Microbial Ecology of Halo-Alkaliphilic Sulfur Bacteria

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Chapter 1

General introduction

1

Soda Lakes

Soda lakes are a specific type of saline lakes characterized by moderate to high salinity and extremely high alkalinity. Saline lakes can be defined as bodies of water with a concentration of salts and other minerals significantly higher than most lakes and often higher than sea water salt (30 g/l). They can be classified in thalassohaline and athalassohaline lakes, depending on the origin of their salts, which can be marine or continental, respectively. Salinity can also originate from ancient salt deposition by gradual dissolution.

Saline lakes are spread all over the world and are generally confined to arid and semi-arid regions, where evaporation rates exceed inflow rates, favoring the accumulation of salt in local depressions. Beside the climatic factor, the topography and the geochemistry of the area play an important role in the genesis of saline lakes as well. The topography is responsible for the existence of a hydrological enclosure with a permanent body of water, while the geochemistry and groundwater chemistry determine which will be the major ions in solution. In saline lakes the major cations in solution are sodium (Na^+), which is usually also the most abundant ion, calcium (Ca^{2+}) and magnesium (Mg^{2+}), while chloride (Cl^-), sulfate (SO_4^{2-}) and carbonate/bicarbonate (CO_3^{2-} / HCO_3^-) are the major anions. Depending on the total salt composition different types of salt lake are formed (Fig. 1.1), with different predominant evaporates (Jones and Deocampo, 2003).

Soda lakes are closed basins and their source of water is percolating groundwater, which takes in various elements from the solid phase. Therefore, in order to predict which ions will be predominant, it is very important to know the geology of the area in which the soda lakes are located. In soda lakes carbonate and bicarbonate are among the major anions in solution followed by chloride and sulphate in different proportions. Sodium is the most abundant cation, whereas Mg^{2+} and Ca^{2+} are almost absent or depleted as carbonate precipitate. In the solid phase carbonates are usually present as sodium sesquicarbonate, Na_2CO_3. $NaHCO_3$. $2H_2O$, also known as `trona`. The combination of high concentrations of soluble carbonates with the absence of calcium confers a high alkaline buffering capacity, keeping the pH stable up to 10.5. Another effect of calcium depletion is the abundance of phosphate in soda lakes, contributing to a high primary production.

There are several theories concerning the formation of alkalinity in soda lakes. One of these suggests the contribution of Na_2CO_3 via volcanism, whereas another one links the rise of pH to sulfate reduction in anaerobic basins (Grant, 1992). However most likely the alkalinity is due to climatic and geological conditions, such as the low concentrations of Mg^{2+} and Ca^{2+} and a high CO_2/HCO^-_3 level in ground waters.

Soda lakes are widely distributed throughout the world and the most well- known lakes are located in the East African Rift Valley (e.g., Lake Bogoria, Lake Natron and Lake Magadi), in the Libyan Desert in Egypt (Wadi Natrun system), in north America (California, Nevada and Washington State), and central Asia (from south-east Siberia to north-east China).

Most of the soda lakes are shallow and well-mixed, but there are also a few examples of deep stratified soda lakes, such as Mono Lake, Big Soda Lake and Soap Lake, located in North America. Mono Lake is situated on the eastern slope of the Sierra Nevada mountain range, California, and its origin is probably volcanic. It is a saline lake (~90 g/l) with a pH around 10. Calcium carbonate is the principal precipitate (Melack, 1983; Hollibaugh *et al.*, 2001; Oremland *et al.*, 2004) and it causes the formation of picturesque tufa towers which reach a height of almost one meter above the water. Besides the major ions commonly present in soda lakes, Mono Lake is especially rich in phosphate and sulfate whereas the

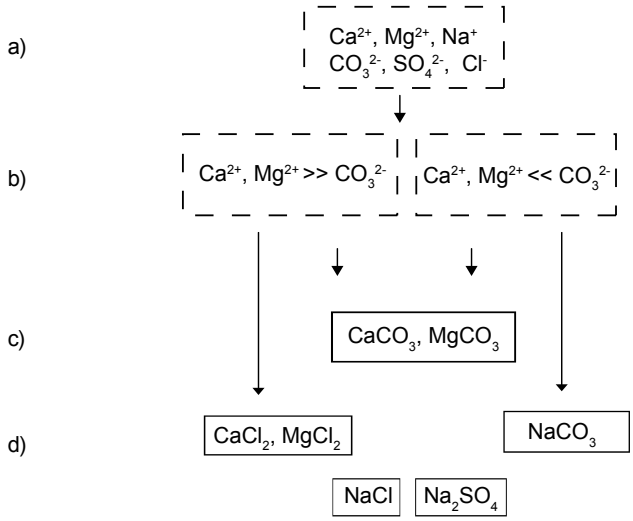

Figure 1.1. Schematic flowchart representing the sequential mineral precipitation in saline basins (Jones and Deocampo, 2003 modified). a) Ions reservoir; b-c) excess or limitation of Ca^{2+} or Mg^{2+} determine whether the solution will be carbonate rich or carbonate poor; d) in carbonate poor solution calcium-magnesium chloride are dominating, whereas in carbonate rich solution sodium carbonate is the dominant salt. Sodium chloride and sodium sulfate are ubiquitous. Solutes are indicated by dashed boxes and mineral precipitate by solid boxes.

inorganic nitrogen concentration is quite low, causing a nutrient limitation (Melack, 1983). It contains also very high concentration of arsenic and selenium. In contrast to many other soda lakes, Mono Lake is a deep lake with a salinity favoring stratification and other meromictic phenomena. Meromixis generates a depletion of nutrients by reduced vertical fluxes, which in turn causes a somewhat reduced primary production. However this is still in the same range as observed for other soda lakes (269-1060 gC $m^{-2}yr^{-1}$) (Hollibaugh *et al.*, 2001).

Soap Lake is another meromictic soda lake situated in central Washington State (USA), at the end of a chain of lakes with increasing salinity and alkalinity. Characteristic of this lake are its sharp stratification and its extreme high sulfide concentration (200 mM) in the monimolimnion, i.e., the bottom layer of the lake. The salinity goes from 15 g/l in the mixolimnion, i.e., the top layer of the lake, to 140 g/l in the monimolimnion and the pH is round 10. Despite the high alkalinity, salinity, stratification and the extremely high sulfide concentration, the lake is highly productive (391 gC m^{-2} yr^{-1}). There is not much information available in the literature regarding the microbial diversity in Soap Lake. However in this thesis we have been able to make a contribution to a better understanding of a specific group of microorganisms, i.e., the sulfur-oxidizing bacteria. The extraordinary high sulfide concentration present in this lake emphasizes the importance of the sulfur cycle and the organisms that are involved.

The best studied shallow soda lakes are probably those of the East African Rift Valley (Kenya and Tanzania), an arid tropical zone where tectonic activity has created a series of depressions (Jones and Grant, 1999). The salinities of these lakes go from 50 g/l to saturation and chloride is one of the major anions besides carbonate and bicarbonate.

Lake Magadi, one of the oldest lake of the Rift Valley, is located in Kenya, close to Tanzania and it is an example of hyper saline alkaline lake. Precursor of Lake Magadi was Lake Oronga, a vast deep fresh water lake which existed in the Middle Pleistocene (800 000 years

ago). In the past 12,000 years it turned into a saline lake mainly due to climate changes (Baumagarte, 2003) and it is now at the stage of maximum evaporate productivity. The surrounding rocks of predominantly volcanic origin are rich in sodium and low in calcium and magnesium. The lake consists mostly of trona deposits (up to 50 m thick), also called Evaporite Series, the formation of which is still going on today. Most of the studies investigating the microbial diversity have been conducted in Lake Magadi and other soda lakes located in the East African Rift Valley (Jones *et al.* 1998, Zavarzin 1999, Grant *et al.* 2004, Baumgarte 2003).

Other well-studied lakes are those of the Wadi Natrun in Egypt. The Wadi Natrun lies in a geographical depression North West of Cairo and along this depression there is a chain of evaporate lakes. Most of them are completely dry in summer time resulting in saline and hypersaline trona brines, rich in chloride and sulfate (Shortland, 2004).

The present thesis especially focuses on the shallow soda lakes from the Kulunda steppe, located in South East Siberia, Russia. These lakes are located near the border with Kazachstan, about 320 km south-west of Barnaul near the village Mikhaylovskiy. Except for Cock Lake, the lakes belong to three large geological systems: the Salt-lake steppe system, the Tanatar system and the Bitter lake system (Fig.1.2). The systems lie closely together covering an area with a diameter of approximately 25 km and they contain so many lakes that not all of them have official names. This restricted area is characterized by the presence of many shallow soda lakes, whose microbiology has not studied before. They vary in alkalinity, salinity and major ions present in solution. Most of the Kulunda soda lakes contain high concentration of Na_2CO_3 and $NaHCO_3$; the salinity varies from 50 g/l (i.e. Cock Lake and few lakes of the Salt Lake Steppe systems) to almost 500 g/l (lakes belonging to the Tanatar and Bitter Lake systems), depending on the lake and the season. In some other lakes the predominant salt is NaCl, such as Stamp Lake, and they can be defined as hypersaline alkaline lakes. Except for the study conducted by Issachenko in the 1930`s (Issachenko, 1951), very little is known about the microbial diversity of these lakes. Therefore, we investigated the general microbial

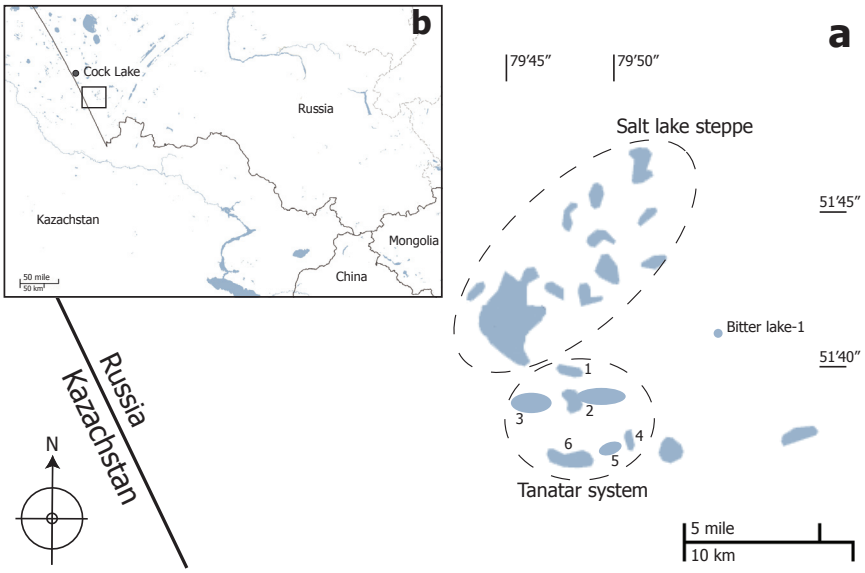

Figure 1.2 Legend:location of the investigated soda lakes in the Kulunda Steppe (Altai, Russia). **a)** Detailed map; **b)** large scale map

community as well as the SRB community in several soda lakes from the Kulunda steppe in collaboration with Russian researchers, who studied the microbial activity *in situ*.

Microbial diversity in soda lakes

Organisms inhabiting soda lakes are defined as halo-alkaliphilic, which means salt and alkali loving organisms, living at high salt and pH conditions. This term was first introduced for halophilic Archeae found in soda lakes. However they prefer sodium chloride instead of sodium carbonate as salt. For organisms preferring soda the term natronophiles or natrophiles should be used.

Very often soda lake brines are green, red-orange or red-purple colored. This phenomenon is due to the presence of prokaryotes, such as cyanobacteria, purple sulfur bacteria or Archeae, inhabiting the water or trona deposits of soda lakes. Indeed, despite the double extreme conditions soda lakes harbor a high variety of microorganisms and almost all ecophysiological groups are present (Fig.1.4). However the high alkalinity and salinity rule out higher eukaryotes, except for some protozoa, such as *Flagellatae*, algae which are mostly represented by *Dunaliella*, and grazers, represented by the brine shrimp *Artemia*. The latter has specific adaptation systems, similar to the prokaryotic organisms, to live in alkaline brines.

The high alkalinity and salinity not only affect the organisms inhabiting soda lakes, but it also imposes other limitations like the availability of metal cofactors at high pH. However, some of the metals, which are indispensable for the bacterial growth, form alkaline complexes with carbonate, being even more soluble than their respective hydroxides (Johannesson and Lyons, 1994). Furthermore, the high alkalinity influences the chemical element cycling in soda lakes (Fig.1.3) and therefore the microbial ecosystem as well. For example, CO_2 becomes limiting at pH above 8 and HCO_3^- at pH above 10, while at pH>9 NH_4^+ is mostly converted to the toxic and volatile NH_3. On the other hand nitrite and sulfide become less toxic under neutrophilic and alkaliphilic conditions to the benefit of the nitrite-metabolizing bacteria and sulfide producing/consuming organisms. As already mentioned, phosphate is present at a higher concentration due to the precipitation of calcium as carbonate (Sorokin and Kuenen, 2005b). All these factors, along with the high salt concentrations, have a big impact on the prokaryotic community inhabiting this double extreme environment.

Several ecological studies have been conducted on thalassic saline environments and their populations, but much less is known about the prokaryotic communities inhabiting soda lakes , which have to deal with a different salt, sodium carbonate rather than sodium chloride, originating from the athalassic inland water bodies, with different physical-chemical properties.

Despite their extreme conditions, most of the soda lakes are highly productive (Melack, 1983) because of the high light intensities, unlimited supplies of Ci and the high content of phosphate. This high productivity, with rates above 10 gC m^{-2} day^{-1} (Melack, 1983), is the driving force behind all biological processes occurring in soda lakes (Jones *et al.*, 1998).

Major contributors to primary production are the autophototrophic cyanobacteria, mostly represented by the planktonic *Spirulina* spp.. *Cyanospira*, *Choococcus* and *Synechococcus* may also be present, but their importance is probably minor in comparison with *Spirulina* spp. (Jones *et al.*, 1998). Cyanobacteria are not only responsible for primary production, but also for the fixation of N_2 and the production of O_2. However, it is still uncertain which organisms in soda lakes are responsible for N_2 fixation, since *Spirulina* spp. is not capable of doing that. A molecular study made on Lake Magadi (Baumgarte, 2003) showed the domination of cyanobacteria among the planctonic prokaryotic community, mostly represented by members

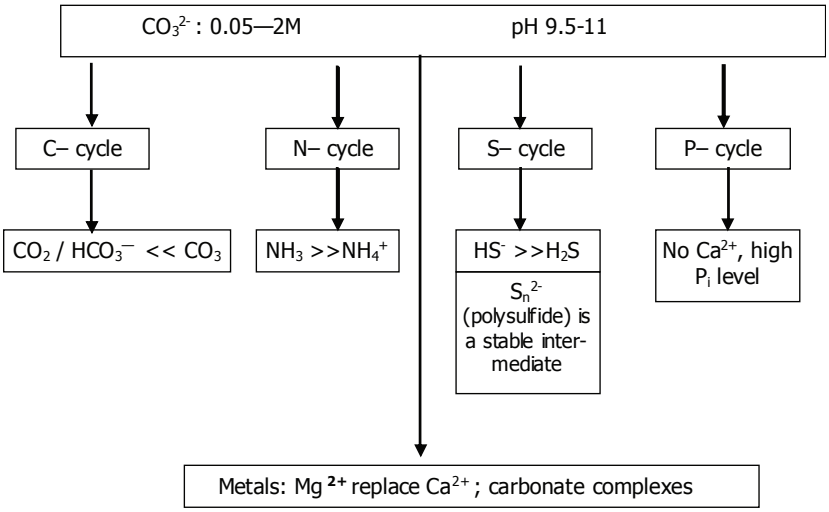

Figure 1.3. Influence of carbonate alkalinity on microbial elemental cycling in soda lakes (Sorokin and Kuenen, 2005b, modified)

of the order *Chroococcales*. This order belongs to a new group based on physiological characteristics (Garcia-Pichel *et al.*, 1998), the *Halothece* cluster, which can stand extreme high salinity conditions and probably is the most important primary producer in hypersaline lakes. However the members of this group cannot fix nitrogen either. In Mono Lake only benthic N_2 fixation was detected (Herbst, 1998), due to the activity of the nonheterocystous filamentous *Oscillatoria*-like cyanobacterium. The same study showed the inhibition of the nitrogenase activity by increased salinity, where nitrogen fixation is probably almost completely attributed to anaerobic heterotrophs (Herbst, 1998). Therefore, it is clear that more research has to be done to understand what is responsible fot the nitrogen fixation and to confirm whether ammonia is mainly produced by this process.

Anoxygenic phototrophic purple sulfur and non sulfur bacteria are secondary producers. Purple sulfur bacteria, such as *Ectothiorodospira*, *Halorhodospira* and *Thiorhodospira*, are not only important for their role in primary production, but also for being a link between the S- and C- cycle, oxidizing sulfide producing intermediate extracellular sulfur deposition. Recently a purple non-sulfur bacterium, *Rhodobaca bogoriensis* has been isolated from Lake Bogoria, African Rift valley (Milford *et al.*, 2000). If *R. bogoriensis* is a low salt organism, *Halorhodospira* can stand high salt concentration and it is one of the dominant organisms in, i.e., Wadi Natrun lakes.

The various polymers produced by primary producers are subsequently utilized by aerobic bacteria, such as *Bacillus* spp. and *Halomonas* spp., and anaerobic hydrolytic bacteria, i.e. *Clostridia*. Haloalkaliphilic halomonads are also involved in the N- and S-cycles, being capable of denitrification (Jones and Grant, 1999) and oxidation of thiosulfate and sulfide (Sorokin, 2003). Members of the Euryarcheota, like *Natronobacterium* and *Natronococcus* (Kamekura *et al.*,1997) can also utilize products of photosynthesis, especially in the alkaline brines of hypersaline lakes. Only recently hydrolytic cellulose degraders have been isolated (Jones *et al.*, 2005; Zhilina *et al.*, 2005b). Cellulose is produced by algal blooms and allochtonous input and is therefore an abundant substrate for cellulolytic organisms. *Clostridium alkalicellum* (Zhilina *et al.*, 2005b) has been isolated from Lake Verkhnee Beloe, Russia. It is a strict alkaliphilic anaerobic bacterium, slightly halophilic, also able to fix nitrogen. *Cellulomonas bogoriensis*,

an aerobic high GC Gram positive bacterium, was isolated from lake Bogoria and it is slightly halophilic and alkaliphilic. Soluble oligomers formed by these cellulolytic bacteria are used by fermenting bacteria like the recently described *Alkaliflexus imshenetskii* (Zhilina *et al.*, 2004), which is the first cultivated alkaliphilic anaerobic organism from the Bacteroidetes (formerly known as CFB: Cytophaga/Flavobacterium/Bacteroides) phylum.

Monomers and oligomers produced by the hydrolytic bacteria are then further utilized by haloalkaliphilic anaerobic fermentative bacteria, such as *Spirochaeta* spp. (*S. alkalica*, *S. africana* and *S. asiatica*) and the genus *Amphibacillus*, able to utilize a wide variety of sugars producing hydrogen, acetate, ethanol and lactate. These, in turn, are substrates for secondary anaerobes, like acetogenic (i.e. *Natrionella acetigena*), methanogenic (i.e. *Methanolobus orgonens*, *Methanohalophilus zhilinaea*) and sulfate reducing bacteria (*Desulfonatronum lacustre* and *Desulfonatronovibrio hydrogenovorans*). These groups are also known as hydrogenotrophic organisms and they play a crucial role because of thermodynamic reasons. In fact complete degradation of organic matter by chemotrophic anaerobes is only possible if hydrogen is completely removed (Zavarzin, 1999). Sulfate reducers are of special importance as they are responsible for the last steps of organic matter degradation and because the S cycle is one of the most active cycles in soda lakes.

Reduced gases generated by secondary anaerobes, i.e. H_2, H_2S, NH_3 and CH_4, are then oxidized by aerobic bacteria. Methane is utilized by methanotrophic bacteria, such as *Methylomicrobium alcaliphilum* and *Methylomicrobium buryatense* (Kaluzhnaya *et al.*, 2001). A new species of *Methylomicrobium*, sp. AMO1, isolated by Sorokin *et al.* (2000b) from Kenyan soda lakes, is also capable of oxidizing ammonia to nitrite. The product of methane oxidation is CO_2, which is then re-utilized for primary production. Recent molecular studies investigated the methane oxidizing community from Transbaikal soda lakes (Lin *et al.*, 2004) and Mono Lake (Carini *et al.*, 2005), both moderate saline lakes. They used the functional gene pmoA (i.e., the gene encoding the alfa subunit of the membrane bound methane monooxygenase) and the 16S rDNA gene specific for methane oxidizers as targets, respectively. Both studies confirmed the dominance of the type I methanotrophs, *Methylomicrobium*.

Oxidation of sulfur compounds in soda lakes is apparently done by aerobic chemolithoautotrophic bacteria, i.e. *Thioalkalivibrio* spp. and *Thioalkalimicrobium* spp., and anaerobic purple sulfur bacteria. The first genus is very broad and contains several versatile species, such as thiocyanate oxidizers (*T. thiocyanoxidans*) and denitrifiers (*T. denitrificans* and *T. thiocyanodenitrificans*). Anaerobic bacteria, such as anoxygenic phototrophic bacteria members of the genus *Ectothiorhodospira* and *Halorhodospira*, are also involved in the oxidation of sulfur compounds. The sulfate generated by this oxidation process is re-utilized by sulfate-reducing bacteria (SRB) in sulfidogenesis processes, closing in this way the sulfur cycle. Recently, a sulfur reducing bacterium *Desulfurispirillum alkaliphilum* (Sorokin *et al.*, 2007a) has been isolated from a sulfide-removal bioreactor. This bacterium is a first example of a haloalkaliphilic bacterium capable of both dissimilatory of sulfur reduction to H_2S to and nitrate-ammonification to NH_3.

The nitrogen cycle is also active in soda lakes. Ammonifiers are represented by *Natronoincola* spp. and *Tindallia magadii* (acetogenic ammonifier), both isolated from Lake Magadi. Ammonia is also produced by N_2-fixing cyanobacteria or anaerobic organisms, i.e. *Clostridium alkalicellum*. Ammonia is then oxidized to nitrite by, e.g., *Nitrosomonas halophilus* or by the methane oxidizer *Methylomicrobium* spp. AMO1. The nitrite is further oxidized to nitrate by *Nitrobacter* spp. (*Nitrobacter alkalicus*). Nitrate is then reduced to nitrogen gas by haloalkaliphilic Gammaproteobacteria denitrifiers, such as *Thioalkalivibrio denitrificans*, *Halomonas*

spp. and *Natronincola lacisaponis*. The last has been isolated from Soap Lake and belongs to the Gamma subdivision of the Proteobacteria (Dimitriu *et al*., 2005). Since nitrification is inhibited at salinity above 1M Na^+ the N-cycle might not be closed in hypersaline soda lakes.

Hence, despite the harsh conditions, soda lakes have been proven to be complete functional ecosystems and to harbor a diverse microbial community. This was confirmed by molecular studies, in which the microbial diversity was investigated using culture independent techniques, i.e. clone libraries targeting the 16S rRNA gene (Baumgarte, 2003; Duckworth, 1996). Most of the studies of the prokaryotic diversity in soda lakes showed similar results. Both in Mono Lake (Scholten *et al*., 2005) and in Lake Magadi (Baumgarte, 2003) the majority of the retrieved clones were *Clostridia* spp. and *Bacillus* spp.. Within the phylum Proteobacteria, the Gamma and Alfa subdivisions were the predominant groups. Most of the clones were related to the sulfur oxidizers related to the *Thioalkalivibrio* spp. and *Thioalkalimicrobium* spp. or to members of the *Rhodobacterales* group. These results were also confirmed by studies made on different Kenyan (Rees *et al*., 2004) and Siberian lakes (Foti, unpublished data). In addition, these studies showed the presence of new phylogenetic lineages, e.g. in the *Halobacteraceae* -(Baumgarte, 2003; Grant *et al*., 1999) and in the SRB group (Foti *et al*., 2007). Moreover, most of the cultivable alkaliphilic organisms are only moderately salt-tolerant and very little is known about the microbial community inhabiting hypersaline soda lakes.

Adaptation to haloalkaliphilic conditions

Soda lakes are unique environments with an extreme high pH and high to extremely high salinity. Organisms inhabiting such places developed different strategies to cope with both alkalinity and salinity.

Since the cell membrane is permeable to water, it is important that the same osmotic pressure is maintained between the cytoplasm and the external environment to prevent loss of water. Halophilic organisms adopt either the so-called “salt-in” or the “compatible-solute” strategy (Oren, 1999). The first is based on the complete adaptation of the intracellular enzyme systems to high salt concentration and it is, in terms of bioenergy, the cheapest solution. However, it seems to be used only by two groups of microorganisms: the aerobic extremely halophilic Archea of the order *Halobacteriales*, and by the anaerobic halophilic bacteria of the order *Haloanaerobiales*. Since this solution has a lower energetic cost than the “compatible-solute” strategy, it is unclear why it is not utilized by a broader range of prokaryotes. However, it is likely that the adaptation of the entire intracellular system to high salt conditions might be a long and complex evolutionary process, that probably can be achieved only by obligate extreme halophiles.

The second strategy is based on producing, or taking up from the environment, small organic molecules that can compensate the osmotic pressure. This strategy doesn’t require the adaptation of the complete enzymatic machinery. Different sorts of compatible solutes can be produced and utilized, such as glycerol, sugar derivatives, ectoine and glycine betaine. Banciu *et al*. (2005) showed the salt-dependent production of glycine-betaine in *Thioalkaliovibrio versutus* strain ALJ15, whose production goes from 1.5% of the total dry weight at 0.6M Na^+ to 9% at 4M Na^+.

This strategy is energetically expensive and 20 to 100 ATP molecules may be required, depending on the compatible solutes produced (Oren, 1999). Probably this is the reason why some metabolic groups with a low energy yield, such as methanogens, complete oxidizing sulfate reducers and nitrifiers, cannot be detected at high salt concentrations, as they produce too little energy to make compatible solutes (Oren, 1999).

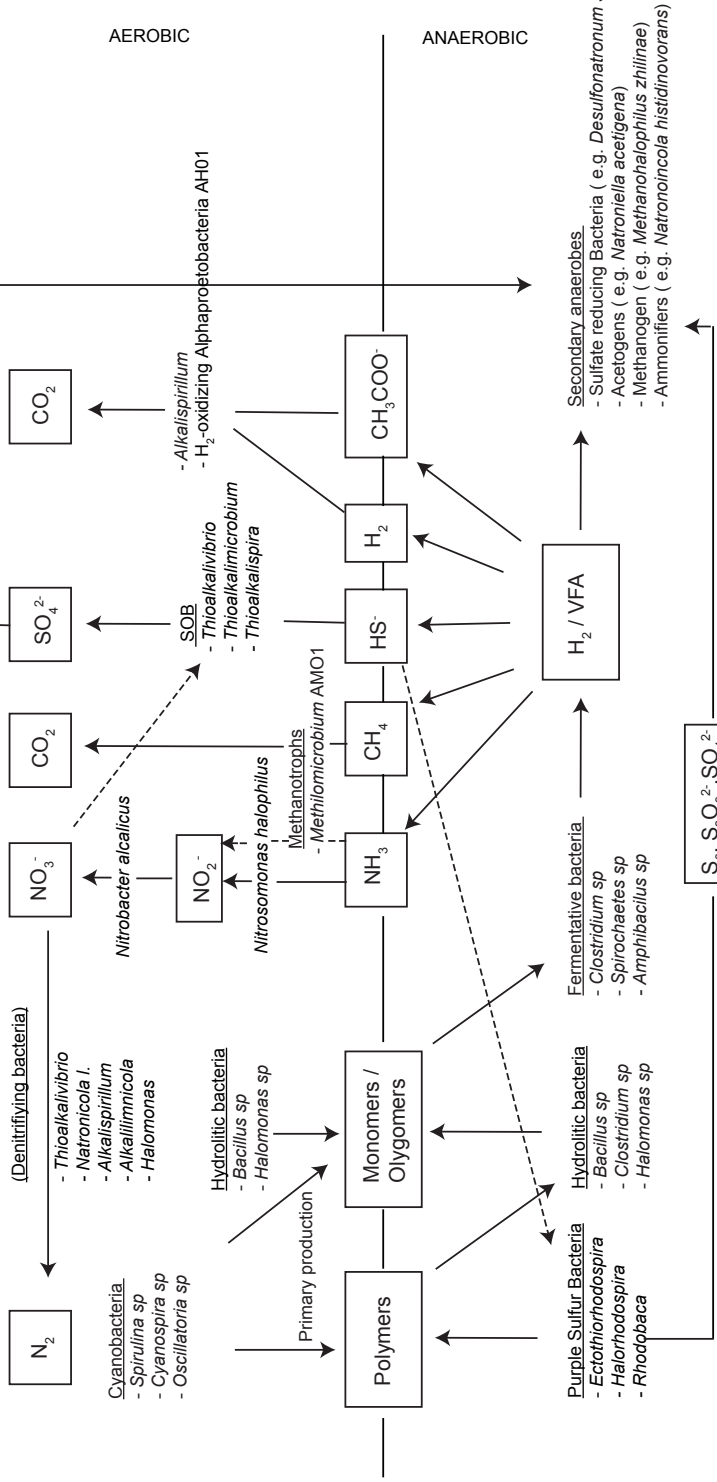

Fig. 1.4 Ecophysiological groups active in soda lakes. Some representatives are presented. Note that denitrifiers in the aerobic zone are facultative anaerobic.

Besides these two strategies, organisms living at high salinities show also biochemical and biophysical adaptations, i.e., modification at the membrane level. Squalene, a non-polar lipid, has been detected in halophilic organisms and it seems to play an important role in osmoadaptation. It is located within the lipid bilayer and it prevents the leakage of Na^+ and H^+ ions. It has been observed (Banciu *et al.*, 2005) that the concentration of this isoprenoid does not increase with salinity, indicating that it is most likely constitutively produced by haloalkaliphilic bacteria.

Another adaptation to high salt concentration is the probable utilization of sodium pumps for the build up of a PMF in addition to the proton pumps. In this way the excess of Na^+ ions is exploited for the bioenergetic purposes.

Interesting is that these last strategies are also typical for the pH homeostasis (Padan *et al.*, 2005; Horikoshi, 1999), to the benefit of the bacteria inhabiting soda lakes. Besides membrane modification, cell surface modifications are also necessary, since the membrane is highly instable at alkaline pH. Therefore alkaliphilic cell walls are enriched in acidic polymers, which infer a negatively charged matrix able to decrease the proton activity already at the surface level. A very important role in pH and osmotic homeostasis is played by the electrogenic Na^+/H^+ antiporter, which exchanges a larger number of entering H^+ than the exiting of Na^+. This removes the Na^+ in excess from the cell, which would be otherwise toxic, and acidifies the cytoplasm, keeping an intracellular pH that is 2-2.3 units below the external pH, with pH 11.5 as highest external pH. However it is still unclear where the cell can capture the required H^+ for the antiporter system in alkaliphillic environments. One hypothesis is the so-called “hand-to-mouth” utilization of protons, an internal proton cycle, in which the H^+ released by the oxidases are kept close to the membrane by acid polymers, i.e. cardiolipins, present in the membrane self. These polymers act like a trap for protons that are then captured by secondary pumps, such as the electrogenic Na^+/H^+ antiporters and ATPases.

It is obvious that living at double extreme conditions, such as halo-alkaliphilic microorganisms do, is energetically costly and stressful. A lot is still unknown about adaptation mechanisms and more research has to be done. Here it has also to be stressed that NaCl and Na_2CO_3 /$NaHCO_3$ are different salts, with different physical and chemical properties. Sodium carbonate is two times weaker as electrolyte than sodium chloride and it has been shown that higher adaptation levels are required in presence of NaCl rather than Na_2CO_3 in terms of osmotic stress (Sorokin and Kuenen, 2005b). Furthermore at concentrations higher than 2M total Na^+, sodium carbonates are present only in their undissociate form, causing less stress than sodium chloride which is fully dissociated up to saturation (5M Na^+). This might explain the surprising richness of prokaryotic life in the observed hypersaline soda lakes.

The sulfur cycle

Sulfur is a very abundant element in nature. It exists in both organic and inorganic form, and in different oxidation states, from -2 to +6 (Table 1.1). The main sources of sulfur are volcanic activity (H_2S), biogenic emissions (H_2S, dimethyl sulfide–DMS-), sulfur storages (gypsum-$CaSO_4^-$, metal sulfides and elemental sulfur) and anthropogenic activities (SO_2, SO_3).

The most reduced and oxidized states are sulfide and sulfate, respectively.

(Poly)sulfide is highly reactive and it can be both chemically and biologically oxidized. It is a polluting, corrosive and highly cytotoxic compound, because it binds preferentially to the iron atoms present in cytochromes and to other intracellular iron containing molecules. The main anthropogenic sources of sulfide are industrial wastes, such as petrochemical plants and paper mills, agriculture and traffic emissions. Nevertheless it can be either chemically

or biologically removed, i.e. by metal precipitation (ZnS or FeS_2) or as elemental sulfur, by sulfur-oxidizing bacteria. Despite its toxicity, reduced sulfur atoms are necessary for the cell functioning. For examples, the –SH (sulfhydryl) group is present in amino acids, vitamins and coenzymes, and the disulfide bonds (S-S bonds) formed between cysteine residues in peptide chains are very important in protein assembly and structure. Incorporation of the sulfhydryl group into the cell is possible via the assimilatory sulfate reduction, which allows the introduction of sulfane atoms avoiding its toxicity. However at high alkaline conditions, as in soda lakes, sulfide becomes less toxic, because it exists only in the dissociate form, which cannot freely diffuse through the cell membrane.

In contrast to sulfide, sulfate is highly inert and it needs to be activated for further utilization by, either dissimilatory or assimilatory reduction. Two ATP equivalents are necessary for the activation of sulfate, making the reaction energetically expensive. In the dissimilatory sulfate reduction, the dissimilatory (bi)sulfite reductase (Dsr) is a key enzyme, catalyzing the six-electron reduction of (bi)sulfite to sulfide. It consists of two subunits and it is encoded by two genes (*dsr*AB) that are adjacent on the same operon. The *dsr*AB gene is highly conserved among the prokaryotic domain and it is nowadays used as a molecular marker for the identification of sulfate reducing prokaryotes (Wagner *et al*., 1998).

Besides the inorganic form of sulfur, there are also several organic sulfur compounds entering into the biogeochemical sulfur cycle. The most abundant is dimethylsulfide (DMS), mainly present in marine environments and originating from dimethylsulfoniopropionate (DMSP), an osmoregulatory compound used by marine algae (de Zwart and Kuenen, 1992). Methylsulfide (CH_3SH), carbonylsulfide (COS) and carbon disulfide (CS_2) are among the industrially relevant compounds.

The sulfur cycle consists of a continuous chemical/biological oxidation and reduction of sulfur compounds (Fig. 1.5). When the quantity of oxidized sulfide corresponds to the quantity of produced sulfate, the cycle is in balance. A good example of an ecosystem in relative balance is a ‘sulfuretum’ (Jorgensen, 1982). Anthropogenic activities have resulted in an unbalanced global cycle, causing a variety of environmental pollution problems, such as acid rain and corrosion.

Microorganisms involved in the sulfur cycle can be assigned in two major groups: the sulfur-oxidizing bacteria and the sulfate-reducing bacteria. The first group consists of organisms capable of oxidizing compounds including elemental sulfur, sulfide and thiosulfate, and from now on they will be defined as sulfur-oxidizing bacteria (SOB). The second group consists of organisms capable of reducing for instance sulfate, thiosulfate and sulfur, and they will be defined as sulfate-reducing bacteria (SRB) (see Fig. 1.6). They are mainly belonging

Table 1.1: Oxidation states of sulfur in various compounds

Form	Compound	Oxidation state
S^{2-}	Sulfides, Mercaptans	- 2
$S_2O_3^{2-}$, S_n^{2-}, SCN	Thiosulfate sulfane atom, Polysulfides, Thiocyanate	- 1
S_0 , $S_4O_6^{2-}$	Elemental sulfur, Tetrathionate sulfur atoms	0
$S_2O_4^{2-}$	Dithionite	+ 3
SO_3^{2-}, SO_2	Sulfite, sulfur dioxide	+ 4
$S_2O_6^{2-}$, SO_3^{-}, $S_4O_6^{2-}$ $S_2O_3^{2-}$	Dithionate,Thiosulfate sulfone atom, Tetrathionate sulfone atoms	+ 5
SO_4^{2-}	Sulfate	+ 6

to the Alfa-, Beta-, Gamma, Epsilon- and Delta-subdivisions of the phylum Proteobacteria. However, there are also low GC Gram positive SRB, like the genera *Desulfotomaculum* and *Desulfosporosinus*, and the genera *Thermodesulfobacterium* (phylum *Thermodesulfobacteria*), *Thermodesulfovibrio* (phylum *Nitrospirae*), the thermophilic gram-negative members of the 'SRP association' and the crenarcheal *Archaeoglobus*.

According to their phylogeny, six generic/suprageneric groups of SRB can be differentiated (Daly *et al*., 2000): *Desulfotomaculum, Desulfobulbus, Desulfobacterium, Desulfobacter, Desulfococcus-Desulfonema-Desulfosarcina* and *Desulfovibrio-Desulfomicrobium.*

According to their physiology SRB can be divided into complete and incomplete oxidizers. The complete oxidizers, like members of the family *Desulfobacteraceae* and the genus *Desulfotomaculum*, are able to oxidize fatty acids, e.g., acetate, completely to CO_2. The incomplete oxidizers cannot oxidize their substrate completely to CO_2, but only to acetate.

