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Review

Arsenics as bioenergetic substrates ☆

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ABSTRACT

Although at low concentrations, arsenic commonly occurs naturally as a local geological constituent. Whereas both arsenate and arsenite are strongly toxic to life, a number of prokaryotes use these compounds as electron acceptors or donors, respectively, for bioenergetic purposes via respiratory arsenate reductase, arsenite oxidase and alternative arsenite oxidase. The recent burst in discovered arsenite oxidizing and arsenate respiring microbes suggests the arsenic bioenergetic metabolisms to be anything but exotic. The first goal of the present review is to bring to light the widespread distribution and diversity of these metabolizing pathways. The second goal is to present an evolutionary analysis of these diverse energetic pathways. Taking into account not only the available data on the arsenic metabolizing enzymes and their phylogenetical relatives but also the palaeogeochemical records, we propose a crucial role of arsenite oxidation via arsenite oxidase in primordial life. This article is part of a Special Issue entitled: The evolutionary aspects of bioenergetic systems.

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1. Introduction

Although present at low concentration in the Earth's crust, Arsenic (As) is commonly found in rocks, minerals and water as inorganic oxyanions, arsenite (H_3AsO_3) and arsenate ($HAsO_4^{2-}/H_2AsO_4^-$), but also exists as methylated organoarsenicals (e.g. methylarsonic, methylarsonus, dimethylarsinic acids) [1]. Both soluble arsenate (As^V) and arsenite (As^{III}) are strongly toxic to life [2]. The biological study of As has therefore focused in the past on the deleterious interactions between As compounds and cellular mechanisms. As^V, a phosphate analog, interferes with normal phosphorylation processes by replacing phosphate, whereas As^{III} binds to sulfhydryl groups of cysteine residues in proteins, thereby inactivating them [1]. Organisms of all the three domains of life have consequently evolved detoxifying energy-consuming systems to limit intracellular levels of these oxyanions [2]. The detoxifying conversion of As^V is the best studied As metabolism. This system allows reduction of As^V to As^{III} in the cytoplasm via the arsenate reductase ArsC, and excretion of As^{III} via an As^{III}-specific transporter, ArsB. Nearly two decades ago the first As^V-respiring bacterium was discovered [3] and in the course of the last 10 years, the widespread role of As as bioenergetic substrate for microorganisms has become apparent. Arsenic metabolizing enzymes have indeed been shown to contribute to the generation of chemiosmotic potential by coupling exergonic electron transfer to proton translocation with arsenics playing the role of reducing or oxidizing substrates [4,5].

A number of prokaryotes have been demonstrated to use As^V as electron acceptor for respiration via arsenate reductase (Arr) whereas several other prokaryotes use As^{III} as electron donor for energy conserving systems, via arsenite oxidase (Aio) [4,5] or alternative arsenite oxidase (Arx) enzymes; Arx is a variant of Arr working in reverse. The large majority of characterized As^{III} oxidizers are mesophilic aerobes. In these cases, Aio transfers electrons arising from the oxidation of As^{III} towards a periplasmic soluble electron carrier ultimately reducing O_2 [4,5]. Arr, by contrast, operates at the opposite end of energy conserving chains by funneling reducing equivalents from organic matter to the terminal acceptor As^V in an anaerobic respiration involving the quinol pool. Anaerobic oxidation of As^{III} has been revealed recently [6–15]. Although scarce information is available on these pathways they have been shown to use either Aio or Arx. Nothing is presently known on the latter enzyme's functioning but the reversion of Arr's directionality would suggest that Arx transfers electrons arising from the oxidation of As^{III} towards the quinone pool.

As supported by the recent burst in discovered As^{III} oxidizing and As^V respiring microbes, the small number of species known to date to metabolize As for bioenergetic ends probably reflects a sampling bias rather than actual abundance (see for example the recent work of Sultana et al. [16]). The above described diversity of energetic pathways involving As metabolism reinforces the idea that it is anything but exotic. The first goal of the present article is to bring to light the widespread distribution and diversity of energetic As metabolizing pathways. Genomic screenings [17,18] furthermore have revealed

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ancestral roots for the oxidation of As^{III}, mediated by the enzyme Aio. The second goal of the article is therefore, through an evolutionary analysis of these diverse energetic pathways, to analyze the hypothesis of a crucial role of As^{III} oxidation in primordial life. Against the backdrop of these two ends we will briefly present a molecular description of the enzymatic systems, followed by an exhaustive inventory of pathways involving As oxidizing or reducing enzymes. Taking into account not only the available data on the As metabolizing enzymes and their phylogenetical relatives but also the palaeogeochemical record, we will try and establish a timeline for the evolution of As bioenergetic metabolism.

2. Molecular description of arsenic bioenergetic enzymes

In contrast to the substrate versatility of bioenergetic chains stands the relatively homogenous architecture of the enzymes involved. These enzymes appear as variations on only a small number of fundamental structural units [19]. In line with this idea, the three enzymes bioenergetically metabolizing As are molybdoenzymes belonging to the vast group of complexes commonly referred to as the DMSO-reductase superfamily and newly renamed complex iron sulfur molybdoenzymes (CISM) [20]. This superfamily is a textbook example of the above mentioned construction kit. The large catalytic molybdenum containing-subunit, present in all members of the group, is supplemented by a variable number of additional subunits to spawn the multitude of individual subfamilies.

2.1. Description of arsenite oxidase

2.1.1. Structural description of Aio

Aio (formerly Aox, Aro or Aso; see [21]) is a heterodimeric enzyme first purified and structurally characterized from *Alcaligenes faecalis* [22,23]. It has since been purified and/or characterized from several bacteria [17,22,24–29]. Its catalytic subunit AioA (formerly AoxB, AroA or AsoA) (about 825 residues) carries the molybdenum-*bis*(pyranopterin guanine dinucleotide) (Mo-*bis*PGD) cofactor together with a [Fe–S] center. Within the CISM superfamily, the AioA distinguishes itself by the facts that no amino acid coordinates to the molybdenum and that it binds a [3Fe–4S] center instead of the more common [4Fe–4S] center [23]. A total of four domains make up the large subunit of Aio (Fig. 1a). Domain I binds the [3Fe–4S] cluster, whereas the structural domains II and III are related to each other by a pseudo two-fold axis of symmetry and both possess homologous dinucleotide-binding folds [23]. The AioA and AioB subunits are held together in the heterodimer structure by a network of hydrogen bonds at the interface between the two subunits and also by AioA's C- and N-terminal stretches that entwine the AioB protein [23].

AioB (around 170 residues), is a member of the Rieske protein superfamily by virtue of its [2Fe–2S] center and protein fold (see also [17,23,30,31]). A common Cys-X-His-X_n-Cys-X_2-His sequence motif, observed in other Rieske proteins, binds the Rieske-type [2Fe–2S] cluster. In the *A. faecalis* Aio structure, the two loops containing both Cys/His couples are held together by a disulfide bridge between Cys60 and Cys80 [23]. This disulfide bridge, strictly conserved in the Rieske/cyt*b* complexes and considered to be crucial for redox and catalytic properties of the [2Fe–2S] center in this complex [32,33] is not fully conserved in the Aio. Neither the redox nor the catalytic properties of the enzyme seem to depend on this disulfide bridge [31,34]. The AioB subunit, harboring a typical Tat signal sequence, is responsible for targeting the Aio to the periplasm. All presently characterized enzymes, except those from NT-14 and NT-26 [24,25], were observed to be bound to the cytoplasmic membrane [17,22,27–29]. It is not clear at present whether the Tat signal sequence, which would then remain uncleaved, could be responsible for this membrane association in the same manner as observed in the Rieske/cyt*b* complex (see also [29,34,35]), but heterologously expressed harboring Tat signal sequence AioAB is also observed as bound to the cytoplasmic membrane [34].

