

Arsenic, microbes and contaminated aquifers

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The health of tens of millions of people world-wide is at risk from drinking arsenic-contaminated well water. In most cases this arsenic occurs naturally within the sub-surface aquifers, rather than being derived from identifiable point sources of pollution. The mobilization of arsenic into the aqueous phase is the first crucial step in a process that eventually leads to human arsenicosis. Increasing evidence suggests that this is a microbiological phenomenon.

Introduction

One does not normally associate arsenic with life, but it is now apparent that various types of microorganisms gain energy for growth from this toxic element [1] (Box 1). These organisms are taxonomically diverse and metabolically versatile (Figure 1). For example, aqueous arsenic in the +3 oxidation state, arsenite [H_2AsO_3^- or As(III)], can be oxidized to arsenate [HAsO_4^{2-} or As(V)] by chemoautotrophic arsenite-oxidizing bacteria (CAOs; see Glossary). These organisms use oxygen or, in some cases, nitrate as their terminal electron acceptor during the fixation of inorganic carbon (CO_2) into cell material. There are also heterotrophic arsenite oxidizers (HAOs), but they need organic carbon as their source of energy and cell material. On the reductive side are microbes that use As(V) as an electron acceptor in anaerobic respiration. These prokaryotes oxidize a variety of organic (e.g. lactate, acetate, formate and aromatics), or inorganic (hydrogen and sulfide) electron donors, resulting in the production of As(III). We refer to these prokaryotes as dissimilatory arsenate-respiring prokaryotes (DARPs) and their As(V) reductase as Arr. Many microbes reduce As(V) to As(III) as a means of resistance. These arsenate-resistant microbes (ARMs) do not gain energy from the process, but use it as a means of coping with high arsenic in their environment. Arsenate that has entered the microbe's cytoplasm is converted to As(III) through a process mediated by a small polypeptide (ArsC) and expelled out of the cell by an As(III)-specific transporter (ArsB). Although the arsenite oxidases of CAOs and HAOs have notable similarities, the arsenate reductases of DARPs and ARMs are very different [2].

Groundwater arsenic and arsenicosis

What does this curious microbiological phenomenon have to do with outbreaks of arsenicosis in countries such as Bangladesh? It started with an effort to eliminate the high

incidence of water-borne diseases caused by consumption of untreated surface waters contaminated with high bacterial counts. The government of Bangladesh, in cooperation with the World Health Organization of the

Box 1. Toxicity of Arsenic Compounds

Arsenic occurs in four oxidation states: As^{+5} , As^{+3} , As^0 and As^{-3} . The two highest oxidation states are the most common in nature, whereas the two lowest are rare.

Arsenate

This oxyanion is an analog of phosphate, and as such it is a potent inhibitor of oxidative phosphorylation, the key reaction of energy metabolism in metazoans, including humans.

Arsenite

The most toxic of arsenic oxyanions. It readily binds to reactive sulfur atoms ((SH groups) of many enzymes, including those involved in respiration.

Arsenic trioxide (As_2O_3)

The most common form of arsenic used for a variety of agricultural, manufacturing and medical purposes. It is highly toxic, and being soluble in water, as well as colorless and tasteless, it has proved useful in criminal homicide. During the 18th century it gained so much notoriety that it was referred to as 'inheritance powder'.

Methylated forms of arsenate and arsenite

Compounds, such as methylarsonic acid (MMA^{V}), monomethylarsonous acid (MMA^{III}) and dimethylarsenic acid (DMA^{V}) are produced by algae and as excretory products of animals. They have varying degrees of toxicity, depending on their chemical form and the oxidation state of the arsenic that they contain. They occur in low concentrations in the environment.

Arsines

Arsenic in the -3 oxidation state, occurring as highly toxic gases, such as H_3As and $(\text{CH}_3)_3\text{As}$. Very little is known about the natural cycles of these substances, as they occur at very low concentrations in the environment.

Organoarsenic compounds

Naturally occurring substances, such as arsenobetaine, are molecular analogs of osmotic-regulating compounds, such as betaine, where arsenic substitutes for the original nitrogen atom. They commonly occur in several marine animals, including shellfish and elasmobranchs. Their physiological role in these organisms is unknown, but they are benign and are not toxic to animals that eat these organisms, including humans.

