A tale of adaptation

Diversity and stress responses in the haloalkaliphilic sulfur-oxidizing bacteria of the genus Thioalkalivibrio

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Chapter 6

Synthesis and outlook
Aim of this research

Living in an environment with different extreme physico-chemical conditions urges organisms to acquire an all-round package of mechanisms to persist under these conditions. Adaptations to the extreme haloalkaline conditions of soda lakes have so far been relatively well studied, yet other stressors also found in soda lakes have received much less attention. The aim of my research was to unravel the adaptation mechanisms to some of these other additional stressors in *Thioalkalivibrio*, one of the most abundant and functionally important bacterial genera in soda lakes. As the isolates of these sulfur-oxidizing bacteria reflect a high diversity, we first performed a taxonomic *in silico* analysis to classify the different strains into species and subspecies with the aim to obtain a clear overview of the diversity within this genus.

To answer these aims, components of the systems biology approach were used, which allowed to perform standardized and reproducible experiments. These components included the analysis of steady state cultures obtained under well controlled conditions in chemostats when possible and using state-of-the-art omics analyses. During my research, I benefited from 76 sequenced *Thioalkalivibrio* genomes, which enabled to conduct a taxonomic *in silico* study, a comparative sequence analysis as well as transcriptomics.

In this chapter, the findings of my research are discussed in the frame of the currently known literature. This includes a synthesis on the taxonomy of *Thioalkalivibrio* and an overview of the known “*Thioalkalivibrio* tool kit” to survive harsh conditions. In the end, an outlook is given on the possible future steps in the research on the fascinating microorganisms from soda lakes in general and on the members of the gammaproteobacterial genus *Thioalkalivibrio* in particular.

Reconsidering the taxonomy of the genus *Thioalkalivibrio*

*In silico* analysis of 76 *Thioalkalivibrio* genome sequences proved that the diversity within this genus was largely underestimated with only ten validly described species until now compared to the 25 species found in our analysis (chapter 2). This diversity will surely increase when we continue to include genomes of additional isolates, for instance, the strains found in the desulfurization bioreactors [strains isolated by Sorokin et al. (2008d)], which would be especially interesting as only two strains isolated from bioreactors were included in our study (chapter 2). Another rich source of potentially novel candidate species are reconstructed genomes from
metagenomic studies, which are integrated in the Genome Taxonomy DataBase project (GTDB) (https://gtdb.ecogenomic.org/; citation date: 02/01/2021). In this database, nine novel candidate species, which were retrieved from different metagenomes of hypersaline soda lakes in the Kulunda steppe (Russia) (Vavourakis et al., 2018, 2019), were already attributed to the *Thioalkalivibrio*-like group. The GTDB builds its phylogeny on the concatenation of 120-122 conserved single-copy marker proteins of almost 200,000 bacterial and archaeal genomes in its present version (Parks et al., 2018, 2020).

