Microbial Arsenic Metabolism: New Twists on an Old Poison

During the early anoxic phase on Earth, some microbes depended on arsenic to respire

John F. Stolz, Partha Basu, and Ronald S. Oremland

Imagine a planet with an atmosphere lacking oxygen, its landscape dotted with volcanic craters, caustic oceans, and basins of brine. Yet, amazingly, these oceans and brines teem with life, albeit very different from our own—microorganisms that breathe arsenic. That description may describe the Earth during the early Archean eon.

Although breathing arsenic may sound alien, microorganisms that use arsenic oxyanions for anaerobic respiration are found in environmental niches ranging from garden soil and subsurface aquifers to more extreme landscapes such as Mono Lake and Searles Lake in California. Diverse microorganisms metabolize both inorganic and organic forms of arsenic, and their activities are part of a robust biogeochemical cycle. What began more than 15 years ago as our quest to learn how bacteria use arsenate as a terminal electron acceptor led us to discover novel respiratory and photosynthetic pathways.

Summary

- Phylogenetically diverse microorganisms metabolize arsenic despite its toxicity and are part of its robust biogeochemical cycle.
- Respiratory arsenate reductase is a reversible enzyme, functioning in some microbes as an arsenate reductase but in others as an arsenite oxidase.
- As(III) can serve as an electron donor for anoxygenic photolithoautotrophy and chemolithoautotrophy.
- Organoarsenicals, such as the feed additive roxarsone, can be used as a source of energy, releasing inorganic arsenic.

The Biogeochemical Cycle of Arsenic

Arsenic has both toxic and therapeutic properties. For instance, the ancient Greek Hippocrates treated ulcers with realgar, an arsenic sulfide mineral, while the 1908 Nobel Prize was awarded to Paul Ehrlich for his development of salvarsan, an organoarsenical, as a treatment for syphilis. Currently, arsenic trioxide is used for treating acute promyelocytic leukemia.

However, arsenic is perhaps better known as a poison in life and in fiction. Its toxicity depends on both its chemical form, whether inorganic or organic, and its oxidation state. Arsenic occurs in four oxidation states, arsenate [As(V)], arsenite [As(III)], elemental [As(0)], and arsenide [As(-III)], with As(V) and As(III) more abundant in nature. These latter two forms are readily soluble in water, with $H_2AsO_4^-$ and $HAsO_4^=$ typically found in oxidized environments, whereas $H_3AsO_3^0$ and $H_2AsO_3^-$ are more typical of anoxic environments.

Because arsenate is a phosphate analogue, it can enter bacterial cells via phosphate transport systems (Pst and Pit). Once inside, arsenate uncouples oxidative phosphorylation from energy production and also interferes with glycolysis by forming 1-arseno-3-phosphoglycerate in place of 1,3-bisphosphoglycerate. Arsenite enters cells at neutral pH via aqua-glyceroporins, which ordinarily transport glycerol molecules into cells, and binds to sulfhydryl

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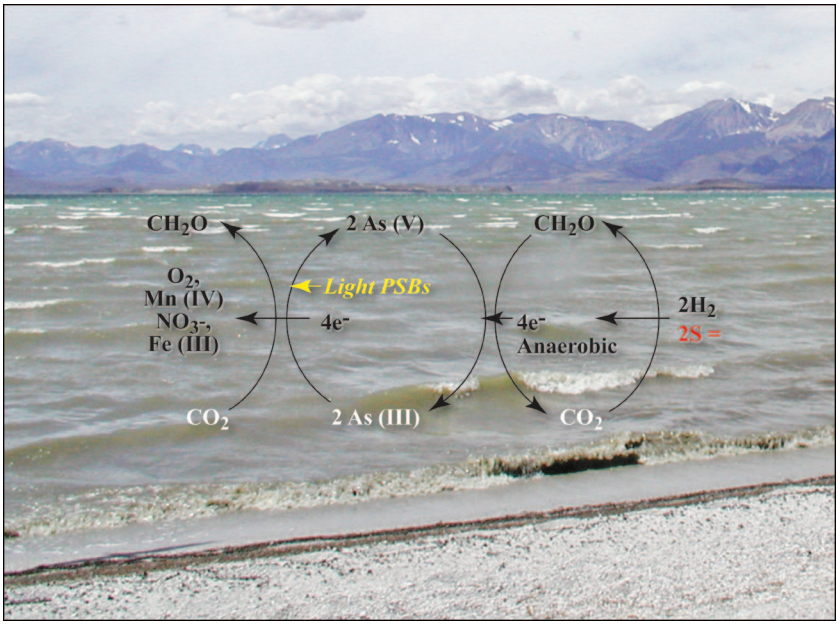
FIGURE 1



