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Unveiling microbial life in the new deep-sea hypersaline Lake *Thetis*. Part II: a metagenomic study

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Summary

So far only little is known about the microbial ecology of Mediterranean deep-sea hypersaline anoxic lakes (DHALs). These brine lakes were formed by evaporite dissolution/brine seeps and are important model environments to provide insights into possible metabolisms and distributions of microorganisms on the early Earth. Our study on the Lake *Thetis*, a new thalassohaline DHAL located South-East of the Medriff Corridor, has revealed microbial communities of contrasting compositions with a high number of novel prokaryotic candidate divisions. The major finding of our present work is co-occurrence of at least three autotrophic carbon dioxide fixation pathways in the brine–seawater interface that are likely fuelled by an active ramified sulphur cycle. Genes for the reductive acetyl-CoA and reductive TCA pathways were also found in the brine suggesting that these pathways are operational even at extremely elevated salinities and that autotrophy is more important in hypersaline environments than previously assumed. Surprisingly, genes coding for RuBisCo were found in the highly reduced brine. Three types of sulphide oxidation pathways were found in the interface. The first involves a multienzyme Sox complex catalysing the complete oxidation of reduced sulphur compounds to sulphate, the second type recruits SQR sulphide:quinone reductase for oxidation of sulphide to elemental sulphur, which, in the presence of sulphide, could further be reduced by polysulphide reductases in the third pathway. The presence of the latter two allows a maximal energy yield from the oxidation of sulphide and at the same time prevents the acidification and the accumulation of S^0 deposits. Amino acid composition analysis of deduced proteins revealed a significant overrepresentation of acidic residues in the brine compared with the interface. This trait is typical for halophilic organisms as an adaptation to the brine's extreme hypersalinity. This work presents the first metagenomic survey of the microbial communities of the recently discovered Lake *Thetis* whose brine constitutes one of saltiest water bodies ever reported.

Introduction

The world's deepest hypersaline anoxic lakes (DHALs) belong to the most interesting environments for research in extreme anaerobic halophilic microorganisms. These brine lakes occur on the seafloor of the Eastern Mediterranean Sea, and have been established by dissolution of evaporites from the Miocene period (Jongsma *et al.*, 1983). The DHALs are situated at depths below 3200 m, and their salinity exceeds that of the seawater by a factor of five to ten (Daffonchio *et al.*, 2006). The known Eastern Mediterranean DHALs differ in their ion compositions (Camerlenghi, 1990; De Lange *et al.*, 1990; Van der Wielen *et al.*, 2005; Cita, 2006) and the sharp density gradient between their hypersaline brines and the upper seawater acts as a stable barrier for the exchange of oxygen and sulphide (for example, see Levin, 2003). The combination of above factors suggests the DHALs belong to most extreme environments on Earth rather hostile to

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'common' marine species (Vaquer-Sunyer and Duarte, 2008; Oren, 2011). Nevertheless, according to recent findings, in spite of being so harsh, the DHALs contain diverse prokaryotic and eukaryotic [including metazoans (Danovaro *et al.*, 2010)] assemblages with a surprisingly high number of novel candidate divisions (e.g. Danovaro *et al.*, 2005; van der Wielen *et al.*, 2005; Daffonchio *et al.*, 2006; van der Wielen and Heijs, 2007; Alexander *et al.*, 2009; Borin *et al.*, 2009; Edgcomb *et al.*, 2009; La Cono *et al.*, 2011). Apart from the data on the taxonomic composition of microorganisms assessed in DHALs in previous studies, our knowledge about their role in biogeochemical functioning in these ecosystems remains largely scarce. Recent functional analyses performed on Bannock, Discovery, l'Atalante and Urania basins revealed the presence of sharply stratified prokaryote network in the oxic–anoxic chemocline that separates the anaerobic hypersaline brines and overlying seawater (Daffonchio *et al.*, 2006; Hallsworth *et al.*, 2007; Yakimov *et al.*, 2007; Borin *et al.*, 2009). As stated elsewhere, high salinities usually tend to reduce biodiversity (Benlloch *et al.*, 2002; Oren, 2008); however, when anoxic brines come into the contact with less-salted and oxygenated waters, emerging chemoclines provide a greater variety and combinations of electron donors and acceptors that could enhance microbial diversity, activity and biogeochemical cycling within relatively narrow spatial boundaries (D'Hondt *et al.*, 2004; Parkes *et al.*, 2005). Indeed, the increase of prokaryotic cell numbers (up to hundredfold) was observed in chemoclines of DHALs compared with seawater or brines (Daffonchio *et al.*, 2006; Yakimov *et al.*, 2007; Borin *et al.*, 2009). Detailed analyses performed through PCR-amplification of key functional genes, activity measurements and cultivations revealed that sulphur cycling and methanogenesis are likely the predominant metabolic processes maintaining the life in DHALs brine and driving the elevated biomass in their chemoclines. Besides that observation, all what we know now is that *Gamma-* and *Epsilonproteobacteria* usually dominate the chemoclines of Mediterranean DHALs and are likely involved into sulphur oxidation, while *Deltaproteobacteria* are responsible for sulphate reduction processes detected in the brines (Daffonchio *et al.*, 2006; Yakimov *et al.*, 2007; Borin *et al.*, 2009). The members of archaeal candidate division MSBL1 (van der Wielen *et al.*, 2005) were predominant archaea in the majority of DHAL brines and their potential involvement in methanogenesis was proposed recently (Borin *et al.*, 2009).

Thanks to metagenomic and metatranscriptomic (OMICs) approaches, our knowledge of abundance, diversity and gene content of marine microbes has been fundamentally advanced over the past decade (Venter *et al.*, 2004; DeLong *et al.*, 2006; Martin-Cuadrado *et al.*, 2007; Rusch *et al.*, 2007; Frías-López *et al.*, 2008; Poretsky *et al.*, 2009; Shi *et al.*, 2009; Feingersch *et al.*, 2010). This rapidly growing field is promoting our understanding on the functions of microbial populations in marine environment and leads to unveiling important mechanisms of microbial metabolism on the single-cell and community levels, genetics and evolution of deep-sea microbes. However, there are very few examples of OMICs studies, mainly metagenomics, applied so far to deep-sea environments (DeLong *et al.*, 2006; Martin-Cuadrado *et al.*, 2007; Konstantinidis *et al.*, 2009). A recent shotgun metaproteomics study of the bathypelagic realm of the Mediterranean Sea has identified a number of key microbial proteins and linked them to essential metabolic processes and environmental adaptations (Yakimov *et al.*, 2011), additionally emphasizing the importance of OMICS approaches to understand the biogeochemical intricacies in the deep sea. The aim of the present study was therefore to gain insights into the genomics of microbial communities in the deep-sea hypersaline anoxic Lake *Thetis*, a new thalassohaline DHAL located in Ionian Sea, SE of the Medriff corridor, which belongs to the saltiest water bodies on Earth (La Cono *et al.*, 2011).

Results and discussion

General features and prokaryotic community structures of the Thetis brine and interface

On September 2009, upper interface and brine samples were collected from the *Thetis* DHAL. The seawater–brine interface of this new lake is located at 3258 m below the sea level, with a maximum brine thickness of 157 m. The maximum microbial cell density was observed in the interface above the brine ($(2.11 \pm 0.13) \times 10^6$ cells ml^{-1}), which gradually decreased with depth and reached almost constant ($7.11 \pm 0.67) \times 10^4$ cells ml^{-1} within the *Thetis* body brine. Total salt concentrations were measured to be of 48–110‰ (upper interface) and 348‰ (brine) (La Cono *et al.*, 2011). Total DNA was extracted for each microbial community, directly sequenced and assembled (for details see Table 1 and Figs S1 and S2). Even when spurious hypothetical ORFs $\leq$ 150 bp were excluded, still 24.5% of the protein sequences deduced from the interface and 45.3% from the brine metagenome were hypothetical proteins not exhibiting any sequence similarity to known proteins in public databases; another 5.2% from interface and 3.5% from brine, exhibited similarity to proteins of unknown function (conserved hypothetical). Thus, a substantial fraction of genes in these ecosystems, in particular within the brine, were entirely novel with yet unknown functions.

A total of 403 sequencing reads from the interface constituted partial 16S rRNA gene sequences with lengths

Table 1. General features of the metagenome of the deep-sea hypersaline anoxic Lake *Thetis*.

