CHAPTER 1

MICROORGANISMS INVOLVED IN BIOLEACHING AND NUCLEIC ACID-BASED MOLECULAR METHODS FOR THEIR IDENTIFICATION AND QUANTIFICATION

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1. INTRODUCTION

Bioleaching is the biological conversion of an insoluble metal compound into a water soluble form. In case of metal sulfide bioleaching, metal sulfides are oxidized to metal ions and sulfate by aerobic, acidophilic Fe(II) and/or sulfur-compound oxidizing Bacteria or Archaea. Bioleaching involves chemical and biological reactions. Despite molecular oxygen being the final electron acceptor for the overall metal sulfide bioleaching process, Fe(III) ions are the relevant oxidizing agent for the metal sulfides. The metal sulfide oxidation itself is a chemical process in which Fe(III) ions are reduced to Fe(II) ions and the sulfur moiety of the metal sulfide is oxidized to sulfate, and various intermediate sulfur compounds, e.g. elemental sulfur, polysulfide, thiosulfate, and polythionates. For example the oxidation of sphalerite (ZnS) to elemental sulfur is given in the following equation:

$$(1) \qquad ZnS + 2Fe^{3+} \rightarrow Zn^{2+} + 0.125S_8 + 2Fe^{2+}$$

Because of two different groups of metal sulfides exist, two different metal sulfide oxidation mechanisms have been proposed, namely the thiosulfate mechanism (for acid-insoluble metal sulfides, such as pyrite) and the polysulfide mechanism (for acid-soluble metal sulfides, e.g. sphalerite or chalcopyrite, $CuFeS_2$). These mechanisms explain the occurrence of all inorganic sulfur compounds which have been detected in the course of metal sulfide oxidation (for review see: Sand *et al.*, 2001; Rohwerder *et al.*, 2003; Schippers, 2004; Chapter 2).

The role of the microorganisms in the bioleaching process is to oxidize the products of the chemical metal sulfide oxidation (Fe(II) ions and sulfur- compounds) in order to provide Fe(III) and protons, the metal sulfide attacking agents. In addition, proton production keeps the pH low and thus, the Fe ions in solution.

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Aerobic, acidophilic Fe(II) oxidizing Bacteria or Archaea provide Fe(III) by the following equation:

$$(2) \qquad 2Fe^{2+} + 0.5O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O$$

Aerobic, acidophilic sulfur-compound oxidizing Bacteria or Archaea oxidize intermediate sulfur compounds to sulfate and protons (sulfuric acid). Most relevant is the oxidation of elemental sulfur to sulfuric acid since elemental sulfur can only be biologically oxidized under bioleaching conditions:

$$(3) \qquad 0.125S_8 + 1.5O_2 + H_2O \rightarrow SO_4^{2-} + 2H^+$$

The sulfur-compound oxidizing Bacteria or Archaea produce protons which dissolve metal sulfides besides pyrite which is not acid-soluble. Pyrite is only attacked by Fe(III) ions (not by protons) and therefore only dissolved by Fe(II) oxidizing Bacteria or Archaea.

This book chapter gives an update of previous excellent reviews on microorganisms involved in bioleaching (e.g. Harrison, 1984; Rossi, 1990; Rawlings, 1997, 2002; Johnson, 1998; Hallberg & Johnson, 2001). In the first part of this chapter, the metal sulfide oxidizing microorganisms are described. In the second part, acidophilic microorganisms which do not oxidize metal sulfides and their importance for bioleaching are reviewed. In the third part, nucleic-acid based methods for the identification and quantification of these microorganisms are introduced.

2. METAL SULFIDE OXIDIZING MICROORGANISMS

The most described acidophilic metal sulfide oxidizing microorganisms belong to the mesophilic and moderately thermophilic Bacteria. The Archaea are usually extremely thermophilic (besides the genus *Ferroplasma*). Most industrial heap and tank bioleaching operations run below 40 °C but operations at higher temperatures promise higher reaction rates (Olson *et al.*, 2003; Batty & Rorke, 2006). All acidophilic metal sulfide oxidizing microorganisms oxidize Fe(II) and/or sulfur compounds. Most of these microorganisms fix CO_2 and grow chemolithoautotrophically. A list of the metal sulfide oxidizing Bacteria or Archaea, their phylogeny and some of their physiological properties is given in the Tables 1–3.

The organisms can be separated in three groups according to their temperature optimum for growth: Mesophiles up to ~ 40 °C, moderate themophiles between ~40 – ~55 °C, and extreme thermophiles between ~55 – ~80 °C.

2.1. Mesophilic and Moderately Thermophilic Bacteria

2.1.1. Proteobacteria

***Acidithiobacillus* spp.** The genus *Acidithiobacillus* was proposed by Kelly & Wood (2000) after reclassification of some species of the genus *Thiobacillus*. The affiliation of the genus *Acidithiobacillus* to the β- or γ-Proteobacteria is not clearly

Table 1. Phylogeny of metal sulfide oxidizing, acidophilic microorganisms

Species[#]	Phylum	G+C (mol%)
Mesophilic and moderately thermophilic Bacteria		
Acidimicrobium ferrooxidans	Actinobacteria	67-69
Acidithiobacillus albertensis	Proteobacteria	61.5
Acidithiobacillus caldus	Proteobacteria	63-64
Acidithiobacillus ferrooxidans	Proteobacteria	58-59
Acidithiobacillus thiooxidans	Proteobacteria	52
Alicyclobacillus disulfidooxidans	Firmicutes	53
Alicyclobacillus tolerans	Firmicutes	49
"*Caldibacillus ferrivorus*"	Firmicutes	51
"*Ferrimicrobium acidiphilum*"	Actinobacteria	51-55
Leptospirillum ferriphilum	Nitrospira	55-58
"*Leptospirillum ferrodiazotrophum*"	Nitrospira	na
Leptospirillum ferrooxidans	Nitrospira	52
Sulfobacillus acidophilus	Firmicutes	55-57
"*Sulfobacillus montserratensis*"	Firmicutes	52
Sulfobacillus sibiricus	Firmicutes	48
Sulfobacillus thermosulfidooxidans	Firmicutes	48-50
Sulfobacillus thermotolerans	Firmicutes	48
"*Thiobacillus plumbophilus*"	Proteobacteria	66
"*Thiobacillus prosperus*"	Proteobacteria	64
Thiomonas cuprina	Proteobacteria	66-69
Mesophilic and moderately thermophilic Archaea		
"*Ferroplasma acidarmanus*"	Euryarchaeota	37
Ferroplasma acidiphilum	Euryarchaeota	36.5
"*Ferroplasma cupricumulans*"	Euryarchaeota	na
Extremely thermophilic Archaea		
Acidianus brierleyi	Crenarchaeota	31
Acidianus infernus	Crenarchaeota	31
Metallosphaera hakonensis	Crenarchaeota	46
Metallosphaera prunae	Crenarchaeota	46
Metallosphaera sedula	Crenarchaeota	45
Sulfolobus metallicus	Crenarchaeota	38
Sulfolobus yangmingensis	Crenarchaeota	42
Sulfurococcus mirabilis	Crenarchaeota	$\sim$ 44
Sulfurococcus yellowstonensis	Crenarchaeota	45

[#]Listed in alphabetical order; G + C = mole% guanine+cytosine content of genomic DNA; na = data not available; species without standing in nomenclature (http://www.bacterio.cict.fr/) are given in quotation marks

defined in the literature (Lane *et al.*, 1992; McDonald *et al.*, 1997; Kelly & Wood, 2000; Hallberg & Johnson, 2001). Species of the genus *Acidithiobacillus* are obligately acidophilic (pH $<$ 4.0), Gram-negative, motile rods. CO_2 is fixed by means of the Benson-Calvin Cycle. The genus comprises the following species: *At. ferrooxidans, At. thiooxidans, At. caldus,* and *At. albertensis*.

