intra- or extracellular conversion of the metal and the efflux out of the cell by active pumps or permeability barriers. These systems are generally finely regulated at transcriptional level by specific protein factors (81).

4.1.1. Metal reductases

Metal reductases are key enzymes for a number of reactions inside a microbial cell. Many element transformations are key steps in biogeochemical cycles, other may result in their incorporation into cell biomass, in the generation of energy or in detoxification.

In bacteria, there are multiple mechanisms involving different enzymes for metal detoxification that indicate the occurrence of several evolutionary pathways. Genome analysis in some archaea confirmed the previous idea of a convergent evolution to provide solutions to the problem of environmental toxic metals (85).

In the case of arsenic resistance for example, three different classes of arsenate reductases have evolved able to enzimatically reduce the arsenate [As(V)] to arsenite [As(III)] which is the substrate of the ArsB protein, the membrane located arsenite transporter (85). There are three families of arsenate reductases, two found in bacterial systems and a third identified in *S. cerevisiae*. At least thirty strains representing nine genera, including also the Chrenarchaeota belonging to the genus *Pyrobaculum* (86), are arsenite oxidising prokaryotes (87).

A similar strategy is adopted by microrganisms to recover from toxic selenium or mercury; specific reductases are able to reduce selenite to elemental selenium and Hg(2+) to non-toxic Hg(0) and subsequent diffusional loss from the cell (88).

Among archaea, it has been reported that the crenarchaeon *P. aerophilum* was able to grow organotrophically under anaerobic conditions in the presence of arsenate, selenate and selenite. During growth on selenite, elemental selenium was formed as final product thus confirming the presence of a specific reductase (86).

In a recent study, a mercuric reductase gene (*merA*) has been identified on the genome of the crenarchaeon *S. solfataricus*. It has been established that *merA* transcription responds to mercury and, interestingly, gene disruption of the *merA* gene determines mercury sensitivity when compared to the wild type cells (89).

4.1.2. Efflux pumps, membrane-bound transporters

Efflux systems have been described in the three kingdoms of life and grouped in four main protein families (90): the heavy metal Resistance, Nodulation and cell Division (RND), Cation Diffusion Facilitators (CDF), P-type ATPase and other transport systems. Distribution of members of heavy metal exporting protein families identified in the genomes of archaea is shown in table 1.

Members of the first family are proteins involved in transport of heavy metals, hydrophobic compounds, nodulation factors, and in protein export (91). Functional studies, mainly developed for bacteria, for which genetic analyses are well developed, have evidenced that RND mediate the active part of the transport process and determine the substrate specificity. The first identified member of the RND family was the CzcA protein from the bacterium *Ralstonia metalidurans*. The encoding gene is included in an operon *czcCBA* that is transcribed tricistronically (92). Expression of the genes confers resistance to Co^{2+}, Zn^{2+}, and Cd^{2+}. Biochemical studies demonstrated that CzcA is a proton-cation antiporter depending on the Z-ΔpH portion of the proton motive force. The protein is composed of two related halves that are probably the result of an ancient gene duplication and fusion, each containing a periplasmic loop flanked by 12

Table 1. Distribution of members of heavy metal exporting protein families identified in the genomes of archaea (90)

	CDF proteins	P-type ATPases	Other transport systems
Chrenarchaeota			
Aeropyrum pernix	nd	2	nd
Sulfolobus solfataricus	2	2	nd
Sulfolobus tokodaii	2	1	nd
Pyrobaculum aerophilum	3	1	nd
Euryarchaeota			
Archaeoglobus fulgidus	3	3	nd
Halobacterium sp.	2	4	nd
Methanothermobacter thermoautotrophicum	3	6	nd
Methanocaldococcus jannashii	2	2	1
Pyrococcus abysii	2	1	nd
Pyrococcus furiosus	3	1	nd
Pyrococcus horikoshii	2	nd	nd
Thermoplasma acidophilum	nd	3	nd
Thermoplasma volcanium	nd	2	nd

RND proteins have not been found yet in the archaea reported above, nd: not detected

transmembrane alfa-helices (93, 94). No homologues of RND proteins could be found analysing genome sequences of crenarchaea and euryarchaea (90).