Synthetic organoarsenic compounds

Substances, such as roxarsone (4-hydroxy-3-nitrophenylarsonic acid), are used as palliatives included in the feed of mass-raised swine and poultry. They are benign, do not accumulate in these organisms, and are ultimately excreted. However, their subsequent breakdown by bacteria in soils will release As(V) into the environment.

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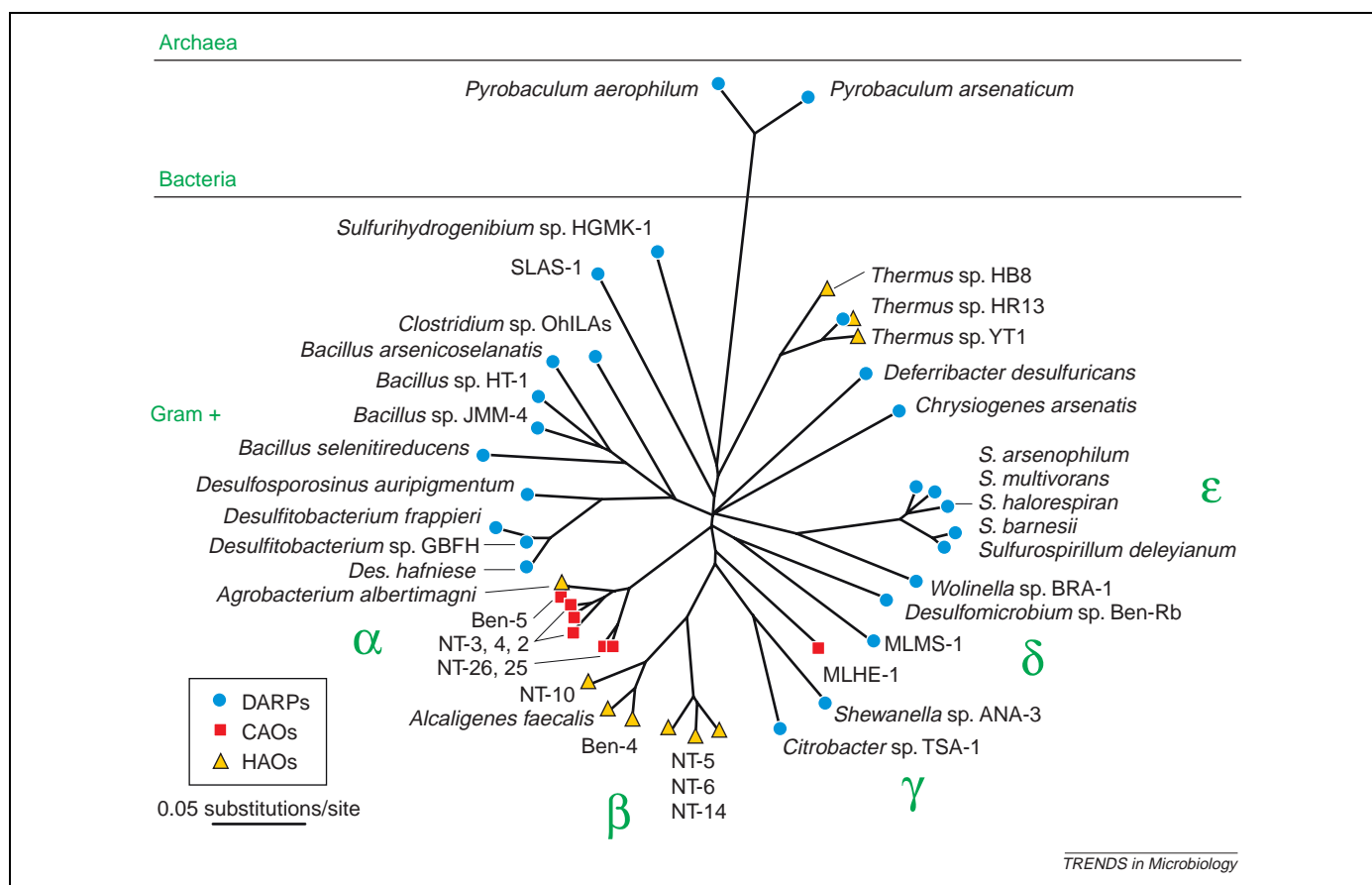


Figure 1. Phylogenetic diversity based on 16S rRNA gene sequences of known species of prokaryotes from the Domains Archaea (top) and Bacteria (bottom) that either reduce or oxidize arsenic oxyanions. Illustrated are dissimilatory arsenate-respiring prokaryotes (DARPs) as indicated by the blue circles, chemoautotrophic arsenite oxidizers (CAOs) as indicated by the red squares, and heterotrophic arsenite oxidizers (HAOs) as indicated by the gold triangles. Not shown are the arsenate-resistant microbes (ARMs).