2.1.2. Genetic organization of Aio

Although genomic screening suggests that As^{III}-oxidation using Aio is phylogenetically and ecologically widespread, as detailed below, few functional As^{III}-oxidation gene clusters have been characterized (for more details see also a recent review [36]). In all studied As^{III}-oxidase gene clusters, mainly from aerobic Proteobacteria, the *aioA* and *aioB* genes are in the same orientation, with *aioB* located upstream of *aioA* (Fig. 2). Based on genomic analysis, *aioBA* are the only constant elements of the *aio* gene cluster. A gene coding for a *c*-type cytochrome occurs in most, but not all, of the known proteobacterial *aio* clusters [27,28,37,38], and does not occur in the clusters of species from any other phylogenetic lineages. In several proteobacterial strains,

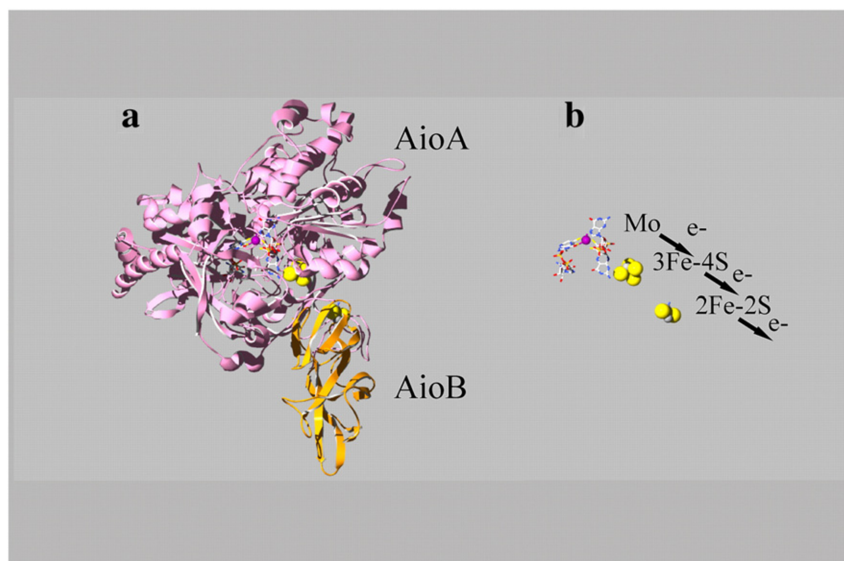

Fig. 1. Structure of *Alcaligenes faecalis* Aio. (a) Crystal structure of Aio enzyme from *A. faecalis* [Ellis et al. [23]]. The large α-subunit is shown in pink, the small β-subunit in orange. (b) Arrangement of the metal centers involved in electron transfer (Mo → [3Fe–4S] → [2Fe–2S]), the electrons are then transferred to a periplasmic electron acceptor (only *c*-type cytochromes have so far been shown to be physiological carriers).

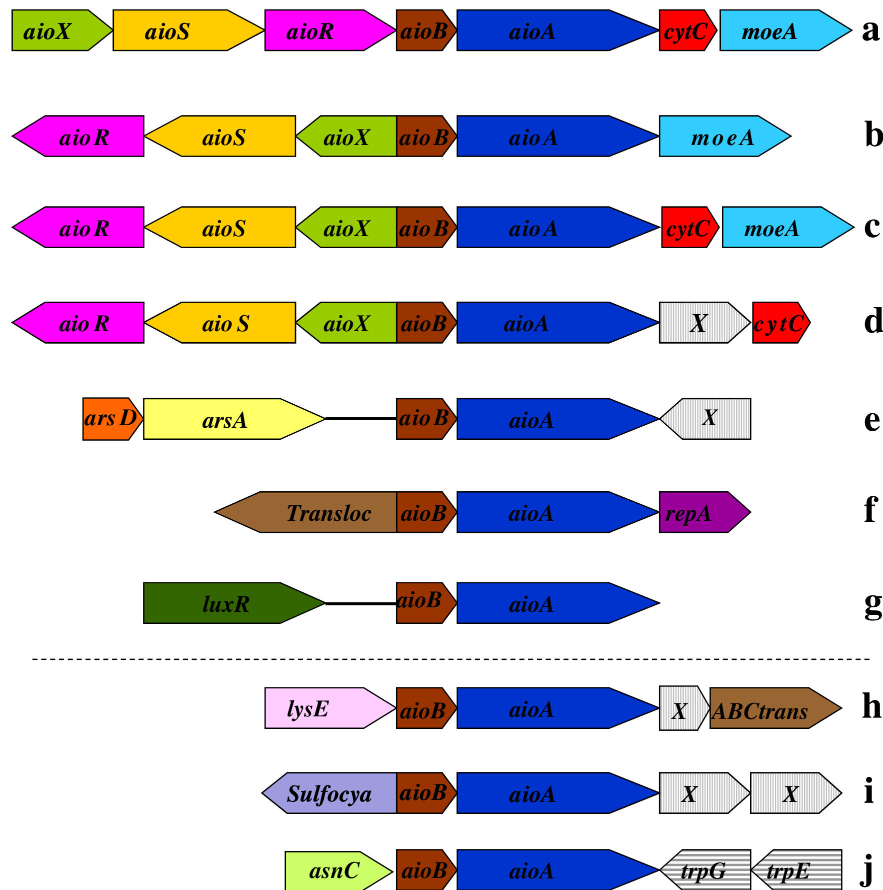

Fig. 2. Organization of observed prokaryotic *aio* gene clusters. Organizations a–g are from demonstrated As^{III}-oxidizing Bacteria. a: *Sinorhizobium* sp. str. M14, NT-26, *Agrobacterium tumefacies* st. 5A and *Ochrobactrum tritici*; b: *Alcaligenes faecalis*; c: *Achromobacter* sp. str. SY8; d: *Herminiimonas arsenicoxydans* str. ULPAs1; e: *Pseudomonas* sp. st. TS44; f: *Thermus thermophilus* str. HB8; g: *Chloroflexus aurantiacus* fl10. Organizations h–j are from putative As^{III}-oxidizing Archaea. h: *Pyrobaculum calidifontis*; i: *Sulfolobus tokodaii*; j: *Aeropyrum pernix*. *aioS*, sensor histidine kinase gene; *aioR*, transcriptional regulator gene; *aioX*, As(III)-binding protein gene *aioA*, As^{III} oxidase large subunit gene; *aioB*, As^{III} oxidase small subunit gene; *cytC*, cytochrome *c* gene; *moeA*, molybdenum cofactor biosynthesis gene; *arsA*, As^{III}-pump ATPase gene; *arsD*, As resistance operon repressor gene; *X*, hypothetical protein gene; *transloc*, translocase gene; *luxR*, transcriptional regulator gene; *ABCtrans*, ABC transporter metal-binding protein gene; *lysE*, lysine exporter protein gene; *sulfocya*, sulfocyanin gene; *asnC*, transcriptional regulator gene; *trpE*, anthranilate synthase component I gene; *trpG*, anthranilate synthase component II gene.
Adapted from Osborne and Santini [36].

the operon contains *aioS* and *aioR* genes, coding for a two-component system, AioS (sensor kinase)/AioR (regulator), which regulates the expression of the *aio* genes [37–41]. However, these genes are not present in other phyla (see Fig. 2f–g; for a recent review see [42]). In addition to the experimentally confirmed *aio* clusters, several Archaea were observed to contain *aio* genes (Fig. 2h–j) for which, however, no As^{III}-oxidation was demonstrated so far. Only *Sulfolobus acidocaldarius* BC, a close relative of *Sulfolobus tokodaii*, has been proposed to do so [43]. Unfortunately, no genome sequence is yet available for this strain.

2.1.3. Redox and functional properties of Aio

The two electrons coming from the oxidation of As^{III} to As^{V} are assumed to reduce the molybdenum (Mo) center (which can accept up to two electrons), from where they pass through the [3Fe–4S] center towards the [2Fe–2S] center (see Fig. 1b). The [3Fe–4S] cluster lies ~12 Å from the Mo atom [23]. The first electron transfer step has been proposed to be mediated by a complex network of hydrogen-bonding interactions, between the pyrazine system of the pterin cofactor and the [3Fe–4S] cluster [23]. The two Fe–S clusters are separated by a distance of ~13 Å. The shortest pathway considered for the passage of electrons from the [3Fe–4S] center to the [2Fe–2S] center is through Ser99 of AioA and His62 of AioB [23] both conserved in all AioA and AioB proteins, respectively [31,34].

The co-purification of cytochromes with the enzyme [22,25,29], the presence of cytochrome-encoding genes in *aio* gene clusters (see above), and the fact that As^{III} oxidation in *Ochrobactrum tritici* requires the cytochrome encoded in the *aio* operon [39], all suggest the As^{III} oxidation to result in the ultimate reduction of a soluble cytochrome at least in Proteobacteria. The physiological electron acceptor of Aio in other organisms has not been proven. Auracyanin may be the electron acceptor in *Chloroflexus aurantiacus* during photosynthesis, since azurin, another cupredoxin-type copper protein, has been shown to be reduced by several Aio [22,29]. Photooxidation of both auracyanin and AioB has indeed been observed [44,45]. We also observed the presence of a gene coding for another cupredoxin-type protein, sulfocyanin, in the *aio* cluster from *Sulfolobus* (Fig. 2i). Kinetic data either using artificial (DCPIP) or proteinaceous (cytochrome or azurin) electron acceptors, have been determined (Table 1). The K_m for As^{III} appears to be an order of magnitude smaller when using proteinaceous (*A. faecalis* and *Ralstonia* studies) instead of artificial electron acceptors (NT-14, NT-26 or *Arthrobacter* studies) and the V_m determined using physiological cytochrome of *Ralstonia* [29] exceeds the values obtained, with the same enzyme (from *Ralstonia*), using non-physiological electron acceptors by an order of magnitude. These results suggest that the experimentally determined activity of Aio is highly dependent on the electron acceptor and a more generalized use of physiological electron acceptors might be considered for future kinetic analyses of Aio. Proteobacterial Aio was furthermore observed to display strong selectivity between various cytochromes and cupredoxins [22,29,46]. In line with all these

Table 1
Comparison of published properties of Aio As^{III} oxidases and Arr As^{V} reductases determined in solution.