Table 2. Optimum and range of growth for pH and temperature of metal sulfide oxidizing, acidophilic microorganisms

Species[#]	pH optimum	pH minimum-maximum	Temperature optimum (°C)	Temperature minimum-maximum (°C)
Mesophilic and moderately thermophilic Bacteria				
Acidimicrobium ferrooxidans	~ 2	na	45-50	<30-55
Acidithiobacillus albertensis	3.5-4.0	2.0-4.5	25-30	na
Acidithiobacillus caldus	2.0-2.5	1.0-3.5	45	32-52
Acidithiobacillus ferrooxidans	2.5	1.3-4.5	30-35	10-37
Acidithiobacillus thiooxidans	2.0-3.0	0.5-5.5	28-30	10-37
Alicyclobacillus disulfidooxidans	1.5-2.5	0.5-6.0	35	4-40
Alicyclobacillus tolerans	2.5-2.7	1.5-5	37-42	<20-55
"*Caldibacillus ferrivorus*"	1.8	na	45	<35->55
"*Ferrimicrobium acidiphilum*"	2-2.5	1.3-4.8	37	<10-45
Leptospirillum ferriphilum	1.3-1.8	na	30-37	na-45
"*Leptospirillum ferrodiazotrophum*"	na	<1.2<	na	<37<
Leptospirillum ferrooxidans	1.5-3.0	1.3-4.0	28-30	na
Sulfobacillus acidophilus	~ 2	na	45-50	<30-55
"*Sulfobacillus montserratensis*"	1.6	0.7->2	37	<30-43
Sulfobacillus sibiricus	2.2-2.5	1.1-3.5	55	17-60
Sulfobacillus thermosulfidooxidans	~ 2	1.5-5.5	45-48	20-60
Sulfobacillus thermotolerans	2-2.5	1.2-5	40	20-60
"*Thiobacillus plumbophilus*"	na	4.0-6.5	27	9-41
"*Thiobacillus prosperus*"	~ 2	1.0-4.5	33-37	23-41
Thiomonas cuprina	3.5-4	1.5-7.2	30-36	20-45
Mesophilic and moderately thermophilic Archaea				
"*Ferroplasma acidarmanus*"	1.2	<0-1.5	42	23-46
Ferroplasma acidiphilum	1.7	1.3-2.2	35	15-45
"*Ferroplasma cupricumulans*"	1-1.2	0.4-1.8	54	22-63
Extremely thermophilic Archaea				
Acidianus brierleyi	1.5-2.0	1-6	~ 70	45-75
Acidianus infernus	~ 2	1-5.5	~ 90	65-96
Metallosphaera hakonensis	3	1-4	70	50-80
Metallosphaera prunae	2-3	1-4.5	~ 75	55-80
Metallosphaera sedula	2-3	1-4.5	75	50-80
Sulfolobus metallicus	2-3	1-4.5	65	50-75
Sulfolobus yangmingensis	4	2-6	80	65-95
Sulfurococcus mirabilis	2-2.6	1-5.8	70-75	50-86
Sulfurococcus yellowstonensis	2-2.6	1-5.5	60	40-80

[#]Listed in alphabetical order; na = data not available; species without standing in nomenclature (http://www.bacterio.cict.fr/) are given in quotation marks

Table 3. Physiological properties of metal sulfide oxidizing, acidophilic microorganisms

Species[#]	Pyrite	Oxidation of other *MS	Fe(II) ions	Sulfur	Growth
Mesophilic and moderately thermophilic Bacteria					
Acidimicrobium ferrooxidans	+	na	+	-	F
Acidithiobacillus albertensis	-	+	-	+	A
Acidithiobacillus caldus	-	+	-	+	F
Acidithiobacillus ferrooxidans	+	+	+	+	A
Acidithiobacillus thiooxidans	-	+	-	+	A
Alicyclobacillus disulfidooxidans	+	na	+	+	F
Alicyclobacillus tolerans	+	+	+	+	F
"*Caldibacillus ferrivorus*"	+	na	+	+	F
"*Ferrimicrobium acidiphilum*"	+	na	+	-	H
Leptospirillum ferriphilum	+	+	+	-	A
"*Leptospirillum ferrodiazotrophum*"	na	na	+	na	A
Leptospirillum ferrooxidans	+	+	+	-	A
Sulfobacillus acidophilus	+	+	+	+	F
"*Sulfobacillus montserratensis*"	+	na	+	+	F
Sulfobacillus sibiricus	+	+	+	+	F
Sulfobacillus thermosulfidooxidans	+	+	+	+	F
Sulfobacillus thermotolerans	+	+	+	+	F
"*Thiobacillus plumbophilus*"	-	+	-	+	A
"*Thiobacillus prosperus*"	+	+	+	+	A
Thiomonas cuprina	-	+	-	+	F
Mesophilic and moderately thermophilic Archaea					
"*Ferroplasma acidarmanus*"	+	na	+	-	F
Ferroplasma acidiphilum	+	na	+	-	F
"*Ferroplasma cupricumulans*"	na	+	+	+	F
Extremely thermophilic Archaea					
Acidianus brierleyi	+	+	+	+	F
Acidianus infernus	+	+	+	+	A
Metallosphaera hakonensis	na	+	na	+	F
Metallosphaera prunae	+	+	+	+	F
Metallosphaera sedula	+	+	+	+	F
Sulfolobus metallicus	+	+	+	+	A
Sulfolobus yangmingensis	na	+	na	+	F
Sulfurococcus mirabilis	+	+	+	+	F
Sulfurococcus yellowstonensis	+	+	+	+	F

[#]Listed in alphabetical order; *MS = Metal sulfides other than pyrite; A = autotroph; F = facultative autotroph and/or mixotroph; H = heterotroph; na = data not available ; species without standing in nomenclature (http://www.bacterio.cict.fr/) are given in quotation marks

Acidithiobacillus ferrooxidans (type strain ATCC 23270 = CIP 104768 = DSM 14882). *At. ferrooxidans* (formerly *Thiobacillus ferrooxidans*) was the first described (Colmer & Hinkle, 1947; Temple & Colmer, 1951: Kelly & Wood, 2000) and is the most studied metal sulfide oxidizing organism. *At. ferrooxidans* is an obligate autotrophic, and derives energy from the oxidation of Fe(II) ions, various sulfur compounds, e.g. elemental sulfur, thiosulfate, trithionate, tetrathionate and sulfide, and hydrogen. It has been reported to oxidize arsenopyrite (AsFeS), bornite (Cu_5FeS_4), chalcocite (Cu_2S), chalcopyrite ($CuFeS_2$), covellite (CuS), enargite ($3Cu_2S.As_2S_5$), galena (PbS), millerite (NiS), orpiment (As_2S_3), pyrite (FeS_2), marcasite (FeS_2), sphalerite (ZnS), wurtzite (ZnS), stibnite (Sb_2S_3), pyrrhotite ($Fe_{1-x}S$ (x = 0 to x = 0.2)), and tetrahedrite ($Cu_8Sb_2S_7$), as well as gallium sulfide and the synthetic metal sulfides CdS, CoS, CuS, Cu_2S, FeS, MnS, MoS_2, NiS, PbS, SnS and ZnS (Silver & Torma, 1971; 1974; Sakaguchi *et al.*, 1976; Torma & Gabra, 1977; Torma, 1978; Torma & Sakaguchi, 1978; Tributsch & Bennett, 1981; Lizama & Suzuki, 1991; Bhatti *et al.*, 1993; Pistorio *et al.*, 1994; Tuovinen *et al.*, 1994; Garcia *et al.*, 1995a; 1995b; Schippers *et al.*, 1996; Fowler & Crundwell, 1998; Fowler *et al.*, 1999; Ehrlich, 2002). The ability to oxidize Fe(II) ions (and pyrite) is the key characteristic of this *Acidithiobacillus* species. During growth on sulfur compounds, *At. ferrooxidans* has been observed to accumulate fine sulfur deposits, which are predominantly associated with the cell wall (Hazeu *et al.*, 1988). Models of the electron transport pathways of Fe(II) and sulfur oxidation have recently been updated (Rohwerder *et al.*, 2003; Rawlings, 2005). The organism also grows anaerobically with sulfur compounds or hydrogen as electron donor and Fe(III) ions as electron acceptor (Brock & Gustafson, 1976). N_2-fixation has been demonstrated (Mackintosh, 1978; Norris *et al.*, 1995). Since *At. ferrooxidans* lives in an environment with high metal ion concentrations, it is resistent to many cationic metals, though metals which occur as oxyanions (such as molybdate) tend to be highly toxic (Dopson *et al.*, 2003; Rawlings, 2005). For example, metal or arsenic concentrations whereby metabolic activity of *At. ferrooxidans* still occurs are 84 mM As(III), 800 mM Cu(II), 1071 mM Zn(II), 500 mM Cd(II), and 1000 mM Ni(II). *At. ferrooxidans* strains have been often isolated from different acidic environments. The organism seems to be more abundant in heap leaching environments than in column or tank leaching operations (Groudev & Groudeva, 1993; Schippers *et al.*, 1995; Brierley, 1997; Olson *et al.*, 2003; Rawlings, 2005).