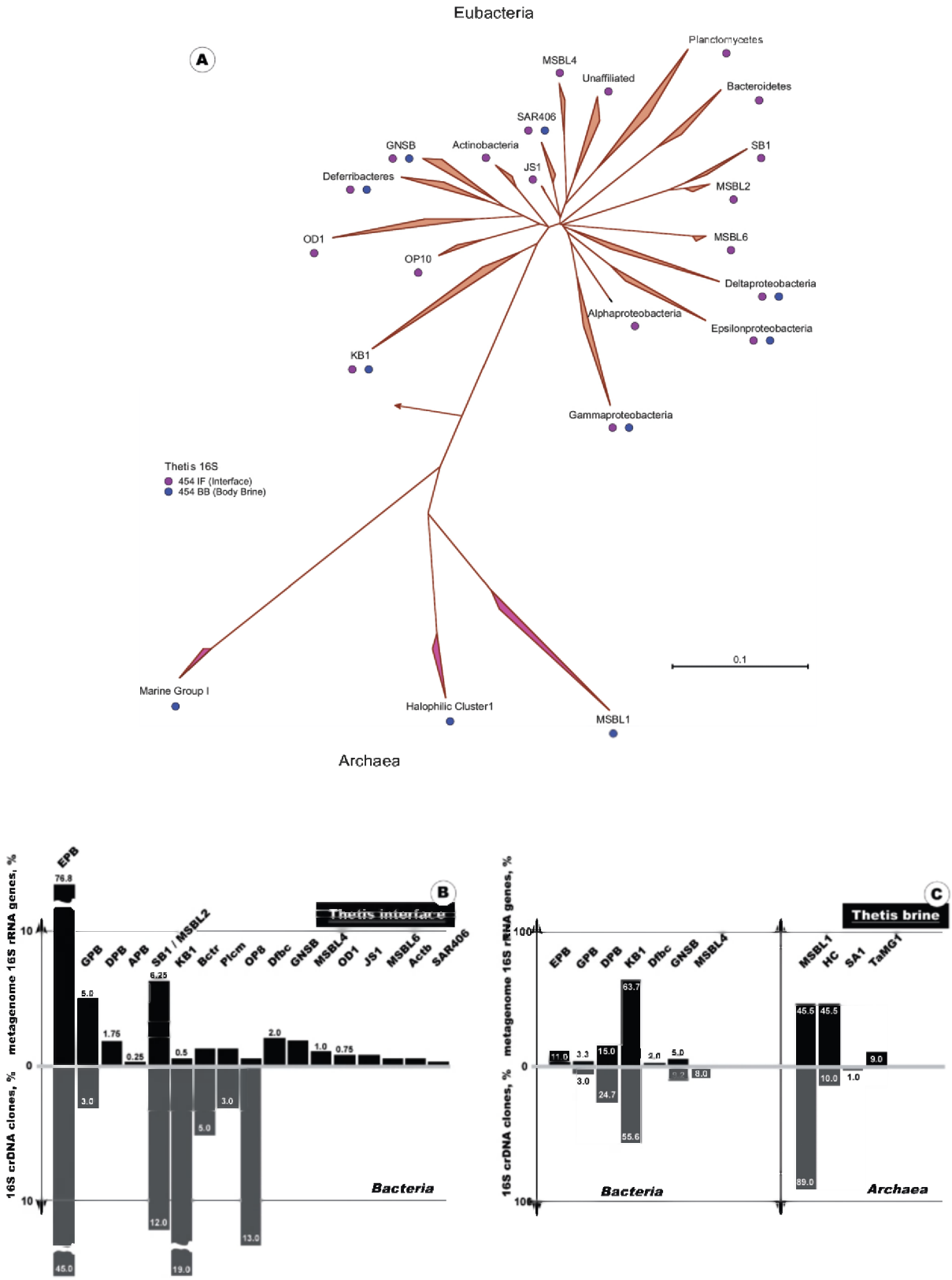
Parameter	Interface	Brine
Total contigs	12 105	15 850
Contigs > 2.5 kbp	967	306
Total bp	13 356 043	9 719 733
Mbp > 500	12.1	7.4
Average contig size (bp)	1 103	613
Average GC content	38.1%	45.9%
Total 16S rRNA tags	555	184
ORFs with predicted function	22 408	14 248
Hypothetical proteins	7 783	12 610
Conserved hypothetical proteins	1 638	977
ORFs assigned to COGs	11 444	8 688
ORFs with KEGG ortologues	6 974	5 350
KEGG ortologues unique for interface/brine	828	267

and quality sufficient to unambiguously taxonomically affiliate with source organisms (Fig. 1A). A surprisingly low number of these sequences (less than 1%) were affiliated with *Archaea* exclusively represented by members of Marine Group 1 of *Thaumarchaeota*. Thus, the interface separating hypersaline anoxic brine from overlaying oxygenated seawater likely acts as a barrier hampering vertical migration of archaea adapted to the different compartments. Similarly to previous results from 16S crDNA clone libraries (La Cono *et al.*, 2011), *Proteobacteria* constituted the bulk of assigned 16S rRNA genes in the interface community (76.8% *Epsilonproteobacteria*, 1.8% *Deltaproteobacteria*, 5.0% *Gammaproteobacteria*), with 69% related to *Sulfurovum*-like epsilonproteobacterial species. The latter finding was also corroborated by the taxonomic binning of the interface metagenome where almost 62% of the assembled contigs were attributed to *Sulfurovum*-like species. Further 16S rRNA partial genes clustered with 15 different clades from the phyla *Actinobacteria*, *Bacteroidetes, Deferribacter*, Green non-sulphur bacteria, *Planctomycetes* and candidate divisions Kebrit Deep Bacteria 1 (KB1), Shaban Deep Bacteria 1 (SB1), Obsidian Pool 8 (OP8), Obsidian Pool 10 (OP10), OP11-derived 1 (OD1), Japan Sea 1 (JS1), SAR406 and Mediterranean Sea Brine Lakes 2, 4 and 6 (MSBL2, MSBL4 and MSBL6) (Figs 1A–C and S3). Besides *Epsilon-* and *Gammaproteobacteria*, members of only five taxa seemed to be metabolically active in the interface as detected by analysis of 16S rRNA from the pool of total RNA by RT-PCR (La Cono *et al.*, 2011). Notably, eight 16S rRNA gene sequences from the interface were related to *Deferribacteres,* and the deferribacterium *Flexistipes sinusarabici* was the first validly described moderate halophile isolated from a deep-sea anoxic brine pool (Fiala *et al.*, 1990; Ludwig *et al.*, 1991).

A rather contrasting prokaryotic community composition was found in the brine. Seventy-one unique 16S rRNA tag sequences were retrieved from the metagenome and 16% of them were affiliated with *Archaea*. Considering that the average occurrence of rRNA operons in bacterial vs. archaeal genomes is approximately four to one, the *Thetis* brine community likely contains an almost equal proportion of bacterial and archaeal members. As shown in Figs 1A–C and S4, two euryarchaeal groups from the brine metagenome, MSBL1 and Halophilic Cluster (HC), were found to be predominant among actively metabolizing *Archaea* (La Cono *et al.*, 2011). The metagenome analysis of bacteria from the brine and the previous 16S crDNA clone library (Fig. 1B and C) yielded congruent results and demonstrated a strong prevalence of organisms belonging to the KB1 candidate division followed by *Deltaproteobacteria*.

Overall, the brine-inhabiting microbial community, dominated by *Aquificae*-related KB1 candidate division, was less complex than that in the interface where *Epsilonproteobacteria* were strongly prevalent. In case of *Sulfurovum*-related organisms we can assume the occurrence of sulphur-driven autotrophy, while the metabolic preferences of KB1 members remain enigmatic. The *Thetis* interface layer with salinities between 48 and 110‰ represents an intermediate zone where the environmental

Fig. 1. Overview on prokaryotic diversity (A), stratification and relative abundance (B, C) of phylogenetic groups recovered from the different compartments of *Thetis* Lake. Comparison of the Lake *Thetis* phylotypes (> 97% sequence identity cut-off) based on 16S rRNA tag sequences extracted from the interface (B) and brine (C) metagenome with those of conventional 16S crDNA clone library. EPB, *Epsilonproteobacteria*; GBP, *Gammaproteobacteria*; DPB, *Deltaproteobacteria*; APB, *Alphaproteobacteria*; Bctr, *Bacteroidetes*; Plcm, *Planctomycetes*; Actb, *Actinobacteria*; Dfbc, *Deferribacter*; SB1, Candidate Division Shaban Deep Bacteria 1; KB1, Candidate Division Kebrit Deep Bacteria 1; OP8, Candidate Division Obsidian Pool 8; GNSB, Green non-sulphur bacteria; SAR406, Candidate Division Sargasso Sea 406; MSBL2, MSBL4, MSLB6, Candidate Divisions Mediterranean Sea Brine Lakes 2, 4 and 6; OD1, Candidate Division OP11-derived 1; JS1, Candidate Division Japan Sea 1; HC, *Euryarchaeota* Halophilic Cluster; TaMGI, *Thaumarchaea* Marine Group I.


Eubacteria
A
Planctomycetes
MSBL4
Unaffiliated
Bacteroidetes
SAR406
GNSB
Actinobacteria
SB1
JS1
Deferribacteres
MSBL2
OD1
MSBL6
OP10
Deltaproteobacteria
Alphaproteobacteria
Epsilonproteobacteria
KB1
Gammaproteobacteria
Thetis 16S
454 IF (Interface)
454 BB (Body Brine)
0.1
Marine Group I
Halophilic Cluster1
MSBL1
Archaea
B
Thetis interface
metagenome 16S rRNA genes, %
16S crDNA clones, %
EPB
GPB
DPB
APB
SB1 / MSBL2
KB1
Bctr
Plcm
OP8
Dfbc
GNSB
MSBL4
OD1
JS1
MSBL6
Actb
SAR406
Bacteria
C
Thetis brine
MSBL1
HC
SA1
TaMG1
Archaea

settings do not sustain flourishing of marine aerobic *Thaumarchaeota* but still prevent the propagation of anaerobic halophilic *Euryarchaea* from the brine.

