Growth and Survival of Acidithiobacilli in Acidic, Metal Rich Environments

Stefanie Mangold
In Memoriam Aviarum Mearum
Toni Klebingat, Barbara Mangold
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Abstract

Acidithiobacilli are acidophilic microorganisms that play important roles in many natural processes such as acidification of the environment, influencing metal mobility, and impacting on global sulfur and iron cycles. Due to their distinct metabolic properties they can be applied in the industrial extraction of valuable metals. Acidithiobacilli thrive in an environment which is extremely acidic and usually low in organic carbon but highly polluted with metals. In the quest to gain insight into how these microorganisms can thrive in their extreme environment, relevant facets of metabolism, metal resistance, and pH homeostasis were explored with the focus on two model organisms, *Acidithiobacillus caldus* and *Acidithiobacillus ferrooxidans*. Understanding these fundamental aspects of an acidophilic lifestyle will help to eventually control detrimental effects on the environment due to acidification and metal pollution as well as improving metal extraction utilizing acidophilic microorganisms.

Bioinformatics can give information about the genetic capacity of an organism. Likewise, ‘omics’ techniques, such as transcriptomics and proteomics to study gene transcription profiles and differentially expressed proteins can yield insights into general responses as well as giving clues regarding specific mechanisms for adaptation to life in extreme environments. This approach was used to investigate the sulfur metabolism of *At. caldus* which is an important sulfur oxidizer for industrial metal extraction. It was found that sulfur oxidation pathways were diverse within acidithiobacilli and a model of *At. caldus* sulfur oxidation was proposed. Furthermore, *At. ferrooxidans* anaerobic sulfur oxidation coupled to ferric iron reduction was studied which can be of importance for industrial processes. It was shown that anaerobic sulfur oxidation was, at least in part, indirectly coupled to ferric iron reduction via sulfide generation. Moreover, metal toxicity and resistance mechanisms in acidophiles are of major interest. Thus, zinc toxicity in three model organisms, *At. caldus*, *Acidimicrobium ferrooxidans*, and ‘*Ferroplasma acidarmanus*’, was explored. An important finding was that the speciation of metals and other chemical influences were of great importance for zinc toxicity in acidophiles. Additionally, the three organisms showed distinct responses to elevated zinc levels. Finally, the response of *At. caldus* to various suboptimal growth pH was evaluated to gain insights into pH homeostasis mechanisms. The results indicated that *At. caldus* used acid resistance mechanisms similar to those described for neutrophilic microorganisms. Analysis of fatty acid profiles demonstrated an active modulation of the cytoplasmic membrane in response to proton concentration, likely resulting in a more rigid membrane at lower pH.
<table>
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<th>Abbreviation</th>
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<td>AMD</td>
<td>Acid mine drainage</td>
</tr>
<tr>
<td>ARD</td>
<td>Acid rock drainage</td>
</tr>
<tr>
<td>ars</td>
<td>Operon encoding arsenic resistance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BLM</td>
<td>Biotic Ligant Model</td>
</tr>
<tr>
<td>CBB</td>
<td>Calvin Benson Bassham cycle</td>
</tr>
<tr>
<td>ΔpH</td>
<td>pH gradient across a membrane</td>
</tr>
<tr>
<td>ΔΨ</td>
<td>Membrane potential</td>
</tr>
<tr>
<td>DMMM</td>
<td>Dynamic multi-species metabolic modeling</td>
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<tr>
<td>F</td>
<td>Faraday constant</td>
</tr>
<tr>
<td>FAME</td>
<td>Fatty acid methyl ester</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-amino butyric acid</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
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<tr>
<td>GSSH</td>
<td>Sulfane sulfur</td>
</tr>
<tr>
<td>Hdr</td>
<td>Heterodisulfide reductase</td>
</tr>
<tr>
<td>HMM</td>
<td>Hidden Markov Model</td>
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<tr>
<td>ICP-MS</td>
<td>Inductively coupled plasma mass spectrometry</td>
</tr>
<tr>
<td>IPB</td>
<td>Iberian Pyrite Belt</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
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<tr>
<td>Maldi-ToF</td>
<td>Matrix assisted laser desorption ionization Time of Flight</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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<tr>
<td>MW</td>
<td>Molecular weight</td>
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<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Reduced NAD</td>
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<tr>
<td>NMR</td>
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<tr>
<td>NGS</td>
<td>Next generation sequencing</td>
</tr>
<tr>
<td>nif</td>
<td>Operon encoding nitrogen fixation</td>
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<tr>
<td>OMP</td>
<td>Outer membrane protein</td>
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<tr>
<td>pH&lt;sub&gt;opt&lt;/sub&gt;</td>
<td>Optimum growth pH</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>PMF</td>
<td>Proton motive force</td>
</tr>
<tr>
<td>polyP</td>
<td>Polyphosphate</td>
</tr>
<tr>
<td>PTM</td>
<td>Post translational modification</td>
</tr>
<tr>
<td>R</td>
<td>Gas constant</td>
</tr>
<tr>
<td>rus</td>
<td>Operon encoding rusticyanine</td>
</tr>
<tr>
<td>Sat</td>
<td>ATP sulfurylase</td>
</tr>
<tr>
<td>sec pathway</td>
<td>Secretory pathway</td>
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<tr>
<td>semiQ RT-PCR</td>
<td>Semi quantitative reverse transcriptase polymerase chain reaction</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>SDO</td>
<td>Sulfur dioxygenase</td>
</tr>
<tr>
<td>SMF</td>
<td>Sodium motive force</td>
</tr>
<tr>
<td>Sor</td>
<td>Sulfur oxygenase reductase</td>
</tr>
<tr>
<td>sox</td>
<td>Operon encoding sulfur oxidation genes</td>
</tr>
<tr>
<td>SQR</td>
<td>Sulfide quinone reductase</td>
</tr>
<tr>
<td>STB</td>
<td>Stirred tank bioreactor</td>
</tr>
<tr>
<td>T</td>
<td>Absolute temperature</td>
</tr>
<tr>
<td>tat pathway</td>
<td>Twin arginine translocation pathway</td>
</tr>
<tr>
<td>Tth/TetH</td>
<td>Tetrathionate hydrolase</td>
</tr>
<tr>
<td>T&lt;sub&gt;opt&lt;/sub&gt;</td>
<td>Optimum growth temperature</td>
</tr>
<tr>
<td>TQR</td>
<td>Thiosulfate quinone oxidoreductase</td>
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<tr>
<td>2D-PAGE</td>
<td>Two dimensional polyacrylamide gel electrophoresis</td>
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</table>
List of Papers

Paper I

Paper II
*Authors contributed equally.

Paper III
Stefanie Mangold, Joanna Potrykus, Erik Björn, Lars Lövgren and Mark Dopson (submitted) Extreme zinc tolerance in acidophilic microorganisms from the bacterial and archael domains.

Paper IV
Stefanie Mangold, Venkateswara rao Jonna and Mark Dopson (manuscript) Response of Acidithiobacillus caldus towards suboptimal pH conditions.

Paper not included in this thesis:
Introduction

In 2009, the Society for General Microbiology (SGM) awarded the Colworth Prize Lecture to Prof. Geoffrey M. Gadd, University of Dundee, for his contribution to geomicrobiology and understanding metal microbe interactions (Gadd, 2010). This demonstrates the recognition and appreciation of the importance that microbial actions have in geological processes. In Gadd's words, “The ubiquity and importance of microbes in biosphere processes make geomicrobiology one of the most important concepts within microbiology, and one requiring an interdisciplinary approach to define environmental and applied significance and underpin exploitation in biotechnology” (Gadd, 2010). Microorganisms interact with metals and minerals in various ways and are involved in global cycling of all elements. One group of microorganisms which especially impact the iron and sulfur cycles as well as heavily influencing metal mobility in the geosphere are acidophiles (optimum growth pH: pH_{opt} < 5) and extreme acidophiles (pH_{opt} < 3). Previously, acidophiles mainly attracted interest due to their use in industrial metal extraction, termed biomining. However, lately they have also received attention due to their ability to live in an extreme environment which might give clues to how extraterrestrial life could be like (Parro et al., 2011). Acidophiles are also of high interest regarding the origin of life on Earth as volcanic, low pH environments may have played an important role for those processes (Holm and Andersson, 2005).

1. Acidithiobacilli and Their Environments

In this thesis, fundamental aspects of acidophilic lifestyle were studied by investigating typical representatives of chemolithoautotrophic bacteria from the Acidithiobacillacieae family. The acidithiobacilli inhabit extremely acidic environments where they are part of a microbial community comprising organisms from all domains of life. In the quest to gain some insight into the question of how these microorganisms can thrive in their extreme environment, relevant facets of metabolism, as well as metal resistance, and pH homeostasis were explored with the focus on two model organisms, Acidithiobacillus caldus and Acidithiobacillus ferrooxidans. In the following sections the Acidithiobacillacieae family, especially At. caldus and At. ferrooxidans, will be characterized in more detail, followed by a description of typical acidic environments, and an overview of the acidophile microbial community.
1.1. *Acidithiobacillus caldus* and *Acidithiobacillus ferrooxidans*

In an effort to systemize their taxonomy, acidophilic species from the genus *Thiobacillus* were renamed *Acidithiobacillus* (Kelly and Wood, 2000). The genus *Acidithiobacillus* belongs to the class of γ-proteobacteria and today comprises several species, i.e. *At. albertensis* (Bryant et al., 1983), *At. caldus* (Hallberg and Lindström, 1994), *At. ferrooxidans* (Colmer et al., 1950), *At. thiooxidans* (Waksman and Joffe, 1922), and the recently described cold-tolerant *At. ferrivorans* (Hallberg et al., 2010). The *acidithiobacilli* are rod-shaped Gram negative lithoautotrophs that fix carbon dioxide from the atmosphere by the Calvin Benson Bassham cycle (CBB) (Elbehti et al., 2000; Levican et al., 2008; Esparza et al., 2010). All *acidithiobacilli* can gain energy from the oxidation of inorganic sulfur compounds but only *At. ferrooxidans* and *At. ferrivorans* can utilize ferrous iron as well. Additionally, other energy substrates such as molecular hydrogen (Drobner 1990) and formate (Pronk 1991) have been described.

Of all *acidithiobacilli* the mesophilic *At. ferrooxidans* (optimal growth temperature, $T_{\text{opt}} = 30^\circ\text{C}$, and $pH_{\text{opt}} = 2.5$) is the most studied species and it is also one of the most studied acidophiles in general. The reason for this is its early discovery and description (Colmer et al., 1950) and the fact that it was thought to be the dominant species in many biomining processes and natural environments (Brierley and Le Roux, 1978). More recent studies have shown that *At. ferrooxidans* is not necessarily the dominant species but rather, it is relatively easy to cultivate on solid medium which is why it was selected for in many classical studies (reviewed in Johnson, 2001; Baker and Banfield, 2003). As mentioned above, *At. ferrooxidans* can oxidize ferrous iron as well as sulfur compounds and models of the respective metabolic pathways have been established in some detail (Quatrini et al., 2009 and references therein).

The first report of the moderate thermophilic *At. caldus* ($T_{\text{opt}} = 45^\circ\text{C}$, $pH_{\text{opt}} = 2.5$) was a description of its isolation from coal spoil heaps (Marsh and Norris, 1983). Later it was characterized and announced as a new species (Hallberg and Lindström, 1994). *At. caldus* grows lithoautotrophically with inorganic sulfur compounds as source of energy but also mixotrophically with tetrathionate as electron donor along with glucose and yeast extract (Hallberg and Lindström, 1994) as well as pyruvate (Aston et al., 2011). Since its discovery *At. caldus* has been shown to efficiently oxidize sulfur compounds during bioleaching (Dopson and Lindström, 1999), making it an important species involved in industrial bioleaching using stirred tank bioreactors (STBs) at temperatures $> 40^\circ\text{C}$ (reviewed in Baker and Banfield, 2003; Rawlings, 2005). Okibe et al. (2003) showed that *At. caldus* was the dominant sulfur oxidizer in early stages of bioleaching in
continuously operated STB and more recent studies on batch STB confirmed these results (Dopson and Lindström, 2004; Hao et al., 2010; Zeng et al., 2010). The important role in biomining spurred the interest to further study At. caldus. Recently, the genome sequence of the type strain (ATCC 51756, Valdes et al., 2009) and strain SM-1 (You et al., 2011) have been reported which opened the door for a variety of investigations such as bioinformatic and proteomic studies. Apart from its application in biomining, At. caldus was also tested for possible application in remediation as a sorbent for metals (Aston et al., 2010). Furthermore, its function as a biosensor for the detection of toxic chemicals was demonstrated (Hassan et al., 2010).

1.2. Acidic Environments and Associated Microorganisms

Typical environments where acidophiles thrive are sulfate and metal rich, acidic or extremely acidic sites worldwide. These sites can occur naturally or be anthropogenic in origin. Acidic areas are often the result of the microbial oxidation of sulfur or inorganic sulfur compounds and ferrous iron eventually leading to the formation of sulfuric acid. Volcanic areas or environments with high thermal activity are naturally occurring acidic sites where elemental sulfur is formed by a condensation reaction of the geothermal gases sulfur dioxide and hydrogen sulfide according to equation (1).

\[ \text{SO}_2 + 2 \text{H}_2\text{S} \rightarrow 2 \text{H}_2\text{O} + 3 \text{S}^0 \]  

Whether microbial oxidation of sulfur results in acidification of the whole site depends on the occurrence of basic minerals such as silicates or carbonates which can neutralize the generated sulfuric acid. This also holds true for environments which have been directly or indirectly influenced by human activities to become acidic. Such places are typically rich in metal sulfides which have been exposed to oxygen and water due to human mining endeavors, e.g. abandoned mine sites or coal spoils. The most abundant sulfide mineral is pyrite (FeS2) which can be efficiently dissolved to yield sulfuric acid in a process catalyzed by microbes. Equation (2) describes the overall reaction which simplifies the naturally occurring processes (described in more detail in chapter 2.1).

\[ 4 \text{FeS}_2 + 14 \text{H}_2\text{O} + 15 \text{O}_2 \rightarrow 4 \text{Fe(OH)}_3 + 8 \text{H}_2\text{SO}_4 \]  

A range of metal sulfides contain other metals than iron which are mobilized by this dissolution process. At low pH, cationic metal ions are usually more soluble than at higher pH values yielding the characteristic acid liquors with a high content of dissolved metals. Another typical property of these
environments is their oligotrophic nature with a low content of soluble organic carbon. Such anthropogenic acidic environments often found at abandoned mining sites are termed acid mine drainage (AMD). One extensively studied AMD site with an extremely low pH of below zero is Iron Mountain in California, USA (Nordstrom and Alpers, 1999). Another well studied man-made acidic environment is Rio Tinto in Spain where mining activities date back to the ancient Romans. Besides the described environments there are extremely acid, low-temperature and sulfur rich environments such as sewers and sulfide enriched caves that are less well studied (reviewed in Dopson and Johnson, 2012). Despite substantial geochemical differences of sulfide rich caves and AMD environments, the inherent microbial communities show remarkable resemblance (Jones et al., 2012).

An unexpectedly large diversity of microorganisms from all domains of life is able to populate these apparently hostile habitats. However, the diversity in any particular acidic microenvironment is usually small with only a few prokaryotic taxa comprising the microbial communities (reviewed in Baker and Banfield, 2003; Schippers et al., 2010) and the same is true for commercial biomining communities (reviewed in Rawlings, 2005; Schippers et al., 2010). Most important for the microbial communities are sulfur and iron oxidizing bacteria and archaea since they are responsible for the prevailing acidity. However, acidophilic eukaryotes have also been detected (reviewed in Baker and Banfield, 2003; Schippers et al., 2010) and their roles and interactions with other community members are slowly being unraveled (Amaral Zettler et al., 2002). Acidophilic flagellates, a ciliate, and an amoeba were isolated from coal spoils and studied in vitro where they were shown to predate acidophilic bacteria (Johnson and Rang, 1993). Furthermore, a rather large eukaryotic diversity was found in Rio Tinto River with photosynthetic algae contributing 60% of the biomass (Amaral Zettler et al., 2002). Additionally, acidophilic fungi – including yeasts – were isolated from AMD sites (Gadanho and Sampaio, 2006; Russo et al., 2008).