CDF proteins form a protein family of metal transporters occurring in all the three domains of life (95, 96) Primary substrate is mainly Zn^{2+} but also Co^{2+}, Ni^{2+}, Cd^{2+}, and Fe^{2+} can be exported through this system. Usually, bacteria contain one or a few CDF-encoding genes, while in eukarya the number of CDF-encoding genes increases in parallel with evolution from yeast to animals and plants. Most CDF proteins exhibit six transmembrane spans and are rich in histidine residues responsible for Zn^{2+} binding. CzcD from *R. metallidurans* is an efflux pump driven by the proton motive force. The distribution of CDF proteins in sequenced bacterial genomes has been analysed and clustered in three subgroups on the basis of multiple alignments (90). Group 2 contains most of the known zinc transporting CDF proteins in bacteria, eukaryotes and archaea suggesting that these protein may have originated very early in evolution. To date, no functional studies have been carried out on archaeal members but evidence is provided on the importance of having evolved system for Zn^{2+} efflux.

P-type ATPases constitute a superfamily of transport proteins driven by ATP hydrolysis (97). Members of this family occur in all three kingdoms of life. Substrates are inorganic cations such as H^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, Cu^+, Ag^+, Zn^{2+}, and Cd^{2+} (91). Members of this huge family carry a conserved proline residue, preceded and/or followed by a cysteine residue, whose role would be binding and releasing of heavy metal-thiolate cations (98).

Only a few P-type ATPases have been studied in archaea from the crenarchaeon *A. ambivalens* (99) and the euryarchaeaons *A. fulgidus* (100, 101) and *M. jannashii* (102). The CopA protein from *A. fulgidus* drives the outward movement of Cu^+ or Ag^+ and is characterised by a putative metal binding site CPC and cytoplasmic metal binding sequences also containing cysteine residues at its N- and C- terminus. Replacement of C residues by A evidenced a regulative role for the Cu^+ metal binding domain located at the N-terminus of the protein whose function could be to accelerate cation release (101).

4.1.3. Transcriptional regulatory proteins

Two general classes of transcriptional regulators allow prokaryotes to respond to stress induced by metal toxicity and are the MerR and SmtB/ArsR families.

MerR-like proteins generally function as repressors in the absence of metal ions and become activators upon metal binding, by driving a conformational switch that converts a sub-optimal promoter into a potent one (103). The archetype of the family is the regulator of Gram-negative mercury resistance operons found on the transposable elements Tn21 and Tn501 (104, 105), but subsequent data obtained for the regulators SoxR from *E.coli* and TipAl from *Streptomyces lividans*, indicated that they were also members of the family and that they activated gene expression in a similar manner. Main feature of a member of this family is the high degree of sequence similarity in the N-terminal DNA binding region containing a helix-turn-helix motif. A subset of the family, showing C-terminal sequence similarity, responds to metal ions for the presence, in this region, of potential metal coordinating residues. MerR from Gram-negative bacteria is divergently transcribed from the major regulated promoter that transcribes the genes encoding for the transporter MerT, for a periplasmic protein MerP, for an additional transporter, for the mercuric reductase enzyme MerA, for a putative repressor and for a putative further transporter (106, 107). Open reading frames with sequence similarity to MerR family of regulators are common and are present also in archaea, although very few have been studied in detail. Very recently, a *mer* locus has been identified in the genome of the archaeon *S. solfataricus* consisting of two ORFs arranged in opposite directions and spaced by a 300 nt sequence, encoding a putative mercuric reductase (*merA*) and a mercury transcriptional regulator (*merR*). Protein phylogenetic analysis clarified their identity as orthologs of

MerA and MerR also if the latter shows several key differences from its bacterial counterparts. The functional role of the two genes to the sensitivity of *S. solfataricus* to challenge by the heavy metal mercure has been further investigated and confirmed by gene disruption (89). No further MerR-like proteins have been characterised from other sequenced archaeal genomes.

The SmtB/ArsR members, perhaps distantly related to the MerR family, function only as transcriptional repressors bound to operator/promoter DNA; metal binding strongly inhibits or negatively regulates DNA binding and the regulatory protein does not actively participate in transcription. Individual members of the family clearly derive from a common evolutionary origin, and have evolved to sense distinct metal ions (108).

Arsenic resistance has been studied extensively in a small number of bacterial species, while very little is known about the arsenic tolerance mechanisms of archaea (109). The *E. coli* plasmid R773 contains an operon for the *arsRDABC* genes where *arsR* and *arsD* encode for two transcriptional repressors, ArsA is an ATPase functioning as the catalytic subunit of ArsB, the membrane located arsenite transporter. Resistance to arsenate is conferred by ArsC, a reductase for the conversion of arsenate to the substrate of the efflux pump. In the archaeon *Ferroplasma acidarmanus*, genes encoding for ArsR and ArsB homologues have been found located on a single operon while the gene encoding for an ArsA relative was found apart. No homologues of the ArsC reductase and the ArsD regulator were identified by genome sequence analysis, and no functional studies have been carried out (110).