At. ferrooxidans is phylogenetically heterogene. The phenotypically indistinguishable strains of *At. ferrooxidans* are divided into at least four genomovars, and it may turn out that these represent separate species. The acidophilic Fe(II) oxidizing strains SPIII/3 and m-1 (DSM 2392) have shown not be related to the genus *Acidithiobacillus* (Harrison, 1982; Karavaiko *et al.*, 2003; Karsten *et al.*, 2005). Early reports of facultatively autotrophic *At. ferrooxidans* strains are now believed to have been due to the presence of facultatively autotrophic (see *Acidiphilium acidophilum*) or heterotrophic contaminants (*Acidiphilium* sp.), some of which have been difficult to remove, as their presence can stimulate the growth of *At. ferrooxidans* (Harrison, 1984).

Acidithiobacillus thiooxidans (type strain ATCC 19377 = CIP 104597 = DSM 14887 = JCM 3867 = NCIMB 8343) was described by Waksman and Joffe (1922). It grows obligately autotrophically with various sulfur compounds, e.g. elemental sulfur, thiosulfate, and tetrathionate. Growth on the following metal sulfides has been reported: covellite, galena, sphalerite, wurtzite (Lizama & Suzuki, 1991; Pistorio *et al.*, 1994; Curutchet *et al.*, 1995; Garcia *et al.*, 1995a; 1995b; Schippers & Sand, 1999; Pogliani & Donati, 2000). *At. thiooxidans* does not oxidize pyrite (Bacelar-Nicolau & Johnson, 1999). A scheme of the sulfur compound oxidation pathways has recently been presented by Kamimura *et al.* 2005. *At. thiooxidans* strains have been isolated from different acidic environments such as soil, sulfur deposits, and mine waste. The organism occurs in tank bioleaching operations (together with Fe(II) oxidizers, Battaglia *et al.*, 1994; Goebel & Stackebrandt, 1994; Rawlings, 2002, 2005; Rzhepishevska *et al.*, 2005).

Acidithiobacillus caldus (type strain: strain KU = DSM 8584 = ATCC 51756). The species was described by Hallberg and Lindström (1994). Like *At. thiooxidans, At. caldus* grows autotrophically with various sulfur compounds (elemental sulfur, sulfide, sulfite, thiosulfate, and tetrathionate, Hallberg *et al.*, 1996), and not with Fe(II) ions), but has a higher temperature growth optimum at 45 °C, and can also grow mixotrophically with yeast extract or glucose. *At. caldus* has shown to oxidize arsenopyrite (Dopson & Lindström, 1990). *At. caldus* has been found in tank bioleaching operations (together with Fe(II) oxidizers, Rawlings, 2002, 2005; Okibe *et al.*, 2003), and other acidic environments suchs as geothermal sites and acid mine drainage. One *Acidithiobacillus* strain has proposed to be a new species ("*Acidithiobacillus cuprithermicus"*) despite phylogenetically closely related to *At. caldus* according to 16S rRNA gene sequence analysis (Karavaiko *et al.*, 2003).

Acidithiobacillus albertensis (type strain ATCC 35403 = DSM 14366) was described by Bryant *et al.* (1983). Although physiologically similar to *At. thiooxidans*, it must be considered as a separate species. The cells have a condensed glycocalyx and, like *At. ferrooxidans*, accumulate internal sulfur deposits. *At. albertensis* was isolated from an acidic soil adjacent to a sulfur stockpile. Unfortunately, the original isolate has been lost (Kelly & Wood, 2000).

"Thiobacillus prosperus" (strain DSM 5130). The species was proposed by Huber and Stetter (1989). Cells are acidophilic, obligately chemolithoautotrophic, Gram-negative, motile rods. They grow aerobically on metal sulfides such as pyrite, sphalerite, chalcopyrite, arsenopyrite and galena, and on Fe(II) ions, elemental sulfur, and H_2S. Thiosulfate, tetrathionate, and the synthetic sulfides Ag_2S, CuS, MoS_2, Sb_2S, SnS and ZnS do not serve as substrate. The halotolerant not validly described species grows at seawater salt concentrations (up to 3.5% NaCl). Concentrations of 0.9 mM Ag, 1.3 mM As, 0.009 mM Cd, 170 mM Co, 16 mM Cu, 0.05 mM Hg, 1 mM Mo, 850 mM Ni, 8 mM Sb, 0.04 mM U, and 1500 mM Zn are tolerated. No significant homology with *Acidithiobacillus* was detected by DNA-DNA hybridization. The organism was originally isolated from a geothermal marine area off Italy.

"Thiobacillus plumbophilus" (strain DSM 6690) was isolated from an uranium mine in Germany by Drobner *et al.* (1992). Cells are strictly aerobic, mesoacidophilic, rod-shaped, obligately chemolithoautotrophic bacteria which are able to grow on H_2S, galena (PbS) or H_2, but not on Fe(II) ions, sulfur, thiosulfate or many other metal sulfides. No significant homology with *Acidithiobacillus* or *Thiomonas* was detected by DNA-DNA hybridization.

Thiomonas cuprina (type strain DSM 5495). The species was originally described by Huber and Stetter (1990) as *Thiobacillus cuprinus (ex),* and reclassified by Moreira and Amils (1997). The newly described genus *Thiomonas* contains many of the facultatively autotrophic members of the previously classified as *Thiobacillus* spp. Strains of *Thiomonas cuprina* are motile, Gram-negative, facultatively chemolithotrophic and mixotrophic, moderately acidophilic rods capable of growth on some metal sulfides including arsenopyrite, galena, sphalerite, chalcopyrite, and synthetic CdS and FeS, as well as on H_2S and elemental sulfur. No growth was obtained on thiosulfate, tetrathionate, Fe(II) ions, pitch blend or the following metal sulfides: bornite, chalcocite, covellite, pyrite, cinnabar, and synthetic Ag_2S, CuS, MoS_2, Sb_2S, SnS and ZnS. Organotrophic growth occurs e.g. on yeast extract, peptone and pyruvate. Resistance to arsenic and heavy metals during growth on ore was observed up to 1.3 mM As, 0.09 mM Cd, 170 mM Co, 7.9 mM Cu, 1 mM Mo, 170 mM Ni, 0.04 mM U, and 150 mM Zn. *Thiomonas cuprina* was isolated from solfatara fields in Iceland and an uranium mine waste heap in Germany.

