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Review Article

REVIEW ARTICLE

The biology of thermoacidophilic archaea from the order *Sulfolobales*

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One sentence summary: The natural habitat and biological features of the *Sulfolobales*.

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ABSTRACT

Thermoacidophilic archaea belonging to the order *Sulfolobales* thrive in extreme biotopes, such as sulfuric hot springs and ore deposits. These microorganisms have been model systems for understanding life in extreme environments, as well as for probing the evolution of both molecular genetic processes and central metabolic pathways. Thermoacidophiles, such as the *Sulfolobales*, use typical microbial responses to persist in hot acid (e.g. motility, stress response, biofilm formation), albeit with some unusual twists. They also exhibit unique physiological features, including iron and sulfur chemolithoautotrophy, that differentiate them from much of the microbial world. Although first discovered >50 years ago, it was not until recently that genome sequence data and facile genetic tools have been developed for species in the *Sulfolobales*. These advances have not only opened up ways to further probe novel features of these microbes but also paved the way for their potential biotechnological applications. Discussed here are the nuances of the thermoacidophilic lifestyle of the *Sulfolobales*, including their evolutionary placement, cell biology, survival strategies, genetic tools, metabolic processes and physiological attributes together with how these characteristics make thermoacidophiles ideal platforms for specialized industrial processes.

Keywords: Archaea; Thermoacidophiles; *Sulfolobales*

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INTRODUCTION

Thermoacidophiles are microorganisms that have developed mechanisms to successfully persist in unusually hot, acidic environments, with optimal conditions of pH ≤4 and temperature ≥55°C. In fact, thermoacidophiles have been isolated from some of the most inhospitable environments on earth, such as acidic hot springs and volcanic solfataras. In 1972, Thomas Brock isolated the thermoacidophile *Sulfolobus acidocaldarius* from a sulfur hot spring in Yellowstone National Park (left image, Fig. 1) and designated the genus *Sulfolobus* (Brock *et al.* 1972). The natural habitat of this microbe, a member of the *Crenarchaeota*, was Locomotive Spring, an extremely hot acidic environment with a pH of 2.4 and temperature of 83°C. Likewise, in 1980, Wolfram Zillig described *Sulfolobus solfataricus* (renamed *Saccharolobus solfataricus*) isolated from a volcanic hot spring in Italy (Zillig *et al.* 1980) and *Desulfurolobus ambivalens* (renamed *Acidianus ambivalens*) from a solfatara in Iceland in 1986 (Zillig *et al.* 1986). Zillig also discovered the first Japanese isolate belonging to this group in 1990—*Sulfolobus shibatae* (renamed *Saccharolobus shibatae*) (Grogan, Palm and Zillig 1990). Beyond these discoveries, Zillig also isolated the first thermoacidophile virus (Martin *et al.* 1984) (see the section 'Viruses and CRISPR systems of thermoacidophiles') and was the first to describe the eukaryotic-like archaeal RNA polymerase from *S. acidocaldarius* (Zillig, Stetter and Janekovic 1979) (see the section 'Genetic mechanisms'). In 1986, Karl Stetter established the genus *Acidianus* with the isolation of *Acidianus infernus* from a solfatara crater in Italy, which consequently led to the renaming of *Sulfolobus brierleyi* as *Acidianus brierleyi* (Segerer *et al.* 1986). Stetter also established the genus *Metallosphaera* with the isolation of *Metallosphaera sedula* in 1989 from a solfataric field in Italy (Huber *et al.* 1989).

Thermoacidophiles not only thrive in thermal acidic biotopes but also encounter other biologically deleterious conditions, such as oxidative stress caused by high levels of metals in mining environments. For instance, *Metallosphaera prunae* was isolated from a uranium mine in Germany (Fuchs *et al.* 1995) and uses an interesting stress response mechanism to withstand high levels of soluble uranium (see the section 'Extreme thermoacidophily and stress response'). Figure 1 (right) shows the features of the isolation site of *M. prunae*. In addition to *Sa. shibatae*, several other thermoacidophiles have been isolated from hot springs in Japan, such as *Sulfurisphaera ohwakuensis* in 1988 (Kurosawa *et al.* 1998), *Sulfolobus hakonensis* (renamed *Metallosphaera hakonensis*) in 1996 (Takayanagi *et al.* 1996) and *Sulfolobus tokodaii* (renamed *Sulfurisphaera tokodaii*) in 2002 (Suzuki *et al.* 2002), to name a few. It has become clear that thermoacidophiles are globally distributed in hot, acidic features; for example, recent isolates have come from the Copahue volcanic region in Argentina—*Acidianus copahuensis* in 2014 (Urbieta *et al.* 2014), and Indonesian hot springs—*Sulfurisphaera javensis* in 2018 (Tsuboi *et al.* 2018). Recently, *Saccharolobus caldissimus* was isolated from an acidic Japanese hot spring, establishing the *Saccharolobus* genus which, as mentioned above, led to the renaming of both *Sulfolobus solfataricus* and *Sulfolobus shibatae* to *Saccharolobus solfataricus* and *Saccharolobus shibatae*, respectively (Sakai and Kurosawa 2017). Figure 1 illustrates the timeline of these thermoacidophile isolations. Many thermoacidophiles have leveraged the chemistry of metal and sulfur deposits for bioenergetic benefit through chemolithotrophy (see the section 'Metabolism'). As such, chemolithotrophic metabolism in hot acid can be exploited for biomining applications (see the section 'Potential and current uses of thermoacidophiles in biotechnological applications').

The study of thermoacidophiles was originally restricted to observational microbiology focused on phenotypic characteristics, such as cell morphology and growth physiology. However, following the sequencing of the *Sa. solfataricus* genome in 2001 (She *et al.* 2001), several other *Sulfolobales* genomes were reported, including *S. acidocaldarius* in 2005 (Chen *et al.* 2005) (Fig. 1). Genome sequences opened up prospects for transcriptomics (Auernik *et al.* 2008; Ortmann *et al.* 2008; Koerdt *et al.* 2011; Kozubal *et al.* 2011; Maezato *et al.* 2012; Ulas *et al.* 2012; Kouril *et al.* 2013b; Wolf *et al.* 2016), proteomics (Ellen *et al.* 2009; Koerdt *et al.* 2011), metabolomics and systems biology (Ulas *et al.* 2012; Kouril *et al.* 2013b; Wolf *et al.* 2016), and metagenomics (Inskeep *et al.* 2013; Campbell *et al.* 2017) with these archaea, offering further insights into life in hot acid. The development of genetic systems for thermoacidophiles was challenging, given their unique characteristics and practical considerations related to their growth conditions. In 2003, soon after the availability of its genomic sequence, Paul Blum generated the first *Sulfolobales* mutant in *Sa. solfataricus* based on lactose autotrophy (Worthington *et al.* 2003). Later in 2009, a genetic system was developed for *Sulfolobus islandicus* (renamed *Saccharolobus islandicus*), based on uracil auxotrophy and the ability to generate uracil through *pyrEF* as a selectable marker (She *et al.* 2009). Similarly, in 2012, Wagner *et al.* developed a genetic system based on a uracil auxotrophic parent strain and 5-FOA toxicity that is widely used today (Wagner *et al.* 2012) (see the section 'Potential and current uses of thermoacidophiles in biotechnological applications'). Genetic engineering capabilities for thermoacidophiles have expanded over the past decade that have not only supported fundamental microbiological studies but also fueled prospects for biotechnological processes.

While there are moderately thermoacidophilic bacteria (Norris *et al.* 1996; Goto *et al.* 2002; Johnson, Okibe and Roberto 2003), most thermoacidophiles are archaea. However, life in thermal, acidic environments is not limited to the order *Sulfolobales*. There are thermoacidophilic *Euryarchaeota* belonging to the order *Thermoplasmatales*. Species in the genus *Picrophilus*, such as *Picrophilus oshimae* and *Picrophilus torridus* from solfataras in Japan (Schleper *et al.* 1995), have an optimum growth temperature of 60°C and a pH optimum near 0. *Thermoplasma acidophilum*, isolated from a coal refuse pile, grows optimally at 59°C and pH of 1–2 (Darland *et al.* 1970). Here, the focus will be on thermoacidophiles from the order *Sulfolobales* and an examination of what is currently known about their diversity, growth physiology, cell biology and biotechnological prospects.

THE DIVERSITY OF THERMOACIDOPHILIC LIFE

Thermoacidophilic biotopes are ubiquitously distributed across both terrestrial and marine environments, closely associated with volcanic outflows or calderas resulting from tectonic activity. In terrestrial realms, these environments are often isolated features, presenting as steam-saturated/superheated discharges (fumaroles) in the form of geysers, solfatara and pools, and on occasion mixing with soils to form mineral-heavy mud pots. In marine environments, these vents are distinguished by their rapid mixing with dramatically cooler, saline waters (Kelley, Baross and Delaney 2002), resulting in sharp gradients of temperature, pH, oxygen and solutes, and abrupt dislocated

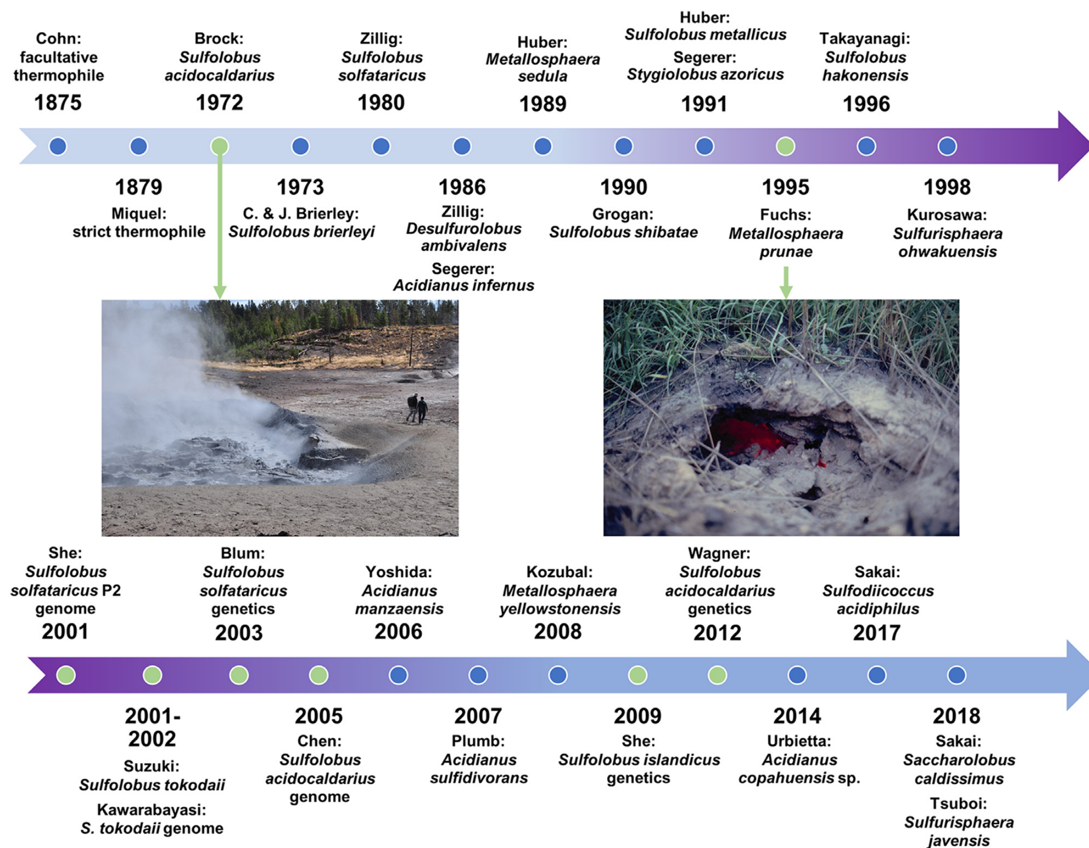


Figure 1. Timeline of thermoacidophile isolations and major events. Timeline contains the organism's name at the time of the associated event. The following are the current classifications: *Sulfolobus brierleyi* (f. *Acidianus brierleyi*), *Saccharolobus solfataricus* (f. *Sulfolobus solfataricus*), *Acidianus ambivalens* (f. *Desulfurolobus ambivalens*), *Saccharolobus shibatae* (f. *Sulfolobus shibatae*) *Sulfuracidifex metallicus* (f. *Sulfolobus metallicus*), *Metallosphaera hakonensis* (f. *Sulfolobus hakonensis*), *Sulfurisphaera tokodaii* (f. *Sulfolobus tokodaii*) and *Saccharolobus islandicus* (f. *Sulfolobus islandicus*).

niches (Reysenbach *et al.* 2000). In both environments, water chemistry is shaped by transformation of sulfur species from highly reduced metal sulfides and hydrogen sulfide to highly oxidized sulfate, with concomitant production of protons (i.e. acid) (Nordstrom, McCleskey and Ball 2009). Despite the incredibly exogenic nature of reduced inorganic species, their abiotic transformation at elevated temperatures and low pH is minimal (Chen and Morris 1972), pointing to the importance of sulfur biooxidizers in constructing and occupying this extremophilic niche (Odling-Smee, Laland and Feldman 1996).

While sulfur oxidation is a potential bioenergetic source in these environments, strategies to handle thermal stress, acid stress, high levels of aqueous heavy metals and minimal organic carbon availability must be employed (see the section 'Extreme thermoacidophily and stress response'). These biotopes are dominated by archaeal chemolithoautotrophs (Inskeep *et al.* 2013; Ward *et al.* 2017) that have been intrinsically tailored by evolution to inhabit and thrive in these highly selective niches (Valentine 2007; Colman *et al.* 2018). In contrast, many bacterial and eukaryotic organisms in thermal, acidic biotopes are limited to acid- or temperature-tolerant microorganisms, as opposed to obligate/sustained thermoacidophily (pH < 3.5; T $> 65^{\circ}$C).

Diversity of eukaryotic and bacterial thermoacidophiles

Previous efforts have identified the limitations of organisms at the cusp of thermoacidophily. Specifically, in eukaryotes, it appears that the boundary stems from a temperature limitation. As far back as the 1970s, exhaustive sampling and culturing have demonstrated the inability to cultivate eukaryotes (specifically, fungi and algae) from geothermal features in excess of 60°C, despite growth at slightly lower temperatures (Tansey and Brock 1972). Further work showed that algae are limited to ~60°C (Boyd *et al.* 2012), and protists to below 70°C (Brown and Wolfe 2006). For unicellular organisms that inhabit more thermophilic locales, hydrogen sulfide levels can be inhibitory. For many more complex organisms that depend upon gaseous compounds for cellular processes, growth is limited by the solubility of many gases (oxygen, carbon dioxide, etc.), which diminishes with rising temperatures (Rothschild and Mancinelli 2001).

As is the case with the Eukarya, there are few lineages of Bacteria that are thermoacidophilic. Some bacteria grow at extreme temperatures, in excess of 70°C (e.g. the genera *Thermotoga*, *Caldicellulosiruptor*, *Aquifex*), albeit at neutralophilic conditions (Counts *et al.* 2017). Conversely, there are also a number of acidophilic bacteria, primarily from the genera *Leptospirillum* and *Acidithiobacillus*, that are also autotrophic and are found in acidic features with low organic carbon concentrations. But these bacteria grow optimally at temperatures far below anything considered thermophilic (i.e. 28–45°C); however, *Leptospirillum thermoferrooxidans* grows at temperatures up to 50°C (Kondrat'eva *et al.* 2012; Dopson 2016). As temperatures increase, bacteria from the thermotolerant and acidotolerant genera *Sulfobacillus*, *Alicyclobacillus* and *Hydrogenobaculum* are most common (see Fig. 2).

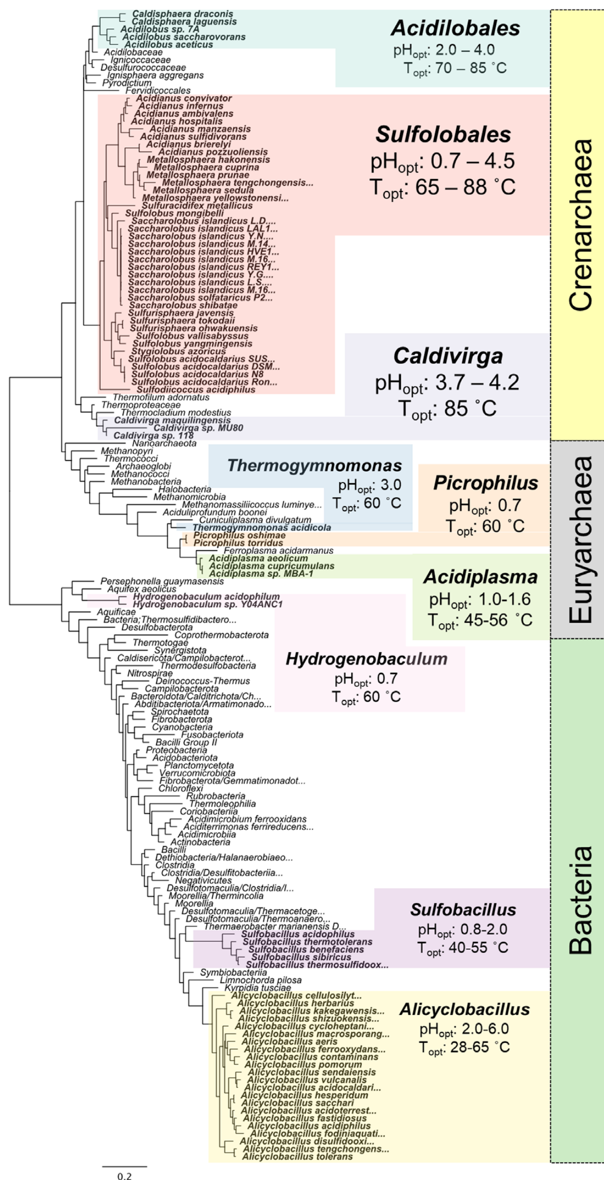


Figure 2. 16S phylogeny tree of thermoacidophilic organisms.

Archaeal thermoacidophilic diversity

In contrast to the other domains of life, the Archaea predominate in extremely thermoacidophilic features. While culture-independent techniques are rapidly expanding the number of recognized species in acidic hydrothermal environments [e.g. candidate phyla *Geoarchaeota* (Kozubal *et al.* 2013) and *Marsarchaeota* (Jay *et al.* 2018)], most of the isolated thermoacidophiles to date originate from the archaeal phyla *Crenarchaeota* and *Euryarchaeota*. In both phyla, almost all isolated and named species are native to terrestrial hot acid environments. Currently, the only thermoacidophile with a validly published name from a marine environment is the deep-sea Euryarchaeon *Aciduliprofundum boonei*, an anaerobic heterotroph growing optimally at 70°C and pH 4.2–4.8, that utilizes sulfur and iron as electron acceptors (Reysenbach *et al.* 2006).

Other thermoacidophilic *Euryarchaeota* originate from the order *Thermoplasmatales*, which includes the thermotolerant *Acidiplasma*, as well as the moderately thermophilic *Picrophilus* and *Thermogymnomonas*. The genus *Acidiplasma* contains a few moderately thermophilic acidophiles (pH optimum 1–2, T_{opt} 45–55°C), including the cell wall-lacking *Acidiplasma aeolicum* (Golyshina *et al.* 2009). While these organisms grow chemoorganotrophically, they also oxidize iron; for example, *Acidiplasma cupricumulans* (f. *Ferroplasma cupricumulans*) originates from a copper mine heap (Hawkes *et al.* 2006), and the recently sequenced *Acidiplasma* sp. strain MBA-1 originates from a pyrite–arsenopyrite gold-bearing concentrate bioleaching reactor (Bulaev, Kanygina and Manolov 2017). In contrast, the thermophilic genus *Picrophilus* contains two members, *Picrophilus oshimae* and *Picrophilus torridus*, which are aerobic, heterotrophic organisms from solfatara in Hokkaido, Japan, exhibiting remarkable acid tolerance (pH optima < 1.0), with optimal growth near 60°C (Schleper *et al.* 1996). While most organisms maintain a near circumneutral intracellular pH, *P. oshimae* actually maintains an intracellular pH of ~4.6, making it a reservoir for acid-stable cytoplasmic proteins (van de Vossenberg *et al.* 1998). Additionally, sequencing revealed that *Picrophilus* spp. have some of the smallest genomes (around 1.5 Mb) isolated from free-living organisms (Futterer *et al.* 2004). Finally, in addition to the well-studied obligately aerobic heterotrophs from the genus *Thermoplasma*, *Thermoplasma acidophilum* and *Thermoplasma volcanium* (Segerer and Stetter 1988), there is a cell wall-less species, *Thermogymnomonas acidicola*, that grows near 60°C, but at slightly higher pH (around 3.0 optimally) (Itoh, Yoshikawa and Takashina 2007).

While the temperature optima of the euryarchaeal thermoacidophiles is limited to around 60°C, the thermoacidophiles from the crenarchaeal phylum all grow at temperatures ranging from 65°C to 88°C. These organisms are composed of three major clades, spanning three orders: *Acidilobales*, *Sulfolobales* and *Thermoproteales*. While the *Sulfolobales* are a well-studied archaeal lineage (over 30 named species, across 7 genera and >20 distinct genomes), the other two lineages, *Acidilobales* (containing *Caldisphaera* and *Acidilobus*) and *Thermoproteales* (only the *Caldivirga* are thermoacidophiles), contain just a few named, characterized strains. From the order *Acidilobales*, there are just two genera, *Acidilobus* and *Caldisphaera*, belonging to families derived from the same names. Both groups consist of anaerobic heterotrophs, growing optimally at pH ranging from 2.5 to 4.0 (mostly moderate acidophiles) and temperatures around 70–75°C for the *Caldisphaera* and slightly elevated temperatures of 50–80°C for the *Acidilobus* spp. (Prokofeva *et al.* 2000; Itoh *et al.* 2003; Boyd *et al.* 2007; Prokofeva *et al.* 2009). Meanwhile, the genus *Caldivirga* is represented by a single member, *Caldivirga maquilingensis*, isolated from the Philippines, which is capable of anaerobic (and microaerophilic) growth on heterotrophic substrates at moderate pH (optimum 3.7–4.2) and extremely thermophilic conditions (85°C) (Itoh *et al.* 1999).

The *Sulfolobales*

As mentioned previously, one of the first archaeal lineages discovered was the *Sulfolobales*, named for their presence and perceived usage of sulfur by Thomas Brock from his excursions to Yellowstone in the 1960s (Brock *et al.* 1972). Over the course of the following decades, a number of intriguing microorganisms emerged from terrestrial hot springs throughout the world, representing the seven named genera today from the order: *Acidianus*, *Metallosphaera*, *Saccharolobus* (f. *Sulfolobus*), *Stygiolobus*, *Sulfodiicoccus*, *Sulfolobus*, *Sulfuracidifex* (f. *Sulfolobus*) and *Sulfurisphaera* (f. *Sulfolobus*) (Counts, Willard and Kelly 2020). These include organisms with a broad array of physiological traits,

ranging from extreme to moderate acidophily (0.7–4.5), thermophily (65–88°C), obligate and facultative aerobes, obligate anaerobes, metal oxidizers, sulfur reducers/oxidizers, chemoheterotrophs and chemolithoautotrophs.

Species in the thermoacidophilic genus *Acidianus* grow anaerobically, reducing sulfur in its various forms, or aerobically, oxidizing sulfur (Segerer *et al.* 1986). The genus contains the most acidophilic *Sulfolobales* member to date: *Acidianus sulfidivorans* ($pH_{opt} \sim 0.7$), and the most thermophilic member: *Acidianus infernus* ($T_{opt} \approx 88°C$) (Segerer *et al.* 1986; Plumb *et al.* 2007). The order also contains several members with metal biooxidation capabilities (Huber *et al.* 1989; Huber and Stetter 1991). *Acidianus ambivalens* (f. *Desulfurolobus ambivalens*) has long served as a model for the study of sulfur biotransformation within the *Sulfolobales* (Laska, Lottspeich and Kletzin 2003; Müller *et al.* 2004; Brito *et al.* 2009; Protze *et al.* 2011).

The genus *Metallosphaera* was named for the perceived ability of its members to biooxidize iron (and by proxy release other metals from ores, e.g. copper) (Huber *et al.* 1989). The type species, *Metallosphaera sedula*, along with the recently isolated *Metallosphaera yellowstonensis*, serve as model systems for metal biooxidation by extremely thermoacidophilic archaea (Auernik *et al.* 2008; Kozubal *et al.* 2011). Further, *M. sedula* has also been examined for autotrophy catalyzed by the 3-hydroxypropionate/4-hydroxybutyrate cycle (Berg *et al.* 2010a), which has shown promise for metabolic engineering of biosynthetic pathways (Hawkins *et al.* 2013; Keller *et al.* 2013; Lian *et al.* 2016; Straub *et al.* 2018).

In contrast to the other genera of the *Sulfolobales*, the genus *Saccharolobus* appears to depend less on lithotrophic pathways and more, as its name suggests, on sugar catabolism (Sakai and Kurosawa 2018). Members of the genus *Saccharolobus* are mostly aerobic and are among the most thermophilic ($T_{opt} \geq 80°C$) and least acidophilic organisms in the order ($pH_{opt} \geq 3.0$) (Zillig *et al.* 1980; Grogan 1989; Sakai and Kurosawa 2018). Their original taxonomical placement in *Sulfolobus* was changed following growing genomics information pointing to evolutionary divergence (Sakai and Kurosawa 2018). In fact, a number of unnamed and informally named species, e.g. '*Sulfolobus islandicus*', appear to be much more closely related to other members of the genus *Saccharolobus*, which is fitting given that they use pentoses, hexoses, and di-, tri- and polysaccharides (Grogan 1989).

The main representative of the genus *Sulfolobus*: *S. acidocaldarius*, has a much narrower range of carbohydrate utilization. *S. acidocaldarius* grows best at 75°C and pH 3.0, using only amino acids, sucrose, dextrin and starch (Grogan 1989). This archaeon was originally named for its perceived capability to oxidize sulfur in the sulfur-rich pools of Yellowstone National Park (Brock *et al.* 1972). Despite these early reports, sulfur biooxidation capacity in strains that are currently available from culture collections is limited. However, recent studies showed that sulfur oxidation can be restored in *S. acidocaldarius* DSM 639 by inserting genes encoding sulfur oxygenase reductase (SOR) and thiosulfate:quinone oxidoreductase (TQO) (Zeldes *et al.* 2019), perhaps reflecting an evolutionary connection to this process. *S. acidocaldarius* has emerged as a tractable genetic platform to understand the physiological features of the *Sulfolobales*, such as pili structure controlling motility (Albers and Jarrell 2015), UV-stress response (Wagner *et al.* 2012), biofilm formation (van Wolferen *et al.* 2020), and cellular division (Pulschen *et al.* 2020). In addition to *S. acidocaldarius*, *Saccharolobus solfataricus* and '*Sulfolobus islandicus*' are currently the only *Sulfolobales* with tractable genetic systems (Straub *et al.* 2018).

The remaining genera are represented by only a few named species, but vary dramatically in some of their observed traits. For example, the genus *Stygiolobus* contains a single member, *Stygiolobus azoricus* (T_{opt} 80°C and pH 2.5–3.0), and is the only obligate anaerobe from the order to date, capable of sulfur reduction in the presence of hydrogen (Segerer *et al.* 1991). The genus *Sulfurisphaera* contains three species: *Sulfurisphaera javaensis*, *Sulfurisphaera tokodaii* and *Sulfurisphaera ohwakuensis* (the genus type species), all of which are extremely thermophilic (optima 80–85°C), but vary with respect to acidophily (optima 2.0–4.0) (Tsuboi *et al.* 2018). *Sulfurisphaera* species are facultative anaerobes and oxidize sulfur and iron to varying extents, and grow on complex organic substrates (Kurosawa *et al.* 1998; Tsuboi *et al.* 2018). In contrast, the two current members of the genus *Sulfuracidifex: Sulfuracidifex* (f. *Sulfolobus*) *metallicus* and *Sulfuracidifex tepidarius*, are less thermophilic acidophiles (temperature optimum: 65°C; pH optima: 2.0–3.5) and obligately aerobic chemolithoautotrophs, capable of mixotrophic growth in the presence of reduced sulfur compounds (Huber and Stetter 1991; Itoh *et al.* 2020). *Sulfuracidifex metallicus* has served as a model system for metal biooxidation studies (Bathe and Norris 2007). The genus *Sulfodiicoccus* is another single-member genus (type species *Sulfodiicoccus acidiphilus*), growing optimally at 65–70°C and pH 3.0–3.5. This archaeon is different from other *Sulfolobales* in that it is not only unable to oxidize elemental sulfur, but is possibly inhibited by it (Sakai and Kurosawa 2017). Furthermore, *S. acidiphilus* also lacks key components for carbon dioxide fixation by the 3-hydroxypropionate/4-hydroxybutyrate cycle and apparently does not grow autotrophically (Sakai and Kurosawa 2017, 2019). See Table 1 for a listing of thermoacidophilic microorganisms.

VIRUSES AND CRISPR SYSTEMS OF THERMOACIDOPHILES

Thermoacidophiles share their natural habitat with viruses (Munson-Mcgee, Snyder and Young 2018). A recent survey of the viral communities in thermal hot springs in Yellowstone National Park showed that >60% of cells were infected by viruses and that the majority even contained two or more virus types at the same time (Munson-Mcgee *et al.* 2018). Consequently, viruses represent an important evolutionary pressure in these archaeal dominated environments. The ongoing arms race between viruses and their hosts has led to the development of anti-viral defense strategies and mechanisms from viruses to circumvent them (Borges, Davidson and Bondy-Denomy 2017; Hwang and Maxwell 2019; Hampton, Watson and Fineran 2020). However, the fact that in many cases cells carry multiple virus types suggests that viruses can also have beneficial relationships with their microbial hosts. Viruses shape microbial populations, are a major driver of microbial evolution and impact host ecology. An excellent example is the virus–host mutualism by which chronically virus infected-*Sulfolobus* cells kill the virus-resistant cells in the population (DeWerff *et al.* 2020).

Viruses of thermoacidophiles

In comparison with known bacterial and eukaryotic viruses, only a modest number of archaeal viruses have been isolated to date (Prangishvili *et al.* 2017). However, thermoacidophiles, especially members of the *Sulfolobales*, have proven to be a very rich source of archaeal viruses (Prangishvili, Stedman and Zillig

Table 1. Thermoacidophile organisms.

Kingdom	Phylum/division	Genus/species	T_{opt} (°C)	pH_{opt}	Isolation site (locale, country)	Reference
Eukarya	Rhodophyta	*Galdieria sulphuraria* (Merola)	45	2–3	Solfatara (Pozzuoli, Campania, Italy)	(Merola *et al.* 1982)
Bacteria	Proteobacteria	*Acidithiobacillus* (*A. caldus*)	25–45 (45)	2.0–4.0 (2.0–2.5)	Coal spoil enrichment (Belfast, Northern Ireland, United Kingdom)	(Dopson 2016)
	Nitrospirae	*Leptospirillum* (*L. ferriphilum*)	30–43 (30–37)	1.4–3.0 (1.4–1.8)	Bioleaching Tank (South Africa)	(Coram and Rawlings 2002)
	Firmicutes	*Sulfobacillus thermosulfidooxidans*	50–55	1.7–2.4	Copper-zinc-pyrite ore (Nikolaev Mine, East Kazakhstan, Kazakhstan)	(Bogdanova *et al.* 2006)
		Sulfobacillus sibericus	55	2.0–2.5	Nezhdaninskoe ore deposit (East Siberia, Republic of Sakha, Russian Federation)	(Melamud *et al.* 2003)
		Alicyclobacillus (*A. acidocaldarius*)	35–65 (60–65)	1.5–4.5 (3.0–4.0)	Hot spring (Yellowstone NP, Wyoming, USA)	(Darland and Brock 1971; Karavaiko *et al.* 2005)
	Aquificae	*Hydrogenobaculum acidophilum*	65	3.0–4.0	Solfatara (Tsumagoi, Gunma, Japan)	(Shima and Suzuki 1993)
Archaea	Candidate *Geoarchaeota*	Uncultured	60–78	3.5	Norris Geyser Basin (Yellowstone, Wyoming, USA)	(Kozubal *et al.* 2013)
	Candidate *Marsarchaeota*	Uncultured	50–80	3.0–3.5	Thermal springs (Yellowstone, Wyoming, USA)	(Jay *et al.* 2018)
	Euryarchaea	*Aciduliprofundum boonei*	70	4.2–4.8	Deep sea vents (Mariner, Lau Basin, near Tonga)	(Reysenbach *et al.* 2006)
		Acidiplasma cupricumulans	53.6	1.0–1.2	Mineral bioleaching heap (Undisclosed, Myanmar)	(Hawkes *et al.* 2006)
		Acidiplasma aeolicum	45	1.4–1.6	Hydrothermal pool (Vulcano Island, Messina, Italy)	(Golyshina *et al.* 2009)
		Picrophilus (*P. torridus*/*P. oshimae*)	60	0.7	Solfatara (Hokkaido, Japan)	(Schleper *et al.* 1996)
		Thermoplasma volcanium	60	2.0	Solfatara (Vulcano Island, Messina, Italy)	(Segerer and Stetter 1988)
		Thermoplasma acidophilum	59	1.0–2.0	Coal refuse pile (Friar Tuck Mine, Indiana, USA)	(Darland *et al.* 1970)
		Thermogymnomomonas acidicola	60	3.0	Solfatara (Ohwaku-dani, Hakone, Japan)	(Itoh, Yoshikawa and Takashina 2007)
	Crenarchaea (non-*Sulfolobales*)	*Acidilobus aceticus*	85	3.9	Thermal spring (Moutnovski, Kamchatka, Russia)	(Prokofeva *et al.* 2000)
		Acidilobus saccharovorans	80–85	3.5–4.0	Thermal spring (Uzon Caldera, Kamchatka, Russia)	(Prokofeva *et al.* 2009)
		Caldisphaera laguensis	70–75	3.5–4.0	Hot spring (Mt Maquiling, Laguna, Philippines)	(Itoh *et al.* 2003)
		Caldivirga maquilingensis	85	3.7–4.2	Hot spring (Mt Maquiling, Laguna, Philippines)	(Itoh *et al.* 1999)
Archaea	*Crenarchaea* (*Sulfolobales*)	*Acidianus ambivalens*	81	2.5	Solfatara (Leihnukur, Iceland)	(Zillig *et al.* 1986)
		Acidianus brierleyi	70	1.5–2.0	Thermal spring drainage (Yellowstone, Wyoming, USA)	(Brierley and Brierley 1973; Segerer *et al.* 1986)
		Acidianus infernus	90	2.0	Mud pot (Naples, Campania, Italy)	(Segerer *et al.* 1986)
		Acidianus sulfidivorans	74	0.8–1.4	Solfatara (Lihir Island, Papua New Guinea)	(Plumb *et al.* 2007)
		Metallosphaera cuprina	65	3.5	Thermal spring (Tengchong, Yunnan, China)	(Liu *et al.* 2011)
		Metallosphaera hakonensis	70	3.0	Thermal spring (Ohwaku-dani, Hakone, Japan)	(Takayanagi *et al.* 1996)

Table 1. Continued

Kingdom	Phylum/division	Genus/species	T_{opt} (°C)	pH_{opt}	Isolation site (locale, country)	Reference
		Metallosphaera prunae	75	2.5	Uranium slag heap (Ronneburg, Hesse, Germany)	(Fuchs et al. 1995)
		Metallosphaera sedula	75	2.5	Thermal pool (Naples, Campania, Italy)	(Huber et al. 1989)
		Saccharolobus caldissimus	85	3.0	Thermal spring (Ohwaku-dani, Hakone, Japan)	(Sakai and Kurosawa 2018)
		Saccharolobus shibatae	81	3.0	Mud pot (Kyushu, Japan)	(Grogan et al. 1990)
		Saccharolobus solfataricus	87	4.5	Thermal spring (Agnano, Campania, Italy)	(Zillig et al. 1980)
		Stygiolobus azoricus	80	2.5–3.0	Solfatara (São Miguel Island, Azores, Portugal)	(Segerer et al. 1991)
		Sulfodiicoccus acidiphilus	65–70	3.0–3.5	Solfatara (Ohwaku-dani, Hakone, Japan)	(Sakai and Kurosawa 2017)
		Sulfolobus acidocaldarius	70–75	2.0–3.0	Thermal spring (Yellowstone, Wyoming, USA)	(Brock et al. 1972)
		Sulfuracidifex tepidarius	65	3.5	Solfatara (Ohwaku-dani, Hakone, Japan)	(Itoh et al. 2020)
		Sulfuracidifex metallicus	65	2.0–3.0	Solfatara (Krafla, Iceland)	(Itoh et al. 2020)
		Sulfurisphaera ohwakuensis	84	2.0	Thermal spring (Ohwaku, Hakone, Japan)	(Kurosawa et al. 1998)
		Sulfurisphaera tokodaii	80	2.5–3.0	Hot spring (Beppu, Kyushu, Japan)	(Suzuki et al. 2002)
		Sulfurisphaera javensis	80–85	2.5–4.0	Thermal spring (Java, Indonesia)	(Tsuboi et al. 2018)

2001; Prangishvili et al. 2017; Munson-Mcgee, Snyder and Young 2018). These viruses are characterized by a large genetic and morphological diversity, including many unique shapes that are not found in viruses infecting bacteria and eukaryotes (Pina et al. 2011; Prangishvili et al. 2017). The evolutionary origin of archaeal viruses is not clear, but the high diversity might have originated during the early stages of evolution of cellular life, maintained in Archaea, and lost in bacterial and eukaryotic lineages (Prangishvili, Forterre and Garrett 2006; Prangishvili 2015; Prangishvili et al. 2017). All isolated viruses from *Sulfolobales* have DNA genomes, and the majority of their gene products have unknown functions (Prangishvili et al. 2017). Metagenomic analysis indicated the presence of viruses with RNA genomes in high-temperature acidic hot springs (Bolduc et al. 2012). However, viral particles were not isolated, and the exact host remains unknown (Bolduc et al. 2012; Stedman, Kosmicki and Diemer 2013).