The sulfur cycle is mostly active in environments rich in sulfide and sulfate, such as marine sediments, in which sulfate is the second most abundant anion in solution. In extreme environments, like acidic coal-mining sediments (Kusel, 2003) the sulfur cycle can also be highly active.

In soda lakes the sulfur cycle is one of the most active cycles. The activity of SRB has been observed by measuring the sulfate reduction rates (SRR) (Foti *et al.*, 2007; Sorokin *et al*., 2004; Gorlenko *et al*., 1999) and several new genera of SOB (Sorokin and Kuenen, 2005a) and SRB (Pikuta *et al*., 1998; Pikuta *et al.* 2000; Zhilina *et al*., 1997) have been recently described.

It is necessary to remind that there are several particular features of the chemistry of sulfur under highly alkaline conditions compared to neutral conditions. At pH above 8.5 the formation of polysulfides (S_n^{2-}) occurs due to a spontaneous reaction between sulfur and sulfide.

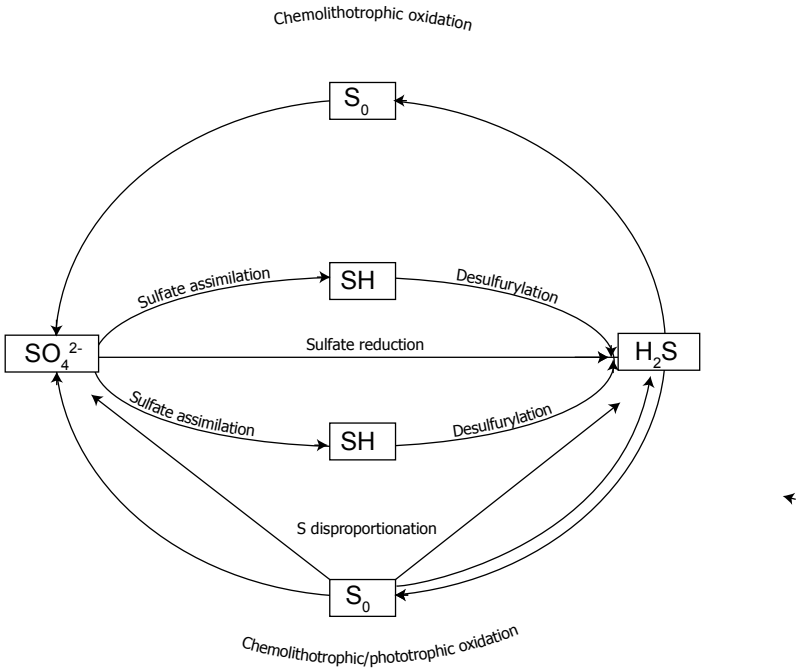

Figure 1.5: The sulphur cycle

This reaction plays a significant role because a highly reactive intermediate, i.e. polysulfides, is produced from the inert sulfur ring, which can be used for bacterial energetic processes. In alkaline conditions also thiosulfate formation is increased, resulting from the chemical reaction between sulfur and sulfite and the chemical oxidation of polysulfides. However, sulfur becomes unstable at pH>10 and at temperatures above 40 °C, meaning that sulfide/polysulfide and thiosulfate become important substrates for the sulfur compound oxidizing community in microoxic\anaerobic and aerobic conditions, respectively (Sorokin and Kuenen, 2005b).

Figure 1.6 shows, to the best of our knowledge, the organisms involved in the haloalkaliphilic inorganic sulfur cycle. In aerobic conditions the main contributors for the oxidation of sulfur compounds are members of the genera *Thioalkalivibrio*, *Thioalkalimicrobium* and *Thioalkalispira* (Fig. 1.6). All of them belong to the Gamma- subdivision of the Proteobacteria and are chemolithoautotrophic organisms, using inorganic compounds as carbon and energy source. The first two genera will receive a special attention in this paragraph, since they have been isolated and characterized in our laboratory. They both completely oxidize sulfide to sulfate under normal aerobic conditions, whereas in microaerophilic conditions the oxidation terminates at the level of elemental sulfur.

To date, within the *Thioalkalimicrobium* genus four species have been characterized: *Thioalkalimicrobium aerophilum*, *Thioalkalimicrobium sibiricum*, *Thioalkalimicrobium cyclicum* and the most recent *Thioalkalimicrobium microaerophilum*. All the strains belonging to this group are moderately halophilic alkaliphiles, with an optimum of salt concentration of 0.6M total Na^+ and possess extremely high sulfide oxidation activity. Several sulfur compounds can be used as electron donors, such as thiosulfate, sulfide and polysulfide. The highest respiration rates were observed with sulfide and thiosulfate, while no growth was observed with sulfite or sulfur. In contrast to members of the genus *Thioalkalivibrio*, members of the genus *Thioalkalimicrobium* show an opportunistic or so-called `R-strategy`, with high growth rates and low growth yields. It outcompetes members of the genus *Thioalkalivibrio* under favorable conditions, i.e. low salt conditions, but has low survival under starvation conditions. Recently it has been shown that members of the genus *Thioalkalimicrobium* dominate the North American soda lake Soap Lake (Sorokin *et al.*, 2007b).

On the other hand, members of the genus *Thioalkalivibrio* are characterized by high salt tolerance (up to 4M total Na^+) and moderate sulfide oxidation activity. These organisms display a 'K-strategy', with low growth rate, high growth yield and long survival during starvation. This genus is highly heterogeneous and so far includes nine species: *Thioalkalivibrio jannaschii*, *T. versutus*, *T. nitratis*, *T. thiocyanoxidans*, *T. halophilis, T. nitratireducens, T. paradoxus, T. denitrificans* and *T. thiocyanodenitrificans*. It also belongs to the Gamma-subdivision of the Proteobacteria and is related to the genus *Ectothiorhodospira* (family *Ectothiorhodospiraceae*).

The *Thioalkalivibrio* genus comprehends versatile organisms, including those capable of denitrification and growing on thiocyanate as sole source of energy and nitrogen. This versatility gives them an important role not only in the sulfur cycle, but in the nitrogen cycle as well.

In fact, three species are capable of denitrification. *Thioalkalivibrio nitratireducens* can reduce nitrate to nitrite, *T. denitrificans* can further reduce nitrite or nitrous oxide to nitrogen gas, and *T. thiocyanodenitrificans* can reduce nitrate or nitrite completely to nitrogen gas. This last species can utilize thiocyanate as electron donor (Fig. 1.6), like *T. thiocyanoxidans* and *T. paradoxus*, producing sulfide, ammonia and CO_2. Furthermore, versatility is expanded in terms of pH (this genus includes facultative alkaliphilic species) and oxygen tolerance (microaerophilic species are also present).

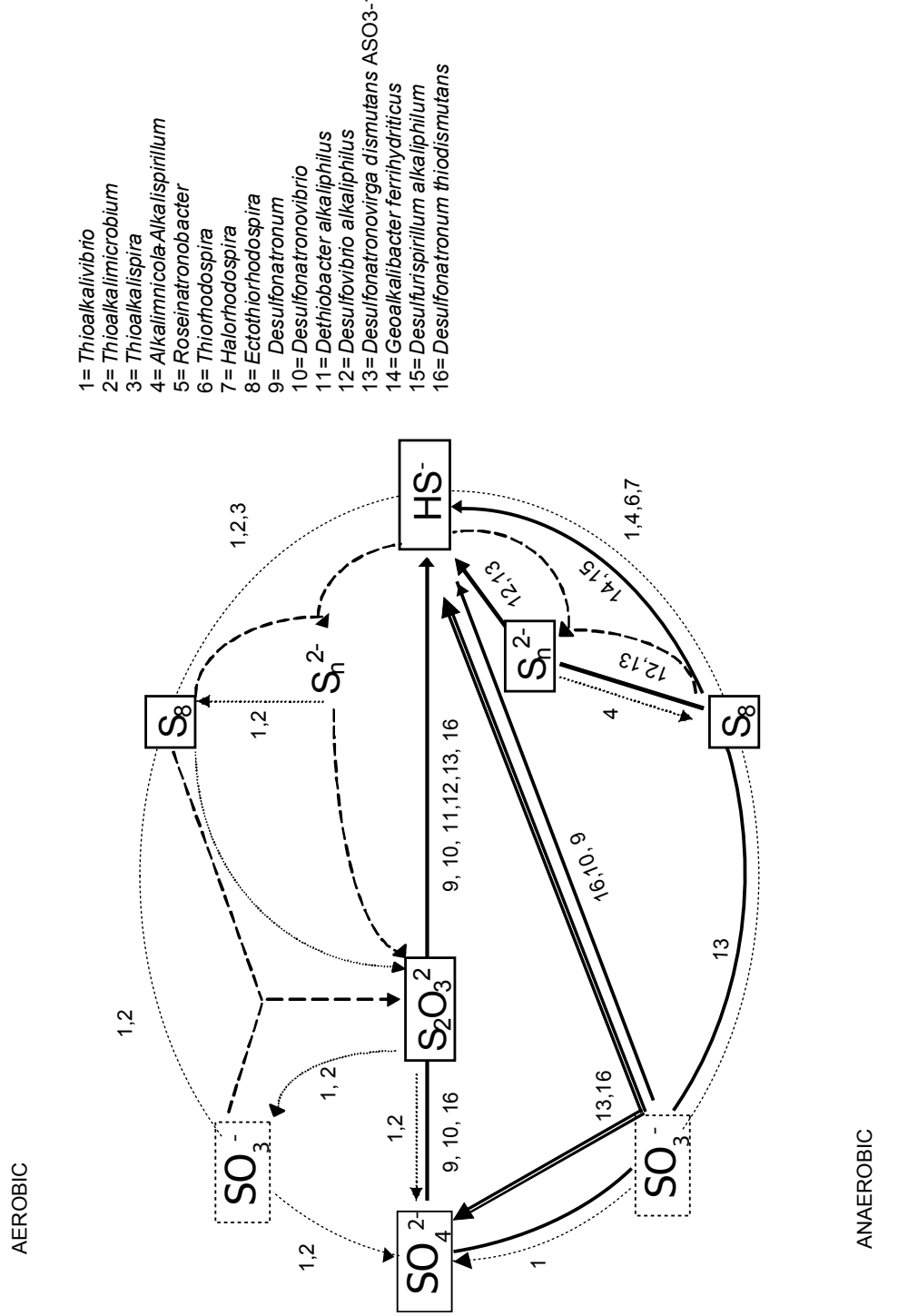

Figure 1.6 Sulfur cycle in soda lakes. Oxidation reaction are indicated by dotted lines; reduction reactions by solid lines; chemical reactions by dashed lines and disproportionation by double solid lines.

More than 100 strains belonging to this genus have been isolated from different places around the world, such as Mono Lake, Soap Lake and Searles Lake (USA), Kenya (Lake Bogoria, Magadi and Crater Lake), Egypt (lakes of the Wadi an Natrun system), Mongolia and Siberia. A study about the genetic diversity and biogeography of strains of the *Thioalkalivibrio* genus has been also conducted in this PhD research. Recent molecular studies showed the presence of this genus in the bacterial clone libraries from different soda lakes, like Mono Lake and Lake Magadi (Rees *et al*., 2004; Humanyoun *et al*., 2003; Baumgarte, 2003) and other environments that might have haloalkaliphilic microniches, such as marine environments.

Besides the autotrophic SOB, soda lakes also host heterotrophic sulfur oxidizers, i.e. the alfaproteobacterium *Roseinatronobacter thiooxidans* (Sorokin *et al*., 2000a) and *R. monicum* (Boldareva *et al*., 2007) and the members of the Gammaproteobacteria *Alkalispirillum–Alkalimnicola* group (Sorokin *et al*., 2006; Oremland *et al*., 2004). *R. thiooxidans* was isolated from soda lakes of the Kunkurskaya steppe (Chita, Russia) and it is strictly aerobic and heterotrophic, able to grow on acetate and thiosulfate at pH 10. *R. monicum* has been isolated form Mono Lake and it can oxidize thiosulfate, sulfide and polysulfide to sulfate during heterotrophic growth. Isolates belonging to the *Alkalispirillum–Alkalimnicola* group were obtained from sediment samples from Lake Magadi (Kenya), from hypersaline lakes from North-Eastern Mongolia and from several Egyptian Wadi Natrun lakes. These bacteria are facultative autotrophs able to oxidize sulfide and polysulfide, but not thiosulfate, to elemental sulfur, both under aerobic and denitrifying conditions.

Another important group involved in the oxidation of sulfide under anoxic condition is the purple sulfur bacteria. The anoxygenic phototrophs, like *Ectothiorhodospira*, *Halorhodospira* and *Thiorhodospira*, are responsible for the oxidation of sulfide with production of elemental sulfur that is deposited outside the cells when light is available. These microorganisms belong to the genus *Ectothiorhodospira* which is, together with the genus *Halorhodospira*, members of the family *Ectothiorhodospiraceae*. Although these two genera were previously allocated in the family *Chromatiaceaea*, they have been recently removed since sulfur is deposited outside the cells, in contrast to the members of the *Chromatiaceaea* (Imhoff and Suling, 1996).

Ectothiorhodospira and *Halorhodospira* are both highly salt tolerant, as the members of the genus *Thioalkalivibrio*. They also belong to the same family, raising the question whether they have a common ancestor. On the other hand, members of the *Thiorhodospira* group are less halotolerant and more similar to *Thioalkalimicrobium*.

The dominant process in the anoxic part of the cycle is dissimilative sulfate reduction. This is carried out by sulfate-reducing bacteria, like members of the genera *Desulfonatronum* (Pikuta *et al*., 1998, 2003) and *Desulfonatronovibrio* (Zhilina *et al*.,1997) and the novel sulfur-reducing isolate *Desulfuspirillum alkaliphilum* (Sorokin *et al*., 2007a). So far, four species of SRB from soda lakes have been described. They all belong to the Delta proteobacteria and are members of the families *Desulfohalobiaceae* and *Desulfonatronumaceae*, within the order *Desulfovibrionales*. *Desulfonatronum lacustre* (Pikuta et al, 1998), *Desulfonatronum thiodismutans* (Pikuta *et al.* 2003) and *Desulfonatronum cooperativum* (Zhilina *et al*., 2005a), belong to the last family, whereas *Desulfonatronovibrio hydrogenovorans* (Zhilina *et al*., 1997), the first described halo-alkaliphilic SRB, is the only species present in the *Desulfohalobiaceae*. They have been isolated from different soda lakes, like Lake Khadyn (Tuva, Russia) (*D. lacustre* and *D. cooperativum*), Mono Lake (*D. thiodismutans*) and Lake Magadi (*D. hydrogenovorans*).

All described haloalkaliphilic SRB can use sulfate, sulfite and thiosulfate as electron acceptor and H_2 and formate as electron donor; ethanol can also be utilized as electron donor

by *D. lacustre* and *D. thiodismutans* (also capable to dismutate thiosulfate or sulfite) and lactate by *D. cooperativum*. It is very likely that these SRB are playing an important role in the hydrogen sink in soda lakes, an important function to complete the anaerobic degradation of organic matter.

All isolates are obligate alkaliphilic and low salt tolerant organisms, with an optimum growth at pH 9.5 and circa 1M total Na^+. In the few culture-independent based studies available in literature, the presence of these organisms in soda lakes has been observed (Humayoun *et al*., 2003; Foti *et al*., 2007; Scholten *et al*., 2005). In this PhD research we observed the presence of new lineages related to *Desulfonatronovibrio hydrogenovorans* and to the complete oxidizers *Desulfatibacillum aliphaticivorans* in sediments from siberian soda lakes (Foti *et al*., 2007). This shows how little is known about the SRB community in soda lakes and that a lot of research has still to be conducted on this topic. In our laboratory a new bacterium, strain ASO3-1, not yet described, has been isolated from a mixture of sediments of hypersaline soda lakes in the Kulunda Steppe (Foti *et al*., 2007). It is an extremely natronophilic SRB, able to grow from 1.5 to 4M Na^+, with an optimum at 2-2.5 M and pH between 9 and 10.5. It is capable of inorganic fermentation, using sulfite as the only electron donor and acceptor. However, it can also grow using H_2 and sulfite/thiosulfate as electron donor and acceptor respectively (Fig.1.6) Disproportionation is a process in which one compound is split into two new compounds, one more oxidized and the other more reduced than the original compound.

Sulfur can also be used as electron acceptor. From sediment of Mongolian and Egyptian soda lakes two bacteria, "*Desulfurivibrio alkaliphilus*" and "*Dethiobacter alkaliphilus*" (D.Sorokin, personal communication), have been isolated. They are facultative autotrophic bacteria able to reduce sulfur, polysulfide and thiosulfate to sulfide in the presence of hydrogen. Zavarzin *et al.* (2006) isolated from a Russian soda lake, Khadyn, an alkaliphilic Deltaproteobacterium of the *Geobacteraceae* family, *Geoalkalibacter ferrihydriticus*, which can reduce sulfur to sulfide, along with iron hydroxide, manganese and other electron acceptors. Very recently in our laboratory a new moderate haloalkaliphilic sulfur-reducing bacterium, *Desulfurispirillum alkaliphilum*, has also been described (Sorokin *et al*., 2007). It has been isolated from a sulfide removing bioreactor and it is a member of a deep bacterial lineage, distantly related to *Chrysiogenes arsenatis*. In addition to sulfur, it can also utilize nitrate as electron acceptor, reducing it to ammonia. This is the first halo-alkaliphilic sulfur-respiring and dissimilatory nitrate-reducing bacterium so far described which plays a special role in both the sulfur and nitrogen cycle. Remarkably this new bacterium was also able to oxidize sulfide to sulfur when both sulfide and nitrate were present in the anaerobic medium.

Applications of haloalkaliphilic bacteria

Haloalkaliphilic microorganisms are of special interest for their application in biotechnology. In the last thirty years several exo-enzymes, such as proteases, lipases, amylases, etc. have been isolated from alkaliphilic organisms, especially from alkaliphilic *Bacillus* strains by Hirokoshi and coworkers (Hirokoshi, 1999). Alkalistable proteases, cellulase and lipases are mostly used in the laundry industry as detergent additives and in the leather tanning industry. Lipases hydrolyze triglycerides to fatty acids and glycerol and are interesting for various applications, from the food to the cosmetic industries. Alkalistable lipase-producing bacteria related to the *Bacillus* cluster have been isolated from Kenyan soda lakes (Vargas *et al*., 2004). Amylases are applied in starch-degrading industry, and very recently a novel amylase (Wang *et al*., 2006) was isolated from *Alkalimonas amylolitica*, a Gammaproteobacteria from a Chinese soda lake (Ma *et al*., 2004). Cellulases and xylanases are used as laundry detergent additives

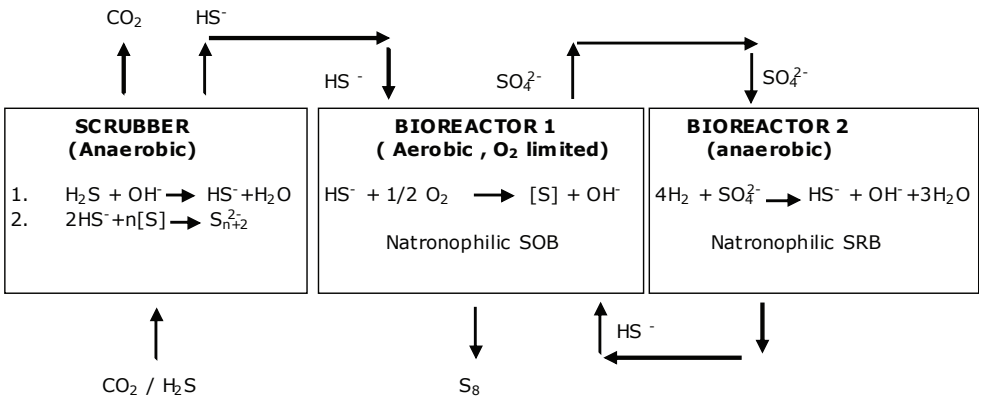

Figure 1.7: Block diagram of the biological process responsible for the removal of H_2S (Buisman *et al.,* 2000*).*

and in the paper mill industry. Cellulase activity has been observed in several haloalkaliphilic bacteria from soda lakes (Jones *et al*., 2005; Zvereva *et al*., 2006).

Besides the interest for the production of enzymes, haloalkaliphilic organisms are recently becoming of interest for desulfurization processes. This PhD research was part of a bigger project aiming to develop a biological process for the removal of hydrogen sulfide from natural gases under halo-alkaliphilic conditions (2 M Na^+ and pH 10). The advantages in this process are the higher scrubbing efficiency of H_2S at high pH and the reduction of bleed streams of the aerobic reactor at high salt conditions. In figure 1.7 a schematic drawing of the process is shown. In the anaerobic scrubber hydrogen sulfide is chemically converted to sulfide, which enters in the first aerobic bioreactor. Here the sulfide is oxidized by SOB to elemental sulfur under oxygen-limited conditions. However a small percentage of sulfate might be still produced, which is then reduced back to sulfide by SRB in the second anaerobic bioreactor. The sulfide produced by the SRB is then reoxidized in the first aerobic bioreactor. Since this process requires halo-alkaliphilic conditions, which are naturally occurring in soda lakes, the investigation of the `sulfur microbial diversity` in such environments was the natural consequence. The most suitable candidates for the first aerobic bioreactor are organisms belonging to the genus *Thioalkalivibrio* sp., due to their capability of respiring inorganic sulfur compounds and their ability to grow at salt concentration up to saturation. For the anaerobic reactor the SRB described so far are not suitable because they do not grow at salt concentrations above 0.6-1 M Na^+. During this PhD research an extreme halo-alkaliphilic SRB has been isolated; strain ASO3-1, which might be the right candidate for the second anaerobic reactor. However still little is known about haloalkaliphilic SRB growing at extreme high salinity and it seems that the isolation of such organisms from soda lakes are especially difficult due a high competetiveness of acetogens.

Scope and outline of the thesis

The present PhD was part of a bigger project: “Application of the biological sulfur cycle under halo-alkaliphilic conditions for high-pressure natural gas desulfurization”. It was funded by The Dutch Foundation for Applied Research (STW), and by the companies Shell Global Solutions Int. B.V. and Paques B.V.. The aim of this project was the development of a biological process for the removal of hydrogen sulfide from natural gas under haloalkaliphilic

conditions. To this goal, a better understanding of the diversity, activity and dynamics of the haloalkaliphilic bacteria involved in the sulfur cycle was required. This PhD research contributed with the detection and identification of these organisms in both natural (soda lakes) and bioengineered (bioreactors) environments, using molecular techniques.

Chapter 1 gives an overview about soda lakes and the organisms inhabiting this ecosystem with special attention to those organisms playing a role in the sulfur cycle. Chapter 2 - 4 are devoted to the investigation of haloalkaliphilic SOB. In Chapter 2 the biogeography and the genetic diversity of 85 *Thioalkalivibrio* isolates has been investigated. In our laboratories more than 100 *Thioalkalivibrio* strains from soda lakes all over the world were isolated. The identification of all isolates by sequencing the 16S rDNA gene was not possible, since the resolution level of this gene as a molecular marker is limited at the subspecies level. To analyze the diversity of *Thioalkalivibrio* strains a strain-specific fingerprinting technique based on the presence of repetitive elements in the bacterial genome (rep-PCR) was used. In addition, a relation between the strains and their location of origin was also investigated.

Chapter 3 focuses on the discrimination between *Thioalkalivibrio* strains in mixed cultures, like bioengineered or sediment samples. One of the tasks was to follow the dynamics of these strains in a bioreactor. The Internal Transcribed Sequence (ITS), which is more variable than the 16S rDNA gene, of these *Thioalkalivibrio* isolates was used as a molecular marker for DGGE analysis.In Chapter 4 the chemolithoautotrophic SOB community from Soap Lake (Washington State) has been investigated using both culture-dependent and –independent techniques. Soap Lake is a meromictic soda lake with an exceptional high sulfide concentration (200 mM) in the anoxic monimolimnion. This study revealed the dominance of the genus *Thioalkalimicrobium* at low salt conditions and a new species, *T. microaerophilum*, has been described.

Chapters 5 to 7 are devoted to the investigation of the reductive part of the haloalkaliphilic sulfur cycle. In chapter 5 the diversity and abundance of the SRB from different (hyper)saline soda lakes from the Kulunda Steppe (South-East Siberia, Russia) has been investigated. For this purpose the functional gene responsible for dissimilatory sulfate reduction (*dsr* gene) has been used as molecular marker. A moderate SRB diversity and a quite high activity, measured by $^{35}S\text{-}SO_4$ method (sulfate-reduction rates), were observed. In addition, new lineages related to the known haloalkaliphilic *D. hydrogenovorans* and *D. aliphaticivorans* were detected. This showed that the diversity related to *D.hydrogenovorans* is higher than anticipated and the possibility of the existence of complete oxidizers adapted to extreme high salt concentrations.

The general bacterial and SRB diversity along a salinity gradient in four different soda lakes from the Kulunda Steppe (South East Siberia, Russia) is presented in Chapter 6. It was analyzed by DGGE targeting the 16S rDNA gene. The major active groups were investigated analyzing the extracted RNA and measuring the SRR. We did not observe any decrease of the bacterial diversity with the increase of the salinity as previously hypothesized for neutral saline habitats. Among the few SRB detected the dominant sequences were related to the complete oxidizers within the *Desulfosarcina* group.

In Chapter 7 a novel sulfur reducing bacterium, *Desulfurispirillum alkaliphilum*, isolated from a sulfide removal bioreactor operated at moderate haloalkaliphilic conditions is described. It is a novel species and genus able not only to reduce sulfur but also to reduce nitrate.

Conclusions and remarks can be found in the last chapter (Chapter 8).

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Chapter 2

Genetic diversity and biogeography of haloalkaliphilic sulfur-oxidizing bacteria belonging to the genus *Thioalkalivibrio*

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ABSTRACT

A group of 85 isolates of haloalkaliphilic obligately chemolithoautotrophic sulphur-oxidizing bacteria belonging to the genus *Thioalkalivibrio* was recently obtained from soda lakes in Mongolia, Kenya, California, Egypt and Siberia. They have been analyzed by repetitive extragenic palindromic (rep)-PCR genomic fingerprinting technique with BOX- and (GTG)5- primer set. Cluster analysis was performed using combined fingerprint profiles and a dendrogram similarity value (r) of 0.8 was used to define the same genotype. Fifty six genotypes were found among the isolates, revealing a high genetic diversity. The strains can be divided into two major clusters, including isolates from the Asiatic (Siberia and Mongolia) and the African (Kenya and Egypt) continents, respectively. The majority (85.9%) of the genotypes were detected in only one area, suggesting an endemic character of the *Thioalkalivibrio* strains. Furthermore, a correlation between fingerprint clustering, geographical origin and the characteristics of the lake of origin was found.

Introduction

Soda lakes are the only habitats on Earth that maintain a stable extreme alkaline condition (pH 9.5-11) due to the high buffering capacity of sodium carbonate. Most of these exotic ecosystems are located in arid areas of Central Asia, Eastern Africa, Central Australia and the North-western part of the USA. In general, soda lakes harbour an enormous diversity of organisms, mostly prokaryotes (Humayoun *et al.*, 2003; Jones *et al.*, 1998). The sulfur cycle is among the most active microbial processes in soda lakes. This cycle is driven by haloalkaliphilic representatives of anaerobic phototrophic purple sulfur bacteria, such as *Ectothiorhodospira*, and sulfate-reducing bacteria, such as *Desulfonatronovibrio* and *Desulfonatronum* (Jones *et al.*, 1998; Zavarzin *et al.*, 1999).

Until recently, the potential of aerobic chemolithoautotrophic sulfur-oxidizing bacteria to grow at extremely alkaline conditions was not recognized. At present, however, more than 100 isolates of chemolithoautotrophic haloalkaliphilic sulfur-oxidizing bacteria have been obtained from sediment samples of different soda lakes. The strains are described as 3 new genera within the gamma subdivision of the Proteobacteria, i.e., *Thioalkalimicrobium*, *Thioalkalivibrio* and *Thioalkalispira*, with more than 13 novel species (Sorokin and Kuenen, 2005). Among these isolates, the most abundant are those belonging to the genus *Thioalkalivibrio*. These have been found in samples from all soda lakes, but dominate especially the hypersaline soda lakes from Kenya, Egypt, south Siberia and California (Sorokin and Kuenen, 2005). This finding raised our interest on the genetic diversity and biogeography of *Thioalkalivibrio* isolates.

Recently, the postulate “Everything is everywhere, but the environment selects” (Beijenrink, 1913; Baas-Becking, 1934) regained interest and has been the subject of several discussions. The common belief was that unicellular microorganisms were uniformly dispersed (Finlay, 2002). Recent studies, however, showed that this can not always be proven, especially in the case of extremophiles, whose growth requires habitats that are often discontinuous and distant (Castenholz, 1996; Staley and Gosink, 1999).

In order to investigate the endemic or cosmopolitan nature of closely-related organisms, different molecular methods have been applied, such as fragment length analysis of the internal transcribed spacer (ITS) region (Papke *et al.*, 2003), AFLP (Amplified-Fragment Length Polymorphism) analysis (Rademaker *et al.*, 2000), ARDRA (Amplified Ribosomal DNA Restriction Analysis (Cho and Tiedje, 2000; Fulthorpe *et al.*, 1998)), MLEE (Multi-locus Enzyme Electrophoresis (Whitaker *et al.*, 2003; Petursdottir *et al.*, 2000)) and rep-PCR genomic fingerprinting (Rademaker *et al.*, 2000; Cho and Tiedje, 2000; Fulthorpe *et al.*, 1998). They differ from each other in resolving power (from genus to strain level) and reliability. Papke and coworkers (Papke *et al.*, 2003) used ITS analysis to study the biogeography of *Synechococcus* strains in microbial mats from North America, New Zealand, Japan and Italy. Cho *et al.* (Cho and Tiedje, 2000) compared three different molecular typing methods (ITS analysis, ARDRA and rep-PCR genomic fingerprinting) to investigate the degree of dispersal of fluorescent *Pseudomonas* strains in soils. Genomic fingerprinting based on repetitive elements was found to be most effective in analyzing microbial geographic dispersal, revealing endemicity, in contrast to coarser resolution. Additionally, these genotypic methods provide insight into microheterogeneity of a population structure, which is defined as phylogenetically closely related, but genetically and physiologically differentiated populations coexisting in the same habitat (Moore *et al.*, 1998). For specific habitats such as soda lakes, highly specialized similar phenotypes might be expected to dominate. In such cases, local variations in conditions,

Table 2.1: *Thioalkalivibrio* isolates used in this study and some characteristics of their habitat.

Continent	Region	Lakes	Number of lakes	Total salts g l^{-1}	pH	Alkalinity M	Number of low salt-tolerant isolates	Number of high salt-tolerant isolates	Strain abbreviation
Africa	Kenya	Nakaru,Magadi, Bogoria, CraterLake, Elmenteita	6	20-220	9.5-11.0	0.12-1.16	20	6	ALJ, ALJT, ARh 2, ARh 3, ALRh
	Egypt	Wadi Natrun (Gaara, Umm-risha, Hamra, Beida, Fazdah, Zugm, Ruzita, Khadra)	8	200-380	9.5-10.3	0.11-0.75	1	28	ALE
North America	California	Mono Lake	1	90	9.7	0.40	0	1	ALM 2
Asia	Northeastern Mongolia	Hotontyn, Dzun-undziin, Shar-burdiin, Golyn-tsagan, Gurvany Behiin	16	50-360	9.2-10.5	0.10-1.20	0	20	ALMg
	Siberia (Tuva, Chita, Altai Region and Kulunda Steppe)	Hadyn,TantarIII, Elongated lake, Narrow Lake, Borzinskoe, Stamp Lake, Tsaidam	26	10-380	9.2-10.6	0.05-5.2	3	17	AL 2, AL 5, AL 21, AKL

such as ion composition of the brines, temperature fluctuations and presence of various toxic trace elements might form a basis for microdiversity in addition to geographic isolation.

Here, we describe the use of rep-PCR genomic fingerprint analysis to determine the genetic diversity and biogeography of 85 *Thioalkalivibrio* strains isolated from soda lakes around the world.

Materials and methods

Sampling and lake description

Samples of sediment (10 cm depth) and adjacent water were collected from different soda lakes in 5 different regions (Mongolia, Siberia, Kenya, Egypt and California (Table 2.1)). Each lake was sampled in 3 different locations, preferably at opposite shores. Whenever possible, the samples were kept in the dark and at low temperature. Mongolian lakes varied greatly in salinity and alkalinity, most being only moderately saline (<60 g/l) (Sorokin *et al.*, 2004). In Siberia three different locations were sampled: Kulunda steppe in Altai (along the south-eastern border with Kazakhstan), Tuva Republic and Chita region-Buriatia (Transbaikal). The last can be also considered as the northern part of the Mongolian site, because it is located in the northeastern part of Mongolia. Soda lakes in Kulunda steppe are located mostly in its southern part; they are small (usually 1-2 km^2), very shallow, mostly hypersaline and with very high carbonate alkalinity, in contrast to hyposaline lakes in other Siberian sites. One of the hypersaline lakes in Kulunda (Stamp Lake) differed from the other by domination of Cl^- among the anions and relatively low carbonate alkalinity (Zavarzin *et al.*, 1999). Both Siberia and Mongolia regions are also characterized by a long, freezing winter period. East African Rift Valley lakes in Kenya are, in general, bigger and deeper than the Siberian-Mongolian lakes, from moderately saline to hypersaline and with high carbonate alkalinity (Jones *et al.*, 1998). The main characteristic of the hypersaline and shallow lakes in Wadi Natrun, Egypt, is domination of Cl^- among the anions and relatively low carbonate alkalinity, similar to the Stamp Lake (Taher, 1999). Mono Lake in California is the only deep and stratified soda lake among those investigated. In most of the cases the distance between the individual lakes is only several kilometers.

Strains and culture conditions

Pure cultures of haloalkaliphilic sulfur-oxidizing bacteria belonging to the genus *Thioalkalivibrio* were obtained previously from soda lakes in Central Asia (Siberia and Mongolia), Africa (Kenya, Egypt) and California (USA). Strains were divided into two groups according to their salt tolerance. Approximately 30% of the strains can grow at salt concentrations between 0.3 and 1.5 M total Na^+, are referred to as low salt tolerant and were isolated mostly from hyposaline lakes. A second, more dominant group (approximately 70%), isolated mostly from hypersaline lakes, is referred to as extremely salt-tolerant, growing optimally in highly concentrated sodium carbonate brines (from 1.5 M up to 4.3 M Na^+) (Sorokin and Kuenen, 2005) (Table 2.1). The strains were grown in a sodium carbonate-based mineral medium at pH 10, with thiosulfate as a substrate (Sorokin *et al.*, 2001). The low salt-tolerant *Thioalkalivibrio* strains were grown at 0.6 M Na^+ and 30°C, and the extremely salt-tolerant strains at 2.5 M Na^+ and 35°C. Growth was monitored by measuring thiosulfate consumption using a standard iodimetric titration (Roy and Trudinger, 1970).

DNA extraction

Cells from 2 ml portions of late logarithmic phase cultures of *Thioalkalivibrio* strains were harvested by centrifugation at 13,000 rpm for 5 min. The pellet was washed twice with 0.5 and 2 M NaCl, for low and high salt strains respectively, and stored at –80°C. Genomic DNA was extracted from the cells using the UltraClean Soil DNA Extraction Kit (MoBio Laboratories, USA), following the manufacturer's instructions. Briefly, the cells were lysed by a combination of detergents and mechanical disruption. The released DNA was bound to a silica spin-filter. Subsequently, the filter was washed, and the DNA was recovered in Milli-Q water. The quality of the extracted DNA was examined on 1% (w/v) agarose gels in 1x TBE-buffer (90 mM Tris-Borate; 2 mM EDTA, pH 8) after staining with ethidium bromide. Images were obtained using the Gel Doc 2000 system (BioRad, Hercules, CA, USA). DNA concentration was measured using the PicoGreen dsDNA Quantitation Kit (Molecular Probes, Europe BV). PicoGreen is an ultra-sensitive fluorescent stain for the quantification of double-stranded DNA in solution, using a standard spectrofluorometer with excitation and emission wavelengths of 480 nm and 535 nm, respectively.

rep-PCR genomic fingerprint analysis

Four variations of rep-PCR genomic fingerprinting were performed i.e. using the BOX A1R (5`-CTACggCAAggCgACgCTgACg-3`), (GTG)5 (5`-gTggTggTggTggTg-3`), REP 1R (5`-IIIICgICgICATCIggC-3`), REP 2I (5`-ICgICTTATCIggCCTAC-3`), ERIC 1R (5`-ATgTAAgCTC-CTggggATTCAC-3`) and ERIC 2 (5`-AAgTAAgTgACTggggTgAgCg-3`)(Versalovic *et al.*, 1994). The amplification reactions were performed in a final volume of 25 µl with 50 ng genomic DNA, 5 µl 5X Gitschier buffer, 0.2 µl BSA (20 mg/ml), 2.5 µl DMSO 100% (v/v), 1.25 µl dNTPs (25 mM each), 1 µl primer (0.3 µM), 0.4 µl Taq DNA polymerase (5 U µl-1), using a thermocycler (Biometra, Germany). The following PCR conditions were used: 2 min at 95°C, followed by 30 cycles of 94°C for 3 s, 92°C for 3 s, 55°C, 49°C, 46°C and 40°C for 1 min for BOX-, (GTG)5-, ERIC- and BOX-primers respectively, 65°C for 8 min, and a final extension at 65°C for 8 min (Rademaker *et al.*, 2004).