2.1.2. Nitrospira

***Leptospirillum* spp.** The genus *Leptospirillum* was validly described by Hippe in 2000, though the first bacteria of this genus were isolated and described by Markosyan in 1972. Phylogentically, the genus belongs to the phylum Nitrospira. *Leptospirillum* spp. are obligate acidophilic (pH < 4.0) and aerobic. Cells are Gram-negative, motile vibroid to spirilla-shaped, but cocci or pseudococci can be formed. They grow obligately chemolihoautotrophically and derive energy only from the oxidation of Fe(II) ions but not from sulfur compounds. CO_2 is fixed by means of the Benson-Calvin Cycle (Balashova *et al.*, 1974; Tyson *et al.*, 2004). The oxidation of pyrite, sphalerite and chalcopyrite in pure culture has been reported (Norris, 1983; Sand *et al.*, 1992; Schippers *et al.*, 1996; Okibe & Johnson, 2004). *Leptospirillum* spp. are regularly found in bioleaching operations where they oxidize metal sulfides in co-culture with sulfur-oxidizers (Battaglia *et al.*, 1994; Goebel & Stackebrandt, 1994; Rawlings, 2002, 2005; Okibe *et al.*, 2003; Kinnunen & Puhakka, 2004; Rzhepishevska *et al.*, 2005; Hawkes *et al.*, 2006a, 2006b). They also occur in acid mine drainage environments (Schrenk *et al.*, 1998; Bond *et al.*, 2000a, 2000b; González-Toril *et al.*, 2003, 2005). The genus *Leptospirillum* comprises the following species: *L. ferrooxidans, L. ferriphilum, "L. ferrodiazotrophum"* and *L. thermoferrooxidans. L. thermoferrooxidans* was described as a thermophilic organism (Golovacheva *et al.*, 1992; Hippe, 2000). Since *L. thermoferrooxidans*

could oxidize Fe(II) ions but not metal sulfides (pyrite and chalcopyrite) in pure culture, and the culture has been lost, the species is not further considered here.

Leptospirillum ferrooxidans (type strain L15 = ATCC 29047 = DSM 2705 = VKM B-1339). The mesophilic species was isolated by Markosyan (1972) and validly described by Hippe 2000. N_2-fixation has been shown (Norris *et al.*, 1995; Parro *et al.*, 2003). The biochemistry of the Fe(II) ion oxidation is different to that of *Acidithiobacillus ferrooxidans* (Rohwerder *et al.*, 2003; Rawlings, 2005) which allows *L. ferrooxidans* to oxidize Fe(II) ions at a high redox potential (Rawlings *et al.*, 1999). The following metal ion concentrations were tolerated: 7.5 g/L Al, 1 g/L Co, 25 g/L Cu, >30 g/L Mn, 7.5 g/L Ni, and 30 g/L Zn (Hallmann *et al.*, 1992).

Leptospirillum ferriphilum (type strain P3a = ATCC 49881 = DSM 14647). *L. ferriphilum* was described by Coram and Rawlings (2002) as a thermotolerant (up to 45°C) mesophilic Fe(II) ion oxidizer. The organism seems to be dominant in tank bioleaching operations at 35 – 50 °C. It is able to oxidize Fe(II) in high rate at pH < 1 (Kinnunen & Puhakka, 2005). *L. ferriphilum* does not have the nitrogen fixing (*nif*) operon (Tyson *et al.*, 2004) in contrast to *L. ferrooxidans* and *"L. ferrodiazotrophum"*.

"Leptospirillum ferrodiazotrophum" (strain ATCC BAA-1181) was recently isolated from a subsurface acid mine drainage biofilm (Iron Mountain, California, USA, Tyson *et al.*, 2005). Due to its ability to oxidize Fe(II) ions and to fix nitrogen, the name "*L. ferrodiazotrophum"* has been proposed (although *L. ferrooxidans* also fixes nitrogen). The ability to oxidize metal sulfides has not yet been demonstrated for this recently proposed species.

2.1.3. Actinobacteria

Acidimicrobium ferrooxidans (type strain ICP = DSM 10331). *Am. ferrooxidans* was described by Clark & Norris (1996) as a species of the single genus *Acidimicrobium* of the family Acidimicrobiaceae. The strains recognized so far were isolated from a copper leaching dump in the USA (strain TH3; Brierley, 1978; Norris *et al.*, 1996) and a pyrite enrichment from an Icelandic geothermal site (type strain DSM 10331; Norris *et al.*, 1996). The two strains differ in their morphology in that strain TH3 grows in filaments in liquid media. The rod-shaped Gram-positive *Am. ferrooxidans* cells are acidophilic and moderately thermophilic. Autotrophic growth occurs on Fe(II) ions (not on sulfur compounds), heterotrophic growth on yeast extract during which cells are motile. Facultative anaerobic growth via reduction of Fe(III) has been reported (Bridge & Johnson, 1998). Mixed cultures of *Am. ferrooxidans* and *Sulfobacillus* oxidize Fe(II) ions more extensively than either strain does in pure culture (Clark & Norris, 1996).

"Ferrimicrobium acidiphilum" (strain T23). The proposed type strain of this acidophile was isolated from an abandoned mine in north Wales, and similar isolates were obtained from mines in the USA and elsewhere (Johnson *et al.*, 1995, 2001; Johnson & Roberto, 1997). It is closely related to *Am. ferrooxidans* but does not fix CO_2, thus, only grows heterotrophically (e.g. with yeast extract). Cells are motile

rods but may form filaments. The mesophilic, acidophilic, Fe(II) ion and pyrite oxidizing actinobacterium does not grow on sulfur compounds. Mixed cultures of the Fe(II) ion oxidizer *"Ferrimicrobium acidiphilum"* with a sulfur oxidizer (*Acidithiobacillus thiooxidans* or *Acidiphilium acidophilum*) oxidize pyrite in higher rates than pure cultures of *"Ferrimicrobium acidiphilum"* (Bacelar-Nicolau & Johnson, 1999).

2.1.4. Firmicutes

Metal sulfide oxidizing Gram-positive bacteria that have low G+C mol% in their chromosomal DNA belong to the genera *Alicyclobacillus, Sulfobacillus,* and the as yet non-validated genus *"Caldibacillus"*. Physiologically, these bacteria are versatile, being able to grow lithotrophically with Fe(II) ions and/or sulfur compounds and/or organotrophically with different organic substances. Growth may be autotrophic (CO_2-fixation), heterotrophic (e.g yeast extract) or mixotrophic (CO_2 + yeast extract). Endospores may be formed. Mesophilic and moderately thermophilic species have been isolated from sulfidic heaps or thermal springs, many of them are not validly described yet.

Alicyclobacillus disulfidooxidans (type strain SD-11 = ATCC 51911 = DSM 12064). The facultativ anaerobic, mesophilic, physiologically versatile species was originally described as *Sulfobacillus disulfidooxidans* (*ex*) by Dufresne *et al.* (1996) and recently reclassified by Karavaiko *et al.* (2005). It was isolated from an enrichment of wastewater sludge. Cells are rod-shaped and non-motile. Yeast extract is necessary as growth factor for elemental sulfur oxidation. It has been doubted that the original culture was pure. Thus, Fe(II) ions and metal sulfides are probably not oxidized by *Alicyclobacillus disulfidooxidans.*

Alicyclobacillus tolerans (type strain K1 = VKM B-2304 = DSM 16297). The thermotolerant mesophilic, physiologically versatile species was originally described as *"Sulfobacillus thermosulfidooxidans* subsp. *thermotolerans"* by Kovalenko and Malakhova (1983) and recently reclassified by Karavaiko *et al.* (2005). It was isolated from lead-zinc ores of a deposit in Uzbekistan. Cells are rod-shaped and non-motile. Facultative anaerobic growth via reduction of Fe(III) is described.

Sulfobacillus acidophilus (type strain NAL = ATCC 700253 = DSM 10332). *Sb. acidophilus* was described by Norris *et al.* (1996) as a rod-shaped moderately thermophilic organism, isolated from a coal spoil heap, UK. Limited motility was observed. Facultative anaerobic growth via reduction of Fe(III) has been described (Bridge & Johnson, 1998).

"Sulfobacillus montserratensis" (strain L15) was isolated from a geothermal area of the Caribian island Montserrat (Yahya *et al.*, 1999; Johnson, 2001). The mesophilic, motile, rod-shaped bacterium tolerates 500 mM Fe^{2+}, 100 mM Fe^{3+}, 100 mM Cu^{2+}, >300 mM Zn^{2+}, 0.2 mM MoO_4^{2-} and an extremely low pH of 1. Growth on Fe(II) ions and sulfur compounds was enhanced in presence of yeast extract.

Sulfobacillus sibiricus (strain N1 = DSM 17363 = VKM B-2280) was isolated from an ore deposit in east Siberia and described by Melamud *et al.* (2003). The rod-shaped, moderately thermophilic bacterium oxidizes metal sulfides, Fe(II) ions and sulfur in presence of yeast extract.

