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Survival mechanisms of microorganisms occurring in acid mine drainage: sulfur, iron, carbon, and nitrogen metabolic pathways

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Highlights
- AMD environment has low pH, high concentration of metals and low availability of nutrients
- Iron and sulfur are the major energy sources for microorganisms living in AMD environments, characterizing them as chemolithotrophs
- Special metabolic mechanisms allow AMD microorganisms to survive and thrive in conditions of low pH, high metal concentrations and lack of nutrients
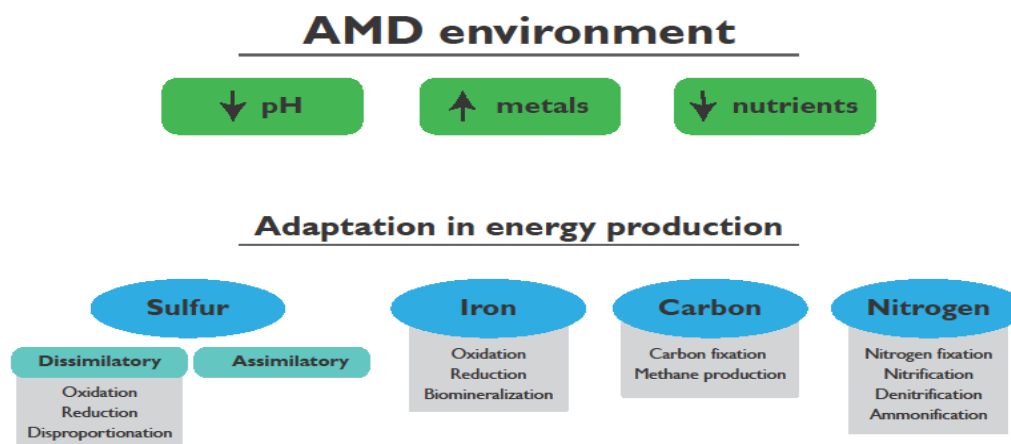
Abstract: Despite its economic importance, mining usually generates intense environmental degradation. The excavation process carried out in mining activities exposes minerals to atmospheric oxygen and water, conditioning a series of biogeochemical processes that can lead to the production of acid mine drainage (AMD). AMD has low pH and high concentrations of sulphates and heavy metals, creating environments with extreme conditions for life. These environments are usually inhabited by microorganisms able to acquire energy from iron and sulfur, using limited sources of carbon and nitrogen. In addition, these microorganisms need mechanisms to resist to extremely low pH and high concentration of heavy metals that can be toxic and lethal to the cellular structure. Acid stress tolerance involves active mechanisms to maintain intracellular pH at adequate levels despite low external values, and adaptive processes against acid stress allowing microorganisms to operate metabolically at low pH. The set of these adaptations give microorganisms the possibility of surviving in AMD environments and, consequently, represent potential for bioremediation and other biotechnological applications like biomining and search for biomolecules for industrial processes. The purpose of this review was to compile the metabolic and adaptive mechanisms involved in the survival of microorganisms occurring in AMD environments, focusing on how they utilize sulfur, iron, carbon and nitrogen metabolic pathways.

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Graphical Abstract

AMD environment

↓ pH   ↑ metals   ↓ nutrients

Adaptation in energy production

Sulfur
Dissimilatory   Assimilatory
Oxidation
Reduction
Disproportionation

Iron
Oxidation
Reduction
Biomineralization

Carbon
Carbon fixation
Methane production

Nitrogen
Nitrogen fixation
Nitrification
Denitrification
Ammonification

Introduction

The increase in population and economic activities in the current industrial system implies a growing demand for energy and minerals. Despite the environmental restrictions agreed in recent decades (Dong et al., 2019), mining continues to be widely exploited economically in many countries. According to a survey carried out in 2018 (Maus et al., 2020), the active mining area in the world comprises a total of 57,278 km^2, with China, Australia, the United States and the Russian Federation being the countries with the largest areas (Figure 1A). The same study pointed to 9,889 active mines of different types of ores, with coal, gold and silver being the most frequent (Figure 1B).

In places without mining activities, the rocks located deep in the soil are protected from chemical oxidation. In the absence of oxygen, sulfide minerals, for example, have low chemical solubility and are stable. However, the excavation process carried out in mining activities causes the minerals to be exposed to atmospheric oxygen and water, initiating a series of biogeochemical processes that can lead to the production of acid mine drainage (AMD) (Ayangbenro et al., 2018; Wu et al., 2021). The low pH and the high concentration of metals make AMD an environment with extreme conditions for life. However, some specific groups of microorganisms have developed adaptation mechanisms, enabling them to survive and thrive in environments affected by AMD.

This review will describe and exemplify the biochemical and physiological mechanisms that evolved in microorganisms occurring in AMD environments, enabling them to survive in these harsh conditions. Understanding these mechanisms is essential to envision possible remediation processes and eventually explore the biotechnological potential of these microorganisms. Mechanisms of tolerance to heavy metals will not be addressed, as they are the subject of another specific review of our research group.

AMD formation

Mining activities involve excavation, mechanical and chemical processing of the exploited material, generating a large number of residues that are toxic, corrosive or flammable materials and devoid of economic value. There is a general estimate that at least one ton of mining waste is generated for every ton of ore extracted (Ayangbenro et al., 2018). In the past, many activities were carried out without any waste management planning, resulting in huge environmental problems. The release of these wastes into the environment can have a significant impact on surface, underground, air and land water resources (Rambabu et al., 2020). In recent decades, environmental concerns have required mining companies to present plans for environmental protection, waste reduction, waste disposal and environmental recovery (Hudson-Edwards et al., 2011).

Factors that influence AMD generation are degree of saturation with water, oxygen, pH, temperature, chemical activity of Fe(III), chemical activation energy to initiate acid generation, sulfide minerals, surface area of exposed metal sulfide and presence of iron oxidizing bacteria (Ayangbenro et al., 2018; Sánchez-Andrea et al., 2014). Most metals are associated with pyrite (FeS_2) and occur primarily as sulfide ores. Metallic sulfides are reduced to ferrous in the reaction with pyrite by ferric iron, the main oxidizing agent. This reaction is oxygen-independent and is the most important step in the oxidation of sulfide minerals. The oxidation of ferrous iron can be mediated biologically or chemically by molecular oxygen at a pH above 4. Specific groups of sulfide and metal oxidizing microorganisms, such as *Acidithiobacillus ferrooxidans*, are able to obtain the energy from pyrite and other sulfide metals (Wu et al., 2021). The chemical oxidation rate of ferrous iron is negligible below pH 4, so the activity of acidophilic iron-oxidizing bacteria plays a crucial role in generating acid drainage. At low pH, heavy metals are stable in solution and mobile, and with increasing pH, they become adsorbed and therefore immobile (Ayangbenro et al., 2018; Sandy & Butler, 2009). Drainage composition can differ drastically from one region to another due to local geology, microclimate, group of microorganisms and water source (Rambabu et al., 2020; Simate & Ndlovu, 2014). The main chemical equations related to common processes in acid mine drainage are presented in Table 1.