Electrophoresis of rep-PCR products

Six hundred ng of PCR product was analyzed on 1.5% (w/v) agarose gel containing 0.5X TAE-buffer (200 mM Tris-Acetate, 0.5 mM EDTA, pH 8). The electrophoresis was performed for 14 hours at 4°C, at a constant voltage of 3.5 V cm^{-1}. The 1-kb Smart ladder (Eurogentec, Belgium) was used as a size marker. The gel was stained for 30 min in 0.6 µg ml/l ethidium bromide solution in 0.5X TAE-buffer, and de-stained for 30 min in 0.5X TAE-buffer. Subsequently, gels were photographed with the Gel Doc 2000 system (BioRad, Hercules, CA, USA) and analysed by using the BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium).

Computer-assisted analysis of genomic fingerprints

Computer-assisted analysis of the genomic fingerprints was performed using the commercially available BioNumerics 4.00 software (Applied Maths, Sint-Martens-Latem, Belgium), as described previously (Rademaker *et al.*, 2004). Briefly, digitized gel images were converted, normalized using the 1 kb size marker and analyzed with BioNumerics. The "rolling disk" background subtraction method was applied. For (GTG)5 and BOX-PCR pattern analysis, alone and in combination, the similarity between pairs of genomic fingerprint profiles was calculated using the pair-wise Pearson's product-moment correlation coefficient (r-value; for

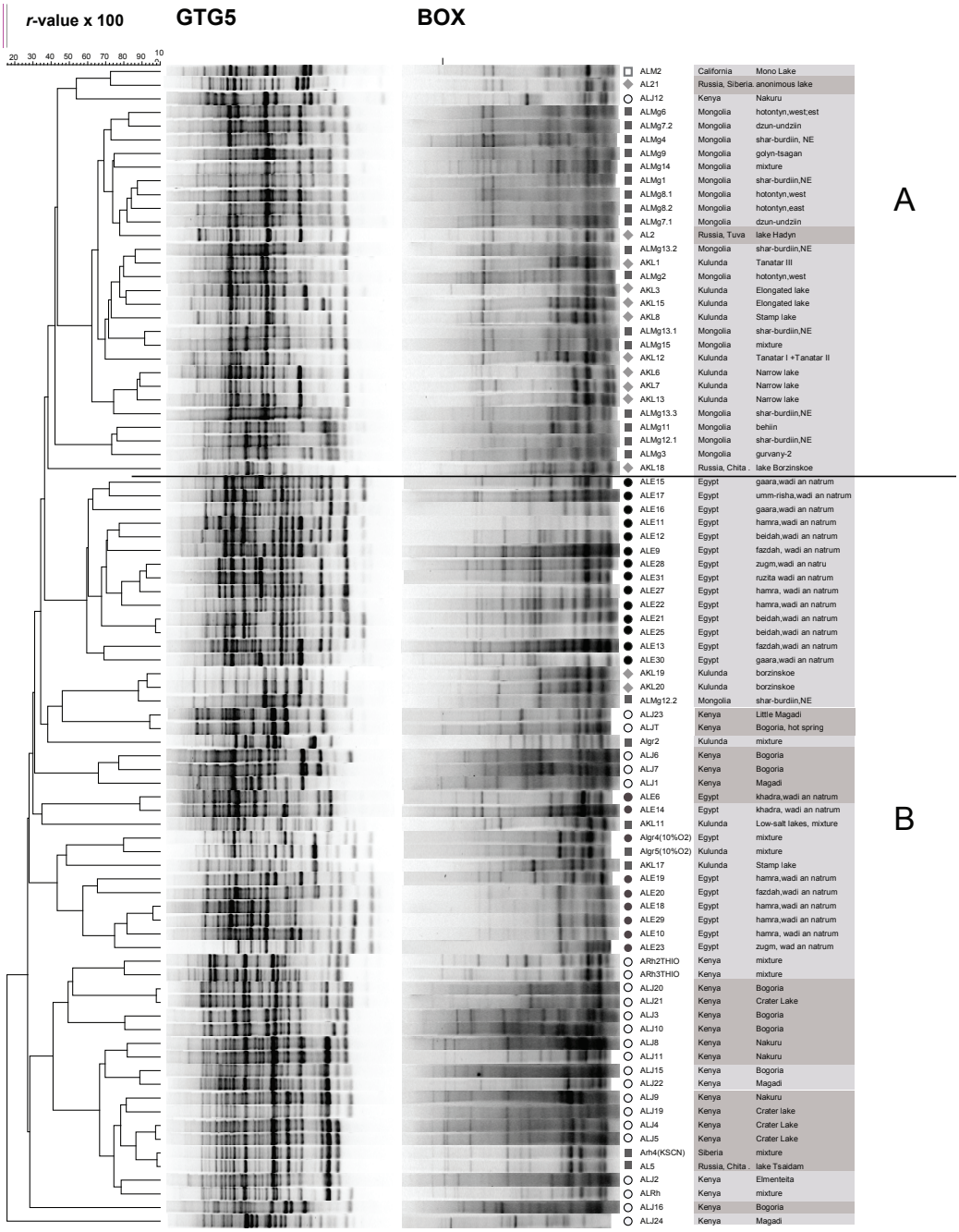

Figure 2.1: UPGMA/product-moment cluster analysis of 85 linearly combined BOX- and (GTG)5- PCR genomic fingerprints of *Thioalkalivibrio* isolates. An arbitrary similarity value (r) of 0.8 was used to determine the same genotypes. In color the isolates origin and salinity characteristic: ● Egypt ○ Kenya □ California ■ Mongolia ◆ Siberia high salt low salt

convenience, these values are often represented by % similarity, r-value 1 equals 100%). This approach compares the whole densitometric curves of the fingerprints (Häne *et al.*, 1983). For this type of analysis zones of gels containing no useful information, such as primer-bands, were excluded by defining appropriate “active zones“ on the digitized images to be included in the analysis. Comparisons were made between (GTG)5-PCR genomic fingerprint profiles from 0.2 kp to 9 kb and BOX-PCR profiles from 0.2 kb to 4 kb. Cluster analysis of similarity matrices was performed using the UPGMA method (Unweighted Pair Group Method using Arithmetic averages (Sokal and Michener, 1958)).

Results

Four different sets of primers, REP, ERIC, BOX and (GTG)5, were tested to assess which was most suitable for analysis of *Thioalkalivibrio* isolates. REP and ERIC primers gave complex profiles, but with low reproducibility (unpublished data), and were therefore omitted from this study. In contrast, BOX and (GTG)5 primers gave complex and reproducible fingerprints (Fig. 2.1), generating profiles with on average 8 and 18 bands, respectively, with DNA fragment sizes from 0.2 to 5.0 Kb (Fig. 2.1).

Fingerprint profiles were analyzed using the Pearson`s product-moment correlation matrix and UPGMA cluster analysis to group isolates. An arbitrary similarity value (r) of 0.8 was chosen to define the same genotype. Dendrograms derived from BOX and (GTG)5 PCR fingerprint profiles gave similar clusters. However, due to the higher resolving power of (GTG)5-PCR profiles, compared to the BOX-PCR profiles, in this collection more genotypes were found in the former case. In fact, 55 genotypes were found using (GTG)5-PCR fingerprinting in contrast to 45 types observed using BOX-PCR fingerprinting at a 0.8 dendrogram similarity cut off. Figure 2.1 shows the dendrogram created from the combined profiles obtained with the (GTG)5 and BOX primers. A total of 56 unique genotypes were identified, including 10 genotypes (15 isolates) from Mongolia, 16 genotypes (22 isolates) from Egypt, 15 genotypes (22 isolates) from Kenya, 10 genotypes (13 isolates) from Siberia and 1 single-strain genotype from California. In addition, 2 genotypes including Mongolian and Siberian isolates (3 strains per each region), 1 genotype including 2 Kenyan and 2 Siberian isolates and 1 genotype including 1 Siberian and 1 Egyptian isolate were present.

The genotypes can be grouped into bigger clusters according to their geographical origin. The upper part of the dendrogram (part A in Fig. 2.1) contains isolates from Central Asia, while the lower part (part B in Fig. 2.1) includes African isolates. The majority of genotypes (85.9 %) were found to be unique to one region (either Mongolia, Siberia, Kenya, Egypt or California) except for 4 genotypes that included isolates from different regions.

Discussion

A collection of 85 *Thioalkalivibrio* isolates from different regions has been analyzed for genetic diversity and geographical distribution by rep-PCR fingerprint analysis using (GTG)5 and BOX primers. The first showed higher resolving power than the latter, resulting in 55 versus 45 genotypes, respectively. The cluster analysis of combined (GTG)5-BOX-PCR fingerprint profiles revealed a higher diversity resulting in 56 different genotypes at a cut off level of 0.8. Rademaker *et al.* (Rademaker *et al.*, 2000) compared similarity values derived from combined BOX-, ERIC- and REP-PCR genomic fingerprints and from DNA-DNA hybridization of *Xanthomonas* strains. A high correlation (0.808) was observed, suggesting that the level of similarity between fingerprints reflected the genetic relatedness of closely related bacteria. This

genetic diversity might originate from different strategies, such as internal (Aber, 2000) (i.e., neutral mutations, or genome rearrangement) and external (Aber, 2000;Ochman *et al*., 2000) (i.e., genetic exchange mediated by plasmids and phages) DNA modification combined with selection pressure. These mechanisms could result in population diversification, with a high degree of microdiversity in the ecosystem (Ochman *et al*., 2000). An example of this was recently showed with *Brevundimonas albas* (Jasper and Overmann, 2004), whereby 11 strains with identical phylogeny exhibited different genotypes representing distinct populations, with genetically determined physiological adaptations and occupying different ecological niches.

This number of obligately chemolithoautotrophic isolates has not previously been studied and it was a surprise to find such a high level of genetic diversity among *Thioalkalivibrio* isolates, which have a relatively restricted phenotype. The genus *Thioalkalivibrio* currently includes nine species and at least 5 distinct phenotypes can be recognized, such as extremely salt-tolerant strains with yellow pigmentation (numerically the most dominant group), nitrate-reducers, denitrifiers, thiocyanate oxidizers and facultative alkaliphiles (Sorokin and Kuenen, 2005). The core group, containing more than 60 strains from different geographical locations, is relatively difficult to differentiate on the basis of its phenotype. Its genetic diversity might therefore result from neutral mutations, which are recognized as an important mechanism of molecular evolution (Kimura, 1983). In chemolithoautotrophic bacteria the role of neutral mutations in genome change might be significant due to relatively primitive central metabolism. It might be interesting to investigate evidence in this group of both functional (due to selection and adaptive pressure) and neutral mutations in more detail by using defined set of variable conditions for a single strain, following a change in its genetic fingerprint after many generations. In fact, preliminary data from variation in the composition of the enrichment medium indicate that the fingerprints obtained from rep-PCR products might reflect the functional changes in the genome of *Thioalkalivibrio*. Particularly, we noticed that replacement of sodium carbonate by potassium carbonate and use of higher than usual (0.5-1 M instead of 0.1 M) chloride content selected populations of high salt-tolerant *Thioalkalivibrio* with different REP-profiles (unpublished data)

Moreover the branching patterns of the (GTG)5-BOX-PCR dendrogram, which provides no information on evolution, might yet give a first indication of the genetic population structure. It was found that a clonal population structure, in which the genetic exchange and variation is mainly due to mutations, is characterized by limited deeply branches lineages (Istock *et al*., 1992; Whittam, 1992;Wise *et al*., 1995). In contrast, freely recombining populations, in which the diversity is generated by parasexual mechanisms of gene transfer through transduction, transformation and conjugation, is characterized by extensive, but shallow branching patterns. The branching patterns of the (GTG)5-BOX-PCR dendrogram might indicate the genetic structure of the *Thioalkalivibrio* population as analogous to the genetic structure of a freely recombinant population. Therefore further investigation is necessary to understand the reasons for the high genetic diversity within *Thioalkalivibrio* genus.

One of the other interesting topics in this work was the investigation of geotypes, defined as genotypes linked to a certain geographical location, within the examined genus. Study of bacterial biogeography (i.e., global distribution of bacterial species) can give important information about the abundance of bacterial species on the planet and their ecological role (Staley and Gosink, 1999). Free-living organisms, which can be found in more than one geographic location on Earth, are defined as cosmopolitan, while organisms that can be found only in one single location are defined as candidate endemic. In defining an organism as endemic to one area, sampling and isolation procedures have to be taken into account, since

they can influence the outcome of the diversity investigated.

Recent studies have demonstrated the endemic property of some bacterial populations (Cho and Tiedje, 2000; Fulthorpe *et al.*, 1998), disproving (at least partially) Baas-Becking's postulate "Everything is everywhere, but the environment selects" (Baas-Becking, 1934). In particular, the ubiquitous distribution of extremophiles had been doubted. Whitaker *et al.* (Whitaker *et al.*, 2003) showed that the hyperthermophilic archaeal Sulfolobus strains clustered geographically, rather than by hot-spring features. In agreement with this result, we also found that the majority of *Thioalkalivibrio* genotypes were associated with a certain geographical location. In this respect, they can be considered as candidate endemic strains, although, a few identical genotypes were found in distant areas, such as Kenya and Siberia, supporting a cosmopolitan distribution of those genotypes. However, this might be explained by the fact that many *Thioalkalivibrio* strains, in contrast to Sulfolobus isolates, possess resting forms that are highly resistant to desiccation and starvation (Loiko *et al.*, 2003). Furthermore, all the extreme salt-tolerant *Thioalkalivibrio* strains produce a yellow pigment whose structure indicates the protecting function from high-light stress (tAKAICHI *Et al.*, 2004). These mechanisms could make it possible for these bacteria to be transported over long distances on salt, sand or dust particles by wind or by migrating water birds, which are frequent visitors of the soda lakes, without lethal light stress. Also, the genotypes containing isolates from different areas share the same salinity characteristic, being tolerant to either high or low salt concentration or the same oxygen demand (one genotype includes Algr4 and Algr5, originating from Egypt and Siberia respectively, which are the only two microaerophilic isolates among the investigated). This demonstrates that local lake characteristics are also important in defining populations. Furthermore, the *Thioalkalivibrio* population from the Wadi Natrun soda lakes in Egypt with high chloride concentration, in contrast to the other isolates, exhibited a high chloride requirement for growth. This unique property is correlated with the (GTG)5- and BOX-PCR genomic fingerprint type, since the Egyptian isolates form a single distinct group in the dendrogram.

No other correlation was found between genotypes and phenotype. Unfortunately, since the 16S rRNA gene sequence was obtained for only few strains, it is difficult to find a correlation between dendrograms derived from the BOX- and (GTG)5-PCR fingerprints and phylogeny of *Thioalkalivibrio* isolates. However, in general a high correlation between fingerprint profile similarity values and DNA-DNA homology was found, supporting the observation of Rademaker *et al.* (Rademaker *et al.*, 2000). Exceptions, included ALJ2 and ALJ3, which show 87% DNA-DNA homology, but only approximately 40% dendrogram similarity.

In conclusion, our results show that REP-PCR genomic fingerprinting is a powerful technique for analysis of genetic microdiversity within the genus *Thioalkalivibrio*. A high degree of genomic diversity was demonstrated in these extremophilic bacteria as well as a tendency for endemism, indicating that geographical barriers might play a significant role in diversification of bacterial populations.

Acknowledgments

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Chapter 3

Discrimination of closely related *Thioalkalivibrio* strains by DGGE analysis targeting the ITS region

ABSTRACT

A new biotechnological process for the removal of H_2S has been recently proposed. The process is operating under halo-alkaliphilic conditions and therefore organisms capable of withstanding high salinity and pH were required. Halo-alkaliphilic sulfur-oxidizing bacteria (SOB) of the genus *Thioalkalivibrio* have been chosen because they comply with such conditions.

The *Thioalkalivibrio* genus is phylogenetically heterogeneous and it contains nine species and more than hundred strains. It is characterized by the high physiological versatility, salt tolerance and moderately high sulfide oxidation activity. Within this heterogeneous group is present a core of extremely salt tolerant and closely related strains. For a better functioning of the process, it is important to identify the most successful strains in the bioreactor under varying conditions, i.e. sulfide and oxygen concentrations. In order to do this, a strain-specific molecular tool is necessary for the discrimination of closely related *Thioalkalivibrio* strains. This should fulfill three requirements: strain-level resolution power, reproducibility and less time consuming. Denaturing Gradient Gel Electrophoresis (DGGE) of the Internal Transcribed Sequence (ITS) region was chosen.

The goal of this study was the development of a new DGGE protocol targeting the specific ITS region for *Thioalkalivibrio* strains.

Introduction

In the last decades, different PCR-based molecular techniques have been developed for the detection and identification of microorganisms from both pure and mixed cultures. For identification and typing purposes, methods like AFLP (Amplified Fragment Length Polymorphism) (Savelkoul *et al.*, 1999), rep-PCR (Rademaker *et al.*,2004) and ITS-PCR (Internal Transcribed Region) (Gürtler *et al.*, 1996) are widely used, as their resolution power may go up to the strain level. However, they are not often suitable for the identification of microorganisms from mixed cultures. For this purpose other methods are commonly applied, like DGGE (Denaturing Gradient Gel Electrophoresis) analysis (Muyzer *et al.*, 1996) and T-RFLP (Terminal Restriction Fragment Length Polymorphism) (Marsh *et al.*, 1999).

DGGE analysis allows the investigation of the major players in a microbial community as well their dynamic and their temporal succession. Principle of DGGE is the separation on a polyacrylamide gel of DNA fragments of the same size but with different sequences. The separation is due to the combination of a DNA denaturant gradient (urea and formamide) and melting properties of the different DNA fragments. Once the domain with the lowest melting temperature reaches its melting temperature at a particular position in the gel, the fragment changes its configuration and will stop its migration along the gel (Muyzer *et al.*, 1996). In order to determine the optimal denaturing conditions and duration of the electrophoresis, a perpendicular gel and a so-called 'time travel' experiments are performed. Variants of DGGE are the CDGE (Constant Denaturant Gel Electrophoresis) and the TTGE (Temporal Temperature Gel Electrophoresis), in which a constant denaturant concentration is used instead of a linear denaturing gradient. In addition, in the TTGE the temperature increases gradually and uniformly, resulting in a linear temperature gradient over the length of the electrophoresis run.

The main target of DGGE is the highly conserved 16S rRNA gene. The major advantage of using this gene as molecular marker is the availability of an extended sequence database. However, it does not display enough resolution power to discriminate between closely related strains. A more variable DNA fragment is the ITS (Internal Transcribed Sequence) region, which is located between the 16S and the 23S rRNA genes. It may encode for one or two tRNAs, depending on the bacterial species, and shows variability in copy numbers, length and nucleotide sequence, even between closely related strains (Gürtler *et al.*, 1996). Since its taxonomic resolution may go, depending from the bacterial species, up to the strain level, the ITS region has been widely used as molecular marker for bacterial typing (Rocap *et al.*, 2002; Daffonchio *et al.*, 1998), but less often as target for the study of microbial communities (Jansen *et al.*, 2003).

The goal of this study was the development of a molecular tool for the discrimination of closely related *Thioalkalivibrio* strains from a mixed culture. Organisms belonging to the genus *Thioalkalivibrio* are haloalkaliphilic chemolithoautotrophic sulfur-oxidizing bacteria (SOB), which have been isolated from soda lakes and grow at an optimum pH of 9.5-10 and are high-salt adapted (up to 4 M Na^+) (Sorokin *et al.*, 2005).

Recently, a mixed culture of extremely halophilic and closely related *Thioalkalivibrio* strains, together with a mixture of sediment samples from soda lakes, were used as a starting biomass for two laboratory-scale aerobic H_2S-oxidizing bioreactor experiments (Van den Bosch *et al.*, 2007).

The newly proposed process consists of a scrubber and two integrated bioreactors, which are operating under halo-alkaliphilic conditions. Briefly, in the scrubber, hydrogen sulfide gas (H_2S) is absorbed by an alkaline carbonate solution. The dissolved sulfide (HS^-) is subse-

quently oxidized under oxygen limited conditions to elemental sulfur (S_0) by sulphur-oxidizing bacteria (SOB). Up to 10% of the sulfide is further oxidized to the unwanted thiosulfate ($S_2O_3^{2-}$) and sulfate (SO_4^{2-}), which are fed to the second anaerobic bioreactor. Here they are reduced back to sulfide by halo-alkaliphilic SRB. Because both bioreactors are integrated, the sulfide can be subsequently recycled to the first, sulfur producing, reactor.

For a better functioning of the process, it is important to identify the most successful strains in the bioreactor under varying conditions, i.e. sulfide and oxygen concentrations. To do this, a new DGGE protocol targeting the ITS region of *Thioalkalivibrio* strains was developed.

Materials and methods

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from 12 *Thioalkalivibrio* strains (Table 3.1) as described in Foti *et al.* (2006). The ITS fragment was amplified using primers 16S-1492f and 23S-115r (Gurtler *et al.*, 1996), and primers pHr and p32SR01 (Massol-Deya *et al.*, 1995) (See Table 3.2). DNA amplification was performed in a 25 µl final volume with 12.5 µl Master Mix Qiagen (Qiagen, Hilden, Germany) and 0.25 µl (0.5 mM final concentration) of both primers. The following PCR conditions were used: 5 min at 94°C, followed by 30 cycles of 94°C for 1 min, 51°C for 1 min, 72°C for 90 s, and a final extension at 72°C for 7 min. The annealing temperature was chosen after amplification performed with a series of different annealing temperatures (i.e. from 47°C to 55°C). The yield and quality of the PCR products were examined on 1% (wt./vol.) agarose gel stained with ethidium bromide. PCR products were then excised from the agarose gel and purified with the Qiaquick gel extraction kit (Qiagen, Hilden, Germany). PCR products were sent for sequencing to BaseClear BV (the Netherlands).

Table 3.1: *Thioalkalivibrio* strains and their place of origin. GC content and melting temperature of the ITS region were determined from the sequences.

Strain	Location	Physiological Type	% GC	T_m°C
ALE6	Egypt	Halo-alkaliphilic	53.4	85
ALE10	Egypt	Halo-alkaliphilic	53.3	86
ALGr3	Egypt	Halo-alkaliphilic	53.4	85
HL17	Egypt	Halo-alkaliphilic	54.9	86
ALJD	Kenya	Natronophilic	54.2	86
ALJ15	Kenya	Natronophilic	53.85	86
ARH2	Kenya	Natronophilic	53.7	86
ALM2	California	Natronophilic	53.1	86
ALMg1	Mongolia	Natronophilic	53	85
ALMg7.1	Mongolia	Natronophilic	53	85
ALMg14	Mongolia	Natronophilic	53.2	85
AKL3	Kulunda steppe, Russia	Natronophilic	53	85

Table 3.2: Primers used in this study

Primer	Sequence 5`-3`	Position (E. coli)	Annealing T°C	Reference
16S-1492f	AAGTCGTAACAAGGTARC	1492-1509	51	Gurtler et al., 1996
23S-115r	GGTTBCCCCATTCRG	115-130	51	Gurtler et al., 1996
pHr (reverse)	TGCGGCATCACCTCCTT	1518-1541	51	Massol-Deya et al., 1981
P23SR01	GGCTGCTTCTAAGCCAAC	1069-1052	51	Massol-Deya et al., 1981
Tav-ITSf	TTCAAATCCACCCAGACCCA		56	This study
Tav-ITSr	GTCCTTCATCGCCTCCTACC		53	This study

Sequence alignment, primers design and amplification

The full length ITS sequences were aligned using ClustalW software (Fig. 3.1). After alignment, the primer sequences were visually searched. Specific ITS primers for *Thioalkalivibrio* were designed. Melting temperature, GC content and secondary structures, i.e. hairpins and dimers, were determined with Netprimer software (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html). Amplification was performed using the developed primers Tav-ITSf and Tav-ITSr (Table 3.2). The same PCR conditions mentioned above were used with an annealing temperature of 56°C. GC clamps were added for DGGE analysis. Also in this case, PCR products were excised from the agarose gel and purified with the Qiaquick gel extraction kit (Qiagen, Hilden, Germany). PCR products were sent for sequencing to BaseClear BV (the Netherlands).

tRNA genes

Scanning for tRNA genes within the ITS sequences was performed using a tRNASCAN-SE program (version 1.21; Lowe *et al*., 1997; http://lowelab.ucsc.edu/tRNAscan-SE/)

DGGE Profiling

All DGGE were performed as described by Schäfer and Muyzer (2001) using the D-Code system (Bio-Rad Laboratories, CA). Electrophoresis was performed with 1 mm thick 6% w/v (or 8% w/v) polyacrylamide gels (ratio of acrylamide to bisacrylamide, 40:1) submerged in 1X TAE buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA, pH 7.5). After electrophoresis, the gels were incubated for 30 min in Milli-Q water containing ethidium bromide (0.5 µg/ml), rinsed for 20 min in Milli-Q water, and photographed using a Bio-Rad GelDoc station (Bio-Rad, CA).

The perpendicular gel was assembled following the manufacturer's instructions (Bio-Rad Laboratories). The PCR product obtained for strain ALE6 was run for 2 hours at constant temperature of 60°C and constant voltage of 150 V, using a linear 0% to 80% denaturant gradient of urea-formamide (UF).

To determine the duration of the electrophoresis run, the ITS fragments of two relatively distantly related strains, ALE10 and ALJ15, were run for 4 hours at 150 V, using a gradient of 25-55 % UF. Every 15 min a mixture of the two amplicons was loaded.

The ITS fragments of the 12 strains have been run on DGGE for 6 hours at 150 V, at a constant temperature of 60°C using a gradient of 30-50% UF. For CDGE, amplicons were run for 6 hours at 150 V, using a constant gradient of 40% UF, and temperature of 60°C. For TTGE, a constant gradient of 30% was used and the temperature varied from 55 °C to 65°C in 6 h (1.7°C/h).

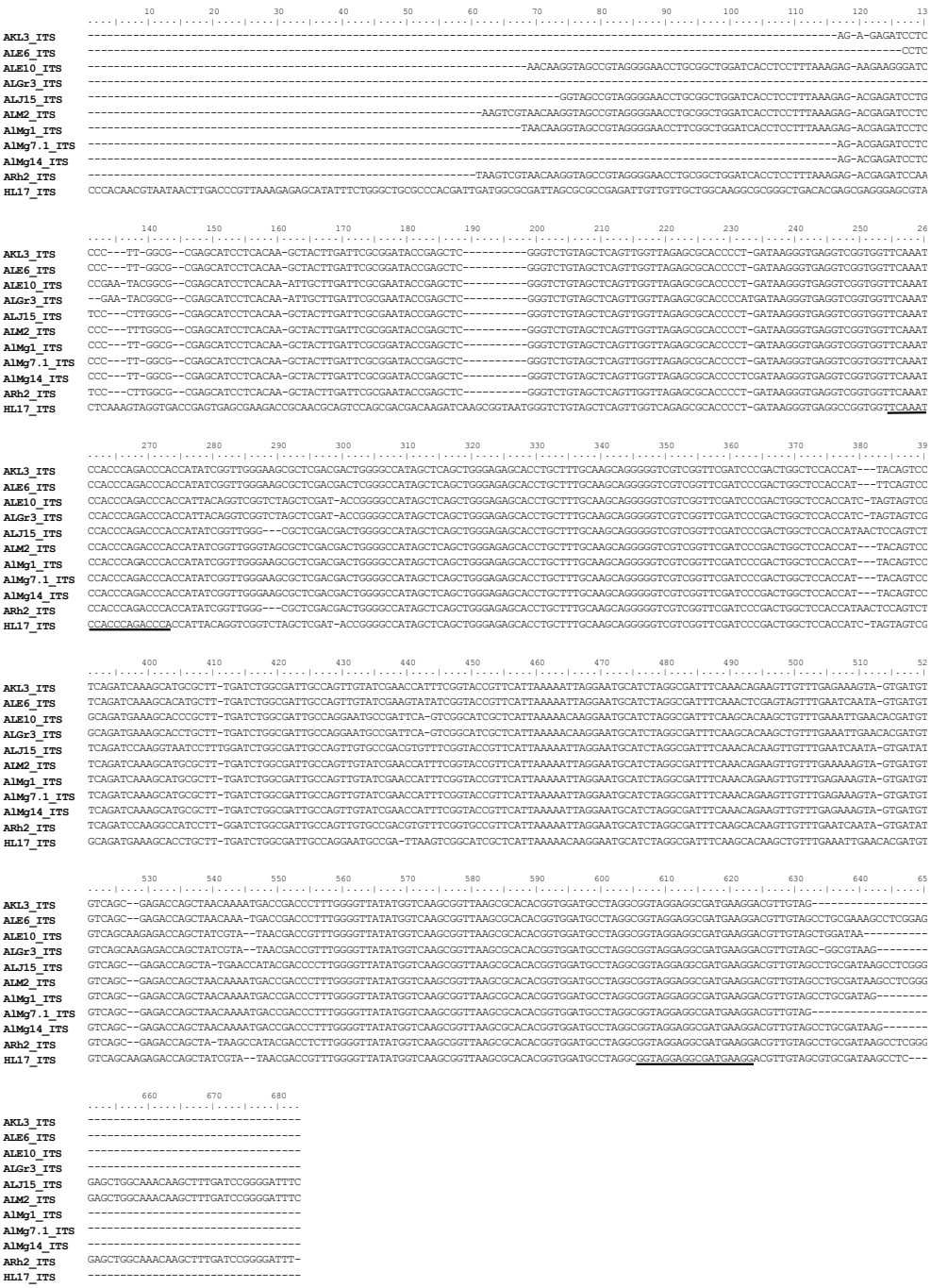

Figure 3.1: Clustal W Alignment of the ITS sequences. The selected primer sequences are underlined.

Results and Discussions

Primer design and amplification

Two sets of universal bacterial primers were tested for the amplification of the ITS fragment. Both primers gave positive results using strains ALJ15 and ALJ12. Primers pHR and p32SR01 were not selected for further analysis, as they umplified unnecessarily longer tails at the end of the 16S and at the beginnig of the 23S rRNA genes. Primers 16S-1492f and 23S-115r were then used to amplify the ITS region of the 12 *Thioalkalivibrio* strains, and positive amplification was observed with all of them (Fig. 3.2). In order to design specific primers for *Thioalkalivibrio* strains, ITS fragments have been sequenced and aligned using the software program Clustal W. In the last year much many sequences have been deposited in the BLAST database, revealing that our primers were not as specific as believed. However, beside the search in BLAST database, the developed primers were tested on both related (*Thiomicrospira crunogena*, *Thiomicrospira pelohila* and *Thioalkalimicrobium*) and non related organisms (*Bacillus* sp.) to check their specificity (Fig. 3.2). No amplification was observed with *Bacillus* sp., whereas for the related bacteria, PCR products of 790 bp, 600 bp and 590 bp, respectively, were detected. In this way, despite the unwanted positive amplification, it was already possible to discriminate between the *Thioalkalivibrio* strains and the related organism.

ITS sequences and tRNAs

All the analyzed strains possess only one ITS copy of the same size (~350 bp), with the exception of strain ALJD, which displays a longer fragment (~550 bp). Strains ALMg14, ALMg7.1, ALMg1 and AKL3 showed identical ITS sequences and strain ALGr3 and ALE 10 differ by four nucleotides, only.

Analyzing the ITS sequences we observed an agreement between the results obtain in this study and the one obtained by rep-PCR (Foti *et al*., 2006). The same genotypes were observed when a less stringency (cut-off of 70% instead of 80) is used in the rep-PCR dendogram. This shows that these two techniques are reliable for the genotyping of our strains and that rep-PCR fingerprinting technique show a higher resolution power than the ITS region.

A search of tRNAs in the *Thioalkalivibrio* ITS sequences showed the presence of two tRNA genes for each strain, i.e. $tRNA_{ALA}$ of 76 bp and $tTRNA_{ILE}$ of 77 bp. The same result was observed in a study conducted on *Roseobacter*–related bacteria, belonging to the Alpha-subdivision of the Proteobacteria (Söller *et al*., 2000). Therefore the tRNA genes cannot be considered as a specific property of the group of organisms in which the gene has been detected.

DGGE analysis

In order to determine the optimal denaturing conditions and the length of the elctrophoresis run, a perpendicular gel and a so-called ‘time travel’ experiment have been performed. The perpendicular gel (Fig. 3.3) showed a stable melting profile, as can be observed by the very sharp line. The inflexion point at about 40% denaturant corresponds to the melting behavior of the lowest melting domain.

The optimal length of the run was determined by loading a mixture of two PCR products on the time travel experiment (Fig. 3.4). A very clear separation was observed after 3 hours. However, since at 4 hours the separation was still slightly increasing, a run of 6 hour was selected for further DGGE analysis, using a denaturing gradient of 30%-50%, chosen after perpendicular DGGE.

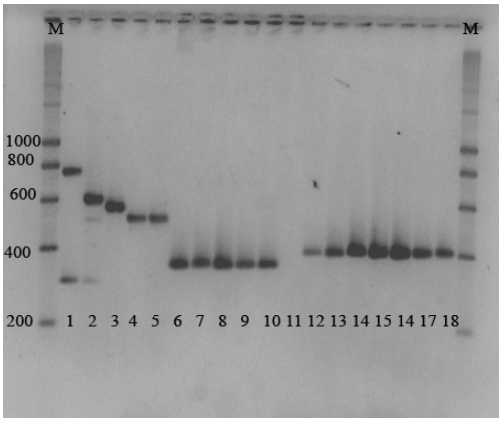

Figure 3.2: Ethidium bromide-stained agarose gel with PCR products obtained with primers specific for the *Thioalkalivibrio* ITS region of different bacteria. 1. *Thiomicrospira crunogena* 2. *Thiomicrospira pelophila* 3. *Thioalkalimicrobium* 4. *Thioalkalivibrio denitrificans* strain ALJD 5. *Thioalkalivibrio denitrificans*-like strain col1-2; 6-10: *Thioalkalivibrio* strains using primers without GC clamps; 11. *Bacillus* sp.; 12-18: *Thioalkalivibrio* strains using primers with GC clamps. M: marker

The 12 *Thioalkalivibrio* stains were then run on DGGE applying the presumed optimal conditions (denaturing gradient and time of electrophoresis run) obtained from the preceeding experiments. Despite of this, it was not possible to discriminate between the strains (Fig. 3.5). Only two bands were observed, corresponding to the so-called halo-alkaliphilic strains and the so-called natronophilic strains (see Table 3.1). In contrast to the latter, the former are chloride dependent and have been isolated from Egyptian soda lakes. A narrower denaturing gradient was also used to enhance the resolution, but no improvement was observed (results not shown).

In order to increase the discrimination between the *Thioalkalivibrio* strains, TTGE (temporal temperature gel electrophoresis) and CDGE (constant denaturant gel electrophoresis) have been performed. These two variants of the DGGE have been often used when fragments display small sequence differences, i.e. point mutation (Yoshino *et al.*, 1991; Börresen *et al.*, 1991). Advantage of CDGE is that DNA fragments melt partially into a certain configuration immediately after entering the gel. This configuration is kept during the entire run in which the fragments migrate with a constant rate. Therefore, it is easier to separate highly similar fragments (Börresen *et al.*, 1991). However, since a unique denaturing gradient is applied, this method is not suitable for the detection of DNA fragments with multiple melting domains. Our strains revealed to be highly similar and with almost identical GC content (see Table 3.1), therefore this method seemed to be appropriate for our purpose. As shown in figure 3.6, this was not the case. On the contrary, less discrimination and less sharp bands were observed. This might be due to non optimal denaturing conditions for CDGE. The perpendicular gel (Fig. 3.6) showed a very steep S-shaped curve, which might have led us to a wrong estimate of the optimal gradient of UF.

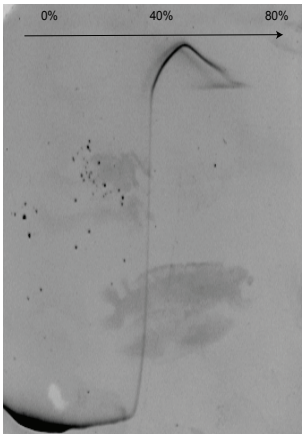

Figure 3.3: Negative image of an ethidium bromide-stained perpendiular gel.

TTGE analysis was then also performed to see if any improvement could be obtained. Here a single concentration of urea/formamide was used, as in CDGE, but the temperature was gradually increased during the run. In this way fragments with different melting behaviour can be analyzed. Unfortunately, TTGE showed only two bands, as observed for DGGE.

Concluding, the discrimination between closely related

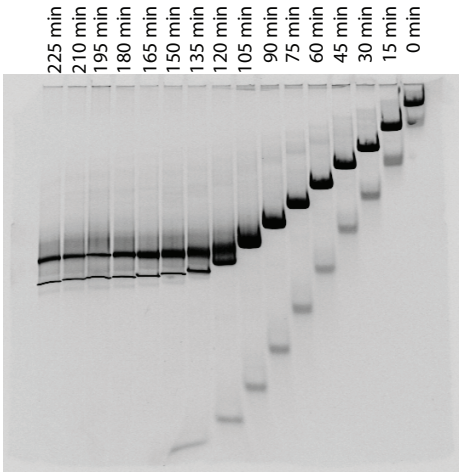

Figure 3.4: Negative image of an ethidium bromide-stained parallel DGGE separation pattern of a mixture of ITS fragments from two *Thioalkalivibrio* strains (ALE10 and ALJ15), which have been loaded every 15 min for 4 hours.