Sulfobacillus thermosulfidooxidans (type strain AT-1 = DSM 9293 = VKM B-1269). The moderately thermophilic, facultative anaerobic (reduction of Fe(III)) strain was isolated from dumps in Russia and described as the first species of the genus *Sulfobacillus* (Golovacheva & Karavaiko, 1978). Some strains grow as coryneforms. Limited motility was observed. Metal concentrations whereby metabolic activity of *S. thermosulfidooxidans* still occurs are 6 mM Cu(II), 43 mM Zn(II), and 5 mM Ni(II) (Dopson *et al.*, 2003).

Sulfobacillus thermotolerans (type strain Kr1 = VKM B-2339 = DSM17362). Cells are straight to slightly curved rods. The thermotolerant, Gram-positive, aerobic, endospore-forming, acidophilic bacterium was isolated from a gold-recovery plant (Siberia). Growth is mixotrophic by oxidizing Fe(II) ions and sulfur compounds in the presense of yeast extract or other organic substrates (Bogdanova *et al.*, 2006).

"Caldibacillus ferrivorus" (strain GSM) was isolated from mine spoil material, Montana, USA (Johnson *et al.*, 2001). The moderate thermophile strain grows autotrophically and mixotrophically, and is facultative anaerobic (reduction of Fe(III)). As substrates, Fe(II) ions, sulfur compounds, and various organic compounds are used. The strain is phylogenetically closely related to the genus *Alicyclobacillus* (Karavaiko *et al.*, 2005). It appears likely that the genus *"Caldibacillus"* will not therefore be validated.

2.2. Mesophilic and Moderately Thermophilic Archaea

2.2.1. Euryarchaeota

All metal sulfide oxidizing, mesophilic and moderately thermophilic Archaea belong to the genus *Ferroplasma* within the family Ferroplasmaceae of the order Thermoplasmatales of the archaeal phylum Euryarchaeota.

***Ferroplasma* spp.** The genus *Ferroplasma* was first described by Golyshina *et al.*, 2000. Species of the genus *Ferroplasma* are acidophilic Archaea that oxidize Fe(II) ion, pyrite and other metal sulfides. Cells lack a cell wall and are pleomorphic (irregular cocci, varying from spherical to filamentous, forming duplex and triplex forms). In contrast to the original description as aerobic and obligately chemolithoautotrophic cells, growth may also be mixotrophic or organotrophic, and facultatively anaerobic via Fe(III) reduction (Dopson *et al.*, 2004). *Ferroplasma* species are widespread in very acidic mining environments (Bond *et al.*, 2000a, 2000b; Edwards *et al.*, 2000; Burton and Norris, 2000; Johnson & Hallberg, 2003; González-Toril *et al.*, 2003; Golyshina and Timmis, 2005). *Ferroplasma* species are regularly found in bioleaching operations as well (Okibe *et al.*, 2003; Okibe & Johnson, 2004; Rawlings, 2005; Rzhepishevska *et al.*, 2005; Hawkes *et al.*, 2006a).

The genus *Ferroplasma* comprises the following species: *Ferroplasma acidiphilum, "Ferroplasma acidarmanus"* and *"Ferroplasma cupricumulans"*.

Ferroplasma acidiphilum (type strain Y = DSM 12658 = JCM 10970) was isolated from a bioleaching bioreactor in Russia and described by Golyshina *et al.* (2000). Aerobic growth may be lithoautotrophic (Fe(II) ions + CO_2), organoheterotrophic (e.g. on yeast extract) or mixotrophic (Fe(II) and an organic carbon source). Anaerobic growth occurs on Fe(III) in the presence of yeast extract as electron donor (Dopson *et al.*, 2004).

"Ferroplasma acidarmanus" (type strain Fer1) was isolated from the AMD site Iron Mountain (California, USA) by Edwards *et al.* (2000) and described by Dopson *et al.* 2004. The type strain has been deposited in the American Type Culture Collection (ATTC) in the patent pending collection. Cells grow aerobically either organoheterotrophically (e.g. on yeast extract) or mixotrophically on Fe(II) and an organic carbon source, and anaerobically on Fe(III) in the presence of yeast extract as electron donor. Metal or arsenic concentrations whereby metabolic activity of *"F. acidarmanus"*still occurs are 13 mM As(III), 16 mM Cu(II), and 9 mM Cd(II) (Dopson *et al.*, 2003).

"Ferroplasma cupricumulans" (strain BH2 = DSM 16651) was isolated from a chalcocite heap bioleaching operation in Myanmar (Hawkes *et al.*, 2006a, 2006b). Mixotrophic growth with Fe(II) and yeast extract was observed. Cells grow anaerobically on Fe(III) in the presence of tetrathionate and yeast extract as electron donors.

2.3. Extremely Thermophilic Archaea

2.3.1. Crenarchaeota

All extremely thermophilic metal sulfide oxidizing Archaea belong to the family Sulfolobaceae within the order Sulfolobales of the archaeal phylum Crenarchaeota. Members of the Sulfolobales have optimal growth temperatures between 65 and 90°C and pH optima of around pH 2. Cells are motile or non-motile cocci, which occur usually singly or in pairs. Characteristically, they are highly irregular in shape, and often strongly lobed or edged, and stain Gram negatively. They grow either aerobically, facultatively anaerobically, or anaerobically. Under autotrophic conditions they gain energy by oxidation of elemental sulfur, thiosulfate, metal sulfides, or molecular hydrogen (H_2). Alternatively, heterotrophic growth occurs by aerobic respiration or anaerobic sulfur respiration or by fermentation of organic substrates (Segerer & Stetter, 1992; Huber & Stetter, 2001; Huber & Prangishvili, 2004).

Several isolates of Sulfolobales are able to extract metal ions from sulfidic ores. Dissolution of the metal sulfides chalcocite, chalcopyrite, molybdenite, pentlandite, pyrite, pyrrhotite and sphalerite has been reported (Brierley & Murr, 1973; Brierley & Lockwood, 1977; Brierley & Brierley, 1986; Huber *et al.*, 1986; Norris & Parrott, 1986; Norris *et al.*, 1988; Huber *et al.*, 1989; Huber & Stetter, 1991; Tobita *et al.*, 1994; Vitaya *et al.*, 1994; Fuchs *et al.*, 1995; Konishi *et al.*, 1995; Norris *et al.*, 2000).

The metal sulfide oxidizing species belong to the following genera of Sulfolobales: *Acidianus, Metallosphaera, Sulfolobus* and *Sulfurococcus.* Whereas some strains only oxidize metal sulfides weakly, others, e.g. *Acidianus brierleyi*, *Sulfolobus metallicus* and *Metallosphaera* species are very efficient ore leachers. *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus* do not oxidize sulfur compounds including metal sulfides in contrast to several reports (Kargi & Robinson, 1985; Vitaya *et al.*, 1994; Tobita *et al.*, 1994). Either the cultures were not pure or they lost their ability to oxidize sulfur compounds (Marsh *et al.*, 1983; Huber *et al.*, 1989; Grogan, 1989, 1991; Hallberg & Johnson, 2001; Huber & Prangishvili, 2004).

Acidianus brierleyi (type strain DSM 1651 = IFO (now NBRC) 15269 = JCM 8954) was isolated from a solfataric spring in Yellowstone National Park, USA (Brierley & Brierley, 1973), one year after the genus *Sulfolobus* was established by Brock *et al.* (1972). The species was originally described as *Sulfolobus brierleyi* (*ex*) by Zillig *et al.* (1980) and reclassified as *Acidianus brierleyi* by Segerer *et al.* (1986). The facultative anaerobic, facultative chemolithoautotrophic organism uses metal sulfides, elemental sulfur, H_2, and organic compounds as substrates.

Acidianus infernus (type strain So4a = DSM 3191 = IFO (now NBRC) 15270 = JCM 8955) was isolated from a solfataric spring in Italy and described by Segerer *et al.* 1986. The facultative anaerobic, obligate chemolithoautotrophic organism uses metal sulfides, elemental sulfur and H_2 as substrates.