The results of chapter 2 also showed that four *Thioalkalivibrio* strains (*Tv. sulfidophilus* HL-EbGr7\(^T\), ALJ17, *Tv. denitrificans* ALJD\(^T\) and *Tv. thiocyanodenitrificans* ARhD1\(^T\)) were separated from the core *Thioalkalivibrio* group around the type species *Tv. versutus* AL2\(^T\) by type strains from other genera in the phylogenetic and phylogenomic trees. Therefore, we recommended to classify these four strains as a novel genus (chapter 2). In its present version, the GTDB actually even reclassified the core *Thioalkalivibrio* strains [genomic species groups 1-21 (chapter 2)] into the novel candidate family “*Thioalkalivibrionaceae*” with two proposed genera. The first genus includes the core genomic species groups 1-19 (chapter 2) with *Tv. versutus* AL2\(^T\) as its type species, and a second novel proposed genus “*Thioalkalivibrio B*” containing *Tv. nitratireducens* ALEN2\(^T\), *Tv. paradoxus* ARh1\(^T\), and eight candidate species reconstructed from metagenomes (Vavourakis et al., 2018, 2019). The four remaining strains (*Tv. sulfidophilus* HL-EbGr7\(^T\), ALJ17, *Tv. denitrificans* ALJD\(^T\) and *Tv. thiocyanodenitrificans* ARhD1\(^T\)), which were separated in our study from the core *Thioalkalivibrio* group (chapter 2), were kept by GTDB within the family *Ectothiorhodospiraceae*, in which they will need to be reclassified into a novel genus as they are currently named “*Thioalkalivibrio A*”. The six strains that were removed from the genus *Thioalkalivibrio* by GTDB (*Tv. sulfidophilus* HL-EbGr7\(^T\), ALJ17, *Tv. denitrificans* ALJD\(^T\), *Tv. thiocyanodenitrificans* ARhD1\(^T\), *Tv. nitratireducens* ALEN2\(^T\), *Tv. paradoxus* ARh1\(^T\)) have also been shown to be different from the other *Thioalkalivibrio* strains based on their key sulfur oxidation genes (Berben et al., 2019). Especially, from all *Thioalkalivibrio* strains, only these six strains possess the rDsr system (Berben et al., 2019). Moreover, *Tv. nitratireducens* ALEN2\(^T\) and *Tv. paradoxus* ARh1\(^T\) were the only *Thioalkalivibrio* strains encoding the arrA gene for arsenate reduction (chapter 3), and the sulfur oxygenase-reductase (SOR) for sulfur disproportionation (Berben et al., 2019; Rühl et al., 2017). These two later species also branched separately from the core *Thioalkalivibrio* strains on all phylogenetic and phylogenomic trees, and they were even on a common branch with *Thiorhodospira sibirica* A12\(^T\) and *Thiohalospira halophila* HL3\(^T\) in the whole-genome GBDP phylogeny based on nucleotide data (chapter 2). Thus, based on all these indices, the genus separation of *Thioalkalivibrio* at the level of *Tv. nitratireducens* ALEN2\(^T\) and *Tv. paradoxus* ARh1\(^T\) would be an
excellent way to rearrange the genus of *Thioalkalivibrio*.

The GBDT also delineated the *Thioalkalivibrio* strains in the same strain to species assignment as proposed in chapter 2 with the exception of the combination of two times two genomic species groups. This concerns the genomic species groups 1 (Tv. versutus) and 2 (Tv. jannaschii), as well as the group 16 (Tv. halophilus) and 17 (groups as defined in chapter 2), respectively. Both of these two clusters scored an ANIb value between 94-96% amongst each other, which indicated no clear species delineation by this analysis, but these clusters were delineated into two genomic species groups by the whole genome nucleotide based GBDP analysis (chapter 2). For the genomic species group 1 and 2, both are already validly described species with its respective type strains *Tv. versutus AL2* and *Tv. jannaschii ALM2* (chapter 2; Sorokin et al., 2001b, 2002a). Importantly, *Tv. jannaschii ALM2* showed in the experimental DNA-DNA hybridization a score of less than 32% with other *Thioalkalivibrio* strains, amongst which was also *Tv. versutus AL2*. This strengthens the argument of its own species attribution (Sorokin et al., 2002a). Another argument for their separation into two species is the possession of the arxA gene in *Tv. jannaschii ALM2* (chapter 3), and with this its potential to use arsenite oxidation as energy metabolism (Zargar et al., 2010, 2012). The presence of this gene is also the case for all strains of the genomic species group 16 (*Tv. halophilus*) but not for the strains of group 17 (chapter 2 and 3). For these reasons, we would suggest that the genomic species groups 1 (*Tv. versutus*) and 2 (*Tv. jannaschii*) as well as the groups 16 (*Tv. halophilus*) and 17 remain in their current strain to species attribution and that they should not be combined into one species, respectively. However, in the case of the genomic species groups 16 and 17, it should be considered to perform a proper investigation of the strains of both groups to take a final decision.