	Aio *A. faecalis*[a]	Aio NT-14[b]	Aio NT-26[c]	Aio *Arthrobacter*[d] str. sp.	Aio *Ralstonia*[e] sp. 22	Arr *C. arsenatis*[f]
Location	Membrane	Periplasm	Periplasm	Membrane	Membrane	Membrane
Native mol. wt (kDa)	100	309	219	100	110	123
Subunit composition	α 85	α 86	α 98	α 85	α 97	α 87
	β 15	β 16	β 14	β 14	β 16	β 29
Oligomeric state	$\alpha_1\beta_1$	$\alpha_3\beta_3$	$\alpha_2\beta_2$	$\alpha_1\beta_1$	$\alpha_1\beta_1$	$\alpha_1\beta_1$
Cofactors	Mo, Fe, S	Mo, Fe, S	Mo, Fe, S	n.d.	n.d.	Mo, Fe, S, Zn
K_m (μm)	8[g]	35[h]	61[h]	26[h]	7[i]	300[j]
V_{max} (μmol min^{-1} mg^{-1})	2.88[g]	6.1[h]	2.4[h]	2.45[h]	5.7[h] 140[i]	7013[k]

n.d. not determined.
[a] From [22].
[b] From [25].
[c] From [24].
[d] From [28].
[e] From [29].
[f] From [49].
[g] Calculated using azurin co-purified with Aio from *A. faecalis*.
[h] Calculated using the artificial electron acceptor 2,6-dichlorophenolindophenol (DCPIP).
[i] Calculated using cytochrome c_{554} from *Ralstonia* str. sp. S.22.
[j] For Arr K_m is given towards As^{V}, whereas for Aio K_m is given towards As^{III}.
[k] For Arr V_m is given in As^{V} reduced, whereas for Aio V_m is given in As^{III} oxidized.

results, higher k_{cat}-values were reported using cyclic voltammetry (independent of any electron acceptor [47]) than for the enzyme in solution using DCPIP. Irrespective of experimental conditions, the V_m values reported for Aio are astonishingly low if compared to usual enzyme turnover values, for example those measured on its sister enzyme Arr (Table 1). As detailed below, this may be related to the electrochemical properties of Aio's cofactors.

Since the three centers of Aio feature optical absorptions and possess paramagnetic states, both optical and EPR spectroscopies have been used to characterize the redox properties of these cofactors. The obtained results suggest an unusual situation in the Aio's electron transport pathway. First of all, to date, no Mo^{V} state has been detected [22]. This result may suggest that the only stable redox states of this center are Mo^{IV} and Mo^{VI}, which are both EPR silent. The observed reduction of Mo^{VI} to Mo^{IV} in *A. faecalis* Aio, in cyclic voltammetry experiments, as a cooperative 2-electron process [47], favors this hypothesis. It is of note that this view was challenged in an independent study on Aio from NT-26 by cyclic voltammetry, interpreted as supporting a one-electron rate-limiting step during turnover [48]. A strongly cooperative 2-electron transition, implying a very low stability constant for the Mo^{V} state, would suggest the possibility that electrons might bifurcate from the Mo-pterin center towards each of the iron–sulfur clusters which can only receive a single electron at a time. The second remarkable fact consists in the redox potential values measured so far for the Mo-center ($E_{mpH6} = +270$ mV), the [3Fe–4S]-cluster ($E_{mpH6} = +270$ mV), the [2Fe–2S]-cluster ($E_{mpH6} = +150$–220 mV) and the soluble carrier ($E_{mpH6} = +240$–250 mV), [29,31,46,47] suggesting a globally isopotential electron transport chain except for the transfer from As^{III} ($E_m = +60$ mV) to the Mo center. This low driving force for forward electron transfer may rationalize the very low turn-over of the enzyme (Table 1). It is noteworthy that so far the enzyme Aio has not been shown to be able to perform, even in vitro, the reverse reaction, i.e. reduction of arsenate from oxidation of an artificial electron donor whereas its sister enzyme Arr readily works both ways. This particularity is probably related to specific (still unknown) properties of the cofactors.

2.2. Description of arsenate reductase and alternative arsenite oxidase

2.2.1. Structural description of the Arr/Arx

As compared to Aio, only scant information is yet available on the Arr enzyme. Three representatives of Arr have been studied biochemically: the enzymes from *Chrysiogenes arsenatis* [49], *Bacillus selenitireducens* [50] and *Shewanella* sp. ANA-3 [51]. All three Arrs have been shown to be more or less tightly associated with cytoplasmic membranes and to be facing the periplasm but to be isolated as soluble heterodimeric ArrAB enzymes (Table 1). Recently, an enzyme closely related to Arr with respect to amino acid sequence but physiologically performing As^{III} oxidation instead of As^{V} reduction, named Arx, has been isolated from *Alkalilimnicola ehrlichii* MLHE-1 as an ArxAB heterodimer and has been partially characterized [52,53].

Sequence and metal analyses (no 3D-structure is presently available) [49,50] have established that Arr/ArxA (around 850 residues) also belongs to the CISM family. The protein indeed exhibits the conserved motifs of the Mo-*bis*PGD- and the [4Fe–4S]-binding domains. A conserved Cys, thought to be the protein ligand to the Mo-atom, is observed in all characterized sequences. This Cys-residue structurally seems to correspond to the Cys173 ligating the Mo in the homologous enzyme polysulfide reductase (Psr, see below) [54]. Arr/ArxA contains a Tat signal peptide which allows for its translocation to the periplasm [49–51]. From sequence analysis, Arr/ArxB (about 230 residues) is predicted to contain four [4Fe–4S] clusters, or one [3Fe–4S] plus three [4Fe–4S] clusters [52,55]. Genomic analysis of As^{V}-respiring and As^{III}-oxidizing bacteria using Arr/Arx, identified several strains where a third gene named *arr/arxC* (see Fig. 3), coding for a membrane-integral protein, is part of the *arr/arx* cluster ([4,18,56] and below). It has therefore been proposed that this membrane-integral subunit, devoid of any cofactor, might serve as anchor to the Arr/ArxAB-diade and promote electron transfer between the quinone pool and the enzyme (see [4,18]). However, no heterotrimeric Arr/ArxABC has yet been purified. As inferred from the similarity of all three of their constitutive subunits, Arr/Arx are closely related to the polysulfide reductase Psr, from which one representative (from *Thermus thermophilus*) has been crystallized [54] (Fig. 4a). The overall sequence homology of Arr/Arx with Psr suggests the Arr/ArxC to be part of the complex, similar to the structure of Psr for the cases where an Arr/ArxC gene is observed in the gene cluster.

2.2.2. Genetic organization of arr/arx

Numerous prokaryotes demonstrated to perform As^{V} reduction have not yet been characterized genetically. The *arr* clusters of species demonstrated to physiologically reduce As^{V} all contain *arrA* and *arrB* genes in an *AB* order. In the *arx* clusters of As^{III} oxidizers, two *arxB* homologs are observed flanking the *arxA* gene (Fig. 3f–h). Biochemical characterization of the ArxAB enzyme from *A. ehrlichii* [52] identified the gene downstream *arxA* as coding for the B subunit of the