A large part of the environmental problems found in mining is directly linked to poor waste management. The most common errors are dam failures, seepages, tailings spills, unrehabilitated sites and cases of direct discharge into
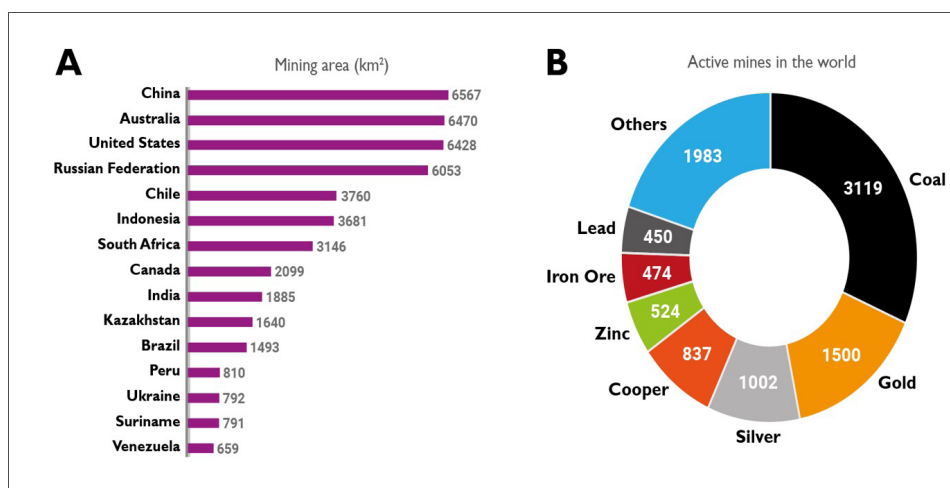
Figure 1. Data set of mining areas around the world. (A) Top 15 country in Mining area (km²), the complete list has 121 countries with a total of 57,278 km². (B) List of commodities from active mines in the world (Maus et al., 2020).

Table 1. Main processes and chemical equations occurring in acid mine drainage (after Wu et al., 2021).

Process	Chemical Equation
Weathering of pyrite in the presence of oxygen and water	$2FeS_2 + 7O_2 + 2H_2O \rightarrow 2Fe(II) + 4SO_4^{2-} + 4H^+$
Oxidation of Fe(II) to Fe(III) (rate limiting step)	$4Fe(II) + O_2 + 4H^+ \leftrightarrow 4Fe(III) + 2H_2O$
Fe(III) hydrolysis and Fe(III) partial precipitation if pH>3.5	$Fe(III) + 3H_2O \leftrightarrow Fe(OH)_3 + 3H^+$
Additional pyrite oxidation (cyclic and self-propagating step)	$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe(II) + 2SO_4^{2-} + 16H^+$
Global reaction	$4FeS_2 + 15O_2 + 2H_2O \rightarrow 4Fe(III) + 8SO_4^{2-} + 4H^+$

watercourses (Franks et al., 2011). All of this can result in serious and long-term environmental consequences. The large amount of waste generated by mining activities destroys the landscape at the exploration site and disturbs ecosystems, altering flora, fauna and microbiota (Ayangbenro et al., 2018). The mining wastes are usually dumped in the surrounding land, being a great source of heavy metals and acids. As a result, there is no topsoil with nutrients and vegetation (Tripathi et al., 2016). When nearby groundwater and surface waters are contaminated, it becomes toxic, affecting ecosystems and public water supply (Rambabu et al., 2020).

The acidity and toxicity conditions found in AMD environments cause microorganisms to induce stress responses, exhibiting changes in cell morphology and assembly (Chakravarty & Banerjee, 2008). Due to these characteristics, only organisms that somehow adapted to these conditions are able to develop, resulting in lower growth and biodiversity when compared to non-impacted environments (Rambabu et al., 2020).

Microbial metabolism in AMD

The energy for the growth or reproduction of microorganisms can be obtained through phototrophy (photosynthesis) or chemotrophy (Pepper & Gentry, 2015). In most cases of phototrophy and chemotrophy, electron movement occurs through the electron transport chain that generates the proton motive force and adenosine triphosphate (ATP), the biochemical unit of energy.

Phototrophic microorganisms such as microalgae and some bacteria contain light-sensitive pigments that absorb energy from sunlight and transfer electrons from water in the presence of oxygen, or from H_2S, $S(0)$, $S_2O_3^{2-}$, H_2, or Fe(II) in anaerobic environments (Konhauser, 2007). In consequence light energy can be assimilated into organic molecules.

Chemotrophs produce energy during a series of redox reactions in which electrons are transferred from a primary organic or inorganic electron donor, to a terminal electron acceptor through a series of enzyme-catalyzed intermediate steps. Oxygen is the most energetic electron acceptor and is used in aerobic respiration. If oxygen is available, microorganisms that are able to use oxygen are likely to grow rapidly and outnumber those that cannot. In a diffusion-limited environment, such as waterlogged soils, oxygen will be consumed and anaerobic respiration will take over, causing microorganisms that can reduce alternative electron acceptors, such as archaea and bacteria, to predominate. In order of highest available energy, at pH 7, these terminal electron acceptors include nitrate, Mn (IV), Fe (III), and

sulfate. Certain metals and metalloids in mine wastes can also act as electron acceptors and be transformed through metal reduction. In addition to sulfate reduction, methanogenesis is often the last step of anaerobic metabolism. Strictly anaerobic archaea can gain energy from disproportionation of acetate or methyl-containing compounds to produce methane, or by producing methane from bicarbonate reduction coupled with H_2 oxidation (Konhauser, 2007). It is quite common for bacteria to be able to use multiple metabolic pathways to work under different environmental conditions, such as switching from aerobic respiration to nitrate reduction in the suboxic zone (facultative anaerobes). Similarly, H_2-oxidizing bacteria can switch to heterotrophic metabolism when organic compounds are available (facultative chemolithoautotrophs), and further fermentation of organic matter when terminal electron acceptors are limited (Konhauser, 2007; Roane et al., 2015). This flexibility allows these bacteria to benefit from more energetically favorable metabolisms under varying environmental conditions.

Chemoheterotrophs obtain energy by using organic compounds as electron donors, oxidizing them to CO_2 or CH_4 coupled with the reduction of a terminal electron acceptor (or light for photoheterotrophs). The availability of organic matter may therefore be a limiting factor for aerobic and anaerobic respiration. Some chemolithoautotrophic bacteria do not need organic matter in their metabolism, and gain energy from the oxidation of inorganic compounds such as H_2, sulfur or Fe (II). In mine wastes, sulfur and Fe (II) oxidizing bacteria degrade sulfide minerals, generating acidity and, consequently, controlling the metal behavior in these environments. While heterotrophs obtain their carbon and energy from organic matter, chemolithoautotrophs and photoautotrophs use energy to fix carbon from inorganic sources, such as CO_2 (Newsome & Falagán, 2021). Although the reduction and oxidation of microbial metals can clearly affect the behavior of the metal in mine waste, the metabolism of organic carbon by aerobic heterotrophs can affect metal mobility. For example, mineralization of organic carbon generates organic acids that are eventually broken down into CO_2, a process that increases acidity and can cause metals to dissolve from minerals. Calcium carbonate minerals are particularly susceptible to acid attack. Aerobic (O_2) respiration is coupled with organic carbon metabolism and causes the redox potential to decrease under limited diffusion, leading to suboxic or anoxic conditions and potentially altering the solubility of metallic oxide minerals (Levicán et al., 2008).

In addition to requiring energy and carbon to grow, microorganisms need essential nutrients such as nitrogen, phosphorus and sulfur. The biogeochemical cycles of these elements are crucial for all life on Earth. Microbial activity in mine wastes may be limited by the availability of these essential nutrients (Craw & Rufaut, 2017; Rashid et al., 2016). The mechanisms used by microorganisms to obtain essential nutrients can also affect the metal behavior in mine wastes, for example, by secreting chelating agents. Although microorganisms have a number of mechanisms to deal with metal toxicity, metal contamination can affect soil functioning, negatively interfering with microbial activity. Evidence for this is obtained by measuring soil enzymatic activities, along with microbial biomass, basal diversity and substrate respiration rates (Alkorta et al., 2003).