The arsenic cycle. As(III) oxidation is driven by light (by phototrophic bacteria, PSBs) or the reduction of O_2, NO_3^-, Mn(IV), or Fe(III). As(V) reduction is coupled to organic matter, H_2S or H_2 oxidation. The background is the view from the northern shore of Mono Lake.

groups of cysteine residues, inactivating the proteins that it modifies. Meanwhile, arsine gas binds to red blood cells, damaging membranes and causing hemolysis. High doses can cause respiratory failure and death, whereas low chronic exposures cause cardiovascular stress, liver disease, and cancers.

Arsenic in the drinking water which resulted in widespread poisoning in Bangladesh and West Bengal led to greater awareness of this element in natural systems. Arsenic-contaminated water sources have since been uncovered in Vietnam, Cambodia, Ghana, Mexico, Chile, Argentina, and Eastern Europe. In the United States, arsenic in drinking water is a concern for about 3,000 municipalities, as the concentration surpasses the maximum allowable concentration (10 ppb).

What are the sources of this arsenic? Although only 0.0001% in crustal abundance, arsenic is distributed widely in weathered volcanic rocks, marine sedimentary rocks, alkaline soils, fossil fuel deposits, iron hydroxides, and in sulfidic minerals such as realgar, orpiment, and arsenopyrite. Anthropogenic sources include mine drainage and tailings, combustion of fossil fuels, irrigation runoff from farm lands treated with arsenic-containing pesticides and herbicides, waste products from the manufacture of glass, pigments, and medicinals, and the wood preservative chromated copper arsenic.

The arsenic in Bangladeshi drinking water, for example, derives from eroded Himalayan sediments. In New England, geologic forces created fingerlings of arsenic-laden granitic rocks that are part of an arsenic crescent running from northern coastal Maine through New Hampshire and central Massachusetts to Rhode Island.

Several processes contaminate aquifers with arsenic, including oxidation of arsenic-containing pyrites; release of As(V) when autochthonous organic matter, including when it is dissolved in recharge water, reduces iron oxides; exchange of adsorbed As(V) with fertilizer phosphates; and recharge with water contaminated with inorganic arsenic from wood preservatives, pesticides such as calcium arsenate, and organoarsenicals such as roxarsone and monomethyl arsonic acid.

Microbial activities are either directly involved or enhance these processes. For instance, in Mono and Searles Lakes in California, arsenic concentrations are high enough to support a rigorous, microbially driven biogeochemical cycle. At the heart of the cycle are oxidation-reduction reactions involving As(V) and As(III) as well as methylated and thioarsenicals (Fig. 1). Because As(V) reduction can be coupled to sulfide oxidation and As(III) oxidation to nitrate reduction, these redox reactions do not depend on oxygen.