Functional differences between the brine and interface microbial communities

Of the non-hypothetical genes, 39.3% could be assigned to COGs (interface: 11 444; brine: 8688), and 24.0% could be assigned to KEGG orthologues (interface: 6974; brine: 5350) (Table 1). Both, the COG and KEGG profiles exhibited no prominent overall differences (data not shown), but key genes and their activities were found to be distinct: 828 (in brine) and 267 (in interface) KEGG orthologues were found to be unique, i.e. not shared between both microbial communities.

No less than nine F_0F_1-ATPase genes (seven distinct orthologues) were found exclusively in the interface, the majority of those apparently associated to single (or several) species of genomes of epsilonproteobacterial origin. Presence of additional ATPases might facilitate survival through elevated ATP-dependent ion efflux at high salinities. Likewise, several enzymes for oxygen and hydrogen utilization, namely cytochrome *c* oxidases (9 genes, 7 orthologues) and hydrogenases (7 genes, 6 orthologues) were found only in the interface. These enzymes are involved in the aerobic respiration and oxidation of H_2, respectively, thus indicating that, in addition to reduced sulphur compounds, molecular hydrogen may serve as an electron donor within the interface layer. Genes preventing oxidative damage (superoxide dismutases, superoxide reductases, catalases, peroxidases and NADPH P450 reductases; 31 distinct genes, 11 orthologues) were found in the interface as well, suggesting that radical scavenging is important, in particular, for the microaerophilic species in the yet oxic interface, whereas the anoxic brine was devoid of such genes.

A partial metabolic reconstruction, although incomplete due to the limited metagenome coverage, revealed conclusive evidences for differences in key metabolisms, which are discussed below.

Autotrophy (CO2 fixation). Autotrophic carbon fixation genes for the reductive tricarboxylic acid cycle (rTCA) were detected in the interface, namely ATP-citrate lyase (2 hits), malate dehydrogenase (1 hit), fumarate hydratase (5 hits), fumarate dehydrogenase (3 hits), succinyl-CoA synthethase (6 hits), 2-oxoglutarate synthase (2 hits), isocitrate dehydrogenase (8 hits) and aconitate dehydratase (2 hits), with 26 (or 90%) of them affiliating to *Epsilonproteobacteria*, and the remaining three (10%) to *Deltaproteobacteria*. In addition, three genes were found encoding subunits of CO dehydrogenase/acetyl-CoA synthase complexes of the reductive acetyl-CoA pathway (r-acetyl-CoA pathway), which catalyse the reduction of CO_2 to CO and the subsequent synthesis of acetyl-CoA. These genes were classified as deltaproteobacterial ones. This suggests the presence of rTCA and r-acetyl-CoA-fixing capacities (Hügler and Sievert, 2011) within the *Thetis* interface, with contributions from different bacterial taxa (Table S1; Fig. 2A). No genes were found for the 3-hydroxypropionate/4-hydroxybutyrate (3-HP/4-HB) and 3-HP bicycle, or for the Calvin-Benson-Bassham (CBB) cycle, suggesting rTCA as the dominant carbon fixation pathway within the *Thetis* interface.

The situation was found to be different in the brine, where a RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase), most likely associated to *Gammaproteobacteria*, the key enzyme of the CBB cycle, was found. This finding was unexpected for an anaerobic environment and might be explained either by settling of bacterial biomass from the overlaying seawater or by the presence of organisms similar to the endosymbiont of the giant tubeworm *Riftia pachptila*, which has been reported as the first bacterium simultaneously operating with two different carbon fixation pathways (rTCA and CBB) (Markert *et al.*, 2007). The second scenario seems be more plausible, as the seawater is separated from the brine by the interface where this enzyme was not detected and, especially, as several eukaryotic organisms were found in the hostile DHAL brines recently (Alexander *et al.*, 2009; Edgcomb *et al.*, 2009; Danovaro *et al.*, 2010). So far, symbiotic interactions in DHAL environments have not been studied at all, which opens a new opportunity of interesting research. Autotrophy under hypersaline anoxic conditions was presupposed by detection of three *Aquificae*-derived (members of KB1 candidate division?) ORFs in the *Thetis* brine metagenome coding for the rTCA cycle key enzyme ATP-citrate lyase. However, we could not identify other bacterial genes related to this cycle. In contrast, a number of pyruvate:water dikinases (6 hits), phosphoenolpyruvate carboxylases (2 hits), malate dehydrogenases (3 hits), fumarate hydratases (7 hits) and succinyl-CoA synthases (2 hits), all associated to *Euryarchaea,* were identified in the *Thetis* brine. The first two enzymes might be involved in the synthesis of oxaloacetate via formation of phosphoenolpyruvate from pyruvate, which is a major and essential CO_2 fixation reaction in some hydrogeno-, aceto- and methylotrophic methanogens and bacteria (Fuchs and Stupperich, 1978; Simpson and Whitman, 1993; Sako *et al.*, 1997). These organisms can use an incomplete rTCA cycle that starts with oxaloacetate and terminates at 2-oxoglutarate via consequent formation of malate, fumarate and succinyl-CoA. Alternatively, these enzymes could be involved in the dicarboxylate/4-hydroxybutyrate (DC/4-HB) cycle; however, because the life in deep hypersaline sea must

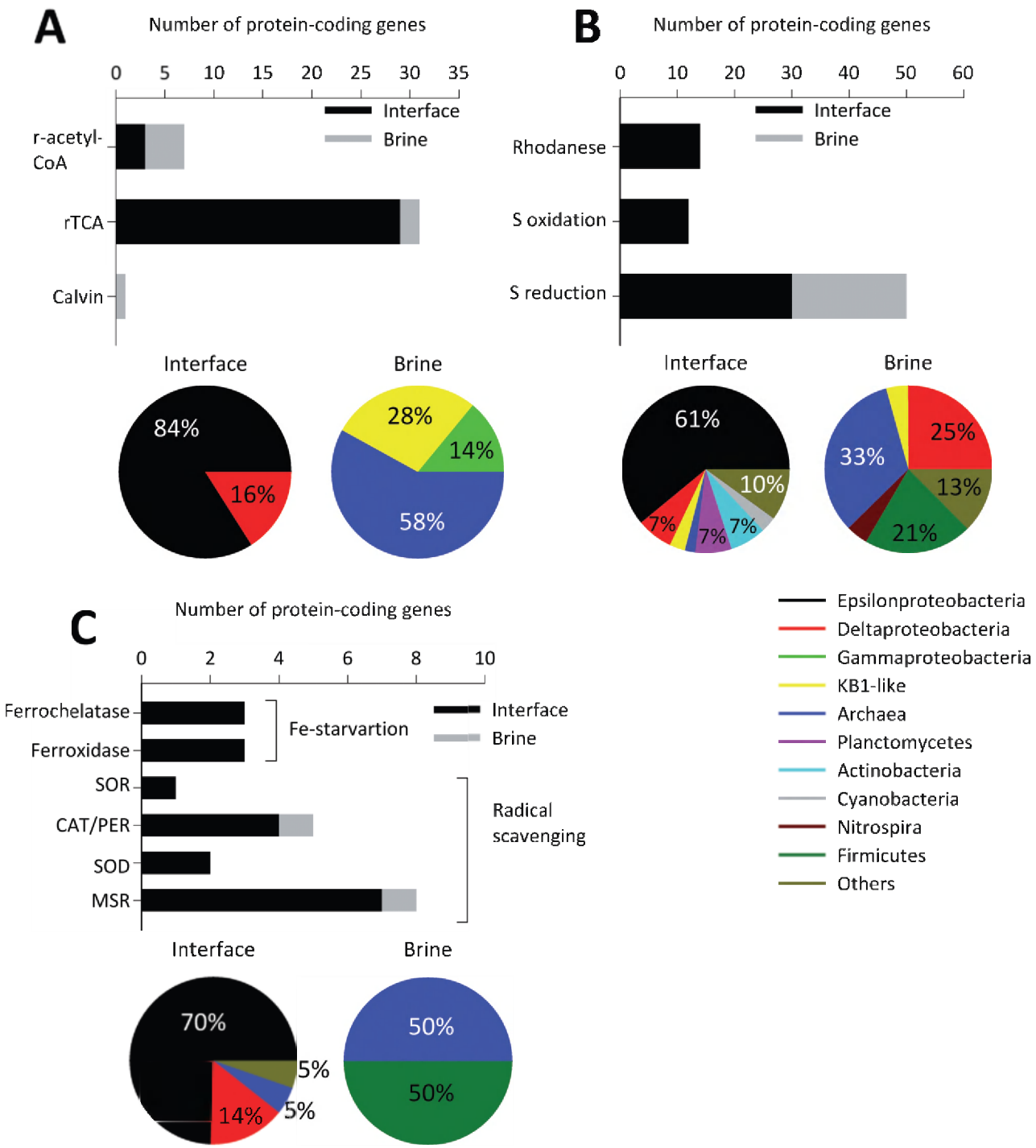


Fig. 2. Distribution of genes encoding enzymes for: (A) autotrophic CO_2 fixation (B) sulphur metabolism and (C) radical-scavenging and iron starvation. MSR, methionine sulfoxide reductase; SOD, superoxide dismutase; CAT/PER, catalase/peroxidase; SOR, superoxide reductase. Phylogenetic distribution based on taxonomic affiliation of coding sequences of the communities in the *Thetis* interface and brine metagenomes, performed as described in *Experimental procedures*, is specifically shown for each relevant metabolism. For details see Table S1.