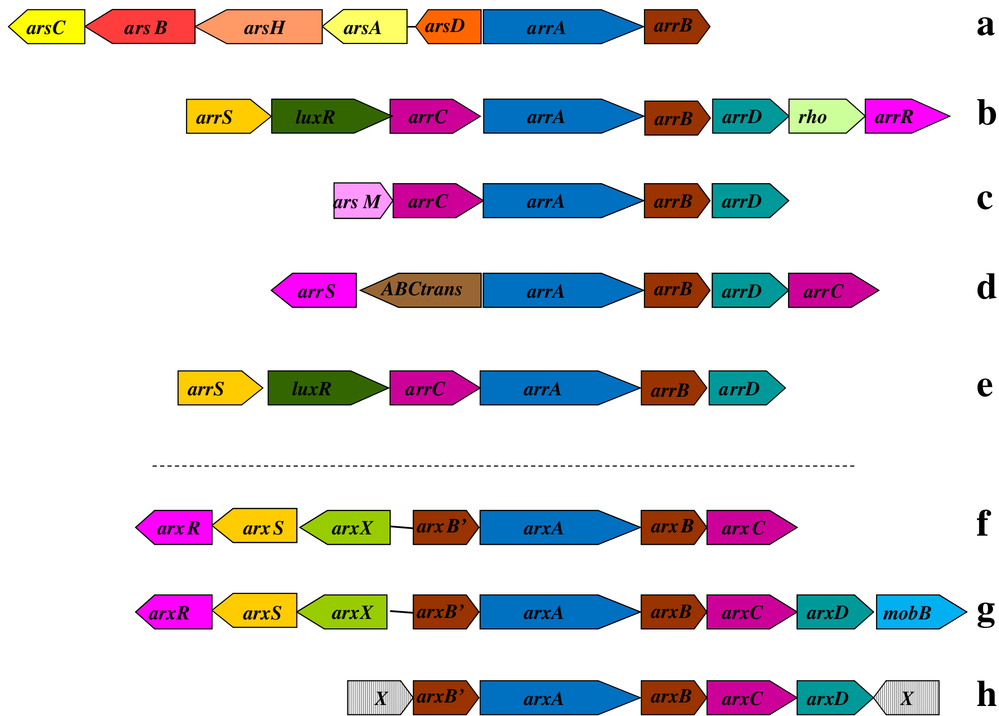

Fig. 3. Organization of observed prokaryotic *arr/arx* gene clusters. Organizations a–e are from demonstrated As^{V}-reducing Bacteria, whereas organizations f–h are from demonstrated As^{III}-oxidizing Bacteria. a: *Shewanella* species; b: *Desulfitobacterium hafniense*, c: *Alkaliphilus oremlandii*, d: *Bacillus selenitireducens*, e: MLMS-1, f: *Alkalilimnicola ehrlichii*, g: PHS-1, h: *Halorhodospira halophila*. *arr/arxA*, arsenate reductase large subunit gene; *arr/arxB* and *arxB'*, arsenate iron–sulfur subunit gene; *arr/arxC*, arsenate reductase membranous subunit gene; *arr/arxD*, arsenate reductase chaperon gene; *arsA*, As^{III}-pump ATPase gene; *arsD*, arsenical resistance operon trans-acting gene; *arsB*, As^{III}-pump protein gene; *arsC*, As^{III} reductase gene; *rho*, rhodanese gene; *arr/arxS*, sensor histidine kinase gene; *ABCtrans*, ABC transporter metal-binding protein gene; *luxR*, transcriptional regulator gene; *arr/arxR*, transcriptional regulator gene; *arxX*, As^{III}-binding protein gene; *mobB*, molybdenum cofactor biosynthesis gene; *X*, hypothetical protein gene.

functional complex, i.e. forming an *arxAB* order. The function of *arxB'* is presently unknown. Only in *A. ehrlichii* does the gene sequence suggest the encoded protein to harbor a cytochromic domain [57]. In several *arr/arx* gene clusters, a gene coding for the above mentioned membranous-subunit has been observed. In the presently known *arr* clusters, this gene is upstream of the *arrA* gene (except in the case of *B. selenitireducens*, Fig. 3d) whereas in the presently known *arx* clusters this gene is downstream of the *arxB* gene (Fig. 3f–h). An *arr/arxD* gene, coding for a chaperon, has often but not always been observed. All other components of the cluster are highly variable (Fig. 3). In several clusters, genes strongly similar to *aioS*, *aioR* and *aioX* genes are present, correspondingly annotated as *arr/arxS*, *arr/arxR* and *arxX*, that may regulate the expression of the *arr/arx* genes. Genes homologous to *luxR*, a transcriptional regulator gene, may

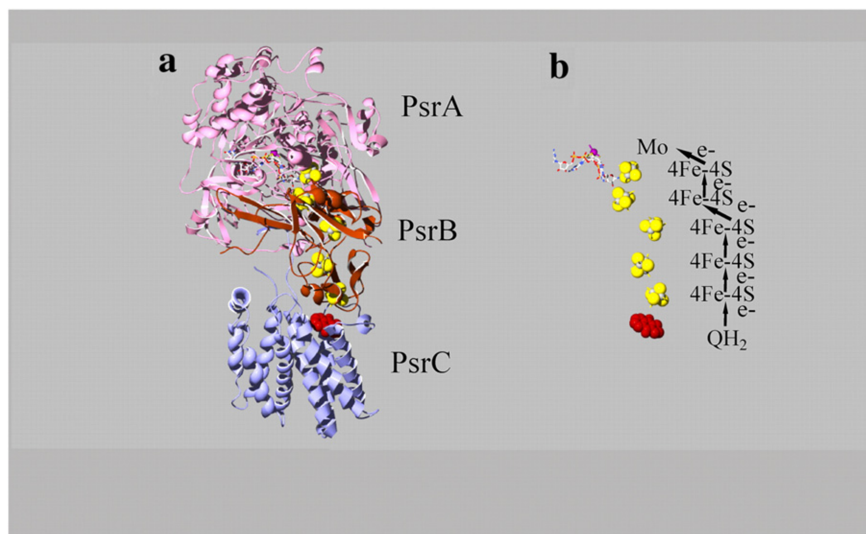

Fig. 4. Structure of *Thermus thermophilus* Psr. (a) Crystal structure of the Psr enzyme from *T. thermophilus* [Jormakka et al. [54]]. The large catalytic α-subunit is shown in pink, the small iron–sulfur β-subunit in deep orange and the membranous γ-subunit in blue. (b) Arrangement of the metal centers involved in electron transfer (4×[4Fe–4S]→[4Fe–4S]→Mo), the electrons are originally coming from liposoluble QH_2 and are finally transferred to As^{V}.

also play a role in this regulation. In *Shewanella* strains (Fig. 3a), the *arr* cluster is adjacent but with opposite directionality to the *ars* operon encoding the arsenic resistance system [55].

2.2.3. Redox and functional properties of Arr and Arx

No redox data is presently available, neither for Arr nor for Arx. Structural homology between Arr and Psr suggests homologous functioning, even similar intra-enzyme electron transfer. That implies that electrons would be transferred from liposoluble menaquinols (MKH_2) via the four Fe–S centers in the ArrB subunit to the [4Fe–4S] and Mo centers of the catalytic A subunit and then onto As^V (Fig. 4b). The oxidation of MKH_2 would therefore be the role of the membranous subunit ArrC, similarly to what has been established biochemically, genetically and structurally for PsrC [54,58]. In *Shewanella* species which do not contain the gene coding for the Arr/ArxC-subunit (Fig. 3a) [55], CymA, a membrane-attached MKH_2 oxidizing protein, has been genetically shown to be required for As^V reduction in the cell [59]. Although no biochemical study presently confirms the oxidation of quinone by the isolated complex, these observations suggest that Arr's function is linked to the liposoluble MK-pool. This MK-pool is replenished by membranous quinone-reductases as demonstrated by the dependence of As^V-reduction to the oxidation of organic electron donors: lactate, acetate, succinate or pyruvate (to mention only the main) [4].

The Arx enzyme has been demonstrated to act as alternative arsenite oxidase, during the anaerobic respiration of either nitrate [6] or selenate [8], or even in anoxygenic photosynthesis [9]. If Arx corresponds to Arr with reverse directionality, as in the case of the succinate dehydrogenase/fumarate reductase couple of enzymes [60], the fate of the electrons resulting from As^{III}-oxidation would be to ultimately reduce liposoluble quinones. However, no direct experimental data testing this hypothesis are presently available.

Although detailed kinetic studies have neither been performed with Arr nor with Arx, both enzymes have been shown to be bi-directional [52] (see although [49]). We thus hypothesize that the electrochemical potential of the quinone available in a given cell determines the enzyme's directionality. Several chemically distinct types of pool-quinones, such as MK-, ubi (UQ)-, plasto-, rhodo (RQ)-, caldariella- or *Sulfolobus*-quinones have been identified so far in the living world [61–63]. The As^{III}/As^V redox couple has an electrochemical midpoint potential of $+60$ mV (with respect to the standard hydrogen electrode, SHE [64]). To be competent for electron transfer, a quinone must be substantially more reducing than the As^{III}/As^V couple in the As^V reduction pathway and substantially more oxidizing than this couple in the As^{III} oxidation pathway. Whereas MK- and RQ-quinones feature a similar redox midpoint potential of -70 mV, UQ-, plasto-, caldariella- and *Sulfolobus*-quinones have more positive redox midpoint potentials at around $+100$ mV. Only the first two quinones therefore appear energetically favorable to function in As^V reduction. The latter four quinones, by contrast, seem energetically well-suited for As^{III} oxidation.

We have performed a genomic survey searching for quinone biosynthesis pathway genes in order to establish the types of quinones used in the various species containing *arr*/*arx* genes (Table 2). While two distinct MK biosynthesis pathways are known in prokaryotes (*men* and *mqn* pathways), only one such pathway is known for UQ/RQ biosynthesis (*ubi* pathway, for a recent review see [65]). We therefore searched for the respective genes in available sequenced genomes. When genomes were not available (*Sulfurospirillum barnesii*, *Halarsenatibacter silvermanii*, *C. arsenatis*, *Bacillus arseniciselenatis*) or when pathways could not clearly be identified in the genome (*Alkaliphilus oremlandii*, *Natranaerobius thermophilus*) the quinone content was determined biochemically. Only the *ubi* pathway was detected in the genome of *A. ehrlichii*. Although a gene coding for an enzyme (YP_428309) involved in the transformation of UQ to RQ has recently been identified in *Rhodospirillum rubrum* [66], it is not certain whether this is the only enzyme involved in this transformation and whether the pathway is

Table 2
Distribution of quinone, among Arr and Arx harboring species. When neither MK nor UQ/RQ biosynthesis pathway was clearly identified by genomic analysis, we performed biochemical analysis of bacterial membranes. In the case of *Alkalilimnicola ehrlichii*, a biochemical analysis was performed in addition to genomic analysis in order to search for eventual RQ (see the text). Only the group of Arr containing bacteria presents species synthesizing MK alone; only the group of Arx containing bacteria presents a species synthesizing only UQ. Question marks denote currently unknown.