Microbial Metabolism of Arsenic

Some microbes not only are resistant to arsenic, but actively metabolize it via methylation, demethylation, oxidation, and reduction reactions, using some of these steps to generate energy. Methylation reactions convert As(V) or As(III) into compounds such as monomethyl arsonate (MMA(V)), methylarsonite (MMA(III), dimethylarsinate (DMA(V)), dimethylarsinite (DMA(III)), and trimethylarsine oxide as well as several volatile arsines, including monomethylarsine, dimethylars-

Stolz: Interested in Arsenic from an Old Place

John F. Stolz grew up in a green, that is, environmentally sensitive household before the term was coined. His parents shunned food additives, cultivated an organic garden, composted kitchen wastes, banned sodas, and promoted recycling. "My dad's favorite saying was 'dump oil,' and that was in the late sixties," Stolz says. Those early practices continue to influence him in both his personal and professional activities. At home, he says, "I go to a butcher that has local beef, buy local produce and free range chickens and eggs." In his lab, he wrestles intellectually with the dangers of arsenic, the compound he studies.

"There is a legacy of environmental arsenic contamination," including from the use of inorganic arsenic compounds as pesticides and herbicides, as wood preservatives, and for raising poultry, Stolz says. Natural processes also mobilize arsenic, helping to account for its presence in drinking water. "We now understand that arsenic can be readily mobilized from rocks and sediments by microbial activity," he says. "These activities can result in the contamination of drinking water. It has been estimated that the number of people affected is in the tens of millions. These compounds are not inert and can be readily transformed to free arsenic. So what started out as a curiosity has taken on more socio/political implications."

Stolz, 54, directs the Duquesne University Center for Environmental Research and Education and is a professor of environmental microbiology. He describes himself as a geomicrobiologist interested in microbes, minerals, and metals. His research, primarily funded by the National Aeronautics and Space Agency (NASA) and the National Science Foundation (NSF), focuses on microbial metabolism of arsenic. "We have been investigating microbial arsenic cycling and the possibility of arsenic-based ecosystems on other planetary systems," he says. "We have been isolating and characterizing microbes that are capable of growing on arsenic. Although it does seem a bit farfetched, you could base an entire ecosystem on arsenic cycling."

Stolz was raised in Syosset, N.Y., on Long Island, and is one of four children. His father, who died nearly five years ago, was a professional pianist and organist. His mother lives in Pittsburgh. "It seems I always wanted to be a scientist," he says. "I was a nerd in high school and did a lot of reading, mostly science fiction, Heinlein, Arthur C. Clarke, C. S. Lewis. I got a telescope in my early teens and that got me excited about space. Growing up during the heyday of the space program and the moon missions helped fan the flames.

"I was a big fan of Carl Sagan in high school," Stolz continues. "There was a chapter in the book on early life on Earth, and I liked the idea of studying past life on Earth to gain insight into what we might find on other planetary systems." But, he adds, "my high school biology teacher, Mrs. Jill Matulewich—who looked a lot like Kim Novak—turned me on to biology." Stolz earned his B.S. in 1977 from Fordham University, and his Ph.D. in 1984 from Boston University. He calls his Ph.D. mentor, Lynn Margulis, his greatest influence. "She taught me the value of collaboration and free thinking," he says. "As students, we read *Genesis of a Scientific Fact* by Ludwig Fleck, and watched endless hours of movies of termite hindgut microbes. I still marvel at the opportunities I had as a graduate student, doing field work in Baja California, Mexico, attending international meetings, participating in NASA and MBL [Marine Biological Laboratory in Woods Hole, Mass.] summer courses, and meeting scientists.

"My first plane flight was a trip to the [San Francisco, California] Bay area to attend the second post-Viking symposium at NASA, Ames," he says. "Lynn lent me her bicycle, and I commuted to NASA from the Stanford University dorms. For a kid who grew up reading about space exploration, it was really exciting to actually be at NASA."