Metallosphaera hakonensis (type strain HO1-1 = IAM 14250 = JCM 8857 = DSM 7519 = ATCC 51241) was originally described as *Sulfolobus hakonensis (ex)* by Takayanagi *et al.* (1996) and reclassified as *Metallosphaera hakonensis* by Kurosawa *et al.* (2003). The species was isolated from hot springs in Hakone, Japan. The aerobic, facultative chemolithoautotrophic organism uses metal sulfides, elemental sulfur, tetrathionate, H_2S, and organic compounds as substrates.

Metallosphaera prunae (type strain Ron 12/II = DSM 10039) was isolated from a smoldering slag heap of an uranium mine in Germany and described by Fuchs *et al.* (1995). The aerobic, facultative chemolithoautotrophic organism uses metal sulfides, elemental sulfur, H_2 and organic compounds as substrates.

Metallosphaera sedula (type strain TH2 = ATCC 51363 = DSM 5348 = IFO (now NBRC) 15509 = JCM 9185) was isolated from a solfataric field in Italy and described by Huber *et al.* (1989). The aerobic, facultative chemolithoautotrophic organism uses metal sulfides (pyrite, chalcopyrite, sphalerite and the synthetic sulfides CdS, SnS, ZnS), elemental sulfur, and organic compounds as substrates. Metal or arsenic concentrations whereby metabolic activity of *M. sedula* still occurs are 1.3 mM As, 0.9 mM Cd, 0.85 mM Co, 16 mM Cu, 0.0005 mM Hg, 0.1 mM Mo, 0.8 mM Sb, 0.4 mM U, and 150 mM Zn.

Sulfolobus metallicus (type strain Kra 23 = DSM 6482 = IFO (now NBRC) 15436 = JCM 9184) was isolated from an Icelandic solfataric field and described by Huber & Stetter (1991). The aerobic, obligate chemolithoautotrophic organism uses metal sulfides (pyrite, chalcopyrite, sphalerite and the synthetic sulfides CdS, ZnS), and elemental sulfur as substrates.

Sulfolobus yangmingensis (type strain YM1) was isolated from a geothermal vent in Yang-Ming National Park in northern Taiwan and described by Jan *et al.* (1999). The aerobic, facultative chemolithoautotrophic organism uses FeS, elemental sulfur, tetrathionate and organic compounds as substrates.

Sulfurococcus mirabilis (type strain INMI AT-59) was isolated from a crater of the Uzon volcano in Kamchatka, Russia. The strain has no deposition number and is preserved at the Institute of Microbiology, Russian Academy of Sciences, Moscow, Russia (Golovacheva *et al.*, 1987a; Golovacheva *et al.*, 1987b). The aerobic, facultative chemolithoautotrophic organism uses metal sulfides, elemental sulfur and organic compounds as substrates.

Sulfurococcus yellowstonensis (type strain Str6kar) was isolated from a thermal spring in Yellowstone National Park, USA. The strain has no deposition number and is preserved at the Institute of Microbiology, Russian Academy of Sciences, Moscow, Russia (Karavaiko *et al.*, 1994). The aerobic, facultative chemolithoautotrophic organism uses metal sulfides, Fe(II) ions, elemental sulfur and organic compounds as substrates.

3. ACIDOPHILIC MICROORGANISMS THAT DO NOT OXIDIZE METAL SULFIDES

Most of the metal sulfide oxidizers described in the previous chapter are lithoautotrophic microorganisms. Some metal sulfide oxidizers grow facultatively autotrophically, mixotrophically, or heterotrophically, such as *Acidithiobacillus caldus*, *Thiomonas cuprina*, *Acidimicrobium ferrooxidans*,*"Ferrimicrobium acidiphilum"*, Firmicutes, *Ferroplasma*, and several extremely thermophilic Archaea. In this chapter further acidophilic microorganisms are introduced. Most of them grow heterotrophically. Some of them oxidize sulfur compounds, thus, have the potential to oxidize acid-soluble metal sulfides. However, since the ability to oxidize metal sulfides has not been demonstrated for these species, they are introduced in this chapter. The phylogenetic affiliation and physiological properties of the non-metal sulfide oxidizing, mainly heterotrophic, acidophilic Bacteria and Archaea are listed in Table 4 and 5 .

3.1. Mesophilic Bacteria

These bacteria are heterotrophs and belong to the genera *Acidiphilium* (Harrison, 1981; Kishimoto *et al.*, 1996), *Acidocella* (Kishimoto *et al.*, 1996), *Acidomonas* (Urakami *et al.*, 1989; Yamashita *et al.*, 2004), *Acidisphaera* (Hiraishi *et al.*, 2000), and *Acidobacterium* (Kishimoto *et al.*, 1991). The species have mostly been isolated from acidic mining environments or cultures of *Acidithiobacillus ferrooxidans.*

The mesoacidophilic, heterotrophic Bacteria are Gram-negative, and grow as motile or non-motile rod-shaped cells. They oxidize various organic substrates but not Fe(II) ions. Some of them also oxidize sulfur compounds such as *Acidiphilium*

Table 4. Phylogeny of acidophilic microorganisms that do not oxidize metal sulfides (mainly heterotrophs)

Species[#]	Phylum	G+C (mol%)	Sulfur oxidation
Mesophilic Bacteria			
Acidiphilium acidophilum	Proteobacteria	63-64	+
Acidiphilium angustum	Proteobacteria	67	-
Acidiphilium cryptum	Proteobacteria	64-70	+
Acidiphilium multivorum	Proteobacteria	66-68	-
Acidiphilium organovorum	Proteobacteria	64	-
Acidiphilium rubrum	Proteobacteria	63	+
Acidisphaera rubrifaciens	Proteobacteria	69-70	-
Acidobacterium capsulatum	Acidobacteria	60	-
Acidocella aminolytica	Proteobacteria	59	-
Acidocella facilis	Proteobacteria	65	-
Acidomonas methanolica	Proteobacteria	63-65	-
Moderately thermophilic Bacteria			
Acidicaldus organivorans	Proteobacteria	72	+
Alicyclobacillus acidiphilus	Firmicutes	54	na
Alicyclobacillus acidocaldarius	Firmicutes	61-63	-
Alicyclobacillus acidoterrestris	Firmicutes	53	-
Alicyclobacillus cycloheptanicus	Firmicutes	54-57	-
Alicyclobacillus herbarius	Firmicutes	56	na
Alicyclobacillus hesperidum	Firmicutes	53-54	na
Alicyclobacillus pomorum	Firmicutes	53	na
Alicyclobacillus sendaiensis	Firmicutes	62	na
Alicyclobacillus vulcanalis	Firmicutes	62	-
Hydrogenobaculum acidophilum (autotroph)	Aquificae	35	+
Archaea			
Acidianus ambivalens (autotroph)	Crenarchaeota	33	+
Picrophilus oshimae	Euryarchaeota	36	-
Picrophilus torridus	Euryarchaeota	na	-
Sulfolobus acidocaldarius	Crenarchaeota	37	-
Sulfolobus shibatae	Crenarchaeota	35	+
Sulfolobus solfataricus	Crenarchaeota	35	-
Sulfolobus tokodaii	Crenarchaeota	33	+
Sulfolobus yangmingensis	Crenarchaeota	42	+
Sulfurisphaera ohwakuensis	Crenarchaeota	33	+
Thermoplasma acidophilum	Euryarchaeota	46	-
Thermoplasma volcanicum	Euryarchaeota	38-40	-

[#]Listed in alphabetical order; G + C = mole% guanine+cytosine content of genomic DNA; na = data not available

acidophilum (formerly *Thiobacillus acidophilus* (*ex*), reclassified by Hiraishi *et al.* (1998)), *Acidiphilium crytum*, *Acidiphilium rubrum* and *Acidiphilium* sp. strain SJH (Hallberg *et al.*, 2001).