Often acidophilic bacteria and archaea are grouped according to their optimal growth temperature into psychrotolerant (T_{opt} < 15°C), mesophilic (T_{opt} 15 - 40°C), moderately thermophilic (T_{opt} 40 - 60°C), and extremely thermophilic (T_{opt} > 60°C). Table 1 gives a short summary of selected acidophiles together with their characteristics. Since acidic environments are typically oligotrophic and often lack photosynthetic community members, lithoautotrophic bacteria are frequently responsible for carbon fixation e.g. the acidithiobacilli. But also heterotrophic and facultative autotrophs/heterotrophs are usually part of acidophilic microbial communities. Another element which might be scarce in acidic environments and therefore, possibly growth limiting is nitrogen.
Table 1. Overview of selected acidophiles from all domains of life.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Primary carbon source</th>
<th>Temperature range</th>
<th>Energy substrate</th>
<th>Domain/ Phylum</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidianus ambivalens</td>
<td>Heterotroph</td>
<td>Thermophile</td>
<td>S</td>
<td>Archaea/ Crenarchaeota</td>
<td>Fuchs et al., 1996</td>
</tr>
<tr>
<td>Acidianus brierleyi</td>
<td>Mixotroph</td>
<td>Thermophile</td>
<td>S, Fe$^{2+}$</td>
<td>Archaea/ Crenarchaeota</td>
<td>Fuchs et al., 1996</td>
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<tr>
<td>Acidimicrobium ferrooxidans</td>
<td>Mixotroph</td>
<td>Mod. thermophile</td>
<td>Fe$^{2+}$</td>
<td>Bacteria/ Actinobacteria</td>
<td>Clark and Norris, 1996</td>
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<td>Acidiphilium cryptum</td>
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<td>Mesophile</td>
<td>Org. comp.</td>
<td>Bacteria/ α-Proteobacteria</td>
<td>Kusel et al., 1999</td>
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<td>Acidithiobacillus albertensis</td>
<td>Autotroph</td>
<td>Mesophile</td>
<td>S</td>
<td>Bacteria/ γ-Proteobacteria</td>
<td>Hallberg and Lindström, 1994</td>
</tr>
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<td>Acidithiobacillus caldus</td>
<td>Autotroph</td>
<td>Psychrotolerant</td>
<td>S</td>
<td>Bacteria/ γ-Proteobacteria</td>
<td>Hallberg et al., 2010</td>
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<td>Acidithiobacillus ferrivorans</td>
<td>Autotroph</td>
<td>Mesophile</td>
<td>S, Fe$^{2+}$</td>
<td>Bacteria/ γ-Proteobacteria</td>
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<td>S</td>
<td>Bacteria/ γ-Proteobacteria</td>
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<td>Org. comp.</td>
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<td>Org. comp.</td>
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<td>Mesophile</td>
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<td>Archaea/ Euryarchaeota</td>
<td>Golyshina et al., 2000</td>
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<td>Fe$^{2+}$</td>
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<td>Leptospirillum ferriphilum</td>
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<td>Mesophile</td>
<td>Fe$^{2+}$</td>
<td>Bacteria/ Nitrospirales</td>
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<td>Mesophile</td>
<td>Fe$^{2+}$</td>
<td>Bacteria/ Nitrospirales</td>
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<td>Thermophile</td>
<td>S</td>
<td>Archaea/ Euryarchaeota</td>
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<td>Archaea/ Euryarchaeota</td>
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<td>Archaea/ Crenarchaeota</td>
<td>Fuchs et al., 1995</td>
</tr>
<tr>
<td>Vahlkampfia spp.</td>
<td>Heterotroph</td>
<td>Mesophile</td>
<td>Org. comp.</td>
<td>Eucarya/ Percolozoa</td>
<td>Norris et al., 1996</td>
</tr>
</tbody>
</table>

*a* S - inorganic sulfur compounds, Fe$^{2+}$ - ferrous iron, Org. comp. - organic compounds.
This makes nitrogen fixation an important trait for acidophilic communities. *At. ferrooxidans* as well as some members of the leptospirilli contain the nitrogen fixing (*nif*) operon which encodes the enzyme nitrogenase (NifHDG). Leptospirilli are mesophiles or moderate thermophiles belonging to the order of Nitrospirales. They can only oxidize ferrous iron and have a high affinity for this substrate which is one of the reasons why they often outcompete *At. ferrooxidans* in commercial STB processes (reviewed in Rawlings et al., 1999). Several species of leptospirilli have been isolated from acidic environments: *L. ferrooxidans* (group I), *L. ferriphilum* (group II) and *L. ferrodiazotrophum* (group III). It has been shown that *L. ferriphilum* is not able to fix nitrogen whereas the opposite is true for the other species (Coram and Rawlings, 2002; Tyson et al., 2005; Levican et al., 2008). Another acidophilic iron oxidizer is the Gram positive *Acidimicrobium ferrooxidans* which is moderately thermophilic and belongs to the Actinobacteria. Likewise, sulfobacilli are Gram positive and moderately thermophilic but they belong to the Firmicutes phylum and can only oxidize inorganic sulfur compounds. Most of the above mentioned organisms are autotrophs whereas the mesophilic *Acidiphilium* spp. belonging to the α-proteobacteria are facultative heterotrophs. Those play an important role in the microbial community as they can degrade organic compounds which might be toxic to autotrophic members of the community. Furthermore, archaea are often found in acidic environments especially at elevated temperatures. Important members include several genera of the extremely thermophilic, sulfur oxidizing family Sulfolobaceae, i.e. *Sulfolobus, Acidianus, Metallosphaera*. Those belong to the Kingdom of Crenarchaeota whereas the iron oxidizers *Ferroplasma* spp. and *Picrophilus* spp. belong to the Thermoplasmatales in the Euryarchaeota kingdom. Some of the archaea are heterotrophs, e.g. *S. acidocaldarius* and *Ferroplasma* spp..

### 2. Applications and Implications

The Rio Tinto mine in the Iberian Pyrite Belt (IPB) of southwest Spain is an example of both the application of acidophiles for the benefit of humans as well as detrimental environmental impact due to the same microbes. Already the Romans extracted metals in this area without knowing that microorganisms were involved in the solubilization of the metal ores. Nowadays, Rio Tinto is known for its pollution due to AMD releasing acid and high concentrations of metals into the environment. In both cases, the same processes are responsible for the effects and in the following section these processes as well as their applications and implications are described. Understanding and ultimately manipulating the microorganisms involved in those processes is key to improving applications and avoiding detrimental effects and thus, an important aim of the research on acidophiles.
2.1. Biomining

The hunger of humans for metals dates back millennia to when we first used metals to craft tools and ornaments. Since then the need for metals has depleted the easily accessible high grade mineral ores and made it necessary to explore ways to extract low grade and often refractive ores. Industrial application of acidophilic microorganisms allows the extraction of low grade ores when smelting is not cost efficient, is too environmentally costly, or yields can be significantly increased by a microbial pre-treatment. This process is generally called biomining. Strictly, biomining includes two more specific processes, bioleaching and biooxidation, however, these terms are often not used according to their specific definitions. Bioleaching comprises all processes where acidophiles are utilized to catalyze the solubilization of sulfide minerals and thereby release the metal of interest as soluble ions into solution. A prominent example is copper extraction from the refractive metal sulfide chalcopyrite (CuFeS₂). In contrast, the term biooxidation describes methods where the target metal is not part of the mineral but rather a microbial pre-treatment makes it more accessible for extraction by other methods. An example is gold extraction where biooxidation breaks down the arsenopyrite (FeAsS) mineral lattice making gold particles more accessible for extraction with cyanide, therefore yielding enhanced gold recovery.

The overall, simplified equation (2) for the dissolution of a typical metal sulfide, pyrite (FeS₂), is given in chapter 1.2. However, in the environment or during biomining this is a complex multistep process which includes reactions catalyzed by microorganisms as well as chemical reactions. Schippers and Sand deciphered the mineral dissolution processes and suggested two pathways for acid soluble (e.g. chalcopyrite) and acid insoluble (e.g. pyrite) sulfide minerals (1999). The pathway for insoluble metal sulfides was named according to the first sulfur intermediate, thiosulfate, and is described with pyrite in equations (3) and (4). In this pathway, sulfuric acid (sulfate (SO₄²⁻) and protons (H⁺)) is the main end product.

\[
\begin{align*}
\text{FeS}_2 + 6 \text{Fe}^{3+} + 3 \text{H}_2\text{O} & \rightarrow \text{S}_2\text{O}_3^{2-} + 7 \text{Fe}^{2+} + 6 \text{H}^+ \\
\text{S}_2\text{O}_3^{2-} + 8 \text{Fe}^{3+} + 5 \text{H}_2\text{O} & \rightarrow 2 \text{SO}_4^{2-} + 8 \text{Fe}^{2+} + 10 \text{H}^+ 
\end{align*}
\]

Essentially, pyrite is dissolved by the chemical attack of ferric iron (Fe³⁺). Iron oxidizing microorganisms catalyze this process by oxidizing the derived ferrous iron (Fe²⁺) and thereby constantly regenerating the leaching chemical as described in equation (5).

\[
2 \text{Fe}^{2+} + 0.5 \text{O}_2 + 2 \text{H}^+ \rightarrow 2 \text{Fe}^{3+} + \text{H}_2\text{O}
\]
In the case of acid soluble metal sulfides (MeS), the dissolution takes place by ferric iron as well as protons and the main intermediates are polysulfides and elemental sulfur. This pathway is called polysulfide pathway and is illustrated in equations (6) and (7).

\[
\text{MeS} + \text{Fe}^{3+} + \text{H}^+ \rightarrow \text{M}^{2+} + 0.5 \text{H}_2\text{S}_n + \text{Fe}^{2+} \quad (n \geq 2) \quad (6)
\]
\[
0.5 \text{H}_2\text{S}_n + \text{Fe}^{3+} \rightarrow 0.125 \text{S}_8 + \text{Fe}^{2+} + \text{H}^+ \quad (7)
\]

In this pathway iron oxidizing microorganisms also regenerate the ferric iron (5). But additionally sulfur oxidizers are important to oxidize the rather stable sulfur intermediate as in equation (8) thereby also contributing to acidification.

\[
0.125 \text{S}_8 + 1.5 \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{SO}_4^{2-} + 2 \text{H}^+ \quad (8)
\]

These processes have been applied to the biooxidation of gold (reviewed in Rawlings et al., 2003) and uranium (Lange and Freyhoff, 1991; Lange et al., 1991) as well as the bioleaching of copper (reviewed in Rawlings et al., 2003), nickel (Riekkola-Vanhanen, 2010), and cobalt (Briggs and Millard, 1997). Although the underlying processes are identical the composition of the metal sulfide is unique for each concentrate/ore and there are two commonly utilized industrial set-ups for biomining, i.e. heap/dump leaching and tank leaching. Consequently, the microbial communities responsible for biomining vary in their composition according to the specific process (reviewed in Johnson, 2001; Rawlings, 2005). Additionally, other industrial processes have been employed, such as in situ leaching for uranium (reviewed in Rawlings and Silver, 1995; Bosecker, 1997), but the two processes mentioned above are those most commonly applied today. Bioleaching heaps are comparably cheap and rather low technology processes which make them an attractive method for the recovery of metals from lower grade ores. Heap leaching has been mainly employed for the recovery of copper (reviewed in Watling, 2006). The heaps can be anything from simple waste dumps to sophisticatedly constructed heaps for improved bioleaching. In any case the crushed ore is piled on an impervious base, and supplied with an irrigation system for the acidic leach solution as well as a collection system for the metal rich leachate. Aeration of the heap can be passive or for improved oxygen supply the heap can be aerated by actively blowing air into the base of the heap. Even with active aeration the oxygen supply is commonly not homogeneous possibly leaving anaerobic zones within the heap. Although bioleaching heaps do not need to be inoculated as biomining microorganisms are inherently associated with the ore, inoculation can shorten lag times and improve heap performance. Due to the make-up of the heap leaching process microorganisms face gradients in pH,
temperature, and oxygen concentrations within the heap that are possibly also influenced by seasonal changes. Thus, there are distinct ecological niches within a bioleaching heap which will lead to a more diverse microbial community compared to the more controlled tank leaching operations. The latter are highly aerated, continuous flow stirred tank reactors which are temperature and pH controlled. Usually a series of tanks are connected where the first one is fed with the finely ground concentrate together with an acidic phosphate and nitrogen enriched medium. Subsequently, the suspension flows through the series of tanks within which the microbial community catalyzes the dissolution of the metal sulfide. As the dissolution proceeds in each tank of the series the conditions that the microbes face change tank by tank leading to variations in the microbial community (Okibe et al., 2003; Hao et al., 2010; Nancucheo and Johnson, 2010). Tank leaching processes are most commonly applied for the biooxidation of gold but have also been used for the bioleaching of cobalt and the applicability for bioleaching of nickel has been demonstrated (reviewed in Norris et al., 2000; Rawlings et al., 2003).

2.2. Acid Mine Drainage

The processes described in the former section can give rise to detrimental environmental effects after mining sites are abandoned leading to AMD or when metal sulfide rich deposits are naturally exposed to oxygen and water leading to acid rock drainage (ARD). In the following, the term AMD will be used but the same concepts generally apply to ARD. Similar to bioleaching liquids, AMD liquors are rich in sulfate, ferric iron, and other metals as well as being acidic, sometimes extremely so. AMD liquors are often discharged into the environment without control. This can lead to acidified and metal polluted groundwater, rivers, streams, and sediments, threatening aquatic life as well as vegetation. The mining industry has recognized AMD as a serious problem related to their activities and efforts for risk assessment and AMD control are made (Akcil and Koldas, 2006). The generation of AMD as well as its dimensions are not only dependent on the chemical and microbial processes mentioned above, but are influenced by many factors including mineralogy, hydrology, geology, and geochemistry making risk assessment and AMD predictions a complex and difficult task (reviewed in Nordstrom, 2011). Especially important for the risk assessment of AMD is the consideration of metal toxicity for the surrounding biota as large amounts of metals can be mobilized. For example, the rivers Rio Tinto and Odiel impacted by AMD from the IPB transport about 37% and 15% of global gross flux (from rivers to the ocean) of dissolved zinc and copper, respectively (Nieto et al., 2007). To aid this risk assessment the mobility of metals at AMD sites can be predicted taking into account metal chemical, physical,
and geochemical information (Smith, 2007, see also chapter 4.1). The contamination in the affected areas constituting high toxicity for the biota has been described (Schippers et al., 2000; Nieto et al., 2007).

Due to its damaging effects several strategies for control and remediation of AMD have been developed (reviewed in Johnson and Hallberg, 2005). In principle, the strategies can be divided into source- and migration control where the former is preferred but often is not feasible. An attempt to control AMD generation at the source is, for example, the application of biocides to inactivate the AMD generating microorganisms (for a review on the microbial communities in AMD see Baker and Banfield, 2003; Schippers et al., 2010), however this proved not to be very effective (Loos et al., 1989; Sand et al., 2007). If it is not possible to avoid the generation of AMD at the source there are a range of strategies to control and remediate it such as chemical treatments to neutralize the AMD liquors (e.g. addition of lime), or biological treatments (e.g. wetlands) (Johnson and Hallberg, 2005).

3. Substrate Metabolism

As mentioned in chapter 1, the main energy substrates utilized by acidithiobacilli are inorganic sulfur compounds and ferrous iron. Some details about the metabolic pathways of those substrates are known, especially for At. ferrooxidans. The following sections give a summary of aerobic iron and sulfur oxidation as well as anaerobic sulfur oxidation coupled to ferric iron reduction in acidithiobacilli.

3.1. Aerobic Iron Oxidation

Iron in the environment is mostly in two oxidation states, ferrous (Fe$^{2+}$) and ferric iron (Fe$^{3+}$). The role of prokaryotes in the iron cycle including ferrous oxidation and ferric reduction is reviewed in Bird et al. (2011). The dominant form of iron in the environment depends on chemical parameters such as oxygen concentration, pH, and redox potential. Ferrous iron is stable in anoxic conditions but rapidly oxidized to ferric iron at neutral pH in the presence of molecular oxygen. On the other hand, ferrous iron oxidation rates are very low at acidic pH. Furthermore, the redox potential of the ferrous/ferric couple depends on the solubility of the ions and therefore, it strongly depends on pH and the presence of chelating agents. The solubility of ferric iron is particularly influenced by pH as it is only completely soluble at pH < 1.6 or in presence of chelating agents such as citrate. Yet it is the redox potential that fundamentally impacts on the utilization of iron as an energy substrate. At acidic pH with soluble ferrous and ferric iron the redox potential of the couple is + 780 mV. At neutral pH the redox potential of the soluble, ligand bound species is much more negative making iron a more
favorable substrate at neutral pH. However, at the same time, the redox potential of the oxygen/water couple is also more positive at low compared to neutral pH, i.e. oxygen becomes a more favorable electron acceptor at acidic pH. All in all aerobic iron oxidation at low pH can only harness a potential of around 350 mV and can thus yield a small amount of energy. Despite this *At. ferrooxidans*, *At. ferrivorans*, and many other acidophiles from various phyla are iron oxidizers, possibly due to the high abundance of iron in their natural habitat (for a general review on iron oxidizing prokaryotes see Hedrich et al., 2011; while acidophiles are reviewed in Bonnefoy and Holmes, 2012).

**Figure 1.** Aerobic iron oxidation in *At. ferrooxidans*. Electron transport components involved in the ‘downhill’ pathway from ferrous iron to oxygen are: (i) Cyc2, a cytochrome c located at the outer membrane; (ii) a periplasmic blue copper protein rusticyanin A (RusA); (iii) Cyc1, a cytochrome attached to the periplasmic side of the cytoplasmic membrane; and (iv) a type *aa*3 terminal cytochrome oxidase. The ‘uphill’ pathway components include: (i) a cytochrome *bc* complex (encoded within operon petI); (ii) the quinone pool; and (iii) the NADH1 dehydrogenase complex. Dotted lines indicate the electron pathway and arrows show the proton fluxes.