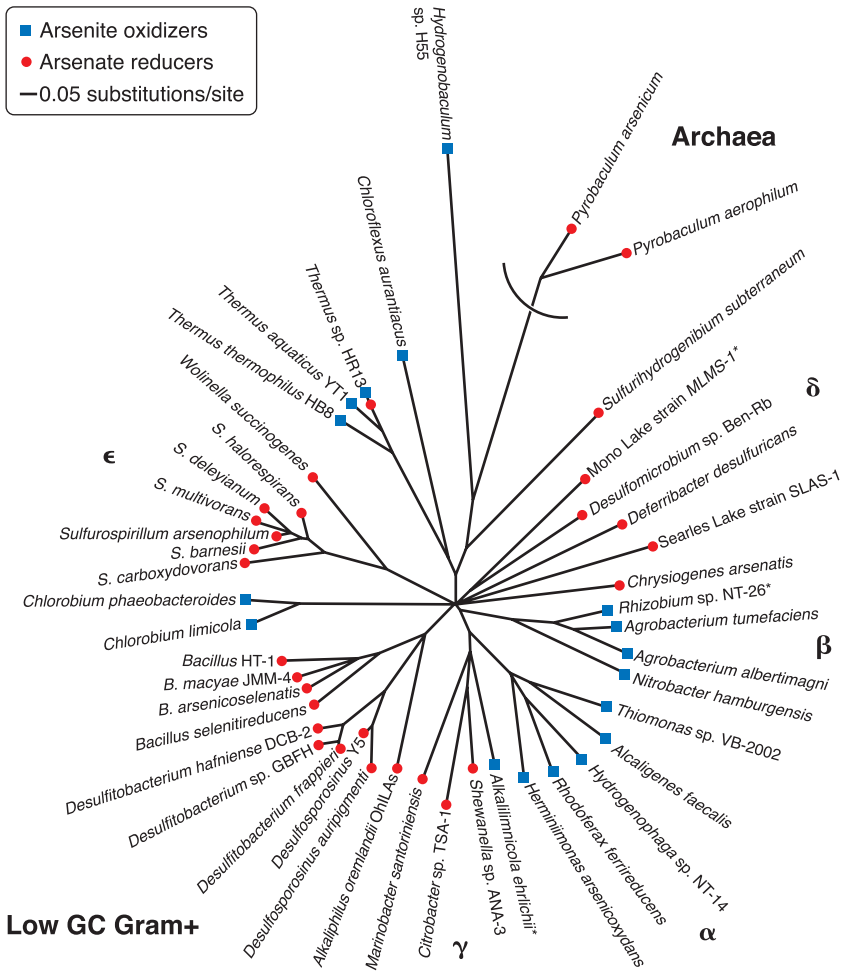
Stolz spent 1984–86 in his first postdoctoral fellowship at the NASA Jet Propulsion Lab in Pasadena, Calif., where he worked with Joe Kirschvink in the department of geology and planetary sciences. "Joe's lab was populated by a remarkable group of students working on bio- and paleo-magnetics," he says. "I worked on magnetotactic bacteria and magnetofossils." From 1987 to 1990, Stolz was a postdoctoral fellow in plant biology at the University of Massachusetts at Amherst, in the lab of Clint Fuller, where he studied photosynthesis in green sulfur bacteria. "I learned about purifying proteins and cloning genes, and to always be open to new research opportunities," he says.

His wife, Donna, is an associate professor at the University of Pittsburgh cell biology department and associate director of its center for biological imaging. She studies liver regeneration. Their daughter is a freshman at the University of Dayton, majoring in environmental biology. Their son, a sophomore at the Pittsburgh High School for Creative and Performing Arts, is studying tuba, piano, and cello.

In his spare time, Stolz enjoys cooking, playing darts, kayaking, and shooting hoops with his son. The two—father and son—also are taking piano lessons. "My dad taught me as a kid, but I hadn't played in decades," he says. "Before he passed away, he started giving my son and me lessons. After he died, it took a while, but we found a really wonderful piano teacher. It's been a great challenge, but I really enjoy tickling the ivories on the Steinway upright."

Marlene Cimons
Marlene Cimons lives and writes in Maryland.