Species	Enzyme-type	Genomic analysis	Biochemical analysis
Alkalilimnicola ehrlichii	Arx	UQ	UQ
Halorhodospira halophila	Arx	UQ/MK	UQ/MK
Ecthothiorhodospira PHS-1	Arx	UQ/MK	
Wolinella succinogenes	Arr	MK	
Sulfurospirillum barnesii	Arr	?	MK
Desulfosporosinus youngiae	Arr	MK	
Desulfitobacterium hafniense Y51	Arr	MK	
Desulfitobacterium hafniense DCB-2	Arr	MK	
Bacillus arseniciselenatis	Arr	?	MK
Bacillus selenitireducens	Arr	MK	
Denitrovibrio acetiphilus	Arr	MK	
Chrysiogenes arsenatis	Arr	?	MK
Desulfurispirillum indicum	Arr	MK	
Geobacter lovleyi	Arr	MK	
Burkholderiales bacterium 1-1-47	Arr	MK	
Parasutterella excrementihominis	Arr	MK	
Desulfonatronospira thiodismutans	Arr	MK/UQ	
Alkaliphilus oremlandii	Arr	?	MK
Alkaliphilus metalliredigens	Arr	?	
Halarsenatibacter silvermanii	Arr	?	
Natranaerobius thermophilus	Arr	?	?
Ferrimonas balearica	Arr	MK	
Shewanella piezotolerans	Arr	UQ/MK	
Shewanella sp. ANA-3	Arr	UQ/MK	
Shewanella putrefaciens	Arr	UQ/MK	
Shewanella sp. W3-18-1	Arr	UQ/MK	
MLMS-1	Arr	MK	

unique. We therefore confirmed the quinone content of *A. ehrlichii* biochemically. In both the Arr and the Arx clades, several species synthesize both UQ and MK (Gammaproteobacteria such as *Halorhodospira halophila*, *Shewanella* strains). However, species exclusively synthesizing MK are restricted to the Arr clade (e.g. *Burkholderiales bacterium* 1-1-47, *Wolinella succinogenes*). On the other hand, the sole species that uses only the UQ biosynthesis pathway, i.e. *A. ehrlichii*, is a member of the Arx clade.

We thus stipulate that all Arr-harboring strains oxidize MK pool-quinols via an As^V reduction process whereas the Arx-harboring strains reduce UQ pool-quinones via an As^{III} oxidation process (Fig. 5). This would therefore be fully analogous to the behavior of quinol-fumarate reductase in *Escherichia coli*, which uses MK for reduction of fumarate and UQ for oxidation of succinate [60].

3. Diversity of pathways involving arsenic bioenergetic enzymes

3.1. Pathways involving Aio

Studies dedicated to establish the precise role of Aio in diverse bioenergetic chains are scarce. We have therefore used thermodynamics, electrochemical and mechanistic arguments to propose possible roles in Fig. 5. Bacteria using Aio to oxidize As^{III} can be divided into two groups: (i) chemolithoautotrophs (aerobes or anaerobes, using As^{III} as the electron donor and CO_2/HCO_3^- as the sole carbon source) and (ii) heterotrophs, growing in the presence of organic matter (for reviews see [4,26]).

Most As^{III} oxidizers characterized so far are mesophilic aerobes, in which the final electron acceptor of the metabolic pathway is O_2, probably via a cytochrome *c*/HiPIP/cupredoxin O_2-reductase (see [25]; Fig. 5a). There are furthermore several bacterial strains containing *aio* genes which oxidize As^{III} anaerobically using various electron acceptors [7,13,14]. Under such conditions, Aio is likely to be part of the anaerobic

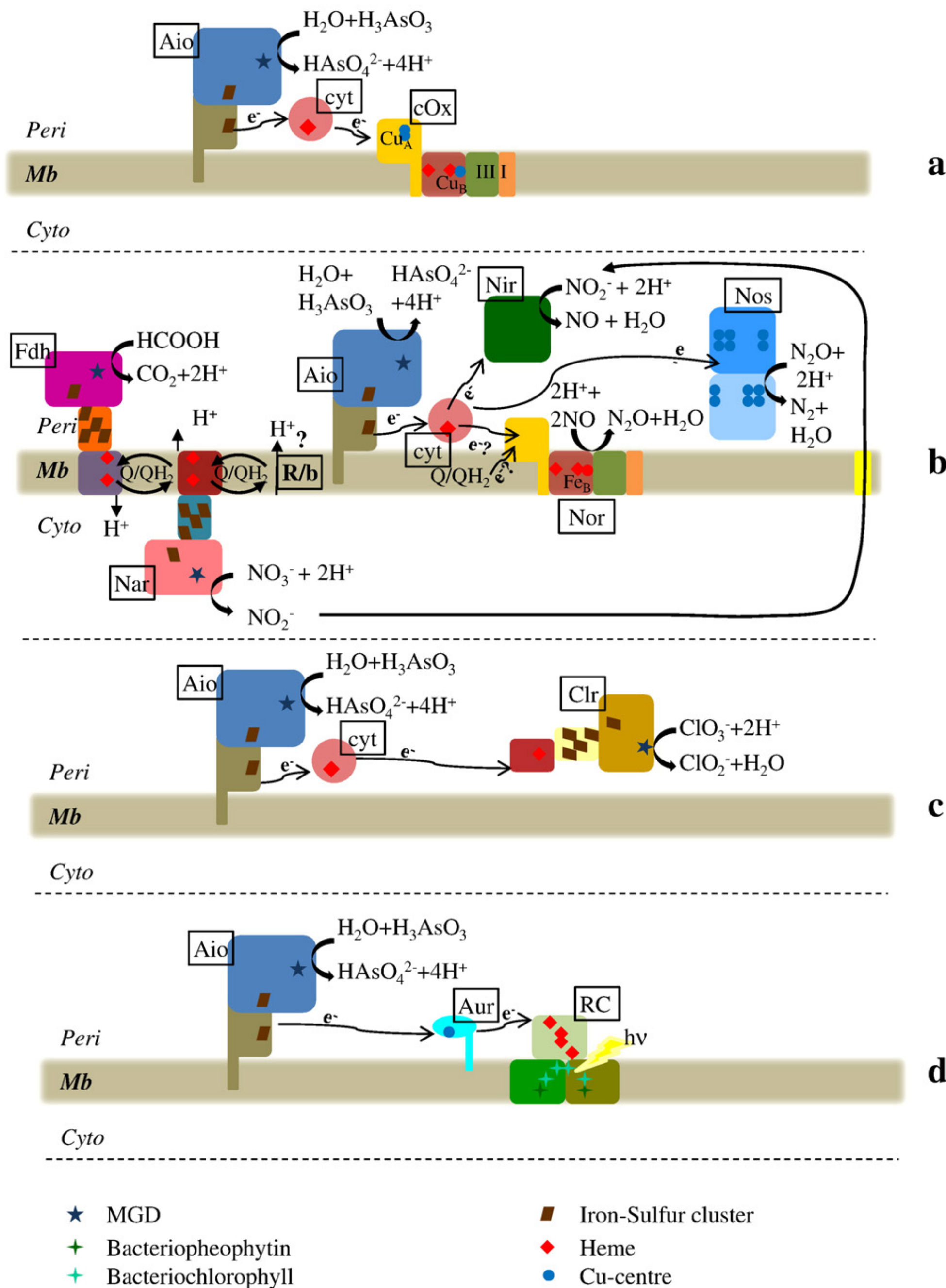

Fig. 5. Possible metabolic pathways involving Aio. (a) Putative aerobic chain involving Aio in *Herminiimonas arsenicoxydans*. Aio, soluble cytochrome (cyt) and cytochrome-oxygen oxido-reductase (cOx) (YP_001101152–YP_001101153) would take part in this chain. (b) Proposed denitrification chain involving Aio in DAO10 strain. Formate dehydrogenase (Fdh), membrane-bound nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor) and nitrous oxide reductase (NosZ) would take part in this chain. The participation of the Rieske/cyt*b* (R/b) complex in this chain would enhance production of the proton gradient. (c) Possible chlorate reduction linked to Aio. Aio, soluble cytochrome and cytochrome-chlorate oxido-reductase (Clr) would take part in this chain. (d) Proposed photosynthetic chain involving Aio in *Chloroflexus aurantiacus* fl-10. The electrons coming from the oxidation of As^{III} would reduce the membrane-bound auracyanin which in turn would serve as reductant to the photosynthetic reaction center (RC).

respiratory pathway. For example, two Proteobacteria are known to use Aio to oxidize As^{III} coupled to nitrate (NO_3^-) reduction [7,11] suggesting a link between Aio and the denitrification pathway. Neither biochemical nor genomic data are presently available on these strains that would allow drawing a precise picture of the pathway. The fact that Aio reduces periplasmic soluble electron carriers (see Fig. 3), however, suggests soluble periplasmic nitrite reductase as the most probable denitrification enzyme deriving its reductant from As^{III} oxidation (Fig. 5b). This would suggest that NO_2^- rather than NO_3^- was the direct substrate of the chain. In the case of the DAO10 strain, this NO_2^- may have been produced from NO_3^- reduction linked to formate oxidation via a formate dehydrogenase/nitrate reductase (Fdh/Nar) redox loop. Since N_2 was found to be produced and since furthermore a *nosZ* gene was identified, coding for N_2O reductase, it has been proposed that the full denitrification chain participates in this metabolism [7]. We therefore propose that in this strain As^{III} oxidation links to denitrification at the level of nitrite- rather than the nitrate-reduction (Fig. 5b). The participation of the Rieske/cyt*b* complex in this chain would enhance the proton gradient production.