4.2. Drug detoxification

Microorganisms have developed various ways to resist to the toxic effects of antibiotics and other drugs. One of these mechanisms involves the production of enzymes that inactivate antibiotics by hydrolysis or by the formation of inactive derivatives (111). Well known examples are beta-lactamases and enzymes that modify aminoglycoside antibiotics (112). A second mechanism of resistance is target alteration. Cellular targets can be altered by mutation or enzymatic modification in a way that the affinity of the antibiotic for the target is reduced (113). A third, more general mechanism of resistance is the inhibition of drug entry into the cell. Also if cellular membranes are selective, they cannot prevent the drugs from exerting their toxic action once entered the cell; for this reason, the active efflux of drugs is essential to ensure significant levels of drug resistance. The so-called multidrug transporters can handle a wide variety of structurally unrelated compounds (114, 115). They can be divided into two major classes: i) ATP Binding Cassette, which uses the free energy of ATP hydrolysis to pump drugs out of the cell; ii) secondary multidrug transporters which use the electrochemical gradient of protons or metal ions to drive the outward transport of the drugs. The wide specificity of multicomponent multidrug efflux systems suggests that their expression may be carefully controlled, because their overexpression might result in the efflux of essential metabolites. Multidrug transporter gene regulation has been pursued in some microorganisms and general and complex systems have been identified in some cases.

Using the available genomic data, Saier and Paulsen in 2001 (116) have analysed complete genomes, identified all predicted transport genes and classified them on the basis of sequence similarities, bioenergetics and substrate specificity. Some of the multiple multidrug transporters expressed in the same cell appear to be entirely redundant; nevertheless, the expression patterns of these proteins may differ, due to different control mechanisms by transcriptional regulators. This evidence suggests that many multidrug transporters have been selected for specific purposes and transport drugs merely opportunistically. Recently, structures of multidrug efflux transporters and their regulators have been reported, providing new insights about the evolution of multidrug transporters, their transport mechanism and their natural physiological roles (117).

4.2.1. The ATP binding cassette superfamily

ATP-binding cassette transporters are ubiquitous membrane proteins that couple ATP hydrolysis to the energy-dependent transport of a wide variety of molecules across lipid bilayers. They comprise the single largest gene family in several sequenced prokaryotic genomes (118, 119). The ABC transporters share an invariant domain organization of two conserved cytoplasmic nucleotide binding cassettes associated with two transmembrane (TM) domains (119). The cassettes contain three highly conserved motifs required for nucleotide binding and hydrolysis: the Walker A and the Walker B sites (120), which reside in the $\alpha\beta$ core of the cassette, and the LSGGQ signature sequence (118, 119), which lies more toward the periphery of the cassette in a α-helical subdomain (121). The TM domains that mediate the movement of the structurally diverse solutes exhibit less sequence conservation (118, 119). The organization of prokaryotic transporter operons and of single polypeptide chain transporters suggests that the minimal functional unit consists of at least two cassettes and two TM domains (121). From genome analysis, it has emerged that transporters potentially active in the efflux of toxic metabolites or other drugs cannot be clustered in a unique family and in most cases, the mechanism of drug resistance induced by ATPases is not known (116).

Among archaea, an ATP-binding cassette from *M. jannashii* (MJ0796) has been biochemically characterised for its ATPase activity indicating that at least two binding sites participate in the catalytic reaction and in domain association (122, 123), but its physiological significance remains to be elucidated. Furthermore, an ATP driven, sugar activated multidrug efflux activity has been found in cells of *Haloferax volcanii* (124). Analysis of the efflux system using rhodamine 123, which is a potent substrate for the P-glycoprotein, the first identified mammalian ATP-dependent multidrug transporter(125), has evidenced that this archaeon might also express a P-glycoprotein-like protein (126). Several different ABC ATPaes have been identified and characterised in

hyperthermophilic archaea but mainly involved in sugar uptake (127).