Members of at least eight different viral families infect thermoacidophilic archaea: bottle-shaped *Ampullaviridae* (Haring et al. 2005), tailed *Bicaudaviridae* (Häring et al. 2005), spindle-shaped *Fuselloviridae* (Schleper, Kubo and Zillig 1992), droplet-shaped *Guttaviridae* (Arnold, Ziese and Zillig 2000), filamentous *Lipotrixviridae* (Bettstetter et al. 2003), polyhedral *Portogloboviridae* (Liu et al. 2017), rod-shaped *Rudiviridae* (Prangishvili et al. 1999) and the icosahedral *Turriviridae* (Rice et al. 2004) (Fig. 3). The diversity of morphotypes encountered among viruses infecting thermoacidophilic *Crenarchaea* is in stark contrast to that found for euryarchaeal or bacterial viruses, which are dominated by head–tail morphologies (Pietilä et al. 2014; Prangishvili et al. 2017). Interestingly, recently available cryo-EM (cryogenic electron microscopy) structures have shown that several viruses infecting members of the *Sulfolobales* package their dsDNA genome in A-form (DiMaio et al. 2015; Wang et al. 2019b). A-form DNA was at first thought to be an artifact and have no biological significance, but the widespread usage of A-form DNA by archaeal viruses suggests that this packaging helps to protect the viral genomes against adverse conditions in thermal hot springs (Wang et al. 2019b).

Among the thermoacidophiles, *Sa. islandicus* and *Sa. solfataricus* are model systems for the study of virus–host interactions in *Crenarchaea* (Pina et al. 2011; Prangishvili, Koonin and Krupovic 2013; Dellas et al. 2014). The valuable, but still limited, knowledge on infection strategies of crenarchaeal viruses mainly derives from viruses infecting these organisms. For initial attachment and entry into the cell, the various surface appendages with which *Sulfolobales* are covered (see the section 'Cell cycle and modes of growth') are important for multiple viruses. *Sulfolobus* turreted icosahedral virus (STIV1) binds with its turrets to thin filaments of unknown identity on the surface of *Sa. solfataricus* (Hartman et al. 2019). *Saccharolobus islandicus* rod-shaped virus (SIRV) particles use the three tail fibers that are present at the distal parts of the virion for attachment to adhesive type IV pili on the its surface (Quemin et al. 2013; Deng et al. 2014; Rowland et al. 2020). Like SIRV, the *Sulfolobus* spindle-shaped virus SSV requires pili for infection, but the particles do not directly attach to the pili, and the role of pili in viral entry is unresolved (Rowland et al. 2020). Primary attachment to filamentous surface structures is a common strategy of bacterial viruses, which can increase the chances of successful infection (Poranen, Daugelavičius and Bamford 2002; Quemin and Quax 2015). The mechanisms by which viruses move along archaeal filaments to the cell surface are unknown and are likely different from those of bacterial viruses, since the archaeal surface filaments have different structural organization (see the section 'Cell cycle and modes of growth') (Quemin and Quax 2015; Chaudhury, Quax and Albers 2018).

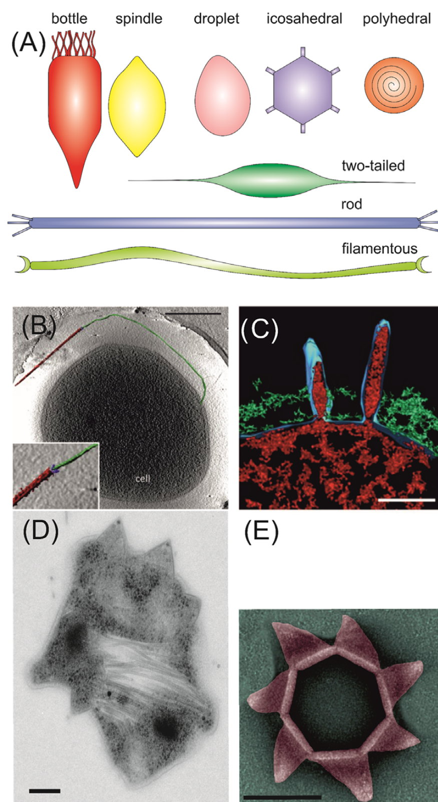


Figure 3. Thermoacidophilic archaeal viruses and their infection mechanisms. **(A)** Schematic representation of virion morphologies of viruses infecting thermoacidophilic archaea as described in the text. **(B)** Segmented tomographic volume of an SIRV2 virion (red) attached to a surface filament of *Sa. islandicus* (green) with help of the three terminal virion fibers (blue). Inset depicts a magnification of the interaction between the tail fibers and the surface structure. Scale bar, 500 nm. **(C)** Volume segmentations of electron microscopy tomograms showing Sulfolobus spindle-shaped virus 1 maturation and release by budding. Scale bar, 50 nm. **(D)** Transmission electron micrograph of a thin section of a SIRV2-infected *Sa. islandicus* cell displaying several pyramidal egress structures. Scale bar, 100 nm. **(E)** Transmission electron micrographs of an isolated pyramidal egress structure in open conformation isolated after SIRV2 infection of *Sa. islandicus*. Scale bar, 100 nm. Adapted from Bize *et al.* (2009); Quax *et al.* (2011); Quemin *et al.* (2013, 2016).

Once virions have attached, their genomes can enter the cell. Some viruses, such as SSV, can integrate their genome into that of the host (Muskhelishvili, Palm and Zillig 1993; Serre *et al.* 2002; Clore and Stedman 2007). Circularization and replication of the integrated SSV genome is induced by UV light (Schleper, Kubo and Zillig 1992; Fröls *et al.* 2008). SSV has been employed to develop a genetic manipulation system for *Sa. solfataricus* (see the section 'Potential and current uses of thermoacidophiles in biotechnological applications'). Several other viruses do not integrate, instead replicating directly after entry. The replication mechanism of only a few thermoacidophile model viruses has been studied. For example, replication of the dsDNA genome of *Acidianus* Filamentous Virus 1 (AFV1) relies on recombination events for initiation and termination and has a terminally bound protein (Pina *et al.* 2014). Replication of the linear dsDNA genome of SIRV requires a virus-encoded dimeric Rep protein that initiates replication by making single stranded nicks and the virus-encoded Holliday junction resolvase (Hjr) to resolve viral genome concatemers (Blum *et al.* 2001; Peng *et al.* 2001; Oke *et al.* 2010, 2011). SIRV Hjr interacts with proliferating cell nuclear antigen (PCNA), a key replication protein in archaea (Gardner *et al.* 2014). Interestingly, SIRV forms a distinct replication focus in the cell to which viral and host replication proteins are specifically recruited (Martínez-Alvarez, Deng and Peng 2017).

After genome replication, virions are formed in the cytoplasm. Virion maturation can occur (i) before, (ii) during or (iii) after release. (i) Several lytic viruses infecting *Sulfolobales*, such as STIV and SIRV, were shown to mature in the cytoplasm and employ an unusual lysis mechanism that relies on the formation of 7-fold symmetric pyramidal egress structures, of which a dozen form during viral infection on the host cell surface (Bize *et al.* 2009; Brumfield *et al.* 2009; Prangishvili and Quax 2011; Quax *et al.* 2011; Quax and Daum 2017) (Fig. 3). These ∼150-nm structures consist of one viral protein and protrude through the protective S-layer (see the section 'Cell cycle and modes of growth') (Fu *et al.* 2010; Quax *et al.* 2010; Snyder *et al.* 2011; Daum *et al.* 2014). They open outward at the end of the infection cycle to allow for the release of virions (Bize *et al.* 2009; Brumfield *et al.* 2009; Fu *et al.* 2010; Daum *et al.* 2014). (ii) SSV matures upon egress, as the virions are released via budding and are covered in a lipid layer during this process (Quemin *et al.* 2016). In fact, this is the first case of budding observed for a prokaryotic virus. Budding viruses allow for a continuous release of virions and the cells remain alive throughout the infection cycle (Schleper, Kubo and Zillig 1992; Quemin *et al.* 2016). The ESCRT-III system could play a role in the budding of archaeal viruses (Fig. 3) (Liu *et al.* 2017). (iii) *Acidianus* two-tailed virus (ATV) and *Sulfolobus* monocaudavirus (SMV1) are exceptional viruses for which virion maturation (the lengthening of the tails) happens outside and independent of the host cell, after viral release (Häring *et al.* 2005; Prangishvili *et al.* 2006; Scheele *et al.* 2011; Uldahl *et al.* 2016). These tails consist of helically arranged globular subunits that develop from the two pointed ends of the virion when it is outside the host cell (Prangishvili *et al.* 2006). High temperatures are required for this morphological transformation. In summary, viruses of thermoacidophiles are unique because of their diverse morphologies and the model virus–host systems of the *Sulfolobales* have provided important insights into the infection strategies of crenarchaeal viruses in general.

CRISPR-Cas mediated viral defense in thermoacidophiles

The omnipresence of viruses in archaeal habitats has resulted in the development of several anti-viral defense strategies, of which CRISPR-Cas is without doubt the best known. CRISPR (clustered regularly interspaced short palindromic repeats) systems in *Sulfolobales* have been studied since the 'early days' of CRISPR research (Vestergaard *et al.* 2008; Han and Krauss 2009; Held and Whitaker 2009; Lillestøl *et al.* 2009; Garrett *et al.* 2011; Zhang and White 2013), just after these systems were suggested to play a role in defense against viruses in bacteria and archaea (Bolotin *et al.* 2005; Mojica *et al.* 2005; Pourcel, Salvignol and Vergnaud 2005; Makarova *et al.* 2006). CRISPR arrays consist of a series of ∼30 bp genomic repeats, which are interspaced by unique sequences that can match foreign genetic elements (van der Oost, Jackson and Wiedenheft 2014). CRISPR-associated proteins (Cas) are usually encoded in the proximity of the CRISPR array. Upon a viral infection, new spacers, exactly matching the genome of the infecting virus, are integrated between two repeats. The arrays are then processed by Cas proteins, and the spacer is used as a guide to specifically target and interfere with the matching sequences in the viral genome (Barrangou *et al.* 2007; Barrangou and Horvath 2017; Jackson *et al.* 2017). Thus,

CRISPR-Cas provides specific and inheritable immunity (Barrangou *et al.* 2007; Brouns *et al.* 2008). CRISPR arrays can also be used as a map to track previous encounters with viruses and to indicate viral host range (Bautista *et al.* 2017; Munson-Mcgee *et al.* 2018; Pauly *et al.* 2019).

CRISPR-Cas systems are present in ∼40% of bacteria, most archaea (85%), and almost all extreme thermophiles (97%) (Makarova *et al.* 2019). Based on the Cas proteins, the CRISPR systems have been divided into several different groups, and this division keeps evolving as new systems are being discovered (Makarova *et al.* 2019). *Crenarchaea*, such as the *Sulfolobales*, usually harbor multiple CRISPR systems in their genome, and generally have longer CRISPR arrays than bacteria (Zhang and White 2013). Crenarchaeal genomes are substantially enriched for type III systems of class 1 (Zhu *et al.* 2018; Makarova *et al.* 2019), which rely on transcription-dependent (specific RNA binding and cleavage) and subsequent (non-specific) DNA degradation (Deng *et al.* 2013; Goldberg *et al.* 2014; Zhu *et al.* 2018). Type III systems typically possess a Cas10 protein with a Palm polymerase domain that can cyclize ATP to generate cyclic oligoadenylate (cOA) to act as a second messenger (Kazlauskiene *et al.* 2017; Niewoehner *et al.* 2017; Rouillon *et al.* 2018). Formation of cOA leads to signal amplification that activates other defense mechanisms, including host and viral DNA degradation that results in immunity or cell dormancy (Rouillon *et al.* 2018; Rostøl and Marraffini 2019). In *Saccharolobus*, cyclic tetra-adenylate (cA_4) can be degraded by host-encoded ring-nucleases to reset the signal (Athukoralage *et al.* 2018). Interestingly, some archaeal viruses encode a potent ring nuclease that acts as an anti-CRISPR (Arc) (Athukoralage *et al.* 2020a,b). More Arcs have been identified in archaeal viruses, such as those that bind and inhibit type III-B or I-D CRISPR systems (He *et al.* 2018; Bhoobalan-Chitty *et al.* 2019). Different viral families probably use different strategies to evade CRISPR-Cas immunity, as natural populations of *Sulfolobus* have developed CRISPR-Cas immunity with a different structure and diversity in response to SIRV and SSV infections (Pauly *et al.* 2019).

Despite the viral strategies to evade CRISPR-Cas mediated defense, this seems an effective immune system in extreme thermophiles ($T_{opt} \geq 70°C$), illustrated by the presence of CRISPR-Cas in nearly all of their genomes. It remains to be seen why CRISPR-Cas systems are so ubiquitous, specifically in extremely thermophilic archaea. One possible explanation relates to the lower mutation rate of viruses in extreme environments that, combined with the lower population sizes of extreme thermophiles compared with mesophiles, gives extremely thermophilic viruses limited possibility to escape immunity (Weinberger *et al.* 2012; Prangishvili *et al.* 2017).

Besides CRISPR-Cas, *Crenarchaea* are specifically enriched in toxin–antitoxin (TA) systems, which play a role in abortive infection in bacteria (Koonin, Makarova and Wolf 2017). Viral infection of different *Sulfolobales* induces expression of TA systems (Ortmann *et al.* 2008; Quax *et al.* 2013; León-Sobrino, Kot and Garrett 2016). Furthermore, several thermoacidophiles encode an Argonaute protein, which has been implicated in defense against foreign genetic elements (Makarova *et al.* 2009; Swarts *et al.* 2014a,b; Willkomm *et al.* 2017). However, it needs to be verified experimentally if archaeal TA or Argonaute systems provide immunity to viral infection. Certainly, thermoacidophiles encode novel viral defense mechanisms that are awaiting discovery (Doron *et al.* 2018).

GENETIC MECHANISMS

Effective packaging and organization of genomic DNA into the confined space of the nucleus or nucleoid, while at the same time enabling a dynamic and reliable genome replication and gene expression, is essential for every living organism. The underlying molecular mechanisms (replication, transcription and translation) are central to life. The study of genetic mechanisms in archaea lags behind those focused on bacteria and eukaryotes. However, it is clear that archaeal information processing machineries are related to their eukaryotic counterparts, that gene regulation processes are bacteria-like and that chromosome organization has both eukaryote-like and bacteria-like features (Peeters *et al.* 2015; Blombach *et al.* 2019; Lemmens *et al.* 2019a; Greci and Bell 2020).

Thermoacidophilic *Crenarchaea* belonging to the *Sulfolobales* order have served as an archaeal model system to study chromosome organization, DNA replication, transcription and translation processes. Although many insights can be extended further to the entire archaeal domain, there are also unique, lineage-specific aspects. For example, while most archaea harbor eukaryote-like histones involved in chromosome structuring, these are completely absent in thermoacidophilic archaea, namely in all *Crenarchaeota* and in *Thermoplasma acidophilum* (Peeters *et al.* 2015; Hocher *et al.* 2019).

Chromosome packaging and structuring

Thermoacidophilic archaea typically have a single circular, relatively small chromosome with a size between 1.5 and 3 Mbp (Chen and Morris 1972; She *et al.* 2001). This chromosome is packaged into a condensed and organized chromatin structure by the action of different types of chromatin proteins (Fig. 4). There is a large evolutionary divergence in chromatin proteins present in archaea, including the *Sulfolobales*; while histone orthologs are absent in this lineage, an interplay exists between a variety of nucleoid-associated proteins (NAPs) with different levels of conservation (Peeters *et al.* 2015). These proteins are small (between 7 and 10 kDa), basic and highly abundant in the cell, constituting up to 5% of soluble cellular protein (Mai *et al.* 1998). They harbor DNA-binding motifs that are also found in specific transcription regulators (see below the section 'Regulatory transcription factors in *Sulfolobales*'), such as the winged helix-turn-helix (wHTH) motif, and bind DNA with low or no sequence specificity. In case of low sequence specificity, these NAPs typically prefer GC-rich sequences (Kalichuk *et al.* 2016; Hocher *et al.* 2019). The *Sulfolobales* harbor two paralogs of the archaea-universal NAP Alba, which was initially assumed to be an important chromatin structuring protein (Bell *et al.* 2002), with the extent of heterodimerization between the two paralogs determining the architectural effects (Laurens *et al.* 2012). This hypothesis has recently been revisited as Alba was shown to be a general nucleic acid-binding protein interacting with RNA as well (Guo *et al.* 2014). Besides Alba, the NAP Cren7 is highly conserved in not only the *Sulfolobales* but also all *Crenarchaeota*; Cren7 is a versatile architectural protein, bending and also bridging DNA, thereby forming highly condensed chromatin filaments (Guo *et al.* 2008; Zhang *et al.* 2019c, 2020). Sul7d, analogous to Cren7, is a monomeric protein, which is capable of bending DNA; it has been found in many *Sulfolobales* genera: *Sulfolobus* and *Saccharolobus*, *Acidianus*, *Metallosphaera*, *Stygiolobus* and *Sul-*

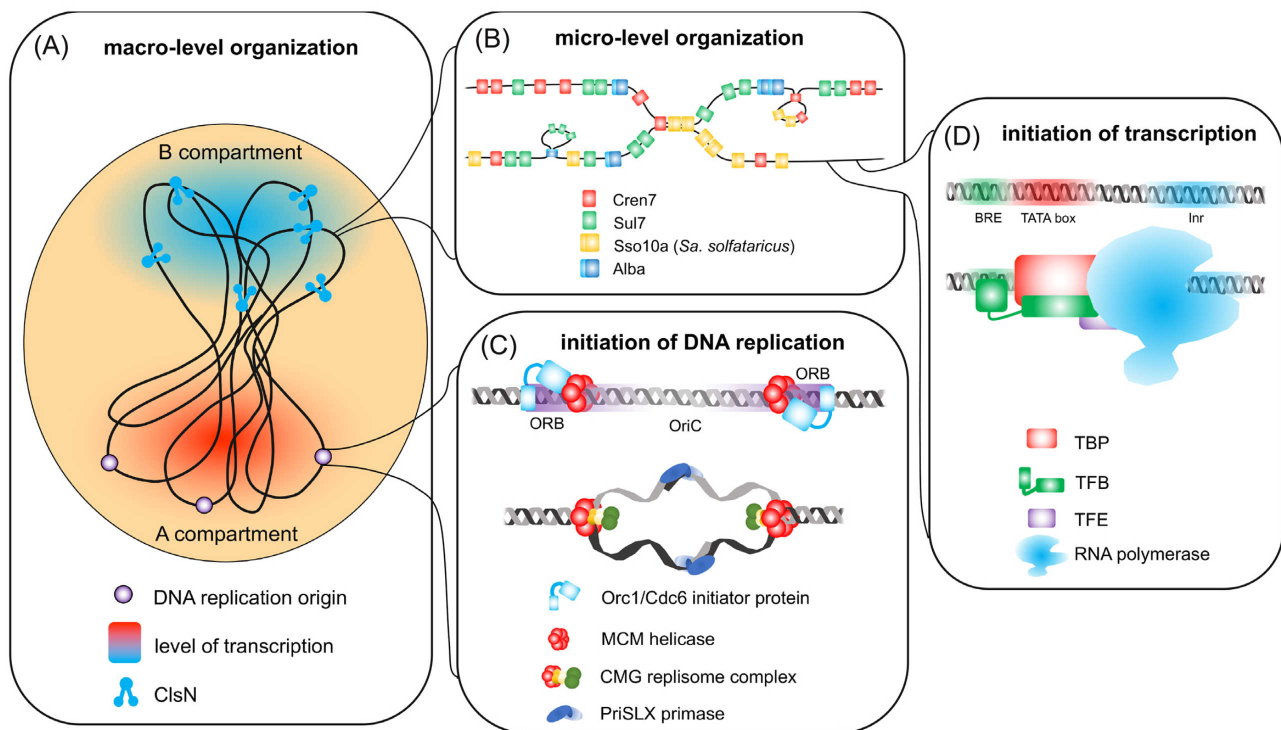


Figure 4. Main principles in genome organization and genetic information processing in the *Sulfolobales*. Conceptual schemes representing the major elements and principles of macro-level organization of the genomic DNA (adapted from Takemata, Samson and Bell 2019) **(A)**, micro-level organization of the genomic DNA (partially adapted from Peeters *et al.* 2015) **(B)**, initiation of replication **(C)** and initiation of transcription **(D)**.

furisphaera (Kalichuk *et al.* 2016). In addition, there are species-specific NAPs, such as the Sso10a parologs in *S. solfataricus*, that are dimeric proteins capable of bending DNA and either bridging it or forming filaments (Driessen *et al.* 2016). Finally, the euryarchaeal *T. acidophilum*, which also lacks histones, harbors a NAP that is homologous to the bacterial HU family (Hocher *et al.* 2019).

The heterogeneity in the NAP protein machinery responsible for packaging DNA in the *Sulfolobales* (Fig. 4B)—when considering a single species—is hypothesized to accommodate differential local chromatin structuring when expression levels or post-translational modifications (PTMs) of the individual NAPs are altered. This in turn might affect transcriptional expression in a polygenic manner (Peeters *et al.* 2015). Indeed, PTMs have been observed for the NAPs Alba, Cren7, Sso7d and Sul7d (Vorontsov *et al.* 2016). Early studies postulated that acetylation of the Lys16 residue of Alba constitutes a global gene regulation mechanism similar to eukaryotic histone modification (Bell *et al.* 2002). This has later been refuted and determined to be an N-terminal acetylation event instead that does not affect the nucleic acid-binding capacity of Alba and is possibly involved in protein turnover regulation (Ma *et al.* 2016; Cao *et al.* 2018a). Besides acetylation, methylation occurs widely on these NAPs (Niu *et al.* 2013; Vorontsov *et al.* 2016) and might be linked to thermostabilization of the chromatin, as well as to epigenetic mechanisms of gene regulation. The existence of such epigenetic mechanisms was recently demonstrated for a strain of *Sa. solfataricus* that was evolved in an adaptive laboratory evolution experiment and displayed a superacid-resistant phenotype. This evolved strain harbored no genomic changes with respect to the original strain (Payne *et al.* 2018; Johnson *et al.* 2019). Instead, the acid resistance appeared to be mediated by a different methylation status of the NAPs Cren7 and Sso7d. Other than NAP methylation, methylation of the genomic DNA itself might also be responsible for epigenetic mechanisms of gene regulation. Recently, the DNA methylome of *S. acidocaldarius* was mapped and shown to consist of base methylations that are more than just a part of restriction modification defense systems (Couturier and Lindås 2018). More specifically, N^6-methyl-adenine methylations were found and hypothesized to be involved in the regulation of the cell cycle or other biological functions (Couturier and Lindås 2018).

Besides NAP-mediated local structuring, the genome is also organized in domains at a higher level. This higher order chromosome organization has recently been elucidated for *S. acidocaldarius* and *Sa. islandicus* using the Hi-C methodology, a combination of chromosome conformation capture (3C) and high-throughput sequencing (Takemata, Samson and Bell 2019). Similar to what has been observed in metazoan eukaryotes and very different from what is observed in bacteria, the *Sulfolobales* genome is organized in two distinct sub-Mbp compartments, each characterized by a different average level of transcription (Takemata, Samson and Bell 2019) (Fig. 4A). While the A compartment, which harbors genes mainly involved in core metabolic processes such as protein biogenesis, is transcriptionally active, the B compartment appears to be in a more silent transcriptional state and harbors genes that function in diverse metabolic pathways and physiological processes. For example, gene expression of the B compartment is typically induced in response to environmental stress conditions, such as the archaellum motility apparatus and a fatty acid metabolism gene cluster. In addition, the B compartment is enriched in mobile genetic elements, such as CRISPR-Cas clusters. Although it is still unclear which proteins are responsible for the active structuring of the chromosome into the two compartments, a major role has been described for the novel chromatin structuring

protein coalescin (ClsN) (Takemata, Samson and Bell 2019). While the *Sulfolobales* do not harbor a homolog of the typical condensin complex belonging to the Structural Maintenance of Chromosome (SMC) family, conserved in bacteria, eukaryotes and most other archaea (Kamada and Barillà 2018), they possess the SMC-like ClsN instead, which is significantly smaller than the SMC subunits of condensin and possibly has a zinc hook domain instead of a hinge (Takemata, Samson and Bell 2019). There is an inverse correlation between the presence of ClsN and the transcriptional machinery, with ClsN being mainly associated with transcriptionally less active genes in the B compartment. It is hypothesized that the protein assists in the higher level compartmentalization by mediating intra- and interdomain interactions (Takemata, Samson and Bell 2019).

DNA replication

One of the most important transactions undergone by genomic DNA is its replication as part of the cell division process. It is striking that, similar to eukaryotes, several archaeal lineages are characterized by the chromosome harboring multiple replication origins, genomic sites at which replication is initiated. In contrast, bacteria only have a single origin. *Sulfolobales* are characterized by three replication origins (OriCs 1, 2 and 3), each of which accommodate a single replication initiation event during the cell cycle (Lundgren *et al.* 2004; Robinson *et al.* 2004; Robinson and Bell 2007; Duggin, McCallum and Bell 2008). Mutagenesis analysis indicated that, while none of the individual OriCs is essential, at least one is required (Samson *et al.* 2013). The observation that all three OriCs are located in the transcriptionally active chromosome compartment A (Fig. 4A) might suggest that DNA replication processes are involved in higher order chromatin structuring. However, this appears not to be the case as OriC mutant strains do not display any differences in their chromatin structure (Takemata, Samson and Bell 2019).

The archaeal replication machinery resembles the eukaryotic machinery, as exemplified by the well-described machinery of *Sa. solfataricus* (Fig. 4C) (Dionne *et al.* 2003b; Greci and Bell 2020). Archaeal initiator proteins, responsible for OriC recognition and assembly of the replisome, are related to the eukaryotic Orc1 and Cdc6 replication initiation proteins, which are characterized by an N-terminal AAA^{+} fold and a C-terminal wHTH domain (Cunningham Dueber *et al.* 2007). *Sulfolobales* encode three Orc1/Cdc6-like paralogs, with Orc1–1 specifically recognizing the origin recognition boxes (ORBs) in OriC1 (Samson, Abeyrathne and Bell 2016). Upon binding ATP, two inversely bound Orc1–1 proteins recruit two MCM homohexamers, which are the 3′-to-5′ helicases (Samson, Abeyrathne and Bell 2016; Meagher, Epling and Enemark 2019). Also, analogous to the eukaryotic system, the MCM helicase associates with additional replication proteins, forming the so-called CMG (Cdc45-MCM-GINS) replisome core (Fig. 4C). In *S. acidocaldarius* and *Sa. islandicus*, MCM recruits a Cdc45 ortholog and two GINS-like proteins Gins23 and Gins15, each protein having a homodimeric composition in the complex (Xu *et al.* 2016). In contrast, in *T. acidophilum* a homotetrameric GINS protein is part of the CMG complex (Ogino *et al.* 2017). Although it is unclear how the melting of the DNA helix is accomplished in *Sulfolobales* after assembly of the replisome at the replication origin, the involvement of a replication-dedicated DNA-dependent RNA polymerase responsible for primer synthesis has been established. This DNA primase initiates leading strand synthesis, or the synthesis of Okazaki fragments for lagging strand synthesis, and is recruited by interacting with the GINS complex (Marinsek *et al.* 2006), with the primase in *Sa. islandicus* being a heterotrimer PriSLX (Liu *et al.* 2015). Finally, the enzyme responsible for DNA synthesis, DNA polymerase, has also been shown to be eukaryote-like. *Crenarchaeota* possess three different B-family DNA polymerases, with PolB1 being essential and PolB2 and PolB3 shown not to be required for cell viability and hypothesized to be involved in DNA damage repair (Greci and Bell 2020). Prior to elongation a sliding clamp, constituted by a heterotrimeric PCNA protein (Dionne *et al.* 2003a), is loaded onto the DNA by replication factor C (RFC) and forms a ring-shaped structure. This clamp functions as a molecular platform to recruit the DNA polymerase and other replication-associated enzymes. In contrast to bacteria, which harbor site-specific mechanisms, replication termination appears to be mediated by passive-fork collision taking place halfway between the active replication origin(s) in a site-unspecific manner (Lundgren *et al.* 2004; Duggin, Dubarry and Bell 2011; Samson *et al.* 2013).

Sulfolobales have an organized cell cycle (see the section '*Sulfolobus* cell division'), with well-defined gap phases and in which the process of DNA replication is temporally separated from the process of chromosome segregation (Bernander and Poplawski 1997). Following the S phase in which the chromosome is replicated, there is a significant post-replicative period (G2 phase) in which sister chromatids remain bound together to form hemicatenane structures (Robinson and Bell 2007). Next, chromosome segregation is accomplished by a bacterial-like ParAB-like system; in *Sa. solfataricus*, this system consists of SegA, an ortholog of the bacterial, Walker-type ParA ATPase protein and an archaea-specific DNA-binding protein named SegB (Kalliomaa-Sanford *et al.* 2012).

Transcription and its regulation

Basal transcription machinery in the Sulfolobales

The small genomes of archaea share their genetic organization with bacteria, with an operonic transcription unit structure that is dense and characterized by short intergenic regions. Similar to other information processing steps, mechanisms of basal transcription have been extensively studied in archaeal species belonging to *Sulfolobales*, ranging from focused biochemical studies with *in vitro* reconstituted transcription systems (Qureshi, Bell and Jackson 1997; Bell *et al.* 1999; Blombach *et al.* 2019) to high-resolution mapping of the transcriptome (Wurtzel *et al.* 2010; Cohen *et al.* 2016; Dar *et al.* 2016). Not long after the isolation of the first thermoacidophilic crenarchaeal isolate *S. acidocaldarius*, Wolfram Zillig performed a biochemical analysis of its RNA polymerase, which is the key enzyme of transcription, thereby concluding that its subunit pattern resembles that of eukaryotic RNA polymerase (Zillig, Stetter and Janekovic 1979). Much later, structural analysis of the *Sa. solfataricus* and *Sa. shibatae* RNA polymerases confirmed that they are complexes consisting of 13-subunit proteins that display an evolutionary relationship with the eukaryotic RNA polymerase II (Hirata, Klein and Murakami 2008; Korkhin *et al.* 2009). Transcription initiation requires a set of additional general transcription factors that are also homologous to eukaryotic factors: TATA-binding protein (TBP), transcription factor B (TFB) and transcription factor E (TFE) (Fig. 4D). A typical promoter region in *Sa. solfataricus* is characterized by a core TATA-box region of which the center is located ~26 base pairs (bps) upstream of the transcription initiation site, which is directly preceded by a purine-rich factor B recognition element (BRE) (Wurtzel *et al.* 2010). Besides these canonical archaeal promoter elements, an additional 6-bp, AT-rich, conserved promoter element was identified in *Sa. islandicus*

just upstream of the transcription start site (TSS), named initiator (Inr) (Ao *et al.* 2013).