A model for *At. ferrooxidans* iron oxidation has been suggested (Ingledew, 1982), studied (Appia-Ayme et al., 1999), and recently refined (Quatrini et al., 2009) and is broadly accepted today (Figure 1). Yet, lately it has been realized that this model cannot serve as a universal acidophile iron oxidation strategy since more than one pathway has evolved in this environment (Amouric et al., 2011; Bonnefoy and Holmes, 2012). The most striking difference between *At. ferrooxidans* and the newly announced species *At. ferrivorans* is the psychrotolerance of the latter. Additionally, both species utilize different iron oxidation pathways (Hallberg et al., 2010; Amouric et
al., 2011; Bonnefoy and Holmes, 2012), a finding that was confirmed by deciphering the *At. ferrivorans* genome sequence (Liljeqvist et al., 2011). This led to the suggestion that the iron oxidizing acidithiobacilli actually comprise four species instead of two (Amouric et al., 2011). Nevertheless, all species face the challenge of the positive redox potential of the ferrous/ferric couple (780 mV) posing particular implications for the acidithiobacilli which fix carbon by the CBB cycle. This pathway requires the reducing power from NADH but the redox potential of NAD+/NADH (-320 mV) is much more negative compared to that of the ferrous/ferric couple at low pH (780 mV). To overcome this discrepancy acidithiobacilli partition the electron flow and drive an ‘uphill’ pathway to gain the reducing power of NADH plus a ‘downhill’ pathway for gaining energy in form of adenosine triphosphate (ATP) by transferring electrons to the final acceptor molecular oxygen (Figure 1). In *At. ferrooxidans*, the electron transport components involved in the ‘downhill’ pathway from ferrous iron to oxygen are: (i) Cyc2, a cytochrome c located at the outer membrane; (ii) a periplasmic blue copper protein rusticyanin A (RusA); (iii) Cyc1, a cytochrome c4 attached to the periplasmic side of the cytoplasmic membrane; and (iv) a type aa3 terminal cytochrome oxidase. It is believed that the splitting of the electron flow into ‘uphill’ and ‘downhill’ pathways takes place at the rusticyanin. The ‘uphill’ pathway components include: (i) a cytochrome bc complex (encoded within operon petI); (ii) the quinone pool; and (iii) the NADH1 dehydrogenase complex. In contrast, *At. ferrivorans* contains a high potential iron sulfur protein, Iro, which is suggested to be the iron oxidase (Amouric et al., 2011) as well as two rusticyanin isoforms, rusA and rusB.

### 3.2. Aerobic Sulfur Oxidation

Sulfur occurs naturally in eight oxidation states. Elemental sulfur has an oxidation state of zero but sulfur compounds range from -2 (sulfide) to +6 (sulfate and sulfuric acid). All acidithiobacilli can oxidize elemental sulfur and inorganic sulfur compounds such as sulfide (S2-), thiosulfate (S2O32-), tetrathionate (S4O63-), or sulfite (SO32-). In contrast to the oxidation of ferrous iron, sulfur oxidation yields much more energy (Pronk et al., 1990). It was realized in the 1980’s that the sulfur oxidation pathways of acidophiles and even within the acidithiobacilli are not the same (Kelly, 1985) which was also highlighted by investigating the genome sequences of *At. ferrooxidans*, *At. caldus*, and *At. thiooxidans* (Valdes et al., 2008a). The sulfur oxidation model suggested for *At. ferrooxidans* (Quatrini et al., 2009) includes a range of enzymes and cytochrome complexes dealing with the various sulfur compounds and delivering electrons to molecular oxygen as the terminal electron acceptor. The periplasmic side of the outer membrane contains tetrathionate hydrolase (Tth or TetH, both designations can be found in
literature) which hydrolyzes tetrathionate to thiosulfate, sulfur, and sulfate. Thiosulfate is converted to tetrathionate by thiosulfate quinone oxidoreductase (TQR) which is located in the cytoplasmic membrane. At the same location is sulfide quinone reductase (SQR) that oxidizes sulfide to yield polysulfides or sulfur while the cytoplasm contains heterodisulfide reductase (Hdr) and ATP sulfurylase (Sat). The enzyme complex HdrABC has been studied in methanogenic archaea where it reversibly reduces the disulfide bond in heterodisulfide (Hedderich et al., 2005). However, in At. ferrooxidans this enzyme complex is proposed to oxidize the disulfide bond of sulfane sulfur (GSSH) derived from elemental sulfur since several characteristics support its role in sulfur oxidation (discussed in Quatrini et al., 2009). How sulfite, produced by HdrABC, is further oxidized to the end product sulfate is not completely clear but Sat is hypothesized to be involved (Quatrini et al., 2009). Electrons derived from GSSH, thiosulfate, or sulfide enter the quinone pool and are subsequently transferred to either the NADH complex to yield reducing power or to the terminal oxidases. There are several cytochromes and cytochrome complexes predicted to be involved in the electron transport chain (Quatrini et al., 2009). The terminal oxidases bd or bo_3 probably receive electrons directly from the quinone pool whereas the aa_3 oxidase possibly receives them indirectly through the bc_1 complex (encoded in the petII operon) and a cytochrome c or a high potential iron-sulfur protein. It is interesting to note that there are two differentially expressed bc complexes in the At. ferrooxidans electron transport chain (Appia-Ayme et al., 1999; Brasseur et al., 2002; Bruscella et al., 2007). The bc complex encoded within the petI operon is expressed during growth on ferrous iron whereas the second complex encoded in the petII operon is up-regulated during sulfur oxidation. Further, it has been shown that the former is involved in the ‘uphill’ pathway towards the NADH complex and the latter in the ‘downhill’ pathway towards the terminal oxidase. Although the current model of sulfur oxidation in At. ferrooxidans is able to explain many experimental observations it is still not understood how the insoluble, hydrophobic elemental sulfur is made accessible for the cell. The current hypothesis is that it is converted to GSSH and then oxidized by Hdr in the cytoplasm, but how this is accomplished in detail remains unclear.

Sulfur oxidation in At. caldus has been previously studied (Hallberg et al., 1996; Dopson et al., 2002; Bugaytsova and Lindström, 2004; Rzhepishevska et al., 2007). In the early studies, the sulfur compounds oxidized and the cellular compartment in which the reaction takes place were revealed (Hallberg et al., 1996). Furthermore, it was demonstrated that ATP production is solely due to electron transport phosphorylation and not substrate-level phosphorylation (Dopson et al., 2002). Those studies yielded a preliminary model showing the oxidation of thiosulfate, tetrathionate, sulfur, sulfide, and sulfite in a cellular context without relating the reactions
to specific enzymes (Hallberg et al., 1996). The subsequent two studies (Bugaytsova and Lindström, 2004; Rzhepishevska et al., 2007) concentrated on Tth and its regulation. Tth was purified and characterized; furthermore, it was shown to be a soluble, periplasmic homodimer (Bugaytsova and Lindström, 2004). The Tth gene cluster is suggested to be transcriptionally regulated (Rzhepishevska et al., 2007). Additionally, a comparison of the At. caldus draft genome sequence with the genome sequence of At. ferrooxidans showed that the former harbors sulfur oxidation enzymes which are not present in At. ferrooxidans (Valdes et al., 2008a). This suggests that both species use quite distinct pathways. A detailed model of At. caldus sulfur is lacking and therefore, requires further investigation. In the meantime, the availability of the At. caldus’ genome sequence favored a global proteomic and bioinformatic approach to study its sulfur metabolism. Consequently, one of the aims of this thesis was to further investigate this pathway and suggest a more detailed model including proposed enzymes. This model will be presented in paper I. Very recently, after publication of paper I, a more refined sulfur oxidation model for At. caldus MTH-04 has been published (Chen et al., 2012). This new model is discussed in more detail in the results section for paper I.

3.3. Ferric Iron Reduction

In contrast to ferrous iron oxidation, the utilization of ferric iron as a terminal electron acceptor by acidophiles, i.e. ferric reduction, has not been well studied. Yet, this reaction is of great importance in the environment as well as for biotechnological applications (reviewed in Johnson, 1998). In AMD environments as well as in heap or stirred tank bioleaching operations soluble ferric iron occurs in high concentrations. At the same time microaerophilic or anaerobic zones might be present especially in abandoned mines or bioleaching heaps (Johnson et al., 1993). As discussed above, the redox potential of the ferrous/ferric couple is 780 mV at pH ~ 2 rendering ferric iron a favorable electron acceptor at acidic conditions. The reduction of ferric iron can be coupled with the oxidation of various energy substrates such as organic compounds, elemental sulfur, inorganic sulfur compounds, and hydrogen (Das et al., 1992; Pronk et al., 1992; Bridge and Johnson, 1998; Ohmura et al., 2002).

Respiration utilizing ferric iron coupled to the oxidation of organic compounds is widespread in pH neutral conditions and has been well studied especially in anaerobic Geobacter spp. and Shewanella spp. (reviewed in Lovley, 2008; Bird et al., 2011). In acidic environments a range of microorganisms have been reported to be capable of utilizing ferric iron as well. However, those acidophilic microorganisms are mostly facultative anaerobes rather than obligate anaerobes and some grow poorly in
completely anoxic conditions (Kusel et al., 1999; Coupland and Johnson, 2008). The first mention of ferric reduction by acidophiles was in 1976 (Brock and Gustafson) although it remained unclear whether the tested organisms, *At. ferrooxidans*, *At. thiooxidans*, and *Sulfolobus acidocaldarius* were able to grow anaerobically. That anaerobic sulfur oxidation with ferric iron as electron acceptor is able to yield enough energy to support growth was subsequently demonstrated for *At. ferrooxidans* (Das et al., 1992; Pronk et al., 1992). Ohmura et al. (2002) showed that both ferric and sulfur reduction could be coupled to hydrogen oxidation as primary energy source for *At. ferrooxidans* under anaerobic conditions. In contrast, Gram positive, moderately thermophilic bacteria such as *Sulfobacillus* spp. and *Acidimicrobium ferrooxidans* performed best when grown heterotrophically or mixotrophically although some species were able to utilize tetrathionate as electron donor as well (Bridge and Johnson, 1998). The ability to couple the oxidation of organic compounds to ferric iron reduction has been reported for the acidophilic heterotroph *Acidiphilum cryptum* (Kusel et al., 1999) and has recently been studied in several other acidophilic heterotrophs such as *Acidocella*, *Acidobacterium*-like, *Acidisphaera*, and *Frateuria*-like spp. (Coupland and Johnson, 2008). The latter study suggested that this trait is widespread in acidophilic heterotrophic bacteria. Additionally, species from the archaeal genus *Ferroplasma* can respire ferric iron with yeast extract as electron donor (Dopson et al., 2007). Although it is now recognized that facultative anaerobic growth by dissimilatory ferric reduction is an important metabolic capacity of several acidophiles, the molecular details remain enigmatic. Attempts to elucidate the anaerobic electron transport chain in acidophiles suggest that cytochromes *c* and *b* are involved in *Ferroplasma* spp. (Dopson et al., 2007). Additionally, a new cytochrome was isolated from anaerobically grown *At. ferrooxidans* which showed specific characteristics supporting its role in the anaerobic electron transport chain (Ohmura et al., 2002). Recently, ferric reduction activity was associated with Tth from *At. ferrooxidans* (Sugio et al., 2009) however, this finding has been questioned. Additionally, ferric reductase activity of the *At. ferrooxidans* ArsH protein was reported (Mo et al., 2011). Although the authors conceded that the biochemical function of ArsH was not unequivocally elucidated they proposed ArsH to be a cytoplasmic ferric iron reductase (Mo et al., 2011). Another recent study applying a proteomics approach to compare aerobic and anaerobic sulfur oxidation of *At. ferrooxidans* suggested that rusticyanin and a cytochrome *c* also encoded in the rus operon are involved in the anaerobic electron transport chain (Kucera et al., 2012). However, until now convincing evidence for the identity of an acidophilic ferric reductase is missing. Therefore, we attempted to expand the knowledge of anaerobic ferric reduction by *At. ferrooxidans* in a study presented with paper II.
Table 2. Metal concentrations of selected metals measured at three different AMD sites. The data exemplifies variations in metal concentrations depending on the AMD site, the geochemical conditions as well as seasonal changes.

<table>
<thead>
<tr>
<th>Location</th>
<th>Fe (total) [mg/L]</th>
<th>Cu [mg/L]</th>
<th>Ni [mg/L]</th>
<th>Co [mg/L]</th>
<th>Zn [mg/L]</th>
<th>As (total) [mg/L]</th>
<th>Sulfate [mg/L]</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Richmond mine, Iron Mountain, USAa</td>
<td>20300</td>
<td>290</td>
<td>0.66</td>
<td>1.3</td>
<td>2010</td>
<td>56.4</td>
<td>118000</td>
<td>0.48</td>
</tr>
<tr>
<td>Richmond mine, Iron Mountain, USAa</td>
<td>86200</td>
<td>2340</td>
<td>2.9</td>
<td>15.5</td>
<td>7650</td>
<td>154</td>
<td>360000</td>
<td>-0.7</td>
</tr>
<tr>
<td>Richmond mine, Iron Mountain, USAa</td>
<td>111000</td>
<td>4760</td>
<td>3.7</td>
<td>5.3</td>
<td>23500</td>
<td>340</td>
<td>760000</td>
<td>-2.5</td>
</tr>
<tr>
<td>Tinto river, Spain; Minimum valuesb</td>
<td>0.07</td>
<td>0.2</td>
<td>16</td>
<td>52</td>
<td>2.2</td>
<td>&lt;3</td>
<td>150</td>
<td>2.22</td>
</tr>
<tr>
<td>Tinto river, Spain; Maximum valuesb</td>
<td>2804</td>
<td>84.3</td>
<td>742</td>
<td>3754</td>
<td>152.3</td>
<td>2290</td>
<td>5547</td>
<td>5.01</td>
</tr>
<tr>
<td>Odiel river, Spain; Minimum valuesb</td>
<td>0.31</td>
<td>0.5</td>
<td>19</td>
<td>33</td>
<td>1.3</td>
<td>&lt;3</td>
<td>110</td>
<td>2.95</td>
</tr>
<tr>
<td>Odiel river, Spain; Maximum valuesb</td>
<td>22.5</td>
<td>17.1</td>
<td>500</td>
<td>938</td>
<td>36.4</td>
<td>22</td>
<td>2379</td>
<td>5.05</td>
</tr>
<tr>
<td>Bozinta tailings heap, Romaniae</td>
<td>0.35 (0.36)</td>
<td>1 (3)</td>
<td>n.d. d</td>
<td>n.d. d</td>
<td>9 (27)</td>
<td>38 (42)</td>
<td>n.d.</td>
<td>7.0 (1.3)</td>
</tr>
<tr>
<td>Bozinta tailings heap, Romaniae</td>
<td>1108 (1229)</td>
<td>25 (47)</td>
<td>n.d. d</td>
<td>n.d. d</td>
<td>116 (112)</td>
<td>58 (52)</td>
<td>n.d. d</td>
<td>3.7 (1.3)</td>
</tr>
<tr>
<td>Bozinta tailings heap, Romaniae</td>
<td>&lt;0.1 (0.07)</td>
<td>0.33 (0.6)</td>
<td>n.d. d</td>
<td>n.d. d</td>
<td>0.21 (0.33)</td>
<td>3.4 (8.2)</td>
<td>n.d. d</td>
<td>8.3 (0.3)</td>
</tr>
</tbody>
</table>


*b Minimum and maximum values from weekly measurements over a period from February 2002 until September 2004 (Nieto et al., 2007).

*c Mean values (standard deviation) from several samples of three different sites sampled in October 1996, May 1997, October 1997 and July 1998 (Schippers et al., 2000).

*d n.d. not determined.
4. Metal Toxicity and Resistance

In section 2.2 it was already touched upon that mobility of metals in the environment depends on many different factors (Smith, 2007; Nordstrom, 2011). Therefore, it is easily conceivable that the concentrations of metals differ largely between various AMD sites and biomining processes. Nevertheless, concentrations of soluble metal ions are typically high in acidic environments, often in the range of mg/L up to g/L (Table 2). Confronted with such high amounts of metals it seems surprising that microorganisms can thrive in those environments. In fact, some acidophiles, especially archaean species, are not very tolerant to some metals and thus their use in biomining operations is hampered (Dopson et al., 2003). In the following chapter some theoretical considerations on metals and metal toxicity as well as a short overview of studies on acidophile metal resistance are given.