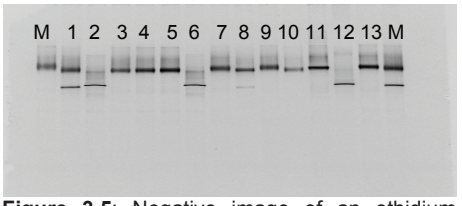

Figure 3.5: Negative image of an ethidium bromide-stained DGGE. M=marker (mixture of ALE10-ALJ15). 1. ALE6; 2. ALE10; 3. ALMg1; 4. ALMg7.1; 5. ALMg14; 6. ALGr3; 7. ALJ15; 8. ALJD; 9. ALM2; 10. AKL3; 11. ARH2; 12. HL17; 13. ALR1-1

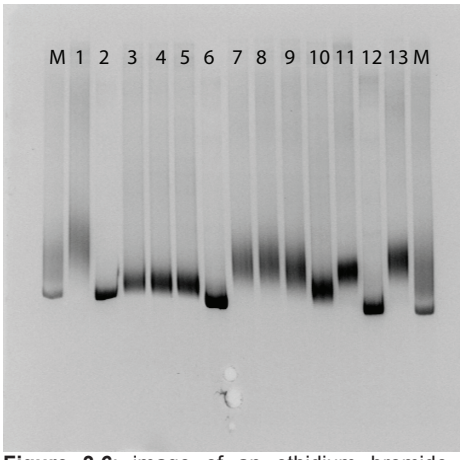

Figure 3.6: image of an ethidium bromide-stained CDGE (see Fig.3.5).

Thioalkalivibrio strains was not possible by DGGE analysis of the ITS region. Janse and coworkers (2003) showed the possibility to discriminate between closely related species of Cyanobacteria using the same approach. However , they also noticed that different organisms may end up at the same position in the gel. Although literature data show that separation of amplicons as little as with only 1 different nucleotide is sometimes possible, other examples showed that multiple sequence differences cannot be discriminated on DGGE (Jackson *et al*., 2000). This might be due to the position of the different nucleotides within the analyzed fragment or to the sharing of the same GC content, both of which can influence the amplicon's melting behaviour (Jackson *et al*., 2000; Kowalchuk *et al*., 1997). In our case it is very likely that the relatively high GC content was the reason of this co-migration effect.

To obtain insight into the diversity and dynamics of mixed populations of *Thioalkalivibrio* strains in bioreactor samples more powerful techniques with a higher resolution are needed. T-RFLP (Marsh, 1999) of the ITS region may be an alternative method. It is also a PCR-based technique in which the amplicons are digested by selected endonucleases producing terminal labelled fragments. Since a fluorescently tagged primer is used, the analysis is limited to the terminal fragments of the digestion. T-RFLP of the ITS region could overcome DGGE limits for some strains, but obviously the discrimination between identical ITS sequences would remain impossible. Therefore the discrimination within a bacterial species with such a high microdiversity remains a very difficult task.

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Chapter 4

Sulfur-Oxidizing Bacteria in Soap Lake (Washington, USA), a Meromictic, Haloalkaline Lake with an Unprecedented High Sulfide Content

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ABSTRACT

Culture-dependent and independent techniques have been used to study the diversity of chemolithoautotrophic sulfur-oxidizing bacteria in Soap Lake (Washington, USA), a meromictic, haloalkaline lake containing an unprecedented high sulfide concentration in the anoxic monimolimnion. Both approaches revealed the dominance of bacteria belonging to the genus *Thioalkalimicrobium*, which are common inhabitants of soda lakes. A dense population of *Thioalkalimicrobium* (up to 10^7 cells/ml) was found at the chemocline, which is characterized by a steep oxygen-sulfide gradient. Twelve *Thioalkalimicrobium* strains exhibiting three different phenotypes were isolated in pure culture from various locations in Soap Lake. The isolates fell into two groups according to 16S rRNA gene sequence analysis. One of the groups was closely related to *Tm. cyclicum*, which was isolated from Mono Lake (California, USA), a transiently meromictic, haloalkaline lake. The second group, consisting of four isolates, was phylogenetically and phenotypically distinct from known *Thioalkalimicrobium* species and unique to Soap Lake. It represented a new species, for which we suggest the name *Thioalkalimicrobium microaerophilum* sp. nov.

Introduction

Soap Lake, located in central Washington (USA), is the terminal lake at the end of a chain of lakes characterized by increasing salinity and alkalinity. It contains high concentrations of sodium carbonate and sulfate, resulting in a high alkalinity and pH (around 10), typical for soda lakes. However, two features distinguish this lake from many other soda lakes: (i) its sharp stratification into two layers with different features, and (ii) an unprecedented high sulfide concentration in the anaerobic layer. The bottom layer of the lake, termed the monimolimnion, is hypersaline (140 g/l), cold (6-8^{o}C), and highly sulfidic, with anaerobic waters containing up to 200 mM sulfide, the highest concentration ever recorded in a natural water. In contrast, the top layer, termed the mixolimnion, is brackish (around 15 g/l) and aerobic. It is separated from the monimolimnion by a chemocline with oxygen concentrations changing from saturation to zero (Anderson, 1958; Rice *et al*., 1988; Walker, 1975). Despite the large difference in density between the two layers, sulfide is diffusing into the aerobic layer and, therefore, the role of sulfur-oxidizing bacteria (SOB) must be particularly important in this unusual lake.

Previous work has demonstrated the presence of obligately chemolithoautotrophic SOB in soda lakes, capable of growth at extremely high pH values and a variable salinity (Sorokin and Kuenen, 2005). The genus *Thioalkalimicrobium* was found mostly in hyposaline lakes of South Siberia and North-Eastern Mongolia, and dominated the enrichments at low-salt conditions (i.e., below 1 M of total sodium). In contrast, the genus *Thioalkalivibrio* was dominant in hypersaline soda lakes, and was the only representative of chemolithoautotrophs known so far capable of growing in saturated soda brines (4 M of total sodium). Use of culture-independent molecular methods allowed the detection of *Thioalkalivibrio* in soda lake sediments, while the presence of *Thioalkalimicrobium* population was obviously below the detection limit (unpublished results).

Here we describe the diversity of SOB in Soap Lake, a haloalkaline, meromictic lake with an unusually high sulfide concentration. We used both culture-dependent and independent techniques to study the SOB present in this lake, and found evidence for members belonging to the genera *Thioalkalimicrobium* and *Thioalkalivibrio*. A group of microaerophilic isolates affiliated to *Thioalkalimicrobium* belongs to a new species, *Thioalkalimicrobium microaerophilum*.

Materials And Methods

Samples

Soap Lake is permanently stratified into two layers, an upper layer (mixolimnion) and lower layer (monimolimnion), separated by a chemocline at 20.5 m (Figure 4.1). Five samples have been obtained from Soap Lake (Washington, USA) in the fall of 2004, including samples from the mixolimnion, the chemocline and the monimolimnion, from the deep sediment, and from the mixolimnion sediment at the fringe of the lake (Table 4.1). Part of the water samples was used for molecular analysis, for sulfide analysis, and for cultivation.

Cultivation of sulfur-oxidizing bacteria

Enrichments and cultivation of haloalkaliphilic SOB were performed using a mineral medium buffered with sodium carbonate/bicarbonate mixture (0.6 and 4 M total Na^{+}) at pH 10-10.1 as described previously (Sorokin *et al*., 2001). Thiosulfate (20-40 mM) served as energy source, and NH_4Cl (4 mM) as nitrogen source. The medium was supplemented with 1 ml/l of trace metal solution (Pfenning and Lippert, 1966) and 1 mM $MgCl_2$. Microaerophilic cultivation

Table 4.1: Samples from the Soap Lake

№	Sample	Depth (m)	Salinity (g L^{-1})	O_2 (mg L^{-1})	HS^- (mM)
SL1	Mixolimnion, water	10	14	9.2	0
SL2	Mixolimnion, sediment	-	-	0*	5.04*
SL3	Chemocline, water	20	20	0.1	1.99
SL4	Monimolimnion, water	23	140	0	175
SL5	Monimolimnion, sediment	25	-	0	143.5*

* - in the pore water

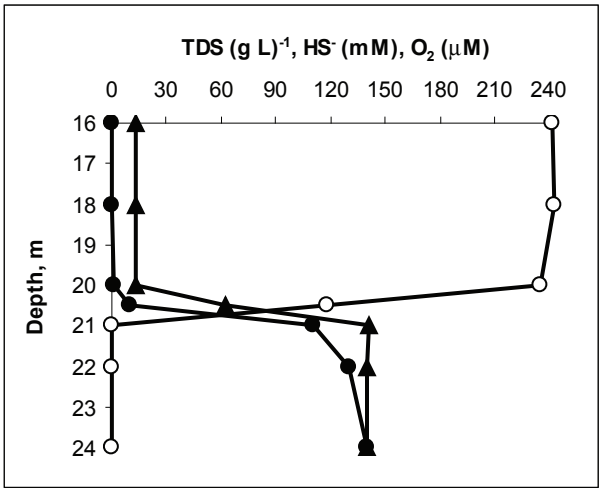

Figure 4.1: Depth profiles of dissolved oxygen (DO, open circles), total dissolved solids (TDS, filled triangles), and sulfide (filled circles).

was performed in 100 ml serum bottles with 10 ml medium in which the air was replaced with argon, containing 1-5% (vol./vol.) O_2. Solid alkaline media with final salt concentrations of 0.6 and 2 M of total Na^+ were prepared by 1:1 mixing of 4% (wt./vol.) agar and double-strength mineral medium at 50°C. The plates were incubated in closed jars in microaerophilic (2% oxygen) conditions. Enrichments for denitrifying SOB were performed in 100 ml serum bottles filled with 50 ml of the alkaline base media and sealed with butyl rubber stoppers. The medium was supplemented either with 20 mM thiosulfate/20 mM nitrate, 20 mM thiosulfate/50 mM N_2O, or with 5 mM HS^-/20 mM nitrate. Anaerobic conditions were achieved by five cycles of evacuation-flushing with argon. The cultivation was performed at 22 (enrichments) and 28 (pure cultures) °C.

Chemical and other analyses

Chemical analysis of sulfur compounds (i.e., sulfide, thiosulfate, sulfur), cell protein, and respiration tests were performed as described previously (Sorokin *et al.*, 2001). The dO_2 in-lake measurements were performed with an oxygen probe associated with a Hydrolab (Hach Environmental, Loveland CO). Phase contrast microphotographs were obtained with a Zeiss Axioplan Imaging 2 microscope (Göttingen, Germany). For electron microscopy, the cells were fixed with glutaraldehyde (final concentration 3% [vol./vol.]) and positively contrasted with 1% (wt./vol.) uranyl acetate. The isolation of the DNA from pure cultures was performed according to Marmur (Marmur, 1961). Determination of the G+C content of the DNA and DNA-DNA hybridization were performed by the thermal denaturation/reassociation technique (De Ley *et al.*, 1970).

DNA extraction and PCR amplification of 16S rRNA genes

Ten ml water samples, 2 cm^3 sediment samples, and 1 ml cultures were used for DNA extraction after centrifugation and washing the pellet with 0.5 M NaCl. Genomic DNA was extracted from the cells using the UltraClean Soil DNA Extraction Kit (Mo Bio Laboratories, West Carlsbad, CA, USA), following the manufacture's instructions.

The partial 16S rRNA gene was amplified using bacterial primers 341F-GC and 907R and a touchdown protocol (Schäfer and Muyzer, 2001). The nearly complete 16S rRNA gene was obtained from pure cultures using bacterial primers GM3F and GM4R (Muyzer *et al.*, 1995).

DGGE of PCR products

DGGE was performed as described by Schäfer and Muyzer (Schäfer and Muyzer, 2001) with minor modifications. The PCR products were separated on polyacrylamide gel with a linear gradient of 35-70% urea and formamide and run at constant voltage of 100 V for 16h. Subsequently, the gels were photographed with the Gel Doc 2000 system (BioRad, Hercules, CA, USA) after 30 min ethidium bromide staining and 30 min rising in Milli-Q water. Bands of interest were excised, reamplified, and checked for purity in a second DGGE. PCR products were then purified using the Qiaquick PCR purification kit (QIAGEN, Hilden, Germany) and sequenced.

Phylogenetic analysis

The sequences obtained in this study were first compared to sequences stored in GenBank using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST). Subsequently, the sequences were imported into the ARB software program (3), aligned, and added to a phylogenetic tree using the QUICK_ADD_TO_EXISTING_TREE tool. The alignment was further corrected by eye, and a tree was calculated using the neighbor-joining algorithm with Felsenstein correction.

Nucleotide sequence accession numbers

All sequences obtained in this work were deposited in GenBank under the accession numbers DQ900619-DQ900627.

Table 4.2: . Isolation of halotolerant alkaliphilic SOB from Soap Lake

Sample №	O_2	Maximal positive dilution	Morphotypes present in dilutions	Pure cultures
SL1	20%	3	Very small and short motile rods (morphotype A)	ASL 9
			Morphotype A	ASL 5, ASL 7
SL2	5%	7	Large motile vibrio with empty patches, (morphotype C)	ASL 8-2
SL3	5%	7	Morphotype A	ASL 4-1, ASL 4-2, ASL 4-3
			Morphotype C – dominating;	ASL 6, ASL 8-1
			Motile curved rod (morphotype B)	ASL 3
SL4	2%	5	Morphotype A	ASL 1
SL5	2%	5	Morphotype C	ASL 2

Results And Discussion

Enrichment and enumeration of haloalkaliphilic SOB

Results of a first screening demonstrated the presence of an active population of aerobic, low-salt tolerant, alkaliphilic, lithoautotrophic SOB in the different samples from Soap Lake, able to grow at 0.6 M of total Na^+ and pH 10 (Table 4.2). The most abundant populations were isolated from the chemocline samples. Smaller populations were detected in the anaerobic, high-sulfide samples from the monimolimnion. This was likely a result of sedimentation of the SOB developing in the oxygen-containing layers of the lake, since no growth of anaerobic (denitrifying) SOB were observed in the enrichments. In this sampling period, there was no evidence of a mass development of anaerobic phototrophic sulfur bacteria, commonly present below the chemocline of meromictic lakes. This was not unexpected since the chemocline does not experience any light penetration (Walker, 1975). Based on these observations, it seems likely that the aerobic chemotrophic SOB are responsible for the most of the biological oxidation of sulfide and other reduced sulfur compounds in the lake.

In contrast to the low-salt medium, enrichments in saturated soda brine at 4 M Na^+ demonstrated much smaller populations, indicating a minor role of these bacteria at the time of sampling.

Isolation and identification of pure cultures

Enrichments at low salt (0.6 M Na^+) yielded twelve pure cultures of obligate chemolithoautotrophic SOB with three different morphotypes (Table 4.2). Seven strains with morphotype A were obtained from all samples except the deep sediments. They were short motile rods. Longer, motile and slightly curved rods of morphotype B were represented by a single strain from the chemocline. Our main target for the isolation, however, was morphotype C, since the cells of this type were seen in water samples of the chemocline and, after concentrating, in samples of the monimolimnion. From four pure cultures of this type, three were long, bent motile rods at pH 10, turning to fat vibrios at pH 8-9. The isolate ASL8-1 was similar in morphology, but with significantly smaller cells.

DGGE analysis of PCR-amplified 16S rRNA gene fragments from pure cultures and samples of different layers of the Soap Lake (Fig. 4.2) demonstrated that: (i) the genetic diversity in Soap Lake was higher in the sediments than in the water column, at least during the sampling period; (ii) dominant DNA fragments from the DGGE analysis detected in the chemocline, mixolimnion and monimolimnion sediments were related to low-salt SOB isolates, while the PCR product obtained from the monimolimnion water layer was related to high salt SOB isolates.

Phylogenetic analysis of the sequences of the dominant DGGE bands from the lake samples, and from the representative SOB isolates with different morphologies isolated at low salt conditions, identified them as members of the genus *Thioalkalimicrobium* (Fig. 4.3). More specifically, the dominant band sequences from the chemocline and the monimolimnion sediments and the morphotypes A and B isolates (see Table 4.2) were closely related to *Thioalkalimicrobium cyclicum*, found previously in the chemocline water of the Mono Lake in California (10) and absent in soda lakes of Central Asia (Siberia, Mongolia) and Africa (Kenya, Egypt) (Sorokin and Kuenen, 2005). It appears that this particular species of haloalkaliphilic SOB is endemic to the Northern American continent. The morpohotype C isolates, together with the dominant sequence from the mixolimnion sediments, formed a separate cluster with a sequence difference of 3% or more with other members of the genus *Thioalkalimicrobium*. The results of DNA-DNA hybridization analysis of the low-salt SOB isolates (Table 4.3) confirmed their phylogenetic identification, demonstrating the intraspecies relation of morpohotype A and B with Tm. cyclicum, and a separate species position of the morphotype C isolates.

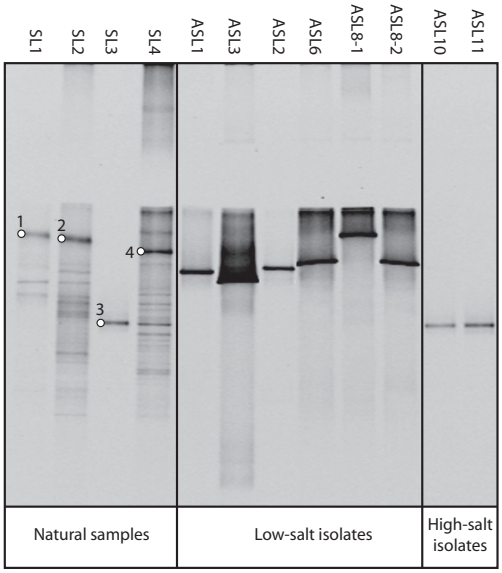

Figure 4.2: DGGE analysis of 16S rRNA gene fragments amplified from water samples (SL) and bacterial isolates (ASL) of Soap Lake. SL1, chemocline; SL2, mixolimnion, sediment pore water; SL3, monimolimnion, water; SL4, monimolimnion, sediment pore water. Sequenced bands that were used for phylogenetic analysis (see Fig. 4.2) are indicated by white circles and numbers.

High salt enrichments at 4M Na^+ resulted in the isolation of two morphologically similar strains from the chemocline and monimolimnion water samples. Both strains contained a yellow pigment with

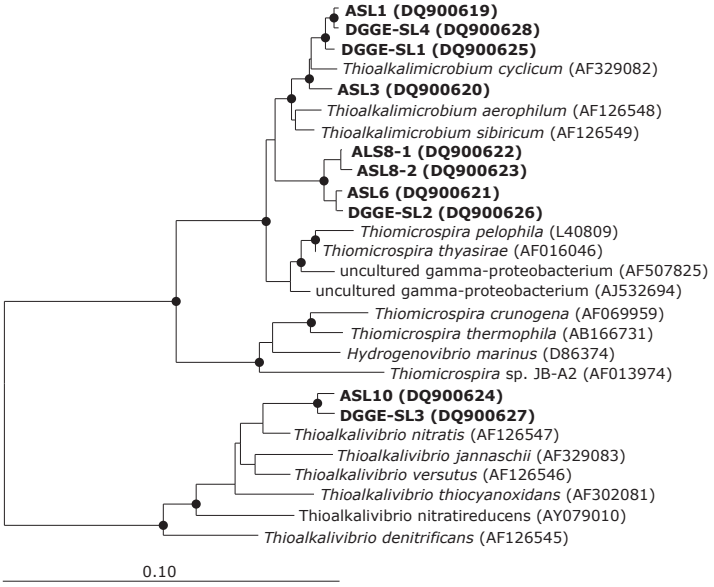

Figure 4.3: Neighbor-joining tree based on 16S rRNA gene sequences, showing the phylogenetic affiliation of bacteria from Soap Lake. Names of the sequences determined in this study are in bold. Sequences with DGGE-SL are obtained from DGGE bands as indicated in Fig. 4.1; sequences with ASL are from pure cultures. The sequence accession numbers are within parentheses. The nodes on the branches indicate bootstrap values above 90% from 1000 replications. The bar indicates 10% sequence variation.

an absorption spectrum typical for the high-salt tolerant representatives of the genus *Thioalkalivibrio* (Sorokin and Kuenen, 2005). The PCR products obtained from these strains had identical migration distance as the dominant band obtained from the monimolimnion water sample (Fig. 4.3, band No. 3). A band at the same position in the gel, although not so intense, was also visible in the profile obtained from the monimolimnion sediment sample (SL4).

Both phylogenetic (Fig. 4.2) and total DNA (Table 4.3) analysis of the two high-salt SOB isolates confirmed their affiliation with the genus *Thioalkalivibrio* and in particular to the core group of this genus, most of which are extremely salt tolerant (Sorokin and Kuenen, 2005). It is interesting to note, however, that there was very low DNA homology between the Soap Lake *Thioalkalivibrio* isolates and *T. jannaschii*, isolated from the Mono Lake (Sorokin *et al.*, 2002).

Some of the other bands from the DGGE profiles of the environmental samples have also been successfully identified. Since general diversity was not a subject of this work, we can only briefly mentioned that most of the sequences belonged to the *Clostridiales*, but with relatively low homology (usually less than 90%) to sequences deposited in the GenBank.

Influence of environmental parameters on growth and activity of the *Thioalkalimicrobium* isolates

Strains ASL1 and ASL8-2, representatives of the two different morphotypes and the genetic groups of the low-salt SOB from the Soap Lake, responded differently to increasing pH. While ASL1 had a pH profile for growth and respiratory activity typical for obligately alkaliphilic *Thioalkalimicrobium* species, with an optimum at 9.5-10 (data not shown), strain ASL8-2 grew and respired best at pH 8.5-9.0. However, no growth occurred at neutral pH values in either strain, similar to the known obligate alkaliphilic *Thioalkalimicrobium* species (Table 4.4). Therefore, strain ASL8-2 can be qualified as a moderate, but obligate, alkaliphile. The respiratory profile of strain ASL8-2 was typical for the representatives of the genus *Thioalkalimicrobium* (Sorokin *et al.*, 2003; Sorokin *et al.*, 2001), with extremely high oxidation rates for thiosulfate

Table 4.3: Total DNA analysis of the haloalkaliphilic SOB isolates from the Soap Lake

Strain	G+C content, mol%	% DNA-DNA homology, %*			
		ASL 1	ASL 3	ASL 8-2	ASL 10
ASL 1	50.0	100	75	23	
ASL 3	49.9	75	100	35	
ASL 2	49.3	30	21	85	
ASL 6	49.8	32	26	72	
ASL 8-2	49.5	23	34	100	
ASL 8-1	49.5	nd	nd	95	
Tm. cyclicum ALM 1	49.6	65	58	32	
Tm. aerophilum AL 3	49.5	43	50	48	
ASL 10	65.5				100
Tv. versutus AL 2	63.7				80
Tv. jannaschii ALM 2	63.7				32

* Average from two experiments, deviation is ± ca. 10%.

and sulfide (1.6 and 2.4 μmol O_2 mg protein^{-1} min^{-1} at pH 10, respectively) and no activity with tetrathionate, elemental sulfur and sulfite. In respect to its salt tolerance, both strains were low-salt tolerant, with a salt range for growth (at pH 9) from 0.2 to 1.2 M total Na^+ (optimum at 0.4-0.5 M). In contrast to ASL1, ASL8-2 (and all other isolates of this type) was incapable of initiating growth at fully aerobic conditions. The best way to grow these bacteria was to start a culture in closed bottles with a gas phase containing 2% O_2. After half of the thiosulfate was consumed, and the biomass level exceeded 30-40 mg protein l^{-1}, oxygen content in the gas phase could be increased up to normal atmospheric concentrations. However, active aeration on the rotary shaker inhibited the growth and eventually caused cell lysis.

Despite extremely high sulfide concentrations in anaerobic parts of the lake, the relevance of extraordinary sulfide tolerance seems to be not of utmost importance for the obligately aerobic SOB, such as *Thioalkalimicrobium* population, dominating in Soap Lake, in contrast to the obvious necessity of such an adaptation among anaerobic inhabitants of the lake. The aerobic SOB populations develop and are active only in the interface, i.e. within a sulfide-oxygen gradient, where sulfide concentration is low. Furthermore, our experiments with soda solutions at pH 10 demonstrated that sulfide at concentrations above 1 mM reacts with oxygen at concentrations above 1 mM. Therefore, the role of SOB in sulfide oxidation in the lake is important at micromolar concentrations. Two other factors are certainly important at so high a sulfide content: (a) the ability to retain activity at short-term exposure to high sulfide, in case of temporary exposure to anaerobic high-sulfide waters, and (b) the extremely high sulfide-oxidizing capacity, which allows this particular group of haloalkaliphilic SOB to cope

with high upward flux of sulfide. In a short-term respiratory experiment at pH 10, strain ASL8-2 showed only slightly higher tolerance to sulfide as compared to the type strain *Thioalkalimicrobium aerophilum* AL3, isolated from a a low-sulfide soda lake (50 and 70% inhibition of sulfide respiration at 2 mM sulfide, respectively). In contrast, tolerance to sulfide of anaerobic haloalkaliphiles can be extraordinary high. For example, strains of fermentative *Amphibacillus* sp., isolated from soda soils can grow at pH 10 in the presence of up 100 mM sulfide (our unpublished data).

Soap Lake harbors relatively dense populations of obligately chemolithioautotrophic haloalkaliphilic SOB which, similar to other soda lakes are represented by the genera *Thioalkalimicrobium* and *Thioalkalivibrio*. The former is clearly dominating the low salinity, aerobic parts of the mixolimnion and seems to play an important role in the oxidation of sulfide diffusing across the chemocline from the extremely concentrated anaerobic brines of the monimolimnion. This is not surprising, owing to the remarkably high sulfide-oxidizing capacity typical for the haloalkaliphilic SOB of the genus *Thioalkalimicrobium* (Sorokin and Kuenen, 2005). The environmental importance of this particular group is confirmed by its detection as a dominant in bacterial population of three out of four analyzed environmental samples from the Soap Lake using PCR-DGGE.

Table 4.4: Influence of pH on growth and respiration of strain ASL 8-2.

Buffering system (total Na^+ is 0.6 M)	pH start	pH final (for growth)	% of maximum*	
			Growth rate	Rate of thiosulfate-dependent respiration
HEPES/NaCl	6.1		nd	20
	7.0			57
	8.0			75
CO_2/$NaHCO_3$/NaCl	6.8	6.85	0	nd
	7.3	7.33	0	nd
$NaHCO_3$/NaCl	8.0	7.5	60	70
$NaHCO_3$/Na_2CO_3/NaCl	8.5	8.3	100	90
	9.07	8.90	90	100
	9.4	9.31	30	92
	10.0	9.75	10	80
	10.3		0	38
	10.5		0	10

* Maximum growth rate was 0.32 h^{-1} and maximum respiration rate was 1.6 μmol O_2 mg $protein^{-1}$ min^{-1}

One of the genotypes of the *Thioalkalimicrobium* (represented by four isolates) detected in the Soap Lake was not detected previously in any other soda lakes. In general, this group has much in common with the other *Thioalkalimicrobium* species. Particularly, it could not grow without reduced sulfur compounds on purely organic medium (acetate, acetate and yeast extract) and could not utilize organic carbon instead of bicarbonate. Only reduced sulfur compounds, such as sulfide and thiosulfate, but not H_2 or formate, could be used as electron donors. On the other hand the group clearly differed from the known species in morphology, phylogeny and some physiological properties, and therefore is proposed to form a new species, *Thioalkalimicrobium microaerophilum*.

Description of *Thioalkalimicrobium microaerophilum* sp. nov.

Thioalkalimicrobium microaerophilum (mic.ro.ae.ro.phi'lum Gr. adj. micros small, little; Gr. masc. noun aër gas; Gr. adj. philum loving; M.L. n. microaerophilum loving low-oxygen conditions). The cell morphology varies from thick vibrios, 1-2 x 2-5 μm, at optimal pH values (pH 8-9) to long rods, 0.6-0.8 x 3-8 μm, which are motile by means of a single polar flagellum (Fig. 4.3c). It is obligately chemolithoautotrophic and microaerophilic. It grows best in soda-buffered culture medium at pH 8.5 - 9. Thiosulfate or sulfide are used as as electron donors. It grows within a pH range from 8 to 10, and at a salt concentration equivalent to 0.2 - 1.2 M of total Na^+. The temperature optimum for growth at pH 9 and 0.6 M Na^+ is between 25 and 28 ^oC. It oxidizes sulfide and thiosulfate to sulfate at high rates within a pH range of 6 - 10.5, with an optimum at 9 - 9.5. The G + C content in the DNA is 49.3-49.8 mol% (T_m). Other properties are similar to those described for the genus. It was isolated from the saline, alkaline Soap Lake (Grant Co.,Washington State, USA). It is represented by four strains with DNA-DNA homology level of more than 70%. The type strain is ASL8-2T (=DSM 17327=UNIQEM U242). The accession number of the 16S rRNA gene sequence is DQ900623.

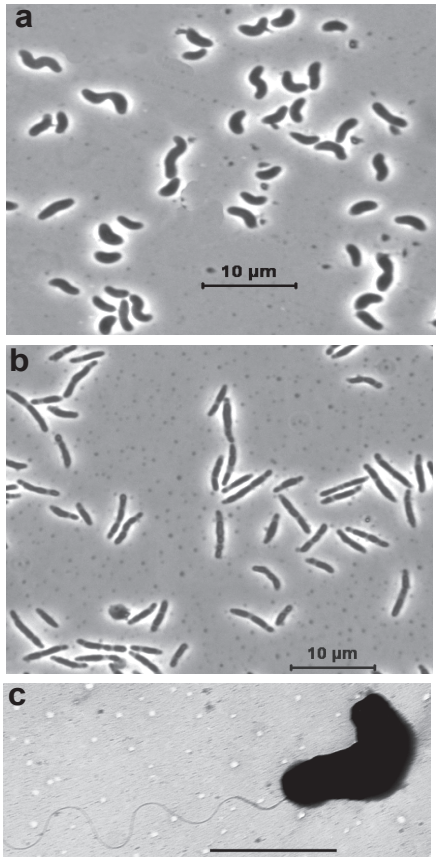

Figure 4.4: Cell morphology of *Thioalkalimicrobium microaerophilum* sp. nov. strain ASL 8-2T. Phase contrast photographs of the cells grown at pH 8.5 (a) and at pH 10.0 (b); c, electron microscopic photograph of the cell, grown at pH 8.5, scale bar is 1 μm.

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Chapter 5

Diversity, Activity and Abundance of Sulfate Reducing Bacteria in Saline and Hypersaline Soda Lakes

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ABSTRACT

Soda lakes are naturally occurring highly alkaline and saline environments. Although the sulfur cycle is one of the most active element cycles in these lakes, little is known about the sulfate reducing bacteria (SRB). In this study we investigated the diversity, activity, and abundance of SRB in sediment samples and enrichment cultures from a range of (hyper)saline soda lakes of the Kulunda Steppe in south-east Siberia (Russia). For this purpose a polyphasic approach was used, including denaturing gradient gel electrophoresis (DGGE) of *dsr* gene fragments, sulfate reduction rate (SRR) measurements, serial dilutions, and quantitative real-time PCR (qPCR). Comparative sequence analysis revealed the presence of several novel clusters of SRB, mostly affiliated to the *Desulfovibrionales* and *Desulfobacteraceae*. We detected sulfate reducers and observed substantial sulfate reducing rates (between 12 and 423 $\mu mol/dm^3\ d^{-1}$) for most lakes, even at a salinity of 475 g/l. Enrichments were obtained at salt saturating conditions (4M Na^+), using H_2 or volatile fatty acids (VFA) as electron donors and an extremely halophilic SRB, strain ASO3-1, was isolated. Furthermore, a high *dsr* gene copy number of 10^8 cells per ml was detected in a hypersaline lake by qPCR. Our results indicate the presence of diverse and active SRB communities in these extreme ecosystems.

Introduction

Soda lakes are extreme environments with pH values up to 11 and salt concentrations up to saturation. These high pH values are maintained due to the high buffering capacity of sodium carbonate/bicarbonate, which are among the major anions in solution. The salinity can vary from 5 - 30 g/l for hyposaline lakes, to up to 500 g/l for hypersaline lakes. Despite these extreme conditions, soda lakes are highly productive and harbor diverse microbial communities (Duckworth et al., 1996; Jones et al., 1998;Scholten et al., 2005; Zavarzin et al., 1999), responsible for the element cycling.

The sulfur cycle, driven by halo-alkaliphilic sulfur-oxidizing (SOB) and sulfate reducing (SRB) bacteria, is one of the most active element cycles in soda lakes. SOB are represented by members of the Gammaproteobacteria belonging to the genera *Ectothiorodospira*, *Thioalkalivibrio*, *Thioalkalimicrobium* and *Thioalkalispira*. Bacteria belonging to the first genus are phototrophic sulfur purple bacteria, while the other three genera are obligate chemolithoautotrophs (Sorokin et al., 2005), utilizing various reduced inorganic sulfur compounds as electron donors. Especially members of the *Ectothiorodospira* and *Thioalkalivibrio* are remarkable in their potential to grow in saturated alkaline brines.

Although more than 100 SOB strains have been isolated from soda lakes (Sorokin et al., 2005), so far only 4 species of SRB, i.e., *Desulfonatronovibrio hydrogenovorans* (Zhilina et al., 1997), *Desulfonatronum lacustre* (Pikuta et al., 1998), *Desulfonatronum thiodismutans* (Pikuta et al., 2003), and *Desulfonatronum cooperativum* (Zhilina et al., 2005) have been isolated, which are all low-salt tolerant alkaliphiles. However, although *Desulfonatronovibrio hydrogenovorans* is a low-salt organism, it was isolated from Lake Magadi, a hypersaline soda lake in Kenya (Africa). According to Oren (Oren, 1999), the upper limit of salt concentration for sulfate reducers appears to be 250 g/l for incomplete oxidizers, and ca. 130 g/l for complete oxidizers, which might be due to the high energy requirements for the synthesis of compatible solutes. So far, the incomplete oxidizers are mainly represented by members of the groups *Desulfovibrionales* and *Desulfobulbaceae*, while the complete oxidizers are mainly represented by SRB belonging to the family *Desulfobacteraceae*. Interestingly, recent in situ measurements (Sorokin et al., 2004) demonstrated sulfate reducing activity in Siberian soda lakes with saturating salinity, indicating the presence of extremely salt-tolerant alkaliphilic SRB species with possible new bioenergetic pathways. The importance of halo-alkaliphilic SRB for element cycling in soda lakes can be illustrated by the fact that anaerobic cellulose degradation in soda lake sediments is only possible in the presence of sulfate (Grant et al., 2004).

Apart from the isolation of pure cultures, culture-independent methods have been used to study SRB community structure in soda lakes. For instance, Scholten and coworkers (Scholten et al., 2005) studied sulfate reducing bacterial communities from Mono Lake, a meromictic moderate saline soda lake in California (USA) by targeting 16S rRNA, apsA and *dsr*AB genes.

Here we describe, for the first time, the diversity, activity and abundance of sulfate reducing bacteria in sediment samples from saline and hypersaline soda lakes located in the Kulunda Steppe, south-east Siberia (Russia). Denaturing gradient gel electrophoresis (DGGE) of *dsr* gene fragments (Dar et al., 2006;Geets et al., 2006) was used to investigate the diversity of SRB in sediment samples and enrichment cultures, while serial dilutions and quantitative real-time PCR (qPCR, Zhang and Fang, 2006) were used to estimate the abundance of SRB. The activity of SRB was investigated by measuring sulfate reduction rates (SRR).

Our results showed the presence and activity of several novel lineages of SRB in the

Table 5.1: . Chemical parameters of soda lakes from the Kulunda Steppe, south-west Siberia, Russia.measured in 2005

Nr Lake	Name	Salinity g/l	pH	Alkalinity (M) CO_3^{2-}	Alkalinity (M) HCO_3^-	SRR µmol/dm^3 d^{-1}	SRR Horizon, cm	SO_4^{2-} (mM)
1KL	Tanatar III	93	10.08	0.84	0.18	ND[b]	ND	ND
2KL	Tanatar II	250	9.78	2.06	0.77	ND	ND	ND
3KL	Tanatar I (east)	475 (520)[a]	10.65 (11.05)[a]	5.0 (4.0)[a]	0	12.7 (100)[b] 113 (93) 44 (78) 12.6 (27)	w[a] 0-3 3-6 6-10	6.7-8.5
4KL	Tanatar I (south)	350	10.0	5.0	0.2	ND	ND	ND
5KL	Tanatar V	50	10.1	0.42	0.12	ND	ND	ND
6KL	Tanatar VI	55	10.1	0.52	0.06	ND	ND	ND
9KL	Elongated Lake	320	9.15	0.32	0.14	ND	ND	ND
10KL	Top Karagay	58	9.7	0.14	0.1	ND	ND	ND
11KL	Anonimous	55	9.76	0.32	0.16	ND	ND	ND
16KL	Stamp Lake	380	9.27	0.38	0.18	ND	ND	ND
18KL	Cock Lake	59 (74)[a]	10.1 (10.13)[a]	0.66 (0.31)[a]	0.14 (0.13)[a]	61 (13) 326 (14) 3.7 (3)	w[a] 0-4 4-12	6.7-8.5
10KL-05	Karacul	30	9.76	0.05	0.04	ND	ND	ND
18KL-05	Picturesque	405	9.95	1.3	0.7	2.8 (0) 47 (43) 423 (0) 12 (0)	w[a] 0-2 2-6 6-18	278-584
22KL-05	Anonimous 2	360	9.46	0.7	0.7	ND	ND	ND
23KL-05	Narrow Lake	110	9.61	0.23	0.26	1.1 (63) 62 (22) 162 (0) 52 (5)	w[a] 0-2 2-17 17-32	235-278
25KL-05	Bitter Lake- 1	120	10.3	0.65	0.49	2.6 (0) 14.7 (19) 2.6 (11) 6 (10)	w[a] 0-1 1-9 9-20	0.6-2.5

soda lakes, mostly affiliated to the *Desulfovibrionales* and *Desulfobacteraceae*. Especially the presence of members of the last group is unexpected, as it was hypothesized that complete oxiders can not grow at salt concentrations greater than 130 g/l (Oren, 1999). In this paper we provide novel insight into the diversity of SRB in soda lakes, which is essential for a comprehensive understanding of the sulfur cycle in these extreme ecosystems.