United Nations, instituted a nation-wide switch to use groundwater. For a period of approximately 20 years, millions of wells were constructed to tap ground water resources that were free of enterics. What was unforeseen, however, was that this would replace one water-quality problem (pathogens) with another one (arsenic). In academic terms, this water-usage switch replaced a problem of medical microbiology with that of geomicrobiology. For an overview of the scope of this human tragedy in Bangladesh, the reader is referred to the recent article by Chowdhury [3].

Where does this aqueous arsenic come from? In the case of Bangladesh and West Bengal, India, it is present as a long-term (i.e. millions of years) consequence of rock weathering, downstream transport and sediment deposition of arsenic-rich minerals originally found in the Himalayas. Hence, the sub-surface arsenic distribution is very much a part of the complex alluvial landscape of the countryside. But in practical terms, the issue boils down to

the question of the mobility of As(V) and As(III) in hydrologic systems that have both an aqueous phase and a solid phase. Very simply stated, the arsenate anion is the prevalent chemical species under oxic conditions and tends to be strongly adsorbed onto several common inorganic mineral surfaces, especially amorphous iron minerals, such as ferrihydrite, as well as the aluminum oxides found in clays. Hence, in oxic sub-surface systems with a high E_h (oxidation-reduction potential) and an abundance of adsorptive minerals, As(V) tends to be immobilized by remaining sorbed onto the solid phase. Conversely, As(III) is the prevalent chemical species under anoxic conditions (low E_h), and because it sorbs to fewer such minerals, it partitions into the aqueous phase and is thus more mobile (and notably more toxic) than As(V).

However, the above statements are clearly an oversimplification. Several laboratory and field investigations have shown the theoretical importance of diverse chemical phenomena at either promoting or decreasing the mobility of arsenic in the subsurface. These can include the formation of arsenic-sulfide solid phases [4], or their carbonate-mobilized leaching into the aqueous phase [5], the enhancement or suppression of the arsenic-sorptive characteristics of iron minerals caused by carbonate ions [6], the competition of As(V) with phosphate (a molecular analog of arsenate) for sorptive sites, and the considerable adsorptive affinity of various Fe-minerals for As(III) [7]. Indeed, there is evidence that as iron minerals themselves

Glossary

DARPs dissimilatory arsenate-reducing prokaryotes. Anaerobic microbes from the domain Bacteria or Archaea that respire As(V), reducing it to As(III).

CAOs: chemoautotrophic arsenite oxidizers: Microbes that gain energy for growth by oxidizing As(III) to As(V), while using CO₂ for cell carbon.

HAO: heterotrophic arsenite oxidizers. Microorganisms that oxidize As(III) to As(V) while respiring oxygen, but cannot grow without organic matter.

ARMs: arsenate-resistant microorganisms. A microbe that reduces toxic As(V) that has entered the cell to As(III), so as to facilitate its export.

become more crystalline over time, they have a decreasing sorptive affinity for either As(V) or As(III) [8]. When such diverse and divergent possibilities are superimposed on the complex hydrology of the alluvial aquifers of Bangladesh [9], it becomes quite difficult to sort out which processes are of primary importance.

On-site hydrochemical investigations of Bangladesh aquifers have led to the hypothesis that the mobility of arsenic is primarily controlled by the availability of organic matter, which drives this process forward. Although this hypothesis has not been rigorously proven, it certainly has guided recent thinking. It is not clear if these organics are derived from decomposing buried peat beds [9,10] or from hydrologic seasonal drawdown of agricultural and other organic wastes from the surface [11]. Regardless of the source, if abundant labile organic matter is present to be degraded (i.e. oxidized to CO₂) then there are always prokaryotes around to do the job. But what role do these prokaryotes play, if any, in the process of arsenic mobilization? Here the story gets even more complicated.