Recently, multiple strains closely related to *Thioalkalivibrio* were revealed within environments without obvious haloalkaline conditions. Those include the detection of candidate *Thioalkalivibrio* strains in metagenomes of the microbiota associated to sponges (Gauthier et al., 2016; Tian et al., 2014; White et al., 2012) or to sub-Antarctic peat moss (Graham et al., 2017), and the isolation of a new *Thioalkalivibrio* strain from brackish estuary sediments contaminated by petroleum (Becarelli et al., 2020). All these strains cluster outside the “core”-*Thioalkalivibrio* group around *Tv. versutus AL2* as defined by GTDB and are related to *Tv. sulfidophilus HL-EbGr7*, *Tv. thiocyanodenitrificans ARhD1*, *Tv. nitratireducens ALEN2* and *Tv. paradoxus ARh1* (Becarelli et al., 2020; Gauthier et al., 2016; Tian et al., 2014; White et al., 2012), although White et al. (2012) also report the presence of a *Thioalkalivibrio* sp. K90mix-like strain. Interestingly, *Thioalkalivibrio* sp. 10fs10, which was isolated from a petroleum-contaminated brackish estuary sediment with an almost neutral pH and 0.51 M Na+, showed a 100% 16S rRNA identity to *Tv. sulfidophilus HL-EbGr7* and was
also able to grow up to a pH of 10 and a Na⁺ concentration of 1.5 M (Becarelli et al., 2020). Moreover, the first Thiopaq reactor, from which *Tv. sulfidiphilus* HL-Ebgr7T was isolated (Sorokin et al., 2012a), was originally inoculated with littoral sediment of the North Sea (personal communication by Cees Buisman). These findings strengthen the importance to officially revise the genus of *Thioalkalivibrio* even more.

### The “*Thioalkalivibrio* tool kit”: Adaptations to stressors present in soda lakes

*Thioalkalivibrio* strains are well equipped to flourish in the extreme environment of soda lakes (Figure 1). These extreme conditions include of course the haloalkaline character of these lakes, but also other stress factors occurring in soda lakes. We define a stressor as a condition, which induces a perturbation of the homeostasis. These stressors can include fluctuating salinities, droughts, high UV radiation, absence of available calcium in solution, high ammonia toxicity, presence of heavy metals like arsenic (chapter 3), presence of antibiotics (chapter 5), as well as high and low temperatures (chapter 4). To face these stressors, the “*Thioalkalivibrio* tool kit” contains a versatile energy metabolism, as well as specific adaptations in their bioenergetics for the maintenance of a neutral cytoplasmic pH and their osmotic pressure (Banciu and Muntyan, 2015). Moreover, *Thioalkalivibrio* takes advantage of compatible solutes, of adaptations within the membrane lipid composition (chapter 4; Banciu et al., 2004b, 2005), of vitamin B₁₂ as an antioxidant (chapter 3 and 4) as well as of heavy metal [including arsenic (chapter 3)] and antibiotic resistance genes (Chakraborty et al., 2020a, 2020b). Finally, *Thioalkalivibrio* are able to produce membrane-bound polyene pigments, which might act as radical traps (Banciu et al., 2005; Takaichi et al., 2004), and to form cyst-like resting cells (Loiko et al., 2003). This diversity of the different adaptation mechanisms did not only allow *Thioalkalivibrio* to persist under these multi-stressor conditions, but also enabled them to thrive and to become dominant community members in soda lakes (Vavourakis et al., 2016, 2018).

#### (1) Diversity of energy metabolism

The different *Thioalkalivibrio* strains possess, for obligate chemolithoautotrophs, a high phenotypical diversity in their energy metabolism in their ability to use different electron donors and acceptors. As previously described, different electron donors can be used depending on the strain, such as different reduced sulfur compounds (Sorokin et al., 2001b), thiocyanate (Sorokin et al., 2001c, 2002c), and potentially also arsenite, since genes responsible for arsenite oxidation have been detected in their
Figure 1. The “*Thioalkalivibrio* tool kit” presented in a conceptual model including the different adaptation mechanisms in *Thioalkalivibrio* against haloalkaline and other stress factors present in soda lakes. RC, respiratory chain; FA, fatty acid; ROS, reactive oxygen species; AB, antibiotic; AR, antibiotic resistance enzyme; EP, efflux pump. The figure was adapted from Banciu and Muntyan (2015) and supplemented with findings presented in chapter 3, 4 and 5. The different mechanisms indicated by the numbers are discussed in detail below.
genomes (chapter 3; Andres and Bertin, 2016; Oremland et al., 2017) and transcripts closely affiliated to *Thioalkalivibrio* in the metatranscriptome of Mono Lake (Edwardson and Hollibaugh, 2017). As electron acceptors, *Thioalkalivibrio* strains can use oxygen (Sorokin et al., 2001b) and certain strains can perform anaerobic denitrification (Sorokin et al., 2001b, 2003b, 2004b), potentially also ammonification (Vavourakis et al., 2018) and arsenate reduction (chapter 3), but so far only the genes for these metabolisms were detected in the genomes and no experimental proof has been given yet. This feature allows the strains with different energy metabolisms to rapidly adapt to changing environmental conditions and to colonize niches in the environment.