Initiation of transcription proceeds by the stepwise assembly of the different components in the pre-initiation complex (PIC), which was first studied with *Sa. shibatae* (Qureshi, Bell and Jackson 1997; Bell *et al.* 1999). First, the highly symmetrical TBP binds the TATA box region followed by the association of TFB to the TBP-DNA complex. By specific recognition of the BRE promoter element, TFB determines the correct orientation of the PIC (Bell *et al.* 1999). Next, the RNA polymerase as well as TFE are recruited, with TFE being a heterodimeric protein consisting of TFEα and TFEβ subunits in *Sulfolobales* (Blombach *et al.* 2015). Although TFE is not absolutely required for *in vitro* transcription reactions to proceed, it stabilizes the PIC and facilitates DNA melting in the Inr promoter region, thereby assisting the formation of an open complex during the transitioning from the initiation to the elongation phase (Bell *et al.* 2001; Blombach *et al.* 2015). During elongation, TBP and TFB dissociate from the RNA polymerase, which is assisted by different transcription elongation factors. Most of these factors, such as the transcript cleavage factor TFS and the processivity factors Spt4/5 and Elf1, are also evolutionarily related to eukaryotic elongation factors (Fouqueau *et al.* 2017; Blombach *et al.* 2019). Finally, although transcription termination remains understudied in archaea, a transcriptome-wide Term-seq approach enabled the mapping of all RNA 3′ termini in *S. acidocaldarius*, revealing a widespread occurrence of multiple terminators. This leads to alternative 3′ isoforms, with U-rich terminator motifs retrieved for 53% of all transcription units (Dar *et al.* 2016).

To some extent, components of the basal transcription machinery are capable of mediating a global regulation of the transcription initiation process. Certain archaea harbor multiple paralogs of TBP and TFB and it is hypothesized that these are employed for global gene regulation in a similar way as alternative sigma factors in bacteria (Facciotti *et al.* 2007). *Sulfolobales* typically harbor a single TBP and three TFB paralogs, with TFB3 being a truncated form. The latter functions as a transcriptional activator in a *trans*-dependent manner on the canonical TFB1 (Paytubi and White 2009). TFB3 activates the expression of Ups pili and the Ced DNA import system in response to UV irradiation (Paytubi and White 2009; Feng *et al.* 2018; Schult *et al.* 2018). On the other hand, TFE might be involved in global regulation in response to oxidative and heat shock stress, as the cellular protein levels were depleted under these stress conditions (Iqbal and Qureshi 2010; Blombach *et al.* 2015).

Regulatory transcription factors in the Sulfolobales

The observation of extensive transcriptome-wide differential gene expression in response to stress conditions or shifts in nutritional conditions (see the sections 'Extreme thermoacidophily and stress response' and 'Metabolism') indicates that transcription initiation is highly susceptible to regulation. It can be assumed that regulatory transcription factors (TFs) play an important role in this regard. In contrast to the eukaryotic basal transcription machinery, archaeal regulatory TFs resemble bacterial regulators pointing to a shared ancestry (Aravind 1999). One-component regulators are characterized by two domains: an N-terminal DNA-binding domain, with a wHTH or an HTH motif, and a C-terminal ligand-binding domain. The structural resemblance between TFs and wHTH-containing NAPs sometimes complicates their distinction, and dual-function DNA-binding proteins can be found within the entire spectrum between a specifically acting regulatory TF and a globally acting chromatin protein (Karr *et al.* 2017; Dorman *et al.* 2020). This is illustrated by the archaea-specific Lrs14 family of DNA-binding proteins, which is widespread in *Sulfolobales* and shown to bind DNA non-specifically and to regulate biofilm formation and motility in *S. acidocaldarius* (Orell *et al.* 2013a) (see the section 'Regulation of biofilm processes'). Given the complete absence of typical bacterial two-component systems in *Crenarchaeota* including the *Sulfolobales* (Galperin *et al.* 2018), these organisms are solely reliant on one-component regulators. Usually, TFs bind in the vicinity of the promoter elements of transcription units and interact with the different components of the PIC, thereby either repressing or activating transcription initiation (Peeters, Peixeiro and Sezonov 2013) (Table 2). In some cases, a single TF can have a dual function, depending on the target gene or in a concentration-dependent manner, as has been shown for the *Sa. solfataricus* TF Ss-LrpB (Peeters, Peixeiro and Sezonov 2013).

The functional understanding of the TFs in thermoacidophilic archaea is still limited and based on a relatively small number of case studies for individual TFs in model species, such as *Sa. solfataricus*, *Sa. islandicus* and *S. acidocaldarius* (Table 2). These TFs are involved in the regulation of various metabolic and physiological processes, such as motility, hetero- or autotrophic growth, metal resistance and detoxification mechanisms, typically in response to interactions with small molecules, e.g. metabolites (see the sections 'Regulation of biofilm processes', 'Extreme thermoacidophily and stress response' and 'Metabolism'). Unfortunately, a system-level approach for mapping TF-mediated gene regulatory networks in relation to common environmental stresses, similar to how it has been performed for the euryarchaeal model organism *Halobacterium salinarum* (Bonneau *et al.* 2007), has not yet been undertaken for a thermoacidophilic archaeal species. An understanding of these networks would be valuable to gain insights into the physiology and stress adaptation of thermoacidophilic archaea and could be exploited for the engineering of metabolism for biotechnological purposes. Most of the characterized TFs in *Sulfolobales* belong to the dominant TF families, Lrp/AsnC and MarR, which together encompass >50% of all TFs in *Crenarchaeota* (Perez-Rueda *et al.* 2018; Lemmens *et al.* 2019a). TFs belonging to the Lrp/AsnC family are responsive to amino acids or related small molecules and display either a global or specific regulatory function of central metabolic pathways (Vassart *et al.* 2013; Liu *et al.* 2014a). BldR and BldR2 of *Sa. solfataricus* are prototypical MarR-family TFs involved in the detoxification of aromatic compounds (Di Fiore *et al.* 2009; Fiorentino *et al.* 2011). Finally, members of archaea-specific TF families are also found in *Sulfolobales*, such as the TrmB family that is typically involved in the regulation of sugar metabolism (Wagner *et al.* 2014).

Intriguingly, archaeal genomes are predicted to harbor a lower fraction of TF-encoding genes as compared with bacterial genomes (Pérez-Rueda and Janga 2010), and *Crenarchaeota* typically have even lower numbers of TFs than *Euryarchaeota* (Coulson, Touboul and Ouzounis 2007). These observations raise the question as to how thermoacidophilic *Crenarchaeota* are capable of efficiently regulating their transcriptome with a limited repertoire of TFs. While this might be partially explained by an extensive specialization to living in niche habitats, alternative mechanisms are hypothesized to exist; for example, cross-interactions exist between paralogous TFs that lead to a combinatorial use of a limited set of regulators, as has been shown for members of the Lrp/AsnC family of TFs in *Sa. solfataricus* and *S. acidocaldarius* (Nguyen-Duc *et al.* 2013; Liu *et al.* 2016). Furthermore, additional layers of regulation might exist, for

Table 2. Overview of characterized transcription regulators in thermoacidophilic archaea and their viruses.

Name	Family	Microbial or viral species	Gene number	Physiological role	Regulatory action	Reference
C68	AbrB	*S. islandicus* plasmid–virus pSSVx	ORFC68	(CRISPR-mediated) virus–host interactions[a]	Activation[a]	(Contursi *et al.* 2011)
MerR	ArsR	*S. solfataricus*	SSO2688	Mercury resistance	Repression	(Schelert *et al.* 2006)
IdeR	DtxR	*T. acidophilum*	TA0872	Iron uptake and homeostasis	Repression	(Yeo *et al.* 2012; Yeo, Park and Lee 2014)
ArnA	FHA	*S. acidocaldarius*	*Saci_1210*	Motility	Repression	(Duan and He 2011; Reimann *et al.* 2012)
		S. tokodaii	ST0829			
YtrA	GntR	*S. acidocaldarius*	*Saci_1851*	Expression of membrane proteins	Repression	(Lemmens *et al.* 2019b)
BarR	Lrp	*S. acidocaldarius*	*Saci_2136*	β-Alanine metabolism	Activation	(Liu *et al.* 2014a)
		S. tokodaii	ST1115			
Lrp	Lrp	*S. acidocaldarius*	*Saci_1588*	Global regulation of metabolism and physiology	Dual	(Enoru-Eta *et al.* 2000; Vassart *et al.* 2013)
		S. solfataricus	SSO0606			
LysM	Lrp	*S. acidocaldarius*	*Saci_0752*	Amino acid transport and metabolism	Activation	(Brinkman *et al.* 2002; Song *et al.* 2013)
		S. solfataricus	SSO0157			
LrpB	Lrp	*S. solfataricus*	SSO2131	Regulation of pyruvate ferredoxin oxidoreductase and permeases	Dual	(Peeters *et al.* 2009; Peeters, Peixeiro and Sezonov 2013)
AbfR1	Lrs14	*S. acidocaldarius*	*Saci_0446*	Biofilm formation and motility	Dual	(Orell *et al.* 2013a; Li *et al.* 2017)
Sta1	Lrs14	*S. solfataricus*	SSO0048	Regulation of SIRV1 viral gene expression	Activation	(Kessler *et al.* 2006)
Lrs14	Lrs14	*S. solfataricus*	SSO1101	N.A.	Repression	(Bell and Jackson 2000)
Csa3a	MarR	*S. islandicus*	*SiRe_0764*	CRISPR spacer acquisition	Activation	(Liu *et al.* 2015)
BldR	MarR	*S. solfataricus*	SSO1352	Detoxification of aromatic compounds	Activation	(Fiorentino *et al.* 2007)
BldR2	MarR	*S. solfataricus*	SSO1082	Stress response to aromatic compounds	N.A.	(Fiorentino *et al.* 2011)
N.A.	MarR	*S. tokodaii*	ST1710	N.A.	N.A.	(Kumarevel *et al.* 2009)
MLPTv	MarR	*T. volcanium*	BAB59904[b]	N.A.	N.A.	(Liu, Walton and Rees 2010)
RbkR	MarR[b]	*T. acidophilum*	*Ta1064*	Riboflavin biosynthesis	Activation[a]	(Rodionova *et al.* 2017)
		M. yellowstonensis	EHP68448.1[d]			
FadR	TetR	*S. acidocaldarius*	*Saci_1107*	Fatty acid and lipid metabolism	Repression	(Wang *et al.* 2019c)
HhcR	TrmB	*M. yellowstonensis*	H2C8P4[d]	Autotrophic metabolism	N.A.	(Leyn *et al.* 2015)
MalR	TrmB	*S. acidocaldarius*	*Saci_1161*	Maltose transport and metabolism	Activation	(Wagner *et al.* 2014)
ArnB	vWA	*S. acidocaldarius*	*Saci_1211*	Motility	Repression	(Reimann *et al.* 2012)
XylR	N.A.	*S. acidocaldarius*	*Saci_2116*	Arabinose/xylose transport and metabolism	Activation	(van der Kolk *et al.* 2020)
ArnR	N.A.	*S. acidocaldarius*	*Saci_1180*	Motility; type IV pili surface structures	Activation	(Lassak *et al.* 2013; Bischof, Haurat and Albers 2019)
ArnR1	N.A.	*S. acidocaldarius*	*Saci_1171*	Motility; type IV pili surface structures	Activation	(Lassak *et al.* 2013; Bischof, Haurat and Albers 2019)
CopR/CopT	N.A.	*S. solfataricus*	SSO2652	Copper homeostasis	Repression	(Ettema *et al.* 2006; Villafane *et al.* 2009)
Fur	N.A.	*T. volcanium*	TVN0292	Oxidative stress	N.A.	(Minoshima *et al.* 2014)
SvtR	N.A.[e]	*S. islandicus* rod-shaped virus 1 (SIRV1)	ORF56b	Viral development	Repression	(Guillière *et al.* 2009)
RIP	N.A.[e]	*Acidianus* two-tailed virus (ATV)	ORF145	Global regulation of host transcription	Repression	(Sheppard *et al.* 2016)
Stf76	N.A.[e]	*S. islandicus* plasmid–virus pSSVx	ORF76	N.A.	N.A.	(Contursi *et al.* 2014)
F55	N.A.[e]	*Sulfolobus* spindle-shaped virus 1	T_{lys}	Viral lysogeny and UV induction	Repression	(Fusco *et al.* 2015)

N.A. = not applicable (unknown based on published information).
[a]Hypothesized;
[b]UNIPROT number;
[c]Multifunctional protein with enzymatic and transcription regulatory domains;
[d]GenBank accession number;
[e]Viral regulators are often difficult to classify into a family because of a lack of homology.

example at the post-transcriptional level (see below the section 'Translation and its regulation'), or by means of PTMs of TFs (see the section 'Protein phosphorylation in *S. acidocaldarius*'; Fig. 6). In this context, it is notable that phosphoproteomic studies have indicated the widespread occurrence of phosphorylation of TFs in *S. acidocaldarius* and *Sa. solfataricus* (Esser *et al.* 2012; Reimann *et al.* 2012). In *S. acidocaldarius*, phosphorylation has been shown to directly affect DNA binding of the Lrs14-type biofilm regulator AbfR1 (Li *et al.* 2017), or ligand interaction in the case of the acyl-CoA-responsive TetR-family regulator FadR (Maklad *et al.* 2020) (see the section 'Protein phosphorylation in *S. acidocaldarius*').

Translation and its regulation

Post-transcriptional regulatory mechanisms in the Sulfolobales

An alternative explanation for the compensation of the rather limited repertoire of TFs in *Sulfolobales* is the existence of gene regulatory mechanisms at alternative levels of information processing, such as the post-transcriptional level (Lemmens *et al.* 2019a). RNA-based regulation is supported by a widespread occurrence of small non-coding RNAs (ncRNAs) in *Sa. solfataricus* and, to a lesser extent, in *S. acidocaldarius* (Tang *et al.* 2005; Zago, Dennis and Omer 2005; Wurtzel *et al.* 2010; Cohen *et al.* 2016). More than 300 ncRNAs were identified in *Sa. solfataricus*, 60% of which are *cis*-acting antisense transcripts (Wurtzel *et al.* 2010). Possibly, these small RNAs assist in the stabilization of mRNA by RNA duplex formation, which is relevant given the thermophilic lifestyle of the organism (Gomes-Filho and Randau 2019). Nevertheless, given that most of these ncRNAs are conserved in closely related *Sa. islandicus* genomes (Reno *et al.* 2009), they likely have functional roles and these antisense ncRNAs may regulate translation in a similar manner as RNA silencing mechanisms in eukaryotes. Besides the observation that they are overrepresented in coding regions of genes involved in ion transport and metabolism (Wurtzel *et al.* 2010), the function of ncRNA-mediated regulation is still unclear as very few ncRNAs have been characterized thus far. A good example is RrrR in *S. acidocaldarius*, an antisense ncRNA that targets two mRNAs, including one that encodes a hypothetical membrane protein that was shown to influence biofilm formation (Orell *et al.* 2018) (see the section 'Regulation of biofilm processes'). In *Sa. solfataricus*, a small ncRNA has been shown to interact with the 3′-untranslated region (UTR) of its target mRNA in a phosphate-responsive gene regulatory process (Märtens *et al.* 2013). This is a logical regulatory site given that most transcripts in thermoacidophilic *Crenarchaeota* are leaderless and lack a 5′-UTR (Brenneis *et al.* 2007; Wurtzel *et al.* 2010), which is the preferred site of small RNA-mediated post-transcriptional regulation in bacteria.

There is a huge variation in the small RNAs found in transcriptomes of *Sulfolobales*; these can have lengths as small as 20–25 nucleotides (nts) or up to 500 nts (Wurtzel *et al.* 2010; Xu *et al.* 2012). Besides the antisense ncRNAs that have a classic regulatory role, *Sa. solfataricus* harbors transposon-associated ncRNAs involved in transposition, CRISPR-associated small RNAs (see the section 'CRISPR-Cas mediated viral defense in thermoacidophiles'), C/D box small nucleolar (sno) RNA that guide methylation sites in rRNAs and tRNAs (Zago, Dennis and Omer 2005), and small RNAs associated with TTSs (TTSaRNAs) (Zaramela *et al.* 2014). Different RNA-binding proteins act as chaperones of ncRNAs, such as members of the L7As/L30 protein family and the Sm superfamily. These proteins are not only involved in the biogenesis and functioning of small RNAs, but also in other aspects of processing of rRNA, tRNA and mRNA species (Gomes-Filho and Randau 2019).

Basal translation machinery in the Sulfolobales

Like replication and transcription, the machinery of translation in archaea shows striking similarities to those in eukaryotes. Studies of translation initiation have been mainly performed in *Sa. solfataricus* as a model for thermoacidophilic archaea (La Teana *et al.* 2013). Two different mechanisms have been discerned: (i) translation is initiated based on a canonical Shine–Dalgarno (SD)/anti-SD interaction analogous to bacteria at internal cistrons of polycistronic mRNAs or (ii) direct pairing of the start codon with the anticodon of the initiator methionine-tRNA occurs at leaderless transcripts devoid of a SD sequence (Benelli, Maone and Londei 2003). The latter mechanism is the most prevalent one, given the observation that most monocistronic mRNAs and the proximal cistrons of polycistronic mRNAs in *Sulfolobales* are leaderless, lacking SD-harboring 5′-UTRs (Brenneis *et al.* 2007; Wurtzel *et al.* 2010). The translation initiation machinery has a complexity reminiscent of their eukaryotic counterpart and consists of multiple translation initiation factors (IFs), some of which have eukaryotic but no bacterial homologs (La Teana *et al.* 2013). A crucial IF, especially for the translation initiation of leaderless transcripts, is aIF2, which is a heterotrimeric protein that forms a ternary complex with GTP and the methionine-loaded initiator tRNA, and binds the small ribosomal subunit. Other initiation factors are aIF1, aIF1a, aIF5b and aIF6. Despite the eukaryotic nature of the machinery, the operational steps in the translation initiation process in *Sa. solfataricus* resemble those in bacteria (La Teana *et al.* 2013).

POST-TRANSLATIONAL MODIFICATION BY REVERSIBLE PROTEIN PHOSPHORYLATION

All living cells, including thermoacidophiles, respond and adjust their cellular processes to a multitude of external and internal signals/cues. Information processing from gene to protein is also time and energy intensive. Therefore, post-translational-modifications (PTMs) are an efficient way to rapidly adjust protein function and coordinate the cellular response to changing needs. One of the best studied PTMs is the reversible protein phosphorylation mediated by protein kinases (PKs) and protein phosphatases (PPs) (Kennelly 2003, 2014; Esser *et al.* 2016; Papon and Stock 2019). Phosphoproteins were first found in the Euryarchaeon *Halobacterium salinarum* (Spudich and Stoeckenius 1980) but, despite this early report, little is still known about reversible phosphorylation in Archaea.

Two distinct phosphorylation systems exist. The two-component system (TCS) involves a histidine sensor kinase (HisK) and response regulator (RR); the covalent modification, i.e. addition of a phosphate group, takes place on histidine (His) and aspartate (Asp) residues, respectively (Galperin *et al.* 2018). It was originally thought that TCS were specific for Bacteria, but it has since been shown that TCS occurs in all three domains of life (Loomis, Shaulsky and Wang 1997; Kim and Forst 2001; Schaller, Shiu and Armitage 2011). The first TCS reported in Archaea was CheA and CheY in the Euryarchaeon *Halobacterium salinarium* (Rudolph *et al.* 1995). In thermoacidophiles, TCS have only been identified in the *Thermoplasmata* within the *Euryarchaeota*, although their function is still unknown (Galperin *et al.* 2018). In other archaeal phyla, i.e. the *Crenarchaeota*, the *Korarchaeota* and the *Nanoarchaeota*, TCSs are largely absent and the organisms solely rely on canonical Hanks-type protein kinases for signal transduction (Hanks 2003; Esser *et al.* 2016; Galperin *et al.* 2018). In these cases, autophosphorylation of the PK takes place on the amino acids Ser and Thr (eSTPKs) or tyrosine (Tyr). Often

multiple PKs are interconnected, leading to a signal transduction cascade with a continuous hierarchical network structure. Hanks-type protein kinases can be broadly split into two groups, which have been well established in Archaea (Leonard, Aravind and Koonin 1998; Kennelly 2014; Esser *et al.* 2016; Hoffmann *et al.* 2017): the conventional Hanks-type protein kinases (ePKs) and the non-canonical, atypical Hanks-type protein kinases (aPKs). The largest group, the ePKs, all share a conserved catalytic domain consisting of twelve subdomains (Stancik *et al.* 2018). The other, smaller group, the aPKs, are distant members of the ePKs superfamily and share only some of the conserved subdomains (Esser *et al.* 2016). Although Tyr phosphorylation has been demonstrated, so far only eSTPKs (phosphorylation on Ser/Thr residues) have been identified and characterized in Archaea, with the responsible tyrosine kinases yet unknown (Smith, Kennelly and Potts 1997; Kennelly 2014).

The counterparts to PKs are PPs, which can remove the covalently linked phosphate residue, thereby making the process reversible. PPs can be classified into different subgroups, depending on their substrate specificity. In archaea, several phosphatases have been reported that differ in substrate specificity and cofactor requirement (Shi 2009). Ser/Thr phosphatases (PPPs) and Mg^{2+}- or Mn^{2+}-dependent protein phosphatases (PPM) act on pSer and/or pThr, whereas protein tyrosine phosphatases (PTPs) are active on pTyr (Shi 2009). Notably, dual activity of the PPM on pTyr and pSer/Thr was demonstrated *in vitro* in the thermoacidophile *Thermoplasma volcanium* (Dahche *et al.* 2009).

Among the thermoacidophilic archaea, the protein phosphorylation pathways of the *Sulfolobales* are the best characterized. When considering protein phosphorylation in the *Sulfolobales*, it is important to know which PKs and PPs have been characterized so far, which proteins are targeted, and, most importantly, what is the physiological and cellular impact of reversible protein phosphorylation. First, homology searches (BlastXP) were performed based on the PKs and PPs identified in *S. acidocaldarius* and *Sa. solfataricus* (Leng *et al.* 1995; Shi, Potts and Kennelly 1998; Lower, Bischoff and Kennelly 2000; Lower and Kennelly 2002, 2003; Lower, Potters and Kennelly 2004; Kennelly 2014; Ray *et al.* 2015), *Sa. islandicus* (Huang *et al.* 2017, 2019) and *Sulf. tokodaii* (Wang *et al.* 2010a) (Fig. 5). In surveying the *Sulfolobales* genomes, *Sulf. tokodaii* has 15 PKs, followed by *S. acidocaldarius* with 13 PKs, and *Sa. islandicus* and *Sa. solfataricus* both with 11 PKs. All strains harbor one typical ePK with an additional tetratricopeptide repeat (TPR-motif) that is known to mediate protein–protein interactions. In *Sa. islandicus*, the ePK was shown to act as a master PK, phosphorylating other PKs *in vivo*. Also, the homolog in *S. acidocaldarius* (ArnC) phosphorylates a variety of different target enzymes (Hoffmann *et al.* 2017; Huang *et al.* 2017; Knüppel *et al.* 2018; Maklad *et al.* 2020). Several PKs have an additional trans-membrane domain, implicating membrane-bound localization. In all *Sulfolobales* genomes, two Rio-like PKs (aPKs) are present. Rio kinases are an ancient conserved family that can be found in all three domains of life and are known to play a role in ribosome biogenesis (Esser and Siebers 2013; LaRonde 2014). All *Sulfolobales* are known to harbor a set of Rio B and Rio 2 PKs, except *S. acidocaldarius*, where Rio 1 and Rio 2 can be found (Esser and Siebers 2013). Rio 1 and Rio 2 are quite similar, except that Rio 2 possesses an additional winged helix-turn-helix domain (wHTH), a structural motif enabling binding of DNA. Recently, further evidence for the role that Rio kinases play in the synthesis of the ribosomal small subunit in archaea was uncovered (Knüppel *et al.* 2018). Furthermore, one piD261-like aPK, of which SSOPK5 is the only one studied in the *Sulfolobales*, one ABC1-like and one AQ578 aPK, both putative, can be found in *Sa. solfataricus*, *Sulf. tokodaii*, *Sa. islandicus* and *S. acidocaldarius* (Fig. 5). Notably, in all of these genomes, only two PPs were found: one annotated as Tyr-phosphatase and one as Ser/Thr-phosphatase.

Protein phosphorylation in *Sa. solfataricus*

The first evidence of protein phosphorylation in the *Sulfolobales* was reported in *Sa. solfataricus* (Kennelly *et al.* 1993). PP activity was detected in soluble extracts of *Sa. solfataricus* using ^{32}P-casein as the substrate and allowed the isolation of the PP from the soluble fraction. Since no activity could be detected with p-Tyr labeled substrates, it was concluded that the PP was a Ser/Thr phosphatase. The amino acid sequence of the SSO-PP was similar to the eukaryotic PP1/2A/2B superfamily (Leng *et al.* 1995).

Sa. solfataricus was also the first *Sulfolobales* species in which PKs were characterized and now include four ePKs and one aPK (SSO-PK1, SSo-PK2, SSO-PK3, SSO-PK4 and SSO-PK5). Their activity on 'artificial' substrates, such as histone, myelin basic protein, p53, casein, reduced carboxyamidomethylated and maleylated lysozyme, in addition to their cofactor dependence, and inhibition by typical ePK inhibitors, have been analyzed (Lower Bischoff and Kennelly 2000; Lower and Kennelly 2002, 2003; Lower, Potters and Kennelly 2004; Haile and Kennelly 2011; Kennelly 2014; Ray *et al.* 2015; Esser *et al.* 2016). SSO-PK4 (encoded by SSO3182), like its eukaryotic homologs, was shown to phosphorylate the *Sa. solfataricus* eukaryotic translation initiation factor (eIF2α) homolog, aIF2α, *in vitro*, but not on the conserved phosphorylation sites known for eIF2α (Ray *et al.* 2015). Inhibition was observed in the presence of 3′,5′-cAMP *in vitro*, whereas a concentration-dependent activation occurred in the presence of oxidized CoA, an indicator of oxidative stress in archaea. Additionally, the aPK of the pID261/Bud32 kinase family (SSO-PK5) was characterized (Haile and Kennelly 2011). SSO-PK5 (encoded by SSO0433) phosphorylated artificial substrates, like p53 and casein, on Ser residues. Autophosphorylation of SSO-PK5 was shown to take place on both Ser and Thr residues and activation was observed in the presence of ADP-ribose (Haile and Kennelly 2011). Phosphohexomutase (SSO0207), first identified in tryptic digests, was shown to be phosphorylated. Site-directed mutagenesis (S309D) was used to mimic the presence of a phosphoryl group, which drastically decreased the V_{max} value of the enzyme. Therefore, it was suggested that protein phosphorylation is used *in vivo* to regulate the phosphohexomutase activity, but the corresponding PKs are yet unknown (Fig. 6) (Ray *et al.* 2005). This appears to be the first report on regulation of central metabolic enzymes by reversible protein phosphorylation in not only the *Sulfolobales*, but Archaea in general.

In 2012, Esser *et al.* performed the first phosphoproteome study in *Sa. solfataricus* where they compared the phosphoproteome of cells grown on D-glucose vs cells grown on tryptone (Esser *et al.* 2012; Dopson 2016). Using a precursor acquisition independent from ion count (PAcIFIC) approach, 540 phosphoproteins in 21 out of 26 arCOGs were found, highlighting the importance of regulation by reversible protein phosphorylation in *Sa. solfataricus*. Interestingly, the phosphorylation profile was dependent on the respective carbon source and a high amount of Tyr phosphorylation (Ser/Thr/Tyr ratio of 26/21/54%) was detected. This was rather unexpected since, so far, no PKs with Tyr-phosphorylation activity had been reported. Due to the significant changes in the phosphoproteome in response to

S. acidocaladrius	S. islandicus	S. solfataricus	S. tokodaii
Protein kinases			
Canonical Hanks type protein kinases (ePKs)			
Saci_1193 (ArnC)	SiRe_2030	SSO3207	STK_08100
Saci_1694 (ArnD)	SiRe_0101	SSO3182 (SSOPK4)	STK_06860
Saci_1041	SiRe_0101	SSO2291	STK_06860
Saci_1869	SiRe_0101	SSO2291	STK_07780
Saci_1181 (ArnS)	SiRe_2056	SSO3182 (SSOPK4)	STK_08990
Saci_1811 (ArnS)	SiRe_2056	SSO3182 (SSOPK4)	STK_24400
Saci_1869	SiRe_0101	SSO2291	STK_15650
Saci_1041	SiRe_2056	SSO3182 (SSOPK4)	STK_19960
Non-canonical/atypical Hanks type protein kinases (aPKs)			
Saci_0796 (RIO2-like)	SiRe_1810 (RIO2-like)	SSO0197 (RIO2-like)	STK_02330 (RIO2-like)
Saci_0965 (RIO1-like)	SiRe_0171 (RIO1-like)	SSO2374 (RIO1-like)	STK_05130 (RIO1-like)
Saci_1289 (AQ578)	SiRe_1057 (AQ578)	SSO1038 (AQ578)	STK_09530 (AQ578)
Saci_0850 (piD261)	SiRe_1570 (piD261)	SSO0433 (SSOPK5)	STK_03640 (piD261)
Saci_1664 (ABC1-like)	SiRe_2600 (ABC1-like)	SSO2605 (ABC1-like)	STK_16520 (ABC1-like)
Saci_2317	SiRe_0181	SSO2387 (SSOPK2)	STK_05220
Saci_0435	SiRe_1531	SSO0469 (SSOPK3)	STK_01810
Saci_1477	SiRe_1639	SSO0361	STK_13820
Protein phosphatases			
Saci_0545	SiRe_0241	SSO2453	STK_05800
Saci_0884	SiRe_1009	SSO1090	STK_23670

Trans-membrane domain
Protein kinase domain
Tetratricopeptide repeat (TPR)
Winged helix-turn-helix domain (wHTH)
Tyrosine-protein phosphatase
Ser-thr-phosphatase

Figure 5. Predicted protein kinase and protein phosphatase homologs in the four different *Sulfolobales* species: *S. acidocaldarius*, *Sa. islandicus*, *Sa. solfataricus* and *Sulfuri. tokodaii*. Depicted are the different canonical and non-canonical Hanks-type protein kinases and protein phosphatases with their correspondent domain structure. (Lower Bischoff and Kennelly 2000; Lower and Kennelly 2002, 2003; Lower, Potters and Kennelly 2004; Haile and Kennelly 2011; Esser and Siebers 2013; Ray *et al.* 2015; Esser *et al.* 2016; Hoffmann *et al.* 2017; Huang *et al.* 2017).

carbon source, it was concluded that reversible protein phosphorylation plays a major role in the regulation of central carbon metabolism (CCM) in *Sa. solfataricus* (Dopson 2016).

Protein phosphorylation in *Sulf. tokodaii*

Detailed characterization of the ePK STK_15650, which comprises all signatures of a canonical Hanks-type kinase in *Sulf. tokodaii*, showed that the important catalytic residues are all located in, or close to, the major functional domains of eSTKs (Wang *et al.* 2010a). It was the first time in Archaea that the interaction between an ePK and a target protein was characterized (Wang *et al.* 2010a). The interaction partner that is phosphorylated is the forkhead-associated (FHA) domain-containing protein (STK_00829). FHA domains are known to act as phosphorylation-dependent protein–protein interaction modules that can bind to pThr residues in their targets (England *et al.* 2009). Additionally, specific interactions of both proteins were demonstrated *in vivo*. Important residues for the protein–protein interaction were identified. It was proposed that STK_00829 might be a transcriptional regulator and, therefore, phosphorylation might play a role in transcriptional regulation in Archaea (Fig. 6) (Wang *et al.* 2010a; Duan and He 2011). Subsequently, the homolog of STK_00829 ArnA was shown to be a repressor of archaellum expression and part of the archaellum regulatory network in *S. acidocaldarius* (see Table 2) (Reimann *et al.* 2012).

Protein phosphorylation in *Sa. islandicus*

Autophosphorylation and cross-phosphorylation activities of the eleven PKs (three ePKs and eight aPKs) from *S. islandicus* REY15A revealed insights into the hierarchy of regulatory networks (Huang *et al.* 2017). The seven PKs (SiRe_0101KD, SiRe_0171, SiRe_0181, SiRe_1570, SiRe_1810, SiRe_2030 and SiRe_2056KD) exhibited autophosphorylation activities, with the highest activity shown for the ePK SiRe_2056KD. Dephosphorylation assays revealed that autophosphorylation mainly proceeds on Ser/Thr residues (Huang *et al.* 2017). To gain more insight into the cross-phosphorylation and cross-talk among the PKs, inactive ePKs were generated. Among the ePKs, SiRe_2030 and the truncated SiRe_2056KD were most active on phosphorylation of the other PKs. Next, to address the importance of ePKs, the effect of PK overexpression was evaluated. Only for SiRe_1531 and SiRe_2056 was there an obvious phenotype detectable, i.e. growth retardation (Huang *et al.* 2017). Therefore, SiRe_2056 and SiRe_2030 are the master PKs, and SiRe_0101 is an accessory kinase at the apex of the phosphorylation hierarchy in *Sa. islandicus* that transduced the signal toward the other substrate kinases. However, more physiological information is needed to elucidate the complex signaling pathways in *Sa. islandicus* (Huang *et al.* 2017).