Materials And Methods

Site description

The Kulunda Steppe stretches from the south-east part of the Novosibirsk region to the south-west part of the Altai region, and along the north–eastern border of Kazah. It harbors numerous saline lakes that differ in size and chemistry. Small and shallow lakes in the southern part of the Kulunda Steppe are characterized by high pH values, high-to-extremely high carbonate alkalinity, and a total salt content from 50 to 500 g/l. The characteristics of the lakes from which sediment samples were obtained are presented in Table 5.1.

Chemical analyses and sampling

Field measurements included pH, conductivity, and carbonate alkalinity (titration with 1 M HCl using phenolphthalein and methyl orange as indicators). The salt content, inferred from conductivity measurements, was verified by gravimetry in the laboratory. For DNA extraction, the top 10 cm sediment was sampled from 3 different places along the littoral and combined in one 50 ml Falcon tube. The tubes were stored at 4°C during transportation to the laboratory, and stored at -20°C until further analysis. For the rate measurements, 20 cm cores were taken and then divided in different layers, based on colour. Sulfate-reduction rates (SRR) were measured in 5 ml syringes capped with butyl rubber stoppers using the 35^{S}-SO_4^{2-} methodology (Lein et al., 2002). After 1 day of incubation at ambient temperature, the sediments were fixed with 10 M KOH and further processed in the laboratory. Additional analysis of total sulfate content in the sediments pore water was performed in the laboratory after centrifugation of 2 cm^3 sample using ion-exchange chromatography (Pimenov and Boch-Osmoloyskaya, 2006). Samples for PCR-DGGE, qPCR and enrichments were taken in the summer of 2003, while sulfate reduction rate measurements and serial dilutions were performed in the summer of 2005. General field measurements were performed during both visit to the sites.

Serial dilutions

Enumeration of SRB was performed in duplicate by decimal serial dilutions in Hungate tubes with the top 5 cm sediment layer as inoculum. Dilutions were done in low-salt (0.6 M total Na^+) and high-salt (4 M total Na^+) carbonate media, pH 10 (Sorokin and Kuenen, 2005) with H_2 or lactate and butyrate as an energy source. The inoculation was done in the field. Growth was monitored within 4-8 weeks incubation at room temperature by estimating sulfide production (Trüper and Schlegel, 1964).

Enrichment of haloalkaliphilic SRB

Enrichments of SRB were obtained by inoculating 30 ml vials filled with 10 ml of carbonate media (1, 2 or 4 M total Na^+, N_2 atmosphere) and 1 ml sediment slurry. Medium composition was similar as described by Sorokin (Sorokin and Kuenen, 2005) with minor modifications. Ammonium chloride (5 mM) was used as a nitrogen source, and trace elements (31), vitamins and a mixture of sodium sulfide (1.2 mM) and sodium dithionite (0.5 mM) were added

as reducing agents to the media (17). The slurries for inoculation were prepared by mixing sediment samples from 3 lakes (i.e., 1KL, 6KL and 18KL, see Table 1) with an equal volume of the appropriate (1, 2 or 4 M) carbonate medium. N_2/H_2 (50/50, v/v), ethanol (30 mM) or a mixture of volatile fatty acids (VFA; formate [30 mM], lactate [30 mM] and acetate [30 mM]) were added as electron donors. Sulfate (30 mM) was added as electron acceptor. Bottles were incubated at 37°C in the dark. After several transfers, dilution series of some of the enrichments were prepared as a first step to obtain pure cultures. Growth of SRB was monitored by determining the concentrations of electron donor and acceptor over time (Scholten et al., 2005).

Special enrichments were made to look at the presence of SRB capable of sulfite fermentation. For this, anaerobic sodium carbonate medium containing 0.6, 2 and 4 M total Na^+, pH 10, was supplemented with 1-2 mM sulfide as reductant, and with 5-10 mM sodium sulfite as the only energy source. The culture was inoculated with 10% (v/v) of sediment sample composed of 10 individual samples from Kulunda soda lakes. The development was monitored by microscopy, increase in sulfide and decrease in sulfite concentration.

DNA extraction

Prior to DNA extraction, the sediments were washed three times with 1 M NaCl and 10 mM Tris (pH 7.5) to lower the pH and the salt concentration. The genomic DNA from 11 sediment samples was extracted using the UltraClean Soil DNA Extraction Kit (MoBio Laboratories, USA), following the manufacturer's instructions with minor modification. Briefly, 200 µl of 0.1 M $AlNH_4(SO_4)_2$ was added to the sediment to remove potential PCR inhibitors, such as humic acids (Braid et al., 2003). Subsequently, the cells were lysed by a combination of detergents and mechanical disruption. The released DNA was bound to a silica spin-filter. The filter was washed, and the DNA was recovered in Milli-Q water. The quality of the extracted DNA was examined on 1% (w/v) agarose gels in 1x TBE-buffer (90 mM Tris-Borate; 2 mM EDTA, pH 8) after staining with ethidium bromide. Images were obtained using the Gel Doc 2000 system (BioRad, Hercules, CA, USA).

PCR of *dsr* gene fragments

In order to perform denaturing gradient gel electrophoresis (DGGE) based on *dsr*B gene, a nested PCR approach was used to increase the sensitivity of the amplification. First the entire *dsr*AB gene of ca. 1900 bp was amplified. Amplification of the extracted DNA was performed in a 25 µl final volume with 12.5 µl Master Mix Qiagen (Qiagen, Hilden, Germany), $MgCl_2$ (1.75 mM final concentration) and 0.5 µl (0.4 µM final concentration) of primer Dsr1F (5`- ACSCACTGGAAGCACG -3`) and Dsr4R (5'- GTGTAGCAGTTACCGCA -3`) (Wagner et al., 1998). The following PCR conditions were used: 5 min at 94°C, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 90 s, and a final extension at 72°C for 10 min. Subsequently, a 350 bp fragment part of the *dsr*B gene was amplified using primers Dsrp2060F (5`- GC clamp - CAACATCGTYCAYACCCAGGG-3`) (Geets et al., 2006) and Dsr4R. Two PCR reactions were performed per each sample in a final volume of 25 µl, with 12.5 µl Master Mix Qiagen (Qiagen, Hilden, Germany), $MgCl_2$ (1.75 mM final concentration) and 1 µl of each primer (0.2 µM final concentration). The reaction was carried out using a so-called 'touchdown' protocol: 5 min at 95°C, followed by 20 cycles of 40 sec at 95°C, a decreasing of the annealing temperature from 65°C to 55°C, 1 min at 72°C. In addition, another 20 cycles of 40 sec at 94°C, 40 sec at 55°C and 1 min at 72°C were performed, followed by a final extension at 72°C for 10 min. The yield and quality of the PCR products were examined on 1% (w/v)

agarose gel stained with ethidium bromide.

DGGE of *dsr*B gene fragments. The *dsr*B-amplicons were analyzed by DGGE as described by Dar *et al.* (Dar et al., 2006) using a gradient of 30 to 65% urea-formamide (UF) in 6% polyacrylamide gel. The electrophoresis was performed in 1X TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8) at 60°C for 5 hours at a constant voltage of 150 V. After electrophoresis, the gels were stained with ethidium bromide and destained in Milli-Q water. Images were obtained using the GelDoc 2000 system (BioRad, Hercules, CA, USA). Bands of interest were excised, re-amplified and run again on DGGE to check their purity. PCR products for sequencing were reamplified using primers without GC-clamp, run on a 1.5% (w/v) agarose gel and purified with the Qiaquick gel extraction kit (Qiagen, Hilden, Germany). The ~350 bp purified PCR products were then sent to a commercial company (BaseClear, Leiden, The Netherlands) for sequencing.

Comparative sequence analysis

Partial *dsr*B sequences were compared with sequences in the GenBank database using the BLAST search tool for the identification of closely related sequences. Nucleic acid sequences were aligned with the most similar sequences using the ClustalW and BioEdit Sequence Alignment Editor software, and subsequently translated into amino acids. Deduced aligned amino acid sequences were then imported into the ARB software package (Ludwig et al., 2004) for phylogenetic analysis.

A consensus tree was calculated from nearly complete sequences using Neighbour Joining, Maximum Parsimony and Maximum Likelihood algorithms and different filters. Subsequently, the partial sequences were added to the tree using the QUICK_ADD parsimony tool and individually removed to avoid long branch attraction. Deletions and insertions were not included in the calculation. In addition, only the positions encoded on the added sequences were considered for the phylogenetic reconstruction. Finally a consensus tree was generated.

Quantitative real time PCR

Quantification of the *dsr*B copy number in the extracted DNA was performed using the I-Cycler (BioRad Laboratories, Veenendaal, The Netherlands). SybrGreen (BioRad Laboratories, Veenendaal, The Netherlands) was used as a dsDNA binding dye, whereby the fluorescence intensity of the dye increases with the amount of amplified dsDNA. Baseline and thresh-hold calculations were performed with the I-Cycler software (version 4).

Amplification was done with SybrGreen Super Mix (BioRad Laboratories, Veenendaal, The Netherlands) and the same set of primers used for the *dsr*B gene at a final concentration of 0.2 µM. The amplification consists of 35 cycles with 1 cycle consisting of denaturation (40 sec at 95°C), annealing (40 sec at 55°C), and elongation (1 min at 72°C).

The standard curve was calculated based on a partial *dsr*B purified PCR product of known copy number. Using the standard curve, we determined the *dsr* copy number per ml sediment. The qPCR measurements were done in triplicate. The optimal DNA dilution was first tested and then used for the final qPCR. The amplification efficiency was calculated from the slope of the standard curve using the formula: % E= (10^{-1}/slope) X 100. Since SybrGreen might also bind to non-specific dsDNA, a melting curve was performed to assure the specificity of the PCR.

Nucleotide sequence accession numbers

The sequences determined in this study were deposited in GenBank under the following numbers: EF055361 – EF055374 for the sediment samples, and EF055375- EF055384 for the enrichment samples.

Results

Sulfate reduction rates in soda lake sediments

SRR were measured in sediments of five lakes with increasing salinity. Sulfate was present at very high concentrations, up to 584 mM, in sediment pore waters of lakes with a salinity of more than 150 g/l, while in less saline lakes, such as Cock Lake and Bitter-1 Lake, it was present at lower concentrations, 8.5 and 2.5 mM respectively (Table 5.1). The maximal sulfate reduction rates were measured for lakes Picturesque and Cock, 423 and 326 µmol / dm^3d^{-1} respectively. Up to 35% of the 35^S- label from sulfate was found in the acid-resistant fraction (i.e., sulfur plus pyrite). Substantial SRR (44 µmol/dm^3d^{-1}) were even found for Lake Tanatar I, which has an extremely high salinity of 475 g/l.

Table 5.2: Enumeration of SRB in sediments of Kulunda soda lakes

Lake	Depth cm	Total [Na^+]	Heterotrophic SRB[a]	Autotrophic SRB[b]
10KL-05	0-5	0.6 M	10^5	10^5
	6-15		10^5	10^4
	16-25		10^2	10^2
25KL-05	0-1		10^6	10^4
	1-9		nd[c]	10^3
18KL	0-4		10^6	nd
	4-12		10^5	nd
18KL-05	0-2	4 M	10^3	10^4
	2-6		10^2	10^2
	6-18		10^2	nd
22KL-05	0-10		10^5	10^5
23KL-05	0-2		10^6	nd

[a] Grown on a mixture of lactate and butyrate.

[b] Grown on a mixture of H_2 and CO_2.

[c] nd, not determined

Serial dilutions and enrichments

Samples from the same sediments were also used for enumeration and enrichments. Enumeration of culturable halo-alkaliphilic SRB was done by serial dilutions using 0.6 and 4 M sodium carbonate-based medium, pH 10, with either H_2 or VFA (lactate and butyrate) as elec-

tron donor (Table 5.2). The results demonstrated the presence of relatively dense populations (up to 10^6 cells per ml sediment) of halo-alkaliphilic SRB in the sediments of the investigated lakes (Table 5.2). Further attempts to grow SRB from primary dilutions were only successful for some of the samples, especially those grown in 4 M sodium cabonate-based medium. The dominant morphotype in all these enrichments was a motile, vibrio-shaped cell.

Incubation in a mineral medium of a mixture of three sediments from lakes of the Kulunda region revealed that sulfate reduction activity was found on all of substrates tested, i.e., H_2, a mixture of VFA's (formate, lactate, acetate) and ethanol. Sulfate-reducing activity was found up to 4 M of sodium for H_2 and ethanol and was highest at 2 M of sodium for all substrates tested. In all enrichments vibrio-shaped cells were dominant.

Enrichments for sulfite fermentation gave a positive enrichment culture with 2 M Na^+ and 10 mM sulfite. From this enrichment a pure culture, strain ASO3-1, was isolated using the dilution to extinction method.

Diversity analysis using DGGE of *dsr*B-gene fragments

The SRB diversity in the sediment samples and enrichment cultures was analyzed by DGGE using the *dsr*B subunit as a molecular marker (Geets et al., 2006). For the sediment samples, as many as 7 distinct bands could be observed (Fig. 5.1, lane 7). Samples 2KL, 3KL and 4KL show patterns similar to each other (Fig. 5.1, lane 2-4). The other samples showed different profiles, indicating a moderate diversity of sulfate reducing bacteria in this habitat.

Regarding the enrichments, the same sediment samples analyzed in this study were used as inoculum. Eleven out of 21 enrichments (results not shown) revealed an identical DGGE profile with a single dominant band, whose sequence (i.e., E_H_2_1_E) [sequence names explained in the legend to Fig.5.2]) corresponds to a novel cluster affiliated to *Desulfonatronovibrio hydrogenovorans* (Fig. 5.2). Sequence E_H_2_1_E was present in all enrichments except for two, in which VFA were used as electron donor at a salinity of 2 M.

Identification of SRB

Altogether 24 representative *dsr*B fragments were sequenced from sediment samples as well as enrichment cultures. Comparative sequence analysis was conducted on both nucleotide and deduced amino acids translated sequences. The resulting topology from DNA (not shown) and protein (Fig. 5.2) sequences was largely congruent, differing only in the exact placement of individual sequences within low branching clusters. All of our sequences fell in the delta-subclass of the Proteobacteria, with a majority in the *Desulfovibrionales* (11 sequences) and the *Desulfobacteriaceae* (11 sequences) (Fig. 5.2). One sequence (KL1_1) fell within the *Desulfobulbaceae*, and showed a similarity of 93% at the protein level with the *dsr* sequence of the glycolate-oxidizing SRB *Desulfofustis glycolicus*. Another sequence (KL10_13), from the low salt lake 10KL, clustered together with sequences of *Desulfobacter anilinii* and the sulfate reducing strain mXyS1 (77% similarity), both belonging to the order *Desulfobacterales*.

Four sequences from sediment and seven from enrichment samples were affiliated to the *Desulfovibrionales*. Nine sequences formed two novel clusters associated with *Desulfonatronovibrio hydrogenovorans* (with similarities between 68% and 86%), and two other sequences were related with *Desulfonatronum lacustre* (with 91% and 94% similarity, respectively). One sequence obtained from the high salt lake Tanatar II (i.e. KL2_3), and two sequences (E_H_2_2_E and E_VFA_2_E) from samples enriched both at 2 M Na^+, but with different electron donors (i.e., H_2 and VFA), were identical and affiliated to *Desulfonatronovi-*

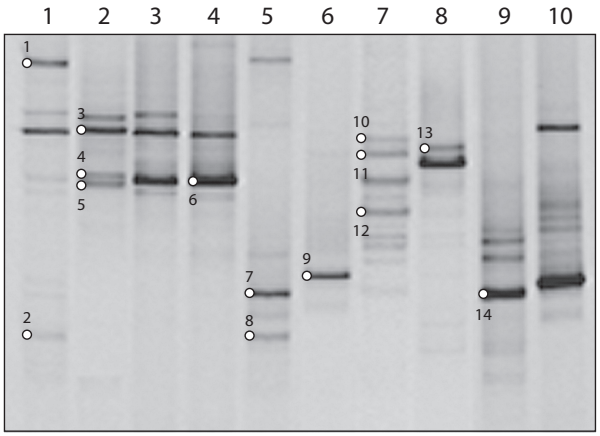


Figure 5.1: DGGE analysis of the partial *dsr*B gene fragments from sediment samples of different soda lakes from the Kulunda steppe (south-west Siberia, Russia). Lane 1, 1KL; lane 2, 2KL; lane 3, 3KL; lane 4, 4KL; lane 5, 5KL; lane 6, 6KL; lane 7, 9KL; lane 8, 10KL; lane 9, 11KL; lane 10, 16KL (see Table 5.1 for more information on the soda lakes). Bands indicated by a white dot and number have been excised from the gel and sequenced.

brio hydrogenovorans (Fig. 5.2). Only sequences from low saline lakes, i.e., KL1_2 from Lake Tanatar III, and E_H_2_1_HDE, obtained from samples enriched at 1 M Na^+ and H_2 as electron donor, were affiliated to *Desulfonatronum lacustre*.

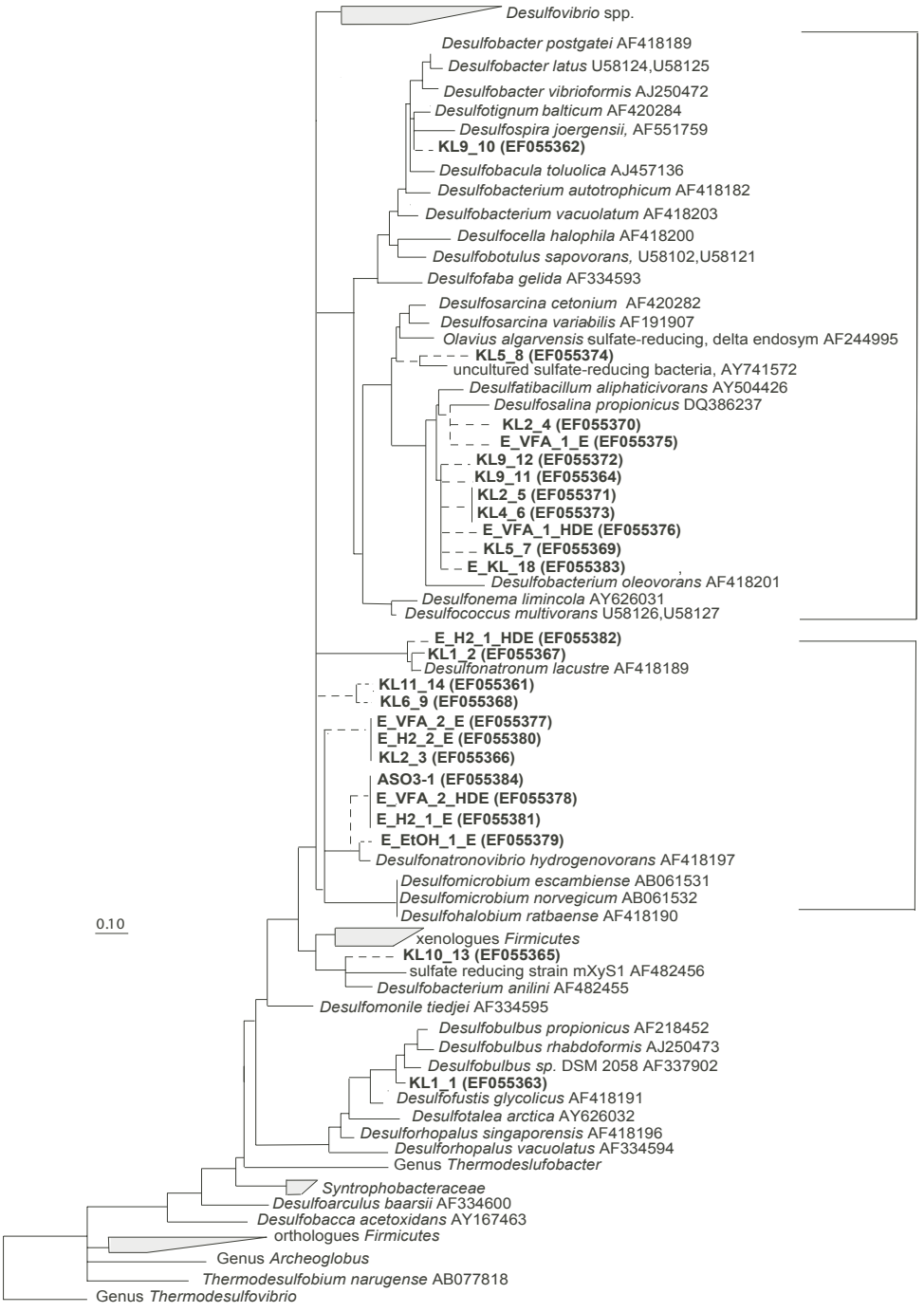
Eleven sequences clustered within the *Desulfobacteraceae*. Nine sequences were associated with *Desulfosalina propionicus*, a so far non-described halophilic SRB isolated from Great Salt Lake (USA), and seven of them formed a new lineage. Two sequences (KL2_5 and KL4_6) obtained from two different lakes, i.e., Lake Tanatar I and Tanatar II (Table 1), had identical sequences. All sequences from sediment samples were obtained from extremely saline lakes (from 250 to 350 g/l Na^+). Another sequence, KL5_8 from lake Tanatar V, clustered together with *Desulfosarcina variabilis* (91% similarity) and another one, KL9_10, was related to *Desulfotignum balticum* (96% similarity), a facultative halophilic organism.

Quantification of SRB by qPCR

We applied qPCR to estimate the abundance of SRB in the sediment samples of different soda lakes. The standard curve was based on a partial *dsr*B purified PCR product, in which the *dsr* copy numbers were known (Fig. 5.3A). All the sediment samples analyzed fit exactly in the standard curve.

In four lakes (i.e., 1KL, 2KL, 4KL and 18KL) the copy numbers of the *dsr* gene have been found to be in the order of 10^6 per ml sediment, while in lakes 5KL, 6KL and 9KL they were found to be one order of magnitude higher (Fig. 5.3B). In lakes 10KL and 11KL, two low salt lakes which have similar conditions, the lowest copy number was found, i.e., 10^5 and 10^4 re-

Figure 5.2: Consensus phylogenetic tree based on amino acid sequences of the *dsr*AB gene. The partial sequences determined in this study are in bold. They were added to the tree using the QUICK_ADD parsimony tool, and individually removed to avoid long branch attraction. Deletions and insertions were not included in the calculation. Branching orders that were not supported by all treeing methods are shown as multiforcations. The band number (see Fig. 5.1) is preceded by the lake number (see Table 5.1) for sediment samples. For enrichment samples the following code was used: E_H_2 /VFA/EtOH (electron donor)_ 1/2 (total Na^+ in M) _ E/HDE/HDO/LDO (type of enrichment). E stays for enrichment obtained by regular transfers of an incubation that was originately incoulated with the sediment mixture sample; HDE are enrichments that were obtained by regular transfer of the highest positive dilution of the initial sediment incubation at that specific condition, wheras HDO and LDO are the first enrichments with the highest and the lowest positive dilutions respectively, in which the original sediment sample was used as inoculum.



spectively (Fig. 5.3B). In contrast the highest *dsr* gene copy numbers (10^8) per ml sediment

were detected in lake 3KL, the most saline lake investigated in this study.

Discussion

Here we describe the diversity, activity, and abundance of SRB in sediment samples from Siberian soda lakes. For this purpose, we used a polyphasic approach, which included both culture-dependent (i.e., enrichment, serial dilutions) and independent (i.e., PCR-DGGE, comparative sequence analysis and qPCR) techniques.

SRB activity

The SRB activity was detected by sulfate reduction rate (SRR) measurements. In sediments of soda lakes from the Kulunda Steppe we observed SRR that are significantly higher than the values measure for less alkaline and saline habitats. For instance, SRR were observed in the range of 0.5 - 13 µmol/dm^3 d^{-1} for fresh water sediments (Li et al., 1999), from 5.6 to 10^4 µmol/dm^3 d^{-1} for a sediment of a Yellowstone hot spring, and from 15 - 20 µmol/ dm^3 d^{-1} for marine sediments (Jorgensen and Back, 1991). The highest SRR reported to the best of our knowledge, 5 10^3 µmol/ dm^3 d^{-1}, was measured in a marine sediment covered by a *Beggiatoa* mat (Boetius et al., 2000).

There are few data are available on SRR in soda lakes; in Big Soda Lake (Nevada, USA) and in Mono Lake (California, USA) the SRR in the anoxic water were significantly lower than in the above mentioned habitats (Oremland et al., 2004;Smith et al., 1987): 0.74 - 3.2 10^{-3} µmol/ dm^3 d^{-1} for Big Soda Lake and 2.3 10^{-3} µmol/ dm^3 d^{-1} for Mono Lake. In the mongolian soda lake sediments (Sorokin et al., 2004), and especially in hyposaline and less alkaline (pH 9.4 - 9.5) lakes in the south-east Transbaikal area the SRR were considerably higher, i.e., 3 10^3 µmol/dm^3 d^{-1} (Gorlenko et al., 1999). For lakes Cock and Picturesque we measured SRR that were only slightly lower (Table 5.1). Especially unexpected was the very high SRR measured in Picturesque Lake, since its extremely high salinity (405 g/l).

Even at a salt concentrations of 520 g/l (lake Tanatar I) substantial SRR were observed (Table 5.1). This indicates again that a yet undiscovered group of extremely halo-alkaliphilic SRB might be active in these hypersaline lakes.

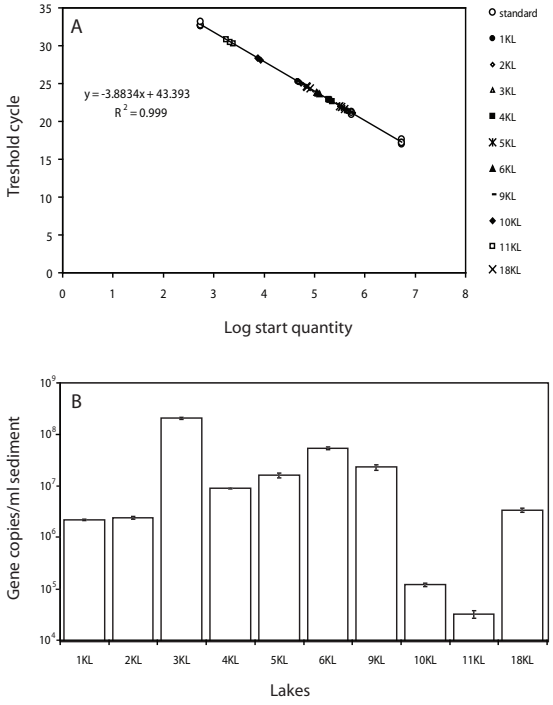


Figure 5.3: Abundance of the SRB in sediment samples by qPCR. (A) Standard curve for qPCR measurements of the *dsr* gene. The standard curve was calculated based on a partial *dsr*B purified PCR product of known copy number. (B) SRB abundance (logarithmic scale) in the different soda lakes. Error bars represent standard deviations.

SRB quantification

Estimation of the number of sulfate reducers was done by both culture-independent, i.e., quantitative real time PCR on genomic DNA, as well as culture-dependent techniques, i.e., serial dilutions analysis. The highest *dsr* gene copy number was detected in lake Tanatar I, with 10^8 cells per ml sediment, assuming that SRB possess only one *dsr* gene copy (Kondo et al., 2004). More than one *dsr* gene copy have been detected in some *Desulfovibrio* species (Kondo et al., 2004); therefore our qPCR analysis might give an overestimation of the SRB population. However, our results are in accordance (Fig. 5.3) with data observed in Mono Lake, a low salt soda lake, in which SRB has been found in the order of $0.5 \times 10^7 - 6 \times 10^7$ cells per ml. Enumerations obtained from serial dilutions were lower than the ones detected by qPCR (Table 5.2), probably due to cultivation biases (Kaeberlein et al., 2002).

SRB diversity in soda lakes

Most of the SRB fell into two major groups, i.e., the *Desulfovibrionales* and *Desulfobacteraceae*. Concerning the first group, sequences were affiliated to *Desulfonatronovibrio hydrogenovorans*, which belongs to the *Desulfohalobiaceae* family and to *Desulfonatronum lacustre*, the only member of the *Desulfonatronumaceae* family (Loy et al., 2002). Both these strains are low salt-tolerant alkaliphiles isolated from soda lakes. They are incomplete oxidizers, able to use hydrogen and a few organic compounds as electron donor, which is a common feature of the members of the *Desulfovibrionales* order. In this study, two novel clusters related to *Desulfonatronovibrio hydrogenovorans* were found, revealing that the diversity within this group is higher than anticipated. The identified SRB belonging to this group originated from both high and low salinity samples. Since we found that two DGGE bands at the same position, but from different enrichment samples resulted to be the same sequence (data not shown), we assume this is generally valid. Band E_H_2_1_E was excised from a sample enriched at 1 M Na^+, but present also at higher salt concentrations. Therefore, we can conclude that this group included SRB, such as E_H_2_1_E, having a broad range of salt tolerance. On the other hand, the group apparently includes highly specialized organisms, such the extremely haloalkaliphilic isolate ASO3-1, which is unique not only by its sodium requirement (2 M Na^+) and tolerance (4 M Na^+), but also for its ability to grow without any organic substrate by sulfite fermentation. This isolate is, to the best of our knowledge, the only sulfate reducer known to be able to grow at this high salt concentration. Therefore it would be interesting to detect this new SRB ecotype with specific probes in other hyper saline environments. The use of probes may, however, not be specific since two sequences originating from low salt enrichments, i.e., E_VFA_2_HDE and E_H_2_1_E, showed 100% identity with the sequence of the obligate extreme high salt isolate ASO3-1 (Fig. 5.2). Clearly, this indicates that physiological differences may not be observable at the *dsr* gene level.

All the sequences within the family *Desulfobacteraceae* were related to halophilic SRB, such as *Desulfotignum balticum*, *Desulfosarcina variabilis*, and *Desulfosalina propionicus*, and almost all of them were detected in hypersaline lakes. *Desulfobacter halotolerans*, isolated from the sediments of Great Salt Lake in Utah (Brandt and Ingvorsen, 1997), can tolerate up to 130 g/l NaCl. We detected sequences related to this family in sediments from lakes with salinity up to 475 g/l. The occurrence of sequences within this group is interesting, because so far all known bacteria belonging to this family are complete oxidizers from which little is known about salinity tolerance.

Life at such extreme conditions is energetically very costly. Oren (Oren, 1999) described

two different microbial strategies that are used by microorganisms to deal with the osmotic stress at this high salinity. One is the so-called “salt-in” strategy, in which the intracellular salt concentration is kept osmotically equivalent to the external one by using K^+. This strategy is energetically relatively less expensive, and so far it is known to be mostly used by organisms belonging to the orders of the aerobic halophilic archaea *Halobacteriales* and the anaerobic bacteria *Haloanaerobiales*. The second strategy is based on organic compatible solutes, such as ectoine, glycerol and glycine-betaine, which are energetically very expensive to produce. This might be one of the reasons that restrict the presence of microorganisms with a metabolism of low energetic yield in hypersaline habitats. Until recently it appeared that the upper salt concentration limit for SRB is ca. 250 g/l for incomplete oxidizers and 130 g/l for complete oxidizers (Oren, 1999). Assuming that all sulfate reducers belonging to the *Desulfobacteraceae* are completely oxidizers, the fact that we detected sequences of organisms within this family at salt concentration of 475 g/l might indicate either the presence of an unknown type of complete oxidizing SRB with an inorganic osmo-adaptation strategy, or the presence of spatial-temporal low salt microniches.

Only one match was found between sequences retrieved from sediment (KL2_3) and enrichment (E_VFA_2_E, E_H_2_2_E) samples, within the order *Desulfovibrionales*. Since the sediment samples investigated in this study were used as inoculum for the enrichments, we expected to find more than one match, although we can not exclude the use of not optimal enrichment conditions (Kaeberlein et al., 2002; Oren, 1999).

We did not detect sequences related to the Gram-positive *Desulfotomaculum* group, as was found in another study (Scholten et al., 2006). Since these SRB have been described as growing best at low salt concentrations (Detmers et al., 2004;Widdel and Back, 1992) they might be not present at all, or only in very low numbers, in the Kulunda soda lakes.

Unfortunately, for different logistic reasons, samples were taken in different years, i.e., in 2003, samples were taken for qPCR, for enrichments, and for PCR-DGGE, and in 2005, samples were taken for SRR and serial dilutions. However, both sampling campaigns were done in the same month (July), which is essential, since lake properties (e.g., total salt content) may vary in different seasons. Furthermore, sampling was performed in the same parts of the lakes, which were marked in 2003. The water chemistry of the lakes was highly similar for both years, and SRR measured in sediments od some of the lakes gave similar values in both years. However, despite these similarities, we cannot exclude the possibility of that there might have been changes in the microbial communities in the 2 years.

In summary, we investigated, for the first time, the SRB communities in saline and hypersaline soda lakes using a polyphasic approach. Different novel clusters were detected, including organisms able to grow at salt concentrations close to saturation, with a higher broad range of salt tolerance than thought before. The presence and vitality of such extremely halophilic SRB has been proved by SRR and enrichment analysis. Overall, our results demonstrated the existence of active SRB communities with a high diversity in soda lakes.

Acknowledgments

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Chapter 6

Bacterial diversity and activity along a salinity gradient in soda lakes from the Kulunda Steppe (Altai, Russia)

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Submitted to Extremophiles

ABSTRACT

Soda lakes are a special type of saline lake with pH values up to 11, which are kept constant by the high buffering capacity of the two major anions present in solution, i.e., carbonate and bicarbonate. It has commonly been observed that the diversity of metabolic types decreases with salinity, probably due to energetic constraints. Several studies investigated the influence of salinity, i.e. NaCl, on bacterial diversity in different environments, but the diversity along a salinity gradient in soda lakes has never been previously studied.

Since the sulfur cycle is one of the most active elemental cycles in these lakes we focused our study on the diversity and activity of sulfate reducing bacteria in four different soda lakes from the Kulunda Steppe (South East Siberia, Russia). For this purpose, a combination of culture-dependent and independent techniques was applied. The general bacterial and SRB diversity was analyzed by denaturing gradient gel electrophoresis (DGGE) targeting the 16S rDNA gene. DNA was used to detect the entire microbial population present in soda lake sediments, whereas ribosomal RNA was used as a template to obtain information on the active populations. Individual DGGE bands were sequenced and a phylogenetic analysis was performed. In addition, the overall activity of SRB was obtained by measuring the sulfate reduction rates (SRR) and their abundance was estimated by serial dilution.

Our results showed the presence of minor, but highly active microbial populations, mostly represented by members of the Proteobacteria. Remarkably high SRR measured at hypersaline conditions (200 g L^{-1}). A relatively high viable count indicated that sulfate reducing bacteria can be highly active in hypersaline soda lakes. Furthermore, the increase of sodium carbonate/bicarbonate seemed to affect the composition of the microbial community in soda lakes, but not the rate of sulfate reduction.

Introduction

Soda lakes are a specific type of salt lake with high to extremely high carbonate alkalinity, a pH from 9 to 11, and a moderate to extremely high salinity. They are spread all over the world, but located, as most inland salt lakes, in arid and semi-arid areas where the evaporative climate favors accumulation of salts in local depressions. The major ions in soda lake brines are sodium, carbonate/bicarbonate, chloride and sulfate, whereas calcium is virtually absent and magnesium only present at very low concentrations. In contrast to other alkaline environments, such as low-salt alkaline ("cement") springs, soda lakes maintain a stable alkaline pH due to the high buffering capacity of the soluble carbonates. These double extreme conditions (i.e. high pH and high salinity) make soda lakes a unique ecosystem.

In the last decade special attention has been given to the investigation of the microbial communities in soda lakes using both traditional isolation methods (Duckworth *et al.*, 1996; Sorokin *et al.* 2004; Sorokin and Kuenen, 2005b) and molecular biology techniques (Humayoun *et al.*, 2003; Rees *et al.*, 2004; Scholten *et al.*, 2005; Foti *et al.*, 2007). Few reviews (Zavarzin *et al.*, 1999; Jones *et al.*, 1998) summarize these results, showing that soda lakes contain representatives of the major trophic and phylogenetic groups of Prokaryotes, and that they can be considered as autonomous systems, in which cycling of nutrients is close to complete. The most well-studied soda lakes are those located in the East African Rift Valley (Duckworth *et al.*, 1996; Rees *et al.*, 2004), in the Libyan desert (Wadi Natrun)(Imhoff *et al.*, 1979) and in North America, i.e. Mono Lake (California) (Humayoun *et al.*, 2003; Scholten *et al.*, 2005a) and Soap Lake (Washington) (Sorokin *et al.*, 2007a). There are also data available on soda lakes in India (Wani *et al.*, 2006) and Central Asia (Sorokin *et al.*, 2004; Ma *et al.*, 2004). This last region accommodates a great number of steppe soda lakes, especially in the Inner Mongolia (North east China) and south Siberia (Transbaikal region, Tuva Republic and Kulunda Steppe).