Sulfur metabolic pathway

Sulfur is one of the most abundant elements on Earth, and it has enormous relevance in mining environments involving sulfide metals. Practically the entire process of AMD formation is involved with the chemical changes of sulfur, which presents different oxidation states (from -II to +VI) and chemical forms (amino acid, sulfide, sulfate, etc.) in the environment (Sánchez-Andrea et al., 2014). In this review, the dissimilatory biological processes (oxidation, reduction and disproportion) and assimilation of sulfur will be described (Figure 2).
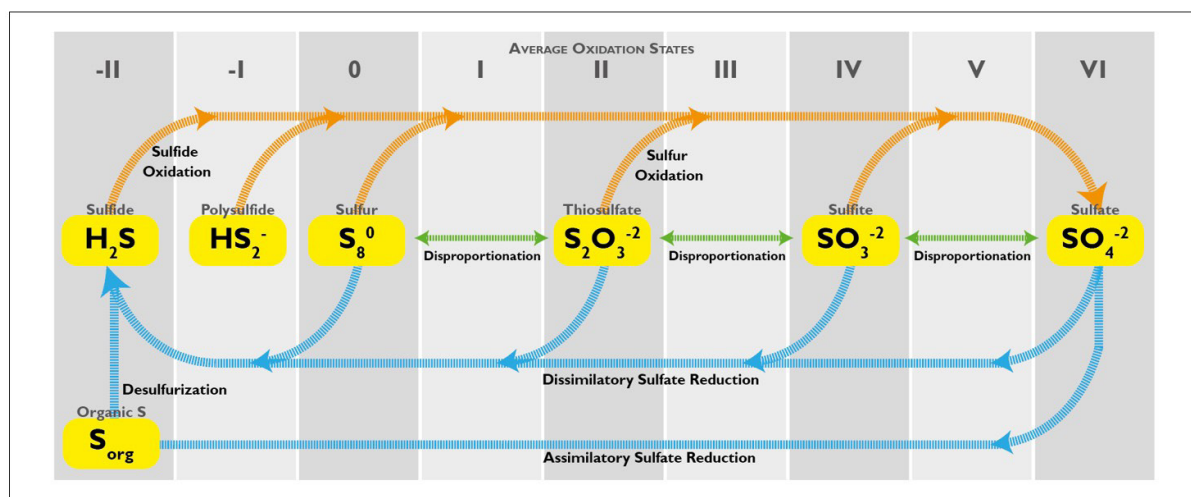

Figure 2. Transformation of sulfur by metabolic pathways mediated by microorganisms, considering different oxidation states, being carried out between inorganic forms of S, converted into organic S. Orange arrows indicate oxidation reactions, blue ones indicates reduction, and green represents disproportionation processes. Adapted from Sánchez-Andrea et al. (2014).

Dissimilatory sulfur processes

The dissimilatory process of sulfur was one of the first energy metabolisms on Earth (Shen et al., 2001). The process changes the valence state of sulfur for energy capture and plays a critical role in the biogeochemical cycle of this element. Based on changes in oxidation states, dissimilatory sulfur processes can be grouped into sulfur oxidation, sulfur reduction, and sulfur disproportion (Fike et al., 2015; Wu et al., 2021).

Oxidation

Sulfur oxidizing microorganisms employ a wide range of genes to mediate the oxidation of sulfide, sulfur, sulfite and thiosulfate conserving energy for cell growth (Wu et al., 2021). The sulfur oxidizing (*sox*) gene cluster is the most studied, and comprises a *sox* gene cluster composed of 15 genes. Seven of these structural genes (*soxXYZABCD*) encode four proteins and complexes (SoxXA, SoxYZ, SoxB and SoxCD) that perform thiosulfate, sulfite, sulfur and sulfide-dependent cytochrome-c reduction (Friedrich et al., 2001). Sulfur dehydrogenase SoxCD, as a key subunit of the Sox complex, is an α2β2 heterotetramer and mediates a unique six-electron oxidative transfer process (Friedrich et al., 2005). Consequently, the Sox complex-catalyzed conversion of thiosulfate to sulfate releases eight electrons, and the released electrons decrease to two when balancing with SoxCD. SoxB is essential for the oxidation of sulfide and thiosulfate, and has been widely used as a phylogenetic marker gene for sulfur oxidizing microorganisms (SOM) (Tourna et al., 2014). However, each of SoxXA, SoxYZ, SoxB and SoxCD alone is catalytically inactive (Wu et al., 2021). Some gammaproteobacterial SOMs have an incomplete Sox system, and perform zero-valence sulfur oxidation using the reverse DSR pathway, common in anaerobic SOMs (Tsallagov et al., 2019). In addition to the *sox* gene cluster, SOMs can employ other genes to mediate sulfite to sulfate oxidation, as it is an energetically favorable process. For example, the sulfite dehydrogenase sorAB (Kappler, 2011), the soluble sulfite dehydrogenase SorT (Hsiao et al., 2018) and the sulfite dehydrogenase Soe (Boughanemi et al., 2020), as well as the indirect oxidation of sulfite to sulfate under the mediation of adenyl sulfate (APS) reductase and ATP sulfurylase (Kappler, 2011).

In addition to sulfur oxidases, other mechanisms catalyze the oxidation of sulfide to sulfur in phototrophic and chemotrophic SOMs, including flavocytochrome-c sulfide dehydrogenase (FCSD) and sulfide-quinone oxidoreductase (Sqr) (Duzs et al., 2018; Friedrich et al., 2001). Another dissimilatory sulfur oxidation pathway is the Hdr (heterodisulfide reductase)-type enzyme complex that can catalyze the oxidation of sulfur to sulfite (Koch & Dahl, 2018). The genes of the *dsr* group also participate in the dissimilatory oxidation of sulfur, where *dsrS* is essential for the oxidation of intracellularly stored sulfur as an obligate intermediate during the oxidation of sulfide and thiosulfate (Grimm et al., 2011). Furthermore, reverse *dsrAB* were also detected in free-living SOMs of the phylum Proteobacteria (Müller et al., 2015). The signature of sulfur oxidizing genes and the *soxB, dsr* and *sqr* genes are used as genetic markers to identify SOMs in environmental samples (Luo et al., 2011).

Since sulfur oxidative processes are among the most important metabolic processes occurring in AMD environments, the bacteria that operate them are among the most common and abundant. *Thiomonas sp.*, *A. ferrooxidans*, and *A. ferrivorans* have been characterized in several mining sites with DAM production (Falagán et al., 2014). Other recurring species are *Acidiphilum cryptum*, *Acidiphillum acidophilum* and *Acidithiobacillus thioxidans* (Bhandari & Choudhary, 2021).

Reduction

Sulfate reduction generally occurs in environments with low redox potential, where oxygen, nitrate, and oxidized metals are less concentrated. In the sulfate reduction process, sulfur-reducing microorganisms (SRMs) use sulfate molecules as electron acceptors. These electrons come from different donors such as hydrogen, methanol, ethanol, volatile fatty acids, sugars and also methane (Liamleam & Annachhatre, 2007).

The first step in sulfate reduction is the transport into the cell, which is also considered a limiting step for the process (Wu et al., 2021). Sulfate is transported across the cytoplasmic membrane via ATP-binding cassette (ABC)-type transporters (SulT family) or main facilitator superfamily (SulP)-type transporters (Kertesz, 2001). Other CysP, DASS and CysZ-type transporters have also been identified as involved in sulfate uptake in SRMs (Marietou et al., 2018). Within the cells of the SRMs, sulfate must first be activated by ATP sulfurylase (encoded by *sat*) to APS, then the APS reductase encoded by *apsAB* catalyzes the reduction of APS to sulfite (Deplancke et al., 2000).