A link between Aio activity and chlorate reduction has also been shown in *Dechloromonas* and *Azospira* strains [13]. Chlorate reductase (Clr) from *Ideonella dechloratans* [67], the only well-known case, is a

soluble periplasmic enzyme receiving electrons from soluble cytochrome [68] (for a general presentation of the system see also [69]). Although the presence of *clr* genes has not been confirmed in these *Dechloromonas* and *Azospira* strains, we hypothesize that a chain involving the Clr enzyme as depicted in Fig. 5c is conceivable. The presence of *cld* genes (coding for chlorite dismutase, the enzyme responsible for the terminal step of chlorate reduction; see also [69] for presentation of this system) has indeed been confirmed in these *Dechloromonas* and *Azospira* strains [13].

Finally, it has been suggested that Aio may be linked to photosynthesis in certain organisms. Genomic surveys have revealed three photosynthetic bacteria containing *aio* genes [17,18]. One of them, *C. aurantiacus*, has been shown to express the enzyme during photosynthetic growth [17,18]. No experimental data have yet unambiguously shown that electrons from Aio-mediated As^{III} oxidation reduce the photooxidized reaction center; however, illumination has been shown to induce oxidation of both auracyanin and Aio-Rieske [44,45], in fragmented systems, suggesting that these components may indeed be involved in the photosynthetic pathway. A possible chain rationalizing these results is depicted in Fig. 5d.

3.2. Pathways involving Arr and Arx

Historically, the enzyme Arr was first shown to play the role of an anaerobic terminal oxidase in prokaryotic energy conversion, and thus to be situated at the opposite end of bioenergetic electron transfer chains with respect to Aio. In all known As^{V}-respirers, various carbon sources (e.g. lactate, succinate, pyruvate) are the initial electron donors. As discussed above, depending on species, ArrC, CymA or further still unknown quinol binding proteins are thought to play the role of the quinol oxidizer for the ArrAB system. Arr is therefore part of a typical anaerobic respiratory chain, with organic donors feeding electrons into the liposoluble quinone pool from which Arr draws the electrons required for As^{V} reduction. In the case of *Shewanella* species for example (Fig. 6a), lactate is oxidized to produced acetate [55], in line with the presence of a lactate dehydrogenase in *Shewanella* ANA-3 genome (Ldh; YP_871138). This reaction produces NADH which we propose to be oxidized by Complex I (ClpxI), thereby reducing the MK pool from which CymA draws electrons to ultimately funnel them towards an ArrAB enzyme.

Whereas respiration of As^{V} using Arr has been discovered as early as 1994 [3], the As^{III}-oxidizing function of Arx has only been discovered in 2007 and shown to be connected to the reduction of nitrate [6]. Since Nar oxidizes quinols to reduce NO_3^- (for reviews see [70,71]), the electron transfer link via quinones between Arx and Nar appears straightforward, though still to be proven experimentally. The two enzymes would form a redox loop reminiscent of the well-known Fdh/Nar system in *E. coli* (Fig. 6b compared to 5b; see [71] for in depth presentations of these systems). The observation [6] that NO_2^-, produced through the reduction of NO_3^- in *A. ehrlichii*, is not further reduced under culture conditions [6] strongly suggests that, in this case, the NO_3^- reduction step is directly linked to the As^{III} oxidation. In line with this hypothesis, *narGHJI* genes (mlg_1003-1000) are identified in the genome, but *nirS*, *nirK* and *nor* genes are all absent.

Although a functional implication is suggested by growth experiments, the detailed molecular make-up of photosynthetic pathways implying Arx still needs to be characterized. *Ectothiorhodospira* sp. PHS-1, a gamma-Proteobacterium of the family Ectothiorhodospiracea, contains Arx which has been shown to oxidize As^{III} during photosynthesis [9]. Our genomic analysis for quinone biosynthesis revealed that PHS-1 contains both MK and UQ biosynthesis pathways (Table 2). UQ-reduction by Arx, energetically allowed in contrast to reduction of the low potential MK, is therefore possible. The electrons derived from the oxidation of As^{III} by Arx, although less reducing than those derived from Na_2S, can be used for CO_2-fixation through a reversed electron flow [9]. The corresponding predicted pathway is depicted by Fig. 6c, with Arx feeding electrons into the liposoluble quinone pool from which Complex I draws the electrons required for NAD reduction using proton motive force to push uphill electron flow. ATP is produced via cyclic photosynthetic electron flow involving a Rieske/cyt*b* complex. Our own results with *H. halophila* [van Lis, unpublished results] show that Arx may feed electrons to a quinone pool, i.e. UQ, which is distinct from the one involved in cyclic electron transfer, i.e. MK, through the Rieske/cyt*b* complex and the photosynthetic reaction center [72].

The question as to how the strain ML-SRAO [8] couples As^{III} oxidation to selenate reduction still remains unanswered. We tentatively propose a pathway similar to that established in *Thauera selenatis*, in which electrons originating from the Arx enzyme are channeled towards selenate reduction via the liposoluble quinone pool, the Rieske/cyt*b* complex and a soluble cytochrome, and eventually to selenate reductase [73] (Fig. 6d). However, the genome of this strain is not yet available for analysis.

4. Evolutionary aspects of arsenic metabolisms

4.1. General principles of phylogenetic analysis

When only a few representatives of an enzyme family have been characterized biochemically, drawing phylogenetic conclusions isn't straightforward. This is the case for Aio and even more for Arr and Arx. Several phylogenetic trees have been published on partial Aio and Arr/Arx sequences amplified from environmental samples. These sequences probably represent true Aio, Arr and Arx enzymes, but they cannot be directly compared with one another since they have been obtained with different primers; nor can they be aligned with paralogous sequences (see below) since they always correspond to less than 150 residues of the full-length sequence. It is tempting, therefore, to retrieve full-length sequences for analyses through BLAST searches of genome databases. However, it would be premature to assign a physiological role to a given gene product only based on sequence homology. In-depth examination of gene cluster organization (presented in Sections 2.1.2 and 2.2.2) and scrutiny of retrieved sequences for conserved motifs (see [18]; presented in Sections 2.1.1 and 2.2.1) can help to lower the chance for misinterpreting gene clusters (see [9] in conflict with [74]).

The reconstruction of the phylogenetic tree is based, in a first step, on the automated multiple alignment ClustalX algorithm. The resulting tree correctly revealed the clades of Aio, Arr/Arx, Psr, and Nar but the phylogenetic relationships between these subfamilies lacked robustness. To improve the reliability of the multiple alignments, sequence alignments were corrected based on available 3D structures when possible. X-ray structures have been reported for members of three of the four enzyme families dealt with in this contribution, i.e. Nar (PDB ID: 1Y4Z), Aio (PDB ID: Q7SIF4) and Psr (PDB ID: 2VPZ). Unfortunately, no structure is yet available for Arr.

For interpretation of the obtained results, the presence of well-separated clusters of archaeal and bacterial sequences in phylogenetic trees, and sometimes even just the existence of both archaeal and bacterial representatives, are frequently taken as evidence for the occurrence of the respective enzyme in the last universal common ancestor (LUCA). However, early but post-divergence horizontal gene transfer between Archaea and Bacteria would yield equivalent phylogenies. To lower this ambiguity, the respective trees must be rooted by paralogous sequences whenever possible. In our case, each of the considered subfamilies (Aio, Arr/Arx, Psr and Nar in Fig. 7) constitutes a root for the other families. A position of the root between Archaea and Bacteria is then used as a criterion for likely pre-divergence origins. Furthermore, the degree of global congruence of a given protein tree with current species trees should also be taken into account.