With regard to targets in *Sa. islandicus* REY15A, phosphorylation of a conserved Holliday junction resolvase (Hjc), an enzyme employed in homologous recombination repair (HRR), by the PKs SiRe_0171 (Rio 1-like aPK), SiRe_2030 and SiRe_2056

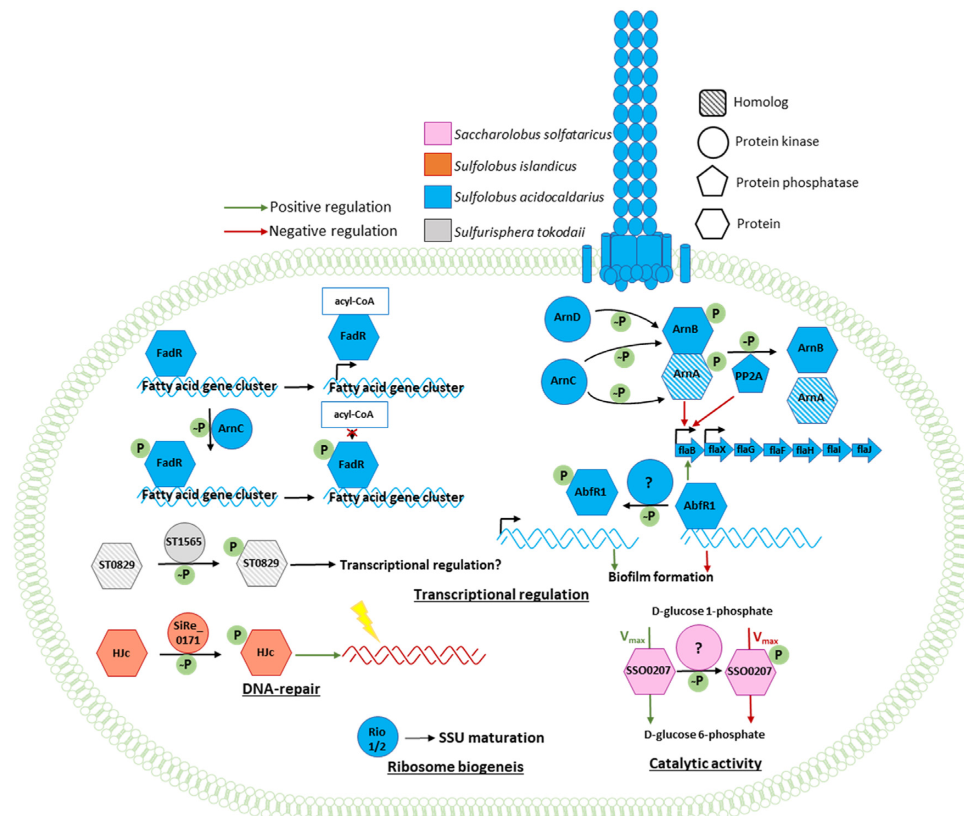


Figure 6. Representation of known phosphorylation-based regulatory networks in different *Sulfolobales* strains (*Sa. solfataricus* in pink, *Sa. islandicus* in orange, *S. acidocaldarius* in blue and *Sulfuri. tokodaii* in gray) with their physiological function. From upper left to right: The FadR transcriptional regulator represses transcription of the fatty acid gene cluster and dissociates from the DNA upon binding to acyl-CoA. Phosphorylation of FadR by the ePK ArnC (Saci_1196) prevents acyl-CoA binding and thus hinders transcription of the gene cluster (Maklad *et al.* 2020). The archaellum regulatory network consists of the gene cluster *arlBXGFHIJ* (*flaBXGFHIJ*), which encodes the motility structure, the archaellum, and is under the control of two promoters, one upstream of *arlB* (*flaB*) being induced under starvation and one weak promoter upstream of *arlX* (*flaX*). The two negative regulators, ArnA (Saci_1210) and ArnB (Saci_1211), were shown to be phosphorylated by the ePKs ArnC and ArnD (Saci_1694) and dephosphorylated by the PP PP2A (Saci_0884). Deletion of the PP2A led to a hypermotile phenotype suggesting a negative influence on the gene cluster (Reimann *et al.* 2012; Hoffmann *et al.* 2017). The DNA-binding protein AbfR1 (Saci_0446) is a positive regulator of the *arlB* (*flaB*) promoter (Orell *et al.* 2013a). Phosphorylation of AbfR1 inhibits DNA binding and thus regulates biofilm formation and motility (Li *et al.* 2017). The FHA domain containing protein ST0829 was shown to interact and be phosphorylated by the ePK ST1565 indicating a role in transcription regulation (Duan and He 2011). The Holliday junction resolvase (Hjc) (SiRe_1431) is phosphorylated by the aPK SiRe_0171 facilitating DNA repair (Huang *et al.* 2019). The phosphohexomutase (SSO0207) exhibited a decreased V_{max} value after being phosphorylated (Ray *et al.* 2005). The Rio kinases (Saci_0796 and Saci_0965) were shown to play a role in the ribosome maturation of the small subunit (Knüppel *et al.* 2018).

(ePKs) was investigated (Huang *et al.* 2019). These PKs phosphorylated different residues *in vitro* and the analysis of the respective phosphorylation-mimic mutants revealed that the phosphorylation of S34 (phosphorylated by SiRe_0171) and S9 (phosphorylated by SiRe_2030) have a strong impact on Hjc activity. To elucidate the *in vivo* significance of Hjc protein phosphorylation, strains expressing the different phosphorylation-mimic mutants were tested for their sensitivity toward DNA damaging agents. The strain expressing S34E (mimicking phosphorylated Hjc) was less sensitive toward high doses of DNA-damaging agents (i.e. UV or cisplatin) indicating a higher DNA repair capability. In addition, deletion of the respective Rio 1 homolog SiRe_0171 (and thus preventing phosphorylation of the S34 residue) resulted in a strain with higher sensitivity toward DNA damaging agents, thus indicating that phosphorylation of S34 in Hjc enhances the DNA repair capability (Fig. 6).

Protein phosphorylation in *S. acidocaldarius*

Protein phosphorylation in *S. acidocaldarius* was initially identified when several proteins were found to be phosphorylated in the presence of [γ-^{32}]ATP *in vivo* (Skórko 1984). Three PKs, ArnC, ArnD and ArnS, are involved in the regulation of the best studied complex signal transduction system in archaea, the archaellum regulatory network (Arn) (Fig. 6). The genes encoding for the archaeal motility structure, the archaellum, are arranged in an operon consisting of seven genes with two promoters. The main promoter upstream of the gene *arlB* (f. *flaB*) was induced upon starvation, whereas the second promoter upstream of *arlX* (f. *flaX*) revealed weak constitutive activity (Lassak *et al.* 2012). The transcriptional regulators, ArnA and ArnB, repress *arlB* (f. *flaB*) expression. *arnA* harbors a zincfinger (ZnF) and a forkhead-associated (FHA) domain and is a homolog to the previously identified regulator STK_00829 in *Sulf. tokodaii*, while ArnB possesses a von Willebrand type A domain (vWA). Deletion of either one of the two genes showed a hypermotile phenotype *in vivo* and protein levels of the archaellin ArlB (f. FlaB) were strongly enhanced compared with the wild-type strain (Reimann *et al.* 2012). The ePKs ArnC (Saci_1193), ArnD (Saci_1694) and ArnS (Saci_1181), and the Ser/Thr PP (Saci_0884) (Saci_PP2A), regulate the archaellum at the post-translational level by reversible phosphorylation. ArnC is able to phosphorylate both regulators

ArnA and ArnB and deletion of the ePK resulted in reduced motility *in vivo*. In contrast, ArnD is only able to phosphorylate ArnB and its deletion resulted in a hypermotile phenotype. These divergent effects on motility suggested that the two PKs have different roles in the regulatory network. Both regulators were dephosphorylated by the addition of the Saci_PP2A (Reimann *et al.* 2012). Since ArnA and ArnB interact with each other in a phosphorylation-dependent manner, protein phosphorylation seems to be the key for their regulatory function in the Arn (Reimann *et al.* 2012). The deletion of the starvation induced ePK, ArnS, which is also located close to the archaellum operon, resulted in reduced motility, indicating an essential role of this ePK as well (Haurat *et al.* 2017). Finally, the deletion of the Ser/Thr PP PPP2A also revealed a hypermotile phenotype, suggesting a negative regulation, although the respective relay mechanism and target protein(s) are still unknown. Another player of the Arn is the transcriptional regulator of the Lrs14 family, the archaeal biofilm regulator 1 (AbfR1) (Orell *et al.* 2013a; Liu *et al.* 2017). AbfR1 binds to its own promoter, as well as the *arlB*, *arlX* (f. *flaB*, *flaX*, respectively) promoter, and has non-specific DNA-binding activity suggesting a general chromatin structuring function. In the non-phosphorylated state, it binds to DNA and increases motility by expression of the archaellum operon and negatively regulates biofilm formation by decreasing the production of extracellular polymeric substance (EPS). Phosphorylation of two residues, S87 and Y84, in the wHTH domain impair stable protein-DNA contacts *in vivo* (Li *et al.* 2017). Thus, AbfR1 phosphorylation promotes the transition from a motile, planktonic growth to a sessile lifestyle in *S. acidocaldarius* (Fig. 6). The PK(s) involved in phosphorylation of AbfR1 are still unknown.

The two Rio kinases, Rio 1 and Rio 2, of *S. acidocaldarius* exhibited ATP hydrolysis activity in a concentration-dependent manner (Knüppel *et al.* 2018) and are non-essential in *S. acidocaldarius*, as demonstrated by creation of single deletion mutants (Hoffmann *et al.* 2017). Both PPs in *S. acidocaldarius* were characterized in detail and the physiological role was addressed by comparison of the phosphoproteomes of the parent strain MW001 and the two PPs deletion mutants ΔSaci_ptp, ΔSaci_pp2a (Reimann *et al.* 2013). Saci-PTP is a dual-specific phosphatase (active with pSer/pThr and pTyr), whereas Saci-PP2A exhibited specific pSer/pThr activity and could be inhibited by okadaic acid (Reimann *et al.* 2013). The study revealed major differences in the phosphorylation and gene expression patterns of the two deletion strains, suggesting important roles for both phosphatases in signal transduction pathways (Reimann *et al.* 2013). Interestingly, the ratio of pSer/pThr/pTyr, varied slightly between the different strains and revealed a high amount of pTyr, also seen in *Sa. solfataricus* (Reimann *et al.* 2013; Dopson 2016).

A very recent study highlighted the physiological effect of protein phosphorylation on central metabolism (Fig. 6) (Maklad *et al.* 2020). The transcription factor, $FadR_{Sa}$, represses a 30-kb gene cluster encoding enzymes involved in the lipid/fatty acid degradation in *S. acidocaldarius* (Wang *et al.* 2019c). Acyl-CoAs act as inducers leading to DNA dissociation of $FadR_{Sa}$ and thus transcription of the gene cluster. $FadR_{Sa}$ repressed the gene cluster by only four binding sites; further studies revealed that $FadR_{Sa}$ might have an additional function in organization of local chromatin architecture, as indicated by the interplay of $FadR_{Sa}$ with the chromosome structuring factor coalescing (Takemata, Samson and Bell 2019). In previous phosphoproteome studies, $FadR_{Sa}$ was found to be phosphorylated on three different residues (Y^{133}, T^{134} and T^{135}) (Reimann *et al.* 2013). Notably, all three residues are located within the binding pocket of acyl-CoA. *In vitro* phosphorylation studies with different PKs confirmed phosphorylation of FadR by ArnC and Saci_1041, whereas ArnD and Saci_0965 were not active. The constructed triple phosphomimetic mutant $FadR_{Sa}$ (Y133D-T134E-T135E) was less sensitive for acyl-CoA. This implies that phosphorylation of $FadR_{Sa}$ may act as an additional control mechanism that keeps the gene cluster in a condensed state and allows transcriptional repression of lipid/fatty acid metabolism in the presence of acyl-CoAs in *S. acidocaldarius* (Maklad *et al.* 2020).

CELL CYCLE AND MODES OF GROWTH

The planktonic lifestyle

The archaellum and other surface structures

Archaea form a multitude of different surface structures that play important roles in diverse processes, such as motility, adhesion, biofilm formation, DNA transfer and probably many more (Chaudhury, Quax and Albers 2018). Many of these structures are type IV pili or type IV pili-like, e.g. the archaellum, the motility structure of archaea (Albers and Pohlschröder 2009; Jarrell and Albers 2012; Makarova, Koonin and Albers 2016). Archaeal type IV pili are similar to those of bacteria in that both pilin precursor proteins exhibit a class III signal peptide at their N-terminus, which is processed by a type IV prepilin signal peptidase (PibD in *Sulfolobales* and *Haloarchaea*) (Albers, Szabó and Driessen 2003; Tripepi, Imam and Pohlschröder 2010). Only after removal of the signal peptide can the pilins be assembled into the pilus filament by the assembly system which is formed by an integral membrane protein and an ATPase (Fig. 7). The basic assembly mechanism of the archaellum resembles that of the type IV pilus. However, the associated accessory proteins (ArlFGH) enable the archaellum filament to rotate and therefore propel the cells forward (Jarrell and Albers 2012; Albers and Jarrell 2015).

Among the *Sulfolobales*, *S. acidocaldarius* is the best studied with respect to surface structures as it exhibits a variety of them (Fig. 7 EM, Model): the Ups pili (UV-induced pili), the Aap pili (archaeal adhesive pili), the threads and the archaellum (Fröls *et al.* 2008; Henche *et al.* 2012a,b; Tsai *et al.* 2020). The threads, the only non-type IV pilus structure on the *S. acidocaldarius* surface, are formed by unknown proteins, but are used as binding sites for viruses (see the section 'Viruses and CRISPR systems of thermoacidophiles') (Hartman *et al.* 2019). The Ups pili are assembled after DNA double-strand breakages and lead to species-specific cell aggregation. During aggregation DNA is exchanged and subsequently used for DNA repair by homologous recombination (Fröls *et al.* 2007; van Wolferen *et al.* 2020). The species specificity is ensured by binding of the pilin subunits to the *N*-glycan trees on the S-layer protein that differ among species like *Sa. solfataricus*, *Sulf. tokodaii* and *S. acidocaldarius* (see the section 'S-layer') (van Wolferen *et al.* 2020). The Aap pili are important for adhesion of *S. acidocaldarius* to surfaces and biofilm formation (see the section 'Biofilms').

Biofilms

Biofilms are the most common form of microbial life. They consist of cells that are attached to a surface (which may or may not serve as a source of nutrients for the microorganism), embedded in extracellular polymeric substances (EPS) that form a matrix produced by the microbial population. This matrix consists of different types of polymers: lipids, polysaccharides, extracellular nucleic acids and proteins (Fröls 2013; van Wolferen, Orell

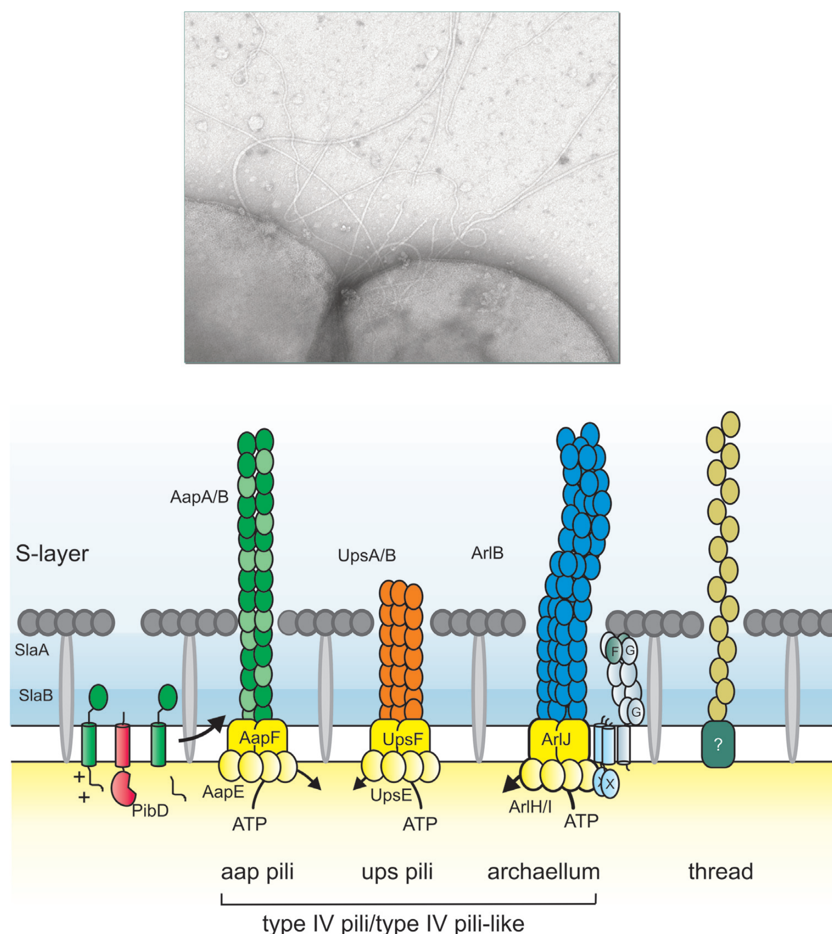


Figure 7. Archaeal cell surface structures involved in planktonic and biofilm growth. Upper image: electronic microscopy image from a *S. acidocaldarius* cell where archaellum and pilus can be seen. The upper image is reproduced from Albers and Meyer 2011. Lower image: schematic model with all proposed cell surface appendages in the *Sulfolobales*: the Aap pili (archaeal adhesive pili), the Ups pili (UV-induced pili), the archaellum and the threads. Also depicted is the S-layer and its proteins, SlaA and SlaB.

and Albers 2018). Biofilms can also be formed at air-liquid and liquid-solid interfaces.

Biofilms are dynamic communities where cells can leave the biofilm structure and swim free in a planktonic lifestyle or attach to another surface to colonize it (Koechler *et al.* 2015). The first biofilm described for archaea was that of the extremely thermophilic euryarchaeon *Thermococcus litoralis* (Rinker and Kelly 1996). Similarly to bacterial biofilms, biofilms protect archaea against diverse kinds of stress, such as changes in pH, desiccation, UV radiation, and high salt and metal concentrations (Laplagia and Hartzell 1997; Fröls 2013). Biofilms also provide an advantage to cells because they form a microenvironment where they share nutrients, water channels, etc. (Petrova and Sauer 2012). In *Sulfolobales* species, stress factors like changes in pH and temperature induce biofilm formation (Koerdt *et al.* 2010), suggesting a role in protection against unfavorable conditions. A method to grow *Sulfolobales* biofilms in microtitrate plates has been standardized (Koerdt *et al.* 2010, 2011). The method consists of growing the species in Brock media pH 3 in microtiter plates covered with a gas permeable membrane at ~75°C for 3–6 days without agitation. Using this method, biofilm from three *Sulfolobales* species were studied (*Sa. solfataricus*, *S. acidocaldarius* and *Sulf. tokodaii*). Also, some *Sulfolobales* have been studied in the context of acid mine drainage (AMD) biofilms, for example *Sulfura. metallicus* (Zhang *et al.* 2015b, 2019b) and *Acidianus* spp. (Zhang *et al.* 2015b) on elemental sulfur and pyrite.

Biofilm formation process

There are three stages in biofilm formation: attachment of cells to the surface, formation of microcolonies and biofilm maturation, and finally, dispersion. In each of these stages, different structures are involved, and diverse morphologies had been described for *Sulfolobales* species (summarized in Table 3). The first stage for biofilm formation is the attachment of cells to a surface. Different cell surface structures play a role in this process, such as type IV pili, in the later stages, the archaellum, and depending on the archaeal species, hami and fimbria are involved (van Wolferen, Orell and Albers 2018). *Saccharolobus solfataricus* mutants that lack ArlB (formerly FlaB), the structural component of the archaellum, or either one of the Ups pili components, are defective in adhesion to surfaces like glass or pyrite (Zolghadr *et al.* 2010). Likewise, the deletion of Ups pili lead to less biofilm formation after 3 days of growth (Koerdt *et al.* 2010). The Aap pili are absent in *Sa. solfataricus* but present in *S. acidocaldarius* and, along with the Ups pili, are important for surface adhesion (Henche *et al.* 2012b). However, the archaellum does not play a role in adhesion in *S. acidocaldarius*, but seems to be involved in biofilm maturation (Henche *et al.* 2012b).

Table 3. Biofilms of the *Sulfolobales*.

Organism	Adhesion	Biofilm morphology	EPS components	Dispersion	References
Sa. solfataricus	Archaella and Ups pili	Carpet-like structure, low density of cells (20–30 mm thick)	Glucose, galactose, mannose and *N*-acetyl-glucosamine residues	Unknown	(Koerdt *et al.* 2010, 2012)
S. acidocaldarius	Aap and Ups pili	Dense biofilm with tower-like structures (25–35 mm thick)	Glucose, galactose, mannose and *N*-acetyl-glucosamine residues, eDNA and proteins	Depends on archaella	(Koerdt *et al.* 2010, 2012; Henche *et al.* 2012b)
Sulfuri. tokodaii	Unknown	Carpet-like structure with towers (25–35 mm thick)	Glucose, galactose, mannose and *N*-acetyl-glucosamine residues	Unknown	(Koerdt *et al.* 2010)
Sa. metallicus	Unknown	Micro- and macrocolonies on elemental sulfur	Galactose, mannose and *N*-acetyl-glucosamine residues, eDNA and proteins	Unknown	(Zhang *et al.* 2015a)

Once attached to the surface, cells start to divide and produce EPS. In *S. acidocaldarius*, microcolony formation was seen after 36 h of inoculation. In *Sa. solfataricus*, *S. acidocaldaricus* and *Sulfura. tokodaii* during the first 3 days of growth, the secreted exopolysaccharides residues were mainly glucose and mannose, while from day five onward galactose and *N*-acetyl glucosamine were predominant (Koerdt *et al.* 2010). The complete path of exopolysaccharides biosynthesis is still unknown. However, an α-mannosidase encoded by SSO3006 in *Sa. solfataricus* affects EPS production (Koerdt *et al.* 2012). The mutant strain *Sa. solfataricus* PBL2025, which lacks a 50 kB region including SSO3006, produced more EPS than the wild-type strain and reverted back to the wild-type phenotype when complemented with SSO3006 (Koerdt *et al.* 2012). In *Sulfura. metallicus* DSM 6482(T), biofilm can form on elemental sulfur with microcolonies and cells clusters of up to 100 μm in diameter. This biofilm was rich in proteins and nucleic acids, in contrast to capsular EPS from planktonic cells, where the EPS mainly contained carbohydrates and proteins (Zhang *et al.* 2015a).

Mature biofilms formed by *S. acidocaldarius*, *Sa. solfataricus* and *Sulf. tokodaii* are morphologically different from each other. *Saccharolobus solfataricus* forms biofilms with a carpet-like structure, with 20–30 μm thick covering the whole surface but with a low density of cells. *Sulf. tokodaii* forms biofilms of 25–35 μm thick and also exhibits a carpet-like structure, but with high cell density and, occasionally, cell aggregates. Finally, *S. acidocaldarius* forms 25–35 μm thick biofilms that contained a high density of cells and large aggregates, forming towering structures above the surface of attached cells (Koerdt *et al.* 2010). Deletion mutant studies in *S. acidocaldarius* showed that the Ups pili and the Aap pili have profound impacts on the morphology of the biofilms (Henche *et al.* 2012b). While deletion of the Aap pilus led to a dense biofilm that was thinner than the wild type, the deletion of the Ups pili led to large aggregates of cells within a 'fluffy' biofilm characterized by a single dense layer of cells at the surface. Clearly, both pili play an essential role in the optimal layering and distancing of the cells in the wild-type biofilm (Henche *et al.* 2012b). Cell–cell connections were also seen extensively for *S. acidocaldarius* and *Sulf. tokodaii*, and to a lesser degree in *Sa. solfataricus* (Koerdt *et al.* 2010). Besides exopolysaccharides, proteins and extracellular (eDNA) can also be found in *S. acidocaldarius* biofilms. Composition analyses revealed several enzyme activities in EPS extracts, but most of them were cytoplasmic proteins (Jachlewski *et al.* 2015), probably derived from cell lyses as these species secrete only small amounts of proteins (Ellen, Albers and Driessen 2010).

Biofilm maturation was followed for several days in *S. acidocaldarius* (Fig. 8, biofilm). From days 3 to 6, an increase in cell density was observed, and in days 7 and 8 dispersion of the cells was seen (Henche *et al.* 2012b; Koerdt *et al.* 2012). In a Δ*arlJ* mutant, however, cell dispersion from the biofilm was decreased, showing that the archaellum is important for cells to leave the biofilm (Henche *et al.* 2012b). It is unknown what triggers dispersion of cells, but this process is important for colonizing other sites along the surface.

Regulation of biofilm processes

The biofilm and planktonic lifestyles differ, as inferred from transcriptomic and proteomic studies comparing both populations (Koerdt *et al.* 2011), with the change from one to another depending on different regulators. In bacteria, it is known that secondary messenger and quorum sensing mechanisms are important for the regulation of biofilm formation, however these have not been described for the *Sulfolobales*. Nonetheless, high throughput proteomics and transcriptomics allowed for a first glance at the factors that might be involved in biofilm formation in three *Sulfolobales* species and a species-specific response was found (Koerdt *et al.* 2011). Among the common differentially regulated proteins were the archaea-specific Lrs14-like regulators (Leucine-responsive Regulator of *Sulfolobus*) (Koerdt *et al.* 2011). Later, deletion mutants confirmed that some of these Lrs14-like proteins are involved in biofilm regulation in *S. acidocaldarius* (see the section 'Regulatory transcription factors in *Sulfolobales*'; Table 2). Knockout mutants for Saci_1223 were impaired in biofilm formation, suggesting that this regulator promotes biofilm formation (Orell *et al.* 2013a). Furthermore, the deletion mutant of Saci_0446 produced more EPS than the wild type, and also showed a non-motile phenotype, where expression levels of the archaellum were downregulated and expression levels of Aap were increased. Therefore, it is thought to act as repressor of biofilm formation and named AbfR1 for Archaeal Biofilm Regulator 1 (see signal transduction in the section 'Post-translational modification by reversible protein phosphorylation'; Fig. 5) (Orell *et al.* 2013a). AbfR1 functions by

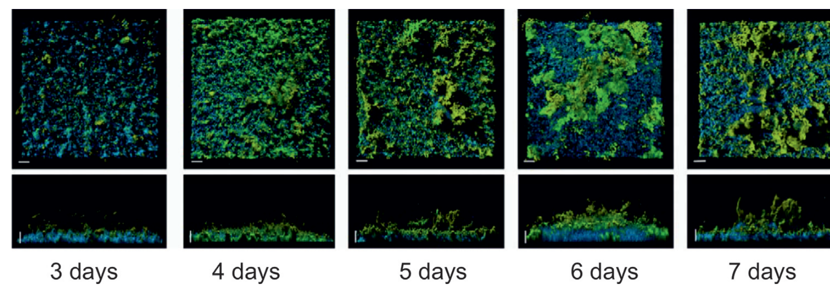


Figure 8. Confocal laser microscopy images from static biofilm from *S. acidocaldarius* in days 3 to 7 of growth. Cells (DNA) stained with 4′,6-diamidino-2-phenylindole (DAPI; blue); extracellular glucose and mannose residues stained with fluorescently labeled concanavalin A (conA; green); and N-acetyl-D-glucosamine residues stained with fluorescently labeled lectin IB4 (yellow). Scale bars: 20 μm. Reproduced from Koerdt *et al.* 2010.

stimulating motility through the induction of the archaellum by binding to the *arlB* promoter region, and repressing EPS production. Finally, detailed studies have shown that AbfR1 is also regulated by phosphorylation through unknown mechanisms, and it cannot bind DNA when phosphorylated (Li *et al.* 2017).

Some non-coding RNAs (ncRNA) also regulate biofilm formation. Sequencing of ncRNAs expressed in planktonic and biofilm cells was performed, and 29 ncRNA were differentially regulated in the latter (Orell *et al.* 2018). One in particular, ncRNA239, named RrrR (RNAse-resistant RNA), was abundant in planktonic cells but further upregulated in the biofilm. Moreover, deletion of this ncRNA led to impairment of biofilm formation. RrrR is a double stranded ncRNA, located in the intergenic region between Saci_1004 and Saci_1005. The sense transcript of this RNA interacts with RNA-binding Lsm proteins, and its antisense RNA binds two mRNAs. The antisense transcript of this RrrR seems to stabilize the sense transcript (Orell *et al.* 2018).

Polyphosphates (PolyP) are polymers of orthophosphate with roles in many cellular functions, including bacterial biofilm formation and related phenomena (Rashid *et al.* 2000; Shi, Rao and Kornberg 2004; Grillo-Puertas *et al.* 2012; Drozd, Chandrashekhar and Rajashekara 2014; Albi and Serrano 2016). In *Escherichia coli*, PolyP is involved in biofilm formation by triggering type II autoinducers (AI-II) synthesis in the stationary phase of growth through PolyP degradation (Grillo-Puertas *et al.* 2012). In archaea, experiments in *Sa. solfataricus* and *S. acidocaldarius* demonstrated that this polymer is also involved in biofilm formation, adhesion and motility (unpublished results), although the mechanism is still unclear.

Cell envelope of *Sulfolobales*

S-layer

The architecture of archaeal cell envelopes can be very diverse (Albers and Meyer 2011; Klingl, Pickl and Flechsler 2019). However, studied *Sulfolobales* species mainly have a cytoplasmic membrane surrounded by a proteinaceous coat, called the S-layer. In contrast to other archaea, which also have an S-layer as the main cell wall component, most *Sulfolobales* have two proteins that form the S-layer: SlaB, which is the membrane anchor, and SlaA forming the outer layer on top of the cell (Grogan 1996; Veith *et al.* 2009). As S-layers are ordered in 2D lattices, they are excellent targets for structural studies. In 1982, Amos and colleagues used electron microscopy to study the structure of the *S. acidocaldarius* S-layer (Taylor, Deatherage and Amos 1982) and related studies then showed that the architecture of the S-layers is species-specific (Prüschenk, Baumeister and Zillig 1987). The *S. acidocaldarius* S-layer was found to be arranged in a conserved lattice with p3 symmetry, with 4.5 nm triangular and 8 nm hexagonal pores (Taylor, Deatherage and Amos 1982). By differential solubilization, the SlaB subunits could be detached from the SlaA lattice, allowing cryo-EM to pinpoint the placement of both subunits in the S-layer lattice (Fig. 9) (Gambelli *et al.* 2019).

As the only cell wall component, the S-layer provides stability to the cell and it has long been assumed that the S-layer is an essential component for *Sulfolobales* cells. However, a transposon library screen in *Sa. islandicus* indicated that both S-layer proteins can be deleted (Zhang *et al.* 2018). When SlaB was deleted in *Sa. islandicus*, the cells were still able to assemble partial SlaA containing S-layer lattices. However, cells lacking SlaA were not only deformed and sometimes very large, but they also had an aberrant number of chromosomes, indicating that the coordinated assembly of the S-layer is important for cell division (Zhang *et al.* 2019a). Furthermore, the S-layer in *Sulfolobales* plays a role in anchoring other surface structures, such as the bindosome, involved in sugar binding and uptake (Zolghadr *et al.* 2011), and the archaellum, where it is absolutely essential for torque generation (Tsai *et al.* 2020). The S-layer also acts as a sieve and as a surface for recognition that is conveyed by extensive *N*-glycosylation (discussed below).

Glycosylation

Many of the extracellular proteins of Archaea are glycosylated (this can be either *O*-glycosylation or *N*-glycosylation). Whereas we know very little about how proteins are *O*-glycosylated, the *N*-glycosylation pathway has been deciphered in halophiles, methanogens and *S. acidocaldarius* (Jarrell *et al.* 2014). Many archaeal extracellular proteins have a so-called ST-linker, which is a stretch of many serine (Ser) and threonine (Thr) residues in a row (Albers *et al.* 2004). In the *Halobacteria* S-layer, these residues were found to be *O*-glycosylated (Lechner and Sumper 1987; Sumper *et al.* 1990) and, thus, it is thought that other extracellular proteins, like SlaB or the sugar-binding proteins that also have these ST-linkers, are also *O*-glycosylated.

A number of proteins have been reported to be glycosylated in the *Sulfolobales*: the S-layer proteins (Peyfoon *et al.* 2010), cytochromes (Zähringer *et al.* 2000), sugar- and peptide-binding proteins (Albers, Konings and Driessen 1999; Elferink *et al.* 2001; Gogliettino *et al.* 2010), pilins (Wang *et al.* 2019a), the archaellum (Meyer, Birich and Albers 2015) and many hypothetical proteins (Palmieri *et al.* 2013). Whereas proteins in other archaea have different glycans or the composition of the glycan changes due to environmental conditions, the *Sulfolobales* have only one kind of glycan decorating all glycosylated proteins in one species. All determined *N*-glycans of the *Sulfolobales* are in their basic structure quite similar to the eukaryotic *N*-glycan, as the glycans always start with two *N*-acetylglucosamines and are branched (Fig. 10) (Zähringer *et al.* 2000; Palmieri *et al.* 2013; van Wolferen

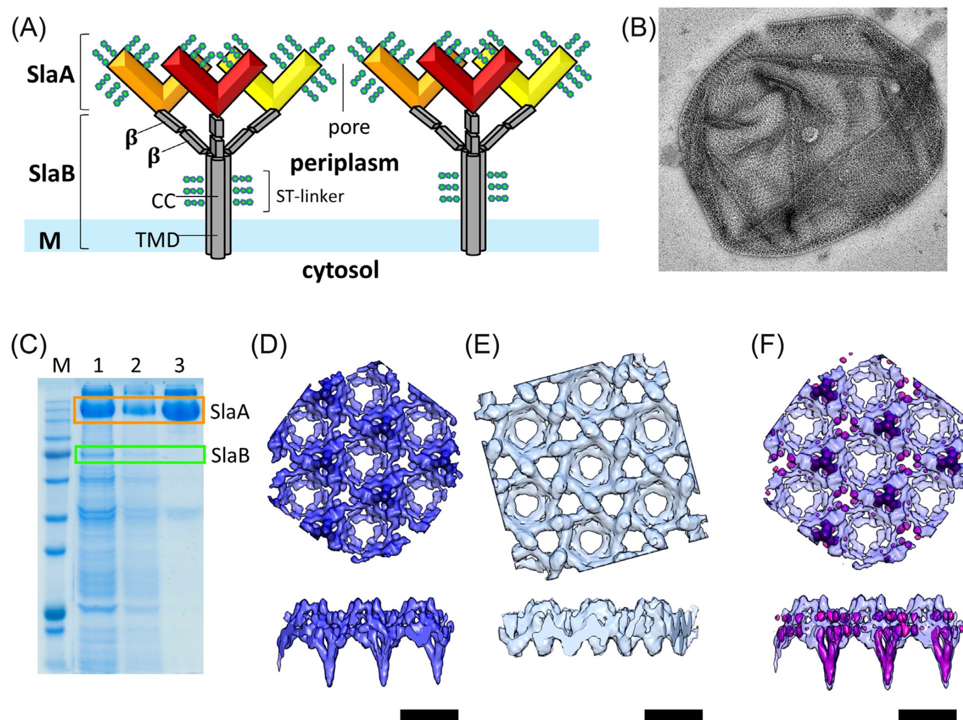


Figure 9. Model of the *Sulfolobales* S-layer. **(A)** The *Sulfolobales* S-layer consists of the two protein subunits. SlaA dimers (red, orange, yellow) form the outer S-layer canopy. Each SlaA protein is predicted to be rich in β-strands. The SlaA dimer has a boomerang-like shape, the angle of which determines the S-layer unit cell size. SlaB trimers (gray) form the membrane anchors of the S-layer. Each SlaB is predicted to consist of an N-terminal transmembrane domain (TMD), a coiled-coil domain (CC) and two to three C-terminal β-sandwich domains (β). SlaA and SlaB proteins are highly glycosylated (green). **(B)** Electron microscopy image from negatively stained isolated S-layer from *S. acidocaldarius*. **(C)** SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) of the isolation of S-layer from *S. acidocaldarius* cells using detergent buffers. Lane 1: pellet after incubating the cells with the detergent buffer the first time, the second time (lane 2) and the third time (lane 3). SlaB is being successively washed off and in the last wash a pure SlaA prep is obtained. **(D)** Subtomogram average of fully assembled S-layer. **(E)** Subtomogram average of SlaB-depleted S-layer. **(F)** Difference map (pink) overlaid with the complete S-layer visualizes location of SlaB. (Scale bars, and C–E, 20 nm). (Figure adapted from Gambelli *et al.* 2019).

et al. 2020). Interestingly, they all contain a sulfated saccharide called 6-sulfoquinovose, a sugar otherwise only found in chloroplast and membranes of photosynthetic bacteria (Meyer *et al.* 2011). However, except for this basic setup, the *N*-glycans of the different *Sulfolobus* species are diverse, as different numbers of mannose and glucose sugars are added to create a species-specific sugar labeling of extracellular proteins (see Fig. 10). This is used for species-specific recognition during DNA exchange (Fröls *et al.* 2008). During the pilin-dependent DNA exchange observed after double strand DNA breakages, the *Sulfolobales* exchange homologous DNA, by a sugar-specific binding of the pilin subunit to the S-layer of the aggregating cells (van Wolferen *et al.* 2020). Although the differences are minor between the *N*-glycans of *Sa. solfataricus*, *Sulf. tokodaii* and *S. acidocaldarius* (see Fig. 10), this is enough to ensure that aggregation and subsequent DNA exchange only happens with the same species (van Wolferen *et al.* 2020).