4.2.2. Secondary Multidrug Transporters

On the basis of size similarities and genome-derived phylogenetic analyses, the secondary multidrug transporters can be further divided into distinc families: the Major Facilitator Superfamily (MFS) (128), the Small Multidrug Resistance family (SMR), (129) the Resistance-Nodulation-cell Division (RND) family (91) and the Multidrug And Toxic compound Extrusion (MATE) family (130).

The MFS family consists of membrane transport proteins which are ubiquitous in the three domains of living organisms and catalyse uniport, symport, or antiport of different substrates such as sugars, polyols, drugs, Krebs cycle metabolites, aminoacids, peptides, nucleosides. Alignment of conserved motifs, and a recent 3D structure available for the *E.coli* lactose permease LacY (131) revealed that these protein can contain either 12 or 14 TM segments. All characterised drug exporters of the MFS family probably function by H^+ antiport. Based on the classification reported by Saier and Paulsen (116), six families are known to export drugs. Two of these are bacterial specific, two eukaryotic specific and two are ubiquitous, the DHA12 and DHA14 drug efflux families which differ from each other for the presence of 12 or 14 TM spanning domains. The functionally characterized members of the DHA12 family, like the DHA14 family, exhibit specificities only for drugs, although the range of drugs transported is remarkable. Interestingly, the range of organisms in which DHA12 family members are found is wider than that for the DHA14 family. Thus, the DHA12 multidrug resistance pumps are found in animals as well as in yeasts and a variety of gram-negative and gram-positive bacteria. Uncharacterized members of this family contain an even wider range of organisms, including humans and archaea (116). Dendograms have been obtained on the basis of multiple alignments for members of both families (128) The multidrug resistance pumps, drug-specific permeases, and uncharacterized proteins did not group together but instead proved to be scattered in an apparently random fashion relative to each other. Thus, in both families, phylogeny does not appear to provide an indication of drug specificity.

Proteins in the SMR family are the smallest secondary drug efflux proteins known consisting of only about 110-amino acids (129). Hydropathy and residue distribution analyses of this family suggested a structural model with only four TM spanning segments of mildly amphipathic alpha-helices. Several SMR proteins have been characterized, purified, and reconstituted in a functional form (132). Most posses a single membrane-embedded charged residue, Glu-14, conserved in more than sixty homologous proteins, which was shown to be part of a binding site common to protons and substrates (133).

The SMR proteins are not as ubiquitous in the archaea as in the bacteria. The first SMR protein identified in the archaeal kingdom is that from the halophilic archaeon *H. salinarum*. Only three other homologues have been identified until now in archaea, all of them in various *Methanosarcinaceae* species. The protein from *H. salinarum* named Hsmr, was identified through sequence similarity to the SMR family, and expressed in *E.coli.* The Hsmr protein contains many of the signature sequence elements of the SMR family and also a high content of negative residues in the loops, characteristic of extreme halophiles; it is able to transport ethidium bromide in an uncoupler-sensitive process and to gain resistance to ethidium bromide and acriflavine (134).

The multidrug and toxic compound extrusion (MATE) family has been recently introduced to group members of a previously unidentified family, which contains more than 30 proteins, including representatives from all three kingdoms of life (130). These proteins mediate resistance to a range of cationic dyes, aminoglycosides and fluoroquinolones and contain 12 predicted TM segments. The new family has been introduced by Brown *et al*. (130) since they do not share significant sequence similarity with any member of the MFS. The MATE family appears to represent a family of transport proteins responsible for protecting cells from drugs and other toxic compounds, although the physiological role of most of its members remains to be elucidated. Unfortunately, no MATE proteins have been functionally characterised in archaea.

4.2.3. Regulation of multidrug transporter genes

In comparison to the limited achievements in understanding the structure-function relationships of the drug transporters themselves, research into the regulatory pathways that govern the expression of drug transporters has progressed relatively rapidly, in particular for a number of bacterial regulatory proteins. The pumps which are known to be subject to regulatory controls typically belong to either the MFS or RND superfamily The requirement for regulatory controls to prevent excessive production of integral membrane proteins is demonstrated by the deleterious effect of constitutive expression of the gram-negative tetracycline/H^+ antiporters TetA(B) and TetA(C); in fact, in the absence of tetracycline, the cells show a severe disadvantage when competing with nonconstitutively expressing strains (135, 136). However, the small multidrug resistance family do not appear to be subjected to any regulatory control that can alter the level at which these proteins are synthesized. It is therefore an intriguing question why some proteins are synthesized under strict regulatory controls, yet others appear to be expressed constitutively. This could well reflect currently unknown physiological roles for the unregulated pumps in normal cellular metabolism that require the constant low-level presence of such transporters. For the drug efflux genes that are known to be subjected to regulation, expression is generally controlled by transcriptional regulatory proteins, repressors and activators of target gene transcription. This process can occur at either the local or global level.