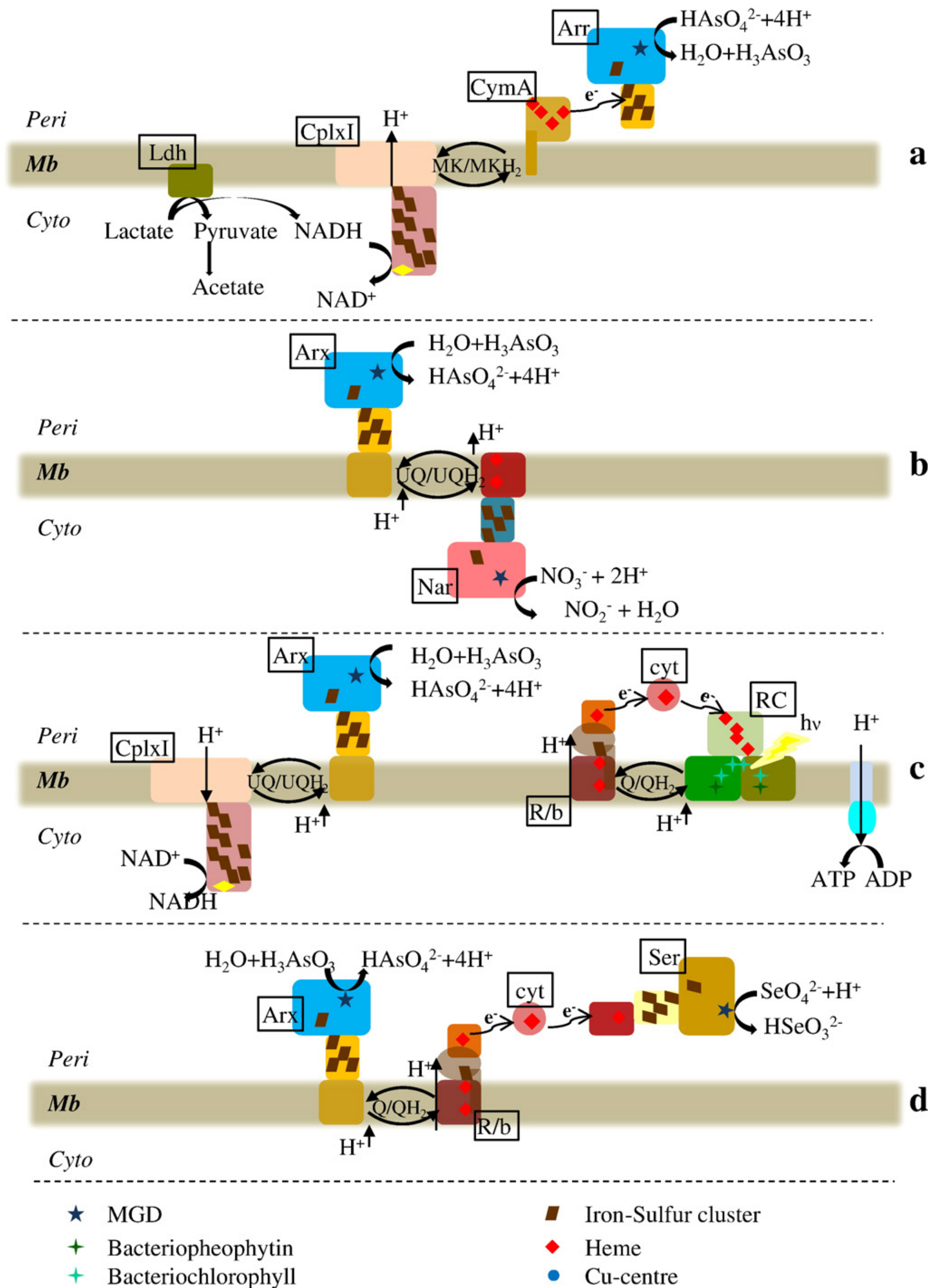

Fig. 6. Possible metabolic pathways involving Arr/Arx. (a) Classic As^V respiration involving Arr. In such a chain, organic carbon compounds are the initial electron donors to quinone reductases (for example *Shewanella* ANA-3 [Saltikov and Newman [55]). Arr is considered to draw the electrons required for As^V from the reduced quinol pool provided by Complex I (CplxI). We propose that the oxidation of lactate by a lactate dehydrogenase (Ldh) produces NADH oxidized in turn via the CplxI. (b) Nitrate reduction linked to As^{III} oxidation by Arx established for *Alkalilimnicola ehrlichii* [Hoeft et al. [6], Richey et al. [52]]. In this chain Arx would reduce UQ which is subsequently oxidized by Nar to reduce NO_3^-. (c) Photosynthetic chain involving Arx as may be imagined for PHS-1. In such a chain, Arx wouldn't be able to provide electrons to photosynthesis (in contrast to Aio), since it is likely to reduce the UQ pool. We propose that UQH_2 resulting from As^{III} oxidation would in turn be oxidized by CplxI (using the proton motive force for reverse electron flow) to produce NADH. This NADH would be the reductant for CO_2 assimilation established by Kulp et al. [9]. The reaction center would provide ATP through photoinduced cyclic electron transfer.

4.2. Phylogenetic analysis of Arr/Arx

A recent molecular phylogeny of selected subfamilies of the CISM superfamily of Mo-*bis*PGD-containing enzymes, including Aio and Arr/Arx, is shown in Fig. 7. This tree includes all (3 Arx and 24 Arr, given in Table SI1) full-length and unequivocal sequences identified by the most recent works published by Zargar et al. [53,57] and Richey et al. [52]. It therefore excludes those from *Geobacter uraniireducens* Rf4 (ZP_01140714), *Magnetospirillum magnetotacticum* (ZP_00053862) and *Desulfosporosinus* sp. Y5 (ABB02056). The corresponding gene clusters of the two first strains in fact do not feature the typical organization whereas the sequence of the third strain is only partial. This latter

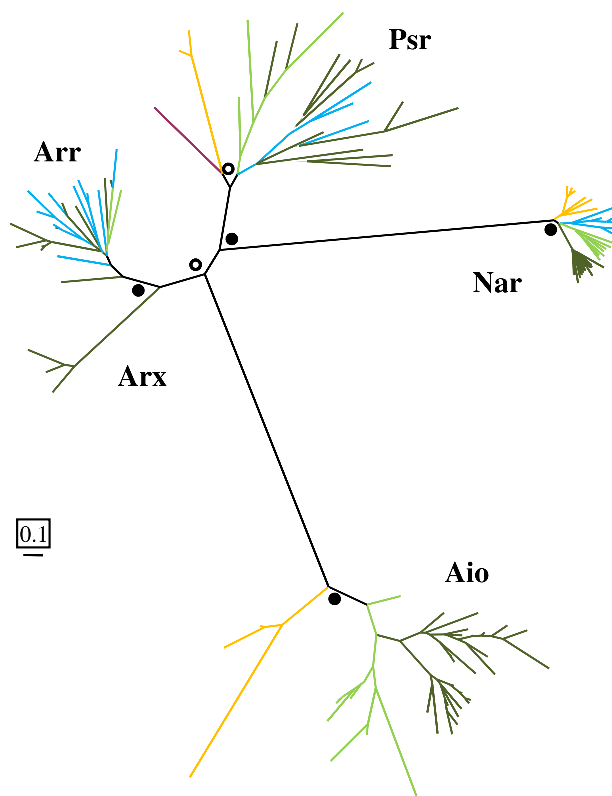

Fig. 7. Phylogenetic tree based on the Mo-*bis*PGD-containing catalytic subunit of selected enzymes from the CISM superfamily. The phylogenetic tree was reconstructed from the alignment of the amino acid sequences (given in Table SI1) based on the ClustalX program improved by structural alignment. The scale bar represents 0.1 changes per amino acid. Open and closed dots indicate bootstrap values for the deep branching exceeding 70 and 90%, respectively. Only the subtrees of As^{III} oxidase (Aio), As^{V} reductase (Arr), alternative As^{III} oxidase (Arx, see below), polysulfide reductase (Psr) and nitrate reductase (Nar) are shown. Violet and orange denote eury- and crenarchaeal branches, dark green, cyan and light green stand for Proteobacteria, Firmicutes and other Bacteria, respectively.

sequence has therefore been replaced by the full sequence from *Desulfosporosinus youngae* (ZP_09653255). The Arr-type sequences branch into two separate clades: "genuine Arr" and Arx. There are a large number of "genuine Arr" sequences coming from beta-, delta-, epsilon-, and gamma-Proteobacteria, Firmicutes, Chrysiogenetes and Deferribacteres, and thus covering a large part of the bacterial domain. However, the topology of the Arr clade differs significantly from that of the bacterial species tree and no archaeal representative has been identified so far. Despite the fact that the Archaea *Pyrobaculum arsenaticum* and *Pyrobaculum aerophilum* have been shown to respire As^{V} [75], no amplification of *arrA* genes has been obtained [56], in line with analyses of archaeal genomes [74].

The Arx clade is typified by the enzyme encoded by the Mlg_0216 gene from the gamma-Proteobacterium *A. ehrlichii*. Further members of this clade are the proteins encoded by the Mlg_2416 gene, also from *A. ehrlichii*, and proteins encoded by the Hal_0353 and ECTPHS_07172 genes from the gamma-Proteobacteria *H. halophila* and *Ectothiorhodospira* sp. PHS-1, respectively [9,52,53,57]. *A. ehrlichii* and *Ectothiorhodospira* sp. PHS-1 have been reported to use Arx to oxidize As^{III} [9,52,57]. Since we recently demonstrated (van Lis, unpublished results) the As^{III} oxidation capacities of *H. halophila* using the enzyme encoded by the Hal_0353 gene, it is highly probable that this phylogenetic clade is homogeneous and contains only enzymes oxidizing As^{III}.