The genes encoding dissimilatory sulfite reductase (Dsr) are "signature" genes in SRMs (Santos et al., 2015; Wu et al., 2021), being composed by *dsrA, dsrB* and *dsrC*. The sulfite is linked to the active site of DsrAB and reduced to the zero-valence sulfur intermediate S (0) linked to DsrC via four electron transfers. Then, the trisulfide bound to DsrC is released from DsrAB, and the S (0) intermediate is further reduced to sulfide by the DsrMKJOP membrane complex. At the same time, DsrC is released from the sulfite reduction process and recycled (Santos et al., 2015).

In addition to the canonical *dsr* genes, there are a variety of other genes involved in sulfur reduction. The *psrABC* (polysulfide encoded reductase) gene cluster, regulated by a global regulator Crp, has been reported in *Shewanella oneidensis* as a mediator in the reduction of thiosulfate to polysulfide (Wu et al., 2015). The Mcc/Sir cytochrome sulfite reductase enzymes allow several bacterial strains of Proteobacteria to perform anaerobic sulfite respiration (Kern et al., 2011). The thiosulfate reductase gene (*phsABC*) in *Salmonella enterica* has been reported to reduce thiosulfate to sulfide (Bang et al., 2000). Oxysulfide reduction genes such as tetrathionate reductase and bifunctional tetrathionate reductase/thiosulfate dehydrogenase TsdA have also been reported (Jenner et al., 2019). A *Sre* gene that can use H_2 to reduce sulfur has been discovered in an archaea (Liu et al., 2012). In another archaea, two genes (*ShyCBDA/SuDH*) that encode two H_2-producing hydrogenases and that are capable of reducing sulfur to sulfide using NADPH as an electron donor have been also reported (Ma et al., 2000).

Among all the genes mentioned above, the most common in microorganisms of AMD is the *dsr*, which coordinates preponderant processes in the metabolism of the SRM. The SRM most commonly recorded in environments contaminated with AMD belong to the genera *Desulfovibrio*, *Desulfosarcina*, *Desulfococcus*, *Desulfobulbus* and *Desulfosporosinus* (Moreau et al., 2010).

Disproportionation

Sulfur disproportionation (SD) is a process in which sulfur, thiosulfate and sulfite act as electron donors and acceptors and generate sulfide and sulfate as end products. Several sulfate-reducing enzymes have been identified as involved in SD, while genes encoding sulfur-oxidizing enzymes are also found in the genomes of sulfur-disproportionation bacteria (SDB) (Finster et al., 2013). The role of PsrA in thiosulfate disproportionation was identified in *Shewanella oneidensis* (Burns & Dichristina, 2009). In contrast, the disproportionation of elemental sulfur is endergonic under standard conditions, and the sulfide produced must be eliminated by metal oxides to keep it in low concentration and make this disproportionation process thermodynamically favorable (Wu et al., 2021). In *Desulfurivibrio alkaliphilus*, sulfide oxidation shows a high level expression of genes that encode a reductive-type *Dsr* and *DsrC* in a disproportional condition, suggesting its fundamental role in SD (Thorup et al., 2017). All these findings were obtained from cultured sulfur dissimilation strains, but no specific functional gene is available as a phylogenetic biomarker of sulfur disproportionation microorganisms.

Several SDB have been identified in AMD environments, such as *Disulfurispira thermophila*, *Caldimicrobium thiodismutans*, *Disulfurimicrobium hydrothermale*, *Disulfuribacter thermophiles*, which offer potential for AMD treatment processes (Zou et al., 2023).

Sulfur assimilation processes

Sulfur assimilatory metabolism is an essential anabolic part of microbial cells, including those found in AMD microorganisms, as sulfur is required to be assimilated into organic compounds as cellular building blocks, e.g. amino acids (Cys and Met), oligopeptides (glutathione/GSH), vitamins (biotin and thiamine) and cofactors (CoA) (Wu et al., 2021). Cys is the central compound for sulfur assimilation and the consequent generation of a variety of downstream metabolites such as Met and GSH. Most microorganisms convert environmental sulfur compounds (e.g. sulfate and thiosulfate) to HS^- via the adenosine 3'-phosphate-5'-phosphosulfate pathway (Cooper, 1983) and then synthesize Cys with key enzymes O-acetyl-l-serine sulfhydrylase (CysK) or O-acetyl-l-serine sulfhydrylase B (CysM) (Kawano et al., 2017).

Iron

Iron is found in two valence states, as oxidized ferric iron Fe (III) and reduced ferrous iron Fe (II). Furthermore, it can adopt different spin states (high or low) in both ferric and ferrous forms, depending on the ligand environment. Iron participates in many important biological processes, such as photosynthesis, N_2 fixation, methanogenesis, H_2 production and consumption, respiration, the trichloroacetic acid (TCA) cycle, oxygen transport, gene regulation, and DNA biosynthesis (Andrews et al., 2003). Its biological functionality is almost entirely dependent on its incorporation into proteins, either as a mono- or binuclear species, or in a more complex form as part of iron-sulfur clusters or heme groups. Inserting iron into proteins allows their local environment to be "controlled" so that iron can adopt the necessary redox potential (ranging from -300 to +700 mV), geometry and spin state necessary to fulfill its designated biological function (Andrews et al., 2003). Despite being essential for most living things, iron can be toxic under some aerobic conditions and in high concentrations such those found in AMD environments. Thus, to achieve effective iron homeostasis, organisms must balance their need to efficiently uptake iron from the environment to ensure maintenance of adequate supplies, with careful management of cellular free iron levels to protect against iron-induced toxicity (Andrews et al., 2003; Touati, 2000). In addition to biotic reactions, various abiotic reactions occur depending on thermodynamic and kinetic conditions. Due to redox reactions, dissolution and precipitation of iron-containing minerals can occur, and have a great influence on the behavior of sorption/desorption and co-precipitation/release of various components, such as phosphate and trace metals (Haese, 2006).

The regulation of iron assimilation is complex and involves mechanisms that act in parallel or hierarchically. The main controller in bacteria is the FUR (ferric uptake regulator) protein, which regulates gene expression in response to iron sufficiency or insufficiency (Sandy & Butler, 2009). When iron is in sufficient condition, it binds to FUR, repressing the transcription of these genes. More than 90 genes are controlled by FUR (Chareyre & Mandin, 2018). In general, the products of these genes act in processes related to iron acquisition, such as heme biosynthesis and transport, and the production of storage proteins. The *fur* gene forms an operon with *fldA*, whose product is a flavoprotein that has the function of keeping iron reduced for the regulatory action of FUR (Fillat, 2014). The *fur* gene is self-regulated through a FUR box that lies between it and *fldA*. It can also be induced under H_2O_2 oxidative stress by OxyR, through its own promoter (Fillat, 2014). Furthermore, the entire operon can be induced in oxidative stress due to superoxide by SoxRS. These responses to oxidative stress result in the binding of FUR to iron, reducing the concentration and consequently the toxicity of this element (Touati, 2000).