4.1. Metals and Toxicity

Chemically metals are defined as “elements which conduct electricity, have a metallic luster, are malleable and ductile, form cations, and have basic oxides” (Atkins and Jones, 1997). This definition includes a large range of elements and further categorization is needed in order to highlight specific properties of metals. Such grouping should be based on chemical or physical qualities to ensure a sound and unambiguous terminology (Duffus, 2002). If used alone, terms like essential metal, trace metal, or toxic metal lack precision since it depends on the organism and the environment whether a metal is essential, available in trace amounts, or toxic. For example, metals such as iron, copper, and zinc are essential for most microorganisms as they are important for the function of many enzymes. Nonetheless, even those metals can exert a toxic effect if they exceed a certain concentration which again depends on the organism. The toxicity of a metal primarily depends on its interaction with the organism, i.e. interactions or reactions with biomolecules. Thus, the chemical and physical properties of the metal can help to predict its toxicity (Newman et al., 1998; Ownby and Newman, 2003). Chemical and physical properties are for example: oxidation state (represents the charge of the element when electrons are taken into account), ionic radius (size of the element) or electronegativity (strength of an element to attract electrons). Based on those properties metals can be categorized and a useful categorization referring to the lewis acid character and preferred ligands has been developed (Ahrland et al., 1958) and later refined (Pearson, 1963, 1968b, a).
Table 3. Characteristics and classifications of selected metals.

<table>
<thead>
<tr>
<th>Element (chemical symbol)</th>
<th>Periodic table</th>
<th>Electronegativity(^a)</th>
<th>Class A/B(^b)</th>
<th>Mobility(^c)</th>
<th>Recovered by biomining</th>
<th>Biological significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron (Fe)</td>
<td>d block</td>
<td>1.7 (Fe(^{2+})),</td>
<td>borderline (Fe(^{2+})),</td>
<td>++</td>
<td>Not commercially</td>
<td>Important trace metal for many organisms</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.8 (Fe(^{3+}))</td>
<td>A (Fe(^{3+}))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copper (Cu)</td>
<td>d block</td>
<td>1.8 (Cu(^+)),</td>
<td>B (Cu(^+)),</td>
<td>+++</td>
<td>Bioleaching (mostly heaps)</td>
<td>Often required in trace amounts</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0 (Cu(^{2+}))</td>
<td>borderline (Cu(^{2+}))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zinc (Zn)</td>
<td>d block</td>
<td>1.65</td>
<td>borderline</td>
<td>+++</td>
<td>Bioleaching (few operations)</td>
<td>Usually required in trace amounts</td>
</tr>
<tr>
<td>Nickel (Ni)</td>
<td>d block</td>
<td>1.91</td>
<td>borderline</td>
<td>+++</td>
<td>Bioleaching (few operations)</td>
<td>Can be utilized in biological systems</td>
</tr>
<tr>
<td>Cobalt (Co)</td>
<td>d block</td>
<td>1.88</td>
<td>borderline</td>
<td>+++</td>
<td>Bioleaching (few operations)</td>
<td>Can be utilized in biological systems</td>
</tr>
<tr>
<td>Arsenic (As)</td>
<td>p block</td>
<td>2.0 (As(^{3+})),</td>
<td>borderline (As(^{3+}))</td>
<td>++</td>
<td>Not commercially</td>
<td>Often toxic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.2 (As(^{5+}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cadmium (Cd)</td>
<td>d block</td>
<td>1.69</td>
<td>B</td>
<td>+++</td>
<td>Not commercially</td>
<td>Often toxic</td>
</tr>
<tr>
<td>Silver (Ag)</td>
<td>d block</td>
<td>1.93</td>
<td>B</td>
<td>+</td>
<td>Biooxidation (tank leaching)</td>
<td>Often toxic</td>
</tr>
<tr>
<td>Lead (Pb)</td>
<td>p block</td>
<td>1.6 (Pb(^{2+})),</td>
<td>B (Pb(^{2+})),</td>
<td>+</td>
<td>Not commercially</td>
<td>Often toxic</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>borderline (Pb(^{4+}))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mercury (Hg)</td>
<td>d block</td>
<td>1.8 (Hg(^+)),</td>
<td>B</td>
<td>++</td>
<td>Not commercially</td>
<td>Often toxic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0 (Hg(^{2+}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gold (Au)</td>
<td>d block</td>
<td>2.54 (Au(^+)),</td>
<td>B</td>
<td>n.a.(^d)</td>
<td>Biooxidation (tank leaching)</td>
<td>Usually no significance</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.9 (Au(^{3+}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Electronegativity according to Pauling scale (Pauling, 1960; data from Smith, 2007).

\(^b\) Classification according to Lewis acidity in class A (hard), class B (soft), and borderline metals (data from Duffus, 2002).

\(^c\) Relative mobility under oxidizing conditions pH < 3 (data from Smith, 2007). Ranking: + somewhat mobile, ++ mobile, +++ very mobile.

\(^d\) n.a. not available.
A Lewis acid is an element which can act as electron acceptor and metals are grouped in three classes according to their preferred ligand: class A or ‘hard acids’, class B or ‘soft acids’, and borderline. Class A (hard) ions have a hard, non-polarizable electron shell and prefer alike hard ligands, such as oxygen donors, with which they assume mostly ionic bonding where they are easily displaced. In contrast, class B (soft) ions are more polarizable and prefer soft ligands, such as sulfur or sulfide donors, with which they form more covalent bonding and are thus less mobile. Since class B metals prefer ‘soft’ ligands such as sulfur or sulfide donors they can interfere largely with cellular components causing toxic effects. However, when using those categories it needs to be kept in mind that the classes apply to a specific ionic form and the borderline metals are difficult to classify and thus the classification is not absolute. Likewise, the knowledge of metal properties can be used to make qualitative statements about relative metal mobilities for a certain geochemical condition (Smith, 2007). Table 3 summarizes some metal characteristics and categorizations as well as giving information whether the metal is recovered by biomining.

![Diagram](https://via.placeholder.com/150)

**Figure 2.** Metal toxicity in microorganisms depends on (i) bioavailability; (ii) interactions with ions at the binding site; (iii) transport into the cell; and (iv) the reactivity of the metal with biomolecules. Usually the free metal cation is the most toxic form of the metal.

It is not only the reactivity with biomolecules that determines the toxicity of a metal. Before the metal can exhibit its effects it needs to enter the cell. Therefore, several other aspects need to be considered (Figure 2) including (i) bioavailability of the metal; (ii) interactions with other ions at the binding site; and (iii) transport into the cell. Bioavailability denotes the accessibility of a substance for organisms, e.g. a solid substance is often unusable for microorganisms. Thus, bioavailability of metals correlates with metal speciation which itself depends on the characteristics of the metal and solution chemistry. Generally, the free metal ion is believed to be the most toxic species as complexed metals are often not bioavailable. Assuming that
the free metal cation is the only toxic species it still needs to be considered that this cation can interact with other cations at the binding or up-take site. Other cations in solution, such as Ca\(^{2+}\) or H\(^{+}\), might compete with the free metal ion and thus mitigate its toxicity. However, care needs to be taken as the assumption that the free metal cation is the only toxic species is not true in all cases. For example it has been shown that the complexed zinc in ZnHPO\(_4\)\(^{\text{aq}}\) could be taken up by *Arthrobacter* and therefore, this species significantly contributed to zinc toxicity (Moberly *et al.*, 2010). These considerations are incorporated in the so-called Biotic Ligand Model (BLM) used for modeling metal toxicity in aquatic organisms (reviewed in Di Toro *et al.*, 2001). In the BLM the biotic ligand replaces the site of toxic action in the aquatic organism, e.g. gills in the case of fish. In the case of microorganisms the toxic metal has to be transported into the cell to show its toxic effect. Thus, the transport of the toxic species over the cell membrane is of great importance requiring special attention. Often unspecific, constitutively expressed importers are the cause of toxicity as influx of the toxic ion through those transporters cannot be stopped (Nies, 1999).

Taking into account all aspects for single metal toxicity already gives a quite complex picture; however, often it is necessary to consider metal mixtures. Especially in the case of AMD or biomining sites multiple metals are the rule and not the exception. Attempts have been made to model the toxicity of metal mixtures and illustrate the complexity of the phenomenon (Ownby and Newman, 2003; Kamo and Nagai, 2008).

### 4.2. Metal Resistance Mechanisms

Microorganisms have evolved mechanisms to counteract metal toxicity, firstly by establishing metal homeostasis and where this is insufficient by resistance mechanisms. Unlike toxic organic compounds, metals cannot be degraded to detoxify them. Consequently, there are limited strategies for metal resistance (Nies, 1999, 2003): (i) efflux of the metal out of the cell; (ii) sequestration; (iii) blocking the permeation/up-take into the cell; and (iv) reduction to a less toxic ionic form. As discussed for metal resistance in neutrophiles such as the extremely metal resistant *Cupriavidus metallidurans* the most important strategy is the transport of excess metals out of the cell (reviewed in Nies, 1999, 2003; von Rozycki and Nies, 2009). Metal sequestration is often unfavorable as it is energetically expensive to either import or synthesize the sequestration ligands. Blocking of metal ions from entering the microbial cell can be achieved by alterations in the cellular membrane to prevent leakage of metal ions into the cell as reported for *At. ferrooxidans* in response to copper and nickel (Mykytczuk *et al.*, 2011). The reduction of metals to a less toxic form is an elegant way for detoxification but only few metal ions can be reduced within the cell due to their favourable
redox potential. Merely in the case of mercury reduction suffices for
detoxification since Hg$_{2}^{+}$ is reduced to the volatile Hg$_{0}$ which can leave the
cell by diffusion. This reduction is carried out by mercury reductase MerA
encoded in the mer operon which is widely distributed among prokaryotes
(Mathema et al., 2011). MerA activity was reported for At. ferrooxidans
(Olson et al., 1981; Booth and Williams, 1984) and the merA gene including
the whole mer operon was further studied (Inoue et al., 1989; Inoue et al.,
1990; Kusano et al., 1990; Velasco et al., 1999). In addition to MerA activity
another mechanism for mercury resistance has been detected in At.
ferrooxidans (Iwahori et al., 2000). This ferrous iron dependent mercury
volatilization mechanism has not been found in neutrophilic microorganisms
so far (Mathema et al., 2011) but was shown to be present in At.
ferrooxidans SUG2-2 (Sugio et al., 2001) and MON-1 (Sugio et al., 2003). In
both cases a cytochrome c oxidase is responsible for the volatilization of
mercury. However, the type aa$_{3}$ cytochrome c oxidase from At. ferrooxidans
MON-1 can also volatilize mercury from organomercurials (Sugio et al.,
2010) which is not the case for cytochrome c oxidase from strain SUG2-2.
The resistance to organomercurials has not been reported before since At.
ferrooxidans does not encode the gene for organomercury lyase merB which
is responsible for this activity in neutrophiles (Mathema et al., 2011).

Metal resistance mechanisms in acidophiles have been reviewed by
Dopson et al. (2003). Mechanisms that have been well studied in acidophiles
include copper resistance (reviewed in Orell et al., 2010) and resistance to
the arsenicals, arsenate (As(V)) and arsenite (As(III)) (reviewed in Dopson et
al., 2003). Arsenic resistance is encoded in the ars operon including genes
for the As(V) reductase, arsC, the As(III) efflux pump, arsB, and the
repressor, arsR. ArsC reduces As(V) to As(III) which is subsequently
exported from the cytoplasm by ArsB. The ars operons from At.
ferrooxidans (Butcher and Rawlings, 2002), At. caldus (de Groot et al.,
2003), and Acidiphilium multivorum (Suzuki et al., 1998) have been cloned
and expressed in Escherichia coli where they conferred resistance to As(III).
Furthermore, resistance to As(V) via genes encoded by the ars operon has
been demonstrated experimentally for At. caldus (Dopson et al., 2001). In
contrast, the archaeon ‘Ferroplasma acidarmanus’ is resistant to As(V) but
it lacks a homologue of known arsenate reductase in its genome. Therefore,
it is hypothesized that a novel resistance mechanism is present (Baker-
Austin et al., 2007). As the ability to withstand high copper concentrations is
important for the application of acidophiles in biomining copper resistance
has also attracted large research interest. Orell and co-workers (2010)
summarize the most important acidophile copper resistance mechanisms
including: (i) many homologues of known copper resistance determinants;
(ii) multiple copies of some resistance genes; (iii) novel copper chaperones;
(iv) polyphosphate (polyP) based resistance mechanism; and (v) a defense
system against oxidative stress. Recently, Völlmecke et al. (2012) demonstrated the role of the ATPases CopA and CopB as copper efflux pumps in copper resistance of *Sulfolobus solfataricus* by deletion mutants. Further, they found indications that both ATPases might be involved in silver resistance.

In contrast to arsenical, copper, and mercury resistance the mechanisms of acidophile zinc resistance are not well studied. Zinc is an essential metal for most organisms. Nevertheless, it can be toxic at comparably high concentrations. In acidophiles zinc resistance was shown to be chromosomally encoded in *At. ferrooxidans* (Kondratyeva et al., 1995) and plasmid encoded in *Acidocella* strain GS19h (Ghosh et al., 1997; Ghosh et al., 2000) and *Acidiphilium symbioticum* (Mahapatra et al., 2002). The resistance mechanisms itself were not elucidated but Mahapatra et al. (2002) suggested that resistance in *Acidiphilium symbioticum* might be due to an unknown mechanism.

Although resistance against metals has been studied in acidophiles the exact molecular mechanisms still remain unknown and reports are often limited to metal tolerances (Dopson et al., 2003; Orell et al., 2010). However, it has been emphasized that acidophiles are generally more tolerant to metals than neutrophiles and that this might be due to their inside-positive membrane potential which is inversed when compared to neutrophiles (Dopson et al., 2003; Baker-Austin et al., 2007; Franke and Rensing, 2007; Orell et al., 2010). So far, this has not been shown experimentally. Furthermore, care must be taken with such comparisons as the solution chemistries of neutrophilic and acidophilic environments are very different. Consequently, toxicity of metal ions might be very different in both cases (as discussed above) making direct comparisons impossible. Toxicity effects due to metal speciation or competition with other ions have hardly or even not at all been considered in acidophile metal resistance literature so far. Due to this and the lack of knowledge in acidophilic zinc resistance mechanisms we studied a range of acidophiles exposed to zinc stress. The results of this study are presented in paper III.

5. **Bioenergetics**

Bioenergetics is a field in biochemistry which considers the status, generation, and flow of energy in organisms. Such considerations are fundamental to understanding biochemical processes within any organism. Acidophiles face particular energetic problems since they are often challenged with low energy substrates and a very low solution pH. Furthermore, pH homeostasis and bioenergetics are tightly connected and pH homeostasis is central to grasp the acidophilic lifestyle. Thus, the principles of bioenergetics will be described in the following section,
followed by an overview of known pH homeostasis mechanisms in neutrophiles and acidophiles.

### 5.1. Principles

The prerequisite for biological energy generation is the ability of organisms to create compartments with various concentrations of specific ions such that their concentration gradients can be used to release energy. The main building blocks for this energy generation are biological membranes which constitute impermeable barriers and protons (in a few cases sodium ions) which are used to build the concentration gradient. Thus, membranes along with proteinaceous pumps and transporters which can bridge the border to create or maintain the gradient are the basis of bioenergetics. Additionally, the F$_{1}$F$_{0}$-ATPase enzyme complex responsible for synthesis of the central energy currency (ATP) is of utmost importance. For prokaryotes those energetic processes take place at the cytoplasmic membrane whereas for higher eukaryotic systems mitochondrial or thylakoid membranes are places of energy generation (the latter will not be considered further). Before energy can be created the energized status of the microbial cell needs to be established. This is achieved by primary proton pumps such as respiratory pumps or ATP-driven proton pumps which extrude protons from the cytoplasm establishing the proton motive force (PMF) over the membrane. The PMF can be envisioned as a force that is acting on protons trying to pull them across the membrane. Likewise, the PMF is an electrochemical gradient consisting of two components: the chemical proton gradient ($\Delta$pH) and the membrane potential, i.e. the electrical difference in charge ($\Delta$$\Psi$) (Mitchell, 1961; West and Mitchell, 1974; Booth, 1985; Macnab and Castle, 1987). Typically (for neutrophiles), the $\Delta$pH is inside alkaline and the $\Delta$Ψ is inside negative. The PMF is calculated according to equation (9) with $R$ being the gas constant, $T$ the absolute temperature, and $F$ the Faraday constant:

$$PMF \ [\text{mV}] = \Delta \Psi - (2.3 \times (R \frac{T}{F}) \times \Delta \text{pH})$$

With standard conditions the formula can be simplified as in equation (10):

$$PMF \ [\text{mV}] = \Delta \Psi - (59 \times \Delta \text{pH})$$

Conventionally, $\Delta \Psi$ and $\Delta$pH are calculated by subtracting the external value from the internal value and – also by convention – the $\Delta \Psi$ is negative when the inner surface of the membrane is negative. For a functioning microbial cell the PMF needs to be maintained at a physiological level which is around -200 mV for most microorganisms (Krulwich et al., 2011). To achieve this
optimal value the two components of the PMF are adjusted to the conditions specific for the microbe and its environment. Generally, there are typical PMF patterns for microorganisms inhabiting different pH niches, i.e. neutrophiles, acidophiles, and alkaliphiles (Figure 3). Neutrophiles can grow at pH values between approximately 5.5 – 9.0 and keep an internal pH of 7.5-7.7 (Krulwich et al., 2011), whereas the cytoplasmic pH of acidophiles is usually slightly lower at 6.0-7.0 (Cox et al., 1979). Extreme alkaliphiles, on the other hand, grow at external pH ≥ 10 keeping a cytoplasmic pH of 7.5-8.3 (Krulwich et al., 2011). For neutrophiles the ΔpH is normally very small and only adds a little to the PMF whereas the major contribution originates from the large inside negative ΔΨ (Figure 3). The situation for acidophiles is substantially different since they need to maintain a circumneutral intracellular pH although facing a very low external pH. Thus, the ΔpH is large and dominates the PMF. Additionally, the ΔΨ of acidophiles is inverted, i.e. inside positive, to counteract the large negative contribution of ΔpH. On the other hand, alkaliphiles face a different problem, i.e. their cytoplasmic pH is more acidic than the external pH resulting in an inverted ΔpH. To counteract the latter, alkaliphiles have a rather large negative contribution from ΔΨ. Still, the PMF of alkaliphiles is much smaller compared to neutrophiles and acidophiles posing specific challenges to alkaliphiles (reviewed in Hicks et al., 2010).