adapt to low energy and given that DC/4-HB cycle requires more ATP equivalents than the r-acetyl-CoA and rTCA cycles (Berg *et al.*, 2010), we suggest that bio-energetic reasons may preclude the DC/4-HB pathway playing a major role in the anoxic brine. Finally, indications for the r-acetyl-CoA pathway, namely genes encoding formylmethanofuran dehydrogenase (2 hits) and subunits of the CO dehydrogenase/acetyl-CoA synthase (2 hits),

all associated with methanogenic *Euryarchaeota*, were also found (Table S1). This finding corroborates the general statement that high salinities favour hydrogeno- and/or methylotrophic methanogenesis (Oren, 1999). Presence of such *Euryarchaeota* in the *Thetis* brine was shown recently by a 16S rRNA survey as well as by direct cultivation (La Cono *et al.*, 2011). Although, further biochemical evidences are required, the present study in the extremely salted *Thetis* brine may suggest that autotrophic pathways are probably more important in hypersaline environments than previously assumed (Yakimov *et al.*, 2007; Joye *et al.*, 2009; La Cono *et al.*, 2011).

Sulphur metabolism. Both *Thetis* interface and brine samples are characterized by strongly reduced conditions and elevated concentrations of sulphide, most likely produced by microbial sulphate reduction (La Cono *et al.*, 2011). Assimilatory sulphate reduction was evident in both metagenomes due to the presence of ATP sulfhydrylase (interface: 15; brine: 13), adenosine 5′-phosphosulphate kinases (interface: 6; brine: 2) and phosphoadenosine phosphosulphate (PAPS) reductases (interface: 2; brine: 4) (Table S1; Fig. 2B). The enzyme cysteine desulfhydrylase (2 hits), which reversibly splits cysteine to pyruvate, NH_3 and sulphide, was also found in the *Thetis* interface metagenome (Fig. S4). This suggests that besides the uptake and assimilatory reduction of sulphate, direct sulphide assimilation occurs in the interface, which is believed to be a common trait of microaerobic sulphur-oxidizing chemoautotrophs inhabiting sulphide-rich environments (Campbell *et al.*, 2006).

Relevance of sulphur-oxidizing chemoautotrophs in the *Thetis* interface was also evidenced by nine *Epsilonproteobacteria*-related genes (three of them in one operon on contig 00001) encoding the components of Sox system, which performs hydrogen sulphide-, sulphur-, thiosulphate- and sulphite-dependent cytochrome *c* reduction (Rother *et al.*, 2001; Bamford *et al.*, 2002; Friedrich *et al.*, 2005). Metagenome analysis revealed furthermore two putative sulphide:quinone reductase (SQR) genes. SQR occurs in a number of phototrophic and chemoautotrophic bacteria and is best characterized in *Rhodobacter capsulatus* and *Thiomicrospira crunogena*. In those bacteria it catalyses the oxidation of sulphide to elemental sulphur, leading to deposition of extracellular sulphur globules. The Sox system, on the other hand, is expected to enable a complete oxidation of sulphide to sulphate. Switching to the production of elemental sulphur rather than sulphate has an advantage in preventing environmental acidification, which ultimately would lead to the cell lysis. The deposited sulphur globules could be consequently remobilized in polysulphides in a reaction with sulphide catalysed by NrfD-like polysulphide reductases found in the metagenome (2 hits). Finally, the non-reducing thiosulphate cleavage driven by RhoD rodanese-like thiosulphate:cyanide sulphurtransferases (14 hits in total) predominated in the interface as well as the reductive thiosulphate/thiosulphonate dismutation to sulphide by MopB-like thiosulphate reductases (1 hit).

Taken together, it is apparent that in the microbial community of the brine–seawater interface three types of sulphide oxidation pathways play a role. The first one involves a multienzyme Sox complex catalysing the complete oxidation of reduced sulphur compounds to sulphate. The second type is implementing sulphide:quinone reductase for the oxidation of sulphide to elemental sulphur, which, in the presence of sulphide, could be further reduced in the third pathway by polysulphide reductases. The presence of the last two pathways may allow yielding the maximal energy from sulphide oxidation while avoiding acidification of the environment and accumulation of S^0 deposits.

Contrasting with the abundance of sulphide oxidation genes, a lower proportion of dissimilatory sulphate reduction genes were found in the *Thetis* interface metagenome. Indeed, no APS reductases were identified and only one *dsrA* gene coding for the dissimilatory sulphite reductase alpha subunit was identified. Low numbers of *dsrA* copies were expected according with our previous study where these genes were not detected by PCR (La Cono *et al.*, 2011). Above observation re-emphasizes that negative PCR results obtained with environmental samples do not necessarily imply the gene absence, as low density and/or high divergence of the gene's sequence(s) can preclude amplification.

It is noteworthy, that sulphur-oxidation enzymes and polysulphide reductases, as well as rhodanese enzymes, could not be found in the metagenome dataset herein obtained from the brine, most likely due to an insufficient energy yield of respective processes to sustain the life in the highly salted brine. The finding of *dsr*A genes in the brine metagenome is in concordance with our previous statement, indicating that sulphate reduction is one of the active respiration pathways in the brine of DHALs (Borin *et al.*, 2009; La Cono *et al.*, 2011). Figure 3 summarizes the overall sulphur cycle between both *Thetis* compartments that includes provision of oxidized sulphur compounds from the interface and reduced sulphur compounds from the brine.

As shown in Table S1, taxonomic sequence binning attributed 75% of all sulphur metabolism-related genes to *Proteobacteria* with sequences affiliating with *Epsilonproteobateria* as most abundant in the interface (60% of the total sequences). In the highly concentrated brine, the sequences were distributed within many different taxa related to *Euryarchaea* (*c.* 33% of all hits) and *Bacteria* (67% hits).

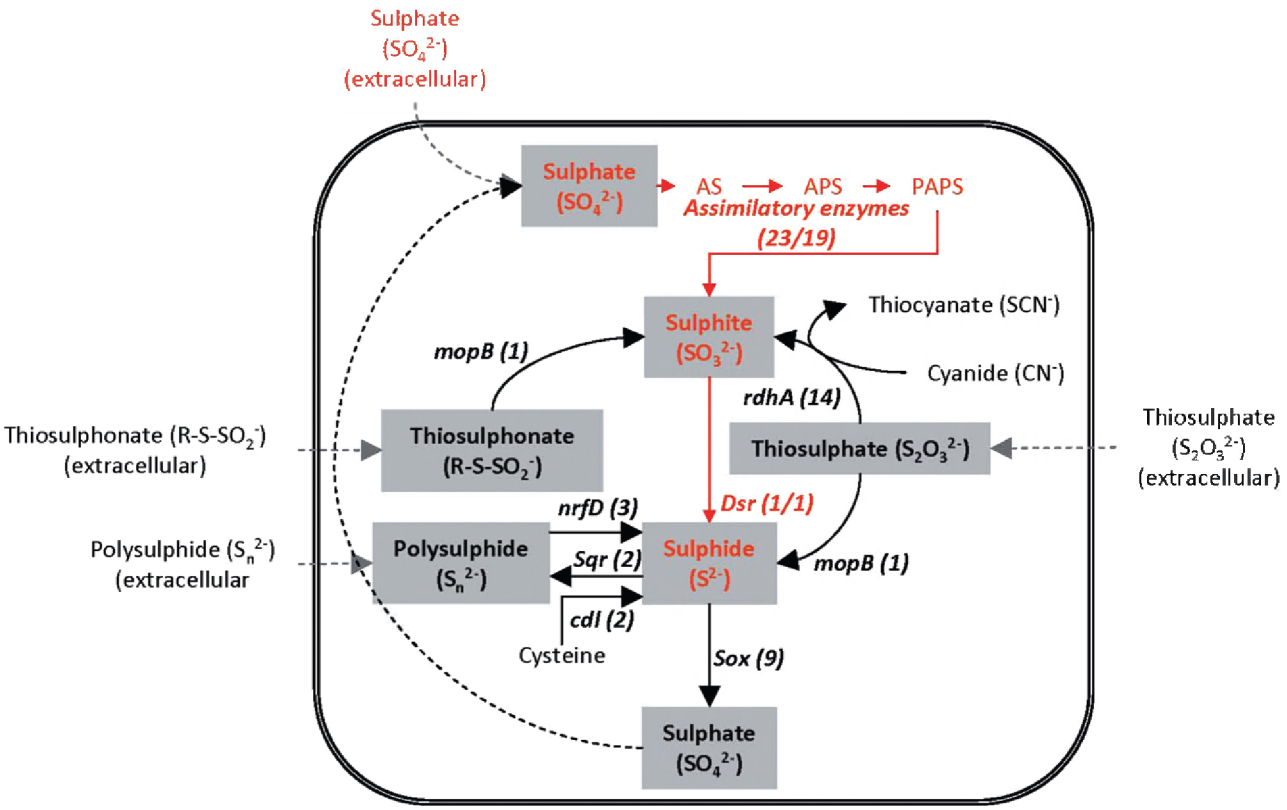


Fig. 3. Proposed sulphur-metabolizing profile of the *Thetis* interface and brine communities based on BLAST hits of protein homologues found in the metagenome data. The number of putative gene encoding for enzymes involved in the potential transformation of each molecule is specifically shown. The enzymes and putative transformations found in the interface and brine compartments are shown in red, while black indicates transformations specific for the interface. Data are based on BLASTx high-scoring sequences paired against the non-redundant GenBank database, according to a maximum *E*-value of 10^{-5}.