Except for the study conducted by Issancheko and coworker in 1933-1935 (Issachenko, 1951), almost nothing is known about the microbial communities in soda lakes of the Kulunda Steppe. In contrast to tropical African lakes and deep stratified North America lakes, the Central Asian lakes are very shallow and usually very small, in which the total concentration of salts and chemistry may differ considerably among nearby lakes as well as during the year. Furthermore, lakes are subjected to unstable water and temperatures regimens between -40°C and +40°C, causing frequent fluctuations of the water level and salinity.

It has been hypothesized that the diversity of metabolic types of Prokaryotes decreases with salinity for thermodynamic reasons (Oren, 1999). Several studies have been performed on systems with saline gradients (Sörensen *et al.*, 2004; Nubel *et al.*, 2000; Casamayor *et al.*, 2002), especially estuaries (Crump *et al.*, 2004; Schultz *et al.*, 2001), to investigate the influence of the salinity on the composition of the microbial community. However, estuaries have very low salinities compared to salt lakes or salterns (Benlloch *et al.*, 2002; Sörensen *et al.*, 2004). Commonly thalassohaline ecosystems are object of investigation, in which sodium, magnesium, chloride and sulfate are the major components of the brines. In soda lakes sodium carbonate/bicarbonate is the major salt in solution, which has different physical and chemical properties, such as being a two times weaker electrolyte than sodium chloride. For this a less energetically expensive osmotic adaptations than demanded for life in NaCl brines (Sorokin and Kuenen, 2005b). Nevertheless, organisms inhabiting soda lakes have to deal with high alkaline conditions, which represent an extra stress factor. Therefore, it was of interest to study the microbial community composition in steppe soda lakes, and how this was

affected by the increase in sodium carbonate/bicarbonate concentrations.

Since the sulfur cycle is one of the most active cycles in soda lakes, special attention was given to the sulfate-reducing bacterial community (SRB). It has been hypothesized (Oren, 1999; Ollivier *et al.*, 1994) that specific groups of SRB, i.e. complete oxidizers, stop growing at salt concentrations above 130 g L^{-1}. Therefore it was of interest to investigate the presence of these different phylogenetic groups of sulfate–reducers along a salinity gradient.

In this study we investigated the bacterial diversity and the major actively growing groups, based on ribosomal RNA determination, in four soda lakes with increasing salinity. For the first time the dominant active populations were detected in these extreme environments by targeting the 16S rRNA gene from the lake sediments. A combination of culture-dependent and -independent techniques was applied to investigate the SRB community. Identification of sulfate-reducers was done by DGGE analysis of the 16S rRNA genes, while the activity was determined by measuring the sulfate reduction rates (SRR).

Our results showed the presence of less abundant, but highly active microbial populations, mostly represented by members of the Proteobacteria. The detection of active SRB from sediment samples by targeting the 16S rRNA gene was not completely successful. However the remarkably high sulfate reduction rates (SRR) measured at hypersaline conditions (200 g L^{-1}) and the relatively high viable counts indicated that this group of organisms was active in these hypersaline soda lakes. Furthermore, an increase of salinity seemed to affect the composition of the microbial community in soda lakes, but did not affect the rate of active processes, such as sulfate reduction.

Material And Methods

Site description

The sampled lakes are located in the southern part of the Kulunda Steppe, in south-eastern Siberia (Altai, Russia), along the north-eastern border with Kazachstan, about 320 km south-west of Barnaul (Altai capital), near the village Mikhaylovskiy (Fig. 6.1). The Kulunda Steppe region is not well known to the scientists due to its remote location, and some lakes are only known by the local population. The only information on the geology and chemistry of this area is available from the results of the Kulunda Expedition of the Russian Academy of Sciences taken place within 1933-1935 (Issachenko, 1951). Lakes present in this region are generally very small and shallow, except for the large chloride-sulfate lakes with neutral pH in the north, such as Kulunda Lake, Lake Burlinskoe and Lake Big Yarovoe. In the south of Kulunda Steppe, the lakes are much smaller and some of them belong to the soda lake type. Salt concentration and salt nature may differ even between closely situated lakes, as well as their sediment structure. Except for Cock Lake, all investigated lakes (see Table 6.1) are located close to each other, i.e., within a diameter of 25 km. They belong to two systems: the Tanatar- and the Bitter Lakes System, and both are part of a larger system, which is locally known as the “Salt Lake Steppe”. Although Cock Lake does not belong to the above mentioned systems of lakes being situated ca 50 Km north-west, it is very similar for its characteristics to the lakes in the Bitter Lake System. Characteristic feature of all the investigated lakes is the mineral deposition of ancient trona (i.e., sodium carbonate/bicarbonate), which confer the high salinity and alkalinity. Cock Lake was the first source of commercial soda in Soviet Union since 1923, while Tanatar system is still actively mined for sodium carbonate. Lakes Cock and Tanatar-3 presented a sandy-clay type of sediment, whereas lakes Tanatar-1 and Bitter-1 presented a

Table 6.1: Chemical parameters of the investigated soda lakes and viable count numbers. CH_4 and SO_4^{2-} concentrations and SRR were measured for the near bottom water (nbw) and for different sediment layers (sediment depth in cm indicated between brackets). In the last 2 columns the enumeration of SRB has been estimated from positive tubes of serial dilutions on H_2 or rich acetate medium, respectively. The top 5 cm of the sediment samples were used as inoculum. 0.6 M Na^+ and 4M Na^+ were used for lakes Tanatar III and Cock, and Tanatar I and Bitter, respectively.

Sediment Sample (depth)	Coordinates		Name	Na^+ g L^{-1}	pH	Alkalinity (M)		CH_4 ml L^{-1}	SO_4^{2-} mM	SRR µmol $dm^{-3}day^{-1}$	H_2 (viable cells cm^{-3})	Acetate (viable cells cm^{-3})
	Longitude	Latitude				CO_3^{2-}	Total					
C1 (nbw) C-1 (0-2) C-2 (3-10) C-3 (11-20)	79.15 °E	52.10 °N	Cock Lake	60	10.30	0.85	0.90	0.44 0.73 0.13 0.07	0.84 0.84 7.9 2.6	0.2 116 7.4 4.8	10^5	10^6
T3 (nbw) T3-1 (0-10) T3-2 (10-20)	79,75 °E	51,65 °N	Tanatar-3	112	10.13	0.90	1.125	0.74 0.44 0.79	74 74 83	0.47 68 236	10^6	10^6
T1 (nbw) T1-1 (0-5) T1-2 (6-20)	79,81 °E	51,66 °N	Tanatar-1	200	10.0	1.30	1.57	0.010 0.016 0.004	221 221 242	12 153 104	10^3	10^6
B (nbw) B-1 (0-4) B-2 (5-11)	79,90 °E	51,67 °N	Bitter Lake-1	200	10.20	2.25	2.825	3.33 0.59 0.70	6.6 6.6 5.6	92 1426 43	10^3	10^4

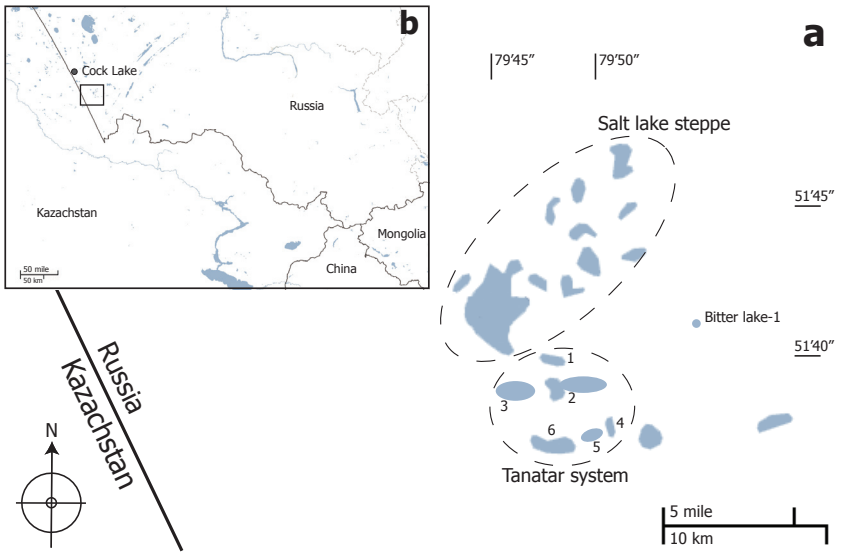


Figure 6.1 Legend:location of the investigated soda lakes in the Kulunda Steppe (Altai, Russia). **a)** Detailed map; **b)** big scale map

black-clay type of sediment, with strong sulfide smells. Sediment samples were taken from the above mentioned lakes with increasing salinity in July 2007. The physical and chemical parameters of the lakes are presented in Table 6.1.

6

Chemical analysis and sampling

Field measurements included pH, conductivity, and carbonate alkalinity in brine water. pH and conductivity were measured using a field meter supplemented with pH and conductivity probes (WTW, model pH/cond340, Weilheim, Germany). The pH probe was calibrated in saline buffers containing 0.5-2 M total Na^+ (Sorokin 2005). The conductivity probe was calibrated in NaCl and carbonate buffer with pH 10 from 10 to 80 g L^{-1}. At higher values the samples were diluted with distilled water. Alkalinity was measured by titration with 1 M HCl using phenolphthalein (carbonate alkalinity) and methyl orange (bicarbonate alkalinity) as indicators. The salt concentration inferred from the conductivity measurements was verified by gravimetry in the laboratory. Samples were taken for nucleic acid extraction, sulfate reduction rate measurements and viable counts. Sediment cores (top 20 cm) and overlaying water were sampled manually along the littoral, using a corer Sediment cores were divided into different layers, based on color, and stored in 50 ml falcon tubes. Samples for nucleic acid extraction were mixed with RNAlater (Ambion, UK) in a 1:1 volume ratio. All tubes were kept at 4°C during transportation to the laboratory, and stored at -20°C until further analysis.

Sulfate reduction rates

Sulfate-reduction rates (SRR) experiments were conducted in situ and measured in 5 ml syringes capped with butyl rubber stoppers using the $^{35}S\text{-}SO_4^{2-}$ methodology (Lein *et al.*, 2002). After 1-2 days of incubation at ambient temperature, the sediments were fixed with 10 M KOH and further processed in the laboratory. Additional analysis of total SO_4^{2-} and CH_4 content in the sediment pore water was performed in the laboratory after centrifugation of 2 cm^3 sample using ion-exchange chromatography (Biotronic) for sulfate and by gas chroma-

tography for methane.

Additional experiments to study the influence of pH/salt and nutrient additions on indigenous SRB populations were performed in the laboratory with sediment slurries (top 5 cm layer) from Cock Lake and Tanatar-1. Nutrient addition experiments were performed with native sediment slurries after addition of nitrogen (100 µM NH_4Cl), phosphorus (10 µM K_2HPO_4) and electron donors such as hydrogen, lactate and acetate (1 mM) as substrates. The influence of pH and salt was examined after separation of the sediment solids from the pore brines by centrifugation. The influence of pH was investigated by resuspending the sediments in buffers containing 5 mM sodium sulfate, varying the pH between 7 and 11 and keeping the original salinity. The influence of salt was investigated at pH 10 with increasing concentration of sodium carbonates. The SRR rates were measured in the same way as for the in situ measurements.

Quantification of sulfate-reducing bacteria by serial dilutions

Quantification of haloalkaliphilic SRB was performed by serial decimal dilutions in Hungate tubes. The top 5 cm sediment layers were used as inoculum. Low-salt (0.6 M total Na^+) and high-salt (4 M total Na^+) carbonate media at pH 10 (Sorokin and Kuenen, 2005a) was used with either H_2 or acetate (20 mM plus 0.5 g L^{-1} yeast extract) as an energy source. The inoculation was done directly in the field. Growth was monitored within 4-8 weeks incubation at room temperature by estimating the sulfide production (Trüper and Schlegel, 1964). Tubes with the highest positive dilutions were then subjected to DGGE analysis.

Nucleic acids extraction

Prior to the nucleic acid extraction, RNAlater was removed by 1 min centrifugation at 5,000 rpm. The sediments were then washed three times with 1 M NaCl and 10 mM Tris (pH 7.5) to lower the pH and the salt concentration. Genomic DNA was extracted from circa 2 g of sediment using the UltraClean Soil DNA Extraction Kit (MoBio Laboratories, USA), following the manufacturer's instructions with minor modification. 200 µl of 0.1 M $AlNH_4(SO_4)_2$ was added to the sediment to remove potential PCR inhibitors, such as humic acids (Braid *et al.*, 2003). For RNA extraction, 7 g of sediments were divided in 2 ml screw cap tubes and washed as described above. After the washing step, bead beating and the addition of 120 µl of preheated MBER® (Max Bacterial Enhancement Reagent, Invitrogen, The Netherlands) were added to each tube and incubated for 4 min at 95°C. Cells were lysed by adding 0.7 ml TRIzol® (Invitrogen, The Netherlands), a mono-phasic solution of phenol and guanidine isothiocyanate and homogenized for 45 sec, followed by 5 min incubation at room temperature to allow the complete dissociation of nucleo-proteins complexes. The TRIzol® reagent maintains the integrity of the RNA during sample homogenization, while disrupting cells and dissolving cell components. After homogenization cold chloroform was added (0.1 ml/0.5 ml TRIzol®). Subsequently, the samples were vigorously shaken manually for 15 sec and incubated at room temperature for 3 min followed by 15 minute centrifugation at 10,000 rpm and 4 °C. The aqueous upper phase was then transferred to a new tube, 0.3 ml cold isopropanol were added and mixed to precipitate the RNA. After 10 min incubation at room temperature, the pellet was collected by centrifugation at 10,000 rpm for 10 min and washed with 75% (v/v) ethanol. Samples were then vortexed and centrifuged 5 min at 8,000 rpm. The supernatant was discarded, while the pellet was air dried and dissolved in 40 µl RNA-free water. Subsequently, DNA traces were removed using the Turbo DNA free kit (Ambion, UK) and the resulting DNA-free RNA was purified with the Qiagen RNAeasy MiniEluteKit (Qiagen, the Netherlands), following

manufacturer's instructions. All reagents, plastic and glass ware were RNAase free.

RT-PCR, PCR and DGGE

RNA was converted to cDNA by reverse transcriptase using the iScript cDNA Synthesis Kit® (BioRad, The Netherlands), following the manufacture`s instructions. The purity of the extracted RNA was checked by 16S rRNA gene amplification using RNA as template; no PCR product was obtained indcating the absence of contaminating DNA. From the genomic DNA and cDNA a fragment of the 16S rRNA gene suitable for DGGE, was amplified. A nested PCR approach was used for the amplification of the 16S rRNA gene. First the whole gene was amplified using the primer GM3F/GM4R (Brinkhoff *et al.*, 1998). The product was then excised from the agarose gel, purified using the Qiagen Gel Extraction Kit (Qiagen, the Netherlands) and used as a template for the amplification of the a gene fragment suitable for DGGE using primer combination 341F+GC/907R (Schäfer and Muyzer, 2001). To detect sulfate-reducing bacteria, six primers pairs specific for the 16S rRNA gene of different SRB groups (Table 2; Daly *et al.*, 2000) were used as described by Dar *et al.* (2005). PCR products of each amplification step were excised from the gel and purified before the following PCR round to avoid non-specific amplification. The obtained PCR products were then subjected to DGGE analysis. DGGE was performed as described by Muyzer *et al.* (1993), using a denaturing gradient of 35 to 60% denaturants in 8% polyacrylamide gel. Individual bands were excised, reamplified, and run again on a denaturing gradient gel to check their purity. PCR products for sequencing were purified using the Qiaquick PCR purification kit (QIAGEN).

Comparative sequence analysis

The sequences were first compared to sequences stored in GenBank using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST). Subsequently, the sequences were imported into the ARB software program (Ludwig *et al.*, 2004), automatically aligned, and added to a phylogenetic tree using the Quick-add tool. Sub-trees were then built using the Neighbor Joining algorithm with automatic selected correction settings.

Nucleotide sequence accession number

The sequences determined in this study were deposited in GenBank under the following accession numbers: EF622422-EF622488.

Results

Physical-chemical characterization of the sediments

The investigated lakes were less than 1 km in diameter and had a maximum depth of 1 m. The salinities varied from 60 to 200 g L^{-1} and the pH from 10 to 10.3 (see Table 6.1). The dominant salt in solution was sodium carbonate/bicarbonate and the total alkalinity was in the range of 0.9 - 2.82 M. Sulfate concentrations in sediment pore waters correlated with total salt and in general were very high except for Bitter Lake in which, despite the extremely high salinity, the sulfate concentration was nearly depleted (Table 6.1). Free sulfide (up to 0.5 mM) was also detectable in the pore waters of the top 5 cm sediment layer in Tanatar-1 and Bitter Lake. In Bitter Lake the highest methane content was measured in the sediments and even in the brines, suggesting that both sulfate reduction and methanogenesis are active.

Nucleic acids extraction and DGGE analysis of 16S rRNA genes

Nucleic acids extraction and PCR amplification of the 16S rRNA gene was successful for all the analyzed samples. PCR amplification using specific primers for SRB groups was successful with most of the samples when targeting group 1 (*Desulfotomaculum*, DFM), group 5 (*Desulfosarcina*, DCC) and group 6 (*Desulfovibrio-Desulfomicrobium*, DVV). One, four and none positive amplifications were obtained targeting group 2 (*Desulfobulbus*, DBB), group 4 (*Desulfobacter* DSB) and group 3 (*Desulfobacterium*, DBM), respectively. In some cases amplification products were identified as non specific, resulting in the detection of different phylogenetic groups than the targeted ones (see Fig. 6.4).

Amplicons obtained from genomic DNA and from cDNA (obtained after reverse transcription of the RNA) were then analyzed by DGGE. An average of 4 bands was observed for both DNA- and RNA-derived profiles (Fig. 6.2) using primers for the 16S rRNA genes of Bacteria in general. The DNA- and RNA-derived profiles were found to be highly different when analyzing the same samples (Fig. 6.2).

DGGE analysis of PCR products obtained with the primer pairs specific for the 16S rRNA genes of different SRB groups (results not shown) gave complex patterns for both group 1 and 6, whereas for group 2 and 4 just one dominant band was detected, corresponding to *Desulfobulbus propionicus* and *Desulfosalina propionicus* respectively. From the highest positive tube of the dilution series for the enrichment of SRB, 1 and 5 bands were obtained, using hydrogen or acetate as electron donor, respectively. Almost all sequences grouped with members of the low G+C gram-positive bacteria and not to SRB of the Deltaproteobacteria.

The DGGE analysis of the different soda lakes along a salinity gradient did not show a decrease in the number of bands at different sediment depths or along the salinity gradient, except for the RNA-derived profiles obtained for Lake Tanatar-1 and Bitter-1 (Fig. 6.2). Rather, the composition of bacterial taxa decreases at higher salinities (Fig. 6.3). In Cock Lake, the lowest saline lake (60 g L^{-1}), 10 phylogenetic groups were detected, in Tanatar-3 (112 g L^{-1}) 8 groups and in Tanatar-1 (200 g L^{-1}) and Bitter Lake (200 g L^{-1}), 3 and 5, respectively (see Fig. 6.3).

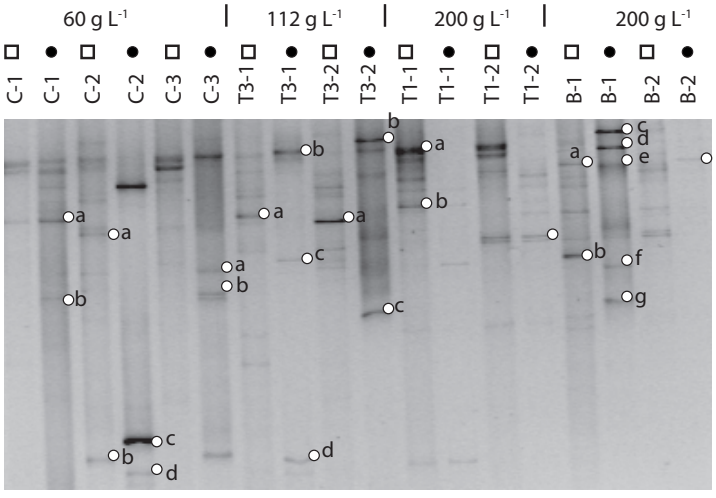


Figure 6.2: DGGE analysis of the bacterial 16S rRNA gene from sediment samples of the investigated soda lakes from the Kulunda Steppe. Lanes 1-6: Cock Lake; lanes 7-10: lake Tanatr-3; lanes 11-14 : lake Tanatar-1; lanes 15-18: lake Bitter-1. Sediment depths have been analyzed for each lake (see Table 1). DNA (□)- and RNA (●)-derived profiles of the same samples are situated close to each other. Bands indicated by a white dot have been excised from the gel and sequenced.

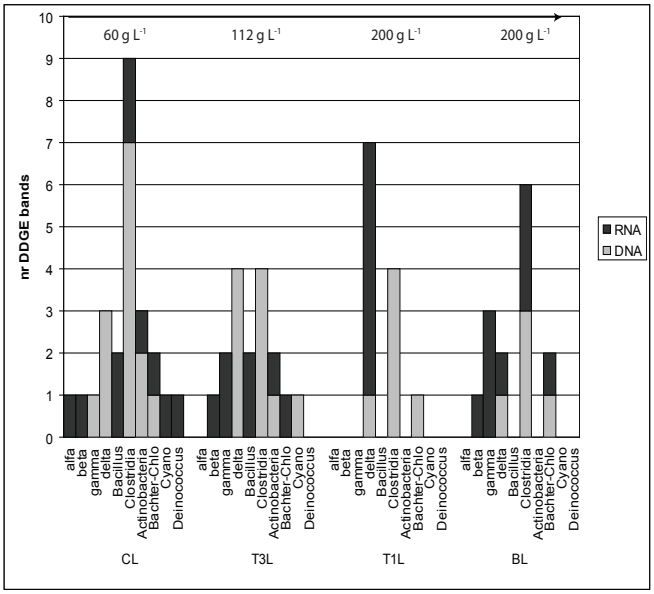


Figure 6.3: Microbial composition along the salinity gradient.

Microbial diversity along a salinity gradient

From the different DGGE gels obtained targeting the 16S rRNA gene, a total of 72 partial sequences has been analyzed phylogenetically. An overview of the obtained results is shown in Table 6.2 and Fig. 6.4. The less saline Cock Lake showed the highest bacterial taxa richness (Fig. 6.3). The top 10 cm sediments were characterized by the presence of sequences affiliated to the halo(alkaliphilic) Deltaproteobacterial SRB, such as *Desulfosalina propionicus* and *Desulfonatronum thiodismutans*, to the Gammaproteobacterium *Acinetobacter junii* and to the Actinobacterium *Propionibacterium acnes*. The sequences retrieved from the RNA-derived DGGE profiles, thus presumed to correspond to the active population, belonged to the groups Cyanobacteria (i.e. *Oscillatoria neglecta*), *Deinococcus*, Actinobacteria and Betaproteobacteria (i.e. *Tepidimonas aquatica* (Fig. 6.2). The deepest sediment layer (10-20 cm) was mostly characterized by sequences related to uncultured low G+C organisms. From RNA-based DGGE`s, sequences related to the Alphaproteobacterium *Brevundimonas* and to the uncultured green sulfur bacteria (Fig. 6.2, lane 6, band a) were observed.

In lake Tanatar-3 (112 g L^{-1}) members of the Alphaproteobacteria and *Deinococcus-Thermus* were detected. Sequences retrieved from DNA-based profiles were affiliated to the Deltaproteobacteria (*D. propionicus* and *D. lacustre*), *Actinobacteria*, Clostridia and Cyanobacteria (*Leptolyngbya* sp. (Fig. 6.2, lane 7, band a)). The sequences presumed to correspond to the active population were related to the Actinobacterium *P. acnes*, the Gammaproteobacteria *M. osloensis* and *Stenotrophomonas* sp., the Betaproteobacterium *Massilia* sp. (Fig 6.2) and to the Bacillus group.

The lakes with highest salinity, i.e. Lake Tanatar-1 and Bitter-1 (200 g L^{-1}), showed the poorest bacterial taxa diversity (Fig. 6.3). Both were mostly represented by the low G+C organisms and the Deltaproteobacterial SRB related to D. propionicus. The latter were predominant

in the RNA-based DGGE`s obtained by targeting the SRB groups specific 16S rRNA gene (data not shown). The top 4 cm sediment of Lake Bitter-1 was found to be particularly rich in sequences belonging to the Proteobacteria (Fig. 6.2, lane 17 and Table 3) and all of them were retrieved from the RNA-derived profiles. Remarkably, almost all identified sequences were related to organisms isolated from halo(alkaliphilic) environments (Fig.6.4).

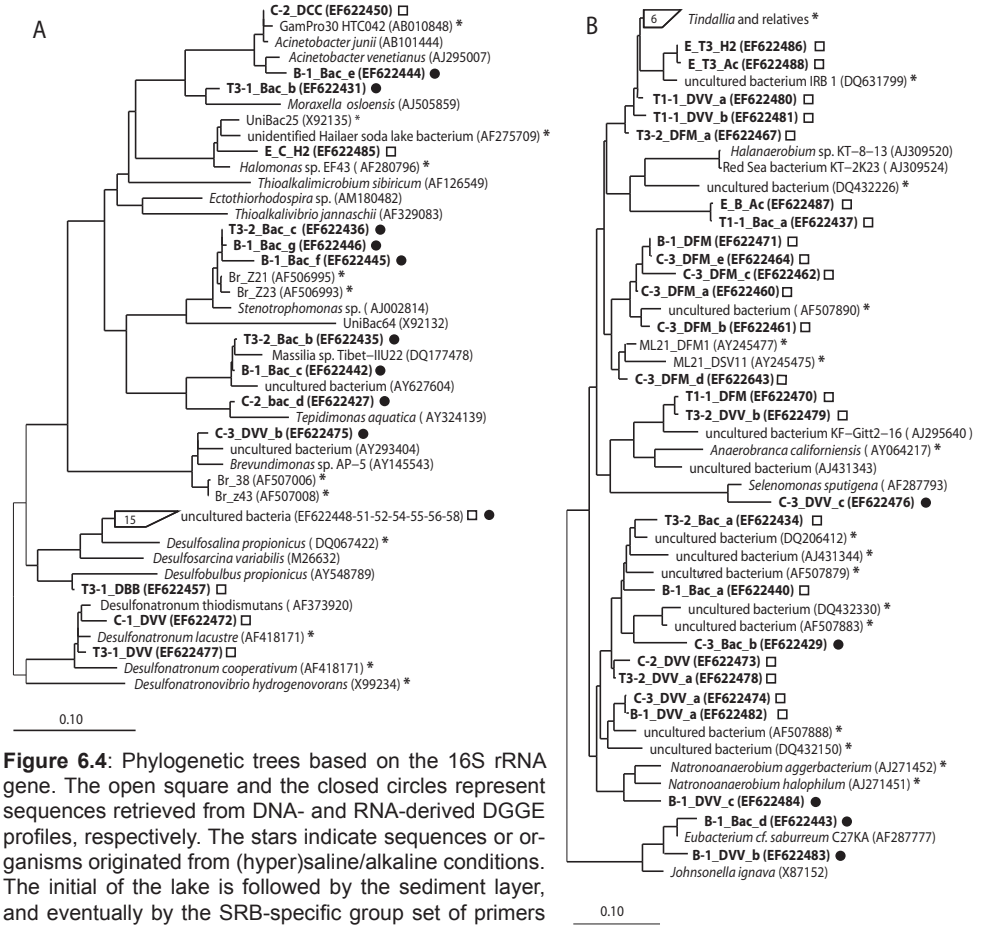
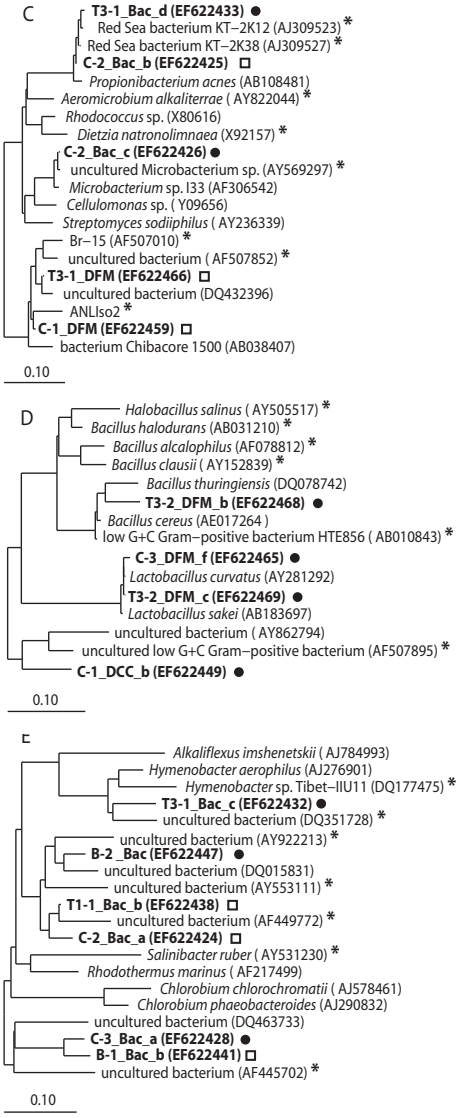


Figure 6.4: Phylogenetic trees based on the 16S rRNA gene. The open square and the closed circles represent sequences retrieved from DNA- and RNA-derived DGGE profiles, respectively. The stars indicate sequences or organisms originated from (hyper)saline/alkaline conditions. The initial of the lake is followed by the sediment layer, and eventually by the SRB-specific group set of primers used (see table 6.2). **a)** Proteobacteria; **b)** Clostridia; **c)** Actinobacteria; **d)** Bacilli; **e)** Bacteroidtes-Chlorobia

Sulfate reducing activity

High sulfate reducing-activity was observed in the near bottom water and in the sediment layers of all four investigated soda lakes (Table 6.1). The rates were comparable with previous measurements (Foti *et al.*, 2007) and, in general, corresponded to characteristic values of eutrophic marine sediments (Lein *et al.*, 2002). Salinity seems to have no effect on the sulfate reduction process in the studied soda lakes. In fact, the highest SRR (1.426 10^3 µmol dm^{-3} day^{-1}) were observed in Bitter Lake, at highest salinity conditions (200 g L^{-1}).

Results of the sediment slurry experiments to investigate the influence of pH and salt concentration suggested that the SRB populations in low-salt Cock Lake were limited by elec-



Figures 6.3c-e Continued from previous page

tron donors, but not by inorganic nutrients , i.e. nitrogen and phosphor, while in high-salt Tanatar-1 lake both inorganic nutrients, and electron donors were moderately stimulating (Fig. 6.5a). Furthermore, both populations were active between pH 8 and 10 (Fig. 6.5b), but differed in salt optimum-tolerance (Fig. 6.5c), although not as much as might be expected from the actual difference in salt content in the two lakes.

The presence of active SRB populations in sediments of the investigated lakes was confirmed by relatively high viable counts obtained both at high and low-salinities (Table 6.1). However, the heterotrophic cultures with acetate and yeast extract proved to be unstable during further transfers. In contrast, autotrophic enrichments with H_2 were active and transferable even at 4 M of total Na^+ with prominent development of relatively large vibrio's resembling the extremely natronophilic sulfite-disproportionating isolate ASO3-1, that previously was isolated from Kulunda soda lakes (Foti *et al*., 2007).

Discussions

Microbial diversity along a salinity gradient

Here we describe, for the first time, the diversity and activity of microbial communities in general and SRB communities along a salinity gradient in different soda lakes. For this purpose we used DGGE analysis of 16S rRNA gene fragments obtained from genomic DNA (as a measure for diversity) and from rRNA (as a measure for activity). In addition, sulfate reduction rates were measured. Salinity did affect the general microbial composition, but not the sulfate reducing activity. As reported in other studies (Wu *et al*., 2006; Bennloch *et al*., 2002), the number of DGGE bands did not show a negative correlation with increasing salinity, rather a decrease in the richness of bacterial genera was observed. This suggests an increase of the microdiversity, within the remaining phylogenetic group, at higher salinities.

At the highest salinities the dominant actively growing populations were belonging to the Delta- and Gammaproteobacteria. The latter were also detected previously in hypersaline lakes (Wu *et al*., 2006; Bennloch *et al*., 2002), together with organisms belonging to the Alphaproteobacteria. In the literature little can be found about the microbial diversity along salt

gradients in athalassophilic habitats and most of the studies have been conducted either on estuaries, with overall salinities 10-100 times lower than in hypersaline lakes, or on sea solar salterns. Furthermore, in most of these studies water and brines were the target and not the sediments, making comparison difficult or impossible.

The highest bacterial diversity was observed in Cock Lake (60 g L^{-1}) and Tanatar-3 (120 g L^{-1}) with actively growing populations affiliated to members of the Proteobacteria and Bacilli. At the lowest salinity, a sequence corresponding to the cyanobacterium *Oscillatoria neglecta* was detected, which is probably responsible of the bulk of primary production. Interesting was the detection of one sequence closely related the N_2-fixing cyanobacterium *Leptolyngbia* sp. PCC7104. So far, no cyanobacteria capable in N_2-fixation have been detected or isolated from soda lakes, and so it is still not clear which organisms are responsible for this process. Hydrolytic (Bacilli, *Bacteroidetes*), lipolytic (*Stenotrophomons* sp.), and fermentative (Propionibacteria, Clostridia, Lactobacilli) bacteria were also detected as actively growing. Interesting was the identification in Cock Lake of two sequences related to the Betaproteobacteria (*Tepidimonas aquatica*) and to an uncultured *Chlorobium*-like organism, both of which might be involved in the sulfur and carbon cycle. Tepidimonas aquatica is a slightly thermophilic, facultatively chemolithoeterotrophic bacterium able to oxidize thiosulfate and tetrathionate, and able to grow up to pH 8.5 (Freitas *et al*., 2003). Since the phylum Green Sulfur Bacteria (GSB) are known to be a homogeneous physiological group, it can be assumed that members of this group inhabiting soda lakes might be involved in the sulfur cycle. This shows the necessity of looking more closely

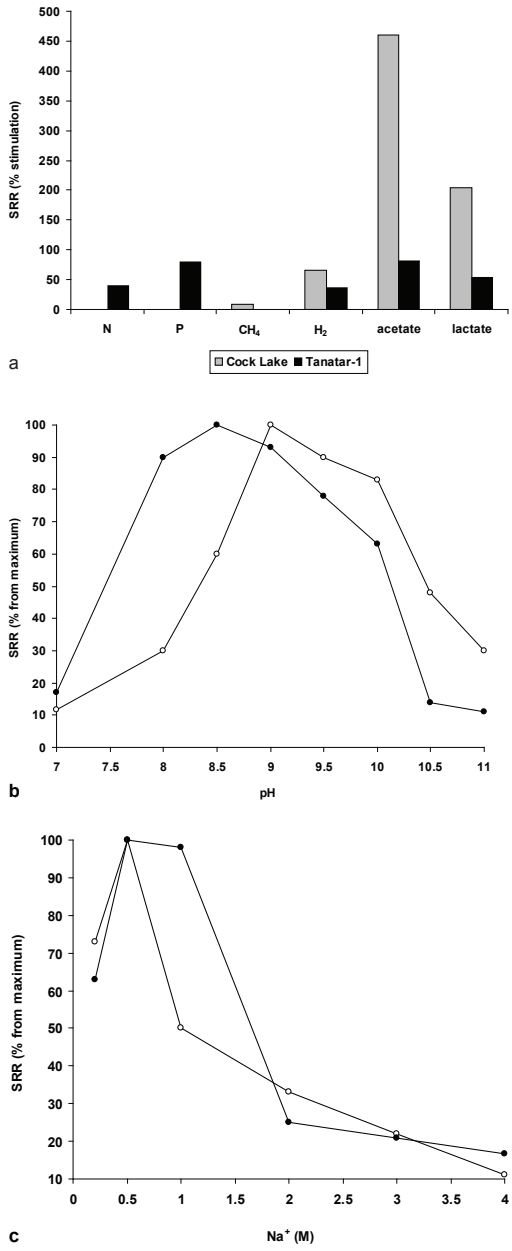


Figure 6.5: Influence of nutrients (a), pH (b) and salt (c) on SRR in the top seiment layer of Cock Lake (gray columns or open circles) and Tanatr-1 (black columns or closed circles), incubated for 48h at 30ºC. pH profiles were measured at 0.6 M total Na^+ for the Cock Lake sediments and at 2 M Na^+ for the Tanatr-1 sediments. Salt profiles were measured at pH 10 with sodium carbonate/ bicarbonate.