**(2) Bioenergetics and membrane transporters**

A major bioenergetic problem of alkaliophiles is the inverted pH gradient across the membrane, which is caused by the necessity of maintaining an almost neutral cytoplasmic pH in an alkaline extracellular environment (Slonczewski et al., 2009). This inverted pH gradient leads to a decrease in the difference of the transmembrane proton potential, the proton motive force (pmf) (Dimroth and Cook, 2004), as the pmf is the result of the electrical membrane potential (Δψ) and the pH gradient (ΔpH) across the membrane. Thus, to compensate for this energetic loss, aerobic alkaliophilic microorganisms keep their membrane electric potential at higher negative values by enhanced respiration rates (Slonczewski et al., 2009). This was also shown to be the case in the *Thioalkalivibrio* strains ALJ15 and AL2T, where the potentials were -228 and -224 mV respectively, and with this, they are even more negative than in most known alkaliophilic bacteria (Muntyan et al., 2012). Furthermore, alkaliophiles use multiple membrane transporters to build up the electric membrane potential (Δψ) and the transmembrane pH gradient (ΔpH), as well as to expel the excess intracellular sodium. The network comprises various ion channels, and primary and secondary pumps forming an ideal intracellular proton and sodium concentration. The ion channels include the Na⁺-solute symporter and the flagellar Na⁺-channel. The secondary membrane pumps contain the H⁺-translocating F₁F₀-type ATP synthase and the Na⁺/K⁺/Ca²⁺-proton antiporters. Finally, the primary proton translocating pumps include the components of the respiratory chain (Banciu and Muntyan, 2015), while the primary sodium-pumps consist of the sodium-pumping *cbb*₃-type terminal cytochrome c oxidase unique for *Thioalkalivibrio* (Muntyan et al., 2015) and possibly also of the sodium-translocating Rnf complex, although its role is still unclear (Banciu and Muntyan, 2015). The cytochrome *cbb*₃ is a sodium-pumping cytochrome oxidase that pumps sodium outside of the cell, and uses the free energy gained by the liberated electrons during the O₂ reduction of the respiration (Muntyan et al., 2015). A similar strategy is used by the sodium-pumping NADH-quinone reductase (Nqr),
present in many heterotrophic alkaliphiles (Reyes-Prieto et al., 2014; Unemoto and Hayashi, 1993), but which is, however, not detected in the *Thioalkalivibrio* strains studied so far (Muntyan et al., 2015).

### (3) Compatible solutes

Effects of haloalkaliphilic conditions on growth were studied in detail in the highly halophilic *Thioalkalivibrio* strain ALJ15. This strain grows well over a broad salinity range and changes could therefore be observed between 0.6 to 4 M Na⁺ (Banciu et al., 2004b, 2005; Takaichi et al., 2004). *Thioalkalivibrio* uses the `salt-out` strategy, in which the osmotic balance is maintained by using compatible solutes. These osmolytes are small, generally uncharged or zwitterionic organic compounds that can be imported or produced *de novo*, and do not interfere with cellular enzymatic reactions. Even though the `salt-out` strategy has as drawback to be energetically expensive, it allows the cell to tolerate a large range of salt concentrations (Oren, 2011). Glycine betaine has been identified as a mayor osmolyte in ALJ15, while sucrose was a minor component (Banciu et al., 2004b, 2005). Moreover, the fact that soda brines are sodium carbonate-based allows their inhabitants to spend less energy on maintaining an osmotic balance in comparison to the neutrophilic halophiles inhabiting sodium chloride-based environments. This originates from the fact that sodium carbonate salts are weak electrolytes in comparison to the strong electrolytic sodium chloride with a nearly two-times difference based on equimolar sodium concentrations (Sorokin et al., 2011b).