Microbe–mineral experimental models

Several laboratory-based studies have been conducted with minerals and sub-surface materials, with or without pure cultures of anaerobes to better understand these dynamics. These are conceptually illustrated in Figure 2. Because iron is very abundant in sediments, and because Fe(III) strongly adsorbs As(V), then its biochemical reduction to the soluble Fe(II) state by iron-respiring bacteria should release As(V) into solution. This was demonstrated [12] in the reductive attack of the mineral scorodite (FeAsO₄·2H₂O) by the iron-reducing bacterium *Shewanella alga* (Figure 2a). However, if the bacterium is a DARP, the release of As(III) occurs rather than As(V). Ahmann *et al.* [13] showed As(III) release mediated by *Sulfurospirillum arsenophilum* from an initial solid phase consisting of ferrous arsenate (Figure 2b). But what happens when As(V) is sorbed to alumina? In this case, the surface As(V) molecules were susceptible to bio-reduction, and because alumina has no adsorptive affinity for As(III), it is released into the aqueous phase (Figure 2b). However, because the bacterium used (*Sulfurospirillum barnesii*) cannot reduce Al(III), any As(V) located in the interior of the alumina matrix is unavailable for further bio-reduction [14]. But what if the DARP is also an iron-reducer (most DARPs can use a variety of electron acceptors)? In this instance, *Sulfurospirillum barnesii* could release both As(III) and Fe(II) from ferrihydrite that was initially co-precipitated with As(V) (Figure 2c). An interesting sidelight was that most of the As(III) formed was re-adsorbed by the un-reacted Fe(III) and only a fraction actually went into solution. If given enough electron donor, however, *S. barnesii* would break up the internal ferrihydrite matrix, eventually liberating most of the material into solution as Fe(II) and As(III).

No harm from ARMs

What happens when the bacterium is an ARM? Curiously, much more is known about the phenomenon of As(V) resistance than As(V) respiration because ARMs were

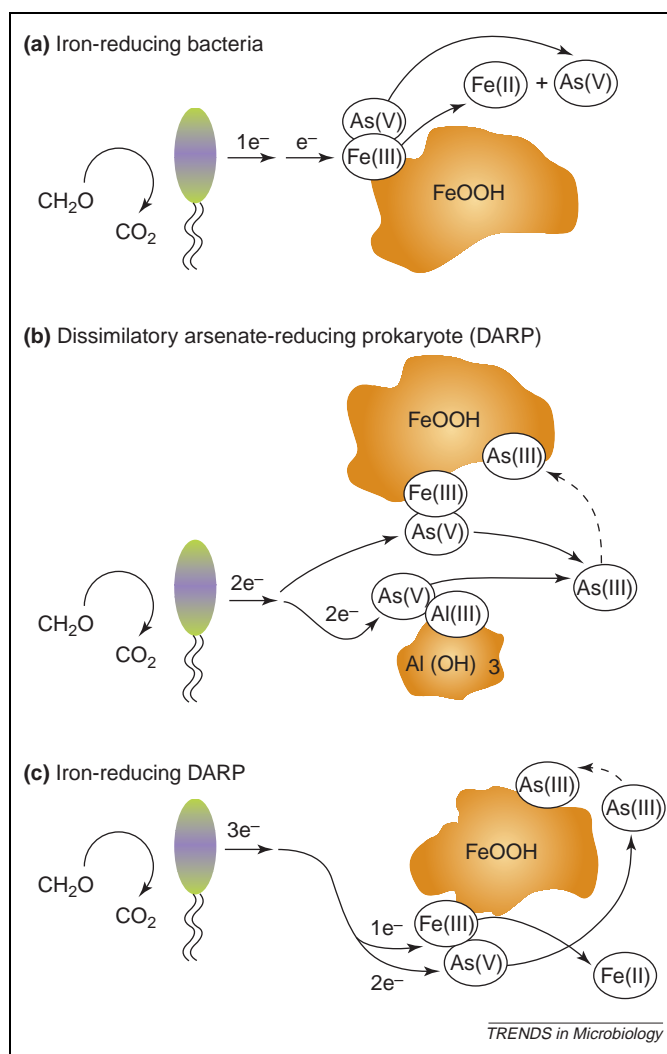


Figure 2. Three possible mechanisms of arsenic mobilization in anoxic sub-surface aquifer materials as mediated by metal-respiring bacteria. The reactions are driven by the oxidation of organic matter carried out by these microbes, which use As(V) or Fe(III) as their terminal electron acceptors. (a) Release of As(V) by iron-reducing bacteria, such as *Geobacter*. (b) Release of adsorbed arsenic from the surfaces of either Fe(III) or Al(OH)₃ minerals via reduction of As(V) to As(III) as mediated by DARPs. Note that some of the released As(III) can re-adsorb to unreacted Fe(III). (c) Reductive release of As(III) and Fe(II) by iron-reducing DARPs, such as *Sulfurospirillum barnesii*. In nature, as opposed to the laboratory conditions illustrated, it is possible that all of these mechanisms are operative in the same aquifer.