Table 3a: Closest relative of the detected sequences. □ = sequence retrieved from DNA-derived profiles; • = sequence retrieved from RNA-derived profiles

	Bands	Closest Relative	Similarity %	Acc nr	Source
Alfa	C-3_DVV •	UncAl431 *Brevundimonas* sp.	99.2 99.15	AY293404 AY145543	Travertine Terraces at Yellowstone Hot Springs; Fresh water
Beta	T3-2•; B-1•	*Massilia* sp.	99.6	DQ177478	Alkaline permafrost
	C-2 •	*Tepidimonas aquatica*	99.6	AY324139	Hot water tank
Gamma	CL-2_DCC□	UncHTC042 *Acinetobacter junii*	100 100	AB010848 AB101444	Deep Sea Activated Sludge
	B-1 •	*Acinetobacter venetianus*	99.2	AJ295007	
	T3-1•	*Moraxella osloensis*	99.1	AJ505859	Lake water
	E_C_H_2□	*Halomonas sp.EF43*niHail3	97 95	AF275709 AF280796	Hailaer soda lake Hungarian soda lake
	T3-2•; B-1•	*Stenotrophomonas* sp UniBac65	~99 ~88.5	AJ002814 X92132	Collemobola gut African soda lakes
	C-1_DCC□; T1-2•; T3-1□ ;T1-1□; B-1_DCC□	ML28_DCC1	94-98.5	AY245462	Mono Lake
Delta	T1-1_DCC•;T1-2_DCC•; B-1_DCC•; T3-1_DSB□; T1-1_DSB □	*Desulfosalina propionicus*	93-94.75	DQ067422	Great Salt Lake
	T3-1_DBB□	*Desulfobulbus propionicus*	99.8	AY548789	Sulfate-reducing fluidized-bed bioreactor
	C-1_DVV □	*Desulfonatronum thiodismutans*	98	AF373920	Mono Lake
	T3-1_DVV □	*Desulfonatronum lacustre*	98.6	AF418171	Soda lake
Actinobacteria	C-2•	UncMicr4 Microbacterium sp.35	99.81 99.81	AY569297 AF306542	Microbial mat Cellulolithic bacteria from Refuse Landfill
	C-2; T3-1•	Re0sea *Propionibacterium acnes*	99.7 98	AJ309523 AB108481	Brine sea-water Blood
	C-1_DFM□; T3-1_DFM□	UncAc655	98	DQ432396	African soda lakes

Table 3b: Continued from table 3a. Closest relative of the detected sequences. □ = sequence retrieved from DNA-derived profiles; • = sequence retrieved from RNA-derived profiles

Bacillus	T3-2_DFM•	*Bacillus thuringensis*	91.8	DQ078742	
	C-3_DFM•; T3-2_DFM•	*Lactobacillus curvatus*	99.3-100	AY281292	Chinese cabbage
	C-1_DCC•	UncLow16	90.5	AF507895	Mono Lake
	E_B_Ac□; T1-1□	UncLow98	87.7/	DQ432226	African soda lakes
Clostridia		*Haloanaerobium* sp.	85.5	AJ309520	Brine-sea water
			85 / 82		
	E_T1_Ac□; E_T1_H_2□; T1-1_DVV□; T1-1_DVV□; T3-2_DFM□	IRB1	99.1-	DQ631799	Soap Lake
		A. metalliredigenes	100	AY137848	Leachate ponds
			97.5		
	C-2_DFM□ ; C-3_DFM□	UncLow 12	92.5-	AF507890	Mono Lake
	C-3_DFM□	ML21_DFM1	97.6	AY245477	Mono Lake
			99.2		
	C-2_DVV□;T3-2_DVV□; B-1□	UncLow69	93-96.7	DQ206412	Mono Lake
	C-3•; B-1_DVV□; C-3_DVV□	UncLow11	95.2	AF507879	Mono Lake
	T1-1_DFM□; T3-2_DVV□	UncB3880	94.5-	AJ295640	Uranium mill tailing
		A. californiensis	94.2	AY064217	Mono Lake
			83-84		
	B-1_DVV•	*N.halophilum*	92.4	AJ271451	Lake Magadi
	B-1; B-1_DVV•	*E.saburreum*	98.4	AF287777	Oral strain
	C-3_DVV•	*Selenemonas sputigena*	94	AF287793	Oral strain
Cyanobacteria	C-1•	*Oscillatoria neglecta*	98.4	AB003168	Hot spring, New Zealand
	T3-1□	*Leptolyngbya* sp.	97.3	AB039012	Long Island, USA
Deinococcus-Thermus	C-1•	IC_M_5a	96.8	DQ028374	Alkaline ikaite columns
		Deinococcus murrayi	83.6	Y13043	Hot spring
	T3-1•; B-2•	*Hymenobacter aerophilus*	90	AJ276901	Air
			95.7	DQ015831	Lake water, Antarctica
Bacteroidetes-Chlorobi	C-2□; T1-1□	Unc25804	95-99.8	AF449772	Mono Lake
	C-3•; B-1□	UncB33772	84	AF445702	Travertine terrace
		UncChl57			

at the anaerobic phototrophic green sulfur bacteria in soda lakes. This Chlorobium-like sequence was detected in the deepest sediment layer, in which low light intensity and high sulfide are favorable for GSB. Unexpectedly the chemolithoautrophic sulfur-oxidizing bacteria of the genera *Thioalkalivibrio* and *Thioalkalimicrobium* have been not detected in this study despite the fact of high viable numbers (up to 10^6 cell cm^{-3}) and the isolation of 12 extremely natronophilic *Thioalkalivibrio* strains in pure culture (D. Sorokin, unpublished results). This clearly shows the limitation of the DGGE analysis method, which indicates major component, but is less suitable than cloning when microbial diversity is analyzed.

At the highest salinities a decrease of the bacterial genera richness was observed, with the actively growing groups in these lakes represented by the Delta- and Gammaproteobacteria, together with a minority of Clostridia (*Natronoanaerobium halophilum*). Saprophytic (*Acinetobacter* sp.), lipolytic (Stenotrophomonas sp.) and saccharolytic (*Eubacterium* sp., *Bacteroidetes*) bacteria were the dominant active groups in the Bitter Lake sediments.

Only few of the identified sequences were related to culturable organisms. Unexpectedly, none of those have been described as tolerant halo-alkaliphilic or halo-alkalitolerant (e.g. *Stenotrophomonas* sp., *Massilia* sp., *Brevundimonas* sp.). Still, the majority (90%) of the identified sequences were related to representatives, i.e. uncultured organisms, from alkaline and saline environments (Fig. 6.4), indicating that many haloalkaliphilic phylotypes remain unknown. One such example is a new cluster of Actinobacteria, from which the first culturable representative recently has been described. Strain ANL-iso2 was enriched and isolated from Kulunda Steppe soda lake sediments using isobutyronitrile as the only substrate. It is specialized in the degradation of C_3-C_6 aliphatic nitriles (Sorokin *et al.*, 2007b).

Sulfate reduction rates (SRR) and sulfate reducing community

Remarkably high SRR values have been observed in all 4 investigated soda lakes, especially in the top organic-rich sediment layer, with the exception of the sandy sediments of Tanatar-3, in which we measured the highest SRR in the deepest layer. The highest SRR were found in Bitter Lake-1, an organic-rich hypersaline lake. In contrast to other studies (Sörensen *et al*., 2004; Brandt *et al*., 2001), in which a remarkable decrease of the sulfate-reducing activity at salt concentrations greater than 12% was documented, we did not observe inhibition of SRR by increasing salinity. Brandt *et al.* investigated three different stations located in the moderate and hypersaline arms of the Great Salt Lake, Utah. A difference of factor 10 was observed between the stations at concentrations 120 g L^{-1} and 274 g L^{-1} of NaCl. Sorensen and coworkers (Sörensen *et al*., 2004) studied the salinity responses of the benthic microbial communities in the Solar salterns (Israel) and they also observed a strong inhibition of the sulfate reduction activity at salinity of 215 g L^{-1}. In case of the soda lakes investigated in this study, the highest SRR were found at the highest salinity. To the best of our knowledge these high SRR have never been measured before in hypersaline soda lakes. Only Sorokin *et al.* (2004) reported a higher measurement in a Mongolian soda lake, but at lower salinity (60 g L^{-1}). Life at high salt concentrations is energetically costly and organisms with a dissimilatory metabolism, which yield low energy, e.g. autotrophic nitrification, methanogenesis and complete-oxidizer SRB, may not be able to deal with high salinities for thermodynamic reasons (Oren, 1999). However, soda lakes are a specific type of saline lake in which $NaHCO_3$/Na_2CO_3 is the major salt in solution, a two times weaker electrolyte than sodium chloride, demanding approximately two times less energy to adapt to osmotic stress (Sorokin and Kuenen, 2005b). Furthermore, at concentrations higher than 2 M total Na^+, sodium carbonates are present only in their undissociated form, causing less stress than sodium chloride, which is fully dissociate

up to saturation (5 M Na^+). This might be one of the reasons of the observed unexpectedly high SRR in the investigated hypersaline soda lakes.

In a recent work conducted on two different soda lakes, i.e. Mono Lake and Searles Lake, Kulp and coworker (2007) found different results. They observed a reduction of the SRR in Mono Lake with increasing salinity and no SRR at all was measured in Searles Lake, in which the sulfate reduction activity seemed to be inhibited by the high concentrations of borate. However, they do imposed the salinity gradients in the laboratory, without analyzing similar lakes with increasing salinities, as we do.

Even if we measured the highest SRR at the highest salt conditions, SRR incubation experiment conducted at different salt concentration (see Fig. 6.5c) showed the optimum at 1 M total Na+, which was two times lower than the actual salt concentration. Furthermore, the optimum pH measured in the similar experiment was also lower (pH 8.5-9) than the natural pH, suggesting that the indigenous SRB population is living under stress in these extreme environments. Spiking experiments demonstrated that the SRB populations are limited by availability of electron donors at both salinities, but especially in low-salt lakes, whereas nitrogen and phosphate were required only in high-salt sediments. These results might be explained by a higher microbial activity and nutrient turnover in the low-salt Cock Lake. Most likely acetate is utilized only by a small, but highly active population, which might correspond to the detected *Desulfosalina propionicus*–like organisms. In fact, at the highest salt concentrations, at which the highest SRR were measured, this group of organisms has been persistently detected by molecular analysis, both by DNA- and RNA-based approaches. *Desulfosalina propionicus*, a complete oxidizing halophilic SRB (Kjeldsen *et al*., 2007), can grow at salinity up to 190 g L^{-1}, using propionate as a carbon source and electron donor. Since Propionibacteria have also been detected in the investigated lakes, there might be a trophic link between these groups of primary and secondary anaerobes in soda lake sediments. In our previous study (Foti *et al*., 2007), the predominance of the *Desulfosalina*-like sequences was also observed when targeting the *dsr*B gene, indicating the relevance of these bacteria in soda lakes of the Kulunda Steppe. So far, complete oxidizing SRB have never been isolated from soda lakes with extreme salinity, therefore it is important to identify these organisms and to infer their functional role in saline lakes.

One of the goals of this research was to determine the diversity and activity of SRB communities along a salinity gradient. In general, this question was only partly answered due to non specific PCR amplification, resulting mainly in the detection of non-SRB low G+C Gram-positive bacteria. This was also observed in another study conducted on Mono Lake (Scholten *et al*., 2005), suggesting that the specific amplification of the 16S rRNA gene from six SRB-groups is not recommended in environments rich in low-G+C organisms. It is possible that preferential amplification of the DNA template from low G+C content occurred due to the easier dissociation into single DNA strand than higher G+C templates, resulting in a bias in the favor of the former group (Suzuki and Giovannoni, 1996). On the other hand, it could also be that low G+C bacteria, like acetogenic clostridia, out-number SRB at hypersaline alkaline conditions, since it is known that the acetogenic haloalkalwiphiles benefit from sodium-based energetics (Häse *et al*., 2001). This was also observed by DGGE analysis and microscopic analysis of the highest positive tubes of the serial dilutions in SRB media, which revealed the predominance of Clostridia even at the highest dilutions. The discrepancy between observed high SRR rates/SRB viable count and the detection of SRB by molecular techniques may in fact imply that sulfate-reducers are represented by very active, but numerically minor populations in soda lake sediments.

Concluding, in this work we investigated the bacterial diversity and activity in sediments along a salinity gradient in different soda lakes of the Kulunda Steppe in south-eastern Siberia. For the first time the actively growing bacterial groups, based on ribosomal RNA determination, have been detected by culture-independent techniques with special attention to SRB representing one of the most active bacterial functional groups in this extreme habitat. A new cluster of complete oxidizing SRB seems to be active in hypersaline soda lakes. In agreement with previous studies a decrease of the general bacterial diversity has been observed with increasing salinity, in contrast to the sulfate reduction activity, which seems to be well adapted even to saturating concentrations of sodium carbonates.

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Chapter 7

Desulfurispirillum alkaliphilum gen. nov. sp. nov., a novel obligately anaerobic sulfur- and dissimilatory nitrate-reducing bacterium from a full-scale sulfide-removing bioreactor

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ABSTRACT

Strain SR 1^T was isolated under anaerobic conditions using elemental sulfur as electron acceptor and acetate as carbon and energy source from the Thiopaq bioreactor in Eerbeek (The Netherlands), which is removing H_2S from biogas by oxidation to elemental sulfur under oxygen-limiting and moderately haloalkaline conditions. The bacterium is obligately anaerobic, using elemental sulfur, nitrate and fumarate as electron acceptors. Elemental sulfur is reduced to sulfide through intermediate polysulfide, while nitrate is dissimilatory reduced to ammonium. Furthermore, in the presence of nitrate, strain SR 1^T was able to oxidize limited amounts of sulfide to elemental sulfur during anaerobic growth with acetate. The new isolate is mesophilic and belongs to moderate haloalkaliphiles, with a pH range for growth (on acetate and nitrate) from 7.5 to 10.25 (optimum 9.0), and a salt range from 0.1 to 2.5 M Na^+ (optimum 0.4 M). According to phylogenetic analysis, SR 1^T is a member of a deep bacterial lineage, distantly related to *Chrysiogenes arsenatis*. On the basis of the phenotypic and genetic data, the novel isolate is placed into a new genus and species, *Desulfurispirillum alkaliphilum* (type strain SR^T = DSM 18275 = UNIQEM U250).

Introduction

The full-scale Thiopaq bioreactor in Eerbeek (The Netherlands) is aimed for the removal of H_2S from biogas, which is produced during anaerobic treatment of wastewater from paper-mill factories. The process is based on recently developed biotechnology of preferential sulfide oxidation to insoluble sulfur by lithoautotrophic sulfide-oxidizing bacteria under oxygen limitation (Buisman *et al.* 1990; Janssen *et al.* 1995). During a first physico-chemical step of the process, H_2S from the biogas is absorbed at a pH of around 8.7 by an alkaline solution, containing $NaHCO_3$. The absorbed alkaline sulphide solution is fed into the bioreactor, operating at 35-45 ^oC and (-300)-(-350) mV under oxygen-limiting conditions to facilitate incomplete oxidation of sulphide to elemental sulphur. This situation creates perfect conditions for a complete sulfur cycle, whereby both aerobic and anaerobic reactions involving sulfur compounds are possible. Lithoautotrophic sulfide-oxidizing bacteria are the dominant populations in such reactors. They are producing elemental sulfur, as a major product, and thiosulfate and sulfate, as by-products, from sulfide, and organic compounds from CO_2. The products then can be utilized by anaerobic sulfur-reducing, thiosulfate-reducing and sulfate-reducing bacteria, which are considered as unwanted components in such reactors (Buisman, 1989). Furthermore, the development of a sulfur-reducing population in the reactor is facilitated by the special properties of biologically-produced sulfur, which, in contrast to chemically-produced sulfur, is hydrophilic and has properties of colloidal particles (Janssen *et al.* 1996). Indeed, anaerobic enrichments at moderately haloalkaline, mesophilic conditions indicated the presence of an active population of the latter type, and resulted in the isolation of a haloalkaliphilic sulfur-reducing (respiring) bacterium strain SR 1, which is described below. Despite the intensive characterization of different functional groups of haloalkaliphilic bacteria (Jones *et al.* 1998; Zavarzin *et al.* 1999; Sorokin and Kuenen 2005), sulfur-respiring and dissimilatory nitrate-reducing representatives have yet not been found among the haloalkaliphilic communities in soda lakes. The bacterium described below represents a first example of such a type.

Methods

Cultivation

Enrichment and routine cultivation of haloalkaliphilic sulfur-reducing bacteria was performed at 30 ^oC on a mineral medium containing sodium-bicarbonate, 0.6-0.8 M, 0.1 M NaCl, and 0.5 g/l of K_2HPO_4. The pH was adjusted to 9.0 by addition of Na_2CO_3. After sterilization, the medium was supplemented with 20 mM acetate as carbon and energy source, 50 mg/l of yeast extract, 4 mM NH_4Cl, 1 mM $MgSO_4$, and 1 ml/l of trace metal solution (Pfenning and Lippert, 1966). Elemental sulfur was obtained from the Eerbeek Thiopaq reactor sludge as a thick suspension. It was separated from the cells by low-speed centrifugation, washed several times with distilled water, sterilized as 20% (v/v) suspension in distilled water, washed again several times with sterile distilled water, and used at a final concentration of 30 mM. Other electron acceptors used were KNO_3 (20 mM), KNO_2, Na_2SO_3, sodium selenate and selenite, sodium arsenate, DMSO (5 mM each), sodium fumarate (20 mM; alone or with 2 mM acetate as carbon source), and freshly prepared ferrihydrite (20 mM). Growth at microoxic conditions was tested with an oxygen concentration in the gas phase from 1% to 5%. Anaerobic cultivation was performed in 60-100 ml serum bottles with 40-80 ml medium whereby air was replaced with argon. In some cases argon was replaced by H_2 with 0.5 bar overpressure. Solid alkaline media with a final salt concentrations of 0.5 M Na^+ was prepared by 1:1 mixing of 4% (w/v) agarose and 1 M Na^+ mineral medium at 50^oC. The plates were incubated in closed jars

under argon atmosphere with an oxygen-scavenging catalyzer (Oxoid). The pH dependence was examined at Na^+ content of 0.6 M, using the following filter-sterilized mineral medium: for pH 6-8, 0.1 M HEPES and NaCl; for pH 8-11, a mixture of sodium bicarbonate/sodium carbonate containing 0.1 M NaCl. Growth resulted in a shift of initial pH values, especially in highly alkaline region; so only final pH values were used as suitable range for growth. To study the influence of salt concentration on growth, mineral media, containing 0.1 and 3.0 M of total Na^+ were mixed in different proportions.

Analytical procedures

Chemical analysis of sulfur (sulfide, polysulfide, sulfur) and nitrogen (nitrate, nitrite, ammonium) compounds, and cell protein were performed as described previously (Sorokin *et al.* 2001; 2004). Phase contrast microphotographs were obtained with a Zeiss Axioplan Imaging 2 microscope (Göttingen, Germany). For electron microscopy, the cells were fixed with glutaraldehyde (final 3% v/v) and positively contrasted with 1% (w/v) uranyl acetate. Respiratory lipoquinones and polar lipids were extracted from 100 mg of freeze dried cells using the method described by Tindall (1990 a,b). Respiratory lipoquinones were separated into their structural classes (menaquinones, ubiquinones etc.) by TLC, bands eluted and further separated and identified by HPLC, using an RP18 column (Tindall, 1996). Polar lipids were separated by two-dimensional chromatography and identified on the basis of their Rf values in combination with their reaction with specific staining reagents (Tindall, 1990a, b). Fatty acid methyl esters were released from 20 mg freeze dried cells using methodologies which release only ester linker fatty acids or ester and amide linked fatty acids (Labrenz *et al*., 2005; Strömpl *et al*., 1999).

Genetic and phylogenetic analysis

The isolation of the DNA and determination of the G + C content of the DNA was performed according to Marmur (1961). Genomic DNA was extracted from the cells using the UltraClean Soil DNA Extraction Kit (MolBio Laboratories, USA), following the manufacture's instructions. The nearly complete 16S rRNA gene was obtained using general bacterial primers GM3f (5'-AGAGTTTGATCCTGGCTCAG-3') and GM4r (5'-TACGGTTACCTT GTTACGACTT-'). Sequencing was performed by the company BaseClear (Leiden, The Netherlands). The sequence was first compared to sequences stored in the GenBank database using BLAST search tool. Subsequently, the sequence was imported into the ARB software program (Ludwig *et al*., 2004), automatically aligned and manually checked. A tree was then generated using maximum likelihood.

Results and discussion

Enrichment and isolation of pure culture

Primary anaerobic enrichment cultures with acetate/sulfur were positive for a pH range from 8 to 10, and salt concentrations from 0.4 to 1.5 M total Na^+, closely matching the reactor conditions. During growth, two stages of sulfur transformation were observed: in the beginning, when sulfur was in excess, greenish-yellow polysulfide (Sn^{2-}) was produced, which accumulated until all sulfur was dissolved. After that, the color started to disappear and all sulfur was finally converted to sulfide. MPN dilution series indicated the presence of up to 10^6 viable cells in 1 ml of reactor sample. From the highest positive dilution, a pure culture was isolated using sulfur-containing plates. Sulfur-reducing colonies formed clearing of the insoluble sulfur

around them with yellow polysulfide as an intermediate stage (Fig. 7.1a). The strain was designated SR 1^T. The new isolate is a thin spirillum (Fig. 7.1b), highly motile with bipolar flagella (Fig. 7.1c).

Growth characteristics and metabolism

SR 1^T grew anaerobically at pH 9 and salt concentration of 0.6 M Na^+ using biologically-produced sulfur as electron acceptor, which was first fully dissolved as polysulfide due to a chemical reaction with produced sulfide under alkaline conditions. At a later stage, polysulfide disappeared and sulfide was the final product (Fig. 7.2a). Chemical analysis demonstrated the presence of S_3^{2-} (greenish) and S_4^{2-} (yellow) as the dominant polysulfide species. The strain also could initiate growth with polysulfide (S_6^{2-}) instead of sulfur, but at a concentration of sulfan (terminal sulfide atoms) not higher than 10 mM. The μ_{max} for growth under sulfur-reducing conditions was 0.12 h^{-1}. From other tested electron acceptors, nitrate and fumarate were utilized. In case of nitrate, ammonia was identified as the final product, so the novel isolate belongs to dissimilatory nitrate reducers. Growth with acetate and nitrate as electron acceptor, instead of sulfur, was faster (μ_{max}=0.19 h^{-1}), but the culture stopped growing before

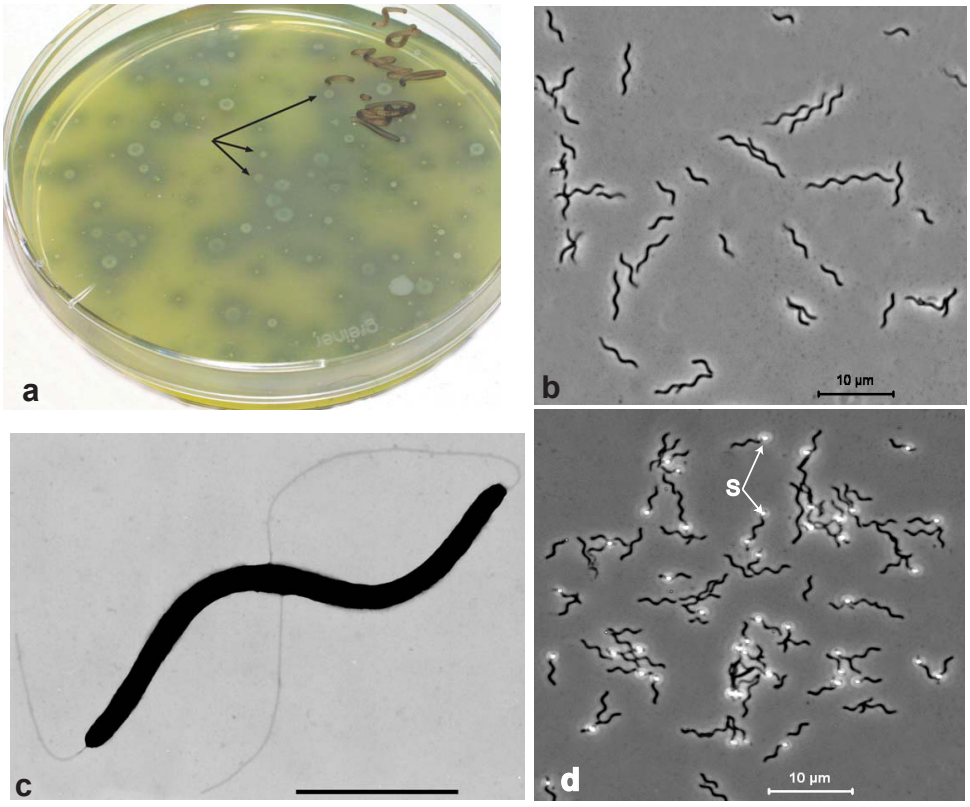

Figure 7.1: Morphology of SR 1. (a) colonies obtained from enrichment culture on sulfur-containing alkaline agar; sulfur was first converted to polysulfide (greenish-yellowish background) and finally to sulfide indicated by clearing zone around the active colonies. (b) phase-contrast microphotograph of cells grown with acetate and nitrate. (c) electron microphotograph of positively stained cell, bar=1 µm. (d) phase-contrast microphotograph of cells accumulating intracellular sulfur (S) during oxidation of sulfide.

all the nitrate was reduced, probably due to toxicity of accumulating ammonia at high pH (Fig. 7.2b). Nitrite was not detected as an intermediate, however, it was the main product of nitrate reduction in experiments with washed cells. Most probably the nitrite-reductase was partially inactivated during cell harvesting under aerobic conditions. In case when both sulfur and nitrate were present as electron acceptors, sulfur reduction was initiated first and only after complete conversion of sulfur to sulfide nitrate reduction started slowly, in contrast to what might be expected from the rule of red-ox potentials. Most probably, the accumulation of polysulfide blocked nitrate reduction. A noticeable difference in color was observed for cells grown at sulfur-reducing and nitrate-reducing conditions: the sulfur-reducing biomass was black, while the nitrate-reducing biomass was bright red, due to a high concentration of a cytochrome c_{554} (data not shown), probably associated with the presence of dissimilatory nitrite reductase *ccNIR* (Simon 2002).

The bacterium could grow anaerobically in presence of fumarate as the only substrate (μ_{max}=0.05 h^{-1}). Although we did not analyze the products, the only possible explanation for the growth at such conditions is fumarate fermentation. Addition of acetate as carbon source did not enhanced the final growth yield in fumarate-grown cultures. Addition of nitrate also did not affect the growth efficiency on fumarate, although up to 5 mM nitrate was finally reduced to ammonia.

SR 1^T can utilize H_2 as an alternative electron donor when acetate was supplied in limited amount (2 mM instead of 20 mM) as the C source both with sulfur or nitrate as electron acceptors. In the presence of H_2, the biomass yield increased 4-5 times with sulfur and 2-3 times with nitrate, respectively. With fumarate as the only substrate, addition of H_2 did not stimulate the growth.

In experiments with washed cells, grown with acetate, nitrate and sulfide, it was H_2, but not acetate, which was utilized as the preferable electron donor, and it was especially active in case of elemental sulfur as the electron acceptor. In case of nitrate, the product was nitrite.

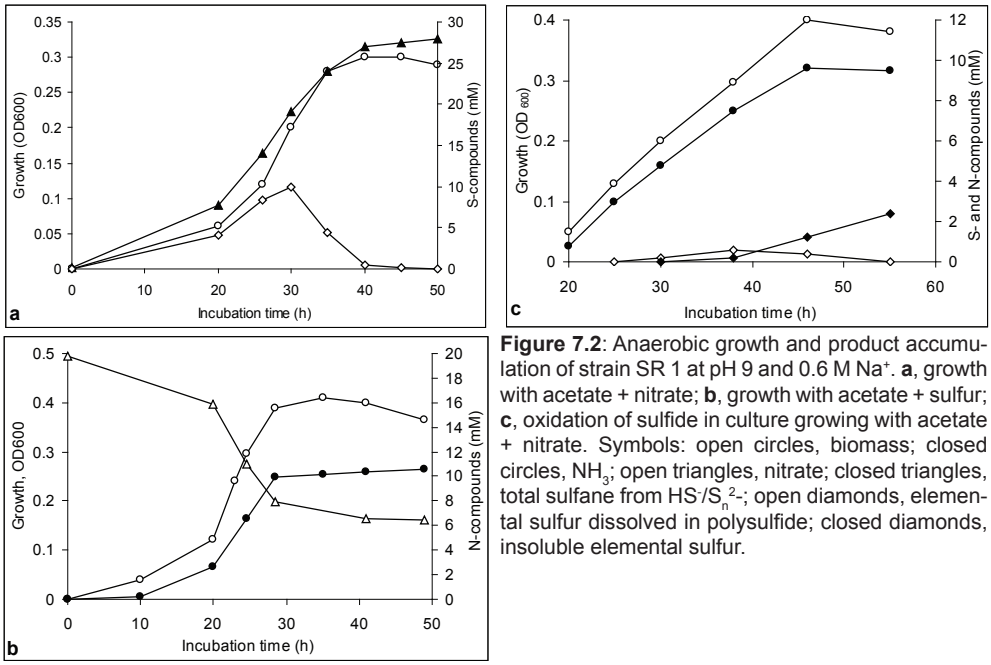

Figure 7.2: Anaerobic growth and product accumulation of strain SR 1 at pH 9 and 0.6 M Na^+. **a**, growth with acetate + nitrate; **b**, growth with acetate + sulfur; **c**, oxidation of sulfide in culture growing with acetate + nitrate. Symbols: open circles, biomass; closed circles, NH_3; open triangles, nitrate; closed triangles, total sulfane from HS^-/S_n^{2-}; open diamonds, elemental sulfur dissolved in polysulfide; closed diamonds, insoluble elemental sulfur.

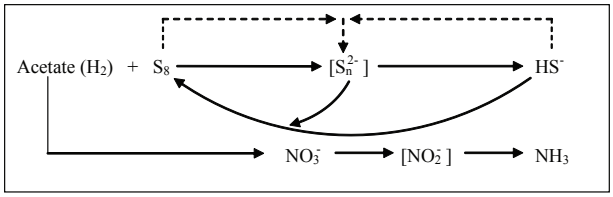

Figure 7.3: Scheme of red-ox interaction of sulfur and nitrogen catabolism in SR 1. Dashed lines indicate spontaneous reaction.

Successful initiation of nitrate-reducing cultures was facilitated by adding sulfide as a reductant at 0.5-1 mM concentrations. At the end of growth sulfide was converted to elemental sulfur. So it seemed that SR 1^T could reverse sulfur reduction reaction. This ability was proven by growing the isolate with 20 mM acetate, 20 mM nitrate and 3 mM sulfide (Fig. 7.2c). Sulfide oxidation started in the late logarithmic phase with polysulfide as an intermediate and elemental sulfur as the final product. First, all elemental sulfur can be seen as tiny droplets inside the cells (Fig. 7.1d), which, on later stage, were excreted into the medium. With fumarate as the electron acceptor, the oxidation of sulfide, either in growing cultures or by washed cells, was not observed. The ability to oxidize sulfide to elemental sulfur was clearly manifested in the experiments with washed cells in presence of both nitrate and nitrite as electron acceptors. The estimated rate of sulfide oxidation in the presence of nitrate at pH 9 was 26 nmol (mg protein min)$^{-1}$.

The results suggest an interesting combination of sulfur and nitrogen red-ox metabolism in SR 1^T as summarized in Fig. 7.3. The dissimilatory nitrate- and sulfur-reducing Epsilonpoteobacteria *Wolinella succinogenes* (Bokranz *et al*., 1983; Macy *et al*., 1986; Klimmeck *et al*., 1991) and *Sulfurispirillum deleyianum* (Schumaher *et al.* 1992; Eisenmann *et al*., 1995) are also capable of reversed reaction of sulfide oxidation to sulfur, for which polysulfide reductase is held responsible (Krafft *et al*., 1995), but the oxidation of sulfide was observed both in the presence of nitrate and fumarate as the electron acceptor. This indicates that the conditions whereby sulfur and nitrogen species can be interconverted within the same ecological niche might be common in certain natural habitats as well as in the industrial bioreactors.

Influence of pH and salts on the growth of strain SR 1^T was examined using nitrate as electron acceptor. The results demonstrated that the reactor isolate is a moderate, but obligate haloalkaliphile (Fig. 7.4). Growth at a salt content between 1.5 and 2.5 M Na^+ was only possible after gradual adaptation, but not directly using the cells from the low-salt cultures.

Genetic analysis

The G + C content in the genomic DNA of strain SR 1^T was 44.8±0.5 mol% (T_m). Phylogenetic analysis based on nearly complete sequence of the 16S rRNA gene placed the novel isolate into a deep bacterial lineage with the arsenate- and nitrate-respiring anaerobic bacterium *Chrysiogenes arsenatis* as the closest described relative (Fig. 7.5). Low sequence similarity (91%) indicated that SR 1^T represents a new genus within the family Chrysiogenetes. Among the unrecognized members of this group, SR 1^T had a much closer relative, strain TE37, which has recently been isolated as an iron reducer from the deep-subsurface alkaline fluids within the Chinese Continental Drilling project (Zhang *et al*., 2005). Sequence similarity of 98% indicated relation on the species level (Fig. 7.5). It seems not a mere coincidence, since the dominant sulfur-oxidizing bacterium from the same bioreactor (*Thioalkalivibrio* sp.) turned out to have a very close relative among one of the clones (CCSD_DF730_B8) identified within the Chinese Continental Drilling project (our unpublished results).

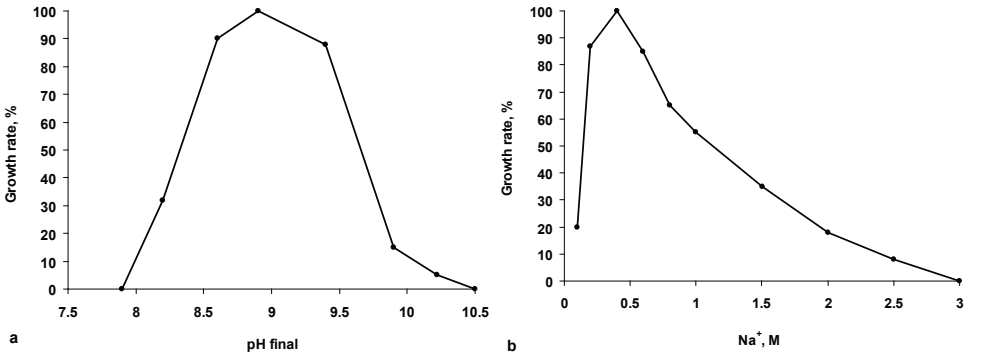

Figure 7.4: Influence of pH at 0.6 M Na^+ (**a**) and sodium carbonate/bicarbonate at pH 9 (**b**) on anaerobic growth of strain SR 1 with acetate and nitrate.

Chemotaxonomy

Examination of the respiratory lipoquinone composition in SR 1 indicated that menaquinones were the sole respiratory lipoquinones present, and were dominated by the MQ-8. The major polar lipids were phospholipids, no evidence being found for glycolipids or amino acid based lipids. The major components were phosphatidylglycerol, and phosphatidylethanolamine, with smaller amounts of an unidentified phopsholipid, diphosphatidylglycerol, together with traces of two additional unidentified phospholipids and an amino-phospholipid. The fatty acids were dominated by the 18:1w7c species. Interestingly, SR 1 also contained multiple isomers of unsaturated 16:1 fatty acids (Table 7.1). In addition a single unidentified derivative of a 3-OH amide-linked fatty acid was detected, which might be an indicative of the presence of a cell wall-bound lypopolysaccharide.

Overall, strain SR 1^T, isolated from sulfide-removing haloalkaline bioreactor, represents a first example of an obligatory anaerobic sulfur- and dissimilatory nitrate- reducing alkaliphile. Although it shares several key physiological properties with some mesophilic members of the Epsilonproteobacteria, its distant phylogenetic position and haloalkaliphily makes it unique. Therefore, the new isolate is proposed to be assigned into a new genus and species *Desulfurispirillum alkaliphilum* within the family *Chrysiogenetes*. The phenotypic comparison of SR 1^T with its closest culturable relative and Epsilonproteobacteria with similar metabolism is given in Table 7.2.

Description of *Desulfurispirillum* gen. nov. sp. nov.

Desulfurispirillum (De.sul.fu.ri.spi.ril' lum. L. pref. *de* from; L. n. *sulfur* sulfur; Gr. n. *spira* spiral; M. L. dim. neut. n. *Spirillum* a small spiral. M. L. neut. n. *Desulfurispirillum* a spirillum that reduces elemental sulfur).

Gram-negative, motile spirillas. Obligately anaerobic with respiratory metabolism. Use elemental sulfur and nitrate as electron acceptors, and short-chain fatty acids and hydrogen as electron donors. Do not grow autotrophically. Alkaliphilic and slightly halophilic. Menaquinones are the dominant respiratory lipoquinones. Phosphatidylglycerol and phosphatidylethanolamine are the dominant polar lipids. Belongs to the family *Chrysiogenetes*. The type species is *D. alkaliphilum*.