Once the PMF is established work can be performed by the energy released when protons are transferred from the high concentration compartment (extracellular space) to the low concentration compartment (cytoplasm). Depending on the needs of the microbial cell this work can be ATP generation by the F$_1$F$_0$-ATPase enzyme complex, transporting solutes, or flagellar movement. The F$_1$F$_0$-ATPase enzyme complex is ubiquitous in all domains of life and well conserved. It is a reversible complex able to synthesize ATP as well as hydrolyzing it coupling these reactions to the PMF by a rotational mechanism (reviewed in Nakanishi-Matsui et al., 2010). Both the F$_1$ and F$_0$ parts consist of several subunits and the F$_1$ part of the complex is membrane extrinsic whereas the F$_0$ section is located within the membrane. Much of the details of its catalytic action as well as its rotational mechanism have been elucidated (see Nakanishi-Matsui et al., 2010 for details). As mentioned above, some microorganisms establish a sodium motive force (SMF) although not all of them also use the sodium gradient for ATP generation by F$_1$F$_0$-ATPase (reviewed in Dimroth and Cook, 2004). For example, some anaerobic microorganisms, e.g. Propionigenium modestum, or Acetobacterium woodii devise a special type of F$_1$F$_0$-ATPase coupled to the SMF for ATP production (reviewed in Dimroth and Cook, 2004). In contrast some marine species like Vibrio spp. are exposed to high salt contents and utilize primary sodium pumps to extrude sodium from the cytoplasm (Hayashi et al., 2001). Nevertheless, those species utilize the PMF
for ATP generation (Krumholz et al., 1990) but can harness the energy from the SMF to import nutrient and drive flagellar movement (Yorimitsu and Homma, 2001).

Figure 3. Proton motive force (PMF) in acidophiles, neutrophiles, and alkaliphiles. Summary (A) of membrane potential ($\Delta \Psi$), pH gradient ($\Delta pH$), and cytoplasmic pH measured for the acidophiles At. ferrooxidans (Cox et al., 1979) and A. acidocaldarius (Michels and Bakker, 1985); the neutrophiles E. coli (Zilberstein et al., 1979) and B. subtilis (Shioi et al., 1978); as well as the alkaliphiles B. pseudofirmus (Sturr et al., 1994) and B. alcalophilus (Guffanti et al., 1978). The figure is redrawn from the original references and a review in the field (Kruwlich et al., 2011). It exemplifies typical PMF patterns for microorganisms inhabiting three different pH niches (acidic, neutral, and alkaline). Schematic overview (B) of acidophile At. ferrooxidans, neutrophile E. coli, and alkaliphile B. pseudofirmus showing typical values for $\Delta pH$ and $\Delta \Psi$ as well as the resulting PMF.

5.2. Mechanisms for pH Homeostasis

As shown in the previous section, $\Delta pH$ is directly connected to the energy status of the microbial cell. In the case of acidophiles the $\Delta pH$ is of even greater importance for bioenergetics as it is the only productive component of the PMF. Not only because of its implications for bioenergetics but also due to fundamental physiological consequences control of internal pH is crucial for all organisms. Acidification of the cytoplasm leads to protonation of biomolecules and thus, e.g. to the denaturation of proteins. Hence, the control and maintenance of the internal pH, i.e. pH homeostasis, is a basic requirement for survival (reviewed in Slonczewski et al., 2009; Kruwlich et al., 2011).
Mechanisms of pH homeostasis have been studied quite extensively in neutrophiles, especially in *E. coli* (reviewed in Foster, 2004) and *Helicobacter pylori* (Sachs et al., 2011) since both species can survive intermittently at very low pH. In comparison, little is known about pH homeostasis in acidophiles (reviewed in Baker-Austin and Dopson, 2007). A possible way to counter excess protons entering the microbial cell is cytoplasmic buffering. Several molecules in the cell can act as buffering substances, e.g. amino acids or the amino acid side chains of proteins, polyamines, inorganic phosphate, and polyP. Apart from being buffering substances polyamines have also been shown to fulfill other roles in acid stress response of *E. coli*, such as blockage of outer membrane porins (Samartzidou et al., 2003). Whether a certain molecule can provide buffering capacity at a specific pH depends on its pKa value. Phosphate and polyP have a pKa of 7.2 making them more suitable for cytoplasmic buffering of neutrophiles than acidophiles. For acidophiles protein amino acid side chains are more likely responsible for buffering as they offer a large range of pKa values (Zychlinsky and Matin, 1983). Cytoplasmic buffering capacity exists in all microorganisms, though levels of the capacity varies between species (Kruulwich et al., 1985) and it was questioned whether this mechanism is part of the global pH homeostasis in alkaliphilic bacilli (Kruulwich et al., 1985). However, it was shown that the buffering capacities of the neutrophile *E. coli* and two acidophiles (*A. acidophilum* and bacterium PW2) are comparable and effective in supporting a constant internal pH upon influx of protons (Zychlinsky and Matin, 1983; Goulbourne et al., 1986).

Although passive cytoplasmic buffering can support pH homeostasis it is believed that active mechanisms are more important. For neutrophiles as well as acidophiles active transport of protons either via primary proton pumps or secondary cation/proton antiporters is of great importance for the maintenance of a stable internal pH (Baker-Austin and Dopson, 2007; Slonczewski et al., 2009; Kruulwich et al., 2011). Respiratory chain primary proton pumps were studied in *At. ferrooxidans* (Chen and Suzuki, 2005; Ferguson and Ingledew, 2008), ‘*F. acidarmanus*’ (Dopson et al., 2005), as well as in several archaeal acidophiles (reviewed in Schäfer et al., 1999). Interestingly, the *At. ferrooxidans* cytochrome c involved in ferrous iron oxidation differs from homologues in neutrophiles as it lacks one of the putative proton channels (Ferguson and Ingledew, 2008). Further, secondary cation/proton antiporters have an important role in modulating the PMF, e.g. the *E. coli* Na+/H+ antiporter NhaA is essential for adaptation to alkaline stress (Padan et al., 1989). Additionally, it has been perceived that potassium ions might play an important role for acidophile pH homeostasis possibly by establishing the inside positive ΔΨ. Until now this has not been shown unequivocally but circumstantial experimental data as well as data
based on genomic sequences support this hypothesis. In *At. thiooxidans* it was demonstrated that potassium ions were the most efficient cations tested to maintain the ΔΨ ([Suzuki *et al.*, 1999]). Further, it was shown that *Sulfolobus* spp. required the presence of potassium ions during respiratory proton pumping ([Schäfer *et al.*, 1996]). Based on sequence data it was found that *Leptospirillum* group II ([Tyson *et al.*, 2004]), *Picrophilus torridus* ([Fütterer *et al.*, 2004]), and *Sulfolobus solfataricus* ([She *et al.*, 2001]) encode a large number of putative secondary cation transporters possibly involved in creation and maintenance of the ΔΨ.

A prevalent mechanism during acid or base stress in neutrophiles is the expression of acid or base consuming enzymes ([Foster, 2004; Slonczewski *et al.*, 2009]). A well-studied system essential for *E. coli* acid stress survival is the decarboxylase system (reviewed in [Foster, 2004]) but other species, such as lactococci and bacilli, have also been shown to express acid dependent decarboxylases (reviewed in Slonczewski *et al.*, 2009). For example, the glutamine decarboxylases GadA and GadB in *E. coli* catalyze the decarboxylation of glutamine which is a proton consuming reaction. The end products are carbon dioxide and γ-aminobutyric acid (GABA). The decarboxylases are co-expressed with an antiporter, GadC, which exports GABA in exchange for glutamine ([Lin *et al.*, 1995; Hersh *et al.*, 1996; Richard and Foster, 2004]). Similar systems are available for the acid dependent decarboxylation of arginine, lysine, or histidine, amongst others. Comparable acid resistance systems have not been detected in acidophiles to date. This might not be surprising as acidophiles typically inhabit environments scarce of organic compounds unlike neutrophiles which can easily import amino acids as substrate for proton consuming decarboxylation. The same system would probably require much more energy for acidophiles due to the necessity to synthesize large amounts of amino acids. However, when it comes to other enzymatic reactions which might be involved in protection against acid it is interesting to note the ability of chemoorganotrophic acidophiles to degrade organic acids. Organic acids are extremely toxic to acidophiles ([Borichewski, 1967; Tuttle and Dugan, 1976; Aston *et al.*, 2009]) as they are protonated and neutral at the external low pH and can easily enter the cell. Within the cytoplasm they rapidly dissociate due to the prevailing circumneutral pH releasing protons and acidifying the cell ([Ingledew, 1982; Alexander *et al.*, 1987]). It has been suggested that the ability to degrade organic acids by extremely acidophilic species, such as *Ferroplasma* spp. ([Dopson *et al.*, 2004]) or *Picrophilus* spp. ([Ciaramella *et al.*, 2005]), might be part of their pH homeostasis system.

Reorganization of the cytoplasmic membrane in response to acid challenge has been reported for neutrophiles ([Chang and Cronan, 1999]) as well as acidophiles ([Mykytczuk *et al.*, 2010]). Studies on *E. coli* have demonstrated the involvement of cyclopropane fatty acids in the low pH
response (Chang and Cronan, 1999). Furthermore, the microbial sterol analogues, hopaniods, are involved in acid stress in neutrophiles (reviewed in Kannenberg and Poralla, 1999). While Mykytczuk et al (2010) studied the fatty acid profiles of several strains of *At. ferrooxidans* and showed that the membrane fluidity was adjusted in response to external pH in a strain-specific manner. The importance of rigid archaean membranes composed of tetraether lipids for inhabiting extreme environments has been pointed out on many occasions (van de Vossenberg *et al.*, 1998; Albers *et al.*, 2000; Konings *et al.*, 2002; Baker-Austin and Dopson, 2007). Additionally, changes due to low pH have been detected in the *At. ferrooxidans* outer membrane (Amaro *et al.*, 1991). Particularly, the *At. ferrooxidans* OMP40 outer membrane pore has specific characteristics, such as small pore size and amino acid composition, which suggest an acid adaptation of the protein (Guiliani and Jerez, 2000). Unfortunately, this is the only report on the subject and it would be interesting to confirm such acid adaptation for further acidophile outer membrane proteins (OMPs). Finally, it has been proposed that the inside positive membrane potential is an intrinsic mechanism of acidophiles to hamper the leakage of protons into the cytoplasm (Baker-Austin and Dopson, 2007). Due to this lack of knowledge in acidophilic pH homeostasis the proteomic response to growth in non-optimal pH conditions was studied in *At. caldus* with paper IV.

6. The Post-Genomic Era

Fifteen years ago the genome sequence of *E. coli*, the most studied microorganism and one of molecular biology’s workhorses, was announced (Blattner *et al.*, 1997) and about ten years ago the human genome was successfully sequenced (Venter *et al.*, 2001). These were the beginnings of vast genome sequencing endeavors which resulted in an exponentially growing amount of sequence data from all domains of life. The technical advances during the last 10 years have also allowed for further new, global methodologies such as proteomics and metabolomics which let us into the so-called post-genomic era. By integrating the various approaches with systems biology, those developments opened the door ajar to glimpse at the big question of what life is. In the following sections the toolkit (genetic tools, ‘omics’ techniques, and use of continuous culture) of the post-genomic era will be explained and put into context with acidophile research. In the end of this section, special notice is given to systems biology with its daunting tasks and its great potential.
6.1. Genetic Tools for Acidithiobacilli

An important tool in molecular biology research is the genetic manipulation of organisms primarily used for evaluating the function of unknown genes. Creating knock-out mutants of the genes in question followed by phenotypic testing and evaluation of the complemented mutant is an elegant way to assess a hypothesized function. The function can be further characterized by cloning the target genes and e.g. heterologous expression of the corresponding proteins in a host such as E. coli followed by subsequent purification. Targeted point mutations or partial deletions can help to study the structure or function of certain domains within the protein of interest. Genetic manipulations of acidophiles, however, have been difficult to realize probably due to their distinct physiology and because the available genetic protocols are optimized for pH neutral conditions (Holmes and Bonnefoy, 2007). Only few reports of successfully created mutants of acidithiobacilli have been published and no standard protocols are available. Major obstacles for an efficient method are, apart from the introduction of exogenous DNA, long generation times and poor growth on solid media which makes enrichment and selection of mutants extremely cumbersome. The most common technique for introducing exogenous DNA to neutrophilic organisms is electroporation (also termed electrottransformation). Remarkably, there are only three reports of electroporation applied to acidithiobacilli. In 1992 Kusano et al. (1992) reported the electroporation of At. ferrooxidans, however, the protocol was not very efficient with about 200 transformants per µg of DNA. Furthermore, the protocol did not work on all the strains tested. Recently a more efficient protocol for At. caldus was presented (Chen et al., 2010) where a maximum transformation efficiency of $3.6 \times 10^4$ transformants per µg DNA was achieved. The authors introduce this as a convenient and robust protocol and suggest the usage as a standard protocol for recombinant gene transfer and creating knock-out mutants. In their study they only show a proof of principle by introducing marker genes in the At. caldus genome without demonstrating a knock-out mutant nor testing several recipient strains. Nevertheless, more recently, the same authors successfully applied their protocol for construction of a sulfur oxygenase reductase (Sor) deletion mutant (Chen et al., 2012). Another method for genetic manipulation is conjugation which was applied for most acidithiobacilli (see Table 4 for an overview and references). In most studies the maximum conjugation frequencies were around $10^{-4}$ and $10^{-5}$ transconjugants per recipient. Though, frequencies were often not very reproducible with large variations for the same protocol (van Zyl et al., 2008), depending on the plasmid (Jin et al., 1992; Liu et al., 2000; Liu et al., 2007), or depending on the recipient strain (Liu et al., 2000). Mostly conjugation was applied to introduce a heterologous gene, such as a metal
resistance gene, to genetically modify the recipient strain (Table 4). Only in three reports conjugation was used to create deletion mutants (Liu et al., 2000; van Zyl et al., 2008; Wang et al., 2012) followed by functional assessment of those genes. In both At. ferrooxidans (Liu et al., 2000), and At. caldus (van Zyl et al., 2008), marker exchange was used to disrupt the target genes and in both cases a kanamycin resistance marker gene was applied. In contrast, a markerfree approach based on the homing endonuclease I-SceI was used to create a knock-out of putative phosphofructokinase in At. ferrooxidans (Wang et al., 2012). Another recent report demonstrated the applicability of a small broad-host-range vector, pBBR1MCS-2, for efficient conjugation of acidithiobacilli (Hao et al., 2012). Unlike vectors previously used for conjugation pBBR1MCS-2 does not belong to the IncQ, IncW or IncP groups. The new plasmid proved to be efficiently transferred and stable and might be a useful future genetic tool (Hao et al., 2012).