Sequencing of entire genomes has identified a number of additional multidrug transporters homologs and

their associated regulatory elements also in archaea, although the functions of the majority of these systems remain to be experimentally investigated (137). In bacteria, the confirmed regulators of drug transporter genes belong to one of four regulatory protein families, the AraC, MarR, MerR, and TetR families. The assignment of these regulatory proteins to their respective families is based solely on similarities detected within their DNA-binding domains, which typically constitute only one third of each polypeptide. Like the majority of bacterial activators and repressors, the drug transport regulators identified to date in archaeal systems possess a α-helix-turn-α-helix (HTH) DNA-binding motifs, which are embedded in larger DNA-binding domains that form a number of different structural environments, such as three-helix bundles and winged helix motifs (138). Importantly, the portions of these proteins not involved in forming the DNA-binding domains have been demonstrated to be capable of directly binding substrates of their cognate pumps, which act as a signal to increase the synthesis of the relevant transport protein(s) in response to the presence of these toxic compounds.

4.2.4. Metabolic detoxification

As discussed above, all living organisms have developed specific, finely regulated efflux systems, to defend themselves from a great variety of toxic compounds; besides this, they have also evolved peculiar pathways or enzymes making them able to biochemically degrade such drugs. Aromatic compounds are environmental pollutants associated with the use of explosives, pesticides and pharmaceuticals. It has been recently demonstrated that some of these compounds can be thoroughly metabolised and utilised as carbon and energy source for anaerobic bacteria (139). A major theme, emerged from studies carried out in bacteria, is that, in anaerobic conditions, diverse aromatic compounds, are metabolised to form benzoate or, more often, benzoyl-coenzyme A as a common intermediate. Benzoyl and benzoyl CoA are degraded to central metabolic intermediates by a series of reactions that involve aromatic ring reduction, ring modification, and ring cleavage (140). In the presence of oxygen, the aromatic compounds can be hydroxilated by multicomponent monooxygenases and dioxygenases.

Multicomponent monooxigenases have been identified in some sequenced genomes. They are always operons coding for four to six polypetides (141, 142). A new cluster has been recently identified in the archaeon *S. solfataricus*. Using comparative genomics it has been shown that this cluster has an architecture distinct from the other characterised operons with a different pool of modular components. Functional data are required to verify expression of these genes (143). Simpler mechanisms have also evolved to relief cells from aromatic molecules mainly involving single enzymes, mostly deyhdrogenases. These latter systems have been better characterised in plants (144), but gene array-based studies have demonstrated that this is also true in bacteria (145). We recently reported about a possible detoxifying role of an alcohol dehydrogenase gene of the archaeon *S. solfataricus*. It had been observed that cells relief from the addition of benzaldehyde by increasing levels of a specific alchol dehydrogenase enzyme able to metabolize the toxic aldehyde (146); in a subsequent study it was evidenced that this response could be mediated by a transcription factor binding specifically at the *adh* promoter and hence postulated that a molecular signaling mechanism could be responsible for the switch of the aromatic aldehyde metabolism in response to environmental changes (147).

5. CONCLUDING REMARKS

A comprehensive understanding of the networks of genes, proteins and molecules underlying environmental stress signaling and defense responses would require the identification and characterisation of all the molecular components involved, but to date, unfortunately, still very little is known. In this review, we have focused on the different strategies adopted by archaea to detoxify from toxic molecules, like oxidants, metals and drugs. Two main strategies have been identified: one involves the metabolic degradation or conversion of the stress agent, the other an efflux (active or passive) toward the cell that can be preceded or not by a biochemical modification of the "stressing" molecule. Local and global regulation mechanisms assure that the involved molecular components are up- or down-regulated in order to increase cell tolerance. Whole genome sequencing projects are revealing more information on the diversity and redundancy of such systems, as well as on the evolution of different genes in different organisms. Neverthless, our basic understanding, especially of metal and aromatic compound stress response in archaea, is still extremely limited. Insights into these new processes may be elucidated using functional and comparative genomics, as well as new powerful tools for high throughput analysis of gene expression in different conditions, like transcriptomics and proteomics. In our opinion, these studies represent a major challenge for the future.

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