Oxidative stress. Deep-sea hydrothermal vents are typified by rapid fluctuations in redox potential that impose a strong selective pressure on resident microbial communities. Many microaerophilic *Gamma-* and *Epsilonproteobacteria*, adapted to thrive in such dynamic environments, possess the numerous genes whose products have redox sensory domains likely function to position these cells in the redoxcline and to enable them to obtain the electron donors and acceptors needed for growth (Scott *et al.*, 2006; Nakagawa *et al.*, 2007; Sievert *et al.*, 2008; Campbell *et al.*, 2009). Additionally, the genome of these organisms encoded several types of enzymes to detoxify reactive oxygen and nitrogen species. Similarly to submarine hydrothermal environments, survival in the oxic–anoxic interface of Lake *Thetis* is likely cope with necessity in the exact positioning in the redoxcline of chemolithotrophs possessing either microaerophilic or anaerobic lifestyles. Logically, that wrong positioning requires a degree of metabolic versatility and occurrence of defence mechanisms against oxidative stress. Accordingly, microaerophilic lifestyle in the interface is based in some cases on the activity of oxygen-sensitive enzymes and thus requires the maintenance of slightly reduced conditions in the cytoplasm via recruitment of oxidative stress proteins. According to that, two superoxide dismutases (SOD), two catalases/peroxidases and one glutathione peroxidase, as three major representative oxygen-detoxifying enzymes, were identified in the interface metagenome (Table S1; Fig. 2C). Also, a superoxide reductase that catalyses the conversion of highly reactive and toxic superoxide (O_2^-) to O_2 and the less toxic H_2O_2, was identified. This suggests that reactive oxygen species (ROS) scavenging is important for the interface microbial community, and that oxidative stress is an important mechanism for microbial adaptation (Table S1). Moreover, seven distinct methionine sulphoxide reductases were identified in the interface metagenome. This enzyme catalyses the thioredoxin-dependent reduction and repair of methionine sulphoxide (MetO) produced by damaging methionine oxidation (Fukushima *et al.*, 2007).

Although the density gradients at the oxic/anoxic interface of DHALs seem to be very stagnant, thus stabilizing the redox interface and preventing mixing, oscillations in the concentration of the oxygen and ROS could occur in this zone at micro-scale level as a consequence of actively swimming and grazing marine invertebrates. The

necessity for chemolithotrophic sulphur-oxidizing epibionts and symbionts to be equipped with oxygen-protection systems could be additional factor explaining the presence of the enzymes related to oxidative stress.

Body brines of DHALs constitute a permanently anoxic environment, protected from any ROS. Consequently, very few ROS-scavenging enzymes were found in the *Thetis* brine metagenome: only two gene sequences encoding methionine sulphoxide reductase (1 hit) and H_2O_2 detoxifying hydroperoxide reductase (1 hit).

Microbial communities in the Thetis interface survive iron droughts. Concentrations of iron in the deep-sea are low enough to limit microbial growth. Therefore numerous systems have evolved in microorganisms to rapidly take up and store iron when it is available and withstand long intervals of limited iron availability (Moore *et al.*, 2002). We have identified ferroxidases (3 hits) and ferrochelatases (3 hits) in the *Thetis* interface, all of them most likely derived from *Delta-* and *Epsilonproteobacteria* (Fig. 2C). These proteins, absent in the brine metagenome, may play two key roles: first, to acquire and store iron and second, to protect cells from potential oxidative damage. Recently, it has been suggested that high levels of such iron storage proteins correlate with high rates of cell division and high CO_2 fixation levels (Marchetti *et al.*, 2009). To the best of our knowledge, the presence of such genes in a DHAL has not been previously reported.

Transport-related proteins. Microbial communities in anaerobic, reducing habitats of deep-sea hypersaline anoxic basins are thought to be limited by carbon substrate availability. We analysed transport-related functions among the identified proteins in order to obtain indications for the type of substrates consumed by community members in the interface and brine. The most prominent group of transport proteins found in the interface consisted of outer membrane beta-barrel proteins [i.e. porins (10 hits)] and TonB outer membrane receptors (47 hits), for which only one hit was found in the brine metagenome. Porins usually facilitate a passive diffusion of small molecules across the outer membranes, while TonB transport systems allow an active transport of substrates larger than 600 Da (Krewulak and Vogel, 2011). The presence of multiple TonB receptors likely reflects a high degree of versatility for scavenging various substrates at low concentrations [including a number of carbohydrates, ions (including iron) and vitamin B_{12}]. This could help to explain the success of a number of microbial groups in the interface as compared with the conditions existing in the brine as has been shown in previous studies (e.g. Schauer *et al.*, 2007; Delmotte *et al.*, 2009). A number of PtsN-like phosphotransferases that likely function to assist in the uptake of sugars and other carbohydrates (Pflüger and de Lorenzo, 2007) were identified in the interface metagenome (8 hits in total) but not in the brine, which also indicates easier accessibility of carbon sources for microbes from the high-energy interface compared with the low-energy brine habitat (Oren, 1999). Alternatively, due to the scarce bio-energetic potential and necessity to spend significant amounts of energy to cope with hyperosmotic stress, different transport systems could be implemented by the brine-inhabiting microbes. Indeed, only one TonB-like receptor and no PtsN-like phosphotransferases were identified in the brine, but a high number of ATPases, which, in parallel with other functions, may likely promote proton motive force-linked transportation of carbohydrates and/or other compounds.

Virulence gene homologues. It has been shown that deep-sea microbes share many virulence genes including genes for virulence factor MviN, haemolysin, invasion antigen CiaB, lytic murein transglycosilase and oligosaccharyltransferases with a conserved catalytic motif (WWDYG) (Nakagawa *et al.*, 2007). In the interface/brine metagenome we identified 12/3 distinct virulence factor proteins (9 and 1 of them homologous to MviN and MCE virulence factors), 3/3 oligosaccharyltransferases with a conserved catalytic motif (WWDYG), 11/3 haemolysin-like proteins and 1/0 invasion antigen protein. The identification of these virulence genes could indicate symbiotic relationships with deep-sea invertebrates, as previously suggested by sequencing of deep-sea symbionts (Kuwahara *et al.*, 2007; Nakagawa *et al.*, 2007). This would also agree with the above-mentioned RuBisCo finding in the brine metagenome. Such symbiosis is expected, as it is hard to imagine that eukaryotes thrive in the hostile brine environment without the help of symbiotic bacterial partners, while many invertebrates in less extreme deep-sea environments rely on close associations with bacteria for their survival.

Insights into protein adaptation in saturated salt brines of the Lake Thetis

A bio-energetically favoured adaptation to survive in anoxic, low-energy hypersaline systems is the 'salt-in' strategy, which means an elevated cation concentration in the cytoplasm. As a consequence, the majority of cytoplasmic proteins exhibit typical halophilic features, i.e. the ability to be active at high intracellular cation concentrations, denaturation at salt concentrations below 1 M and typically exhibiting acidic amino acid (AA) profile and therefore the negative charge. The negative charge of halophilic proteins plays an essential role in maintaining their solubility and stabilization of their functional structures (folding). Many studies have dissected the features of intracellular proteins of hyperhalophilic organisms (for

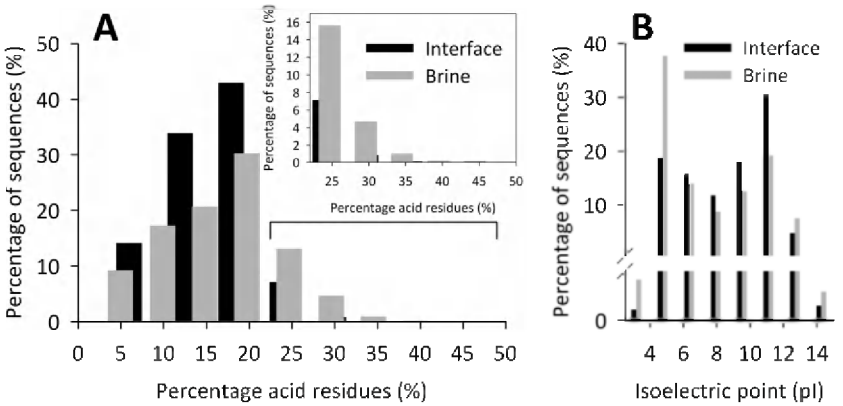


Fig. 4. General amino acid sequence features of proteins of *Thetis* compartments. Results of acidic residues and pI calculations are shown in (A) and (B) respectively. Results of *F*-test: *P*-value < $2.2e^{-16}$ (for acidic residues calculations); *P*-value < $2.2e^{-16}$ (for pI calculations). Results of Welch two sample *t*-test: *P*-value < $2.2e^{-16}$ (for acidic residues calculations); *P*-value < $2.2e^{-16}$ (for pI calculations).