The osmolyte glycine betaine has also been described to have cryoprotectant properties (Deshnium et al., 1997; Hoffmann and Bremer, 2011; Ko et al., 1994). It does this by shielding cytoplasmic proteins from denaturation during freezing (Welsh, 2000) and by decreasing the cytoplasmic freezing point to prevent intracellular ice crystal formation (Knierbein et al., 2019; Yancey, 2004). In agreement with this information, Figure 2 of chapter 4 shows that the cells of the strains *Tv. versutus* AL2ᵀ and *Tv. nitratis* ALJ2 accumulated more glycine betaine during growth at 10°C in comparison to cells grown at 30°C. Moreover, *Tv. versutus* AL2ᵀ, which showed a shorter lag phase and a higher growth rate at 10°C than *Tv. nitratis* ALJ2 (Table 1 of chapter 4), contained a three-fold higher intracellular glycine betaine content. Therefore, we suggested that the cryoprotective effects of glycine betaine are the main drivers for low-temperature resistance in *Tv. versutus* AL2ᵀ (chapter 4).
(4) Membrane lipid composition

Changes in the fatty acid composition of membrane polar lipids are essential as they allow maintaining the membrane fluidity according to the environmental conditions (Siliakus et al., 2017). For *Thioalkalivibrio*, those changes have been demonstrated in adaptation to increasing salinities (Banciu et al., 2005) and to low temperature (chapter 4). At 0.6 M Na⁺, the membrane lipid’s fatty acids in ALJ15 are dominated by unsaturated fatty acids and contain C₁₆:₀, C₁₈:₀, C₁₈:₁ cis∆9 and C₁₉ cyclo. With increasing salt concentrations, C₁₈:₁ cis∆9 decreased and C₁₉ cyclo increased in proportion (Banciu et al., 2005). Moreover, the non-polar lipid squalene is present in high amounts independent to the salt concentrations and probably also the negatively charged cardiolipin (diphosphatidylglycerol) (Banciu et al., 2005). The presence of squalene enhances the membrane’s impermeability for protons and sodium helping to maintain the proton electrochemical gradient (Hauß et al., 2002). Cardiolipin has been previously shown essential in adaption to haloalkaline conditions (De Leo et al., 2009; Padan et al., 2005) as it is involved in the proper functioning of the respiratory chain (Arias-Cartin et al., 2012). To circumvent membrane stiffness induced at low temperature, the strains *Tv. versutus* AL2 and *Tv. nitratis* ALJ2 raised their proportion of unsaturated fatty acids (chapter 4; Sinensky, 1971, 1974). Those included the increase of C₁₈:₁ Δ₁₁ in both strains and of C₁₆:₁ Δ₉ in *Tv. versutus* AL2 as well as the decrease of C₁₉ cyclo in both strains and of 10-Me-C₁₆:₀ in *Tv. versutus* AL2 compared to the fatty acid composition at 30°C (Table 3 of chapter 4). Surprisingly, these two strains did not utilize chain length reduction nor an increase in the branching of the fatty acids commonly used by bacteria to maintain membrane fluidity at lower temperature (Siliakus et al., 2017). The responses to the increase in salinities and the decrease in temperature illustrate the adaptations at the level of the membrane lipid composition to face environmental changes well.

(5) Antioxidant

Vitamin B₁₂ has been proposed as the main antioxidant in *Thioalkalivibrio* against oxidative stress provoked by arsenite (chapter 3) and by low temperature (chapter 4) as its complete synthesis pathway was significantly upregulated when being exposed to these conditions. This ability has already previously been confirmed in prokaryotic and eukaryotic cells under oxidative stress (Alzoubi et al., 2012; Birch et al., 2009; Bito et al., 2017; Ferrer et al., 2016; Moreira et al., 2011; Qin et al., 2018; Suarez-Moreira et al., 2009). Interestingly, *Thioalkalivibrio* did not increase gene expression of known antioxidants under these conditions, e.g., superoxide dismutase or catalase (chapter 3 and 4), nor other common antioxidant pathways provoked by arsenite (chapter 4).
The only exception were multiple upregulated peroxiredoxins in *Tv. nitratis* ALJ2 as well as an upregulated glutathione S-transferase for *Tv. versutus* AL2\(^\dagger\) and *Tv. nitratis* ALJ2 under low temperature (chapter 4).