In contrast to methanogens and halophiles (Jarrell *et al.* 2014), *N*-glycosylation seems to be essential in *Sa. islandicus* and *S. acidocaldarius*, as the enzyme attaching the *N*-glycan to the modified protein, the oligosaccharyl transferase AglB, cannot be deleted (Meyer and Albers 2014; Zhang *et al.* 2018). *N*-glycosylation is initiated by the assembly of the hexasaccharide *N*-glycan on short dolichol pyrophosphate carriers in *S. acidocaldarius* by AglH (Guan *et al.* 2016; Meyer, Shams-Eldin and Albers 2017). Deletion of the glycosyltransferase *agl16* led to a removal of a terminal hexose from the *N*-glycan, whereas deletion of *agl3*, the sulfoquinovose synthase, reduced the *N*-glycan to a trisaccharide (Meyer *et al.* 2011, 2013). Both mutants had difficulties in adjusting to growth in media with elevated salt concentration. The *N*-glycosylation pathway of *S. acidocaldarius* is depicted in Fig. 10.

Sulfolobus cell division

The most investigated archaeal cell division system is the ESCRT-III-based system in the *Sulfolobales*. The proteins involved in cell division (Cdv) are organized in two groups. One group comprises the proteins CdvA, -B and -C organized in a gene cluster, while the other group consists of three CdvB paralogs located at different positions in the genome (Lindas *et al.* 2008; Samson *et al.* 2008, 2011). CdvA is the only protein of these two groups that does not share homologies to the eukaryotic ESCRT-system or any known protein family. CdvA can bind membranes and polymerizes into helical filaments on the outside of liposomes composed of tetraether lipids isolated from *S. acidocaldarius*. Interestingly, the presence of lipids was necessary for CdvA polymerization *in vitro* (Dobro *et al.* 2013). Transcriptional analysis of the *cdv* operon showed that *cdvA* is upregulated around 30 min before the *cdvB* genes are transcribed. Immunofluorescence with antibodies raised against CdvA displayed a ring-like localization pattern at midcell, but also outside the midcell region, before the genome was segregated (Lindas *et al.* 2008; Samson *et al.* 2011). These results suggest that CdvA is the earliest cell

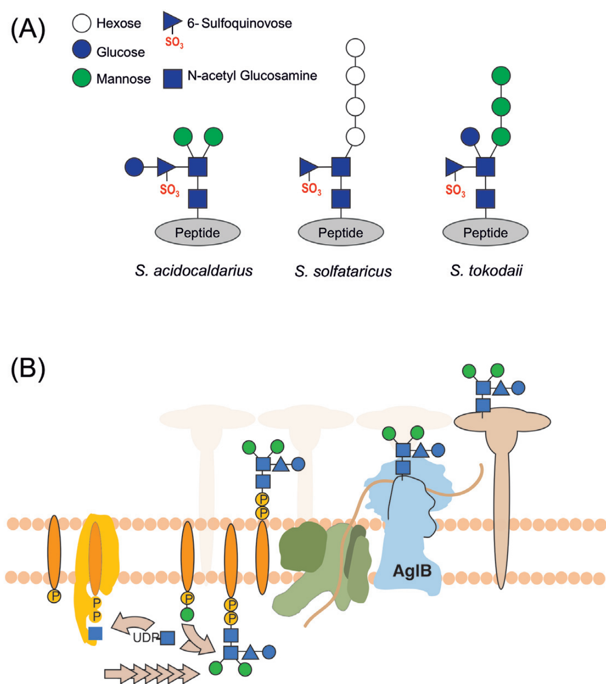


Figure 10. Glycosylation. **(A)** Comparison of the *N*-glycan trees of three different *Sulfolobus/Saccharolobus* species. **(B)** The current understanding of the *N*-glycosylation pathway in *S. acidocaldarius*. The *N*-glycan biosynthesis is initiated by adding nucleotide-activated monosaccharides sequentially to the lipid carrier dolichol phosphate on the cytoplasmic side of the membrane. The fully assembled dolichol pyrophosphate-linked *N*-glycan (hexasaccharide) is translocated across the membrane and then transferred by AlgB on the specific *N*-glycosylation sequons in secreted proteins. Sugar code is shown in (A).

division protein localizing at the future division plane. However, it is unknown how CdvA is positioned. Remarkably, CdvA from *Metallosphaera sedula*, which was heterologously expressed and purified from *E. coli*, formed extended double-helical filaments that strongly interacted with the DNA of *E. coli* (Moriscot *et al.* 2011). This suggests that CdvA participates in the chromosome segregation processes or functions as an inverse nucleoid-occlusion type localization mechanism for the archaeal divisome (Caspi and Dekker 2018). However, the exact function of its DNA binding remains unknown. CdvA can recruit CdvB to preformed liposomes by direct interaction, resulting in extensively deformed vesicles. The β-strand forming C-terminal ESCRT-III-binding (E3B) peptide of CdvA interacts with the C-terminal incomplete winged-helix (wH) domain of CdvB. As such, the E3B peptide complements the 'broken' part of the CdvB wH domain, repairing the domain to a wH-related architecture. Importantly, the wH domain of CdvB, necessary for interaction with CdvA and the recruitment of CdvB to the membrane, is not present in the other three ESCRT-III homologs found in *Sulfolobales* species. Also, no interaction of the other CdvB paralogs with CdvA has been reported (Samson *et al.* 2011).

Interestingly, the CdvB paralogs can interact with themselves and the two other paralogs (Samson *et al.* 2008). A recent study implied that CdvB does not constrict *in vivo* but functions as a scaffold for CdvB1 and CdvB2 in *S. acidocaldarius*. The latter two proteins localize at the cell division plane, forming a ring-like structure after CdvB has already been recruited to midcell. Subsequent proteasomal degradation of CdvB allowed the formed CdvB1/B2 ring to constrict, completing cell division (Risa *et al.* 2019). Furthermore, CdvB1/B2 were shown to be involved in other membrane-remodeling events in the *Sulfolobales*, as they were found in secreted membrane vesicles (Ellen *et al.* 2009). Deletion of *cdvB1* affected growth at 75°C only modestly and resulted in occasional failures in cell division. Remarkably, the Δ*cdvB1* strain was severely impaired at growth at 65°C, suggesting a more important role under stress conditions (Pulschen *et al.* 2020). Additionally, deletion of *cdvB2* showed a loss of cell division symmetry and was severely affected in cell growth (Pulschen *et al.* 2020). Analysis of cell division in the *Sulfoscope* (an inverted fluorescent microscope with a heated chamber) showed that the CdvB and CdvB1 rings were correctly assembled in the Δ*cdvB2* strain, while after proteasome-mediated degradation of CdvB, CdvB1 rings were found at variable positions. This asymmetric division plane resulted in both ghost cells without DNA and cells with a double amount of DNA. This suggested that CdvB2 fixes the position of the ring after the loss of the CdvB scaffold. It was proposed that CdvB2 and CdvB1 are recruited to the cell center by the CdvB ring and that, after proteasome mediated degradation of CdvB, they hold the division ring in position while it constricts (Pulschen *et al.* 2020).

When *cdvB3* was deleted, growth was delayed, colonies on plates were very small and cells had a division defect. In the *cdvB3* deletion strain, CdvB was not localized at midcell in a ring-like structure. Instead, a diffuse CdvB signal was detected in the cytoplasm. Furthermore, CdvA was mislocalized and formed distinct foci at the cell membrane of enlarged cells (Yang and Driessen 2014). On the other hand, in *Sa. islandicus*, deletion of *cdvB1* and *cdvB2* was not successful. The reduction of intracellular CdvB1 protein levels led to a chain-like morphology of the cells, indicating that CdvB1 is important for the final abscission during cell division. In contrast to *S. acidocaldarius*, *cdvB3* deletion in *Sa. islandicus* led to neither a cell division defect nor growth retardation. However, after infection with spindle-shaped virus 2 (STSV2), the Δ*cdvB3 Sa. islandicus* strain no longer developed viral buds on the cell surface (Liu *et al.* 2017). These findings indicate that although all *Sulfolobus* species have four ESCRT-III homologs, their specific function is different among the *Sulfolobales*. Yet, CdvB seems to be the key cell division protein of the four ESCRT-III homologs, providing a platform for later cell division proteins. Recently, ESCRT-I and -II homologs were identified in *Lokiarchaeota*. Though the function of these other ESCRT homologs is unknown, they may be involved in vesicle formation rather than in cell division as ESCRT-III homologs are also present in *Lokiarchaeota* (Caspi and Dekker 2018).

The third gene of the *cdv* operon encodes an ATPase (CdvC) that is homologous to the eukaryotic AAA-type (ATPase associated with various activities) ATPase Vps4 (Hobel *et al.* 2008). In HeLa cell lines, Vps4 was observed to localize at the cell center during cytokinesis simultaneously with ESCRT-III. It was shown that Vps4 is important for the dynamic assembly and disassembly of ESCRT-III filaments, leading to the formation and constriction of intercellular bridges. As such, the dynamic turnover rate of ESCRT-III was dependent on the Vps4 ATPase activity (Mierzwa *et al.* 2017; Caillat *et al.* 2019). With regard to archaea, it is assumed that CdvC also remodels CdvB filaments and is thereby responsible for membrane invagination.

Coinciding with the other two Cdv proteins, CdvC localizes in a band-like structure to midcell during cytokinesis (Lindas *et al.* 2008). Biochemical characterization of the CdvC protein from *Sa. solfataricus* showed (Samson *et al.* 2008; Caspi and Dekker 2018) that it assembles into single hexameric rings, comparable to Vps4 from *Saccharomyces cerevisiae* (Monroe *et al.* 2014). Furthermore, it was shown that CdvC directly interacts with CdvB (Obita

et al. 2007). Important for this interaction is the N-terminal conserved MIT (microtubule interacting and trafficking) domain of CdvC that recognizes a C-terminal peptide motif of CdvB, called MIM2 (MIT domain interacting motif). The MIM2 domain is characterized by a proline-rich end part that is not present in the MIM2 domains of CdvB1 and CdvB2, resulting in a very weak binding affinity of the latter proteins to CdvC. Moreover, CdvB3 completely lacks the MIM2 domain and, hence, does not interact with CdvC (Samson *et al.* 2008; Caspi and Dekker 2018). This indicates that CdvB is the only ESCRT-III-like protein in *S. acidocaldarius*, recruiting CdvC to midcell. In contrast, a yeast two-hybrid assay with ESCRT-III homologs and CdvC from *Sa. islandicus* showed interaction between CdvC and CdvB1/B2 (Liu *et al.* 2017). Possibly, the lack of the proline-rich strand in CdvB1 and B2 is compensated by an additional interaction of the CdvB1/B2 protein with the MIT domain of CdvC (Caspi and Dekker 2018). Moreover, the different interaction pattern of CdvC with the respective CdvB paralogs in *S. acidocaldarius* and *Sa. islandicus* might explain their different influence on cytokinesis. However, the mechanism of how CdvC is involved in cell division is still unknown. See Fig. 11 for the model of cell division in *S. acidocaldarius*.

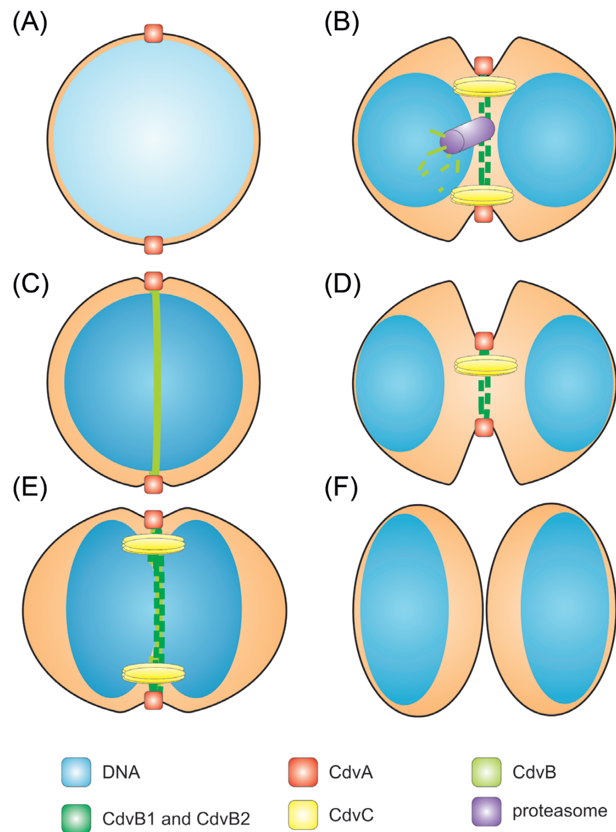


Figure 11. Schematic model of the cell division process in *Sulfolobus acidocaldarius*. **(A)** CdvA (red) is the first protein of the *S. acidocaldarius* cell division machinery that arrives at the future site of cell division, before DNA (light blue) segregation starts. **(B)** During nucleoid condensation (blue), CdvB (light green) forms a ring-like structure at midcell that is anchored to the membrane by CdvA. **(C)** CdvB provides a scaffold for CdvB1 and B2 (green) that are positioned at the cell center in ring-like structures. Additionally, CdvC (yellow), a homolog of the hexameric ATPase Vps4, localizes at the septum while nucleoid segregation and initial membrane invagination start. **(D)** After nucleoid segregation, the CdvB-ring undergoes proteasomal (purple) degradation. **(E)** Upon CdvB removal, CdvB1 and B2 constrict, leading to the final division of the cell. **(F)** Directly after fission, the new born cells have an oval shape that rapidly changes to the typical coccoid shape of *S. acidocaldarius* cells. CdvA and CdvC are organized in a ring-like structure as well. However, for a better overview the organization of both proteins at midcell was only indicated in the figure.

EXTREME THERMOACIDOPHILY AND STRESS RESPONSE

Thermoacidophily

Thermoacidophiles have specialized mechanisms to deal with stressors associated with their unusual temperature and pH optima and the fact that some thermal acidic environments are characterized by metal deposits. Although thermoacidophilic archaea largely do not have unique thermal or pH stress response mechanisms compared with thermophiles or acidophiles, some mechanisms play dual protective roles. Here, the major contributors to thermophily and acidophily will be discussed, with particular emphasis on where these stress responses overlap.

Because G–C bonds in DNA are more heat stable, it was initially hypothesized that thermophiles would have a higher G+C content then their mesophilic counterparts. Surprisingly, evaluation of DNA sequences from both thermophiles and mesophiles demonstrated that this was not the case (Tekaia, Yeramian and Dujon 2002; Wang, Susko and Roger 2006; Zeldovich, Berezovsky and Shakhnovich 2007). However, thermophiles have reduced mutation rates and have DNA repair mechanisms to combat biological damage at high temperatures. Though higher temperatures are correlated with an increase in mutations, base substitutions occur less frequently (Drake 2009), or at an equivalent rate (Grogan, Carver and Drake 2001) in thermophiles compared with mesophiles. Furthermore, DNA repair can also occur by homologous recombination and DNA uptake through type IV pili (reviewed in the section 'Cell cycle and modes of growth').

Even though DNA stability does not depend on G+C content as it does not correlate with higher growth temperatures, thermophiles do have codon/amino acid preferences to support enzyme thermal stability. For instance, thermophiles favor codons AGG and AGA over CGN for arginine (Farias and Bonato 2003; Singer and Hickey 2003) and ATA for isoleucine, instead of ATC or ATT (Singer and Hickey 2003). In addition, thermophiles have a nucleotide bias for the more heat-stable purines A and G; these bases are more frequently neighbors in thermophile compared with mesophile genomes (Zeldovich, Berezovsky and Shakhnovich 2007).

Not only do thermophiles have nucleotide preferences, but they also favor the use of certain amino acid residues. In general, thermophiles prefer amino acids that are charged or hydrophobic, while avoiding polar uncharged residues (Tekaia, Yeramian and Dujon 2002). Intuitively, thermophilic proteins also avoid heat labile amino acids, such as histidine, glutamine and threonine (Tekaia, Yeramian and Dujon 2002; Singer and Hickey 2003). Additionally, one study looking at the proteome amino acid composition of a variety of genomes discovered that the ratio (Glu + Lys)/(Gln + His) was lowest in mesophiles, higher in moderate thermophiles and highest in extreme thermophiles ($T_{opt} \geq 70°C$). This ratio was higher in thermostable chaperonins and DNA ligases in mesophiles compared with the rest of the proteome (Farias and Bonato 2003). Furthermore, another study demonstrated that the occurrence of certain amino acids (IVYWREL) correlated with microbial optimal growth temperature and, thus, was a predictor of thermophily (Zeldovich, Berezovsky

and Shakhnovich 2007). Not only is the codon usage different in thermophiles but also their proteins averaged 283 aa compared with the 340 aa in mesophiles, possibly increasing the thermostability of the protein (Tekaia, Yeramian and Dujon 2002).

Another thermal adaptation is the use of a reverse DNA gyrase, a DNA topoisomerase that introduces positive supercoiling to DNA strands, thereby increasing its heat stability. When the reverse DNA gyrase was deleted in *Thermococcus kodakarensis*, this extreme thermophile was more sensitive to higher temperatures, although the mutation was not shown to be lethal (Atomi, Matsumi and Imanaka 2004). However, though multiple methods were used to mutate the gyrase genes in *Sa. islandicus*, no viable mutants were generated (Zhang *et al.* 2013b), indicating that this mutation can be lethal. In addition, a comparative genomic study showed that the only common gene in all extreme thermophiles sequenced at the time was a DNA reverse gyrase, lending further support to this enzyme's importance to thermophily (Forterre 2002).

In extreme thermophiles, the thermal stress response involves the thermosome (a molecular chaperone also referred to as the rosettasome, or archeaosome), a large HSP60-like protein complex originally discovered in *S. shibitae* (Trent, Osipiuk and Pinkau 1990, Trent *et al.* 1991). In archaea, the thermosome is composed of multiple α and β subunits that form ring structures with either 8- or 9-fold symmetry (Kagawa *et al.* 1995). A third γ subunit has been described in some extreme thermophiles that is downregulated during heat shock (Archibald, Logsdon and Doolittle 1999; Kagawa *et al.* 2003; Tachdjian and Kelly 2006). The thermosome is a Group II chaperonin, conserved among archaea (Kagawa *et al.* 1995), that shares structural and functional similarities to the bacterial Group I chaperonin GroEL complex (Trent *et al.* 1991). In both heat shock complexes, cytosolic proteins are sequestered inside an internal cavity where they can refold. As demonstrated for the *Sa. solfataricus* thermosome, complexes are formed with different subunit compositions in a temperature-dependent manner, which supposedly have different substrate specificities (Chaston *et al.* 2016). However, unlike the GroEL chaperonin of bacteria that has GroES to act as a lid for the complex, the thermosome does not have a separate subunit cap to close its internal cavity. Instead, the thermosome goes through conformational changes to close the apertures at each end (Schoehn *et al.* 2000). These conformational changes are correlated with the binding and hydrolysis of ATP (Bigotti and Clarke 2005). The apo-chaperonin is naturally in an open conformation and binding of ATP allows for the expansion of the thermosome entrance regions. The hydrolysis of bound ATP causes a conformational change to enclose a denatured protein. Finally, the release of ADP and/or P_i liberates the folded protein and reopens the thermosome (Gutsche *et al.* 2000; Gutsche, Mihalache and Baumeister 2000). The archaeal thermosomes are very similar in sequence to the cytosolic TCP1 eukaryotic chaperonins, the major difference being the number of subunits that compose each chaperonin's ring structures (Kagawa *et al.* 1995).

In addition to the thermosome (~60 kDa subunits), thermoacidophiles also have HSP20 small heat shock proteins (sHSP), composed of 12–43 kDa subunits that also serve as chaperones during thermal stress response. HSP20 are common heat shock response proteins that are represented in every phylogenetic kingdom (Haslbeck *et al.* 2005). These small proteins form larger structures of up to 50 subunits that can bind and stabilize proteins upon exposure to supra-optimal temperatures preventing protein aggregation (Wang *et al.* 2010b; Li *et al.* 2012; Baes *et al.* 2020). Similar to the thermosome, the sHSP can also refold denatured proteins.

If denatured or damaged proteins cannot be refolded via chaperones, they will be degraded by the proteasome. In eukaryotes, ubiquitin and the ubiquitin-related modifier-1 (Urm1) mark proteins for degradation by the proteasome. Likewise, in thermoacidophilic archaea, an Urm1-like molecule is covalently attached to proteins by an E1-like enzyme that is reliant on the hydrolysis of ATP. This urmylation designates a protein for degradation by the archaeal proteasome. The archaeal Urm1 protein is also degraded in the process, unlike the Urm1 signal in eukaryotic systems (Anjum *et al.* 2015). The archaeal proteasome consists of two components: the 19S proteasome-activating nucleotidase (PAN) and the 20S proteolytic core particle. PANs are AAA^+ ATPase regulatory particles that unfold proteins in preparation for degradation by the core particle. The 20S core particle of the proteasome is a cylindrical stack of four heptameric rings, which consists of two β rings flanked by two α rings. Within the core particle is an internal channel that contains the proteolytic site for protein degradation located within the two β rings. PAN is a homohexomeric ring that associates with the outside α rings of the core particle. Comprehensive reviews of proteasomes and AAA^+ ATPases are available (Bar-Nun and Glickman 2012; Maupin-Furlow 2012).

Another possible way thermophiles handle thermal stress is through compatible solutes that were originally described only as an osmoprotectant (Brown and Simpson 1972). These organic molecules can accumulate intracellularly without interfering with cell metabolism to protect cytosolic components from other stressors, such as heat. For instance, *Mycobacterium smegmatis* mutants, unable to generate the compatible solute trehalose, exhibited sensitivities to elevated temperatures (Woodruff *et al.* 2004). Though trehalose is present in all domains of life, it has been found in large quantities in several thermophilic archaea, such as *Sa. solfataricus* (Nicolaus *et al.* 1988). On the other hand, the organic solutes di-myo-inositol-phosphate (DIP), di-mannosyl-di-myo-inositol-phosphate, di-glycerol-phosphate, mannosylglycerate and mannosylglyceramide are only observed in thermophilic organisms (reviewed in Santos and Da Costa 2002). Also, cellular quantities of DIP have been shown to increase in response to increasing temperature in the thermophiles *Pyrococcus furiosus* (Martins and Santos 1995) and *Thermotoga neapolitana* (Martins *et al.* 1996). Additionally, di-mannosyl-di-myo-inositol-phosphate also increases in *T. neapolitana* due to thermal stress (Martins *et al.* 1996). Although the exact mechanism of thermal protection by compatible solutes has not been determined, these compounds clearly have a part in the thermal stress response.

Some thermoacidophiles have pH optima near 0 (Schleper *et al.* 1995), but most have cytosols with a near neutral pH, thereby resulting in a large pH differential across the membrane. Even *P. oshimae*, previously mentioned to have an internal pH of ~4.6, has a cytoplasmic pH several pH units above its environmental pH optima of <1. Acidophiles (both thermophilic and mesophilic) must have ways to maintain their cytoplasm near neutral and still generate a proton motor force for energy generation. For instance, acidophiles have a reversed membrane potential, where the intracellular membrane is positively charged and the extracellular is negatively charged. This reverse membrane potential is driven by the active transport of potassium ions into the cell and prevents passive proton transport. Despite the large ΔpH across the membrane, acidophiles still

generate ATP by coupling the influx of protons to the phosphorylation of ADP through a membrane-bound ATP synthase (Moll and Schäfer 1988). However, this necessary influx of protons must be countered to maintain the intracellular pH of the cell. Proton pumps that are uniporters, symporters and antiporters are part of the energy generating respiratory chain that prevents the acidification of the intracellular environment by exporting internalized protons. These proton pumps are dependent on maintenance of the inversed membrane potential, which can be accomplished by pumping cations, such as potassium ions, into the cell (Schäfer 1996). In fact, potassium ions have been shown to be a vital part of proton pumping in the respirations chain of *S. acidocaldarius* (Moll and Schäfer 1988).

Alternatively, if protons do reach the cytoplasm, the cell must have ways to prevent the acidification of the cytosol. Intracellular molecules and proteins have buffering capacities that will neutralize internalized protons (Baker-Austin and Dopson 2007). For example, basic amino acids, such as histidine, arginine and lysine, can add to the buffering capacity of the cytoplasm. As discussed in the section 'Degradation of proteins and amino acids', members of the *Sulfolobales* have amino acid decarboxylases that may produce polyamines that are known contributers to cytoplasmic buffering. In fact, reduction of amino acids in continuous cultures of *M. sedula* caused a decrease in internal pH and an increase in thermosome protein levels (Peeples and Kelly 1995). Furthermore, protonated organic acids can permeate the cell and release a proton once inside the neutral cytoplasm. Heterotrophic acidophiles could possibly degrade organic acids without the production of a free proton to prevent the acidification of the cytoplasm (Baker-Austin and Dopson 2007). As is the case for thermal stress, damaged proteins due to low pH can either be repaired by microbial chaperones or be marked for degradation by the proteasome.

Membrane composition and thermoacidophily

Thermoacidophiles maintain their intracellular pH by having membranes that are more impermeable to protons than neutralophiles (Konings *et al.* 2002). The ether-linked lipids of archaeal membranes are more resistant to acid hydrolysis than the ester-linked lipids of bacterial membranes. Liposomes comprised of ether-linked lipids are more resistant to leakage due to thermal stress (Choquet *et al.* 1994). Furthermore, tetraether lipids that span the microbial membrane make a monolayer that reduces the fluidity of the archaeal membrane, thereby decreasing its permeability to proton penetration and increasing heat tolerance. These tetraether lipids can also have multiple cyclopentyl rings that will further increase the packing of these lipids, making the membrane even less permeable to the diffusion of protons and more resistant to elevated temperatures. This has been demonstrated in liposomes derived from the membranes of *S. acidocaldarius* (Elferink *et al.* 1994; Komatsu and Chong 1998) and *P. oshimae* (van de Vossenberg *et al.* 1998). The most abundant tetraether lipids are the glycerol dibiphytanyl glycerol tetraethers (GDGTs) that can have up to 4 cyclopentyl rings on each chain. The number of ring structures within these tetraether lipids increased with increasing temperature in *Thermoplasma acidophilum* (Uda *et al.* 2001), and with both decreasing pH and increasing temperature in *P. torridus* (Feyhl-Buska *et al.* 2016) and *Acidilobus sulfurireducens* (Boyd *et al.* 2011). These studies exemplify the importance of archaeal tetraether lipids with these cyclic moieties to survival in hot acid environments. However, only thermoacidophiles of the *Sulfolobales* have calditol, a cyclopentyl head group, ether linked to their GDGTs. Recently, the importance of this distinctive head group to acid

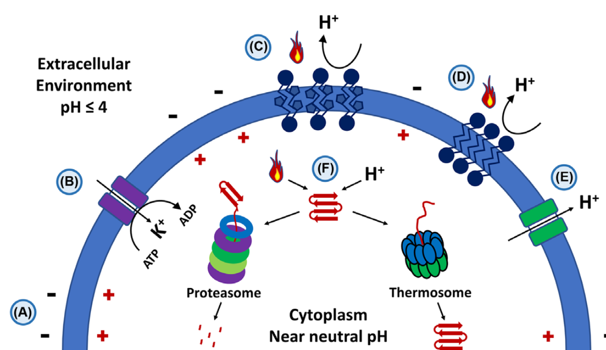


Figure 12. Major mechanisms of thermoacidophily. **(1)** Thermoacidophiles have an inverted membrane potential with a positive charge on the inside of the cellular membrane and a negative charge on the outside to prevent the acidification of the cytoplasm by the passive diffusion of protons. **(2)** The inverted membrane potential is maintained by transporting cations such as K^+ into the cytoplasm. **(3)** Cyclopentyl ring moieties on tetraether lipids increase packing of the tetraether lipids decreasing the permeability of the membrane by protons and increasing cellular heat stability. **(4)** Tetraether lipids make a monolayer that is less permeable to protons and more heat stable than diether lipids. **(5)** Proton pumps export protons from the cytoplasm to prevent the acidification of the cytoplasm. **(6)** Heat-damaged or protonated proteins can either be degraded via the proteasome or properly refolded by the thermosome.

resistance has been demonstrated in *S. acidocaldarius* as deletion of a calditol synthase resulted in a sensitivity to low pH (Zeng *et al.* 2018).

It is clear that thermoacidophiles have several different mechanisms to persist in elevated temperatures and low pH, including the thermosome and proteasome to manage the impact of damaged proteins in the cytosol, membranes made up of tetraether lipids with a reverse potential, and proton pumps to maintain intracellular pH homeostasis (Fig. 12).

Thermal stress response

While the importance of the thermosome, sHSPs and proteasome to thermoacidophile heat tolerance is well known, the transcriptional thermal stress response of their associated genes is somewhat varied in the *Sulfolobales*. Although some sHSPs in other organisms are constitutively expressed, in *Sa. solfataricus* and *S. acidocaldarius* two sHSP are transcriptionally upregulated during thermal stress (Tachdjian and Kelly 2006; Li *et al.* 2012; Baes *et al.* 2020). However, the expression of the α and β subunits of the thermosome is unchanged during thermal stress in *Sa. solfataricus* (Tachdjian and Kelly 2006), while upregulated in *Sa. shibatae* and *S. acidocaldarius* (Kagawa *et al.* 2003; Baes *et al.* 2020). Also, as previously mentioned, transcriptional expression of the γ subunit of the thermosome decreases during thermal stress in most studied species (Archibald, Logsdon and Doolittle 1999; Kagawa *et al.* 2003; Tachdjian and Kelly 2006), with the exception of *S. acidocaldarius* (Baes *et al.* 2020). Although the proteasome plays a vital role in the heat shock response, the transcriptional expression of the 20S core components of the proteasome was unaffected by thermal stress in *Sa. solfataricus*, while the PAN regulatory component was significantly downregulated under these conditions (Tachdjian and Kelly 2006). However, post-transcriptional or -translational regulation, which has not yet been studied in detail, may also play a role in the thermosome's and proteasome's response to elevated temperatures.

The transcription of genes encoding components of the thermosome and proteasome are not significantly impacted by thermal stress, but this is not the case for much of the genome. In

Sa. solfataricus, one-third of the genome, including sHSPs, was transcriptionally responsive to a 10°C temperature shift from 80°C to 90°C (Tachdjian and Kelly 2006). Of note, many type II TA loci, which encode a ribonucleolytic toxin and a corresponding antitoxin protein, were also upregulated. In particular, when the genes for one of these TA loci were deleted, the resulting *Sa. solfataricus* mutant became heat shock labile (Tachdjian and Kelly 2006; Cooper Charlotte *et al*. 2009; Maezato *et al*. 2011).

Thermoacidophiles can also use motility to seek out cooler conditions when thermal stress is encountered. Specifically, a temperature shift from 50°C to 80°C in *S. acidocaldarius* resulted in an increase in swimming speed and run time. Also, when exposed to a temperature gradient, cells migrated away from the higher temperature to regions closer to their optimum temperature. However, a similar response was not seen when the pH was shifted from 2 to 4, indicating that this was a temperature specific stress response for the thermoacidophile (Lewus and Ford 1999). Overall, thermoacidophiles mount a complex response to thermal stress that is varied among the members of the order *Sulfolobales*.

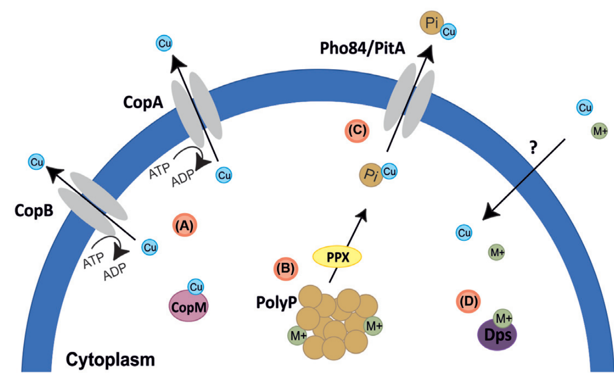


Figure 13. Model of heavy metal resistance in the *Sulfolobales*. **(1)** CopA and CopB export Cu outside the cell with ATP consumption. CopM is a metal chaperone that forms part of the Cop system that also includes a transcriptional factor called CopT (not shown). **(2)** PolyP can sequester cations via its negatively charged surface. **(3)** PolyP can also be degraded by PPX into inorganic phosphate to be exported outside the cell along with cations via PitA or Pho84 transporters. **(4)** Some proteins also act by sequestering metal ions, for example Dps. The mechanism for which metals enter the cell is still unknown.