examples see Frolow *et al.*, 1996; Madern *et al.*, 2000), revealing that they usually contain a large excess of acidic AA residues and low amounts of hydrophobic amino acids as compared with non-halophilic organisms. To prove this, the occurrence of charged AA was analysed in the pools of deduced proteins in interface and brine metagenomes. First, the preponderance of acidic residues (independently of the location) was analysed, revealing that the brine proteins have the highest negative charge density. While less that 8% of all proteins analysed in the brine–seawater interface proteome contained more than 18% of acidic aa residues, this value increased 2.8-fold (circa 22%) for the brine *in silico* proteome (Fig. 4A). According to these observations, the average isoelectric point (pI) of sequences showed a marked difference between both metagenomes (Fig. 4B). Thus, the percentage of proteins with pI ranging from 3.2 to 4.8 is about twofold higher in the brine as compared with the interface (circa 38 vs. 18%, respectively).

Although, further analysis is required to address the effect of amino acid composition of proteins in the *Thetis* compartments, it is clear that proteins from both environments have distinct amino acid compositions, and that the brine possesses a higher number of halophilic enzymes. Enzyme activity-centred metagenomic studies are currently underway to validate above assumptions.

Conclusions

In the present study we performed sequencing of the environmental metagenomes of microbial communities inhabiting the interface and brine of the *Thetis* Lake, a recently discovered thalassohaline DHAL filled by 5 M of NaCl (La Cono *et al.*, 2011). Data analysis generally agreed with the anticipated compositions of microbial assemblages and also pointed at genomic backgrounds for adaptation and survival in the interface and brine compartments. Although, it cannot be excluded that additional genes coding relevant enzymes slipped detection due to low metagenome coverage, the sequence reconstruction herein provided revealed important components of the CO_2 fixation pathways, sulphur reduction and oxidation, iron storage and assimilation of carbon sources. It is apparent that in the interface three types of sulphide oxidation occur, whereas sulphate reduction is one of the principal respiratory processes in the brine. Amino acid composition analyses revealed a significant overrepresentation of acidic aa residues in the brine proteome as compared with that in the interface. This protein feature is typical for the halophilic organisms and seems to be one of the key mechanisms of microbial adaptation to the extreme hypersalinity in the brine. Taken together, this work presents the first metagenomic survey of the microbial communities of the recently discovered Lake *Thetis* whose brine constitutes one of saltiest water bodies ever reported.

Experimental procedures

Sampling of the halocline and brine in the Lake Thetis and DNA extraction

Sampling of the *Thetis* Lake was conducted from the RV *Urania* at location 34.6698°N and 22.1455°E during oceanographic cruises of the RV *Urania* in September 2009. Samples were collected using 12 l Niskin bottles housed on a rosette (General Oceanics, 15 Miami, FL, USA) equipped with SBE-911plus conductivity-temperature-depth (CTD) sensors [for details see La Cono and colleagues (2011)]. For DNA extraction, 200 l of brine sample was filtered by tangential flow concentration using Millipore Pellicon system with 0.1 μm cut-off, in a N_2 atmosphere. The concentration was carried out to 400 ml of the final volume of retentate. The latter was filtered using 100 kDa membrane filtration disk YM-100, 63.5 mm diameter (Millipore) mounted into the Ultrafiltration cell 8200 (Amicon). For interface sample, 10 l was filtered through sterile Sterivex capsules (0.2 μm pore size, Millipore, Billerica, MA, USA) using a peristaltic pump. In both cases, after filtration, DNA was isolated using MetaGnome DNA extraction Kit (Epicentre, Madison, WI, USA).

DNA sequencing, assembly, gene prediction and annotation

Sequencing was performed with a Roche 454 GS FLX Ti sequencer (454 Life Sciences, Branford, CT, USA) at Lifesequencing S.L (Valencia, Spain), with one picotiterplate each for the interface (616 810 reads) and the brine (498 717 reads). Assembly was done with Roche's Newbler assembler v. 2.5.3 with default parameters, resulting in 12 105 contigs amounting to 13.4 Mb of sequence for the interface and 15 850 contigs comprising 9.7 Mb for the brine. The bulk of assemblies were between 500–2500 bp in lengths. Assembly was better for the interface with 8% of assemblies exceeding 2.5 kb as compared with 2% for the brine, indicating a higher biodiversity of the latter. Potential protein-coding genes were identified using a combination of MetaGene (Noguchi *et al.*, 2006) and a brute-force prediction for ORFs exceeding 150 bp in the intergenic regions to find incomplete or overlooked genes. Transfer RNA genes were identified using tRNAScan-SE (Lowe and Eddy, 1997) and ribosomal RNA genes were identified via BLAST searches (Altschul *et al.*, 1997) against public nucleotide databases. The annotation of the genome sequence was performed with a modified GenDB v2.2.1 system (Meyer *et al.*, 2003). For each predicted gene, similarity searches were performed against public sequence databases (nr, SWISSPROT, KEGG) and protein family databases (Pfam, InterPro, COG). Signal peptides were predicted with SignalP v3.0 (Nielsen *et al.*, 1999; Emanuelsson *et al.*, 2007) and transmembrane helices with TMHMM v2.0 (Krogh *et al.*, 2001). Based on these observations, annotations were derived in an automated fashion using a fuzzy logic-based approach (MicHanThi software; Quast, 2006).

Taxonomic classification of metagenome sequences

A consensus from five individual taxonomic prediction tools was used in order to infer the taxonomic affiliation of the metagenome sequences: (a) CARMA (Krause *et al.*, 2008) infers taxonomy of sequences by post-processing genes with HMMER hits to the Pfam database. The basic principle is that it fetches the expert seed alignment underlying a Pfam model, realigns it with inclusion of the matching query sequence and infers a neighbour-joining phylogenetic tree. We used a re-implemented and optimized C++ version of the original PERL code. (b) KIRSTEN (Kinship Relationship Reestablishment, unpublished) infers taxonomy of sequences by post-processing BLAST hits by means of rank-based statistical evaluations on all 27 levels of the NCBI taxonomy with an increasing stringency from the superkingdom down to the species level. (c) TaxSOM (Weber *et al.*, 2011) uses a unique combination Markov model-based oligonucleotide statistics and Self-Organizing Maps (SOMs) to infer taxonomy. We used a large SOM that was trained with all sequences from the NCBI database exceeding the genome size of *Nanoarchaeum equitans* (~ 490 kb). (d) SU tag analysis (Klindworth *et al.*, in preparation) extracts all full and partial 16S ribosomal RNA genes from the de-replicated reads, maps them to a well-curated reference tree provided by the SILVA rRNA database project (Pruesse *et al.*, 2007), and then uses this information to infer the taxonomy of the contigs into which the reads were assembled. (e) SSAHA2 (Ning *et al.*, 2001) was used to map the de-replicated pyrosequencing reads on a well-chosen set of 339 marine reference genomes taken from EnvO-lite environmental ontology. Repetitive regions in the reference genomes were masked with mreps (Kolpakov *et al.*, 2003). For each sequence, the combined mapping information was used to infer its taxonomic affiliation. Finally, a logic was developed (Klindworth *et al.*, in preparation) that consolidates the individual tool's taxonomic predictions into a consensus using a weighted assessment on all existing 27 ranks of the NCBI taxonomy from superkingdom to species. As a result a substantial fraction of the sequences and thus the genes from the *Thetis* metagenome could be classified on some taxonomic level (interface: 79% superkingdom, 63% phylum, 54% class, 42% genus; brine: 45% superkingdom, 39% phylum, 31% class, 23% genus).

Bioinformatic methods in Thetis sequence analysis

Calculation of the isoelectric point and percentage of the residues was done with the program *pepstats*, which is part of the *EMBOSS* toolkit (Rice *et al.*, 2000). Statistical comparisons of the averages of the isoelectric point and percentages of the different types of amino acids were compared using a student's *t*-test. First, we performed a Fisher's test to test for the equality of the variances. In case the variances were equal, we computed a *t*-test to compare the means, but when the variances were different a modification of the *t*-test (Welch *t*-test) was used. All the statistical procedures were performed using the *R* platform (R Development Core Team, 2010). A Perl script was used with the program *pepstats* to calculate the lengths of the sequences and the maximum and minimum values, the distributions by intervals, and the means, variances and standard deviations. A second script was used to export the data from *pepstats* for each protein and process them with *R* platform.