discovered earlier, and have therefore been studied longer than DARPs. Although Arr is either membrane-bound with the catalytic subunit facing the periplasmic space [15] or free in the periplasm [16], ArsC is located in the cytoplasm and thus can only reduce aqueous As(V) that has entered the cell. For example, strain CN8, a fermentative ARM, was capable of reducing aqueous As(V) but incapable of attacking solid-phase Fe(III) or any As(V) sorbed onto the Fe(III) [17]. Importantly, DARPs can reduce either aqueous or solid-phase As(V). Recently, work with *Shewanella* strain ANA-3, an organism that is both a DARP and an ARM [18,19], demonstrated that only Arr was involved in reducing solid-phase As(V) [20], because mutants deficient in *arsC* [the gene encoding the cytoplasmic resistance As(V) reductase] were still capable of bio-reduction of solid-phase As(V). It is probable that only DARPs and Fe(III) reducers

are involved in the process of mineral dissolution and bio-reduction of solid-phase As(V) in aquifer materials, fueled by their oxidation of organic substrates. These microbes ultimately just pass their electrons to As(V) and Fe(III), or both.

Preliminary field studies

It is significant that most of the field work done to date in Bangladesh was by hydro-geochemists and not geomicrobiologists. Hence, some of these researchers speculated about possible microbial involvement [9,10], whereas others described arsenic mobilization in purely chemical or physical terms [11]. Islam *et al.* [21] have begun to address this lack of information by showing the microbial reductive dissolution of solid-phase Fe(III) and As(V) from aquifer materials taken from the Bengal Delta. Furthermore, the release of As(III) and Fe(II) was stimulated with the addition of acetate (as a proxy for autochthonous labile carbon compounds), clearly demonstrating microbial involvement in the arsenic cycling of this system. Curiously, 16S rRNA gene analysis of the microbial populations in the acetate-amended incubations showed a predominance of the δ -Proteobacteria belonging to the *Geobacteriaceae* family. *Geobacter* species are well known for their ability to carry out the dissimilatory reduction of solid-phase Fe(III), but not As(V). This result might be explained by: (i) the activity of new species of geobacters that can also function as DARPs; (ii) direct attack of the adsorbed As(V) by the less numerous DARPs; (iii) an initial release of As(V) into solution by Fe(III) reduction mediated by geobacters, followed by attack of the aqueous As(V) by a combination of DARPs and ARMs; or (iv) some combination of all the above. Which of these scenarios is correct cannot be resolved, especially because DARPs have such a broad phylogenetic distribution that renders moot the amplification of segments of 16S rRNA genes to identify their presence in natural materials. Nonetheless, it is now clear that the mobilization of arsenic in the subsurface aquifer materials of Bangladesh and West Bengal is partly, if not primarily, a microbiological phenomenon. Which specific microbial mechanisms for arsenic dissolution are involved is still very much a matter of debate, but now even experimental hydrochemical studies with these aquifer materials have taken on a distinct microbiological slant [22–24]. What are needed are molecular tools that identify the microbial agents of arsenic mobilization in subsurface materials.

Future perspective

So where is this field heading? The products of several years of research devoted to the biochemistry and genetics of DARPs and CAOs is bearing fruit in the form of molecular tools to amplify functional genes in samples taken from As-contaminated environments. For example, Malasarn *et al.* [20] have recently used a primer specific to a portion of the *arrA* gene of the dissimilatory arsenate reductase to amplify and sequence DNA recovered from arsenic-rich sediments. It is probable that this will be followed by the use of primers for amplification of the arsenite oxidase of CAOs [25]. The future looks bright for the use of modern molecular techniques [26], especially if

the full genomes of representative DARPs and CAOs become known, to enhance investigations of hydrochemistry of arsenic in drinking water aquifers. Perhaps a better understanding of the fundamental mechanisms involved in arsenic mobilization (and immobilization) will eventually yield practical solutions to this grave environmental problem.