FIGURE 2



Phylogenetic diversity of representative arsenic metabolizing Archaea and Bacteria. Dissimilatory arsenate respirers are indicated by red circles. Arsenite oxidizers are indicated by blue squares. The presence of both a square and a circle indicates an organisms found able to both respire As(V) and oxidize As(III). Chemoautotrophic species are denoted by an asterix.

ine, and trimethylarsine. Although many of the enzymes involved remain unknown, a methyl transferase, ArsM, from *Rhodobacter sphaeroides* confers resistance to arsenic and can generate trimethylarsine.

As(V) can be reduced through two distinct mechanisms. The ArsC system, that confers resistance, reduces As(V) but does not generate energy. Although the resistance genes were originally discovered on plasmids, they have since been found on the chromosomes of a diverse group of organisms, including Archaea, Bacteria, yeasts, and protoctists. Three systems, exemplified by *Escherichia coli*, *Staphylococcus*, and yeast, evolved through convergent evolution. Each system has three components: the arsenate reductase ArsC (or ACR2), a low-molecular-mass protein (13–15 kDa) related to tyrosine phosphate phosphatases; ArsB (or ACR3), the arsenite-specific efflux pump; and a source of reducing equivalents provided by either reduced thioredoxin or glutaredoxin. Additional components include an ATPase (ArsA), which forms an ArsAB complex, and regulatory elements (ArsR, ArsD).

The second mechanism, involving dissimilatory reduction of arsenate, is part of a respiratory pathway. Discovered in *Sulfurospirillum arsenophilum* more than 15 years ago, it was subsequently identified in several Crenarcheota and more than 30 species of Bacteria. In each case, As(V) serves as a terminal electron acceptor (Fig. 2). Typically these organisms are metabolically versatile, using organic (e.g., acetate, lactate) and inorganic (e.g., H_2, H_2S) electron donors as well as other electron acceptors (e.g., oxygen, nitrate, Fe(III), sulfate, and thiosulfate).

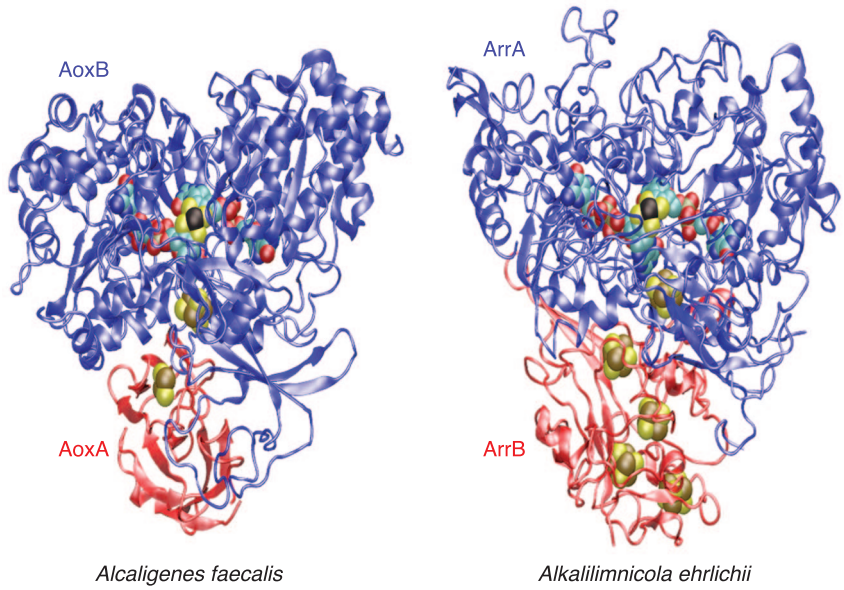
The respiratory arsenate reductase (Arr) is a heterodimer with a catalytic subunit, ArrA, and a smaller electron transfer protein, ArrB. ArrA contains the molybdopterin center and a [3Fe-4S] cluster while the small subunit contains three, possibly four, [4Fe-4S] clusters. The core enzyme, ArrAB, is highly conserved. However, the *arr* operon differs from organism to organism in number of genes. For instance, *Shewanella* sp. strain ANA has only the genes for the core enzyme (*arrAB*), while *Desulfitobacterium hafniense* has a gene encoding a putative membrane-anchoring peptide as well as a multicomponent regulatory system (*arrSKRCAB*).