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References

Alexander, E., Stock, A., Breiner, H.W., Behnke, A., Bunge, J., Yakimov, M.M., and Stoeck, T. (2009) Microbial eukaryotes in the hypersaline anoxic L'Atalante deep-sea basin. *Environ Microbiol* **11:** 360–381.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., *et al.* (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25:** 3389–3402.

Bamford, V.A., Bruno, S., Rasmussen, T., Appia-Ayme, C., Cheesman, M.R., Berks, B.C., and Hemmings, A.M. (2002)

Structural basis for the oxidation of thiosulfate by a sulfur cycle enzyme. *EMBO J* **21:** 5599–5610.

Benlloch, S., López-López, A., Casamayor, E.O., Øvreås, L., Goddard, V., Daae, F.L., *et al.* (2002) Prokaryotic genetic diversity throughout the salinity gradient of a coastal saltern. *Environ Microbiol* **4:** 349–360.

Berg, I.A., Kockelkorn, D., Ramos-Vera, W.H., Say, R.F., Zarzycki, J., Hugler, M., *et al.* (2010) Autotrophic carbon fixation in archaea. *Nat Rev Microbiol* **8:** 447–460.

Borin, S., Brusetti, L., Mapelli, F., D'Auria, G., Brusa, T., Marzorati, M., *et al.* (2009) Sulfur cycling and methanogenesis primarily drive microbial colonization of the highly sulfidic Urania deep hypersaline basin. *Proc Natl Acad Sci USA* **106:** 9151–9156.

Camerlenghi, A. (1990) Anoxic basins of the eastern Mediterranean: geological framework. *Mar Chem* **31:** 1–19.

Campbell, B.J., Engel, A.S., Porter, M.L., and Takai, K. (2006) The versatile epsilon-proteobacteria: key players in sulphidic habitats. *Nat Rev Microbiol* **4:** 458–468.

Campbell, B.J., Smith, J.L., Hanson, T.E., Klotz, M.G., Stein, L.Y., Lee, C.K., *et al.* (2009) Adaptations to submarine hydrothermal environments exemplified by the genome of *Nautilia profundicola*. *PLoS Genet* **5:** e1000362.

Cita, M.B. (2006) Exhumation of Messinian evaporites in the deep-sea and creation of deep anoxic brine-filled collapsed basins. *Sediment Geol* **188–189:** 357–378.

D'Hondt, S., Jørgensen, B.B., Miller, D.J., Batzke, A., Blake, R., Cragg, B.A., *et al.* (2004) Distribution of microbial activities in deep subseafloor sediments. *Science* **306:** 2216–2221.

Daffonchio, D., Borin, S., Brusa, T., Brusetti, L., van der Wielen, P.W., Bolhuis, H., *et al.* (2006) Stratified prokaryote network in the oxic-anoxic transition of a deep-sea halocline. *Nature* **440:** 203–207.

Danovaro, R., Corinaldesi, C., Dell'Anno, A., Fabiano, M., and Corselli, C. (2005) Viruses, prokaryotes and DNA in the sediments of a deep-hypersaline anoxic basin (DHAB) of the Mediterranean Sea. *Environ Microbiol* **7:** 586–592.

Danovaro, R., Dell'Anno, A., Pusceddu, A., Gambi, C., Heiner, I., and Kristensen, R.M. (2010) The first metazoa living in permanently anoxic conditions. *BMC Biol* **8:** 30.

De Lange, G.J., Middleburg, J.J., van der Weijden, K.H., Luther, III, G.W., Hydes, D.J., Woittiez, J.R.W., *et al.* (1990) Composition of anoxic hypersaline brines in the Tyro and Bannock Basins, 23 Eastern Mediterranean. *Mar Chem* **31:** 63–88.

Delmotte, N., Kniel, C., Chaffron, S., Innerebner, G., Roschitzki, B., Schlapbacj, R., *et al.* (2009) Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. *Proc Natl Acad Sci USA* **106:** 16428–16433.

DeLong, E.F., Preston, C.M., Mincer, T., Rich, V., Hallam, S.J., *et al.* (2006) Community genomics among stratified microbial assemblages in the oceans interior. *Science* **311:** 496–503.

Edgcomb, V., Orsi, W., Leslin, C., Epstein, S.S., Bunge, J., Jeon, S., *et al.* (2009) Protistan community patterns within the brine and halocline of deep hypersaline anoxic basins in the eastern Mediterranean Sea. *Extremophiles* **13:** 151–167.

Emanuelsson, O., Brunak, S., Von Heijne, G., and Nielsen, H. (2007) Locating proteins in the cell using TargetP, SignalP and related tools. *Nat Protoc* **2:** 953–971.

Feingersch, R., Suzuki, M.T., Shmoish, M., Sharon, I., Sabehi, G., *et al.* (2010) Microbial community genomics in eastern Mediterranean Sea surface waters. *ISME J* **4:** 78–87.

Fiala, G., Woese, C.R., Langworthy, T.A., and Stetter, K.O. (1990) *Flexistipes sinusarabici* a novel genus and species of eubacteria occurring in the Atlantis II Deep brines of the Red Sea. *Arch Microbiol* **154:** 120–126.

Frías-López, J., Shi, Y., Tyson, G.W., Coleman, M.L., Schuster, S.C., *et al.* (2008) Microbial community gene expression in ocean surface waters. *Proc Natl Acad Sci USA* **105:** 3805–3810.

Friedrich, C.G., Bardischewsky, F., Rother, D., Quentmeier, A., and Fischer, J. (2005) Prokaryotic sulfur oxidation. *Curr Opin Microbiol* **8:** 253–259.

Frolow, F., Harel, M., Sussman, J.L., Mevarech, M., and Shoham, M. (1996) Insights into protein adaptation to a saturated salt environment from the crystal structure of a halophilic 2Fe2S ferredoxin. *Nat Struct Biol* **3:** 452–458.

Fuchs, G., and Stupperich, E. (1978) Evidence for an incomplete reductive carboxylic acid cycle in *Methanobacterium thermoautotrophicum*. *Arch Microbiol* **118:** 121–125.

Fukushima, E., Shinka, Y., Fukui, T., Atomi, H., and Imanaka, T. (2007) Methionine sulfoxide reductase from the hyperthermophilic archaeon *Thermococcus kodakaraensis*, an enzyme designed to function at suboptimal growth temperatures. *J Bacteriol* **189:** 7134–7144.

Hallsworth, J.E., Yakimov, M.M., Golyshin, P.N., Gillion, J.L., D'Auria, G., de Lima Alves, F., *et al.* (2007) Limits of life in MgCl2-containing environments: chaotropicity defines the window. *Environ Microbiol* **9:** 801–813.

Hügler, M., and Sievert, S.M. (2011) Beyond the Calvin cycle: autotrophic carbon fixation in the ocean. *Ann Rev Mar Sci* **3:** 261–289.

Jongsma, D., Fortuin, A.R., Huson, W., Troelstra, S.R., Klaver, G.T., Peters, J.M., *et al.* (1983) Discovery of an anoxic basin within the Strabo Trench, eastern Mediterranean. *Nature* **305:** 795–797.

Joye, S.B., Samarkin, V.A., Orcutt, B.N., MacDonald, I.R., Hinrichs, K.-U., Elvert, M., *et al.* (2009) Metabolic variability in seafloor brines revealed by carbon and sulphur dynamics. *Nat Geosci* **2:** 349–354.

Kolpakov, R., Bana, G., and Kucherov, G. (2003) mreps: efficient and flexible detection of tandem repeats in DNA. *Nucleic Acids Res* **31:** 3672–3678.

Konstantinidis, K.T., Braff, J., Karl, D.M., and DeLong, E.F. (2009) Comparative metagenomic analysis of a microbial community residing at a depth of 4,000 meters at station ALOHA in the North Pacific subtropical gyre. *Appl Environ Microbiol* **75:** 5345–5355.

Krause, L., Diaz, N.N., Goesmann, A., Kelley, S., Nattkemper, T.W., Rohwer, F., *et al.* (2008) Phylogenetic classification of short environmental DNA fragments. *Nucleic Acids Res* **36:** 2230–2239.

Krewulak, K.D., and Vogel, H.J. (2011) TonB or not TonB: is that the question? *Biochem Cell Biol* **89:** 87–97.

Krogh, A., Larsson, B., Von Heijne, G., and Sonnhammer, E.L. (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol* **305:** 567–580.

Kuwahara, H., Yoshida, T., Takaki, Y., Shimamura, S., Nishi, S., Harada, M., *et al.* (2007) Reduced genome of the thioautotrophic intracellular symbiont in a deep-sea clam, *Calyptogena okutanii*. *Curr Biol* **17:** 881–886.

La Cono, V., Smedile, F., Bortoluzzi, G., Arcadi, E., Maimone, G., Messina, E., *et al.* (2011) Unveiling microbial life in new deep-sea hypersaline Lake Thetis. Part I: prokaryotes and environmental settings. *Environ Microbiol* **13:** 2250–2268. doi: 10.1111/j.1462-2920.2011.02478.x.

Levin, L. (2003) Oxygen minimum zone benthos: adaptation and community response to hypoxia. *Oceanogr Mar Biol Annu Rev* **41:** 1–45.