(6) Resistance genes against heavy metals and antibiotics

Soda lakes in eastern California and west Nevada are known to contain high concentrations of arsenic (Oremland et al., 2004), while low levels of this heavy metal were also measured in Lake Nakuru and Lake Bogoria of the East African Rift valley (Jirsa et al., 2013). In addition to the arsenite oxidase (ArxA) and the arsenate reductase (ArrA) found in multiple *Thioalkalivibrio* strains, we also described the presence of the arsenic resistance operon (*ars*) in *Thioalkalivibrio* (Figure 1B of chapter 3). Moreover, various other metal and antibiotic resistant genes have been revealed in metagenomes of the anthropologically polluted Lonar Lake in India (Chakraborty et al., 2020a, 2020b). In those metagenomes, antibiotic resistance genes were found in abundance in strains of *Thioalkalivibrio* (Chakraborty et al., 2020a).

(7) Pigmentation

The yellow pigmentation is characteristic for high salt-adapted *Thioalkalivibrio* strains. The pigment is membrane-bound and composed of three homologues. Two of those, which are called natronochrome and chloronatronochrome, were further analyzed. They represent a C\(_{23}\) compound containing a C\(_{15}\) polyene ending with a methylated phenol and a carboxymethyl group. In contrast to the first compound, the second contains a supplementary chloride in the phenol group. It has been proposed that these pigments could be involved in the protection against UV radiation and oxygen radical stress due to the abundance of double bonds in their backbone (Takaichi et al., 2004).

(8) Resting cell form

The formation of a resting cell form called `cyst-like refractile cell` is another strategy found in *Tv. versutus* AL2\(^\dagger\) for survival under unfavorable conditions. This resting form enhances survival under autolytic conditions, including the stationary phase, as well as thermal, osmotic and acidic shock. Morphological changes include a shrunken protoplast, an increase in the periplasmic space and an acquisition of a certain level of refractility (Loiko et al., 2003). In chapter 5, we also proposed that this resting form enabled the survival during temporal antibiotic stress, after which the cell could recover its normal vegetative form. However, our observations of the storage of the different *Thioalkalivibrio* strains at 4°C showed that certain
Thioalkalivibrio strains like *Tv. sulfidophilus* HL-EbGr7\textsuperscript{T} and *Tv. halophilus* HL17\textsuperscript{T} are more fragile towards long term storage than others as *Tv. versutus* AL2\textsuperscript{T} (data not shown) indicating that maybe not all *Thioalkalivibrio* strains possess the ability to produce resting cells.

**Versatility of the “tool kit”**

As already discussed in chapter 4, the “tool kit” can be applied in a versatile manner against stressors. For instance, the osmolyte glycine betaine is also used as cryoprotectant by *Thioalkalivibrio* under low temperature stress (chapter 4). However, against desiccation stress, often occurring in the soda lake area during hot seasons, glycine betaine only enabled protection at lower desiccation levels in other bacteria (Kets et al., 1996; Kets and de Bont, 1994; Welsh, 2000) and showed even no effect against complete desiccation, which is probably due to the higher removal of intracellular water compared to osmotic or low temperature stress (Welsh and Herbert, 1999). The compatible solute trehalose is known as protector under desiccation stress in other bacteria (Welsh, 2000; Welsh and Herbert, 1999), but it was never detected so far in cells of *Thioalkalivibrio*. Thermotolerance, which would be needed for example in case of higher water temperature next to the hot springs of the Rift valley soda lakes (Jones et al., 1998), could also be provided by trehalose (Welsh, 2000) as well as by the resting form (Loiko et al., 2003). Another important stress factor during the drying out of soda lakes is UV stress. We hypothesize that *Thioalkalivibrio* could also here take advantage of vitamin B\textsubscript{12} (chapter 3 and 4) and from the production of polyunsaturated pigments as proposed by Takaichi et al. (2004) to counteract oxidative stress.