Table 4. Overview of conjugation protocols for acidithiobacilli

<table>
<thead>
<tr>
<th>Organism</th>
<th>Method</th>
<th>Trait studied</th>
<th>Max. Transfer frequency</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>At. ferrooxidans</td>
<td>heterologous gene transfer</td>
<td>arsenic resistance</td>
<td>2.9 x 10^{-4}</td>
<td>Peng et al., 1994a</td>
</tr>
<tr>
<td>At. ferrooxidans</td>
<td>marker exchange, knock-out mutant</td>
<td>recA gene</td>
<td>10^{-3}</td>
<td>Lui et al., 2000</td>
</tr>
<tr>
<td>At. ferrooxidans</td>
<td>markerless gene replacement, knock out mutant</td>
<td>phosphofructokinase</td>
<td>10^{-4}</td>
<td>Wang et al., 2012</td>
</tr>
<tr>
<td>At. caldus</td>
<td>heterologous gene transfer</td>
<td>arsenic resistance</td>
<td>10^{-4}</td>
<td>Zhao et al., 2005</td>
</tr>
<tr>
<td>At. caldus</td>
<td>heterologous gene transfer</td>
<td>arsenic resistance</td>
<td>n.a.</td>
<td>Kotze et al., 2006</td>
</tr>
<tr>
<td>At. caldus</td>
<td>heterologous gene transfer</td>
<td>proof of principle</td>
<td>10^{-4}</td>
<td>Lui et al., 2007</td>
</tr>
<tr>
<td>At. caldus</td>
<td>marker exchange, knock-out mutant</td>
<td>arsenic resistance, tetrathionate hydrolase</td>
<td>10^{-5}</td>
<td>van Zyl et al., 2008</td>
</tr>
<tr>
<td>At. caldus</td>
<td>heterologous gene transfer</td>
<td>mercury resistance</td>
<td>1.5 x 10^{-4}</td>
<td>Chen et al., 2011</td>
</tr>
<tr>
<td>At. caldus and At. thiooxidans</td>
<td>heterologous gene transfer</td>
<td>proof of principle</td>
<td>10^{-7}–10^{-5}</td>
<td>Hao et al., 2012</td>
</tr>
<tr>
<td>At. thiooxidans</td>
<td>heterologous gene transfer</td>
<td>proof of principle</td>
<td>10^{-5}</td>
<td>Jin et al., 1992</td>
</tr>
<tr>
<td>At. thiooxidans</td>
<td>heterologous gene transfer</td>
<td>proof of principle</td>
<td>10^{-5}</td>
<td>Peng et al., 1994b</td>
</tr>
<tr>
<td>At. thiooxidans</td>
<td>heterologous gene transfer</td>
<td>phosphofructokinase</td>
<td>10^{-5}</td>
<td>Tian et al., 2003</td>
</tr>
</tbody>
</table>

*n.a. Not possible to determine the transfer frequency as distinct colonies were not formed.
Molecular cloning of genes and their heterologous expression in a host such as *E. coli* is technically more straightforward and has been reported for genes of acidithiobacilli even prior to the availability of the respective genome sequence (Barros et al., 1985; Shiratori et al., 1989). For periplasmic and OMPs which are naturally exposed to acidic pH it has been reported that heterologous expression in a neutrophilic host can lead to false folding or failure of co-factor binding (Bengrine et al., 1998; Kanao et al., 2010). Interestingly, the heterologous expression of a periplasmic protein secreted through the twin arginine translocation (tat) pathway yielded a protein with properly bound co-factor (Bruscella et al., 2005). This is probably due to the fact that in the tat system proteins are fully folded within the cytoplasm and not in the periplasm, as it is the case for the secretory (sec) pathway (Bruscella et al., 2005).

In conclusion, almost 20 years of efforts in establishing a method for the genetic manipulation of acidithiobacilli has so far not lead to routine use of any of the suggested protocols. Possibly, recent developments such as the protocols of Chen et al. (2010), Hao et al. (2012), or Wang et al. (2012) might establish themselves as standard procedures. In any case, a robust and convenient protocol would greatly advance the basic and applied research in acidophiles. Until such routine genetic analysis is available the use of ‘omics’ (Burg et al., 2011) techniques will continue to provide useful insights into acidophile physiology and community interactions as will be discussed in the following section.

### 6.2. ‘Oomics’ Techniques and Continuous Culture

All ‘omics’ techniques are global approaches which seek to depict the entire collection of a certain biomolecule present under a specific condition and time (Figure 4), i.e. genomics is the collection of all genes encoded by an organism, transcriptomics captures all RNA transcripts of active genes and so on. Initially, all ‘omics’ techniques pertained to single organisms and pure strains, however, it is also possible to study much more complex microbial communities with the same approaches. Usually the prefix ‘meta’ or the terms ‘community’ and ‘environmental’ are used to denote this application (Figure 4).

In recent years DNA sequencing technologies have dramatically improved while costs have dropped which allows fast and inexpensive sequencing of genomes or metagenomes. The traditional Sanger DNA sequencing method is now largely replaced by high throughput next generation sequencing (NGS) approaches (Novais and Thorstenson, 2011; Shokralla et al., 2012). Several commercial NGS platforms are available having individual advantages and drawbacks (Shokralla et al., 2012). One example is a PCR-based technology for short- to medium-reads termed pyrosequencing which
functions by detecting the release of pyrophosphate during DNA synthesis (Novais and Thorstenson, 2011).

Today the genome sequences of all acidithiobacilli except At. albertensis are available (Valdes et al., 2008b; Valdes et al., 2009; Liljeqvist et al., 2011; Valdes et al., 2011). In the first place the availability of the genome sequences enabled in silico analysis of the predicted genes and gene products thereby providing insight into the genetic potential of the organism (Valdes et al., 2008b; Quatrini et al., 2009). Furthermore, with the availability of several acidophilic genome sequences it was possible to compare acidithiobacilli with each other (Valdes et al., 2008a) or specific traits within the acidophile community such as carbon and nitrogen fixation (Levican et al., 2008). Such bioinformatic analyses of several genome sequences is named comparative genomics and it can aid in assessing the genetic capacities of organisms and provide clues on interesting traits which should be studied in more detail. An overview of available acidophile genome sequences and associated bioinformatics studies is given in a recent review by Cardenas et al. (2010). But not only are the genome sequences of pure strains rewarding but also genome sequences of the whole microbial community in a certain environmental niche are of great interest. These sequences are commonly called metagenomes. The immense benefit that metagenomics holds is that it can capture all members of a microbial community even those that are uncultivable (reviewed in Simon and Daniel, 2011). The accessibility of uncultivable microbes has already allowed the discovery of new antibiotics and enzymes and will undoubtedly further provide impetus to biotechnology (reviewed in Schloss and Handelsman, 2003). Additionally, metagenomics can also advance our knowledge in microbial ecology, as well as evolution and diversity (reviewed in Rodriguez-Valera, 2004). However, with the ability to sequence metagenomes new challenges are connected which have been pointed out recently by several researchers (Desai et al., 2012; Dini-Andreote et al., 2012). Their concerns are largely related to computational problems due to large amounts of data and the difficulty to resolve complex community structures on a strain level (Desai et al., 2012; Dini-Andreote et al., 2012). A milestone in metagenomic studies was the random shotgun sequencing analysis of an AMD community from the Iron Mountain Mine, USA, which revealed the near-complete genome sequences of Ferroplasma type II and Leptospirillum group II as well as other partial genomes (Tyson et al., 2004). The low diversity within the AMD community allowed effective genome reconstruction. With the help of the genomic data, information about prevailing metabolic pathways and survival strategies in acidic environments was reconstructed. Recently, pyrosequencing has been applied to study the metagenome of acidic biofilms, called ‘snottites’ that form in hydrogen sulfide rich caves (Jones et al., 2012). Although there are major geochemical differences between the
snottite habitat and AMD sites both acidophilic communities showed remarkable similarities: (i) low microbial diversity; (ii) dominance of acidophilic chemolithoautrotrophs; (iii) lower contribution of heterotrophic microbes; and (iv) membrane adaptations to survival in acidic environments (Jones et al., 2012).

![Diagram of 'omics' techniques and systems biology](image)

**Figure 4.** Overview of ‘omics’ techniques and systems biology. All ‘omics’ techniques can be applied for pure cultures and microbial communities investigating various aspects of organisms or environmental communities. Data generated by ‘omics’ techniques can be used to generate mathematical models of biological systems. The models can be used to test hypotheses about the systems and can further be refined in an iterative way.

Although genome sequences of pure strains as well as metagenomes largely expanded our knowledge of acidophiles and their communities, the type of information available from DNA sequences is limited to putative function only. In order to receive functional information other types of analysis such as transcriptomics and proteomics are required. As transcriptomics is based on nucleotide sequencing the same sequencing methods as for genomics and metagenomics can be applied. The first approaches to transcriptomics were done by extraction of mRNA and reverse transcription into DNA. Analysis was frequently done with microarrays bearing probes for genes of interest usually covering the whole genome (reviewed in Watson et al., 1998). The latest technique is to directly sequence the transcripts using NGS techniques. In contrast to DNA sequences, transcriptomics is based on mRNA sequences and therefore, gene expression information is retrieved. By using microarrays and mRNA extractions from
acidophilic microorganisms grown at different conditions differential gene expression profiles can be accessed and used to learn more about stress responses (Yin et al., 2012), metabolic pathways (Appia-Ayme et al., 2006; Quatrini et al., 2006; Auernik and Kelly, 2008), and also environmental community functions by metatranscriptomics (Parro et al., 2007; Moreno-Paz et al., 2010).

Proteomics takes functional analysis one step further by analyzing the whole set of proteins expressed in a certain condition or environment (metaproteomics/community proteomics). Proteins are non-homogenous molecules having diverse properties, differing in e.g. charge, hydrophobicity, molecular weight (MW), and a large dynamic range of abundance. This complexity makes it difficult to simultaneously analyze all proteins expressed in a certain condition using the same method. Thus, a variety of different methods was developed targeting various subproteomes and protein species thereby attempting to circumvent this problem (Poetsch and Wolters, 2008; Casado-Vela et al., 2011; Ly and Wasinger, 2011). On the other hand, proteomics has a technical advantage over transcriptomics as proteins are more stable than mRNAs thus analysis is more robust. Furthermore, proteins are in most cases the final, functional gene products (although there are exceptions such as genes coding for small regulatory RNA for example) making information derived from proteomics a very strong case. Moreover, it has been noted in many studies trying to correlate transcriptomics and proteomics data that mRNA levels do not always correspond to protein levels which makes it extremely difficult to extrapolate functional information from transcriptomics data alone (Pradet-Balade et al., 2001 and references within). The two main approaches in proteomics are gel-based and gel-free methods. The most common gel-based method is two dimensional polycrylamide gel electrophoresis (2D-PAGE) which can visualize and quantify the protein spots directly on a gel and subsequently spots of interest are identified by mass spectrometry (MS). The disadvantage of this approach is the incompatibility with integral membrane proteins and its limited dynamic range. On the contrary, gel-free approaches mostly based on the mass spectrometric analysis of peptide mixtures in solution have been shown to detect membrane proteins. Both methodologies have strengths and weaknesses, however when it comes to quantification of expression levels the ‘traditional’ 2D PAGE approach still has advantages over quantification by MS (Wilmes and Bond, 2006). Generally, the identification of proteins is much more straight forward if genome data of the studied organism is available. If this is the case most proteins can easily be identified by Matrix assisted laser desorption ionization Time of Flight (Maldi-ToF) MS. If no sequence data is available cumbersome de novo sequencing MS techniques have to be used. Gel-based differential expression proteomics was applied to study various aspects of acidophile physiology. Early work on At.
ferrooxidans included differential proteomics in response to pH stress (Amaro et al., 1991), phosphate starvation (Vera et al., 2003), and different energy substrates (Ramirez et al., 2004). Additionally, periplasmic proteins were investigated using a gel-free approach (Chi et al., 2007). Gel-based proteomics was also used to help elucidate aspects of Ferroplasma spp. physiology such as metal resistance (Baker-Austin et al., 2005; Baker-Austin et al., 2007), anaerobic growth (Dopson et al., 2007), and iron homeostasis (Potrykus et al., 2011). In contrast to the investigation of a single organism, metaproteomics or community proteomics provide the opportunity to gain functional insights into the whole microbial community of a given habitat (reviewed in Wilmes and Bond, 2006; Wilmes and Bond, 2009). The first report of metaproteomics of an acidophilic community was conducted on biofilm samples from AMD sites at Iron Mountain, USA (Ram et al., 2005). The authors used a gel-free, shotgun MS technique as well as metagenomic data for their analysis. They revealed that apart from anticipated metal and acid stress, oxidative stress also plays a role in AMD sites. Further, it was shown that certain members of the community were responsible for specific processes, such as extracellular polymer production or nitrogen fixation (Ram et al., 2005). Other studies of AMD communities also combined metagenomic and metaproteomic data (Lo et al., 2007; Goltsman et al., 2009). Additional work on AMD communities in the laboratory of Jillian Banfield advanced those ‘omics’ approaches by integrating proteomics, geochemical, and biological data of multiple communities and over a period of time (Mueller et al., 2010; Mueller et al., 2011). In the same research group a method for cultivation of AMD biofilms under laboratory conditions was developed which can now be used to test hypotheses under controlled conditions (Belnap et al., 2010; Belnap et al., 2011). Certainly, integrated ‘omics’ approaches such as those mentioned will further help to understand the complex processes and interactions within environmental communities (reviewed in Zhang et al., 2010).

One of the most recent ‘omics’ methods is the investigation of the entire set of metabolites under a given condition (Figure 4), termed metabolomics. Metabolomics is a potent technique which is capable of quantitatively analyzing hundreds of metabolites in one sample. Often the samples are separated by liquid chromatography and subsequently analyzed by MS. Additionally, nuclear magnetic resonance or gas chromatography coupled to MS (GC-MS) are possible alternative methods for analysis. Due to the vast complexity and the possibly very short half-lives of metabolites existing in a microbial cell this type of analysis bears its specific challenges. However, with advancing analytical techniques those challenges are being addressed (Patti et al., 2012). Metabolomics expands the functional studies of transcriptomics and proteomics to yet another level and additionally allows information on pathway regulation and metabolite fluxes (termed fluxomics)
to be collected. When metabolomics is applied to communities of several organisms it is termed environmental metabolomics which is another emerging field (reviewed in Viant, 2008; Bundy et al., 2009). Recently, the first metabolomics study on acidophiles has been published (Martínez et al., 2012). Strains of *At. ferrooxidans* and *At. thiooxidans* were grown on various substrates and their metabolic profiles investigated using capillary electrophoresis and MS. The polyamide spermidine was detected in both strains and the authors suggest a role in biofilm formation whereas the finding that glutathione was present primarily in the sulfur oxidizing species strengthened its suggested role as activator of sulfur during the sulfur oxidation pathway (Martínez et al., 2012). Another interesting approach is the application of metabolomics to study toxicity (reviewed in Booth et al., 2011) which might also be of interest for metal toxicity in acidophiles.

In order to draw valid conclusions from ‘omics’ experiments (as for other biological experiments) the experimental design is of great importance. For the study of pure cultures and model organisms the cultivation of microorganisms in the laboratory is obligatory. Consequently, the experimental design starts with growing the biomass for the experiments. The method of culturing will unavoidably influence the physiology of the organism under study. Thus, the apparently trivial task of growing the biomass for a subsequent experiment turns out to be an essential part of the experimental design. Fundamentally, there are two types of liquid culturing techniques available: (i) the closed system, i.e. batch cultures; and (ii) continuous cultures which are open systems (Figure 5). Often batch cultures are the routine mode of growth in many microbiological laboratories since they are simple and do not require special equipment or maintenance. A certain amount of medium containing all substances necessary for growth is inoculated with microorganisms, then placed at the desired temperature and usually aerated by shaking. During this mode of growth the bacteria continuously adapt to varying conditions as substrates are used up while metabolites and waste products accumulate in the medium (Figure 5A). This is often accompanied by changes in pH and possibly other parameters (such as redox potential in the case of iron rich media for acidophilic iron oxidizers). Those changing conditions are avoided in the open system where a constant culture volume is kept in balance by continuously feeding fresh growth medium and removal of superfluous culture liquid (waste, Figure 5B). After inoculation and equilibration of the continuous culture the microorganisms grow at a fixed, constant rate and all concentrations within the culture are constant as well (Figure 5B). Therefore, the continuous mode of growth avoids the largest disadvantage of batch culturing, i.e. changing growth phases which can possibly mask the physiological response being studied.
Recently, microbiologists have rediscovered the benefits of continuous cultures and realized that in order to address the complex biological questions of today culturing microorganisms in a strictly controlled manner is a prerequisite (Hoskisson and Hobbs, 2005; Pullan et al., 2008; Bull, 2010). Especially, when it comes to applying ‘omics’ techniques samples originating from continuous growths are better suited (Hoskisson and Hobbs, 2005; Pullan et al., 2008), showing for example less variation (Piper et al., 2002). Furthermore, a recent study demonstrated that the toxicity of metals differs significantly for bacteria grown in batch and continuous culture (Şengör et al., 2012) which is important to consider for research and application of acidophiles as they are often faced with high concentrations of metals.

![Figure 5](image_url)

**Figure 5.** Batch (A) and continuous (B) systems for culturing microorganisms. Batch cultures are closed systems where generation time/biomass of microorganisms as well as concentrations of substrate and metabolites change over time. In continuous cultures a constant volume is kept by feeding fresh substrate, and removing waste, thereby constant concentrations of biomass, substrate, and metabolites as well as a fixed generation time are maintained.