Many genes are indirectly regulated by FUR, through the 90-nucleotide sRNA (small RNA) called *RyhB*. The production of this sRNA occurs in the absence of iron. In sufficiency, Fe(II)-FUR inhibits the expression of the *ryhB* gene that encodes it (Chareyre & Mandin, 2018). *RyhB* regulates, at traduction level, the expression of more than 50 genes involved in the acquisition and use of iron. This is reflected in the economy of this nutrient in its absence and can be manifested in three ways: (1) in limiting the production of proteins that require iron, such as enzymes of the Krebs cycle and components of the respiratory chain; (2) in the coordination between the systems responsible for the genesis of Fe-S groups, with

Isc replaced by *Suf*; and (3) in the increased production of siderophores (Chareyre & Mandin, 2018).

In addition to global control by FUR, other mechanisms regulate specific processes of iron metabolism. The Feo Fe(II) transport system, for example, is activated in anaerobiosis (Ong et al., 2015). This reflects greater availability of the reduced form of iron in the absence of oxygen. The *fecIR-fecABCDE* genes encode the proteins that carry out the transport of ferric citrate. This set of genes is repressed by Fe(II)-FUR and activated by the FecIR regulatory system in response to the presence of ferric citrate (Krewulak & Vogel, 2008).

The genesis of Fe-S groups is also regulated by a more specific system, where acts the transcriptional regulator IscR - an iron and sulfur protein encoded by the *iscRSUA* operon. In its mature form, containing Fe-S cofactor, FscR acts by inhibiting the expression of the *iscRSUA* operon. On the other hand, in the immature form, without its cofactor, IscR upregulates the *sufABCDSE* operon.

Iron Oxidation

The ferrous ion can be oxidized by various types of microorganisms. Lithotrophic bacteria and archaea can use Fe (II) as a source of energy and reducing power. These prokaryotes can be separated into two groups. The first contains acidophilic species belonging to genera such as *Acidithiobacillus* (Proteobacteria), *Leptospirillum* (Nitrospirae) and *Ferroglobus* (Euryarchaeota) that live in acidic environments such as AMD (Emerson et al., 2010). The second group comprises neutrophilic bacteria that oxidize Fe (II) at neutral pH. These bacteria belong to the classes Betaproteobacteria and Zetaproteobacteria, and include the genera *Gallionella* and *Mariprofundis*; living in freshwater environments, such as streams and aquifers, and marine environments, especially in underwater hot springs (Emerson et al., 2010).

Iron oxidizing microorganisms obtain energy by oxidizing Fe (II) to Fe (III), even under conditions where the chemical oxidation of Fe (II) is very rapid as, for example, in oxygenated waters with pH close to neutral (Hedrich et al., 2011; Ilbert & Bonnefoy, 2013). At pH close to 7, with several potential electron acceptors such as oxygen, some prokaryotes couple Fe (II) oxidation to nitrate reduction and are able to oxidize Fe (II) under microaerophilic or anoxic conditions (Hedrich et al., 2011). The dissimilatory oxidation of Fe (II) is carried out by bacteria and archaea, where some of them are able to fix CO_2 through the RuBP enzyme, and others need the presence of organic carbon to grow (Hedrich et al., 2011; Ilbert & Bonnefoy, 2013).

At low pH (< 4) commonly found in AMD, chemical oxidation of Fe (II) by oxygen occurs very slowly. However, in such low pH environments, the rate of Fe (II) oxidation actually increases with decreasing pH, demonstrating the significant contribution of microbial Fe (II) oxidation to AMD formation (Larson et al., 2014). The redox potential of the O_2-H_2O pair makes oxygen the most favorable electron acceptor to oxidize Fe (II) at low pH (Hedrich et al., 2011). In acidophilic bacteria such as *Acidithiobacillus ferrooxidans*, the iron oxidation systems involve: several cytochromes that conduct electrons from Fe (II) located outside the cell to the electron acceptor located inside; a cytochrome oxidase that reduces the electron acceptor (oxygen); a high potential iron-sulfur protein (HiPIP) and; a rusticianine (Figure 3). In acidophilic archaea, the pathways differ from those found in acidophilic bacteria and include different cytochromes, HiPIP, copper proteins, and oxidases (Ilbert & Bonnefoy, 2013).

Dissimilative iron reduction

The ferric ion, as well as the oxidized forms of sulfur and nitrogen, can be used as the final electron acceptor in anaerobic respiratory processes. Dissimilatory microbial reduction of Fe (III) is a ubiquitous respiratory pathway in the environment and is carried out by a phylogenetically diverse range of bacteria and archaea called metal-reducing microorganisms (Lovley, 2006). It requires an electron donor which can be H_2, or fermentation products from sedimentary organic matter, for example, simple organic acids such as acetate (Lovley et al., 2004). The Fe (II) product can be soluble, sorb on surfaces or form minerals such as magnetite (Fe_3O_4) (Lovley et al., 1987). Metal-reducing microorganisms can be facultative anaerobes (eg, *Shewanella* spp.) that can also grow with alternative electron acceptors, such as oxygen or nitrate, or be obligate anaerobes (eg, *Geobacter* spp.).

In *Shewanella*, electrons enter the respiratory chain via menaquinone (MQ) or NADH dehydrogenase (NDH). From these molecules, electrons are transferred to cytochrome-c tetraheme CymA, located in the cytoplasmic membrane (Schwalb et al., 2003). From CymA, the electrons pass to the periplasmic carriers Cytochrome-C_3 and MtrA (Gordon et al., 2000). The latter can reduce soluble forms of iron present in the periplasm itself. Alternatively, both carriers, Cytochrome C*3* and MtrA, can transfer electrons to two outer membrane proteins, OmcB and an iron reductase; both will possibly act by reducing Fe (III) minerals (Figure 4) (Lies et al., 2005).

In *Geobacter*, electrons are transferred from an NDH via MQ, both in the cytoplasmic membrane, to a periplasmic cytochrome MacA (Dantas et al., 2017). From this carrier, or from the MQ, electrons pass through PpcA, another periplasmic carrier, to outer membrane proteins such as OmcB, OmcE and OmcS, which reduce different forms of iron, whether chelated or insoluble (Figure 4) (Dantas et al., 2017; Liu et al., 2015). These reductions may involve pili as electron conductors, especially in the case of using iron oxides as final acceptors. It is noteworthy that these models of respiratory chains are proposed and not yet definitively established.

Shewanella and *Geobacter* are commonly found in soils and sediments impacted by AMD (Villegas-Plazas et al., 2019). While *Shewanella* is more abundant at extremely acidic environments (pH < 3), *Geobacter* is more abundant at weakly acidic conditions (pH > 4) (Xu et al., 2020).