The microbial oxidation of arsenite was first demonstrated in a bacillus in 1918. Established as a mechanism for detoxification, it has only recently been linked to energy generation. Also phylogenetically widespread, arsenite oxidation occurs in more than 30 strains representing at least 9 genera, including members of the Crenarcheaota, Aquificales, and Thermus, as well as

α-, β-, and γ- proteobacteria (Fig. 2). In most cases, the organisms are aerobic heterotrophs or chemolithautotrophs, using oxygen as the electron acceptor for As(III) oxidation.

More recently, species of anaerobic chemolithoautotrophic bacteria that couple As(III) oxidation to NO_3^- reduction have been found. Arsenite oxidation is carried out by arsenite oxidase (Aox), a heterodimer with a catalytic subunit, AoxB, and a smaller beta subunit, AoxA (Fig. 3). AoxB contains the molybdopterin center and a [3Fe-4S] cluster, while AoxA contains a Rieske-type [2Fe-2S] cluster containing subunit. Although Aox and Arr are both mononuclear molybdoenzymes, they are distinct enzymes. As with the *arr* operons, there is heterogeneity in the *aox* operon. While the order *aoxAB* is conserved, other elements are generally not. Further, homologs of arsenite oxidase are found in the genomes of the Crenarcheota *Aeropyrum pernix* and *Sulfolobus tokodaii*, as well as green anoxyphototrophic bacteria (*Chloroflexus aurantiacus, Chlorobium limicola*, *Chlorobium phaeobacteroides*), *Nitrobacter hamburgensis*, *Rhodoferax ferrireducens,* and *Burkholderia* species (Fig. 4).

FIGURE 3



Structure of arsenite oxidase from *Alcaligenes faecalis* (left) and the respiratory arsenate reductase from *Alkalilimnicola ehrlichii* (right) that functions as an arsenite oxidase. The large, catalytic subunits containing the molybdenum co-factor (AroB and ArrA) are shown in blue. The smaller Rieske subunit of AoxA and the iron-sulfur cluster subunit of ArrB are shown in red. The iron-sulfur clusters are shown in yellow (S) and gold (Fe). Courtesy of Courtney Sparacino-Watkins.

Respiratory Arsenate Reductase Is a Reversible Enzyme

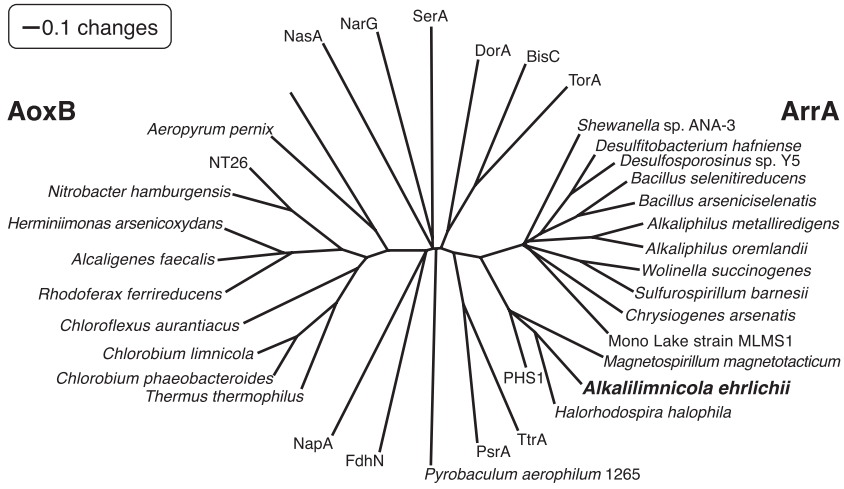
The efforts of one of us (RSO) to dissect the biogeochemical cycle of arsenic in Mono Lake resulted in the isolation and characterization of a number of unique haloalkaliphiles, including As(V)-reducing heterotrophs (*Bacillus selenitireducens* and *B. arseniciselenatis*), As(V)-reducing chemolithoautotrophs (strain MLMS-1), photoautotrophic As(III) oxidizers (strains PHS-1 and MLP-2), and chemolithotrophic As(III) oxidizers (*Alkalilimnicola ehrlichii*).