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Another extreme genome: how to live at pH 0

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Many *Archaea* live under conditions that challenge the physico-chemical limits to life: low or high temperature, extremes of pH, elevated pressure and high salt concentration. A recent paper reports the genome sequence of another record-setting archaeon, *Picrophilus torridus*, that thrives at 65°C and pH 0. The genomic sequence provides several hints of the mechanisms used for adaptation to such hostile environment, but most secrets remain hidden and await further analysis to be disclosed.

Introduction

Archaea comprise the third domain of living organisms, evolutionarily distinct from *Bacteria* and *Eucarya*. Many *Archaea* are extremophiles, meaning that they live in almost any environmental niches previously thought of as insurmountable physical and chemical barriers to life. Functional-structural genomics and phylogenomics can provide answers to central questions concerning the unique adaptive strategies, as well as the evolution of these intriguing microorganisms. Now Futterer *et al.* [1] report the genome sequence of the archaeon *Picrophilus torridus*, which lives optimally at pH 0.7 and can even survive at negative pH values, the lowest pH values reported to support life. To give a handy hint, this is similar to thriving happily in 1.2 M sulfuric acid. Above pH 4.0 cells lyse and lose their viability. And if this was not enough, it also likes moderately high temperatures (60–65°C).

P. torridus belongs to one of the two archaeal sub-domains, *Euryarchaea* (Figure 1), and lives in hot acid solfataric fields, the same habitat that is populated by several other thermoacidophilic microorganisms. Some of them are phylogenetically close to *P. torridus* (e.g. the euryarchaeon *Thermoplasma*), whereas others are evolutionarily distant (some *Bacteria* and the crenarchaeon *Sulfolobus*). *P. torridus* is peculiar even when compared

with its neighbours. The easiest approach for an organism to preserve cell functions in a harsh habitat is to maintain more moderate conditions in the cell. Most organisms that live at extremes of pH are able to do so by maintaining their internal pH close to neutral. By contrast, the intracellular pH value of *Picrophilus* is very low (4.6), suggesting the evolution of peculiar adaptation mechanisms at the level of single macromolecules, sub-cellular structures and metabolic pathways.

When *P. torridus* was isolated for the first time in the Hokkaido island in Japan, two possible strategies were

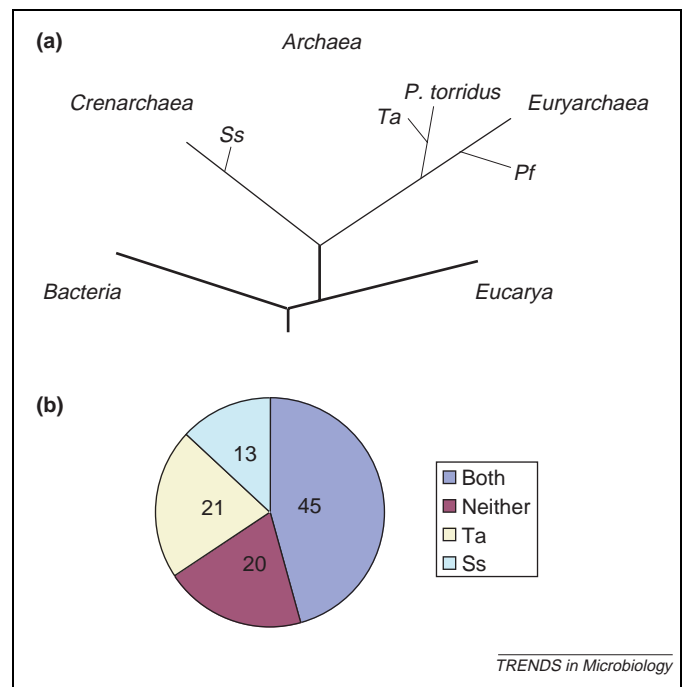


Figure 1. (a) A simplified phylogenetic tree showing the position of *Picrophilus torridus* and other thermoacidophilic *Archaea*. (b) Open-reading frames (ORFs) shared among thermoacidophilic neighbours. Numbers indicate the percentage of *P. torridus* ORFs shared with either the phylogenetically close *Thermoplasma acidophilum* (Ta) or the phylogenetically distant *Sulfolobus solfataricus* (Ss), both, or neither of them.

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