Metal stress response

Not only do thermoacidophiles have to combat the deleterious effects of life in hot acid, they also often encounter toxic heavy metals in their biotopes. In general, certain metal ions are essential for proper function of cells. Metals participate in many cellular processes, including as cofactors bound to proteins, as catalysts of redox reactions and to transport electrons in the respiratory chain. In mesophilic organisms, the focus is on acquiring biologically important heavy metals that are scarce in their environment. But, in the case of acidophilic organisms, especially in mining environments, the aim is to avoid their influx of heavy metals into the cell or at least to reduce the effective intracellular concentration. Mesophilic bacteria do this by using active and passive systems to remove the metal ions from the cell, form complexes with these metals or convert them into a less toxic form. It is also important to note that there are mechanisms associated with metabolic response to the reactive oxygen species (ROS) generated inside the cell after metal uptake (reviewed in Lemire *et al*. 2017). Most of the studies on heavy metal resistance in *Crenarchaeota* have been done in *Sa. solfataricus* and *M. sedula*, although many resistance mechanisms are still unknown, especially in species like *Sulfura. metallicus*, which has unusually high metal resistance levels.

Uranium and type II toxin–antitoxin systems

Some extreme thermoacidophiles have adapted to toxic metals, such as uranium, through unusual resistance mechanisms. *Metallosphaera sedula*, more so than a very similar species, *Metallosphaera prunae*, catalyzes the oxidization of U_3O_8 to soluble U(VI), mediated by Fe^{3+} generated from Fe^{2+} under chemolithoautotrophic conditions. However, the U(VI) so generated is toxic to the microbe (Mukherjee *et al*. 2012). As an adaptation to uranium U(VI) stress, *M. prunae* activates type II TA systems to degrade cellular rRNA during uranium stress, thereby inducing a population-wide dormancy. This response is not seen in *M. sedula*. This resistance mechanism allows *M. prunae* to withstand the toxic effects of living in the uranium-rich environment from which it was initially isolated (Mukherjee *et al*. 2012, 2017).

Active transporters for metal resistance

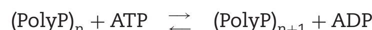
Active transport efflux is one of the most common approaches used for cellular metal resistance and the Cop system, which is involved in copper (Cu) resistance, is the best studied example in archaea. It includes a transcriptional regulator (CopT), a metal-binding chaperone (CopM) and a Cu-transporting ATPase (CopA) (Fig. 13) (Villafane *et al*. 2011). The genes for this system form a cluster in the *Sa. solfataricus* genome (She *et al*. 2001; Ettema *et al*. 2003, 2006), which is also present in *M. sedula*, *S. acidocaldarius*, *Sulf. tokodaii* (Martínez-Bussenius, Navarro and Jerez 2017) and *Sulfura. metallicus* (Orell *et al*. 2013b; Martínez-Bussenius, Navarro and Jerez 2017). Furthermore, in *Sulfura. metallicus*, the *cop* cluster is duplicated in the genome, although one cluster has a lower transcriptional response to copper. This duplication likely relates to the high heavy metal resistance of this organism (Orell *et al*. 2013b).

Besides CopA, there is another copper-exporting ATPase, CopB, which contributes to Cu resistance and is present in some thermoacidophile genomes, including *Sa. solfataricus*. While CopA responds to intracellular Cu levels, CopB is constitutive (Völlmecke *et al*. 2012). The Cop system is also responsive to cadmium (Cd), but apparently not to silver (Ag), as seen in *Sa. solfataricus* (Ettema *et al*. 2006) and *Sulfura. metallicus* (Orell *et al*. 2013b). The *Sulfura. metallicus* Cop system also responds to chalcopyrite (CuFeS2) (Orell *et al*. 2013b) and likely reinforces its Cu resistance in mining environments.

Polyphosphate

Polyphosphates (PolyP) are ubiquitous molecules that play many cellular roles, including heavy metal resistance. These polymers are made of hundreds to thousands of inorganic orthophosphate (P_i) residues, linked by high-energy bonds, similar to those in ATP. There are two major enzymes involved in metabolism of this molecule: polyphosphate kinase (PPK) and exopolyphosphatase (PPX). The first enlarges the PolyP chain, adding a P_i to the end in a reversible reaction, using ATP as substrate, while PPX catalyzes the reverse reaction, hydrolyzing PolyP starting from the terminal P_i residue (there are endo-polyphosphatases as well, that cleaves internal PolyP bonds) (Kornberg, Rao and

Ault-Riché 1999; Albi and Serrano 2016):



$$(PolyP)_n + ATP \rightleftarrows (PolyP)_{n+1} + ADP$$

$$(PolyP)_{n+1} \longrightarrow PPX(PolyP)_n + P_i$$

In the *Crenarchaeota*, the enzyme that synthesizes PolyP has not been identified, since neither PPK1 nor PPK2 has been identified. Several archaea, especially in the *Sulfolobales*, possess a PPX enzyme (Martínez-Bussenius, Navarro and Jerez 2017; Wang *et al.* 2019d). Meanwhile, in *Euryarchaeotes*, a putative PPX was recently identified via bioinformatic analyses (Paula *et al.* 2019). High concentrations of intracellular polyP are related to higher metal resistance in *Sulfolobales* species (Remonsellez, Orell and Jerez 2006). Also, a *Sa. solfataricus* strain lacking PolyP due to overexpression of PPX, exhibited less copper resistance compared with the wild-type strain (Soto *et al.* 2019). Moreover, a spontaneous mutant with a functional PitA transporter, called *M. sedula* CuR1, was more resistant to copper and arsenic than the wild-type strain that carries a truncated *pitA* (McCarthy *et al.* 2014). PitA and Pho84 transporters are related to the uptake of P_i, and the exportation of P_i-Cu outside the cell (Fig. 13). In *M. sedula*, Pho84-like transporters responded to the presence of Cu and are also proposed to be involve in P_i-Cu uptake (Rivero *et al.* 2018). There are two ways in which PolyP helps directly to decrease intracellular effective concentration of metals. Since PolyP has negative charges, metal cations are attracted and complexed in the surface of the granule (Fig. 13). On the other hand, the increase in metal concentration triggers PolyP degradation, and Cu associated with P_i is exported outside the cell by PitA or Pho84 transporters (Fig. 13) (Keasling 1997; Remonsellez, Orell and Jerez 2006; Orell *et al.* 2012; Grillo-Puertas *et al.* 2014).

As mentioned, PolyP plays many roles in archaea and bacteria related to stress response and protein aggregation, the latter as an inorganic chaperone (Gray and Jakob 2015). PolyP can also sequester metals, like Fe, preventing the Fenton reaction and generating more free radicals (Gray and Jakob 2015). Proteomic studies in the *Sa. solfataricus* PolyP (-) mutant revealed upregulation of stress-related proteins when compared with the wild-type strain (Soto *et al.* 2019). Proteins, like peroxidases, were upregulated, and other enzymes from various metabolic pathways exhibited changes that collectively reassembled metabolic oxidative stress response (Soto *et al.* 2019).

Metal sequestration and transformation

Besides sequestration by PolyP granules, there are other molecules, such as DNA-binding proteins from starved cells (Dps) (Fig. 13), that are proposed to bind to metals, thereby avoiding deleterious effects as seen in *Sa. solfataricus* (Wiedenheft *et al.* 2005). *Saccharolobus solfataricus* also contains a *mer* operon, required for Hg (II) reduction. The operon contains a regulator, MerR, that controls production of a mercuric reductase (MerA) along with other components (Table 2). MerR acts as both a repressor and a metal-responsive activator of mercury resistance genes by binding an operator sequence (*merO*) that also forms part of the operon (Schelert *et al.* 2004, 2006).

Metal resistance and bioleaching

The use of mesoacidophiles and thermoacidophiles in biomining applications, that have reduced environmental impact, has driven interest in understanding heavy metal resistance in these organisms (Martínez-Bussenius, Navarro and Jerez 2017) (see the section 'Potential and current uses of thermoacidophiles in biotechnological applications'). The relationship between metal resistance and lithoautotrophy was seen in *M. sedula* when *copA* was disrupted, which lowered copper resistance and consequently chalcopyrite bioleaching (Maezato *et al.* 2012). In contrast, a spontaneous mutant of *M. sedula*, strain CuR1, also showed increased bioleaching capability, associated with its higher Cu resistance (Maezato *et al.* 2012). These examples illustrate that understanding metal resistance can lead to new strategies for bioleaching, where the *Sulfolobales* show great potential (see the section 'Potential and current uses of thermoacidophiles in biotechnological applications').

METABOLISM

Heterotrophic metabolism

As mentioned in the section 'The diversity of thermoacidophilic life', microorganisms adapted to both high temperature and low pH, so called thermoacidophiles, have been identified mainly in the domain of Archaea and most of them belong to the two orders, the euryarchaeal *Thermoplasmatales*, including the genera *Thermoplasma*, *Picrophilus* and the crenarchaeal *Sulfolobales* with the genera *Caldivirga*, *Thermocladium*, *Acidianus*, *Desulfurolobus*, *Metallosphaera*, *Sulfolobus*, *Saccharolobus* and *Sulfurococcus* (Bertoldo, Dock and Antranikian 2004; Zaparty and Siebers 2011; Sakai and Kurosawa 2018). Most of these organisms were isolated from solfataric fields or hot springs and are able to grow heterotrophically, using a variety of substrates as carbon and energy sources. Some have been also described as facultative heterotrophs that are also able to grow autotrophically by sulfur, metal or hydrogen oxidation. Among these species, the *Crenarchaeota Sa. solfataricus* and *S. acidocaldarius* have been most extensively analyzed with respect to the heterotrophic lifestyle and, therefore, represent archaeal model organisms for metabolic network reconstruction.

Saccharolobus solfataricus shows high metabolic versatility and is able to utilize a broad spectrum of substrates, including monosaccharides (e.g. D-glucose, D-galactose, L-fucose, D-fructose, D/L-arabinose and D-xylose), disaccharides (e.g. cellobiose, maltose, sucrose, trehalose and lactose), oligo- and polysaccharides (e.g. β-glucans, starch and dextrin), amino acids (e.g. glutamate), peptides and proteinaceous substrates (e.g. tryptone), and alcohols including aromatics (e.g. ethanol, phenol) (Quehenberger *et al.* 2017; Schocke, Bräsen and Siebers 2019). In contrast, *S. acidocaldarius* has a much narrower substrate spectrum; this could be attributed to its relatively smaller genome, which lacks numerous transport systems for substrate uptake. The metabolism of *S. acidocaldarius* is limited to a smaller range of substrates that include D-glucose, L-arabinose, D-xylose, sucrose, maltotriose, dextrin, starch, wheat bran, several fatty acids, and peptides and amino acids (Grogan 1989; Wang *et al.* 2019c). Over the past several decades, the metabolic network, especially the central carbohydrate metabolism, in these two model organisms has been studied intensely, and is characterized by the presence of unusual and/or unique pathways and enzymes (Bräsen *et al.* 2014; Quehenberger *et al.* 2017). As such, we focus on these two model organisms to provide a prospective on heterotrophy in thermoacidophilic Archaea (see Fig. 14 for central metabolism overview).

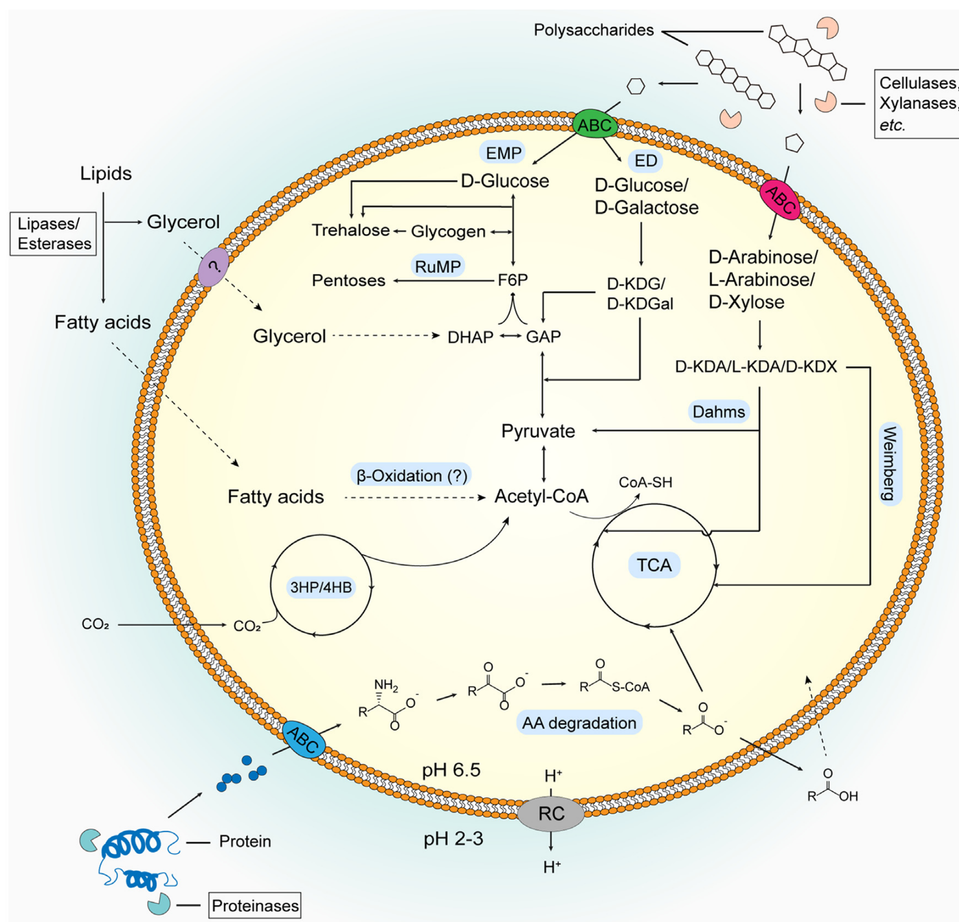


Figure 14. An overview of the central metabolism in *Sulfolobales*. Dashed arrows indicate pathways, which have not yet been experimentally demonstrated. Abbreviations: F6P, fructose 6-phosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; D-KDG, 2-keto-3-deoxy-D-gluconate; D-KDGal, 2-keto-3-deoxy-D-galactonate; D-KDA, 2-keto-3-deoxy-D-arabinoate; L-KDA, 2-keto-3-deoxy-L-arabinoate; D-KDX, 2-keto-3-deoxy-D-xylonate; AA, amino acid; ED, Entner–Doudoroff pathway; EMP, Embden–Meyerhof–Parnas pathway; RuMP, reversed ribulose monophosphate pathway; TCA, tricarboxylic acid cycle; 3HP/4HB, 3-hydroxyproprionate/4-hydroxybutyrate cycle; ABC, ATP-binding cassette transporters; RC, respiratory chain.

Carbohydrates metabolism

Sugar catabolism: The two model organisms, *Sa. solfataricus* and *S. acidocaldarius*, can grow on a range of monosaccharides, disaccharides and also polysaccharides (Table 4). A variety of polysaccharides-degrading enzymes have been studied in *Sa. solfataricus*, such as cellulase (Maurelli *et al.* 2008), glucanase (Limauro *et al.* 2001; Huang *et al.* 2005; Girfoglio, Rossi and Cannio 2012), glucoamylase (Kim *et al.* 2004), β-glycosidase (Noh and Oh 2009), α-glucosidase (Rolfsmeier *et al.* 1998; Wagner *et al.* 2014), β-glucosidase (Moracci *et al.* 1995; Shin *et al.* 2013), α-amylase (Haseltine, Rolfsmeier and Blum 1996; Wagner *et al.* 2014), xylanase (Cannio *et al.* 2004; Maurelli *et al.* 2008) and xylosidase (Moracci *et al.* 2000). The degradation products, i.e. mono-, di- and/or oligosaccharides, are taken up into the cytoplasm primarily by various ATP-binding cassette (ABC) transporters (Albers *et al.* 2004; Choi, Hwang and Cha 2013; Wagner *et al.* 2017). The further processing of the monosaccharides follows a general scheme: the sugars are first oxidized to the corresponding lactone by sugar dehydrogenases, which in most cases belong to the medium chain dehydrogenase/reductase (MDR) superfamily (Lamble *et al.* 2003; Brouns *et al.* 2006; Nunn *et al.* 2010; Haferkamp *et al.* 2011; Wolf *et al.* 2016; Reinhardt, Johnsen and Schönheit 2019), and in only some cases to the short chain dehydrogenase/reductase (SDR) superfamily (Yasutake *et al.* 2007; Kim, Paek and Lee 2012). The sugar lactones are then hydrolyzed either enzymatically via lactonases or spontaneously in a non-enzymatic reaction favored by the extremely thermophilic conditions. The resulting sugar acids are subsequently dehydrated to the key intermediates, the 2-keto-3-deoxy sugar acids. These reactions are catalyzed by dehydratases mainly from the enolase superfamily. The further degradation then varies with respect to phosphorylation and (aldol) cleavage. In the modified branched ED pathway, the 2-keto-3-deoxy sugar acids, 2-keto-3-deoxy gluconate (KDG) and 2-keto-3-deoxy galactonate (KDGal) are either first phosphorylated by KDG kinase to KDPG/KDPGal and then cleaved to glyceraldehyde 3-phosphate (GAP) and pyruvate in the semi-phosphorylative (sp) ED branch, or they are directly cleaved by the same aldolase to yield glyceraldehyde (GA) and pyruvate in the non-phosphorylative (np) ED branch (Ahmed *et al.* 2005; Lamble *et al.* 2005). GAP is then directly oxidized to 3-phosphoglycerate (3PG) by a non-phosphorylating GAP dehydrogenase (GAPN), without coupling the oxidation to ATP generation via substrate level phosphorylation. 3PG is then further converted to a second molecule of pyruvate through the reaction sequence of the common lower Embden–Meyerhof–Parnas (EMP) pathway. In the npED branch, GA is first oxidized in a ferredoxin-dependent manner to glycerate by GA:ferredoxin oxidoreductase and then phosphorylated to 2-phosphoglycerate

Table 4. An overview of the reported substrates and degradation pathways in *Sa. solfataricus* and *S. acidocaldarius*.

Substrate	Organism		Reference
Carbohydrates	Cellulose	*Sa. solfataricus*	(Girfoglio, Rossi and Cannio 2012)
	Xylan	*Sa. solfataricus*	(Cannio *et al.* 2004)
	Glucose	*Sa. solfataricus*, *S. acidocaldarius*	(De Rosa *et al.* 1984; Ahmed *et al.* 2005)
	Galactose	*Sa. solfataricus*	(Lamble *et al.* 2003)
	D-Arabinose	*Sa. solfataricus*	(Brouns *et al.* 2006)
	L-Arabinose	*Sa. solfataricus*, *S. acidocaldarius*	(Nunn *et al.* 2010; Wagner *et al.* 2017)
	D-Xylose	*Sa. solfataricus*, *S. acidocaldarius*	(Nunn *et al.* 2010; Wagner *et al.* 2017)
	L-Fucose	*Sa. solfataricus*	(Wolf *et al.* 2016)
Proteins/peptides/amino acids	Proteins/peptides	*Sa. solfataricus*, *S. acidocaldarius*	(Lin and Tang 1990; Gogliettino *et al.* 2014)
	Glutamate, methionine, leucine, phenylalanine, isoleucine, threonine, alanine, asparagine, glycine, tyrosine and serine	*Sa. solfataricus*	(Stark *et al.* 2017)
Lipids/fatty acids	Olive oil, corn oil, *p*-nitrophenyl (PNP)-butyrate, PNP-caprylate, PNP-palmitate	*Sa. solfataricus*	(Choi *et al.* 2016)
	Tributyrin, tricaproin	*S. acidocaldarius*	(Zweerink *et al.* 2017)
	Butyrate, hexanoate	*S. acidocaldarius*	(Wang *et al.* 2019c)
Other substrates	Ethanol	*Sa. solfataricus*	(Chong *et al.* 2007a)
	Phenol	*Sa. solfataricus*	(Izzo *et al.* 2005)

(2PG) by means of 2-phosphoglycerate kinase. 2PG again enters the lower common shunt of the EMP. The 2-keto-3-deoxy-L-fuconate derived from the 6-deoxy hexose L-fucose is cleaved by the same aldolase to lactaldehyde, and pyruvate and the lactaldehyde are further oxidized in two consecutive steps to lactate and finally to a second molecule of pyruvate (Wolf *et al.* 2016). Also, pentose degradation initially follows the same reaction sequence of oxidation and dehydration, finally leading to the corresponding 2-keto-3-deoxy acids, which are then further processed either in an aldolase-dependent manner, called the Dahms pathway, or in an aldolase-independent manner, referred to as the Weimberg pathway (Nunn *et al.* 2010). The aldol cleavage in the Dahms pathway yields pyruvate and glycolaldehyde, which is then oxidized in two steps to glyoxylate, converted with acetyl-CoA to malate via malate synthase, and finally enters the tricarboxylic acid (TCA) cycle as malate. In the Weimberg pathway, the 2-keto-3-deoxy pentanoates are converted via a second dehydration catalyzed by the 2-keto-3-deoxy xylonate dehydratase (KDXD) to 2-ketoglutarate semialdehyde (KGSA) and then oxidized through KGSA dehydrogenase (KGSADH) to 2-ketoglutarate entering the TCA cycle (Nunn *et al.* 2010; Wagner *et al.* 2017). While D-arabinose is only used as carbon source in *Sa. solfataricus* and degraded via the Weimberg pathway (Brouns *et al.* 2006), L-arabinose and D-xylose are converted by both pathways to the same extent in *Sa. solfataricus* and *S. acidocaldarius* (Nunn *et al.* 2010). However, deletion mutant analyses in *S. acidocaldarius* MW001 demonstrated that the Dahms pathway is dispensable, whereas the Weimberg pathway is essential for D-xylose degradation (Wagner *et al.* 2017). In contrast to the other hexoses, another 6-deoxy sugar, i.e. L-rhamnose, was also proposed to be degraded in an aldolase-independent manner via the so called 2,4-di-keto pathway. The 2-keto-3-deoxy rhamnoate generated by the common initial reactions is then oxidized at C4 to 2,4-keto-3-deoxy-rhamnoate, which is cleaved by a hydrolase to yield lactate and pyruvate (Reinhardt, Johnsen and Schönheit 2019). The lactate is then subsequently oxidized to a second molecule of pyruvate.

An interesting feature of the sugar degradation routes in the *Sulfolobales* is the pronounced substrate promiscuity of the enzymes involved, especially in the upper part of the pathways catalyzing analogous reactions. This has first been described by Danson and co-workers, who found that the glucose dehydrogenase, the gluconate dehydratase and the KD(P)G aldolase from *Sa. solfataricus* also accept the D-galactose derivatives as substrates (Lamble *et al.* 2003, 2004, 2005). Subsequently, it was shown that the same sugar dehydrogenase is also responsible for the oxidation of the pentoses D-xylose and L-arabinose (Nunn *et al.* 2010). However, D-arabinose and L-fucose demonstrated the opposite stereochemistry on C2–C4 compared with L-arabinose oxidized by another dehydrogenase, which also accepted L-rhamnose as a substrate (Brouns *et al.* 2006; Wolf *et al.* 2016; Reinhardt, Johnsen and Schönheit 2019). Interestingly, the dehydratase's conversion of L-rhamnonate is different from the conversion of D-arabonate and L-fuconate, indicating that the promiscuity of the dehydratases is less pronounced than that of the dehydrogenases (Reinhardt, Johnsen and Schönheit 2019). This has also been proposed for the gluconate/galactonate dehydratase, which presumably does not accept the pentose derivatives as substrates suggesting the presence of an alternative dehydratase, although this has not been confirmed (Nunn *et al.* 2010). The KD(P)G aldolase, however, shows by far the most marked promiscuity, accepting 2-keto-3-deoxy acids derived from hexoses and pentoses, i.e. D-glucose, D-galactose, D-xylose, L-arabinose, D-arabinose and L-fucose, as well as the phospho-

rylated derivatives KDPG and KDPGal (Ahmed *et al.* 2005; Lamble *et al.* 2005; Nunn *et al.* 2010; Wolf *et al.* 2016). Thus, this enzyme plays a central role in sugar degradation via the branched ED pathway, comprising the npED and spED branch, as well as the Dahms pathway, both of which are involved in the breakdown of a wide variety of naturally occurring sugars in *Sulfolobales*.

The end products of the glycolytic pathways are pyruvate and the TCA cycle intermediates, α-ketoglutarate and malate. As in all Archaea, the pyruvate produced is then oxidatively decarboxylated to acetyl-CoA by ferredoxin oxidoreductase (Kerscher, Nowitzki and Oesterhelt 1982; Zhang *et al.* 1996; Yan *et al.* 2016). The resulting acetyl-CoA is then completely oxidized to CO_2 in the TCA cycle (Danson 1988). Also, the oxidative decarboxylation of α-ketoglutarate is carried out by a ferredoxin-dependent oxidoreductase. $NAD(P)^+$-dependent dehydrogenase complexes (i.e. pyruvate dehydrogenase complex), known from bacteria and eukaryotes, are not operative in the *Sulfolobales* or, for that matter, Archaea in general (Payne, Hough and Danson 2010).

Sugar anabolism: Sugar degradation in aerobic Archaea, including the *Sulfolobales*, proceeds via a modified branched ED pathway and is, at least in some ways, analogous to the Dahms and/or Weimberg pathways. Notably, for a complete EMP pathway, only a functional phosphofructokinase (PFK) is missing, which highlights the exclusively gluconeogenic function of the EMP in the *Sulfolobales* (Bräsen *et al.* 2014). In addition to the common lower shunt enzymes, gluconeogenesis is initiated by the phosphoenolpyruvate synthetase, bypassing the irreversible pyruvate kinase (PK) reaction (Haferkamp *et al.* 2019), and also involves the classical glyceraldehyde-3-phosphate (GAPDH)/phosphoglycerate kinase (PGK) couple, which bypasses the irreversible GAPN (Kouril *et al.* 2013b). Perhaps, the most striking difference to classical gluconeogenesis is the presence of the bifunctional fructose-bisphosphate aldolase/phosphatase (FBPA/ase), characterized among others from the *Sulfolobales M. sedula* and *Sa. solfataricus*, replacing the classical fructose-1,6-bisphosphate aldolase and F1,6BP phosphatase couple of the gluconeogenesis route in Bacteria and Eukarya (Say and Fuchs 2010). This enzyme catalyzes the conversion of GAP and DHAP to F6P without liberating the F1,6BP intermediate.

All of these modifications, both in sugar catabolism and anabolism, have been discussed as a mechanism of metabolic thermoadaptation, since the formation of extremely thermolabile triose phosphates, e.g. GAP, DHAP and 1,3BPG, is avoided (Say and Fuchs 2010; Kouril *et al.* 2013b; Figueiredo *et al.* 2017; Zhang *et al.* 2017). Also, the altered regulatory properties of the pathways and enzymes could contribute to thermoadaptation, since the classical control points, e.g. HK, PFK and PK, are missing or changed in Archaea, including in the *Sulfolobales*. For example, the main glycolytic control point is the GAPN, instead of the sugar(phosphate) kinases being activated by glucose 1-phosphate (Ettema *et al.* 2008; Kouril *et al.* 2017). Also, the PKs so far characterized, including those from *Sulfolobus* spp., show divergent regulation compared with the enzymes from the classical bacterial and eukaryotic pathways (Haferkamp *et al.* 2019; Johnsen *et al.* 2019).

The phosphoglucose isomerase (PGI) interconverting F6P and G6P and the phosphoglucomutase (PGM) present in the *Sulfolobales* represent the branch point to the synthesis of the storage compound glycogen and trehalose as the only compatible solutes in *Sulfolobus* spp. described so far (Martins *et al.* 1997; Bräsen *et al.* 2014). The additional presence of a hexokinase suggests that these biosynthesis reactions can also be directly initiated from the substrate molecule glucose. However, the spED branch, particularly the KDG kinase as its key enzyme, may be involved in gluconeogenesis by providing triose phosphates for anabolic purposes by directing the flux from the upper branched ED to the hexose phosphate synthesis (Kouril *et al.* 2013a).

The pentose metabolism in the *Sulfolobales* and *Thermoplasmatales* are also characterized by a missing oxidative pentose phosphate pathway (OPPP). Furthermore, the non-oxidative pentose phosphate pathway (NOPPP) is only partially present in the *Sulfolobales* lacking a ribulose-5-phosphate epimerase and a transaldolase, but is entirely present in the *Thermoplasmatales* (*Thermoplasma* and *Picrophilus*) (Bräsen *et al.* 2014). However, the pentose precursor ribulose-5-phosphate, as in most Archaea, is provided by the ribulose monophosphate (RuMP) pathway converting F6P to Ru5P and formaldehyde via D-arabinohexulose 6-phosphate. Ribose 5-phosphate (R5P) and erythrose 4-phosphate (E4P) are precursors of nucleotide and aromatic amino acid biosynthesis, respectively, and are then provided by the remaining reaction of the NOPPP. Thus, F6P is the main source of pentose/tetrose phosphates in most Archaea in general and, particularly, thermoacidophiles (Bräsen *et al.* 2014).

Degradation of proteins and amino acids

Although thermoacidophilic Archaea are routinely grown on proteinaceous substrates, surprisingly little is known about the breakdown of amino acids in these organisms. It appears that certain *Sulfolobales* species grow well on these complex protein substrates, such as yeast extract, casein hydrolysate, N-Z-Amine, and on mixtures of amino acids, but hardly if at all on single amino acids as a sole carbon, energy and nitrogen source (Grogan 1989; Stark *et al.* 2017; Quehenberger *et al.* 2019). The reason for this remains unclear so far. However, *Sa. solfataricus*, when growing on casein hydrolysate, prefers certain amino acids like glutamate, methionine, leucine, phenylalanine and isoleucine and, to a lesser extent, threonine, alanine, aspartate, glycine and tyrosine (Stark *et al.* 2017). Growth of *Sa. solfataricus* on glucose was stimulated most by glutamate and, to a lesser extent, by aspartate (Stark *et al.* 2017). Additionally, growth of *S. acidocaldarius* on glucose is enhanced by glutamate, followed by aspartate, arginine and lysine (Quehenberger *et al.* 2019). For the breakdown of (poly)peptides though, many proteinase and peptidase encoding genes are present in the genomes of the *Sulfolobales*, but few have been studied in detail (Cannio *et al.* 2010; Gogliettino *et al.* 2010). Additionally, ABC transporters for di/oligopeptides have been identified in *Sa. solfataricus*, *Sulf. tokodaii* and *T. acidophilum* (Albers *et al.* 2004).

There are generally three mechanisms known by which amino acid degradation is initiated: decarboxylation, transamination and (oxidative) deamination. Decarboxylation leads to biogenic amines and there are some reports in *Sa. solfataricus* for amino acid decarboxylases, e.g. arginine decarboxylase, which might play a role in the biosynthesis of spermidine and putrescine (Giles and Graham 2008; Esser *et al.* 2013). However, the more common mechanism for amino acid breakdown in Archaea, which was intensively studied for *Thermococcales*, is the transamination with 2-oxoacids, mainly alpha-ketoglutarate, and/or the oxidative deamination using amino acid dehydrogenases, most importantly the glutamate dehydrogenase liberating ammonia and concomitantly reducing $NAD(P)^+$ (Yokooji *et al.* 2013; Awano *et al.* 2014; Scott, Poole and Adams 2014). Both mechanisms ultimately lead to the formation of the corresponding 2-keto acids that are subsequently oxidatively decarboxylated by ferredoxin-dependent 2-oxoacid:Fd oxidoreductases, which are also well known and characterized from *Sulfolobales* species, *Sulf. tokodaii* in particular (Kerscher, Nowitzki

and Oesterhelt 1982; Zhang *et al.* 1996; Park *et al.* 2006; Yan *et al.* 2016). The reaction products are the corresponding CoA esters of the carboxylate backbone of the amino acids. In aerobes, these CoA esters are then completely oxidized to CO_2 by channeling these compounds into the central energy metabolic pathways via pyruvate, PEP, acetyl-CoA and the TCA cycle intermediates. These pathways have also been identified in *Sa. solfataricus* by *in silico* metabolic reconstructions (Ulas *et al.* 2012). In anaerobic archaea, most well studied in *Thermococcales*, the CoA esters play pivotal roles in fermentative energy generation by being the sole source of net ATP via substrate level phosphorylation. The key enzymes are the ADP-forming acyl-CoA synthetases coupling the CoA ester hydrolysis to the ATP formation from ADP and P_i (Awano *et al.* 2014; Scott, Poole and Adams 2014; Weiße *et al.* 2016). However, these ADP-forming acyl-CoA synthetases are also present in aerobic archaea, including the *Sulfolobales*. Specifically, during *Sa. solfataricus* growth on casein hydrolysate carboxylic acids, mainly isovalerate, are excreted into the medium (Stark *et al.* 2017). Although such product formation appears unusual under aerobic conditions, it is well documented in bacterial model organisms, like *E. coli* and *Bacillus subtilis* (Bräsen and Schönheit 2004), and this so-called 'overflow metabolism' has also been observed in halophilic archaea (Bräsen and Schönheit 2004). However, under acidophilic conditions, carboxylic acids at elevated concentrations act as uncouplers/protonophores that lead to the acidification of the cytosol and the breakdown of membrane gradients (Baker-Austin and Dopson 2007). Moreover, the excretion of products results in the loss of carbon, which may account for the less efficient growth of *Sa. solfataricus* on casein hydrolysate compared with glucose. Furthermore, in *Sa. solfataricus* pyroglutamate may form from glutamate at high temperature and low pH to inhibit growth of thermophilic archaea (Stark *et al.* 2017). However, in *S. acidocaldarius*, pyroglutamate is not inhibitory and even serves as a carbon source, making *S. acidocaldarius* a better thermoacidophilic platform organism for applications with glutamate-containing media (Vetter *et al.* 2019). Finally, it has not been determined whether the carboxylic acids formed during exponential growth can be re-used by *Sulfolobus* species, as it has been shown for other aerobic organisms.