Lowe, T.M., and Eddy, S.R. (1997) tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Res* **25:** 955–964.

Ludwig, W., Wallner, G., Tesch, A., and Klink, F. (1991) A novel eubacterial phylum: comparative nucleotide sequence analysis of a tuf-gene of *Flexistipes sinusarabici*. *FEMS Microbiol Lett* **62:** 139–143.

Madern, D., Ebel, C., and Zaccai, G. (2000) Halophilic adaptations of enzymes. *Extremophiles* **4:** 91–98.

Marchetti, A., Parker, M.S., Moccia, L.P., Lin, E.O., Arrieta, A.L., Ribalet, F., *et al.* (2009) Ferritin is used for iron storage in bloom-forming marine pennate diatoms. *Nature* **457:** 467–470.

Markert, S., Arnd, C., Felback, H., Becher, D., Sievert, S.M., *et al.* (2007) Physiological proteomics of th euncultured endosymbiont of *Riftia pachptila*. *Science* **315:** 247–250.

Martin-Cuadrado, A.B., López-García, P., Alba, J.C., Moreira, D., Monticelli, L., *et al.* (2007) Metagenomics of the deep Mediterranean, a warm bathypelagic habitat. *PLoS ONE* **2:** e914.

Meyer, F., Goesmann, A., McHardy, A.C., Bartels, D., Bekel, T., Clausen, J., *et al.* (2003) GenDB–an open source genome annotation system for prokaryote genomes. *Nucleic Acids Res* **31:** 2187–2195.

Moore, J.K., Doney, S.C., Glover, D.M., and Fung, I.Y. (2002) Iron cycling and nutrient limitation patterns in surface waters of the World Ocean. *Deep Sea Res II* **49:** 463–507.

Nakagawa, S., Takaki, Y., Shimamura, S., Reysenbach, A.L., Takai, K., and Horikoshi, K. (2007) Deep-sea vent epsilonproteobacterial genomes provide in- sights into the emergence of pathogens. *Proc Natl Acad Sci USA* **104:** 12146–12150.

Nielsen, H., Brunak, S., and Von Heijne, G. (1999) Machine learning approaches for the prediction of signal peptides and other protein sorting signals. *Protein Eng* **12:** 3–9.

Ning, Z., Cox, A.J., and Mullikin, J.C. (2001) SSAHA: a fast search method for large DNA databases. *Genome Res* **11:** 1725–1729.

Noguchi, H., Park, J., and Takagi, T. (2006) MetaGene: prokaryotic gene finding from environmental genome shotgun sequences. *Nucleic Acids Res* **34:** 5623–5630.

Oren, A. (1999) Bioenergetic aspects of halophilism. *Microbiol Mol Biol Rev* **63:** 334–348.

Oren, A. (2008) Microbial life at high salt concentrations: phylogenetic and metabolic diversity. *Saline Systems* **4:** 2.

Oren, A. (2011) Thermodynamic limits to microbial life at high salt concentrations. *Environ Microbiol* **13:** 1908–1923.

Parkes, R.J., Webster, G., Cragg, B.A., Weightman, A.J., Newberry, C.J., Ferdelman, T.G., *et al.* (2005) Deep subseafloor prokaryotes stimulated at interfaces over geological time. *Nature* **436:** 390–394.

Pflüger, K., and de Lorenzo, V. (2007) Growth-dependent phosphorylation of the PtsN (EIINtr) protein of *Pseudomonas putida*. *J Biol Chem* **282:** 18206–18211.

Poretsky, R.S., Hewson, I., Sun, S., Allen, A.E., Zehr, J.P., *et al.* (2009) Comparative day/night metatranscriptomic analysis of microbial communities in the North Pacific subtropical gyre. *Environ Microbiol* **11:** 1358–1375.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W.G., Peplies, J., and Glöckner, F.O. (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acid Res* **35:** 7188–7196.

Quast, C. (2006) MicHanThi – design and implementation of a system for the prediction of gene functions in genome annotation projects. Master Thesis 2006; available on request.

R Development Core Team (2010) *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing. ISBN 3-900051-07-0, URL http://www.R-project.org.

Rice, P., Longden, I., and Bleasby, A. (2000) EMBOSS: The European Molecular Biology Open Software Suite. *Trends Genet* **16:** 276–277.

Rother, D., Henrich, H.J., Quentmeier, A., Bardischewsky, F., and Friedrich, C.G. (2001) Novel genes of the sox gene cluster, mutagenesis of the flavoprotein SoxF, and evidence for a general sulfur-oxidizing system in *Paracoccus pantotrophus* GB17. *J Bacteriol* **183:** 4499–4508.

Rusch, D.B., Halpern, A.L., Sutton, G., Heidelberg, K.B., Williamson, S., *et al.* (2007) The Sorcerer II Global Ocean Sampling expedition: northwest Atlantic through eastern tropical Pacific. *PLoS Biol* **5:** e77.

Sako, Y., Takai, K., Nishizaka, T., and Ishido, Y. (1997) Biochemical relationship of phosphoenol pyruvate carboxylases (PEPCs) from thermophilic archaea. *FEMS Microbiol* **153:** 159–165.

Schauer, K., Gouget, B., Carrière, M., Labigne, A., and de Reuse, H. (2007) Novel nickel transport mechanism energized by the TonB/ExbB/ExbD machinery. *Environ Microbiol* **63:** 1054–1068.

Scott, K.M., Sievert, S.M., Abril, F.N., Ball, L.A., Barrett, C.J., Blake, R.A., *et al.* (2006) The genome of deep-sea vent chemolithoautotroph *Thiomicrospira crunogena* XCL-2. *PLoS Biol* **4:** e383.

Shi, Y., Tyson, G.W., and DeLong, E.F. (2009) Metatranscriptomics reveals unique microbial small RNAs in the oceans water column. *Nature* **459:** 266–269.

Sievert, S.M., Scott, K.M., Klotz, M.G., Chain, P.S., Hauser, L.J., Hemp, J., *et al.* (2008) Genome of the epsilonproteobacterial chemolithoautotroph Sulfurimonas denitrificans. *Appl Environ Microbiol* **74:** 1145–1156.

Simpson, P.G., and Whitman, W.B. (1993) Anabolic pathways in methano- gens. In *Methanogenesis: Ecology, Physiology, Biochemistry, and Genetics*. Ferry, J.G. (ed.). New York, NY, USA: Chapman and Hall, pp. 445–472.

Van der Wielen, P.W., and Heijs, S.K. (2007) Sulfate-reducing prokaryotic communities in two deep hypersaline anoxic basins in the Eastern Mediterranean deep sea. *Environ Microbiol* **9:** 1335–1340.

Van der Wielen, P.W., Bolhuis, H., Borin, S., Daffonchio, D., Corselli, C., Giuliano, L., *et al.* (2005) The enigma of prokaryotic life in deep hypersaline anoxic basins. *Science* **307:** 121–123.

Vaquer-Sunyer, R., and Duarte, C.M. (2008) Thresholds of hypoxia for marine biodiversity. *Proc Natl Acad Sci USA* **105:** 15452–15457.

Venter, J.C., Remington, K., Heidelberg, J.F., Halpern, A.L., Rusch, D., *et al.* (2004) Environmental genome shotgun sequencing of the Sargasso Sea. *Science* **304:** 66–74.

Weber, M., Teeling, H., Huang, S., Waldmann, J., Kassabgy, M., Fuchs, B.M., *et al.* (2011) Practical application of self-organizing maps to interrelate biodiversity and functional data in NGS-based metagenomics. *ISME J* **5:** 918–928.

Yakimov, M.M., La Cono, V., Denaro, R., D'Auria, G., Decembrini, F., Timmis, K.N., *et al.* (2007) Primary producing prokaryotic communities of brine, interface and seawater above the halocline of deep anoxic lake L'Atalante, Eastern Mediterranean Sea. *ISME J* **1:** 743–755.

Yakimov, M.M., Cono, V.L., Smedile, F., Deluca, T.H., Juárez, S., Ciordia, S., *et al.* (2011) Contribution of crenarchaeal autotrophic ammonia oxidizers to the dark primary production in Tyrrhenian deep waters (Central Mediterranean Sea). *ISME J* **5:** 945–961.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Contigs length distribution of *Thetis* interface metagenome.
Fig. S2. Contigs length distribution of *Thetis* brine metagenome.
Fig. S3. Bacterial phylogenetic tree [proteobacterial (A) and other clades (B)] based on the affiliation of 16S rRNA tag sequences retrieved from the *Thetis* interface (purple) and brine (blue) metagenomes and all 16S crRNA sequences recovered by sequence analysis of 16S crDNA clone libraries (La Cono *et al.*, 2011) (green).
Fig. S4. Archaeal phylogenetic tree based on the affiliation of 16S rRNA tag sequences retrieved from the *Thetis* interface (purple) and brine (blue) metagenomes and all 16S crRNA sequences recovered by sequence analysis of 16S crDNA clone libraries (La Cono *et al.*, 2011) (green).
Table S1. Annotation of genes relevant to the principal metabolic pathways in the brine and interface compartments of the Lake *Thetis*.

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