Under the near-neutral pH conditions commonly found in most environments, Fe (III) oxide minerals are insoluble, therefore, metal-reducing microorganisms must be able to transfer electrons to solid phases through extracellular transport mechanisms. These include direct physical contact between the mineral and C-type cytochromes in the cell membrane or nanowires (Yalcin & Malvankar, 2020), or secretion of extracellular electron transport (Von
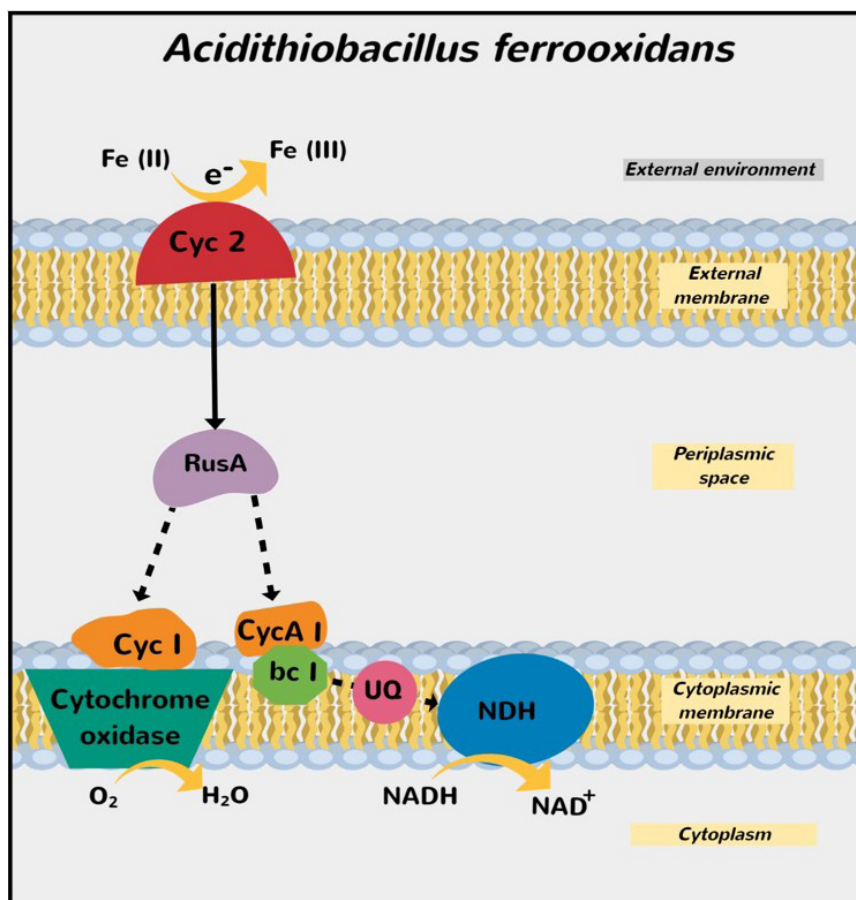
Figure 3. Electron transport chains of *Acidithiobacillus ferrooxidans*,, taking into account a sequence of oxido-reduction reactions. Figure adapted from Ilbert & Bonnefoy (2013). Cyc2: outer membrane embedded cytochrome *c Cyc2*; CycA1: membrane-bound cytochrome *c CycA1*; Cyc1: membrane-bound cytochrome *c Cyc1*; RusA: protein rusticyanin; bc1: cytoplasmic membrane *bc1*; and UQ: ubiquinone.

Canstein et al., 2008). Under acidic conditions found in AMD, high concentrations of Fe (III) are present in solution, and acidophilic microorganisms such as *A. ferrooxidans* can grow by reducing aqueous Fe (III) to Fe (II) coupled with the oxidation of H_2 or S(0), again with type C cytochromes implicated (Das et al., 1992; Ohmura et al., 2002).

Iron biomineralization

As a result of microbial activity, a wide variety of iron minerals can be formed (Konhauser, 1998). There are two mineralization mechanisms associated with microorganisms: biologically induced and biologically controlled mineralization. In the induced biomineralization process, production is associated with the release of metabolites, ions or with the formation of charged structures that act to induce the formation of the mineral. This appears to be the dominant mechanism and occurs in two stages. In the first, the metal in solution reacts with a chemical group in the cell. Then the activation energy required for nucleation is reduced by the metal at the cell surface, and more soluble compounds are produced. Minerals formed in this way include iron oxides and hydroxides, silicates and sulfates.

In the biologically controlled microbial mineralization mechanism, the microorganism dictates the entire process of mineral formation, which occurs inside the cell. An example of this process is the formation of magnetite in magnetosomes. Magnetosomes are inclusions that allow bacteria to guide and align themselves with the Earth's geomagnetic field. It also allows them to seek better growing conditions by aerotaxis and/or chemotaxis in a process called magnetotaxis (Scheffel & Schüler, 2006). For these bacteria, which are microaerophilic or obligate anaerobes, these sites lie just below the transition zone between the oxic and anoxic environments. However, despite the magnetotaxis theory being the most accepted, other functions have been proposed for magnetosomes, such as intracellular detoxification of soluble iron (Scheffel & Schüler, 2006). In AMD, despite the scarcity of oxygen, microaerophilic conditions can occur, and there is full availability of ferrous ion. These conditions are favorable for magnetite formation and magnetotactic bacteria growth, since oxygen acts as an electron acceptor resulting in energy production (Scheffel & Schüler, 2006). Thus, the higher the oxygen concentration, the better the growth, which agreed with the aerobic property of *A. ferrooxidans* (Zhang et al., 2012).
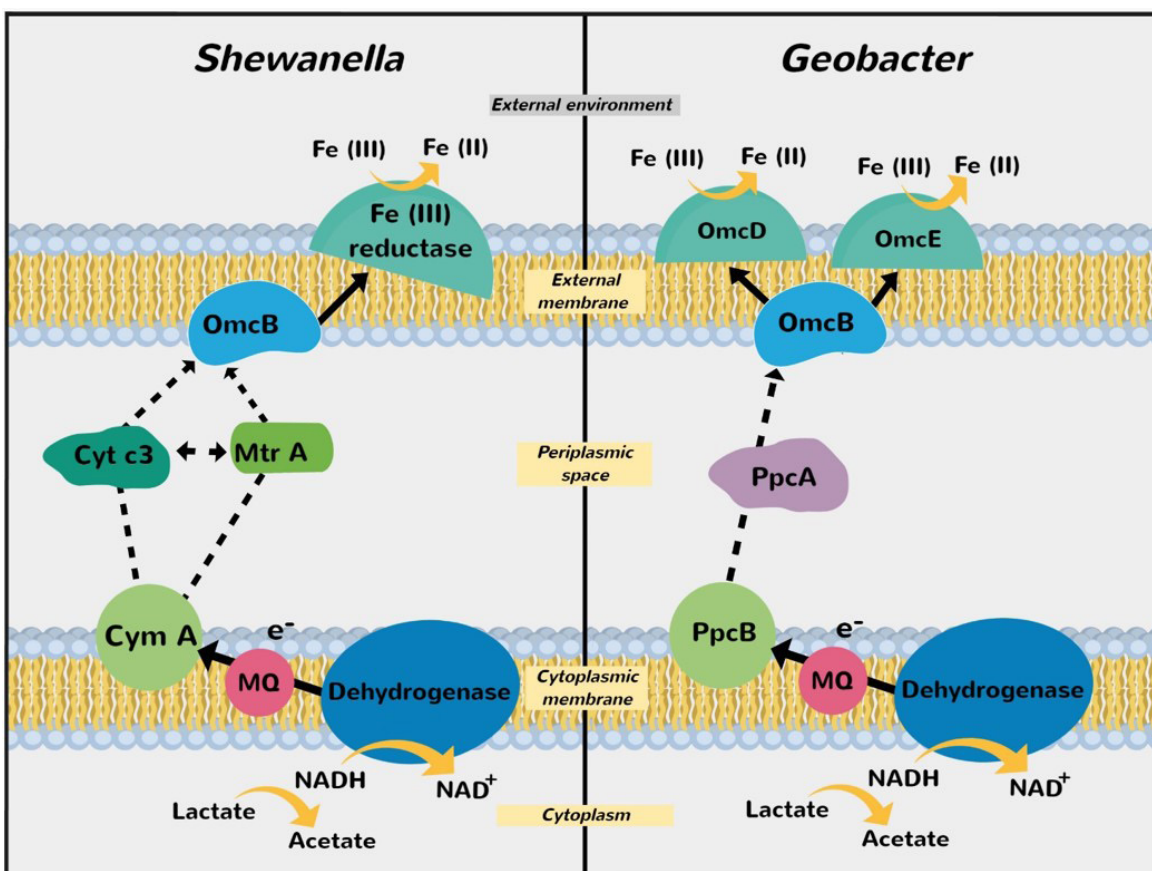
Figure 4. Electron transport chains of iron-reducing organisms. OmcB: outer membrane complex protein *OmcB*; Cyt c3: Cytochrome-C3; Mtr A: decaheme c-type cytochrome *MtrA*; Cym A: cytochrome-c tetraheme *CymA*; OmcD: outer membrane complex protein *OmcD*; OmcE: outer membrane complex protein *OmcE*; PpcA: cytochrome *c* 3 heme-binding sites *PpcA*; PpcB: cytochrome *c* 3 heme-binding sites *PpcB*; And MQ: menaquinone.