Table 5. Optimum and range of growth for pH and temperature of acidophilic microorganisms that do not oxidize metal sulfides (mainly heterotrophs)

Species[#]	pH optimum	pH minimum-maximum	Temperature optimum (°C)	Temperature minimum-maximum (°C)
Mesophilic Bacteria				
Acidiphilium acidophilum	2.5-3	1.5-6.5	27-30	<25-37
Acidiphilium angustum	na	2.5-6	na	na
Acidiphilium cryptum	3	1.9-5.9	35-41	20-41
Acidiphilium multivorum	~ 3.5	1.9-5.6	27-35	17-42
Acidiphilium organovorum	3	2-5.5	37	20-45
Acidiphilium rubrum	na	2.5-6	na	na
Acidisphaera rubrifaciens	4.5-5	3.5-6	30-35	20-40
Acidobacterium capsulatum	na	3-6	na	20-37
Acidocella aminolytica	na	3-6	na	20-37
Acidocella facilis	na	2.5-6	na	25-37
Acidomonas methanolica	na	2-5.5	na	<30-42
Moderately thermophilic Bacteria				
Acidicaldus organivorans	2.5-3	1.8-3	50-55	na-65
Alicyclobacillus acidiphilus	3	2.5-5.5	50	20-55
Alicyclobacillus acidocaldarius	3-4	2-6	60-65	45-70
Alicyclobacillus acidoterrestris	na	2.2-5.8	42-53	<35-55
Alicyclobacillus cycloheptanicus	na	3-5.5	48	40-53
Alicyclobacillus herbarius	4.5-5	3.5-6	55-60	35-65
Alicyclobacillus hesperidum	3.5-4	>2-<6	50-53	>35-<60
Alicyclobacillus pomorum	4.5-5	>2.5-<6	45-50	30-60
Alicyclobacillus sendaiensis	5.5	2.5-6.5	55	40-65
Alicyclobacillus vulcanalis	4	2-6	55	35-65
Hydrogenobaculum acidophilum (autotroph)	3-4	2-na	65	na-~70
Archaea				
Acidianus ambivalens (autotroph)	2.5	1-3.5	80	na-87
Picrophilus oshimae	0.7	0-3.5	60	47-65
Picrophilus torridus	0.7	0-3.5	60	47-65
Sulfolobus acidocaldarius	2-3	1-6	70-75	55-85
Sulfolobus shibatae	3	nd	81	na-86
Sulfolobus solfataricus	3-4.5	2-5.5	85	50-87
Sulfolobus tokodaii	2.5-3	2-5	80	70-85
Sulfolobus yangmingensis	4	2-6	80	65-95
Sulfurisphaera ohwakuensis	2	1-5	84	63-92
Thermoplasma acidophilum	1-2	0.5-4	59	45-63
Thermoplasma volcanicum	2	1-4	59-60	33-67

[#]Listed in alphabetical order; na = data not available

The mesoacidophilic, heterotrophic Bacteria live closely associated with autotrophic metal sulfide oxidizers such as *Acidithiobacillus ferrooxidans*, and are often found in bioleaching operations or sulfidic mining environments together with

the autotrophs. Presumably, the heterotrophs promote the bioleaching activity of the autotrophic metal sulfide oxidizers by consuming inhibitory organic compounds which are produced by the autotrophs. Furthermore, *Acidiphilium* species are able to reduce Fe(III), and thereby deliver Fe(II) as substrate for autotrophic Fe(II) oxidizers. Metal or arsenic concentrations at which metabolic activity of still occurs are up to 30 mM As(III), 30 mM Cu(II), 900 mM Zn(II), 700 mM Cd(II), and 350 mM Ni(II) (Belly & Brock, 1974; Harrison, 1981, 1984; Wichlacz & Thomson, 1988; Johnson & McGinness, 1991; Pronk & Johnson, 1993; Goebel & Stackebrandt, 1994; Schippers *et al.*, 1995; Johnson & Roberto, 1997; Hiraishi *et al.*, 1998; Küsel *et al.*, 1999; Bridge & Johnson, 2000; Hallberg & Johnson, 2001; Dopson *et al.*, 2003; Johnson & Hallberg, 2003; Wenderoth & Abraham, 2005).

3.2. Moderately Thermophilic Bacteria

Acidicaldus organivorans (Johnson *et al.*, 2006) grows heterotrophically on a range of organic substrates, and oxidizes sulfur but not autotrophically. Facultativ anaerobic growth with Fe(III) was observed. The Gram-negative bacterium was isolated from geothermal sites in Yellowstone National Park, USA.

Alicyclobacillus disulfidooxidans and *Alicyclobacillus tolerans* were described as facultatively anaerobic, metal sulfide-, Fe(II) ions- and sulfur compound-oxidizing bacteria (Karavaiko *et al.*, 2005). As far as known, the other *Alicyclobacillus* species (isolated from various environments) only grow aerobically and heterotrophically with organic substrates. Cells are Gram-positive, spore-forming rods.

The Gram-negative, motile *Hydrogenobaculum acidophilum* has been isolated from a solfataric field in Japan, and reclassified (formerly *Hydrogenobacter acidophilus* (*ex*), Stöhr *et al.*, 2001). This organism grows autotrophically (CO_2 fixation via reductive tricarboxylic acid cycle) with H_2 and sulfur compounds as substrates.

3.3. Archaea

Several species of the phylum Crenarchaeota (order Sulfolobales) have been described as metal sulfide-, Fe(II) ions- and sulfur compound-oxidizing extremely thermoacidophilic Archaea. Most of the species of the genera *Acidianus*, *Sulfolobus* and *Sulfurisphaera* listed in Table 4 and 5 oxidize sulfur but metal sulfide oxidation has not been demonstrated. *Acidianus ambivalens* does not grow heterotrophically (obligate autotroph). In addition to the Crenarchaeota, moderately thermophilic Euryarchaeota of the genera *Thermoplasma* and *Picrophilus* are listed.

Thermoplasma was firstly isolated from a coal refuse pile in Indiana, USA (Darland *et al.*, 1970). Further *Thermoplasma* isolates were also obtained from self-heated smoldering coal refuse piles (Belly *et al.*, 1973; Brock, 1978), and from different solfatara fields by Segerer *et al.* 1988, who described the species. *Thermoplasma*

species lack cell walls and are mostly motile, pleomorphic cells. They are facultative anaerobic, obligate heterotrophs and do not oxidize Fe(II) ions and sulfur compounds.

Picrophilus strains were isolated from geothermal solfataric soils and springs in Hokkaido, northern Japan, and described by Schleper *et al.*. (1995, 1996). Cells are irregular cocci. They are strict aerobic, obligate heterotrophs and do not oxidize Fe(II) ions and sulfur compounds.

4. NUCLEIC ACID-BASED MOLECULAR METHODS

To control and optimize metal bioleaching, quick and reliable methods to identify and quantify single species in complex bioleaching communities are needed. Microbial communities can be analyzed using microscopic techniques, cultivation techniques, immunological techniques and nucleic-acid based molecular techniques. The latter are introduced here.

Total cell numbers can be determined by counting cells under a fluorescence microscope after application of nucleic acid-staining fluorochromes (e.g. SybrGreen, acridine orange, DAPI). The drawback of this technique is that these fluorochromes bind unspecifically to nucleic acids and thus, do not provide information on the viability of the cells. Potentially, a major part of the counted cells could be dormant or even dead and yet retain stainable DNA (Kepner & Pratt, 1994; Morita, 1997).

Using classical cultivation techniques, i.e., the most-probable-number (MPN) cultivation method (Schippers & Bosecker, 2005) or the dual-layer agarose plate technique (Johnson *et al.*, 1995), acidophilic autotrophic Fe(II) and sulfur oxidizing bioleaching microorganisms have been enriched from bioleaching communities. By cultivation techniques, however, only a subset of the whole microbial community can be detected, though media have been designed to select different groups on the basis of their physiologies. Furthermore, cultivation techniques are labor-intensive and results are only available after incubation times of several days or even weeks which does not allow a monitoring of bioleaching operations.

Immunoassays with specific antibodies have been applied to enumerate *At. ferrooxidans* and other bioleaching microorganisms (Jerez, 1997; Dziurla *et al.*, 1998), however, their application is time-consuming and requires thorough knowlegde of the microbes occurring in the bioleaching operation.