### 6.3. Systems Biology

Systems sciences are multidisciplinary approaches to complex systems. Data from parts comprising the system of interest are collected, studied, and evaluated. Finally, mathematical tools are used to infer a model describing
the system. This model can be used to make predictions of the system and by testing the model with a new set of data it can subsequently be improved in an iterative way (Figure 4). The great potential of the systems sciences is that they integrate all aspects of the system, and thereby leave the reductionist point of view behind. Systems sciences are for example thermodynamics or ecology (Smith et al., 2012). However, with the advent of ‘omics’ techniques and the large amounts of data that are produced by applying them, systems sciences are also being applied to biological systems (Ideker et al., 2001; Kitano, 2002b, a), and the term systems biology was coined. Systems biology can describe processes at such a small scale as a single cell or expand to larger scales such as a multi-cellular organism or a whole habitat. Since its emergence, systems biology has been praised as the key to understand the principles of life. Although we are still at the beginning of this quest, which started about 10 years ago, scientists still believe that systems biology can provide those answers (Pfau et al., 2011; Ideker and Krogan, 2012; Khatri et al., 2012). Undoubtedly, many challenges still lie ahead but progress has been made (Pfau et al., 2011; Ideker and Krogan, 2012; Khatri et al., 2012). A recent review on systems biology of the model microbe E. coli describes how genome scale models can be applied to five categories: (i) metabolic engineering; (ii) bacterial evolution; (iii) network analysis; (iv) phenotypic behavior; and (v) biological discovery (Feist and Palsson, 2008). The research of lactic acid bacteria has also benefited from systems biology approaches by providing “new knowledge or perspective, often counterintuitive, and clashing with conclusions from simpler approaches” (Teusink et al., 2011). Another interesting review highlights the benefits of systems biology approaches for the study of toxicity, including metal mixtures (Spurgeon et al., 2010). When it comes to metal-microbe interactions two studies are of particular interest, a constraint based model on Geobacter sulfurreducens (Mahadevan et al., 2006) and dynamic multi-species metabolic modeling (DMMM) on Geobacter and Rhodoferax (Zhuang et al., 2011). Geobacter sulfurreducens grows anaerobically by oxidation of organic compounds coupled to the reduction of ferric iron and it can be applied for the remediation of metal or organic loaded water. With the help of the constraint based model, Mahadevan and co-workers (2006) explored the central metabolism and electron transport of G. sulfurreducens. They point out that the iterative modeling together with experimentation can aid in comprehending the physiology of poorly studied organisms which are of environmental interest and eventually this can help in improvement of their application. A great advance for the study of microbial community interactions is the report on the first multiple genome scale metabolic model (Zhuang et al., 2011). Here the DMMM is used to predict the microbial interactions of two ferric iron reducing organisms which are competing in in situ bioremediation of ground water contaminated with uranium. Although
the presented model takes only two members of the microbial community into account this approach has significant potential for future studies of microbial ecology. Also in acidophile research mathematical metabolic models based on genome sequence data have been developed. So far stoichiometric models of *At. ferrooxidans* (Hold *et al.*, 2009) and *L. ferrooxidans* (Merino *et al.*, 2010) have been presented and both showed good behavior when comparing calculated values with experimental data from literature. Certainly, more such approaches are needed to advance our knowledge of acidophile physiology and community structure.
Aims of This Study

The main aim of this study was to elucidate strategies of acidithiobacilli to cope with the extreme conditions prevailing in their typical habitat. In order to meet this aim several aspects of acidithiobacilli physiology were investigated in more detail: (i) substrate metabolism; (ii) metal resistance; and (iii) pH homeostasis.

*At. caldus* is an important sulfur oxidizer within the acidophile community and valuable for industrial biomining. Still its sulfur oxidation pathways are poorly characterized. In paper I we aimed to learn about sulfur metabolism in *At. caldus*.

A lot is known about *At. ferrooxidans* sulfur and iron oxidation. However, the metabolic pathways for anaerobic sulfur oxidation coupled to ferric iron respiration are still elusive. We aimed to elucidate those pathways and find the enzyme responsible for ferric iron reduction in paper II.

Zinc can reach high concentrations in AMD environments or biomining activities. Such high concentrations of this essential metal ion can be toxic. With a comparative study on three acidophilic model organisms we aimed to clarify the mechanisms of zinc tolerance in model organisms in paper III.

The main characteristic of acidophiles is their acidophilic nature which is yet poorly understood. In paper IV we aimed to learn more about pH homeostasis in *At. caldus*.
Results and Discussion

In order to reach the specific aims four studies dealing with the individual aspects have been conducted and are presented within this thesis. As reviewed in chapter 6 of the introduction, tools for genetic manipulation of acidophiles are still not feasible for routine experimentation whereas various ‘omics’ techniques offer great potential for the study of extremophiles. Furthermore, the recent availability of the At. caldus genome sequence offered new possibilities for the investigation of this organism, such as bioinformatics and proteomics. Consequently, a focus of this thesis was to apply ‘omics’ techniques, mostly proteomics, to study acidophilic model organisms. The proteomics experiments were based on differential protein expression assessed by 2D-PAGE and protein identification by Maldi-ToF. In the following the results of the presented papers are summarized and their contribution to the field discussed.

Paper I: Sulfur metabolism in the extreme acidophile Acidithiobacillus caldus

Previous knowledge of At. caldus sulfur oxidation pathways was fragmentary despite the importance of this organism for industrial applications. Consequently, the newly available genome sequence of At. caldus was scrutinized by bioinformatics to predict genes putatively involved in sulfur metabolism. As the bioinformatics survey revealed a different set of sulfur oxidation enzymes compared to At. ferrooxidans a more detailed study was initialized. For the following experiments At. caldus was grown in continuous culture on two different sulfur compounds, solid elemental sulfur and soluble tetrathionate. The first indication that putative sulfur oxidation genes were activated under these conditions was provided by measuring mRNA levels of selected genes with semi-quantitative reverse transcription polymerase chain reaction (semiQ RT-PCR). Furthermore, a proteomics approach was used to pinpoint differentially expressed proteins during the utilization of the two sulfurous substrates. Based on the results, a model for sulfur oxidation in At. caldus was presented.

An overview of the genes putatively involved in sulfur metabolism revealed by the bioinformatics analysis is presented in Figure 1A of paper I. Interestingly, At. caldus harbors homologous genes detected in At. ferrooxidans as well as additional genes which have not been previously shown in acidithiobacilli. The former includes the genes sqr, th, and hdr, whereas the latter were the sulfur oxidation (sox) genes and sor. Except for th and sor, two copies of the respective genes were detected in the At. caldus genome. Most striking was that two sox gene clusters with differing gene
organization were found. Both of them contained soxABXYZ which together encode a protein complex required to oxidize thiosulfate. Thiosulfate oxidation by the Sox system has been well studied in *Paracoccus pantotrophus* (Friedrich et al., 2005) and green sulfur bacteria (Sakurai et al., 2010) and the presented paper is the first report of a Sox system in acidophiles. The enzyme Sor, on the other hand, has been studied in the archaea *Acidianus ambivalens* (Urich et al., 2004) and its activity has been demonstrated in *At. caldus* like strains (Janosch et al., 2009).

Based on the bioinformatics predictions primers were designed specifically targeting a selection of the putatively involved genes. The results of semiQ RT-PCR for *At. caldus* grown on tetrathionate and solid sulfur are shown in Figure 2 of paper I. All sox genes except soxZ from both gene clusters were up-regulated during the growth on tetrathionate and no detectable differential expression was noted with any of the other genes tested. These results suggested that both sox clusters were regulated alike and were possibly involved in thiosulfate oxidation which is a degradation product of tetrathionate (de Jong et al., 1997). The reason why both soxZ copies did not follow the same trends as the other sox genes remains unknown. Additionally, no differential mRNA levels for sor implied that this enzyme was transcribed at similar levels during the conditions tested. The same was true for *tth* and *doxD* which was unexpected as they have been previously reported to be up-regulated upon growth on tetrathionate (Rzhepishevska et al., 2007).

The proteomics study of this paper was divided into two comparisons. Firstly, continuous culture samples grown on tetrathionate or sulfur were compared (Figures 3 and 4 in paper I) and secondly, planktonic as well as sessile cells of sulfur grown batch cultures were harvested and analyzed (Figure 5 in paper I). Complete lists of identified, differentially expressed proteins are supplied in the appendix of paper I. Several proteins predicted to be involved in sulfur metabolism were differentially expressed. Proteins encoded by both sox clusters were up-regulated during growth on tetrathionate which was in accordance with the results from semiQ RT-PCR. Additionally, both orthologues of Sqr were up-regulated on tetrathionate. Several Hdr subunits were differentially expressed whereas neither Tth nor Sor were detected in proteomics which strengthens the semiQ RT-PCR results. These results indicated that the predicted genes were involved in sulfur metabolism and that some of them were differentially regulated depending on the main sulfur compound available. Furthermore, the results from the first proteomics comparison revealed different general trends for cells grown on tetrathionate and sulfur. During growth on tetrathionate proteins involved in central carbon metabolism, cell division, translation, and DNA repair were up-regulated. Up-regulation of proteins from those functional groups might suggest that bacteria grew faster on tetrathionate
than on sulfur. However, since the biomass was produced in continuous cultures with the same flow rates the growth rates were also identical. Therefore, this trend could point to a stress response during growth on sulfur triggering the down-regulation of central functions. Additionally, up-regulation of chaperones and proteases during growth on sulfur suggested high stress levels in this condition. Some proteins involved in carbon dioxide fixation were also expressed in higher levels during utilization of sulfur, however, the reason for this remains unknown.

The interpretation of the results was complicated by the fact that sulfur is a solid substrate and bacteria can grow either attached to the substrate or in a planktonic state. Interestingly, even though sufficient surface area was available a subpopulation of planktonic cells remained (Arredondo et al., 1994). Thus, a proteomic comparison of sessile and planktonic subpopulations was carried out to study potential differences in the subpopulations. Due to low yields of sessile cells which can be extracted from sulfur it was necessary to grow the biomass for this experiment in large batch cultures. The trends found in sessile and planktonic cells were not as clear cut as in the case of the comparison between sulfur- and tetrathionate grown cells. All subunits of Hdr were found to be up-regulated in sessile cells whereas Sqr and proteins encoded by the sox cluster were up-regulated in planktonic cells. Furthermore, proteins involved in amino acid biosynthesis and central carbon metabolism were present in higher levels in sessile cells suggesting that those are less starved than planktonic cells. Nevertheless, chaperones, which usually indicate stress, were also higher expressed in sessile cells. A twitching motility protein and a YVTN family beta-propeller repeat protein up-regulated in sessile cells might be involved in attachment to the substrate. It was suggested that the sessile cells mainly utilized the elemental sulfur and that Hdr was important for its oxidation. In contrast, planktonic cells might be able to use soluble sulfur compounds possibly released from sessile cells and therefore, Sqr and Sox proteins were up-regulated. However, this remains to be proven.

A model of *At. caldus* sulfur metabolism based on the above results is presented in Figure 1B of paper I. The proteomics and semiQ RT-PCR results showed clear indications that proteins encoded in both sox clusters were involved in sulfur metabolism. The same holds true for Hdr which was previously suggested to be involved in *At. ferrooxidans* sulfur oxidation (Quatrini et al., 2009). Although there are still open questions this model provides a good basis for further targeted investigations of implicated enzymes.

In the meantime, Chen et al. (2012) published a refined sulfur oxidation model for *At. caldus* MTH-04 based on transcriptomic analysis of the wild type and a sor deletion mutant grown on elemental sulfur and tetrathionate. While some aspects of the new model are essentially as proposed in paper I,
such as the role of the Sox system, Tth, proposed sulfide oxidation, as well as electron transport through the quinone pool to terminal oxidases $bo_3$, $bd$, or the NADH complex, the recently presented model suggests novel oxidation pathways for elemental sulfur. Three elemental sulfur oxidation pathways are proposed to take place in various cell compartments: (i) a sulfur dioxygenase (SDO) oxidizes the activated sulfur in the periplasm to yield sulfite; (ii) Hdr oxidizes GSSH in the cytoplasm near the inner membrane to regenerate thiol proteins (GSH); and (iii) Sor oxidizes cytoplasmic elemental sulfur originating from periplasmic oxidation processes (e.g. produced by SQR or the Sox system) to yield thiosulfate. The thiosulfate is proposed to be oxidized by rhodanese using GSH as sulfur atom acceptors and yielding GSSH which in turn is the substrate for Hdr. Furthermore, sulfur oxidation by Sor is suggested to be coupled neither to the electron transport chain nor to substrate level phosphorylation. Hence, in the novel model Hdr as well as Sor are responsible for oxidation of cytoplasmic elemental sulfur preventing the accumulation of intracellular sulfur globules. In contrast, SDO is proposed to solely deal with sulfur of extracellular origin. The SDO enzyme activity has been reported for acidithiobacilli but the identity of the SDO protein remained unknown so far (Rohwerder and Sand, 2003). In the bioinformatics analysis of paper I no putative SDO was detected. Unfortunately, the data describing the cloning and characterization of the novel SDO in *At. caldus* is not presented by Chen et al. (2012).

**Paper II: Anaerobic sulfur metabolism coupled to dissimilatory iron reduction in the extremophile *Acidithiobacillus ferrooxidans***

The ability of *At. ferrooxidans* to grow anaerobically was reported many years ago (Brock and Gustafson, 1976; Das et al., 1992; Pronk et al., 1992), yet, the underlying mechanisms are largely unknown. Therefore, in paper II a global transcriptomics and proteomics study of *At. ferrooxidans* sulfur oxidation coupled to ferric iron reduction was conducted. Growth with the electron donor sulfur and ferric iron as electron acceptor was compared to growth with sulfur as electron donor and molecular oxygen as electron acceptor. The biomass for RNA and protein extractions was grown in pH controlled batch cultures and analysis was based on three biological replicates. For complete lists of differentially expressed genes and proteins see Supplemental Tables S1 and S2 of paper II.¹

In aerobic as well as anaerobic condition sulfur is proposed to be oxidized by Hdr to produce sulfite. The HdrB subunit was expressed in both conditions but with increased levels under aerobic conditions. The same was

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¹ Supplemental data tables are not shown due to space limitations.
true for the HdrA subunit and a hypothetical protein encoded within the Hdr gene cluster. In both conditions the sulfite generated by Hdr was proposed to be further converted to sulfate or assimilated for growth according to the aerobic sulfur oxidation model (Quatrini et al., 2009). Differentially expressed genes/proteins from those pathways supported this suggestion (Table 1 of paper II). Interestingly, the iron-binding subunit of sulfur reductase (SreB) was uniquely expressed during anaerobic growth which suggested the production of sulfide from reduction of sulfur by Sre under anaerobic conditions. Furthermore, the proteins DsrE and TusA were up-regulated in anaerobic conditions and thus were proposed to transfer sulfide out of the cell. Taken together this indicated an indirect ferric reduction pathway with the abiotic reduction of the terminal electron acceptor by the generated sulfide. Biological production of sulfide during anaerobic growth was evaluated by anaerobic incubation in the presence of soluble copper. Inoculated cultures showed black precipitates, most likely copper sulfide, and a reduced concentration of soluble copper. While in sterile controls black precipitates were not produced. This was a strong indication of sulfide production during anaerobic growth of At. ferrooxidans and supports the hypothesized indirect ferric reduction pathway. Models for the aerobic and anaerobic electron transfer and energy conservation were presented in Figure 1 of paper II.

Previously, proteins encoded in the rus operon, TtH, or ArsH were suggested to reduce ferric iron under anaerobic conditions (discussed in chapter 3.3). However, the data presented here did not support the role of a ferric reductase for any of the mentioned proteins. Although no alternative putative ferric reductase was detected the possibility remains that ferric reduction is mediated by an unknown ferric reductase through the bc1 complex (encoded by petII) and the cytochrome c CycA2 which were up-regulated during anaerobic growth.

The data suggested that energy conservation takes place at the F_{0}F_{1} ATPase. Interestingly, several of the F_{1} catalytic subunits were up-regulated during aerobic growth whereas some of the F_{0} proton translocating subunits were up-regulated under anaerobic conditions (Table 1 of paper II). This indicated that more ATP was generated during aerobic growth and generation of PMF was more important during anaerobic growth. Up-regulation of the F_{1} subunits, i.e. increased ATP production, may be due to an enhanced demand of ATP during aerobic growth. This in turn was most likely due to the accelerated growth rate in the aerobic batch cultures that were harvested after only 3-5 days compared to 2-3 weeks for anaerobic cultures. The faster aerobic growth rate was also reflected in other trends observed in the transcriptomics and proteomics data. Most prominently, many enzymes of the central carbon metabolism were up-regulated during aerobic growth (Table 1 and Figure 2 of paper II). Furthermore, proteins
involved in the oxidative stress response and molecular chaperone functions were more highly expressed during aerobic growth (Table 1 of paper II) probably reflecting both increased oxidative stress as well as increased growth rate. In contrast, the expression patterns of several genes and proteins involved in carbon dioxide fixation via the CBB cycle (Table 1 and Supplemental Figure S3 of paper II) suggested an increased demand of carbon dioxide during anaerobic growth. This was likely caused by the intermittent supply of carbon dioxide during anaerobic cultivation.