One microbe in particular, *A. ehrlichii* strain MLHE-1^T, can express two completely different physiologies. As an aerobe, it grows heterotrophically, with acetate as the electron donor and carbon source. As an anaerobe, it is a chemolithoautotroph, coupling the oxidation of As(III) to the reduction of nitrate to nitrite. Analysis of its genome revealed several striking features, including operons for nitric oxide reductase (*norDQBC*), nitrous oxide reductase (*nosLYDZR*), and nitrate reductase ($narLXK_2GHJI$). However, the genes for respiratory nitrite reductase (*nirK*, *nirS*) are absent, explaining the organism's inability to denitrify.

More surprising was the failure to find *aox* genes. Using a combination of proteomics and activity assays, we identified an enzyme with arsenate oxidase activity that is a homolog of Arr (Fig. 4). The enzyme with arsenate reductase activity couples the oxidation of As(III) to the reduction of DCIP, benzyl viologen, and methyl viologen. It functions as arsenite oxidase in *A. ehrlichii*, as was confirmed through gene disruption experiments by Kamrun Zargar and Chad W. Saltikov of the University of California, Santa Cruz.

The active Arr is a heterodimer, with a 91-kDa catalytic subunit containing the motif for a

FIGURE 4



Phylogenetic tree (Neighbor Joining) showing relatedness of the catalytic subunit of respiratory arsenate reductase (ArrA) and arsenite oxidase (AoxB) with members of the DMSO reductase family of molybdoproteins. The Arr homolog from *A. erhlichii* forms a separate branch with Arr homologs from PHS-1, *H. halophilus*, and *M. magnetotacticum*. BisC, biotin sulfoxide reductase (*Escherichia coli*); DorA, DMSO reductase (*E. coli*); FdhG, formate dehydrogenase (*E. coli*); NapA, periplasmic nitrate reductase (*E. coli*); NarG, respiratory nitrate reductase (*E. coli*); NasA, assimilatory nitrate reductase (*Klebsiella pneumonae*); PsrA, polysulfide reductase (*Salmonella typhimurium*); SerA, respiratory selenate reductase (*Thauera selenatis*); TorA, trimethylamine oxide reductase (*E. coli*); TtrA, tetrathionate reductase (*Wolinella succinogenes*). The putative arsenate reductase from *Pyrobaculum aerophilum* is also shown.

[4Fe-4S] cluster (C-X_2-C-X_3-C-X_{27}-C) rather than the [3Fe-4S] cluster of AoxB (Fig. 3). The beta subunit, ArrB, is 28 kDa and is predicted to have four [4Fe-4S] clusters (Fig. 3). The *arr* operon from *A. ehrlichii* has additional genes that appear to encode a 45-kDa [4Fe-4S] cluster-containing subunit (ArrB2), a 44-kDa membrane-anchoring subunit (ArrC), and a 33-kDa chaperone TorD (ArrD). Homologs of these genes are found in *arr* operons from other arsenate-respiring bacteria but not *aox* operons.