Over the last years, nucleic-acid based molecular techniques have been increasingly used to identify and quantify microorganisms in the environment and technical applications. Most of them are based on the extraction of DNA from a culture, a bioreactor or an environmental sample, followed by the amplification of DNA using the Polymerase Chain Reaction (PCR), and finally an analysis of the DNA amplification products. In most of the cases, the 16S ribosomal RNA gene (16S rDNA) of prokaryotes (Bacteria and Archaea) is targeted, but also functional genes coding for key enzymes of particular metabolic interest have been analyzed (e.g. the *rus* gene coding for rusticyanin in *At. ferrooxidans*). In the following, several PCR-based techniques for the identification of microorganisms are briefly introduced.

Techniques for the quantification of microorganisms are quantitative, real-time PCR and Fluorescence In Situ Hybridization (FISH) or its modification Catalyzed Reporter Deposition – Fluorescence In Situ Hybridization (CARD – FISH). These quantitative techniques and their application are described afterwards.

4.1. PCR-based Molecular Methods for Identification of Microorganisms

Usually the 16S rRNA gene is analyzed for the PCR-based identification of microorganisms. To address the biodiversity and to identify new species, PCR products can be cloned and the 16S rRNA gene of the various clones in the clone library can be sequenced. The similarities of the sequences can then be shown in a phylogenetic tree, to address the phylogenetic affiliation of the microorganisms in the sample. This approach has been choosen to analyze the microbial communities in natural, acidic environments and bioleaching operations (Goebel & Stackebrandt, 1994, 1995; Bruneel *et al.*, 2005; González-Toril *et al.*, 2005).

Alternatively to clone libraries, the PCR products can be separated in denaturing gradient gel electrophoresis (DGGE) which allows a separation of DNA fragments of the same length but different base-pair sequences. Bands in the DGGE can be excised and the 16S rRNA gene sequenced to address the phylogenetic affiliation of the organisms. This method has been applied to bioleaching communities as well (González-Toril *et al.*, 2003; Dopson & Lindstrom, 2004; Kinnunen & Puhakka, 2004; Demergasso *et al.*, 2005b; Rzhepishevska *et al.*, 2005).

Less labour intensive are DNA fingerprinting techniques which allow only the identification of known organisms. The DNA fingerprinting techniques RFLP (restriction fragment length polymorphism) and ARDREA (amplified ribosomal DNA restriction enzyme analysis) have been applied to identify bioleaching organism (Rawlings, 1995; Selenska-Pobell *et al.*, 1998; 2001; Bond *et al.*, 2000a; Asmah *et al.*, 2001, Bergamo *et al.*, 2004; Coupland & Johnson, 2004; Bruneel *et al.*, 2005; Diaby *et al.*, 2006; Johnson *et al.*, 2005; Bryan *et al.*, 2006a). These techniques include the digestion of the PCR product with one or more restriction enzymes to produce fragments of varying sizes that are resolved on appropriate gels.

Further PCR-based techniques for the identification of bioleaching organisms are: RAPD (randomly amplified polymorphic DNA) and rep-APD (Novo *et al.*, 1996; Selenska-Pobell *et al.*, 1998; 2001; Waltenbury *et al.*, 2005), SSCP (single stranded conformation polymorphism, Battaglia-Brunet *et al.*, 2002), analysis of the PCR-amplified 16S-23S rRNA gene intergenic spacer (Pizarro *et al.*, 1996; Vásquez & Espejo, 1997; Bergamo *et al.*, 2004), and the use of microbe-specific PCR primers (Wulf-Durand *et al.*, 1997).

4.2. Real-time PCR for Quantification of Microorganisms

Real-time PCR (quantitative PCR) is a technique with high sensitivity used in environmental microbiology to quantify different phylogenetic groups and genera,

e.g. Bacteria, Archaea, the Fe(III) reducer *Geobacter*, methanogens or cyanobacteria (Suzuki *et al.*, 2000; Takai & Horikoshi, 2000; Stults *et al.*, 2001; Beller *et al.*, 2002; Nadkarni *et al.*, 2002; Harms *et al.*, 2003; Kolb *et al.*, 2003; Sharkey *et al.*, 2004; Schippers *et al.*, 2005). The technique is based on the online fluorescence detection of PCR products and allows the rapid detection and quantification of gene sequences without the need for labor-intensive post-PCR processing (Heid *et al.*, 1996). DNA is quantitatively extracted from samples, purified, and specifically amplified with a thermocycler using sequence-specific fluorescently labeled probes. There are different chemical assasys for real-time PCR, but the most common are sequence-specific TaqMan probes (Heid *et al.*, 1996) and the intercalating non specific SybrGreen dye (Wittwer *et al.*, 1997). The detection limit of the method depends on the target of interest, sample purity, PCR conditions and other factors, but theoretically allows the detection of a single DNA molecule (Lockey *et al.*, 1998; Smith *et al.*, 2005). Real-time PCR has been applied to quantify Bacteria and Archaea oxidizing metal sulfides in mine heaps (Kock & Schippers, 2006). Furthermore, SybrGreen based protocols have been developed to quantify single species in bioleaching communities such as *Acidianus brierleyi, Sulfolobus* sp., *Sulfobacillus thermosulfidooxidans, Sulfobacillus acidophilus, Acidithiobacillus caldus*, and *Leptospirillum ferrooxidans* (Liu *et al.*, 2006).

In addition to DNA, RNA can be quantified after application of an additional reverse transcription step (real-time RT-PCR), which allows quantification of gene expression in environmental microbiology (Wilson *et al.*, 1999; Wawrik *et al.*, 2002; Sharkey *et al.*, 2004). Concerning bioleaching organisms, gene expression has been studied in pure cultures of *Acidithiobacillus ferrooxidans.* The expression of the *rus* gene coding for rusticyanin, a protein involved in Fe(II) oxidation, and of several additional genes important for Fe(II) and sulfur oxidation as well as for CO_2-fixation has been measured by real-time PCR (Yarzábal *et al.*, 2003, 2004; Quatrini *et al.*, 2006; Appia-Ayme *et al.*, 2006). It has been shown that the biological Fe(II) oxidation is most relevant for metal sulfide oxidation during bioleaching (Sand *et al.*, 2001; Schippers, 2004) and that the *rus* gene in *At. ferrooxidans* is particularly expressed during Fe(II) and metal sulfide oxidation rather than during sulfur oxidation (Yarzábal *et al.*, 2003, 2004; Ramírez *et al.*, 2004). Rusticyanin may not be expressed in all strains classified as *At. ferrooxidans*. However, the *rus* gene should be a good target to monitor the abundance and activity of *At. ferrooxidans* in biomining.

4.3. Fluorescence In Situ Hybridization (FISH) for Quantification of Microorganisms

A powerful technique to quantify microbial cells in environmental samples is FISH (Amann *et al.*, 1990, 1995). Since FISH targets ribosomal RNA (rRNA), which is indicative of actively metabolizing bacteria, FISH can provide quantitative information on living bacteria in an environmental sample. FISH has been successfully applied to quantify acidophilic Fe(II) oxidizing *Acidithiobacillus*,

Leptospirillum, *Ferroplasma* and other microorganisms in acid mine drainage environments (Schrenk *et al.*, 1998; Bond *et al.*, 2000a; Edwards *et al.*, 1999a, 2000; González-Toril *et al.*, 2003; Bernier & Warren, 2005; Mahmoud *et al.*, 2005) and in bioleaching operations (Peccia *et al*., 2000; Okibe & Johnson, 2004; Ebrahimi *et al.*, 2005; Kimura *et al.*, 2005; Coram-Uliana *et al.*, 2006). A drawback of the technique is that a sufficient content of cellular ribosomes is prerequisite for its successful application (Amann *et al.*, 1995; Ludwig & Schleifer, 2000; Schippers *et al.*, 2005). Recently, modified FISH protocols (CARD-FISH = Catalyzed Reporter Deposition - Fluorescence In Situ Hybridization) have been published which allow the detection of less active cells in environmental samples as well (Pernthaler *et al.*, 2002; Sekar *et al.*, 2003; Teira *et al.*, 2004). So far, these protocols have successfully been applied to quantify Bacteria and Archaea oxidizing metal sulfides in mine heaps (Demergasso *et al.*, 2005a; Kock & Schippers, 2006).

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