Degradation of lipids and fatty acids

In general, the mechanisms for fatty acid and, fatty acid-based, (phospho)lipid metabolism are not well understood in Archaea. However, *Sa. solfataricus* P1 can (partially) degrade corn oil as well as olive oil (Choi *et al.* 2016), and *S. acidocaldarius* can cleave triacylglycerols (Zweerink *et al.* 2017). Moreover, several extracellular esterases/lipases have been identified from *Sa. solfataricus*, *S. acidocaldarius* and *Sulf. tokodaii* (Suzuki, Miyamoto and Ohta 2004; Choi *et al.* 2016; Zweerink *et al.* 2017). A TetR-family transcription factor ($FadR_{Sa}$) plays a role in regulation of putative fatty acid metabolism-related genes in *S. acidocaldarius* (Table 2) and growth of this organism on short-chain fatty acids, i.e. butyrate and hexanoate, as sole carbon sources has been demonstrated (Wang *et al.* 2019c).

Genomic analyses revealed that all the genes encoding homologs of the key enzymes involved in the bacterial-like β-oxidation are present in the genomes of several Archaea, including *Sa. solfataricus* and *S. acidocaldarius* (Dibrova, Galperin and Mulkidjanian 2014; Wang *et al.* 2019c), implying that fatty acids could be degraded in these organisms through classical β-oxidation, but it remains unproven. Nonetheless, *S. acidocaldarius* can grow on acetate (as also described for many other Archaea including methanogens, *Pyrobaculum*, and halophilic Archaea) as the sole carbon and energy source. The glyoxylate shunt is operative under these conditions, demonstrating that C2 units can be assimilated by this reaction sequence (Uhrigshardt *et al.* 2002).

As a product of lipid hydrolysis, glycerol can be utilized as a carbon and energy source by many bacteria. In Archaea, glycerol degradation has been examined in halophiles (Sherwood, Cano and Maupin-Furlow 2009; Rawls, Martin and Maupin-Furlow 2011; Williams *et al.* 2017). Glycerol is taken up either by simple diffusion or glycerol transporters (Richey and Lin 1972; Stroud *et al.* 2003; Anderson *et al.* 2011). The haloarchaea employ one of the bacterial-like mechanisms, first phosphorylating glycerol followed by sn-glycerol-3-phosphate oxidation to DHAP. So far, glycerol catabolism has not been studied in thermoacidophilic archaea, although there are some indications that *Sulfolobales* spp. do not utilize glycerol as carbon and energy source (Grogan 1989). In contrast, genome-scale metabolic network reconstruction and modeling suggested glycerol as the most efficient carbon source for *Sa. solfataricus* (Ulas *et al.* 2012).

Degradation of other substrates

In addition to the three major types of nutrients described above, other substrates also support heterotrophic growth of thermoacidophilic archaea. For instance, *Sa. solfataricus* grows on acetoin, citric acid, alcohols and phenol (Izzo *et al.* 2005; Chong *et al.* 2007a; Wolf *et al.* 2016). There are several alcohol dehydrogenases in *Sa. solfataricus*, allowing oxidation of alcohols into aldehydes. The second step is then the oxidation to the corresponding carboxylic acids and subsequent activation of the CoA esters for further degradation (Chong *et al.* 2007a,b). Phenol is degraded in *Sa. solfataricus* in a classic pathway, as reported in some bacteria, e.g. *Burkholderia pickettii* and *Pseudomonas stutzeri* OX1. It is first converted to catechol, which undergoes a series of ring cleavage reactions producing products that finally enter TCA cycle (Izzo *et al.* 2005).

In conclusion, thermoacidophilic (facultative) heterotrophs, especially from the *Sulfolobales*, utilize a variety of substrates for cell growth (reported substrates with degradation pathways are concluded in Table 4). This metabolic versatility includes the potential for autotrophic growth, combined with the thermoacidophilic lifestyle, making them ideal candidates for the development as platform strains for the production of added-value compounds from renewable (waste) materials like lignocellulosics (see the section 'Potential and current uses of thermoacidophiles in biotechnological applications').

Autotrophy and chemolithotrophy

Autotrophy

The inhospitable environments in which the *Sulfolobales* thrive often have a scarcity of organic carbon available. As a result, many of the species within this order rely on the autotrophic fixation of CO_2 to support growth. At present, six mechanisms for CO_2 fixation are known throughout the domains of life. Several of these mechanisms build carbon–carbon bonds by fixing CO_2 using oxygen-sensitive carboxylases, or in the case of the Calvin–Bassham–Benson cycle, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) (Hugler *et al.* 2003). While RuBisCo is not oxygen sensitive, it does have a highly detrimental side reaction with oxygen, and the prevalence of oxygen-reducing terminal oxidases throughout the *Sulfolobales* (anaerobes included) seems to indicate that oxygen is essential for the organisms' ability to survive their extreme conditions. The autotrophic pathway in the *Sulfolobales* circumvents the

dependence on RuBisCo by incorporating bicarbonate molecules rather than CO_2 (Gong *et al.* 2019). This pathway, named the 3-hydropropionate/4-hydroxybutyrate (3-HP/4-HB) cycle after two of its prominent intermediates, generates one acetyl-CoA per cycle (Berg *et al.* 2007). Initially identified in *M. sedula*, the 3-HP/4-HB pathway shares the enzymatic route from succinyl-CoA to acetyl-CoA with the dicarboxylate/4-hydroxybutyrate autotrophy cycle. However, the generation of succinyl-CoA eschews the traditional ferredoxin-powered pyruvate synthase and instead relies on the sequential addition of bicarbonate molecules and coenzyme-A moieties (Fuchs 2011). One full rotation of the 3-HP/4-HB cycle costs the cell 4 ATP and 4 NADPH, making it only a moderately expensive route to carbon fixation (Gong *et al.* 2019).

Since the discovery of the 3-HP/4-HB pathway, the enzymes responsible for each step in the pathway have been identified and characterized (Kockelkorn and Fuchs 2009; Teufel *et al.* 2009; Ramos-Vera *et al.* 2011). *Metallosphaera sedula* has served as the model organism for many of these efforts, and part of the pathway characterization has involved linking the enzymes of the 3-HP/4-HB pathway to open reading frames within *M. sedula*. This has proved to be challenging, considering the promiscuity of enzymes causing overlapping catalytic activities. In the case of 4-hydroxybutyrate-CoA synthetase, five candidate genes were identified based on bioinformatic analysis. However, three of these genes showed no activity for the desired reaction (Ramos-Vera *et al.* 2011), while the remaining two did demonstrate this activity (albeit with orders of magnitude of difference in V_{max} values). Further investigation into these two candidates determined that one was merely a promiscuous acyl-CoA synthetase rather than the genuine 4-hydroxybutyrate-CoA synthetase (Hawkins *et al.* 2013).

As greater understanding of the precise enzymatic path of the 3-HP/4-HB cycle has developed, modeling of the pathway has become a point of interest. This effort has been greatly aided by the quantification of thermodynamic and kinetic parameters associated with each step in the pathway (Ramos-Vera *et al.* 2011; Loder *et al.* 2016). A kinetic model of the system in *M. sedula* revealed differing degrees of rotations the cycle can undergo, resulting in a different distribution of products including acetyl-CoA and succinyl-CoA (Loder *et al.* 2016). This modeling supports previous exploration into the channeling of carbon into various biosynthetic pathways from the 3-HP/4-HB cycle. Based on this isotope distribution, it was determined that the majority of CO_2 taken up during autotrophy generates succinyl-CoA, representing a half-turn or one-and-a-half turns of the full cycle. Acetyl-CoA (one full turn) is generated during this process, but only enough for amino acids directly synthesized from acetyl-CoA. Otherwise, succinyl-CoA dominates as the product of this cycle (Estelmann *et al.* 2011). Extension of 3-HP/4-HB cycle modeling was done to identify the effect of the pathway on carbon isotopes and demonstrated that the source of carbon for the cycle may not be extracellular bicarbonate. Instead, bicarbonate is formed intracellularly as CO_2 is taken up by the cell (Pearson *et al.* 2019).

While this autotrophic cycle requires 16 steps to generate acetyl-CoA from two bicarbonate molecules, only 13 enzymes are involved in the pathway. This inconsistency points toward the unusual redundancy in enzyme function throughout this pathway and it manifests in a variety of ways (Fig. 15). For example, the two-step conversion of crotonyl-CoA to acetoacetyl-CoA is catalyzed by the crotonyl-CoA hydratase. The first step of this conversion, however, can also be catalyzed by 3-hydroxypropionyl-CoA dehydratase, which also serves a separate function of dehydrating 3-hydroxypropionyl-CoA into acryloyl-CoA. Other redundancies include the reduction of malonyl-CoA and succinyl-CoA by the aptly named malonyl-CoA/succinyl-CoA reductase and the catalysis of hydroxypropionate to 3-hydroxypropionyl-CoA by either 3-hydroxypropionyl-CoA synthetase or the promiscuous 4-hydroxybutyryl-CoA synthetase (Loder *et al.* 2016). Notably, these overlaps in function reflect the structural similarity in the 3-hydroxypropionate half of the pathway and the 4-hydroxybutyrate half of the pathway. In fact, the two halves can almost be viewed as the same sequence of reactions, with enzymes acting on the same functional groups and the substrates varying only in length of the carbon chain.

The 3-HP/4-HB cycle is highly conserved within the *Sulfolobales*. In fact, with the exception of *Sulfo. acidophilus*, all genome-sequenced members of the order possess homologs to the characterized enzymes from *M. sedula* (Counts, Willard and Kelly 2020). In spite of this conservation, not all *Sulfolobales* appear to be capable of carbon fixation. A prime example of this is *S. acidocaldarius*, which at the time of its isolation was reported to grow chemolithoautotrophically in the presence of elemental sulfur (Brock *et al.* 1972). More recently, it appears that the commonly used lab strain, *S. acidocaldarius* DSM 639, is a strict heterotroph (Zeldes *et al.* 2019). This example may be explained through an incomplete transcriptional regulation pathway. Recently, a conserved transcriptional regulator, HhcR, was proposed to be a universal autotrophy regulator in the *Sulfolobales* (Leyn *et al.* 2015) (Table 2). Several genes involved in the 3-HP/4-HB cycle appear to be lacking the binding motif for this regulator in *S. acidocaldarius* (Zeldes *et al.* 2019). Whether this lack of promoter-binding regions is a result of strain domestication by extensive heterotrophic growth in the lab remains to be determined. Further complicating the network of autotrophy in the *Sulfolobales* is the existence of all genes necessary for the dicarboxylate/4-hydroxybutyrate in several chemolithoautotrophs, including *M. sedula*. However, these genes are transcribed at a low level even during autotrophic growth conditions (Berg *et al.* 2010b). One area of interest for this pathway is its application in engineered organisms. A partial pathway was engineered into *E. coli* to generate both 3-hydroxypropionate and 4-hydroxybutyrate as a means of generating a block copolymer product (Meng *et al.* 2012). Parts of the cycle from *M. sedula* have been introduced into *Pyrococcus furiosus* in an effort to convert CO_2 into 3-hydroxypropionic acid (Keller *et al.* 2013). Similarly, *E. coli* has again been host to 3-HP/4-HB enzymes in order to produce propionic acid and acrylic acid (Liu and Liu 2016).

Chemolithotrophy at the cell surface

In order to power an autotrophic lifestyle, members of the *Sulfolobales* tend to rely on metals and inorganic compounds that prevail in their primordial environments. The oxidation of ferrous iron (Fe^{2+}) is one such source of energy. Despite a very positive reduction potential (+0.77 V for Fe^{3+}/Fe^{2+}), chemolithoautotrophs are still able to leverage iron oxidation to drive the electron transport chain (ETC) (Amend and Shock 2001). While this pathway has been studied most intensely in mesoacidophiles like *Acidithiobacillus ferrooxidans*, transcriptomic studies of several *Sulfolobales* members have shed light on enzymes responsible for thermoacidophilic iron oxidation (Bathe and Norris 2007; Auernik and Kelly 2008; Kozubal *et al.* 2011). *Metallosphaera sedula* is a prolific iron oxidizer and has served as the organism of study to further elucidate the mechanism for iron oxidation. These results indicate control of iron oxidation by a locus known as the *fox* cluster. The cluster appears to be membrane-bound, with

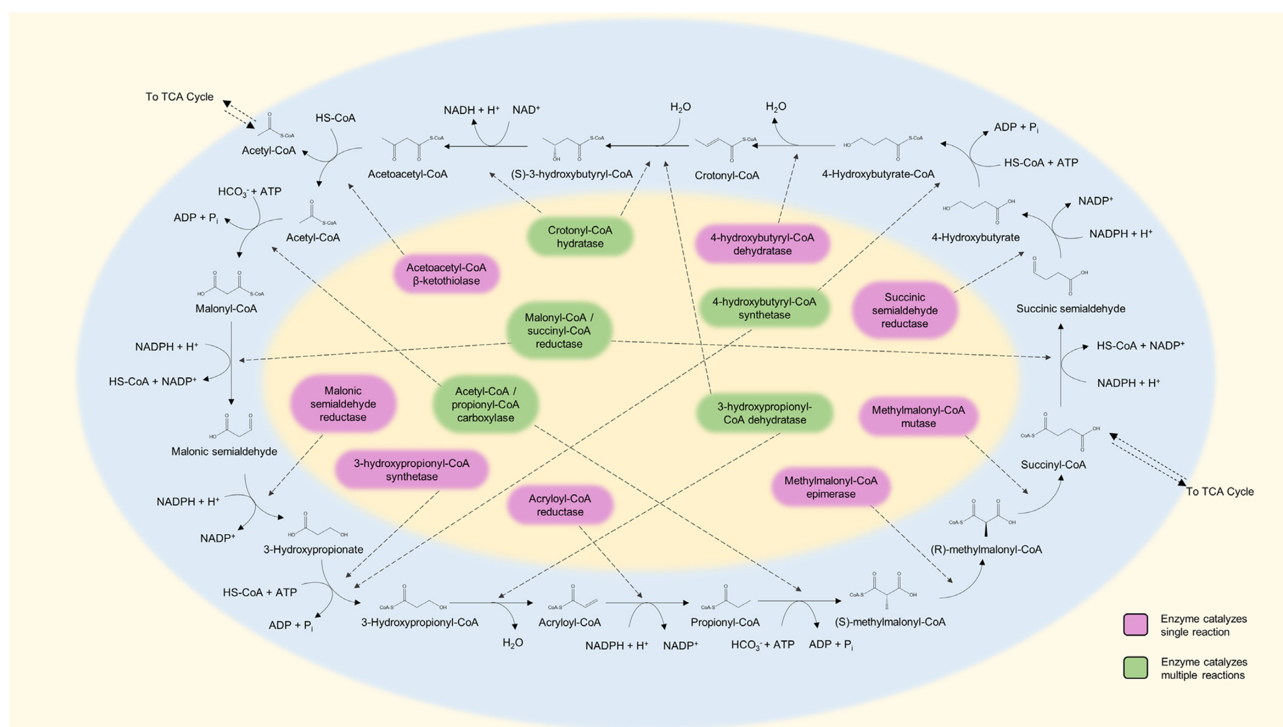


Figure 15. The enzymatic pathway of the 3-hydroxypropionate/4-hydroxybutyrate (3-HP/4-HB) cycle as characterized in *Metallosphaera sedula*. Enzyme names are contained within the yellow oval with arrows indicating reactions for which they have known catalytic activity. Enzymes in pink have shown activity for only a single reaction in the 3-HP/4-HB cycle; enzymes in green exhibit activity on multiple steps in the cycle.

iron oxidation taking place at the surface of the cell membrane and funneling electrons into the ETC.

A variety of sulfur species also persist in the *Sulfolobales*' habitats. Anaerobic *Sulfolobales* reduce zero-valent sulfur to hydrogen sulfide (H_2S) in conjunction with oxidizing diatomic hydrogen. Much like iron oxidation, sulfur reduction takes place entirely at the surface of the cell membrane, where a membrane-bound sulfur reductase acts on zero-valent sulfur (Fig. 16A) (Laska, Lottspeich and Kletzin 2003). The complex achieves this reduction by transferring electrons from reduced quinols (specifically *Sulfolobus* quinol). Quinone cycling links sulfur reduction to hydrogen oxidation, and it both regenerates the oxidized quinone pool and powers proton export (Kletzin *et al.* 2004). The dominant form of zero-valent sulfur under acidic conditions is cyclooctasulfur, but experimental evidence suggests sulfane sulfur from polysulfide chains may be the substrate for sulfur reductase (Blumentals *et al.* 1990).

Chemolithotrophy within the cytoplasm

In contrast to iron oxidation and sulfur reduction, sulfur oxidation takes place largely in the cytoplasm, where it cycles through a number of different reduced inorganic compounds (RISCs) (Fig. 16B). The premier enzyme of sulfur oxidation in the *Sulfolobales* is the cytoplasmic sulfur oxygenase reductase (SOR). The 24-subunit homomeric enzyme disproportionates zero-valent sulfur into H_2S and sulfite (SO_3^{2-}) without the assistance of any cofactors (Kletzin 1989). An abiotic reaction of these two products generates thiosulfate ($S_2O_3^{2-}$) as a by-product (Kletzin 1992). These species are coupled to the ETC through a variety of membrane-bound oxidoreductases: sulfide:quinone oxidoreductase (SQR) for H_2S (Brito *et al.* 2009), thiosulfate:quinone oxidoreductase (TQO) for $S_2O_3^{2-}$ (Müller *et al.* 2004) and sulfite:acceptor oxidoreductase (SAOR) for SO_3^{2-} (Zimmermann, Laska and Kletzin 1999). In the case of SAOR, activity of the enzyme has been detected in *Acidianus ambivalens*, but the enzyme has not been linked to an open reading frame in any of the *Sulfolobales*. Caldariellaquinone is the primary acceptor of electrons for these oxidoreductases.

While the product of SQR is a polysulfide chain that can be recycled to SOR and the product of SAOR is fully oxidized sulfate (SO_4^{2-}), TQO generates tetrathionate ($S_4O_6^{2-}$) as a product (Müller *et al.* 2004). Recent studies have investigated the possibility of tetrathionate acting as the substrate for a set of highly conserved genes in the *Sulfolobales*, the *hdr/dsr/tusA* locus. DsrE3A and TusA both appear to be sulfur-trafficking proteins, which cleave the sulfur–sulfur bond of $S_4O_6^{2-}$ to regenerate $S_2O_3^{2-}$ and form an organic persulfide compound. The persulfide ultimately acts as the substrate for the membrane-bound heterodisulfide reductase (Hdr) complex, which generate SO_4^{2-} (Liu *et al.* 2014b). A tentative role of the Hdr complex is once again the reduction of quinones to conserve energy. However, recent studies in the dimethyl sulfide (DMS)-degrading *Hyphomicrobium denitrificans* have demonstrated the association of a lipoate-binding protein with the Hdr complex and the importance of the *hdr/dsr/tusA* locus for energy conservation from DMS (Koch and Dahl 2018). Homologs of these binding proteins have been identified in some *Sulfolobales*, and the reduction potential of lipoate is sufficient to reduce NAD^+, thereby conserving energy (Cao *et al.* 2018b). While the exact acceptor molecule of Hdr has yet to be confirmed in the *Sulfolobales*, it seems clear that the complex and its traffickers play some role in conserving energy from tetrathionate. In addition, the complex provides a route to total oxidation of $S_2O_3^{2-}$ to SO_4^{2-} and may serve to detoxify the abiotic by-product of SOR.

A final avenue to energy conservation is the phosphorylating pathway of cytoplasmic SO_3^{2-} oxidation. In this pathway sulfite is attached to AMP by adenyl-5′-phosphosulfate reductase (APSR) using an unknown electron acceptor. Adenylylsulfate:phosphate adenylyltransferase (APAT) then replaces

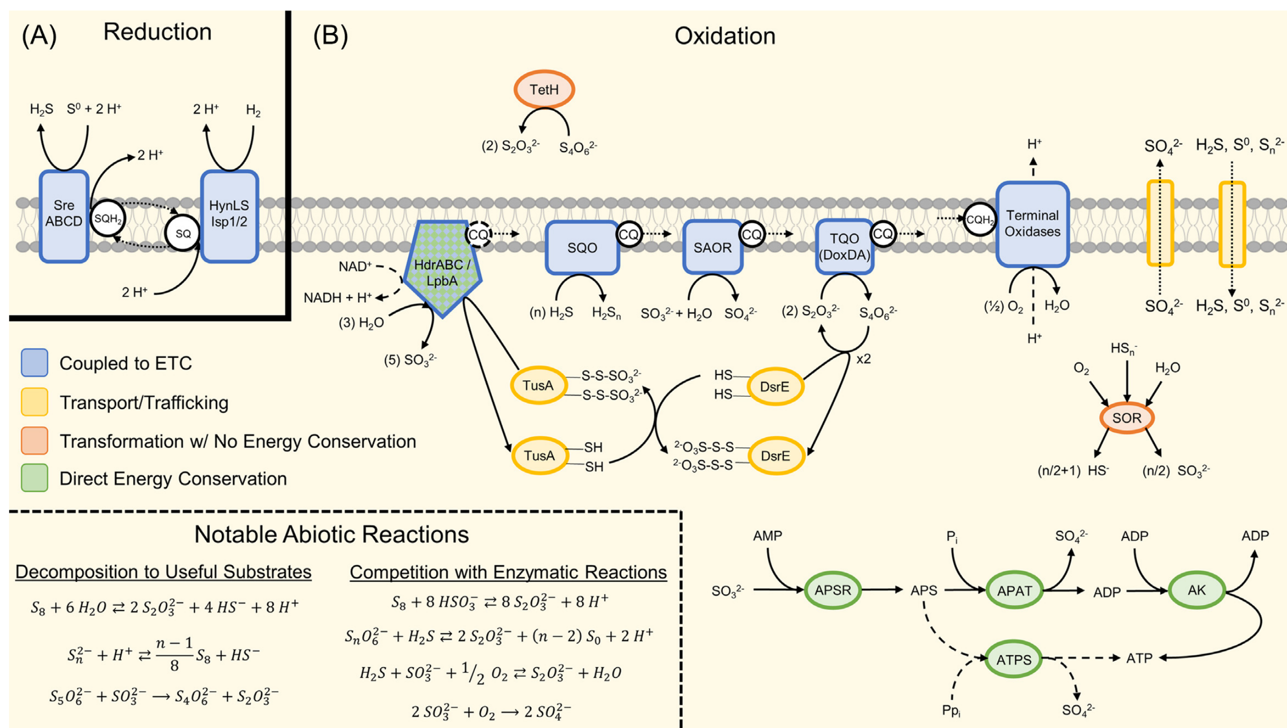


Figure 16. Current knowledge of the mechanism of sulfur oxidation and reduction in the *Sulfolobales*. **(A)** Sulfur reduction and **(B)** sulfur oxidation. Solid arrows indicate involvement in a reaction; dotted arrows represent transport of species; and dashed lines indicate that the function is suspected but has not been demonstrated experimentally in the *Sulfolobales*. Enzyme colors indicate general grouping of function: coupled to electron transport chain (blue), involved in transporting or trafficking sulfur species (yellow), transformation of sulfur species with no energy conservation (orange) and transformation of sulfur species directly coupled to energy-conserving biomolecules (green). Abbreviations: sulfur reductase (Sre), hydrogenase (Hyn), heterodisulfide reductase (Hdr), tetrathionate hydrolase (TetH), sulfide:quinone oxidoreductase (SQO), sulfite:acceptor oxidoreductase (SAOR), thiosulfate:quinone oxidoreductase (TQO), sulfur oxygenase reductase (SOR), adenosine-5′-phosphosulfate reductase (APSR), adenosine-5′-phosphosulfate (APS), adenylylsulfate:phosphate adenylyltransferase (APAT), ATP sulfurylase (ATPS), adenylate kinase (AK).

the sulfite group with phosphate to generate ADP. Finally, two molecules of ADP are converted to ATP and AMP by adenylate kinase (AK), thereby generating ATP directly from SO_3^{2-} oxidation (Kappler and Dahl 2001). Much like SAOR, activity of these enzymes has been detected in *A. ambivalens* cell extracts, but the enzymes have not been purified, characterized or linked to a sequence in the genome (Zimmermann, Laska and Kletzin 1999).

Sulfur transport into the cytoplasm remains something of a mystery in the *Sulfolobales*. No transporter for zero-valent sulfur has been identified yet in any organism from this order, although a possible sulfate transporter in *Metallosphaera cuprina* has been identified in transcriptomic data (Jiang *et al.* 2014). Evidence has been presented, however, for the passive diffusion of H_2S through the membrane (Mathai *et al.* 2009). In addition, an extracellular tetrathionate hydrolase is expressed in the *Acidianus* spp., many of which are prolific sulfur oxidizers (Protze *et al.* 2011). The reaction generates thiosulfate extracellularly, which may undergo abiotic reactions in the acidic environment to generate the appropriate sulfur species for transport across the membrane. Given the wide distribution of sulfur substrates used within the cytoplasm, it is difficult to say what RISC might act as the 'starting point' of sulfur oxidation, and understanding sulfur transport is still a key area of investigation for chemolithotrophy in the *Sulfolobales*.

Terminal oxidases and the electron transport chain (ETC)

The ETC in the *Sulfolobales* deviates from the traditional four-complex structure. While homologs to complexes I and II (NADH dehydrogenase and succinate dehydrogenase, respectively) have been identified (Lemos, Gomes and Teixeira 2001; Melo, Bandeiras and Teixeira 2004), complexes III and IV appear to be combined into single quinone oxidoreductase complexes. In fact, cytochrome c has yet to be found in any of the *Sulfolobales*. As a result, these quinone oxidoreductases are responsible for the reduction of molecular oxygen and pH homeostasis through proton pumping. A diverse array of terminal oxidases is present throughout the *Sulfolobales* and appear to relate to the mode of growth for each organism (Table 5). The DoxBCE complex is conserved throughout the *Sulfolobales*, including in the obligate anaerobe *Stygiolobus azoricus*, and it appears to sometimes co-purify with the DoxDA subunits of TQO (Purschke *et al.* 1997). However, SoxABCDD′L and SoxEFGHIM are more varied in their distribution. SoxABCDD′L, which directly pumps protons out of the cell (Gleissner *et al.* 1997), seems to be associated with aerobic growth. Meanwhile, SoxEFGHIM is absent from known chemolithoautotrophic organisms such as the *Acidianus* spp., and it is associated with heterotrophic growth (Lubben, Castresana and Warne 1994). An additional quinol oxidase, SoxLN-CbsAB, has been identified in *A. ambivalens* and is highly similar to the complex III cytochrome bc_1 (Bandeiras *et al.* 2009), but the electron acceptor of this complex has yet to be definitively established. There is a wide range of binding affinities and kinetic parameters for these structures (Schafer, Moll and Schmidt 2001), and it is possible that the kinetics of quinol oxidation plays a key role in energy conservation from inorganic substrates in the *Sulfolobales*.

Table 5. Distribution of oxidase complexes in the *Sulfolobales*.

Organism	SoxABCDD'L	SoxEFGHIM	DoxBCE	SoxLN-CbsAB
Acidianus ambivalens			X	X
Acidianus brierleyi	X		X	X
Metallosphaera cuprina	X	X	X	X
Metallosphaera sedula	X	X	X	X
Saccharolobus islandicus	X	X	X	X
Saccharolobus solfataricus	X	X	X	
Stygiolobus azoricus			X	
Sulfodiicoccus acidophilus		X	X	X
Sulfolobus acidocaldarius	X	X	X	X
Sulfuracidifex metallicus	X		X	X
Sulfurisphaera tokodaii	X	X	X	X

POTENTIAL AND CURRENT USES OF THERMOACIDOPHILES IN BIOTECHNOLOGICAL APPLICATIONS

Challenges in establishing genetic systems in *Sulfolobales* species

A genetic toolbox is essential to study and understand the function of genes and proteins of an organism and it has to include three essential components: (i) a strain, which is able to take up foreign DNA, (ii) a vector system, allowing for the introduction of genetic material and (iii) a selection/screening system for identification of mutated cells. Much effort has been put into the implementation of established systems from different prokaryotic organisms into the *Sulfolobales*. The main challenge of this objective is the natural growth conditions of this group. The high temperature and acidic environment lead to degradation of antibiotics and their resistance-mediating enzymes, which are mainly used as selectable markers in mesophilic organisms. There have been numerous attempts to adapt the systems to these difficult conditions, but either the attempt failed (Cammarano *et al.* 1985; Grogan 1989, 1991; Aagaard *et al.* 1994; Sanz *et al.* 1994; Ruggero and Londei 1996; Hjort and Bernander 2001; Bini *et al.* 2002; Reilly and Grogan 2002) or positive results could not be reproduced (Aravalli and Garrett 1997; Cannio *et al.* 1998, 2001). Another more promising approach is the use of auxotrophic systems, where metabolically deficient strains are complemented with a functional gene, which becomes a selectable marker. Because these systems use the endogenous metabolic system for selection, they are not affected by the harsh environmental conditions. Therefore, most genetic systems in the *Sulfolobales* make use of these auxotrophies for mutant selection, which are discussed in this section.

Transformation

The fundamental prerequisite for the establishment of a genetic tool is a reliable transformation protocol, consisting of a transformable strain and a way to introduce DNA into it. The first transformation in *Sulfolobales* was carried out by Schleper *et al.* in 1992 using electroporation, to test the infectivity of *S. shibatae* virus 1 (SSV1) (see the section 'Viruses and CRISPR systems of thermoacidophiles') derived shuttle vector system in *Sa. solfataricus* P1 (Schleper, Kubo and Zillig 1992). This protocol has been transferred and improved over the years into various other related strains (Zillig *et al.* 1993; Arnold *et al.* 1999; Aucelli *et al.* 2006). Transformation efficiency was later improved by altering the electroporation procedure as well as introducing a regeneration treatment to the cells after the electric shock (Kurosawa and Grogan 2005; Albers and Driessen 2007).

S. acidocaldarius expresses a restriction-modification enzyme, Sua I (Prangishvili *et al.* 1985), cleaving GGCC sequences lacking N4-methylation on the first cytosine (Grogan 2003). Therefore, the transformation protocol for *S. acidocaldarius* was significantly improved by methylating these sites on the plasmid DNA (Berkner *et al.* 2007). Another possibility is to delete Sua1 and use the resulting strain as a host for further experiments (Suzuki and Kurosawa 2016). While methylation of transformed plasmid DNA in *S. acidocaldarius* is mandatory, it is strain dependent in *Sa. islandicus* and *Sa. solfataricus*, with unmodified transformation possible for *Sa. solfataricus* P1 and *Sa. solfataricus* 98/2 and their derived strains (Stedman *et al.* 1999; Albers and Driessen 2007). In any case, electroporation has proven to be the most efficient means for the transformation of species used for genetic systems in the *Sulfolobales*.

Genetic stability

The most studied *Sulfolobales* species regarding genetic manipulation are *Sa. solfataricus*, *S. acidocaldarius* and *Sa. islandicus*. In contrast to the other two, *S. acidocaldarius* exhibits only a small number of insertion elements (Grogan, Carver and Drake 2001). These are highly mobile constructs resulting in an elevated mutation frequency, which is problematic for genetic studies, as whole sections of the genome can be inverted or rearranged within a couple of generations (Redder and Garrett 2006). These elements are most abundant in *Sa. solfataricus* (Martusewitsch, Sensen and Schleper 2000), making it the most unstable genome. For *Sa. islandicus*, it depends on the strain, some of which have low mutation frequencies (Berkner and Lipps 2008), making them preferable for studies of this species.

Cryptic and virus-based shuttle vectors

A broad spectrum of different viruses and plasmids was discovered for the *Sulfolobales* (reviewed by Prangishvili, Stedman and Zillig 2001; Snyder *et al.* 2003; Prangishvili and Garrett 2004; Lipps 2006), some of which were used to create the first generation of *Saccharolobus/Sulfolobus–E. coli* shuttle vector systems. The first derived genetic tool, used in different laboratories, was pMJ03 that consisted of the virus DNA of SSV1 and parts of the bacterial pUC18 vector (Jonuscheit *et al.* 2003). An advantage of using SSV1 DNA is that no selectable markers are required, as the plasmid can transfect cultures independently. However, this is also the largest disadvantage, since viral infection puts the cells under severe stress, causing other problems down the line (e.g. growth

retardation or contamination of other close cultures). But with no other selection system available at that time, using virus DNA was the only reliable method for vector spreading.

Another approach was the use of naturally occurring cryptic plasmids as scaffolds for vector systems. The basis is the cryptic plasmids pRN1 and pRN2, which were extracted from *Sa. islandicus* (Keeling *et al.* 1996, 1998), with pRN1 as the main plasmid backbone in *S. acidocaldarius* systems (Berkner *et al.* 2007), and pRN2 in *Sa. islandicus* (Deng *et al.* 2009). However, these systems require a selectable marker for propagation. As the development of these marker systems advanced, the existing system based on SSV1 was largely replaced by cryptic plasmids, which today form the basis for the most used systems in *S. acidocaldarius* and *Sa. islandicus* (Deng *et al.* 2009; Berkner *et al.* 2010; Wagner *et al.* 2012).

LacS: first directed mutants in Sulfolobus

The first targeted mutation in *Sulfolobales* was performed by Blum and co-workers (Worthington *et al.* 2003), based on a natural *lacS* deficient strain, *Sa. solfataricus lacS::IS1217*, that had an insertion in the endogenous *lacS* cluster making it unable to grow on lactose as a carbon source. Therefore, growth on lactose could be used as a selection after electroporation of linear fragments containing the *lacS* gene flanked by the region of the DNA where the *lacS* gene had to be inserted. Later, the strain *Sa. solfataricus* PBL2025 proved to be more efficient as the recipient strain. In this natural mutant, a 50 kB region spanning the locus SSO_3004 to SSO3050 were deleted, including the *lacS* gene (Schelert *et al.* 2004). Improved transformation protocols and a 7–14 days adaptation time after the electroporation in minimal lactose medium enabled the more frequent use of this strain for genetic studies (Albers and Driessen 2007). However, it only works for *Sa. solfataricus* and *Sa. islandicus*, since *S. acidocaldarius* is not able to grow on lactose as the sole carbon source. Therefore, other methods were sought for *S. acidocaldarius* on the basis of uracil auxotrophic strains and vector systems containing the *pyrEF* gene cassette as a selection marker.