Description of Desulfurispirillum alkaliphilum sp. nov.

alkaliphilum (al.ka.li.phi'lum M.L. n. alkali soda ash; Gr. adj. phylum loving; M.L. adj. alkaliphilum loving alkaline conditions)

Cells are slender spirals, 0.15-0.20 x 2-5 µm, motile by single bipolar flagella. Gram-negative. Strictly anaerobic with respiratory metabolism. Use elemental sulfur, polysulfide, nitrate and fumarate as electron acceptors. The final products are sulfide and ammonium. Obligatory heterotrophic. Utilizes short-chain fatty acids, such as acetate, lactate, pyruvate, propionate, citrate and fumarate as electron donor, and carbon source; H_2 can be used as electron donor. Formate and methanol are not utilized. Can oxidize sulfide/polysulfide to elemental sulfur intracellularly in the presence of nitrate as electron acceptor. Moderately alkaliphilic with a pH range for growth between 8.0 and 10.2 and an optimum at pH 9.0 and slightly halophilic with a salt range from

Fatty acid	% from total
12:1	3.6
12:0	2.3
3OH 12:1?	2.3
3OH 14:1?*	2.3
16:1a	1.3
16:1w7c	5.3
16:1b	3.9
16:0	15.3
cyclo 17:0	1.3
18:1w7c	57
methyl 18:1	2.9
18:0	3.7

? indicates uncertain identification;
* indicates amide-linked fatty acid

Table 7.1: Fatty acid composition of polar lipids in SR 1

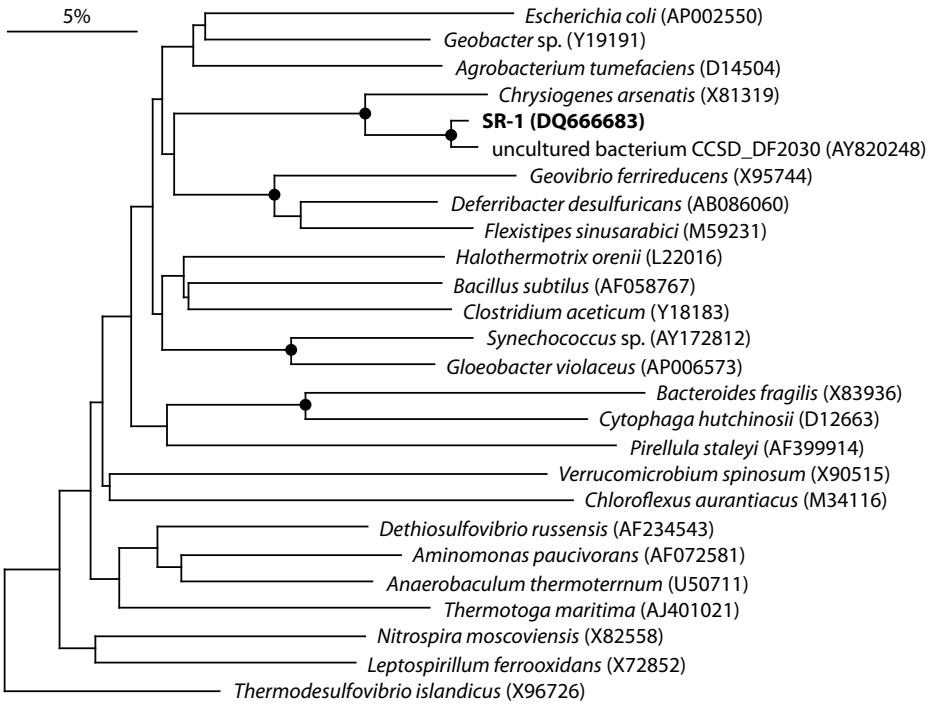

Figure 7.5: Phylogenetic position of strain SR 1^T based on 16S rDNA sequence analysis. The tree was constructed using maximum likelihood method and filter. The scale bar represents 10 nucleotide changes per 100 nucleotides. The percentage of bootstraps was derived from 1000 resampling using neighbour joining algorithm, only values greater than 95 are given. The sequence of *Thermotoga maritima* was used as an outgroup.

Table 7.2: Phenotypic comparison of strain SR 1 and related bacteria

Characteristic	*Chrysiogenetes*		Epsylonproteobacteria	
	SR 1	*Chrysiogenes arsenatis*	*Wolinella succinogenes*	Genus *Sulfurispirillum*
Cell morphology	Spirillum, single polar flagellum	Vibrio, single polar flagellum	Spirillum, single polar flagellum	Spirillum, single polar flagellum
Autotrophic growth	-	-	-	+
Electron donors:				
H_2	+	-	+	+
formate	-	-	+	+
HS^-	+	nd	+	+
acetate, fumarate, succinate, lactate, pyruvate	+	+	+	+
Electron acceptors:				
O_2	-	-	+	+
sulfur (polysulfide)	+	-	+	+
nitrate (>>NO_2^->>NH_3)	+	+	+	+
N_2O	-	-	+	-
fumarate	+	-	+	+
arsenate	-	+	-	+/-
DMSO	-	-	+	+/-
selenate	-	-	-	+/-
Fe^{3+}	-	-	-	+/-
$S_2O_3^{2-}$/SO_3^{2-}	-	-	+	+/-
Oxidation of HS^- with:				
nitrate	+	n.d.	+	+
fumarate	-	n.d.	+	+
pH range (optimum)	8.0-10.2 (9.0)	neutrophilic	neutrophilic	neutrophilic
Salt range (M Na^+)	0.1-2.5	Na-independent	Na-independent	Up to 0.5
Habitat	Bioreactor	Gold mine	Rumen fluid	Marine sediments

n.d., not determined; =, present; +/-, present in some species; - absent

0.1 to 2.5 M Na^+ (optimum at 0.4 M). Mesophilic with a maximum temperature for growth at 45 and an optimum at 35 °C. The main respiratory lipoquinone is MQ-8. The predominant fatty acids in the membrane lipids include 12:1, 12:0, 16:1a, 16:1b, 16:1w7c, cyclo 17:0, 18:1w7c, 18:0 and a methyl 18:1. The G + C content of the genomic DNA is 44.8±0.5 mol% (Tm).

The type strain is SR 1^T (=DSM 18274 = UNIQEM U250). Isolated from a full-scale sulfide-removing bioreactor in the Netherlands. The GenBank 16S rDNA sequence accession number is DQ666683.

Acknowledgements

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Chapter 8

General discussions, conclusions & outlook

General Discussions and Conclusions

Soda lakes are naturally occurring halo-alkaline environments, characterized by high to extremely high carbonate alkalinity and moderate to extremely high salinity. Soda lakes represent a unique ecosystem, which makes them highly interesting from a (micro-) ecological point of view and for industrial application due to their double extreme conditions (Chapter 1). This thesis focuses on the ecology of halo-alkaliphilic sulfur-oxidizing (SOB) and sulfate-reducing bacteria (SRB) in natural and bioengineered ecosystems. In particular, aspects like diversity, activity and biogeography have been investigated. This PhD research was involved in the development of a new biological process for hydrogen sulfide removal under halo-alkaliphilic conditions. The process consists of a scrubber and two integrated biological reactors. In the scrubber hydrogen sulfide gas (H_2S) is absorbed by an alkaline carbonate solution. The dissolved sulfide (HS^-) is subsequently oxidized under oxygen limited conditions to elemental sulfur (S_0) by SOB. Up to 10% of the sulfide is further oxidized to the undesired thiosulfate ($S_2O_3^{2-}$) and sulfate (SO_4^{2-}), which are fed to the second anaerobic bioreactor. Here they are reduced back to sulfide by halo-alkaliphilic SRB. Because both bioreactors are integrated, the sulfide can be subsequently recycled to the first, sulfur producing, reactor. Therefore, for a better functioning of the process, a better understanding of the halo-alkaliphilic SOB and SRB was necessary. A number of fundamental questions had to be answered concerning their (eco)physiology, their competitive capabilities and they response to varying process conditions. In the present thesis we tried to answer some of these questions combining culturable-dependent and –independent techniques: “Does the salinity in soda lakes affect the microbial structure? Can sulfate reduction activity still occur at hypersaline conditions? Are extremophilic organisms endemic to their place of origin? How can this knowledge be used for the improvement of the operation of the reactors in the industrial process?”

Since the process for biological removal of sulfide is operating at extremely halo-alkaliphilic conditions, specialized bacteria that are able to withstand such conditions were needed. Recently two new genera of halo-alkaliphilic SOB, i.e. *Thioalkalivibrio* and *Thioalkalimicrobium*, suitable for the purpose have been described. They have been isolated from soda lakes and grow at an optimum pH of 9.5-10. The genus *Thioalkalivibrio* shows a so called `K-strategy`, with low growth rate, high growth yield and long survival during starvation. The genus *Thioalkalimicrobium* shows an opportunistic `R-strategy`, with high growth rates and low growth yield. Most of the *Thioalkalivibrio* species are high-salt adapted, while the genus Thioalkalimicrobium contains only low salt-tolerant members. These two types of haloalkaliphilic SOB occupy two distinct ecological niches (Chapter 4). Since the process was meant to operate at extremely high salt conditions (2-4 M Na^+), *Thioalkalivibrio* was selected to operate in the oxidation of sulfide/polysulfide in the aerobic step of the process.

In our laboratory more than 100 strains of *Thioalkalivibrio* have been isolated from different lakes located in different countries, i.e. California (USA), Egypt, Kenya, Mongolia and Kulunda Steppe (South-East Siberia, Russia) (Sorokin, Kuenen, 2005). *Thioalkalivibrio* is a very diverse group, with high genetic and phenotypic heterogeneity. Some strains show similar physiological properties but are not closely related phylogenetically, whereas the others are phenotypically and physiologically very similar but genotypically different. Therefore it was of ecological interest and utility to discriminate between the *Thioalkalivibrio* strains originating from different locations, using a molecular technique with high, i.e. strain-level resolution (Chapter 2). This study revealed a very high genetic diversity within this ecophysiological group with a relatively restricted phenotype. The high genetic diversity might be the

result of neutral mutations, which is an important mechanism in molecular evolution, or DNA rearrangements; but most likely this microdiversity is due to micro niche specialization. The majority of the investigated strains were endemic to their place of origin and a correspondence was found between genotypes and properties like salinity or oxygen demands. Therefore we propose that this high genetic diversity is a result of adaptations to highly heterogenous conditions within one ecosystem.

The discrimination between *Thioalkalivibrio* strains within a mixed population was also necessary for the sulfide removal process, in order to determine which particular strains were most successful and therefore most suitable at full-scale conditions. A mixed culture of extremely halophilic *Thioalkalivibrio* strains, together with soda lake sediment sample, was used as a starting biomass for two laboratory-scale aerobic H_2S-oxidizing bioreactor experiments (Pim van den Bosch, WUR). In this case, DGGE (Denaturing Gradient Gel Electrophoresis) analysis of the ITS (Internal Transcribed Region) region was performed (Chapter 3). We observed that even if relatively phylogenetically distant strains of *Thioalaklivibrio* were tested, resolution of the bands was not possible. Literature data show that separation of amplicons with as few as a single different nucleotide is sometimes possible. However, in other examples multiple sequence differences cannot be discriminated with DGGE (Jackson *et al*., 2000). This might be due to the position of the different nucleotides within the analyzed fragment or to the sharing of the same GC content, both of which can influence the amplicon's melting behaviour (Jackson *et al*., 2000; Kowalchuk *et al*., 1997). In our case, it is very likely that the GC content was the reason for this co-migration effect. DGGE analysis was therefore not suitable for our purpose and another less time-consuming molecular technique should be applied to monitor the dynamics of *Thioalkalivibrio* strains in a bioengineered ecosystem.

In the industrial process sulfide/polysulfide are oxidized to sulfur, which is removed by sedimentation. Thiosulfate and sulfate, which are inevitably formed as undesired byproducts, are fed to an anaerobic bioreactor, in which sulfate-reducing bacteria (SRB) are responsible for the reduction of sulfate/thiosulfate back to sulfide. Since the new process will have to operate at high pH and salt concentrations, alkaliphilic halotolerant sulfate-reducing bacteria would be required for the second reactor. However, very little is known about this group at halo-alkaliphilic conditions. Up to date only two genera, i.e. *Desulfonatronum* and *Desulfonatronovibrio* and few species have been isolated from soda lakes and described. They all are low-salt tolerant organisms and therefore are not suitable for the development of the high-salt process. In this thesis we gave our contribution for a better understanding of the SRB community and the sulfate-reduction process in soda lakes (Chapter 5-7). We showed that even at hypersaline conditions the sulfate-reducing community is highly active (Chapter 5-6); for extremely alkaline and saline lakes we measured even higher sulfate-reduction rates (SRR) than for those less extreme environments. Additionally, we also showed that the sulfate reduction process seems not to be influenced by increasing salinity (Chapter 6), as observed in other studies (Benlloch *et al*., 2002; Sörensen *et al*., 2004), probably due to the difference in the electrochemical nature of sodium chloride and carbonate/bicarbonate (Chapter 6). This finding is highly interesting for applications, showing that the anaerobic bioreactor can also operate at hypersaline conditions. The detection and identification of the main organisms involved in sulfate reduction in the soda lake sediments was done by PCR-based molecular techniques, using both functional-, i.e. *dsr*AB (chapter 5), and the 16S rRNA gene (Chapter 6). The *dsr*AB gene encodes for the dissimilatory (bi)sulfite reductase, a key enzyme in the sulphate reduction pathway. It catalyzes the last six-electron reduction step of sulfite to sulfide and it is highly conserved among the sulfate-reducing prokaryotes. For this reason the *dsr*AB gene

has recently being used as a molecular marker for the detection and identification of SRB. By targeting the functional gene, three new clusters of uncultured SRB have been detected. The first two clusters were related to the halo-alkaliphilic *Desulfonatronovibrio hydrogenovorans* (Zhilina *et al.*, 1997), an incomplete oxidizer isolated from a soda lake, revealing that the diversity of this group is much higher than anticipated. This cluster might be very important for the sulfide removal application, since the only culturable high-salt alkaliphilic SRB species, strain ASO3-1, isolated within this project, belongs to the same group. The third cluster was related to the complete oxidizer *Desulfosalina propionicus*, a halophilic non alkaliphilic bacterium isolated from Great Salt Lake, Utah (Kjeldsen *et al.*, 2007). This was unexpected, because all organisms belonging to this family, i.e. Desulfobacteraceae, are complete oxidizers and so far there are no halo-alkaliphilic SRB described belonging to this group. Since living at high salt conditions is energetically expensive, it has been hypothesized that microorganisms with low energetic yield, like complete-oxidizing SRB, cannot grow at hypersaline conditions (Oren, 1999). However the presence of this group was confirmed in a second study (Chapter 6), in which the SRB community from soda lakes was investigated by targeting the 16S rRNA gene along a salinity gradient. Here, both the presence and the actively growing community of six specific groups of SRB (including complete and incomplete oxidizers) were investigated (Daly *et al*., 2000), using DNA- and RNA- based techniques, respectively. Also here a new cluster related to *D. propionicus* was detected and was shown to be actively growing even at the highest salinities. Therefore, it strongly indicates that a new type of complete oxidizing haloalkaliphilic SRB inhabits the investigated soda lakes. The detection of the other SRB groups by targeting the 16S rRNA gene was not as successful as expected, since the PCR amplifications resulted in aspecific products affiliated to phylogenetic groups other than the targeted ones. Therefore, it was not possible to detect the different groups of sulfate-reducers at increasing salinities. The isolation of SRB under halo-alkaliphilic conditions by serial dilutions proved also to be very difficult. Both DGGE and microscopic analysis revealed the predominance of Clostridia even at the highest sulfidogenic dilutions. We may speculate that they employ a sodium-based bioenergetics (Müller, 2003), making them successful in the isolation procedure, i.e. serial dilution, or that their viability in the laboratory procedure is significantly higher than that of the sulphate-reducers.

However, two halo-alkaliphilic representatives of the reductive sulfur cycle have been isolated during this PhD research (Chapter 5 and 7). '*Desulfonatronovirga dismutans'* gen. nov. sp. nov. ASO3-1 (Sorokin *et al*., in preparation) has been isolated from a lake sediment sample of the Kulunda Steppe at high salt/pH and highly selective conditions, i.e. with sulfite as the only electron donor and acceptor. It is the first example of an extremely natronophilic sulfate-reducing bacterium, growing in sodium carbonate brines, with an optimum at 2-2.5 M Na^+ and at pH of 9.5-10. It can grow chemolitho-autotrophically with hydrogen and sulfite/thiosulfate as electron donor and acceptor, respectively, and can also grow autotrophically by disproportionation of either sulfite or thiosulfate (Chapter 5). *Desulfonatronovirga dismutans* might be the most suitable candidate for the second anaerobic reactor in the process, being able to reduce thiosulfate at extremely halo-alkaliphilic conditions.

The second organism, *Desulfurispirillum alkaliphilium*, has been isolated from a full-scale sulfide removing Thiopaq bioreactor (Eerbeek, The Netherlands) using acetate as carbon source and sulfur as electron acceptor (Chapter 7). Nitrate and fumarate can also be used as electron acceptors, the former dissimilatorily reduced to ammonium. In contrast to the currently under development high-salt process, the Thiopaq reactor in Eerbeek consists of a single bioreactor for sulfide oxidation operating under mildly haloalkaline conditions. The low redox

potential creates the perfect conditions for a complete sulfur cycle, in which microorganisms utilizing different sulfur compounds as electron acceptor are active along with the dominant microaerophilic SOB.

Outlook

In the present thesis we tried to give our contribution for a better understanding of the microbial population, especially of the sulfur-oxidizing (SOB) and sulfate-reducing bacteria (SRB), in soda lakes and their possible applications for the removal of hydrogen sulfide. Still, many questions need to be answered and new approaches are necessary. The sulfate-reducing community in soda lakes, for example, remains insufficiently investigated. We showed the presence of new groups of both incomplete- and complete oxidizers at hypersaline conditions, but further research has to be addressed in the isolation of these organisms. Especially for the latter, enrichments using propionate as carbon source should be done, as we detected a new cluster of sequences retrieved from high salt lakes related to D*esulfosalina propionicus*. Additionally, competition studies should be done between acetogens and SRB at halo-alkaliphilic conditions. We observed that the isolation of pure culture of SRB was very difficult or impossible due to the presence, even at highest dilutions, of Clostridia. Literature data show that some acetogenic bacteria, i.e. *Acetobacterium woodii*, make use of Na^+-based bioenergetics (Müller, 2003), whereas the halo-alkaliphilic *Desulfonatronovibrio hydrogenovorans* depends on a proton-translocating ATPase (Sydow *et al.*, 2002). Hypothesizing that these two different bioenergetic strategies might be a specific property of the two groups, it might be that acetogens compete successfully with the sulfate reducers for limiting resources. Despite the fact that we detected high sulfate reduction rates it is possible that the acetogens outnumber the sulfate reducers and then as a result the serial dilution technique is meaningless. FISH analysis should be used to count the representatives of SRB and acetogens in the original samples.

The discrimination between *Thioalkalivibrio* strains remains a difficult issue to be solved. The existence of such high genetic diversity within this genus, and especially within a restricted group of extremely high-salt tolerant strains, indicates a micro-niche specialization with relevant physiological differences. Therefore, the discrimination between those strains, in response to varying process conditions, was of relevance for a better understanding and efficiency of the sulfide removal process. The sulfide-oxidizing bioreactor operates at highly specific conditions: high salt/pH, low oxygen and very low redox potential, due to the high sulfide concentrations. These conditions selected already for a specific 'sulfidic' population. However, changes in the operational settings may select for populations, better adapted to the new conditions. Therefore, the development of yet other molecular tools able to monitor and detect the most successful organisms at specific conditions must remain an important goal of research. For the future research it would be also interesting to study the sulfide-oxidation activity of the selected population by targeting functional genes. The SQR gene, encoding for the sulfide-quinone reductase, and the cytochrome bd, could be possible targets. Both enzymes function under low-oxygen conditions. The former is responsible for the growth on sulfide producing sulfur as final oxidation product, whereas the latter is a terminal oxidase. *Thioalkalivibrio* isolates growing at such conditions have been shown to posses both enzymes (D.Sorokin, personal communication). The sulfide-oxidation activity could then be investigated by quantitative PCR of both genes. However, SQR sequences between different genomes share low identity, posing a challenge to phylogenetic analysis and development of specific

primers. The imminent complete genome sequence of a few *Thioalkalivibrio* strains will be of great utility for the future research.

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Chapter 9

Summary
Samenvatting
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Summary

The scope of this thesis was to obtain a better picture of the halo-alkaliphilic bacterial community with special attention to chemotrophic sulfur-cycle bacteria. Soda lakes are specific types of saline lakes with extremely high alkalinity and pH due to presence of soluble carbonates at high concentrations. Compared to other saline environments, e.g. marine ecosystems and salterns, soda lakes have not been broadly investigated. These doubly extreme environments represent a unique ecosystem, highly interesting for their microbial ecology and possible application of haloalkaliphiles in biotechnology.

This PhD research was involved in the development of a new biological process for hydrogen sulfide removal under halo-alkaliphilic conditions. The process consists of a scrubber integrated with two bioreactors. In the absorber hydrogen sulfide gas (H_2S) is contacted with an alkaline solution. The dissolved sulfide (HS^-) is subsequently oxidized under oxygen limited conditions to elemental sulfur (S_0) by sulFide oxidizing bacteria (SOB). A small percentage of the sulfide is converted to the undesired thiosulfate ($S_2O_3^{2-}$) and sulfate (SO_4^{2-}), which are reduced back to sulfide by halo-alkaliphilic sulphate reducing bacteria (SRB) in the second anaerobic reactor. The investigation and the detection of bacteria involved in this sulfur cycle, i.e. the SOB and the SRB under such conditions, were thus required. For this purpose a polyphasic approach was used, including molecular techniques, traditional cultivation methods and in situ measurements. This was possible thanks to a successful cooperation with other scientist and research groups, i.e. in the Wageningen University and Research Center (WUR) and in the Institute of Microbiology of the Russian Academy of Sciences in Moscow (INMI).

In Nature, halo-alkaline environments are mainly represented by soda lakes. Chapter 1 gives an overview about soda lakes, e.g. their genesis, their microbial community and its sulfur cycle. Furtheremore, strategies utilized to live at halo-alkaliphilic conditions are also described, along with possible industrial applications of haloalkaliphiles. One of the applications is the biological removal of hydrogen sulfide under halo-alkaliphilic conditions, as described in chapter 1 (Fig. 1.7). Best candidates for the sulfide removal process are the chemolithoautotrophic sulfur-oxidizing bacteria (SOB) belonging to the genera *Thioalkalivibrio* and *Thioalkalimicrobium* (Chapter 1). They possess high rates of oxidation of various sulfur compounds and play an important role in the sulfur cycle in soda lakes. The former genus was selected to operate in lab-scale sulfide-oxidizing bioreactor at high-salt conditions due to its extremely high salt tolerance. Currently, more than hundred *Thioalkalivibrio* strains have been isolated by D. Sorokin from sediments of different soda lakes distributed all over the world. In Chapter 2 the genetic diversity and the biogeography of those strains is described. To do this, a fingerprinting technique with resolution power up to the strain level, rep-PCR, has been applied. The results showed a very high genetic microdiversity within this ecophysiological group. The majority of the isolates appeared to be endemic to their place of origin.

The discrimination between the *Thioalkalivibrio* isolates within a mixed culture was necessary for a better understanding and control of the functioning of the aerobic sulfide-oxidizing bioreactor under investigation. Chapter 3, a methodological chapter, describes the application of Denaturing Gradient Gel Electrophoresis analysis of the ITS fragment for the discrimination of closely related *Thioalkalivibrio* strains from samples obtained from a mixed population of *Thioalkalivibrio*. The Internal Transcribed Sequence (ITS) region is located between the 16S- and 23S rRNA genes, and is more variable and less conserved than the other two genes. Thirteen different genotypes of *Thioalkalivibrio* have been investigated. Despite the differences in their ITS sequences, it was not possible to separate them on DGGE, making this technique

not suitable for our purpose.

In Chapter 4 the diversity of chemolithoautotrophic SOB in Soap Lake (Washington State, USA) is presented. Soap Lake is a stratified soda lake with an unprecedentedly high sulfide concentration in the anoxic monimolimnion (bottom layer of the lake). This stratification results both in oxygen/sulfide and saline gradients, which goes from 15 g/L to 140 g/L for salinity and from 0 to 200 mM for sulfide concentration in the mixolimnion (top layer of the lake) and in the monimolimnion, respectively. These two layers are separated by a chemocline, where steep gradients of sulfide and oxygen occur. Culture-independent techniques revealed the predominance of the genus *Thioalkalivibrio* at higher salinities and *Thioalkalimicrobium* at lower salinities (Chapter 1). A highly dense population of the latter has been detected in the chemocline and a new species of *Thioalkalimicrobium*, *Tm. microaerophilum*, has been isolated and described.

In Chapter 5 the attention is shifted to the other part of the the sulfur cycle represented by sulfate-reducing bacteria (SRB). Here the diversity, abundance and activity of this group of bacteria has been investigated in soda lakes of the Kulunda Steppe (South-East Siberia, Russia), using a polyphasic approach, including traditional cultivation and molecular techniques. By targeting the functional *dsr*AB gene, encoding a key enzyme of the dissimilatory sulfate reduction, two major groups of SRB containing three novel clusters have been detected. High sulfate reduction rates have been measured in hypersaline soda lakes (Chapter 5), revealing the functional importance of this group of bacteria even at such extreme conditions. These results were confirmed in Chapter 6, where both the SRB and the general bacterial diversity have been investigated in four soda lakes with increasing salinity. In this case not only the microbial diversity but also the actively growing populations have been described. A different approach was used to investigate the SRB, based on the 16S rRNA gene instead of the functional gene. The obtained results showed a decrease of the general bacterial diversity along the salinity gradient, whereas the sulfate reduction activity seemed unaffected by the salinity.

In Chapter 7 the description of haloalkaliphilic dissimilatory sulfur- and nitrate-reducing bacterium is presented. *Desulfurispirillum alkaliphilum* gen. nov., sp. nov., has been isolated from a full-scale bioreactor aimed at the removal of hydrogen sulfide from biogas (Thiopaq, Eerbeek, NL) under mildly haloalkaline conditions. The development of a sulfur-reducing population has been probably facilitated by the biological production of sulfur, which is more accessible for sulfur-reducing bacteria than the chemically produced sulfur. *D. alkaliphilum* is a moderately haloalkaliphilic, obligately anaerobic bacterium, able to use sulfur, fumarate and nitrate as electron acceptors. In the presence of nitrate, it is also able to oxidize a limited amount of sulfide to sulfur during anaerobic growth with acetate. It is the first example of sulfur-respiring and dissimilatory nitrate-reducing bacteria among the known haloalkaliphiles.

In conclusion, this thesis presents new information about the halo-alkaliphilic microbial community in soda lakes. Special attention has been given to the sulfur cycle and the sulfur-oxidizing and sulfate-reducing bacteria. Both groups are abundant and active even in hypersaline soda lakes and appear to be broader in their ability to cope with extreme conditions than previously anticipated. Further research has to be done to isolate and describe the new groups of extremely salt-tolerant natronophilic SRB, which could be also of great utility for the industrial application.

Samenvatting

De doelstelling van deze dissertatie was het verkrijgen van een beter beeld van de halo-alkalifiele gemeenschap van bacteriën en met name de zgn. chemotropische zwavel- bacteriën. Sodameren vormen een specifiek type zoutmeren met een extreem hoge alkaliniteit en pH als gevolg van de in hoge concentraties aanwezige oplosbare carbonaten (Na_2CO_3/$NaHCO_3$). In vergelijking met andere zoute omgevingen, zoals marine ecosystemen en zoutziederijen, zijn sodameren nog niet uitgebreid onderzocht. Deze extreme omgevingen vormen een uniek ecosysteem met een bijzonder interessante microbiële ecologie en mogelijke toepassing van halo-alkalifielen in de biotechnologie.

Dit promotie-onderzoek was betrokken bij de ontwikkeling van een nieuw proces voor het verwijderen van waterstofsulfide onder halo-alkalische condities. Dit proces wordt gevormd door een zgn. 'scrubber', geïntegreerd met twee bioreactoren. In deze 'scrubber' wordt waterstofsulfide (H_2S) in contact gebracht met een alkalische oplossing. Het opgeloste waterstofsulfide (HS^-) wordt vervolgens onder zuurstof-beperkende condities door sulfide-oxiderende bacteriën (SOB) geoxideerd tot elementaire zwavel (S). Echter een klein percentage van de sulfide wordt omgezet in het ongewenste thiosulfaat ($S_2O_3^{2-}$) en sulfaat (SO_4^{2-}). Deze worden in de tweede bioreactor door halo-alkalifiele sulfaat-reducerende bacteriën (SRB) weer opnieuw omgezet in sulfide. Een optimaal verloop van dit proces vereist het onderzoeken en detecteren van bacteriën die een rol spelen in deze zwavelcyclus, i.e. de SOB en de SRB onder dergelijke omstandigheden. Hiervoor werd een meerfasen aanpak gebruikt, bestaande uit moleculaire technieken, traditionele cultivatie methoden en in situ metingen. Dit werd mogelijk gemaakt door succesvolle samenwerking met wetenschappers van andere onderzoeksgroepen aan de Universiteit van Wageningen (WUR) en het Instituut voor Microbiology (INMI) van de Russische Academie van Wetenschappen in Moskou.

In de natuur worden de halo-alkalische omgevingen hoofdzakelijk vertegenwoordigd door sodameren. Hoofdstuk 1 geeft een overzicht over sodameren, hun ontstaan, microbiële gemeenschappen en de zwavelcyclus. Verder worden strategieën beschreven die worden gebruikt om te overleven onder halo-alkalische condities evenals mogelijke industriële applicaties van halo-alkalifiele bacteriën. Eén van de toepassingen is de biologische verwijdering van waterstofsulfide onder halo-alkalische condities, zoals beschreven in hoofdstuk 1 (Fig. 1.7). De beste kandidaten voor het sulfide verwijderingsproces zijn de chemolitho-autotrophische zwavel-oxiderende bacteriën (SOB), die behoren tot de genera Thioalkalivibrio en Thioalkalimicrobium. Deze zijn in staat verscheidene zwavelverbindingen met grote snelheid te oxideren en spelen een belangrijke rol in de zwavelcyclus van sodameren. Stammen van het eerste genus werden vanwege hun extreem hoge zouttolerantie geselecteerd voor een laboratorium-schaal sulfide-oxiderende bioreactor onder hoge zoutconcentraties. Op dit moment zijn door Dimitri Sorokin van het INMI in Moskou uit sedimenten van verschillende sodameren over de gehele wereld meer dan honderd Thioalkalivibrio stammen geïsoleerd. In hoofdstuk 2 worden de genetische diversiteit en de biogeografie van deze stammen beschreven. Hiervoor werd een 'genetische vingerafdruk'-techniek met een resolutie tot op stam-niveau gebruikt: de zgn. 'rep-PCR'. De resultaten lieten binnen deze ecofysiologische groep een bijzonder hoge genetische microdiversiteit zien en de meerderheid van de isolaten leek inheems te zijn voor de vindplaats.

Voor een beter inzicht en invloed op het functioneren van de aerobe sulfide-oxiderende bacteriën in de betreffende bioreactor was het van belang de Thioalkalivibrio isolaten in een

gemengde cultuur te kunnen onderscheiden. Hoofdstuk 3, een methodologisch hoofdstuk, beschrijft de toepassing van denaturerende gradient gel electrophorese (DGGE) van het ITS fragment voor het discrimineren van nauw-verwante Thioalkalivibrio stammen uit monsters van een gemengde Thioalkalivibrio cultuur. De Internal Transcribed Sequence (ITS) regio bevindt zich tussen het 16S- en 23S rRNA gen en is meer variabel in DNA sequentie dan de twee genen. Dertien verschillende Thioalkalivibrio stammen werden onderzocht. Ondanks de verschillen in de DNA sequenties van het ITS deel was het niet mogelijk ze m.b.v. DGGE te onderscheiden en bleek deze techniek niet geschikt om ons doel te bereiken.

In hoofdstuk 4 wordt de diversiteit van chemolitho-autotrofische SOB in Soap Lake (Washington State, USA) gepresenteerd. Soap Lake is een gestratificeerd (met gelaagde structuur) sodameer met een enorm hoge sulfide concentratie in de zuurstofloze monimolimnio (onderste laag van het meer). Deze stratificatie leidt tot zuurstof/sulfide en zoutgradiënten met concentraties van 15 tot 140 g/L wat betreft het zoutgehalte en van 0 tot 200 mM wat betreft de sulfide in respectievelijk de mixolimnio (bovenste laag van het meer) en de monimolimnio. Deze twee lagen zijn gescheiden door een zgn. chemokliene, waar stijle gradiënten van zwavel en zuurstof voorkomen. Cultuur-onafhankelijke technieken lieten de overheersing van het genus Thioalkalivibrio zien bij hogere zoutconcentraties en van Thioalkalimicrobium bij lagere zoutconcentraties. Een zeer dichte populatie van het laatste genus werd ontdekt in de chemokliene en een nieuwe Thioalkalimicrobium soort (species), Tm. microaerophilum, werd geïsoleerd en beschreven.

In hoofdstuk 5 verschuift de aandacht naar het andere deel van de zwavelcyclus, vertegenwoordigd door de sulfaat-reducerende bacteriën (SRB). Hier werd de diversiteit, het aantal en de activiteit van deze groep bacteriën onderzocht in sodameren van de Kulunda Steppe (Zuid-Oost Siberie, Rusland). Voor het onderzoek werden zowel traditionele cultivatie-, als moleculaire technieken gebruikt. Door te richten op het functionele dsrAB gen, dat codeert voor het enzym dissimilatory sulfide reductase, zijn twee hoofdgroepen van SRB die drie nieuwe clusters bevatten gedetecteerd. Hoge sulfaat-reductie snelheden zijn gemeten in hyper-zoute sodameren (hoofdstuk 5). Dit laat het functionele belang van deze groep bacteriën zien zelfs onder dergelijke extreme condities. Deze resultaten werden bevestigd in hoofdstuk 6, waarin zowel de SRB als de algemene bacteriële diversiteit werden onderzocht in vier sodameren met toenemende zoutconcentraties. In dit geval werd niet alleen de microbiële diversiteit, maar ook de actief groeiende populaties beschreven. Een afwijkende aanpak, gebaseerd op het 16S rRNA gen in plaats van het functionele gen, werd gebruikt om de SRB te onderzoeken. De verkregen resultaten lieten een vermindering van de algemene bacteriële diversiteit zien bij een toename in de zoutconcentratie, terwijl de sulfaat-reducerende activiteit gelijk bleef.

In hoofdstuk 7 wordt de beschrijving van halo-alkalifiele dissimilatory zwavel en nitraat-reducerende bacterium gepresenteerd. Desulfurispirillum alkaliphilum gen. nov., sp. nov., werd geïsoleerd uit een industriële bioreactor bedoeld voor de verwijdering van waterstofsulfide uit biogas (Thiopaq, Eerbeek, NL) onder milde, haloalkalische condities. De ontwikkeling van een zwavel-reducerende populatie werd waarschijnlijk geholpen door de biologische productie van zwavel, die meer toegankelijk is voor zwavel-reducerende bacteriën, dan de chemisch geproduceerde zwavel.

D. alkaliphilum is een gemiddeld halo-alkalifiel, obligaat anaerobe bacterie, die in staat is zwavel, fumarate en nitraat te gebruiken als elektron acceptor. Indien nitraat aanwezig is, is het eveneens in staat een beperkte hoeveelheid sulfide te oxideren tot zwavel gedurende anaerobe groei op basis van acetaat. Het is het eerste voorbeeld van sulfur-respiring en dis-

similatory nitraat reducerende bacteriën in de bekende groep van haloalkalifielen.

Concluderend presenteert deze dissertatie nieuwe informatie over de halo-alkalifiele microbiële gemeenschap in sodameren. Hierbij is speciale aandacht gegeven aan de zwavelcyclus en de zwavel-oxiderende en -reducerende bacteriën. Beide groepen zijn actief en overvloedig aanwezig zelfs in hyper-zoute sodameren en lijken beter in staat om te gaan met extreme condities dan eerder was gedacht. Verder dient er onderzoek te worden gedaan om nieuwe groepen van extreem zout tolerante natronofiele SRB te beschrijven die ook een grote industriële toepasbaarheid zouden kunnen hebben.

Curriculum Vitae

Mirjam Josephine Foti was born on 3rd of July 1976 in Milan, Italy. In 1995 she graduated at the scientific high-school in Cinisello Balsamo, near Milan. In 2002 she obtained her Master Degree in Biology at the University of Milan. She carried out a 10-months internship at the Department of Food Science, Technology and Microbiology (DISTAM), Milan. The project dealt with the study of the degradative pathways of two sulphonylureic herbicides, under the supervision of Dr. Zanardini and Prof. C. Sorlini. In 2003 she joined the Delft University of Technology, Department of Biotechnology, faculty of Applied Science, for her PhD. His doctoral supervisors were Prof. Gijs J. Kuenen, Dr. G. Muyzer and Dr. D. Yu. Sorokin.

Since June 2007 Mirjam Foti is employed a postdoctoral researcher at the Bioconversion group, TNO (De Nederlandse Organisatie voor toegepast-natuurwetenschappelijk onderzoek).

Acknowledgments

Here we are, the time for the acknowledgment is come. I was not sure to write this page, since writing is not my strongest point and I didn't want to end up with a long list of `cliché `(which is, anyhow, exactly what I am going to do!). However, I decided to do it because there are plenty of people I want to thank and because I do think that most of the people, like me, enjoy reading their name in the acknowledgements!

First of all I want to thank my family, and especially my parents (and here the first cliché…), as they are the persons who make possible, in several ways, the realization of my PhD. Pa`, Ma`, Sandra e Claudia, grazie!

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