Carbon metabolic pathway

Autotrophic microorganisms use the energy and reducing power derived from the oxidation of sulfur and/or iron for various metabolic processes, including CO_2 fixation and acquisition of different nitrogen sources (Levicán et al., 2008). In acidic bioleaching environments, dissolved inorganic carbon can reach levels below average atmospheric concentrations. Therefore, some organisms that inhabit these places have mechanisms to store the existing CO_2 (Dobrinski et al., 2005). The most common pathways of CO_2 fixation used by microorganisms occurring in AMD are the Calvin-Benson-Bassham cycle (CBB), the reductive tricarboxylic acid cycle (rTCA), the 3-hydroxypropionate/4-hydroxybutyrate cycle (3HP/4HB) and the Wood-Ljungdahl (WL) pathway.

The CBB cycle is the most commonly found, and the model microorganism studied is *Acidithiobacillus ferroxidans* that grows in the presence of sulfur using ferric iron as oxidant agent (Cárdenas et al., 2010). *Leptospirillum ferrooxidans*, which is an obligate autotrophic chemolithotroph sulfide oxidant uses the rTCA pathway (Goltsman et al., 2009). Many microorganisms found in AMD environments belong to the Archaea group, from which the thermophilic Sulfolobales that uses the 3HP/4HB cycle to fix carbon has been reported (Hügler & Sievert, 2011; Levicán et al., 2008). In the

3-hydroxypropionate/4-hydroxybutyrate (3HP/4HB) cycle, the key enzyme is acetyl CoA carboxylase (accC), which is present in Sulfolobales (Montoya et al., 2012). The bacterium *Desulfotomaculum acetoxidans* uses the WL reductive pathway to fix CO_2 (Levicán et al., 2008). In the Acetyl-CoA pathway, also known as the Wood-Ljungdahl (WL) pathway, the key enzyme is CO dehydrogenase/acetyl-CoA synthase (cdh/acs) (Montoya et al., 2012).

Carbon fixation by the Calvin-Benson-Bassham cycle (CBB)

The CBB cycle is composed of 13 enzymatic reactions, of which 12 are involved in the regeneration of ribulose 1,5-bisphosphate (RuBP) and one is responsible for CO_2 fixation catalyzed by ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Levicán et al., 2008). The main CO_2-fixing enzymes in the CBB cycle are Rubisco, Phosphoribulokinase (PRK) and Sedoheptulose 1,7-bisphosphatase (SBP) (Shively et al., 1998), with Rubisco encoded by *cbb* being the key enzyme (Montoya et al., 2012). Canonical forms of Rubisco were reported in the *Acidithiobacillus ferrooxidans* genome, with Rubisco I encoded by two genes (*cbbSL1* and *cbbSL2*) and Rubisco II by one gene (*cbbM*) (Levicán et al., 2008). *CbbR* is a positive regulator of the *cbb* operon that coordinates

the expression of three Rubisco genes (Toyoda et al., 2005). It is likely that the presence of multiple forms and copies of Rubisco genes and a controlled Rubisco expression system allow this bacterium to respond rapidly to environmental changes in CO_2/O_2 concentrations (Levicán et al., 2008).

Carbon fixation by the reductive tricarboxylic acid cycle (rTCA)

The rTCA cycle or also known as the Arnon-Buchanan Cycle, is essentially the tricarboxylic acid cycle in reverse, which leads to the fixation of two CO_2 molecules to the production of one acetyl-CoA molecule. The acetyl-CoA formed is then reduced by carboxylation to pyruvate, from which all other core metabolites can be formed. The four main enzymes that enable TCA cycle reversal and pyruvate formation are ATP citrate lyase (ACL), fumarate reductase (FDR), 2-oxoglutarate ferredoxine oxidoreductase (OGOR) and pyruvate ferredoxine oxidoreductase (POR) (Campbell & Cary, 2004). The key enzyme for the process is ATP citrate lyase (Montoya et al., 2012).

An important reaction in carbon metabolism is the condensation of acetyl-CoA and oxaloacetate to citrate through the TCA cycle. This reaction is catalyzed by citrate synthase; however, in the case of the rTCA cycle, the reverse reaction is catalyzed by ATP citrate lyase (ACL). ACL is a key enzyme of the rTCA cycle and is unique to organisms that utilize the rTCA cycle. Pyruvate produced from the rTCA cycle is targeted for gluconeogenesis for the biosynthesis of various carbonate intermediate molecules required by the cell. The anabolic conversion of pyruvate to phosphoenolpyruvate (PEP) is typically catalyzed by phosphoenolpyruvate synthetase (PEPS), while the catabolic conversion of PEP to pyruvate is catalyzed by pyruvate kinase (PK). The combined and coordinated action of PEPS and PK allows the cell to control the interconversion of pyruvate and phosphoenolpyruvate according to its metabolic needs (Levicán et al., 2008). Furthermore, in several organisms, including bacteria and archaea, phosphoenolpyruvate dikinase (PPDK) has been reported to interconvert these metabolites (Levicán et al., 2008; Tjaden et al., 2006).

Molecular mechanisms involved in CO_2 concentration

Carbon concentration mechanisms are present in many species of chemolithoautotrophic bacteria, allowing them to grow in the presence of low concentrations of CO_2. They mainly use bicarbonate transporters and CO_2 retention mechanisms to generate high intracellular concentrations of dissolved inorganic carbon (Levicán et al., 2008). Inorganic carbon transporters that provide intracellular HCO_3 represent an important mechanism of carbon concentration in cyanobacteria (Badger & Price, 2003). The carboxysome is a polyhedral microcompartment located in the cytoplasm of most autotrophic bacteria and is surrounded by a protein monolayer that supposedly contains Rubisco and carbonic anhydrase (Badger & Price, 2003). Carbonic anhydrase converts the accumulated cytosolic HCO_3 into CO_2 inside the carboxysome, increasing the CO_2 concentration in the vicinity of Rubisco (Price et al., 2004). Likewise, seven candidate genes potentially involved in carboxysome

formation were identified downstream of the *cbbLS1* genes in *Acidithiobacillus*, which is followed by the *cbbQO* genes, which are involved in the post-translational regulation of Rubisco (Cannon et al., 2003).

Methane production in AMD environments

Biological production of methane (methanogenesis) is common in AMD environments, being carried out mainly by a group of strictly anaerobic methanogenic archea. The steps that lead to the transformation of CO_2 into CH_4 form a set of cascading reactions, which are used partially or totally with other substrates (Deppenmeier & Müller, 2008; Liu & Whitman, 2008; Thauer et al., 2008). These pathways utilize several unusual coenzymes, of which methanefuran (MF), tetrahydromethanepterin (H4MPT), tetrahydrosarcinapterin (H4SPT) and coenzyme M (or HS-CoM) carry the carbon fraction destined to generate methane, while coenzyme F420 (a derivative of deazaflavin), coenzyme B (HS-CoB or HS-HTP), methanophenazine and coenzyme F430 (a tetrapyrrole) transfer electrons that are used in carbon reduction (Deppenmeier & Müller, 2008; Thauer et al., 2008). Direct substrates for methanogenesis are H_2 plus CO_2, acetate, formate, methylamines, methanol, methyl sulfides and ethanol or a secondary alcohol plus CO_2 (Purwantini et al., 2014). Methanogenesis facilitates the mineralization of biopolymers such as carbohydrates, proteins and lipids in numerous anaerobic niches in nature, making methanogens critical players in the global carbon cycle (Purwantini et al., 2014).