**Outlook**

In recent years, multiple novel advances in term of techniques were developed including high throughput sequencing, (meta)-omics and genetic modification. Those also did not just pass unnoticed by the research conducted on the soda lakes environment and their haloalkaliphilic microbial community. In this line, multiple whole-genome sequences were generated of various soda lake isolates, amongst which the genus *Thioalkalivibrio* is the most highly represented. Furthermore, multiple metagenomic (Andreote et al., 2018; Chakraborty et al., 2020a, 2020b; Stamps et al., 2018; Vavourakis et al., 2016, 2018, 2019; Zorz et al., 2019) and metatranscriptomic analyses (Edwardson and Hollibaugh, 2017, 2018; Vavourakis et al., 2019) were performed on soda lakes from all over the world, and the first genetic modifications on *Alkalilimnicola ehrlichii* (Zargar et al., 2010) and *Thioalkalivibrio* sp. (Mu et al.,
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2017; Sharshar et al., 2020) were conducted. The combination of those techniques with the systems biology approach, in which experiments are performed under well-controlled and standardized cultivation conditions and subsequently explained by a metabolic model, will form a powerful approach for future research on soda lakes and on *Thioalkalivibrio.*

**Relevance of studying stress responses in *Thioalkalivibrio* for industrial applications**

To fill the last pieces of the puzzle in the understanding of the ecophysiology of *Thioalkalivibrio,* the remaining stress factors found in soda lakes as well as those encountered in biotechnological processes should be studied. For their natural environment, it would be interesting to investigate the stress response to UV radiation, high temperature and drought. To study the soda lake’s complete community response to stressors, mesocosms should be used in combination with microsensor, chemical, metagenomic and metatranscriptomic analyses.

Next to soda lakes, *Thioalkalivibrio* is also an important driver in full-scale sulfide-removing Thiopaq reactors (Sorokin et al., 2008d, 2012a). These reactors treat sulfide-containing gas- or wastewater streams that are produced in the paper/pulp (Janssen et al., 2009), the refining or the petrochemical industries (Alnaizy, 2008), and generate biosulfur (Janssen et al., 2009). However, these waste effluents also bring multiple toxic compounds in the system, which include volatile organic sulfur compounds such as methanethiol, dimethyl disulfide and dimethyl trisulfide, and aromatic hydrocarbons including benzene and toluene (De Graaff et al., 2011; Janssen et al., 2009; Sipma et al., 2004; Smet et al., 1998; Van Den Bosch et al., 2009; Van Leerdam et al., 2011). Using bioreactors inoculated with soda lake sediments, the compounds methanethiol, dimethyl disulfide and dimethyl trisulfide impacted negatively the sulfide and polysulfide oxidation. However, an improvement of the sulfide oxidation rates could be achieved when the haloalkaliphilic community was pre-treated for almost a month with low amounts of methanethiol (Van Den Bosch et al., 2009). When bioreactors, which were abundant in *Thioalkalivibrio,* were treated with benzene, their microbial community changed in proportion during the run. Interestingly, these changes included that a specific strain, close to *Thioalkalivibrio* sp. K90mix, increased in dominance during higher benzene concentrations compared to the rest of the microbial community and to the other *Thioalkalivibrio* strains (De Graaff et al., 2011). However, it was also observed that the sulfide oxidation by *Thioalkalivibrio* sp. K90mix, *Tv. sulfidophilus* HL-EbGR7T and Thiopaq sludge was strongly inhibited by dimethyl disulfide and even more severely by methanethiol (De Graaff, 2012; Roman et al., 2015). Due to this high vulnerability of *Thioalkalivibrio* to thiols, efforts
were taken to adapt the reactor´s microbial community over time to elevated thiol concentrations. Stable productivity of the bioreactors could be successfully re-established in concert with a drastic reduction in proportion in *Thioalkalivibrio* sp. and with an increase in *Thioalkalibacter* sp. and *Alkalilimnicola* sp. (Roman et al., 2016). With this background, it would be interesting to study more in depth the stress induced by thiol compounds on *Thioalkalivibrio* sp. and on the complete reactor community by metatranscriptomics and/or metaproteomics. Furthermore, once the existing stress responses are well characterized, genetic modification of the sulfide oxidizing bacteria could be imagined in order to increase their resistance towards the toxic compounds found in the system.