Genetic system for S. acidocaldarius

The generation of markerless deletion mutants in archaea was first established in the *Euryarchaeota Haloferax volcanii* using uracil auxotrophy (Bitan-Banin, Ortenberg and Mevarech 2003) and *Thermococcus kodakarensis* using uracil and tryptophan auxotrophic mutants (Sato *et al.* 2005), as well as in the methanogens *Methanosarcina acetivorans* (Pritchett, Zhang and Metcalf 2004) and *Methanococcus maripaludis* (Moore and Leigh 2005). The genes *pyrE* and *pyrF* encode for the enzymes orotate phosphoribosyl transferase and orotidine-5′-monophosphate decarboxylase, respectively, which catalyze the last two steps of the uridine monophosphate synthesis pathway (Grogan and Gunsalus 1993). Upon deletion of one of these two genes, cells lose the ability to grow without uracil supplementation. This deletion can be induced by exposure to the analog substrate 5′-fluoroorotic acid (5-FOA), which is metabolized to cytotoxic products, forcing mutations in the *pyrEF* cluster and generating auxotrophic colonies (Grogan 1991; Kondo, Yamagishi and Oshima 1991). These generated deficient mutants were first used to test horizontal marker transfer and homologous recombination in *S. acidocaldarius* (Grogan 1991; Kurosawa and Grogan 2005). *S. acidocaldarius* is capable of recombining linear DNA fragments into its genome via site specific interactions during rapid growth phases (Grogan and Stengel 2008). The efficiency of the recombination rises proportionally with the length of the fragments used, with 10–30 nt as the minimum length (Kurosawa and Grogan 2005). Additionally, attachment of short flanking sequences to a selectable marker (e.g. *pyrEF*) could lead to integration of the marker into a gene of interest (Sakofsky, Runck and Grogan 2011), similar to a technique used in *S. cerevisiae* (Kelly, Lamb and Kelly 2001) or the one previously described to obtain *Sa. solfataricus lacS::IS1217* (Worthington *et al.* 2003). Wagner *et al.* were able to generate a *pyrE* deficient mutant, called MW001, derived from *S. acidocaldarius* DSM639 using this approach (Wagner *et al.* 2012). *S. acidocaldarius* MW001 contains a deletion of 322 bp (91–412 bp) in the *pyrE* gene and only grows in medium supplemented with uracil. In contrast to the typically used 5-FOA or UV light treatment (Grogan 1991), this method ensures a low probability for additional mutations in the genome.

Generation of markerless deletion mutants in S. acidocaldarius

The general idea of this method is an integration of *pyrEF* into a deficient strain via homologous recombination of target sequences, which flank the marker cassette and can interact with the region around the gene of interest (GOI) (Fig. 17). Positive clones can then be isolated in uracil free medium. The *pyrEF* sequence is derived from *Sa. solfataricus* to avoid homologous recombination between the *pyrEF* in the genome and plasmid. In addition to the auxotrophy, *lacS* from *Sa. solfataricus* was introduced into the vector system as a selectable marker allowing for standard blue/white screening with X-Gal staining, with positive clones exhibiting a blue color.

Following this, colonies are treated with 5-FOA and uracil, which imposes selective pressure, leading to a loop out of the *pyrEF* marker to avoid the formation of toxic by-products. The successful marker deletion can again be additionally tested via blue/white staining, with successfully obtained mutants displaying a white coloring. Depending on the design of the experiment, the GOI can be deleted by two different approaches. The first is by cloning the upstream (US) and downstream (DS) regions around the GOI consecutively next to the *pyrEF* marker cassette. Single-crossover can then occur leading to an integration of the plasmid (Fig. 17A, intermediate state). Upon treatment with 5-FOA, single cross-over can occur again, now between either of the two US or DS regions, respectively, leading to either a deletion of the GOI or the regeneration of the wild-type genotype (Fig. 17A) (Wagner *et al.* 2009, 2012). In an alternative approach, a linearized plasmid or linear PCR fragment is used, which allows for a double-crossover. For the second crossover site, a part of the GOI sequence is used and cloned in front of *pyrEF* (Fig. 17B). As only the US is present in a merodiploid form, subsequent removal of *pyrEF* via loop out generates only mutated colonies (Wagner *et al.* 2009, 2012).

This strategy is useful to test if a gene and its product have essential functions in the cell and, therefore, cannot be deleted. Removal of the *pyrEF* marker cassette by loop out allows for reuse of the marker, resulting in the possibility of multiple gene deletions in a single mutant (Meyer *et al.* 2011; Henche *et al.* 2012; Wagner *et al.* 2012). The *S. acidocaldarius* MW001 strain has so far been used successfully in >100 studies.

Generation of markerless deletion mutants in Sa. islandicus

The starting point in *Sa. islandicus* was the generation of a *pyrEF* deletion mutant, *Sa. islandicus* E233 (She *et al.* 2009), as a recipient strain for the generation of markerless deletion mutants (Deng *et al.* 2009). Deng *et al.* were able to show that their genetic system worked by using the single-crossover (Fig. 17A) and double-crossover (Fig. 17B) strategies, as in *S. acidocaldarius*. Alternatively, the plasmid is introduced as a linear fragment again, with the US and DS region flanking the *pyrEF* marker on

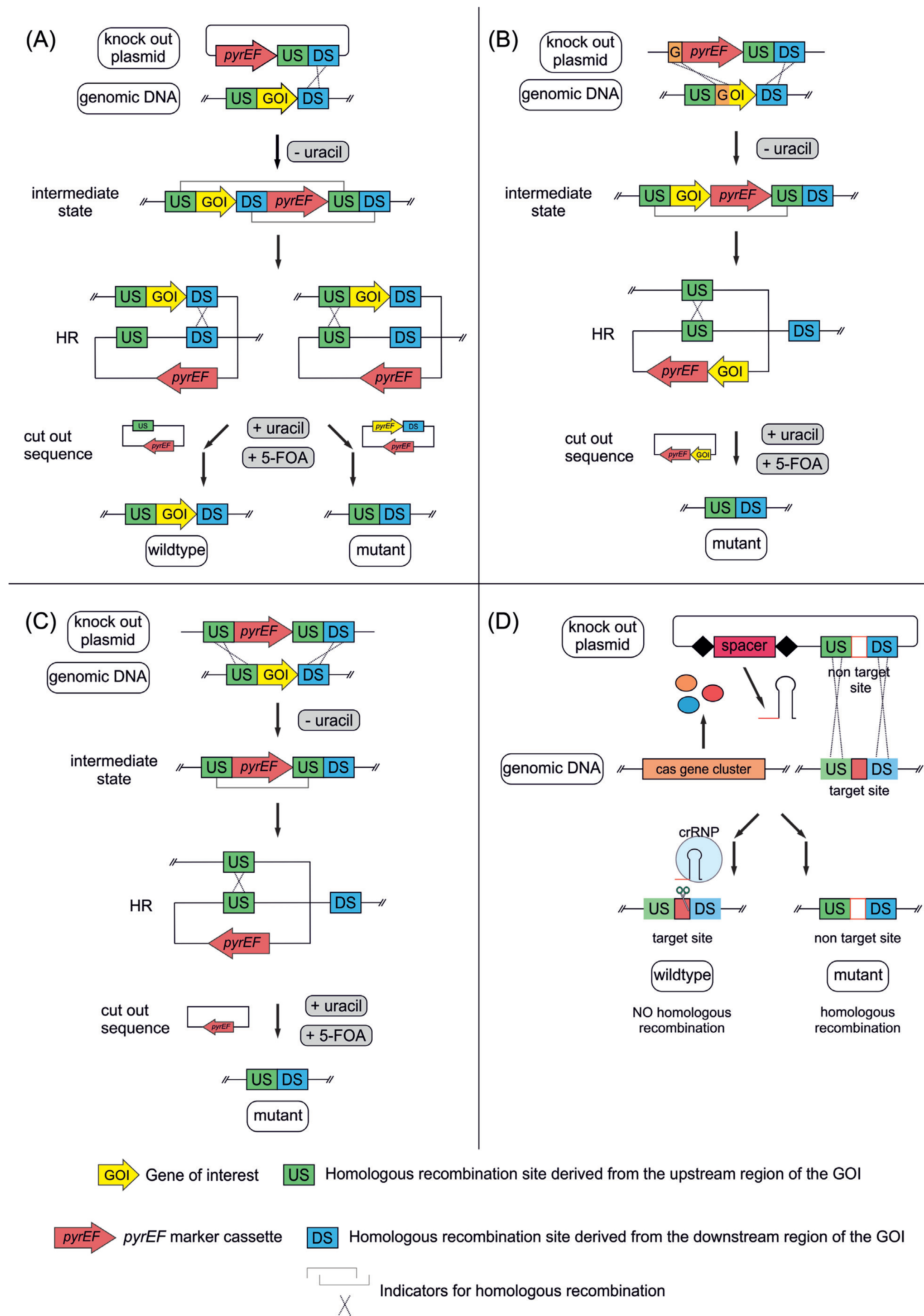


Figure 17. Mechanisms for the generation of markerless deletion mutants. **(A)** Plasmid integration occurs via single crossover, resulting in a merodiploidal form. After counterselection with 5-FOA, the *pyrEF* marker cassette is looped out, either with or without the GOI, resulting in a theoretical ration of one to one in mutated and wild-type cells. Double crossover is feasible by introducing a linearized vector. Depending on the experimental design, either parts of the GOI **(B)** or an upstream (US) region **(C)** are introduced for recombination. Counterselection with 5-FOA produces marker-free deletion mutants. **(D)** A plasmid containing a CRISPR array and a repair fragment with homologous sequences to the GOI are introduced into a recipient strain. Upon induction, crRNA is transcribed and forms a ribonucleoprotein complex with the endogenous Cas protein, scanning the genomic DNA for the spacer sequence and cutting it. Only colonies that conducted recombination with the repair fragment survive. GOI, gene of interest; US, upstream; DS, downstream; *pyrEF*, *pyrEF* marker cassette; crRNA, CRISPR RNA; crRNP, ribonucleoprotein complex consisting of crRNA and Cas protein.

both sites, plus one additional US or DS site, leading to a double cross-over event. Substitution of the GOI with only *pyrEF* (Fig. 17C) leads to mutated cells after 5-FOA treatment (Deng *et al.* 2009). However, the use of *pyrEF* was not successful in other *Sa. islandicus* strains, as the background growth on solid medium is always high (Zhang and Whitaker 2012). Therefore, additional selectable markers had to be introduced to achieve higher selective pressure.

Zheng *et al.* (2012) showed that simvastatin, a thermostable antibiotic, inhibits the growth of *Sa. islandicus*. Shuttle vector systems were developed for *Sa. islandicus* 16.4 (Zhang and Whitaker 2012) and *Sa. islandicus* REY15A (Zheng *et al.* 2012), based on the resistance mediated by the overexpression of the 3-hydroxy-3-methylglutaryl coenzyme A reductase gene (*hmgA*). It became apparent, however, that simvastatin-resistant cultures had fitness issues that could only be counteracted by enriching the mutants in liquid medium, resulting in a time-consuming protocol. Therefore, it was suggested to use the simvastatin selection only as a last resort.

By deleting the *argD* gene encoding for the arginine decarboxylase, Zhang *et al.* were able to generate an agmatine auxotrophic system (Zhang *et al.* 2013a). The methodology follows the uracil auxotrophy strategy, as it is possible to recover growth by supplying agmatine or expressing *argD* in a vector system. With this positive selectable marker, the problems derived from using *pyrEF* could be solved for *Sa. islandicus* (Zhang *et al.* 2013a). In addition, a second counterselectable genetic marker was introduced. Through inactivation of a putative adenine phosphoribosyltransferase, resistance could be mediated to 6-methylpurine (6-MP), a purine analog (Zhang *et al.* 2016a). Like 5-FOA, the metabolism of 6-MP leads to toxic compounds, which forces the cell to mutate the gene of the catalyzing enzyme from the genome. This system has been successfully used to establish a transposon library, revealing the essential genome of *Sa. islandicus* (Zhang *et al.* 2018). This counterselection was also later successfully implemented in *Thermococcus barophilus* (Birien *et al.* 2018). Table 6 summarizes the most frequently cited and applied methods of gene disruption and deletion.

Expression vectors: promoters and tags

The combination of the development of selectable markers and recipient strains stimulated efforts to create vectors for homologous protein expression, using different promoters and protein tags. Several attempts were made, but none were useful for high-level expression of proteins or for the study of promoters using reporter genes (Aagaard *et al.* 1996; Elferink, Schleper and Zillig 1996; Aravalli and Garrett 1997; Cannio *et al.* 1998). The first stable system for homologous expression and tagging of proteins was developed in *Sa. solfataricus* by Albers and co-workers (Jonuscheit *et al.* 2003; Albers *et al.* 2006). As previously mentioned, pMJ03 was designed using the SSV1 virus and pUC18 from *E. coli* (Jonuscheit *et al.* 2003). In this work, the *pyrEF* complementation served as a selectable marker and the heat inducible promoter of the chaperonin tf55α gene was used. The reporter gene was *lacS*, that codes for a β-galactosidase, and the recipient strain was a double *pyrEF/lacS Sa. solfataricus* mutant. Under heat shock conditions (shift from 75°C to 88°C), an increase of greater than 10-fold gene expression was seen, measured by northern blot analyses and activity assays of the enzyme. The promoter also had a strong basal expression. This vector was stable for 40–60 generations when cells were maintained in uracil, but propagation of transformants without selective pressure led to loss of the plasmid (Jonuscheit *et al.* 2003).

Subsequently, this vector was modified. A sugar-inducible promoter (D-arabinose) was added instead of the tf55α promoter along with cloning sites that allowed for the exchange of *lacS* for a gene of interest. Different tags for protein purification and detection, such as 6× His or Strep, were also added, leading to the pSVA plasmids set (Table 7). The strength of the D-Ara promoter was tested using LacS as a reporter resulting in an increase in activity of 13-fold when D-arabinose was added to the medium. The amount of protein obtained was similar to that of the tf55α promoter, but without the previously seen basal expression levels. The low basal expression can prevent adverse effects of high expression of proteins on growth before induction. While the His tag resulted in a 99% homogeneity rate in purification, Strep-tagged proteins co-eluted with a carboxylase from *Sa. solfataricus* when low yields of the recombinant protein were obtained (Albers *et al.* 2006).

An advantage of pMJ03 and derived plasmids was that they were self-spreading, but the production of virus particles had an adverse effect on the transformed cells. A breakthrough came with the use of the plasmid pRN1 in *S. acidocaldarius* (Berkner *et al.* 2007). It was stable in different *Sulfolobus* species and could be easily selected for in *S. acidocaldarius* using the *pyrEF* marker cassette. Several promoters were tested including those from *tf55*α *dps*, *lacS*, *mal*, *gdhA* and *sac7d* (Berkner *et al.* 2010). The promoter from the maltose-binding protein (*mal*) showed low basal activity and increased expression in the presence of maltose or dextrin in the medium, leading to the plasmid pCmalLacS. The other promoters showed low (*dps*, *lacS*) or high constitutive expression (*gdhA* and *sac7d*) and were left aside. The promoter from *copMA* was also tested but exhibited even lower expression levels than the maltose promoter (Wagner *et al.* 2012).

In a recent study, a D-xylose/L-arabinose promoter was tested and showed less basal activity than the widely used maltose inducible promoter (van der Kolk *et al.* 2020). Furthermore, this promoter is also D-arabinose inducible, and can be used as a strong inducer similar to IPTG in *E. coli* since D-arabinose is not a growth substrate for *S. acidocaldarius* (van der Kolk *et al.* 2020). This study led to the availability of several expression plasmids for *S. acidocaldarius*. Using FX cloning (Geertsma 2013) for introduction of the desired gene, it is possible to choose between different promoters (D-xylose/L-arabinose/D-arabinose and maltose inducible) and several tags for proteins (StrepII, His, HA, His + Strep, etc.), either in the N-terminal or C-terminal region of the protein (Table 7) (van der Kolk *et al.* 2020).

Mutants via CRISPR-Cas and gene silencing

CRISPR systems and spacer acquisition are discussed in the section 'Viruses and CRISPR systems of thermoacidophiles'. CRISPR-Cas systems are classified into three groups so far: type I, associated with Cas3 protein; type II, associated with Cas9; and type III, associated with Cas10. Types I and II recognize DNA via PAM sequences in the protospacer in the target genome. Type III does not need a PAM sequence, but instead a seed sequence, corresponding to a mismatch in the 5′ end of the crRNA (van der Oost, Jackson and Wiedenheft 2014).

In the *Sulfolobales*, the idea of using the proper CRISPR-Cas system was first explored in *Sa. islandicus*. Li *et al.* (2016) used the native type IA and IIIB systems of this organism to generate mutants for *lacS*, so the phenotype could be easily tested with X-gal. The pSe-Rp plasmid was used as backbone and an artificial CRISPR array was introduced. The array consisted of two repeat sequences, flanking a spacer designed based on a protospacer sequence from the target gene. Also, the plasmid contained a donor region that does not exhibit DNA interference activity. Mutagenesis through deletion, insertion or point mutation is achieved via recombination of the donor region with the genomic DNA (Li *et al.* 2016).

Table 6. Most used knockout systems in *Sulfolobales*.

Sulfolobus acidocaldarius	Markerless deletion mutants via crossover based on *pyrEF*/5-FOA counterselection (Wagner *et al.* 2012)
Saccharolobus solfataricus	Gene disruption via permanent insertion of *lacS* reporter gene via homologous recombination (Albers and Driessen 2007)
Saccharolobus islandicus	Markerless deletion mutants via crossover based on pyrEF/5-FOA counterselection improved with *argD* selection (Zhang *et al.* 2013a)
	Addition of apt/6-MP counterselection (Zhang *et al.* 2016a)
	CRISPR-based gene knockout (Li *et al.* 2016)

Table 7. Most used expression vectors in *Sulfolobales*.

Organism	Expression vectors				
	Name	Promoter/ Inducer	Selection marker	Feature	Reference
S. acidocaldarius	pCmalLacS	*mal* maltose	*pyrEF*, *lacS*, *amp*	-	(Berkner *et al.* 2010)
	pSVAaraFX	*araS* arabinose	*pyrEF*, *lacS*, *amp*	HA, 6xHis, StrepII, Twin-Strep, 10xHis + StrepII tags at the C and N terminus	(van der Kolk *et al.* 2020)
	pSVAmalFX	*mal* maltose			
	pSVAxylFX	*xylR* xylose			
Sa. solfataricus	pSVA	*araS* arabinose	*pyrEF*, *lacS*, *amp*	6xHis, 10x His, StrepII tags	(Albers *et al.* 2006)
Sa. islandicus	pSeSD	*ara-SD* arabinose	*pyrEF*, *lacS*, *amp*	6xHis tag, 2 protease sites for tag removal	(Peng *et al.* 2012)

The CRISPR RNA (crRNA) generated from the CRISPR array of the plasmid guides the native CRISPR-Cas system to self-targeting DNA from wild-type cells, killing them but not the mutants, which accomplished recombination with the donor DNA. Using a plasmid containing the donor sequence and the CRISPR array led to better results than performing co-transformation using a CRISPR plasmid and a short DNA fragment as donor sequence (Li *et al.* 2016).

In *Sa. solfataricus*, on the other hand, type III CRISPR-Cas was used to develop silencing via RNA interference (Zebec *et al.* 2016). *Saccharolobus solfataricus* has two type IIIB CRISPR complexes, both targeting RNA, and one of them also DNA (Zhang *et al.* 2016b). A plasmid containing a mini-CRISPR array with a spacer designed to target mRNA from β-galactosidase was used to demonstrate that 50% of gene silencing is possible to achieve using the native CRISPR-Cas system from *Sa. solfataricus* (Zebec *et al.* 2014). In later work, silencing up to 90% could be accomplished using a CRISPR array containing five different spacers from the same gene, in this case α-amylase (Zebec *et al.* 2016). Lower levels of silencing were achieved using one to three spacers, resulting in 35–82% gene silencing. Since one of the type IIIB complexes also targets DNA, the protospacers in the genome were chosen to have a flanking region that matches with the 5′-end handle of the crRNA. This inhibits DNA targeting since CRISPR-Cas system uses the repeat sequences to 'protect itself' by recognizing the cells' own genomic DNA (Manica and Schleper 2013).

CRISPR has also been used to edit rod-shaped virus 2 (SIRV-2) from *Sa. islandicus*, using the archaeon as a host and its endogenous CRISPR system as machinery (Mayo-Muñoz *et al.* 2018). This method was also used to investigate the core genome of the virus, generating knockout mutants, useful for probing the details of the infection process. In general, CRISPR-Cas systems are powerful genetic tools, which have had a huge impact in the life science community (van der Oost, Jackson and Wiedenheft 2014; Plagens *et al.* 2015; Mougiakos *et al.* 2016; Quehenberger *et al.* 2017), and also show promise for studying and engineering thermoacidophiles.

The potential of thermoacidophiles as metabolic engineering platforms

As outlined in the section 'Metabolism', representatives of the *Sulfolobales* have been examined for their autotrophic and heterotrophic lifestyles. They have enormous metabolic versatility that differs from species to species, with respect to their growth on a variety of carbon sources that include complex polymers (e.g. polysaccharides, proteins, lipids), monomers (e.g. carbohydrates, amino acids, fatty acids, alcohols, aldehydes) and CO_2. In addition, comprehensive biochemical and functional genomics data are available for members of the *Sulfolobales* (Bräsen *et al.* 2014), including ^{13}C NMR flux analysis for different carbon sources (Nunn *et al.* 2010). Furthermore, systems biology (www.sulfosys.com; Zaparty *et al.* 2009), genome-scale stoichiometric (FBA) (Ulas *et al.* 2012) and detailed kinetic models (e.g. for gluconeogenesis) (Kouril *et al.* 2013a,b, 2017) have been established for one of the model *Sulfolobales*, *Sa. solfataricus*.

Critical for metabolic engineering is the availability of advanced genetic tools, which are described above for several species in the *Sulfolobales* and include in-frame markerless

deletion mutants, ectopic integration of foreign DNA, and a homologous expression system (Wagner *et al.* 2014) (see the section 'Metabolism'). Thus, *S. acidocaldarius*, *Sa. islandicus* and *Sa. solfataricus* are all a potential host 'chassis' on which to build biosynthetic designs of increasing complexity, although *S. acidocaldarius*'s genetic stability might be an advantage. Another important criterion for metabolic engineering and application in biotechnology is ease of cultivation under aerobic conditions. Complex and minimal media have been described, e.g. for *S. acidocaldarius*, and high cell density cultivation has been established for *S. shibatae* and *S. acidocaldarius* (Quehenberger *et al.* 2017, 2020; Schocke, Bräsen and Siebers 2019). The current genetic systems do require further improvement. In particular, the integration of larger gene clusters and the development of new regulatory strategies are needed to fully realize the biotechnological potential of these thermoacidophiles (Crosby *et al.* 2019).

Thermoacidophile biotechnology

As mentioned above, by their very nature, thermoacidophiles are robust microorganisms that can handle industrial processing conditions well and therefore offer potential advantages as metabolic engineering hosts over more established but less extremophilic species, such as *E. coli* and *S. cerevisiae* (Crosby *et al.* 2019). Their cytosolic enzymes are adapted to high temperature and neutral pH, whereas their extracellular enzymes, such as amylases, cellulases and lipases, are also adapted to low pH. Thus, the properties of thermoacidophilic biocatalysts are consistent with process schemes used in lignocellulosic biomass pre-treatments, which are typically done at high temperatures and low pH. The utilization of thermoacidophiles and their enzymes offers certain benefits for industrial biotechnology (Turner, Mamo and Karlsson 2007; Hess 2008; Zeldes *et al.* 2015; Straub *et al.* 2018): at high temperature, reaction rates increase and so does substrate accessibility for biopolymers such as starch and lignocellulosic carbohydrates, thereby enhancing biomass conversion. Since substrate solubility improves at higher temperatures, this enables mixing of otherwise viscous slurries. Furthermore, the energy input for cooling steps in bioreactors and thus production costs can be reduced. Under thermal conditions, volatile products can be removed through gas stripping and evaporation, facilitating product recovery. As such, expensive distillation steps as well as inhibition by toxic products can be minimized, allowing for novel design 'one-pot' strategies (Zeldes *et al.* 2018). Particularly important, microbial contamination is negligible at high temperatures and low pH, so that the use of antibiotics and the need for pharmaceutical-like processing can be avoided (Marhuenda-Egea and Bonete 2002; Champdore *et al.* 2007; Quehenberger *et al.* 2017; Cabrera and Blamey 2018).

There are also some disadvantages and challenges to overcome to fully realize the biotechnological potential of thermoacidophiles as industrial microorganisms. As previously described, central metabolic pathways (e.g. for lipid or glycerol degradation) are still not well understood and further work is needed to unravel the metabolic complexity of promising representatives of the *Sulfolobales*. In particular, networks with regulation at the gene and protein levels require further basic research, and only a few transcriptional regulators have been investigated, and regulation via post-translational modification is also not well understood (see the section 'Genetic mechanisms'). Processes that truly exploit thermoacidophily remain to be developed, although advances in thermoacidophile genetic motivate such efforts.

Biomining applications of thermoacidophiles

The importance of acidophilic organisms in the breakdown of sulfidic ore has been known for many years. Acid mine drainage is a by-product of acidophiles at work on pyritic mine waste and provides an environmental backdrop for studying the mechanism of biological oxidation (Schippers, Jozsa and Sand 1996). However, the same mechanism can be leveraged to extract base and precious metals from sulfidic ores through bioleaching operations. An important distinction to note is that bioleaching refers specifically to the dissolution of metals by bacteria and archaea, while biooxidation simply refers to the oxidation of metal and non-metal substrates by the same organisms.

At first glance, one might suspect that the acidophilic organisms directly attack the solid ore. However, closer inspection of the mechanism shows that the dissolution of both pyrite and other metal sulfides is an indirect result of biooxidation by acidophilic bacteria (Fig. 18). The ore undergoes electrophilic attack in the presence of ferric ions (Fe^{3+}) and protons (Sand *et al.* 2001). Other ions have also been proposed to facilitate this initial attack, including copper and silver (Hiroyoshi *et al.* 2000; Zhao *et al.* 2019). The role of biooxidation in the bioleaching process is then the regeneration of ferrous ions into ferric ions that can again attack the ore and the total oxidation of the freed sulfur to sulfate (Li *et al.* 2013). Two distinct mechanisms for ore dissolution underscore the importance of the distribution of sulfur in the system. In the presence of pyrite, thiosulfate is directly generated during the electrophilic attack (Fig. 18A). This initiates a cycle of abiotic sulfur reactions, in which greater than 80% of the sulfur product is converted to sulfate (Sand *et al.* 2001). In contrast, the polysulfide mechanism applies to dissolution of metal sulfide ores, like chalcopyrite (Fig. 18B). Here, the sulfur product of the initial attack is hydrogen sulfide (H_2S), which then undergoes a series of abiotic chain elongation reactions to form polysulfides. Ultimately, these polysulfides cyclize to form the thermodynamically stable and water-insoluble S_8 ring (Steudel 1996), which accounts for 90–99% of the final sulfur product depending on the metal sulfide species (Sand *et al.* 2001). While pyrite is the dominant form of metal sulfide ores, mining streams generally contain a mixture of these various crystal structures (Neale *et al.* 2009), and thus these mechanisms exist simultaneously.

A key issue associated with the polysulfide mechanism generating solid cyclic sulfur is the passivation of the ore's surface (Zhao *et al.* 2019). Passivation is the formation of an inhibitory film on the surface of the ore that prevents further electrophilic attack (Klauber 2008). In the case of chalcopyrite, a passivating layer of solid sulfur coats the ore and significantly slows the dissolution rate. Another component of this passivating effect is the formation of jarosites, complex ferric sulfate compounds, that is only an issue with long leaching times. However, the slowed dissolution rate caused by passivating sulfur may allow for the accumulation of jarosites, and both modes of inhibition are seen in chalcopyrite bioleaching (Klauber 2008).

There are two primary formats for industrial-scale bioleaching. Heap leaching, as the name implies, is the open-air extraction of metals from large heaps of mine tailings. Leaching solution is percolated throughout the pile, with the metal-laden runoff collected for downstream processing (Schlitt 2006). The capital investment of these operations is generally low and uses low-grade ore to maximize yield from mining operations. These heaps are often self-inoculated and contain a consortium of acidophilic organisms, including mesophilic *Acidithiobacillus* spp.,

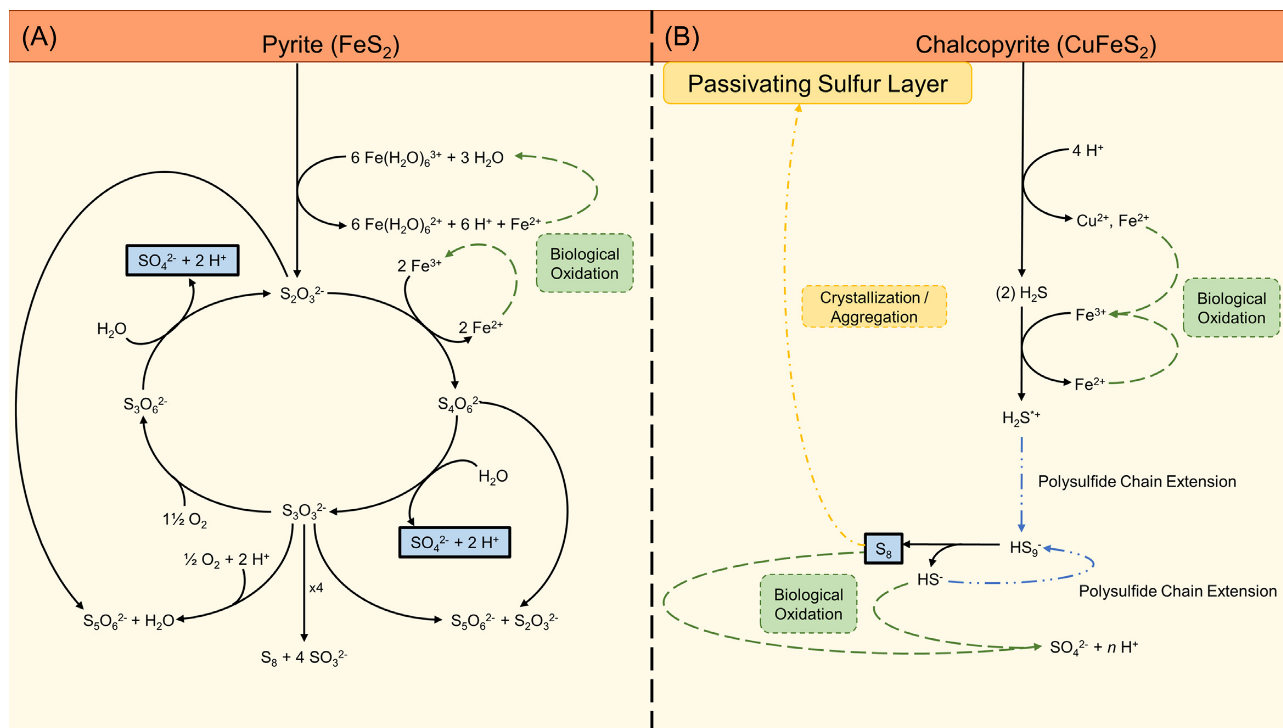


Figure 18. Mechanisms of sulfidic ore dissolution. **(A)** Thiosulfate mechanism **(B)** and polysulfide mechanism. Green dashed arrows indicate biological steps; solid arrows indicate spontaneous abiotic reactions; blue dashed-dotted arrows indicate an overall transformation involving multiple reaction steps; yellow dashed-dotted arrows represent phase transition. Bold box around a species indicates that this is the dominant sulfur product of the dissolution process.

moderately thermophilic *Sulfolobacillus* spp., and extremely thermophilic *Acidianus* spp. and *Metallosphaera* spp. (Pradhan *et al.* 2008). Despite the open-air environment of the heaps, the highly exothermic oxidation of sulfur results in heap temperatures reaching as high as 81°C, which supports the growth of the *Sulfolobales* (Pradhan *et al.* 2008).

The second format is a traditional bioreactor. While the capital cost of this setup is much higher than that of heap leaching, the more controlled environment and improved contact area dramatically reduce the leaching time. While heap operations run for months to years (Schlitt 2006), bioreactor leaching has a retention time on the order of days (Neale *et al.* 2009). These bioreactors are often still self-inoculating and contain a complex microbial landscape. Notably, the acidic conditions provide a natural barrier to contamination, and this barrier is further enhanced when working at the high temperatures of the *Sulfolobales*.

Bioleaching at the high temperatures required by the *Sulfolobales* offers distinct advantages. Given the large exotherm associated with sulfur oxidation, the reactor does not need to be heated to maintain the temperatures necessary for *Sulfolobales*' growth. In fact, cooling is necessary to maintain the constant temperatures (Neale *et al.* 2009), and at higher temperatures less cooling is necessary. Furthermore, chalcopyrite dissolution kinetics are significantly improved at higher temperatures (Watling 2006). In a 10-day lab-scale bioleaching reactor, around 70% copper recovery was achieved with the mesophilic *Acidithiobacillus ferrooxidans* and roughly 85% copper recovery was achieved with the moderate thermophile *Sulfobacillus* (Mousavi et al. 2005). At pilot scale, a thermoacidophile bioreactor containing *Acidianus*, *Metallosphaera* and *Sulfolobus* spp. achieved 95% copper recovery (Neale *et al.* 2009).

As understanding of the mechanisms for iron and sulfur oxidation improves, engineering an optimized bioleaching organism presents an intriguing possibility. In particular, this could overcome the obstacle of surface passivation caused by the accumulation of elemental sulfur and jarosites. Indeed, one means of controlling the passivation of ore is to lower the reduction potential of the reactor. This process controls the ratio of Fe^{3+}/Fe^{2+} ions in order to limit the rate of elemental sulfur and jarosite formation. A variety of approaches to control the redox potential of the system have been explored, ranging from adding reagents to form new redox couples, controlling dissolved oxygen levels and adjusting the microbe composition (Zhao *et al.* 2019). This last option points toward the possibility of an optimized bioleaching organism that manages the redox potential through the relative rates of sulfur and iron oxidation. Efforts to generate an optimized strain of *M. sedula* through laboratory evolution had moderate success in this regard (McCarthy, Ai and Blum 2018). While natural evolution of the ability to breakdown chalcopyrite may provide novel insights, tailoring an organism to mitigate surface passivation may require a more controlled approach. As such, the genetic tools available to select members of the *Sulfolobales* provide a promising platform to expand their bioleaching capabilities.

CONCLUSIONS

The scientific and technological potential of the *Sulfolobales* has come a long way since their isolation more than a half century ago. During this time, molecular biology and genomics came of age, with all of the associated tools that can be brought to bear in understanding the microbiology of these thermoacidophiles. Here, the goal was to provide some historical perspective as well as to give an up-to-date overview of where the world of the *Sulfolobales* stands. Despite the length and breadth of this review, we have likely inadvertently left out important contributions to the field and thank all of those who have studied and reported on facets of these interesting microorganisms.

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