The most striking result of the proteomics analysis was the dominance of protein spots on the outer membrane enriched gels from the anaerobically grown cells (Supplemental Figure S2 of paper II). Those highly abundant proteins were identified to be OMP40 and an OMPP1/FadL/TodX family protein. OMP40 was not expressed at significantly different levels in the two tested conditions. The remaining seven protein spots (OMPP1/FadL/TodX family protein) dominating the 2D gel had approximately the same MW but various isoelectric points (pIs) which might be caused by post translational modifications (PTMs). The fact that the expression levels of the OMPP1/FadL/TodX family protein were so high that it dominated the spot pattern of the 2D gel in such a way suggests that this protein had an important function for anaerobic growth. Previously this protein has been shown to be an OMP and to be up-regulated during aerobic growth on sulfur compared to growth on ferrous iron (Yarzabal et al., 2003). The OMPP1/FadL/TodX family protein was proposed to transport hydrophobic compounds into the periplasm (van den Berg, 2010).

**Paper III: Extreme zinc tolerance in acidophilic microorganisms from the bacterial and archaeal domains**

In this paper a detailed study of zinc toxicity in three acidophilic model organisms was presented. The selected model organisms were the Gram negative sulfur oxidizer *At. caldus*, the Gram positive iron oxidizer *Am. ferrooxidans*, and the iron oxidizing archaeon ‘*F. acidarmanus*’. The basis of this study was a bioinformatics prediction of transporters known to be involved in neutrophile zinc resistance mechanisms. These predictions were based on Hidden Markov Models (HMMs) and a total of seven acidophile genomic sequences including the three model organisms were investigated. The toxicity of zinc towards the three model organisms was assessed by batch growth experiments while zinc speciation modeling in the media containing either tetrathionate or ferrous iron was used to estimate the amount of the presumed most toxic species, free ionic zinc. The biomass for further experiments was grown in continuous culture with media containing trace amounts of zinc and 200 mM zinc sulfate for the control and test cultures, respectively. Inductively coupled plasma mass spectrometry (ICP-
MS) was used to measure accumulation of intracellular zinc. The levels of transcripts of selected predicted zinc transporters were measured with semiQ RT-PCR to check whether the predicted transporters might be involved. The global response on the protein level was analyzed by proteomic comparison.

The results of bioinformatics predictions using HMM were summarized in Table 1 of paper III. The overview shows that the different acidophiles harbor various sets of genes homologous to known zinc transporters. The acidithiobacilli contain predicted homologues of all transporters tested whereas *T. acidophilum* was the organism containing the smallest number of predicted zinc transporters.

Further in-depth analysis was focused on the three model organisms to elucidate their levels of zinc tolerance as well as finding clues on the resistance mechanisms that each organism employs. To test zinc toxicity levels batch growth experiments were carried out and substrate consumption as well as biomass production was measured. Tolerance levels of *Am. ferrooxidans* and ‘*F. acidarmanus*’ were comparable with a cessation of biomass production at a zinc concentration of around 750 mM in the medium although substrate consumption still continued at this zinc concentration (Supplemental Files S5-S8 of paper III). In contrast, *At. caldus* was inhibited at 200 mM zinc (Supplemental File S4 of paper III).

Zinc speciation modeling showed that sulfate was a suitable ligand for zinc at low pH (Figure 1 of paper III). Acidophile media usually contain high concentrations of sulfate that can complex zinc and mitigate its toxicity. The model for zinc speciation in the ferrous iron containing *Am. ferrooxidans* and ‘*F. acidarmanus*’ growth media predicted that only about 45% of total zinc remains available as free zinc cations. Therefore, the amounts of free zinc are much lower than it was assumed. The speciation model for the tetrathionate containing medium of *At. caldus* could not be determined conclusively as information on equilibrium constants was lacking. However, this medium contained 50 mM less sulfate because additional sulfate was added to the iron medium as ferrous sulfate. Hence, it is probable that more free zinc was available in the tetrathionate medium which might partly explain the lower tolerance levels of *At. caldus*. The results of the speciation modeling highlight the importance of solution effects on the toxicity of metals (reviewed in chapter 4 of the introduction), an aspect that has previously not been given much attention in acidophile research. In chapter 4 of the introduction it was also pointed out that other factors, such as competition with cations possibly including protons, can influence metal toxicity. Such influences have not been considered with the presented speciation modeling. Nevertheless, preliminary toxicity experiments at various pH with *At. caldus* showed that zinc toxicity was lower at pH 2 compared with pH 3 suggesting that competitive effects of protons might
play a role in acidophile metal toxicity (Figure 6). This is an important finding which should be kept in mind when dealing with acidophile metal toxicity.

![Figure 6](image)

**Figure 6.** Growth of *At. caldus* on tetrathionate measured by generation of acidity. Preliminary data shows the influence of solution pH on zinc toxicity. Shown are triplicate measurements with standard deviations of cultures with starting pH of 2.0 (circles) and 3.0 (triangles) in the absence of zinc (solid symbols) and in the presence of 25 mM zinc (open symbols). At pH 3, 25 mM zinc inhibits growth of *At. caldus* whereas the same amount of zinc does not influence growth at pH 2.

The results of the transcript profiling analysis were shown in Figure 3 of paper III and demonstrated that all targeted genes, except putative *zntA* of ‘*F. acidarmanus*’, were transcribed at similar levels regardless of the amounts of extracellular zinc. ZntA is a primary zinc export P-type ATPase and the putative ‘*F. acidarmanus*’ *zntA* was 1.4 fold up-regulated during growth in high zinc conditions. This might explain how ‘*F. acidarmanus*’ can avoid intracellular zinc accumulation as demonstrated by ICP-MS (Table 2 of paper III). ‘*F. acidarmanus*’ was the only of the three organisms which kept constantly low amounts of intracellular zinc. The levels of intracellular zinc increased with fold changes of 2.6 and 5.4 (comparing highest extracellular zinc to the control) for *Am. ferrooxidans* and *At. caldus*, respectively.

The proteomics analysis yielded a list of differentially expressed proteins for each model organism presented in Supplemental Files S13-S16 of paper III². The proteomics data did not reveal specific zinc resistance mechanisms, except a putative mechanism for *At. caldus* discussed below, but rather illustrated the global response of the three model organisms to elevated zinc levels. Supplemental File S16 of paper III shows the differentially expressed proteins in each condition classified according to functional groups. As can be seen, no unifying trend could be discerned among the three acidophiles. Interestingly, no chaperones pointing to a stress response were detected as

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² Supplemental data tables are not shown due to space limitations.
differentially expressed. In all tested organisms several enzymes involved in central carbon metabolism were differentially expressed. Yet, those enzymes showed various expression patterns suggesting that carbon flow was individually adjusted in response to high extracellular zinc. Noteworthy are two proteins involved in oxidative stress response: alkyl hydroperoxidase reductase up-regulated in *At. caldus* on high zinc, and OsmC family protein down-regulated in *Am. ferrooxidans* on high zinc (Supplemental File S17 of paper III). This suggests different roles of zinc with respect to oxidative stress in the two acidophiles. Finally, one subunit of a phosphate ABC transporter, PstS was up-regulated during growth on high zinc in *At. caldus* and it was hypothesized that a polyP-based resistance mechanism was in operation similar to the copper resistance mechanism described for acidophiles (Orell et al., 2010). This resistance mechanism is based on sequestration of metal ions by intracellular polyP, cleavage of polyP, and export of phosphate-bound metal ions by phosphate transporters. However, whether *At. caldus* employs such a resistance mechanism in case of zinc could not be shown in the present study and requires further investigation.

**Paper IV: Response of Acidithiobacillus caldus towards suboptimal pH conditions**

In order to study which proteins were important for pH homeostasis in *At. caldus* a proteomics analysis of cells grown at three different extracellular pH values was carried out. *At. caldus* was grown under tightly controlled conditions in continuous culture with a pH control system keeping constant pH at either pH 1.1 (below optimal), optimal pH 2.5 (optimal), or pH 4.0 (above optimal). To further elucidate changes in the cytoplasmic membrane, profiles of membrane fatty acid methyl esters (FAMEs) were analyzed by GC-MS.

The proteomics analysis revealed major adjustments of the proteome in response to extracellular pH. A complete list of identified, differentially expressed proteins was presented in Supplemental Table 1 of paper IV. A summary of the differentially expressed proteins discussed in more detail was shown in Table 1 of paper IV. Various transcription factors showed specific expression patterns upon changes of extracellular pH (examples shown in Figure 2 of paper IV) suggesting that a tight transcriptional control was important. One of the transcription factors that showed highest expression in pH 1.1 was PspA, a protein initially studied in *E. coli* phage shock response. More recently a direct role of PspA in the maintenance of PMF in neutrophiles was reported (Kobayashi et al., 2007) as well as a connection between Psp response and acid stress (Jovanovic et al., 2006).

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3 Supplemental data files are not shown due to space limitations.
Apart from transcriptional control there were also indications of specific PTM in response to extracellular pH (Figure 1 paper IV). Some PTMs like serine, threonine, and tyrosine phosphorylation are detectable in 2D-PAGE gels as they are stable under the experimental conditions and detectably alter the pI of the protein. Thus, proteins modified in such a way are visible as discrete spots with similar MW but shifted pI compared to the unmodified protein. Examples of protein spot patterns that could be explained with this phenomenon were found for the phosphate selective porin O and P (Figure 1 paper IV) and Sqr (Figure 3 paper IV). Clearly, this phenomenon requires further investigations to prove whether protein phosphorylation is its cause and to elucidate the biological function of this modification.

Another obvious trend was up-regulation in low pH of enzymes involved in sulfur metabolism such as Sqr, Hdr, or Sox proteins (Figure 3 of paper IV). All of these proteins showed elevated levels at pH 1.1 which suggested either that cells needed more energy to grow at low pH and therefore, they enhance the oxidation of their energy substrate or that sulfur oxidation enzymes are specifically up-regulated to allow increased outward proton pumping by primary electron transport proton pumps. The latter explanation would comprise a specific pH homeostasis mechanism in *At. caldus* and supporting evidence for the connection between sulfur oxidation and PMF was previously reported (Dopson et al., 2002).

Other differentially expressed proteins were of interest as they had been shown to be involved in acid stress responses of neutrophiles (Figure 4 of paper IV). These included a toluene tolerance protein up-regulated at more basic pH, spermidine synthase detected in two protein spots with different expression patterns, and glutamate decarboxylase which was a unique protein spot at pH 1.1. The toluene tolerance protein was encoded together with other proteins putatively involved in hopanoid biosynthesis. Hopanoids are bacterial sterol analogues which might play a role in membrane adjustments in response to environmental conditions. Spermidine and other polyamines have various roles in bacteria (Wortham et al., 2007), however, reports have shown their involvement in acid stress response (Samartzidou et al., 2003). Although the expression patterns in the present study were not obviously related to an acid stress response the differential expression of spermidine synthase might point to a pH homeostatic role of spermidine in *At. caldus*. However, the most interesting protein in this context was glutamate decarboxylase which is part of the most important acid stress resistance system in *E. coli* whereby protons are intracellularly consumed by decarboxylation of glutamate. Whether a similar system operates in *At. caldus* needs further investigation, however, it is intriguing that glutamate decarboxylase was expressed at high levels only in the low pH 1.1 (Figure 4 of paper IV).
FAME analysis clearly showed a modulation of the cytoplasmic membrane in response to proton concentration with the largest changes at lowest pH (Figure 5 of paper IV). Similar to the low pH response of *At. ferrooxidans* (Mykytczuk *et al.*, 2010) FAMEs C16:0 were increased and C18:1 were decreased in *At. caldus*. Such changes likely brought about a more rigid membrane which would be more efficient in shielding protons from entering the cell. In contrast to *At. ferrooxidans* (Mykytczuk *et al.*, 2010) and *E. coli* (Chang and Cronan, 1999) the *At. caldus* cyclo-C19:0 content was decreased upon growth in low pH. Interestingly, the content of cyclo-C19:0 was highest during growth at optimal pH and reduced to about the same levels at pH 1.1 and 4.0. The biological meaning of this phenomenon remains unknown.

The results of this paper showed very interesting trends in response to suboptimal extracellular pH. So far it has not been shown that neutrophile acid resistance mechanisms could be in operation in acidophiles during growth in very low pH. This was an interesting finding that needs further verification. Therefore, the presented paper supplies a good basis for further studies on pH homeostasis in *At. caldus*. 
Conclusions

Proteomics can yield insights into general responses as well as giving clues regarding specific mechanisms for adaptation to life in extreme environments.

Sulfur oxidation pathways in acidophiles were diverse, even within acidithiobacilli. *At. caldus* used the Sox system as well as Sor which were not present in *At. ferrooxidans*. However, both acidithiobacilli were proposed to oxidize sulfur via Hdr.

Anaerobic sulfur oxidation in *At. ferrooxidans* was, at least in part, indirectly coupled to ferric iron reduction via sulfide generation.

Speciation of metals and other chemical influences were of great importance for the toxicity of zinc in acidophiles. *At. caldus, Am. ferrooxidans*, and ‘*F. acidarmanus*’ showed distinct responses to elevated zinc levels.

Solution pH, especially values below growth optimum, greatly influenced the *At. caldus* protein expression pattern and FAME profiles. Active adjustments of FAME composition likely yielded a more rigid membrane at lower pH. Moreover, indications for the use of acid resistance mechanisms similar to neutrophilic systems were found.
Future Directions

Research on acidophiles is of great importance for human society as insight into geomicrobiological and geochemical processes will lead to benefits for society such as the control of detrimental effect caused by microorganisms and the exploitation of acidophiles for industrial processes. Undoubtedly, acidophile research has taken a leap forward with entering the genomic and post-genomic era. However, we are just beginning to understand the fundamental processes associated with an acidophilic lifestyle and many open questions remain to be answered. As pinpointed within this thesis the application of ‘omics’ techniques has advanced our knowledge concerning many aspects of acidophile microbiology and has created a great deal of hypotheses that await scrutinization. Especially, the recently reported successes with genetic manipulation of acidophilic microorganisms, specifically acidithiobacilli, open up new technical possibilities. This will further spur the research in the field and help to solve important questions. Furthermore, systems biology approaches start to emerge within the field of acidophile research which will help to integrate knowledge and provide deeper comprehension than regarding isolated aspects. Yet, a note of caution is appropriate here as systems biology not only offers great opportunities but also bears pitfalls such as computational limitations in dealing with enormous amounts of data. Hence, research has to develop in the field of bioinformatics as well in order to circumvent technical problems.

Some findings of this thesis are of particular interest and should be further studied to elucidate the underlying processes. The model for *At. caldus* sulfur metabolism presented here was recently further refined but still the sulfide oxidation in the cytoplasm remains an unsolved aspect. Furthermore, the identity and characteristics of the GSSH oxidizing enzyme Sdo, the main novelty of the recent model, remain elusive. This is an extremely important aspect of the sulfur oxidation pathway that has also implications for *At. ferrooxidans* sulfur oxidation since the presence of Sdo in *At. ferrooxidans* was hypothesized earlier. The recent model proposes a compartementalization of sulfur oxidation in *At caldus* and suggests the prevention of sulfur globule formation by cytoplasmic sulfur oxidation carried out by Sor. Clear evidence for the lack of intracellular sulfur globules in *At. caldus* is missing. However, the presence of sulfur globules would also affect the hypothesized metal resistance mechanism based on polyP sequestration and export suggested in this thesis. Therefore, proofing the absence or presence of intracellular sulfur globules in *At. caldus* would add another piece to the puzzle.

Very interesting was the discovery that *At. ferrooxidans* anaerobic sulfur oxidation is, at least in part, indirectly coupled to ferric reduction through
biological production of sulfide. As a matter of fact *At. ferrooxidans* is able to utilize hydrogen oxidation coupled to ferric reduction where sulfide generation would not occur and therefore, alternative pathways for ferric reduction are necessary. Hence, the clarification of ferric reduction by *At. ferrooxidans* is still not complete. Additionally, the proteomics analysis provided an exciting clue on the importance of an OMPP1/FadL/TodX family protein for anaerobic growth. Creating a deletion mutant for this protein might help to reveal its biological function.

Additionally, the proteomics study of *At. caldus* pH homeostasis provided several interesting hypotheses that should be tested in depth. The most exciting finding was the indication that *At. caldus* might utilize a decarboxylase system during acid stress as reported for neutrophilic acid stress systems.

In all the proteomics studies presented in this thesis were indications that protein isoforms played a biological role for the characteristics under observation. Protein isoforms with similar MW but significantly shifted pIs were probably due to PTMs. This is an important observation and investigation of those protein isoforms with specialized proteomics techniques will help to understand cellular processes on a molecular scale.

A significant finding of this thesis was that zinc toxicity in acidophiles was largely influenced by speciation and chemical effects occurring in solution, such as effects due to pH. So far, such aspects were to a large extent not considered for metal toxicity in acidophiles. It will be beneficial to further investigate metal toxicity in dependence of solution chemistry and to bear those influences in mind when making comparisons between metal toxicity of various species. This finding also underscores the importance of interdisciplinary research especially in the field of acidophile research which is heavily interconnected with other fields such as mineralogy, geology, and geochemistry.
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List of References


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Valdes, J., Pedroso, I., Quatrini, R., Dodson, R.J., Tettelin, H., Blake, R. *et al.* (2008b) *Acidithiobacillus ferrooxidans* metabolism: from genome sequence to industrial applications. *BMC Genomics* **9**.