Recent advances put the utilization of *Thioalkalivibrio* sp. in these systems even further in question. Under normal operating conditions, the sulfur oxidation by *Tv. sulfidiphilus* HL-Ebgr7 produces in addition to *S*$_8$, the unwanted by-product *SO$_4$*$_2^-$ (Muyzer et al., 2011a) and due to the chemical reaction of HS with oxygen or polysulfide, also *S$_2$O$_3$*$_2^-$ (Kleinjan et al., 2005; O’Brien and Birkner, 1977). Moreover, desulfurization by *Thioalkalivibrio* sp. is severely hampered under elevated HS levels due to the inhibition of the cytochrome c oxidase (Collman et al., 2009) inducing as a side-effect also a suppression of the flavocytochrome c Fcc responsible for the oxidation of sulfide to elemental sulfur. However, when an additional anaerobic reactor was included between the H$_2$S absorber and the aerated bioreactor of the system, an increase in *S$_8$* formation and a reduction in *SO$_4$*$_2^-$ and *S$_2$O$_3$*$_2^-$ production was observed. This could be explained by the exchange of *Thioalkalivibrio* sp. by *Alkalilimnicola ehrlichii* in the reactor´s community. This exchange probably happened as the sulfide:quinone reductase (SQR), which is another enzyme in addition to Fcc able to oxidize sulfide, is not inhibited by HS (De Rink et al., 2019).

**Genetic modification to improve bio-industrial applications and to unravel gene function**

Recent advances described the protocols for the genetic modification within *Thioalkalivibrio* by conjugational transformation (Mu et al., 2017) and by CRISPR-Cas9 modification (Sharshar et al., 2020). These studies focused on the improvement of the desulfurization activity and of the production of biosulfur, a topic which has recently obtained plenty of attention (Parveen et al., 2020). In addition, the momentousness of these protocols´ establishment could also tremendously contribute to the understanding of the ecology and physiology of *Thioalkalivibrio*. We were able to repeat in our lab with success the conjugational transformation of *Tv. versutus* AL2$^+$ with pBBR-rfp-ptac, which was kindly provided by Prof. Jianmin Xing, and which encoded a streptomycin resistance and a MCherry fluorescence marker (Mu et al.,
Genetic modification is a powerful tool to confirm physiological and transcriptomic observations, and to study the role of proteins. An interesting continuation of the research presented in this thesis would be genetic modification of *Thioalkalivibrio* to test our hypothesis that the soeABC-like genes (cluster 2) are involved in the aerobic oxidation of As(III) or thioarsenate into As(V) (chapter 3), and if this enzymatic complex is not responsible, other candidates could be screened to elucidate the metabolic pathway of As(III) oxidation. However, findings as presented in chapter 4 and by Berben et al. (2017a) decreased the probability of this hypothesis as *Thioalkalivibrio* upregulated soeABC (cluster 2) as well under cold temperature (chapter 4), and also soeA (cluster 2) when cultivated with thiocyanate as a sole electron donor (Berben et al., 2017a). Another interesting follow-up of chapter 3 would be to check with the help of knockouts, whether the porin, which was downregulated in *Tv. jannaschii* ALM2T under As(III) stress, promotes As(III) uptake inside the cell and whether the role of the sulfurtransferases DsrE/F-like genes is essential under As(III) stress. Finally, the verification of the hypothesis that vitamin B₁₂ works as an antioxidant should be further studied and it should be tested whether it is indispensable to resist oxidative stress (chapter 3 and 4).

Figure 2. Conjugational transformation of *Tv. versutus* AL2T (A) on plate and (B) in liquid culture, with each time on the right the strain without and on the left the strain with the MCherry-encoding plasmid.
Concluding remarks

The results described in this thesis advance our understanding of how *Thioalkalivibrio* does not only survive, but thrives in soda lakes under multi-extreme conditions. We revealed a highly diverse stress-resistance tool kit within these haloalkaliphiles, which most likely enabled them to become one of the most abundant bacteria in soda lakes. However, our results are not only relevant for *Thioalkalivibrio*, but also shed light on possible adaptation strategies in other bacteria. Moreover, we were able to propose an *in silico* taxonomic classification based on phylogenetic and phylogenomic analyses, which revealed a high diversity amongst the 76 genome sequences of different *Thioalkalivibrio* strains. This diversity will certainly still increase in future with the help of new cultivation techniques and the reconstruction of genomes by metagenomics. Our analyses also revealed that the genus *Thioalkalivibrio* is not monophyletic and needs a reorganization of strains in novel genera and even families. Combining the recent advances in research techniques like high throughput sequencing, (meta)-omics and genetic manipulation, together with a systems biology approach, will open novel possibilities for future research to study the community of soda lakes and of biodesulfurization reactors in general, and *Thioalkalivibrio* as their fascinating and highly important drivers in these multi-extreme environments in particular.