Sulfate reducing bacteria (SRB) normally outcompete methanogenic archaea for acetate and hydrogen (Sanz et al., 2011). This preferential use of methanogenic substrates may initially inhibit methanogenesis in iron-rich freshwater sediments (Roden & Wetzel, 2003). However, the reduction of Fe(III) in anoxic sediments leads to a decrease in the redox potential and an increase in pH, generating favorable conditions for methanogenesis, which can often occur in microniches (black bands) common in sediments of AMD (Sanz et al., 2011). Thus, SRBs tend to be more common and widely distributed, both in water and sediments associated with AMD. The methanogenic archaea, on the other hand, are more restricted to sediments, with a more heterogeneous distribution.

Nitrogen metabolic pathways

The main nitrogen transformations that occur at AMD environments include nitrogen fixation, ammonification, nitrification and denitrification.

Nitrogen fixation

AMD environments normally receive a limited amount of carbon and nitrogen from external sources, and therefore, the fixation of atmospheric CO_2 and N_2 by microorganisms becomes crucial for the maintenance of these nutrients (Méndez-García et al., 2015; Tyson et al., 2005). Nitrogen fixation is, with few exceptions, mediated by the Mo-Fe nitrogenase

enzyme complex, whose activity is sensitive to the presence of oxygen. The enzymatic structural components are encoded by the *nif* operon (*nifHDKENX* genes) (Méndez-García et al., 2015). The genes that flank this operon (regulators, transporters, oxygen/redox sensors) may also be involved in nitrogen fixation (Parro & Moreno-Paz, 2003; Tyson et al., 2005).

The main N2 fixing microorganisms identified in AMD environments are *Acidithiobacillus ferrooxidans*, *Leptospirillum ferrooxidans*, *Leptospirillum ferrodiazotrophum*, and *Ferrovum myxofaciens*. *Acidithiobacillus ferrooxidans* is abundant in natural environments associated with pyritic ore bodies, coal deposits, and their acidified drainages (Valdés et al., 2008). *Leptospirillum ferrooxidans* occurs in minerals, soils, sediment and water associated to AMD (Parro & Moreno-Paz, 2003). *Leptospirillum ferrodiazotrophum* was found in a biofilm growing in pH 0.8, 37˚C, metal rich acid mine drainage (Tyson et al., 2005). Finally, *Ferrovum myxofaciens* is an acidophile common in AMD flows (Johnson et al., 2014).

Despite these occurrences, most members of the AMD community do not fix nitrogen and must obtain it through ammonium absorption (Méndez-García et al., 2015). Even *A. ferrooxidans*, which is capable of fixing atmospheric nitrogen, contains genes predicted to be involved in ammonium uptake (*amt1*, *amt2* and *amtB*) and a gene encoding a protein that incorporates ammonium into glutamine (Valdés et al., 2008). In fact, it is difficult to say which is the main source of nitrogen in microbial communities of AMD, but the discovery of new genes associated with nitrogen fixation, many of them detected in non-cultivable species. Dai et al. (2014), has increased the sense that fixation can be the most important way.

Nitrification

Ammonium nitrogen can be assimilated directly or after nitrification. Nitrifying microorganisms use molecular oxygen as the terminal electron acceptor, and are sensitive to low pH due to lack and/or toxicity of substrates at these pH values (Jiang & Bakken, 1999). Therefore, the occurrence of nitrifiers in AMD sites strictly depends on the availability of O_2. The two main enzymes involved in ammonium oxidation are ammonium monooxygenase encoded by the *amoCAB* operon (*AmoA* contains the putative active site of the enzyme) and hydroxylamine oxidoreductase encoded by the gene *hao* (Méndez-García et al., 2015). Also, *nxrB* gene encodes the beta subunit of the nitrite oxidoreductase enzyme (Ramanathan et al., 2017).

Despite the limitation that the acidic and metal-rich environment of AMD imposes on the nitrification process, several of the genes mentioned above have already been found in microorganisms present in these environments. These microorganisms can be classified into ammonia oxidizing archaea (AOA), such as the genera *Nitrososphaera*, *Nitrosotalea* and *Nitrosoarchaeum*; ammonia oxidizing bacteria (AOB); such as *Nitrosomonas* and *Nitrosospira* and nitrite oxidizing bacteria (NOB); such as *Nitrospira* (Ramanathan et al., 2017).

Denitrification

Nitrate or nitrite ions can be used as terminal electron acceptors under anoxic or low oxygen (denitrification) conditions. A study on acidic stream sediments with heavy metals suggested that denitrification occurs and can reduce acidity (Baeseman et al., 2006). Denitrification is an active and integral part of the overall metabolism in AMD systems (Xie et al., 2011). Example of AMD denitrifying microorganisms are *Sulfurimonas*, *Thiobacillus* and *Dechloromonas*, which operate in collaboration being responsible for the nitrate removal via complete nitrate respiration even at low pH and high concentrations of ferric iron (Chen et al., 2021).

Ammonification

Ammonification occurs via nitrate reduction followed by nitrite ammonification, and the enzymes involved include *Nas*, *Nar* and *Nap* (nitrate reductases) and *Nir* and *Nrf* (nitrite reductases). In AMD environments, ammonification activity was related to the bacterium *Leptospirillum ferriphilum* "group II" based on the observation of a protein of the cytochrome-c family *NapC*/*NirT* involved in the respiratory ammonification of nitrite (Méndez-García et al., 2015). *Leptospirillum* spp. "group II" and "group III" have genes for a nitrite/sulfite reductase, which are necessary for the assimilatory ammonification of nitrite, being directly channeled to the biosynthesis of amino acids (Goltsman et al., 2009). Once ammonium enters cells, it is assimilated via the glutamine/glutamate synthase (GS/GOGAT) pathway, which appears to be absent in *Leptospirillum*, where ammonium assimilation can occur via the GS pathway similar to that proposed for *A. ferrooxidans* (Tyson et al., 2005; Valdés et al., 2008). Most members of the AMD community do not fix nitrogen and must obtain it through the absorption of ammonium.

Final considerations

The generation of AMD results in an environment with low pH and high concentrations of heavy metals and limited access to nutrients. To inhabit and survive these environments, microorganisms need a metabolism capable of obtaining energy from sulfur, iron and limited sources of carbon and nitrogen. Biodiversity and growth rates of these microorganisms are low, but the multi-stressor condition of AMD environments has evolved a diversity of homeostasis and detoxification mechanisms, making their genomes strategic for bioremediation studies, in addition to other biotechnological approaches like biomining and bioprospection of biomolecules with industrial interest. Several of these mechanisms can be applied to mitigate environmental impacts in mining environments.

It is also important to point out that the AMD environments, which are abundant and impactful around the world, cannot be seen as a matrix where only geochemical processes operate, but rather as an environment where microorganisms are great mediators of biogeochemical transformation processes. The extremophile microbiota that lives in these environments present metabolic modes that go back to the times of the origin of life and almost unthinkable adaptations within conventional microbiology.

Conflicts of interest

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