Mining presently available genomes for the putative presence of Arr/Arx therefore yields a phylogenetic subtree that clearly indicates a post-divergence origin for this enzyme. Phylogenetic proximity of Arr/Arx with polysulfide reductase (Psr) furthermore suggests an evolution of the first enzyme from an enzyme involved in dissimilatory reduction of sulfur compounds (Fig. 7; [18]). In contrast to Arr/Arx, Psr (21 sequences used for this study, given in Table SI1) is a textbook example of a pre-divergence enzyme, with a clear cut separation of archaeal and bacterial clusters, a root in between these two and a global congruence of the analyzed tree with current species trees.

4.3. Phylogenetic analysis of Aio

We have expanded the previously published [4,17,18] set of Aio representatives by new full-length sequences retrieved during our present genomic survey (in total 40 sequences, given in Table SI1) (Fig. 7). The Aio "subtree" of the CISM superfamily's catalytic subunit shows a clear-cut split between archaeal and bacterial species and is rooted by the other molybdenum enzymes right in between the two prokaryotic domains. The topology of branching order in both domains corresponds reasonably well to current species trees providing strong support for a pre-divergence origin of this enzyme followed by predominantly vertical inheritance (Fig. 7). This suggests that Aio was present in the common ancestor of Bacteria and Archaea. These phylogenetic results confirm that, rather than representing recent "exotic" adaptations to challenging environments, As^{III} oxidation by Aio would have been a feature of life from its very early age. At first sight this appears contradictory to the relative paucity (as compared to other types of energy metabolisms) of As^{III} oxidizing species identified hitherto. Our suspicion that there are large numbers of As^{III} oxidizers out there is in fact supported by the strong increase in strains reported in the very recent microbiological literature.

4.4. Rationalization of this analysis

The scenario of a bioenergetic role of As^{III} in primordial life has been suggested early on [2] and makes palaeogeochemical sense since arsenic (a) was probably fed in substantial amounts from the Earth's interior to hydrothermal environments frequently considered as vestiges of primordial biochemistry where (b) they certainly have predominantly occurred in their reduced state, As^{III}, due to the absence of molecular oxygen in the environment and the abundance of the reducing agent ferrous iron, Fe^{2+} [76]. Only the global increase in the oxidation state of the environment dated by palaeogeochemists to about 2.5 billion years ago will have tipped this balance over to the oxidized species, As^{V}. Only then could As^{V}-reducing bioenergetic pathways become energetically viable. The notion of an arsenite oxidase as ancient enzyme can therefore be reconciled with these palaeogeochemical considerations.

As detailed above, although Arx is an enzyme typically working in anoxic metabolisms (Fig. 6), actual Arr/Arx phylogeny excludes Arx as responsible for the As^{III} oxidation in primordial life. Of course, the present failure to identify archaeal Arr/Arx sequences and the internal topology of the Arr/Arx clade might be due to sampling bias. However, UQ-usage by Arx also argues against ancient oxidation of As^{III} by Arx. Analysis of quinone-usage among prokaryotes indicates that MK is evolutionarily older than the higher-redox-potential quinones (see for example [77]). UQ likely appeared in the gamma-Proteobacteria [72] as a response to the global environmental oxidation. The oxidation of As^{III} by Arx using UQ is therefore certainly a post-O_2 process and thus a recent enzymatic process.

Aio's phylogeny on the contrary suggests this enzyme to be a likely candidate for having performed As^{III} oxidation in primordial life. To yield biologically relevant work (that is, participate in membrane-potential build-up), the electron transfer reaction from As^{III} to its ultimate electron acceptor must span a sufficiently high difference of electrochemical potential. This implies that the respective electron acceptor must be substantially more oxidizing (typically a few hundred millivolts) than the As^{III}/As^{V} couple. All processes presented in Fig. 5 seem to be potential sources of

sufficiently oxidizing electron sinks for As^{III} oxidation to become an energetically useful process. O_2 is, however, considered to have been absent from the primordial environment and recent phylogenies of O_2 reductases are in agreement with these palaeogeochemical considerations [78,79].

Palaeogeochemical evidence [80] supporting a very early origin for anoxygenic photosynthesis led several authors [81,82] to assume this mechanism to be older than the Archaea/Bacteria divergence. Based on species distributions and especially on the complete absence of – chlorophyll-based – archaeal photosynthesis, the evolutionary community of photosynthetic research instead tends towards an appearance of this bioenergetic process within the bacterial domain [83–85], early but still post-divergence. This would imply that photosynthetic As^{III} oxidation via Aio is post-divergence.

There is only scant data on chlorate reduction. This reaction occurs in two-steps: chlorate reduction and chlorite dismutation ending with chloride (see [69]). Only the chlorate reductase from *I. dechloratans* has been characterized [67] and using this sequence as template for BLAST searches gave only one other representative of the enzyme in *Alicycliphilus denitrificans* strain BC (YP_004124060; see [69,86]). The topology of the published chlorite dismutase-based phylogenetic trees [87,88] moreover suggests this enzyme to have been propagated predominantly via lateral gene transfer. Finally chlorite, chlorate and perchlorate are contaminants predominantly produced by industries that are unlikely to have been present in primordial life although a natural production pathway for these chemicals has also been proposed (see [69] and references herein). Taken together, this suggests the link between Aio and Clr to be recent.

The possibility that intermediates of the denitrification pathway served as early strong oxidants is presently favored. Our recent re-examination of the functional specificities and phylogenies of the various subgroups of heme/copper oxidases (O_2 reductases) led us to propose that these subgroups represent independent evolutionary conversions of an ancestral enzyme, which wasn't an O_2 but a nitric oxide (NO) reductase (containing iron rather than copper in its active site), into proper O_2 reductases [78]. In striking contrast to the situation concerning O_2, an abiotic source for mass production of NO from electrical discharge-induced reactions between N_2 and CO_2 existed in the early Archaean [89]. This atmospherically produced NO is expected to have dissolved into the ocean where it reacted to produce nitrate and nitrite [79]. Phylogenies of nitrate reductases Nar (51 sequences used, given in Table SI1) (Fig. 7) and cd_1 nitrite reductases [90] indeed suggest that these two enzymes are also ancient and thus corroborate the hypothesis of occurrence of denitrification already in the LUCA (for a conflicting interpretation see [71] in this issue).

The synthesis of the considerations outlined above therefore leads us to postulate that an anaerobic type of As^{III} oxidation, linked to the reduction of nitrogen oxyanions, must have been operating in the early Archaean. The thereby biologically produced As^{V}, however, would slowly be reduced by the abundant Fe^{2+} and therefore wouldn't start to accumulate until the increase in environmental redox state (see below). Such an anaerobic oxidation of As^{III} by Aio persists to the present day in specific habitats as exemplified by the DAO10 strain [7].

5. Conclusion

Although a bioenergetic metabolism based on arsenics may appear exotic, the exponential increase in prokaryotic As metabolizing prokaryotes reported in the literature on one hand, and the phylogeny of Aio on the other hand, suggest As-oxidation to constitute one of the oldest energy metabolisms of life. A counterintuitive feature of this metabolism as seen in extant organisms is that As^{V}, while occurring mainly in aerobic environments ensuring sufficient quantities of the oxidized form, is exclusively converted via anaerobic metabolisms whereas As^{III}, more common in anoxic environments, is oxidized via O_2-respiration in most species isolated so far. Proposing an evolutionary scenario for the biological redox conversion of arsenics therefore isn't straightforward. Two distinct scenarios have so far been proposed to explain the evolutionary history of this process. The scenario proposed by Oremland and co-workers [9] postulates that Arr, originally functioning in reverse as observed with extant Arx, was responsible for anaerobic As^{III} oxidation in the Archaean before acquiring the As^{V} reducing function seen in most currently known prokaryotes. In this scenario anoxygenic photosynthetic reaction centers would have provided the ultimate electron acceptor for As^{III} oxidation. Aio would have appeared later in response to the global accumulation of O_2, this gas providing the commonplace ultimate electron acceptor for this enzyme. In a later stage, Arr would acquire its As^{V} reduction function in response to As^{V} accumulation due to the oxidation of the global environment. As we have pointed out above, the putative ancestral Arx-based bioenergetic chains resulting from this scenario face thermodynamic and phylogenetic inconsistencies. The data assembled in this contribution thus favor a different scenario. Aio, integrated in the anaerobic respiration of nitrogen oxides, would have existed already in the anaerobic early Archaean as far back as LUCA. Only after the divergence of Bacteria and Archaea, has Aio been recruited into more recent pathways such as photosynthesis in Bacteria. Aio activity would have been integrated into aerobic respiration only in the Proterozoic era — i.e. after the appearance of atmospheric O_2. The global oxidation of the environment dated to about 2.5 billion years ago would then have led not only to the accumulation of As^{V} but also to the emergence of high-potential quinones. Subsequently, an enzyme initially dedicated to reduction of sulfur compounds and related to the extant Psr enzyme evolved into the As^{V}-reducing Arr enzyme. At roughly the same time, Arx originated by reversing the catalytic reaction of an Arr/Psr-related enzyme to oxidize As^{III}, resulting in reduction of a UQ pool.

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