The arsenate reductase of *A. ehrlichii* is an enzyme that also runs in reverse, reducing As(V) when coupled to oxidation of benzyl viologen or methyl viologen. With these results in mind, we determined that Arr from two arsenate-respiring bacteria, *Alkaliphilus oremlandii* and *Shewanella* sp. strain ANA-3, could also oxidize arsenite. These results reveal that Arr is an oxido/reductase capable of functioning as an arsenate reductase or arsenite oxidase. Physiologically, however, the enzyme works only as an oxidase or reductase. What determines how it functions could depend on electron potentials of metal centers, additional subunits, and how the electron transfer chain is organized in a particular organism.

Arsenite Oxidation Linked to Photosynthesis

Finding arsenite oxidation in periphyton communities and identifying arsenite oxidase homologs in the genomes of *Chloroflexus aurantiacus* and two *Chlorobium* species (*C. limnicola* and *C. phaeobacteroides*) raises the possibility that As(III) is involved in photosynthesis. In the summer of 2007, members of the U.S. Geological Survey (USGS) team led by one of us (RSO) visited hot springs on Paoha Island in Mono Lake, where pigmented microbial biofilm communities thrive in arsenic-rich waters. Anoxygenic phototrophic bacteria dominate the red biofilms, while the green biofilms are populated with an *Oscillatoria*-like cyanobacterium.

Both biofilm types oxidize As(III) under anaerobic conditions in the light, suggesting that a phototroph growing under anoxygenic conditions was using As(III) as an electron donor in photolithoautotrophy. To investigate this possibility, members of the USGS research team isolated strain PHS-1, a photosynthetic bacterium closely related to *Ectothiorhodospira shaposhnikovii*. They found that it indeed grew anaerobically in the light with As(III) and as in *A. ehrlichii*, the Arr functioned as the arsenite oxidase.

As(III)-linked anoxygenic photosynthesis and the reversibility of Arr provide insights into the origins of microbial arsenic metabolism. As(III) was probably the predominant form of arsenic in the early Archaean period. Dissimilatory arsenate reduction could arise only after sufficient As(V) became available, presumably derived from aerobic arsenite oxidation. The use of As(III) as an electron donor in anoxygenic phototrophy, however, provides a mechanism for generating As(V) in the absence of atmospheric oxygen. The widespread occurrence of Arr and Aox in the bacterial and archaeal domains also suggests that both appeared before those two domains diverged. The ability of Arr to act as an oxido/reductase, however, implies a more ancient origin for this enzyme. Regardless of which

protein came first, arsenic was an important factor during evolution.

Microbial Transformation of Organoarsenicals

Although inorganic arsenic pesticides and herbicides were banned during the 1970s, organoarsenicals still are being widely used. For instance, about 2,500 tons of monosodium methanearsenate (MSMA) and dimethyl arsinic acid (DMA) are used annually for weed control on cotton fields, citrus groves, and golf courses.

Similarly, roxarsone, which is 3-nitro-4-hydroxybenzene arsonic acid, was used widely as a feed additive by the poultry industry until recently. It prevents coccidiosis, and also accelerates chicken weight gain and improves pigmentation. Although little of the roxarsone accumulates in birds, the compound ends up in litter, in which it degrades rapidly. Specifically, clostridial species, including our lab strain *Alcaliphilus oremlandii*, reduce the nitro group, producing 3-amino-4-hydroxybenzene arsonic acid as an end product. We (JFS and PB) find that when *A. oremlandii* is grown with lactate and roxarsone, cell yields are 10-fold greater than if cells are grown on lactate alone. Cells of *A. oremlandii* can also respire arsenate and thiosulfate, suggesting that this species also generates ATP through oxidative phosphorylation linked to roxarsone reduction.

Whether the source is natural or anthropogenic, it is remarkable how microorganisms adapt to grow on compounds containing arsenic. Their metabolic activities affect its chemical state, mobility, and thus its toxicity—in some cases, poisoning water supplies. Thus, the ongoing biogeochemical cycling of arsenic, presumably more prominent during the early anoxic phase of life on Earth, continues to have an important impact on biology in general and human health in particular.

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