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Corresponding Author: Prof. Ulrich Stingl,

Corresponding Author's Institution: King Abdullah University of Science and Technology (KAUST)

First Author: Yue Guan

Order of Authors: Yue Guan; Tyas Hikmawan; André Antunes; David Ngugi; Ulrich Stingl

Abstract: Oceanic deep hypersaline anoxic basins (DHABs) are characterized by drastic changes in physico-chemical conditions in the transition from overlaying seawater to brine body. Brine-seawater interfaces (BSIs) of several DHABs across the Mediterranean Sea have been shown to possess methanogenic and sulfate-reducing activities, yet no systematic studies have been conducted to address the potential functional diversity of methanogenic and sulfate-reducing communities in the Red Sea DHABs. Here, we evaluated the relative abundance of Bacteria and Archaea using quantitative PCR and conducted phylogenetic analyses of nearly full-length 16S rRNA genes as well as functional marker genes encoding the alpha subunits of methyl-coenzyme M reductase (mcrA) and dissimilatory sulfite reductase (dsrA). Bacteria predominated over Archaea in most locations, the majority of which being affiliated with Deltaproteobacteria, while Thaumarchaeota were the most prevalent Archaea in all sampled locations. The upper convective layers of Atlantis II Deep, which bear increasingly harsh environmental conditions, were dominated by members of the class Thermoplasmata (Marine Benthic Group E and Mediterranean Sea Brine Lakes Group 1). Our study revealed unique microbial compositions, the presence of niche-specific groups, and collectively a higher diversity of the sulfate-reducing communities compared to the methanogenic communities in all five studied locations.

***Detailed Response to Reviewers**

Dr. Uli Stingl
Asst. Professor for Marine Science
Red Sea Research Center
Principle Investigator Red Sea Marine Microbial Ecology
King Abdullah University of Science and Technology
Thuwal 23955-6900 Saudi Arabia
http://mme.kaust.edu.sa
http://www.kaust.edu.sa
uli.stingl@kaust.edu.sa
T +966 12 808-2381
M +966 5 447 000 83

Dear Dr. Jebbar,

Please find attached our revised version of RESMIC-D-15-00081 for publication in Research in Microbiology. The only comment from the referee was on the measurements of CO_2 in our samples. As these were done by a commercial service provide by GEOMAR in Germany, we now refer to their website.

We hope that you will find the manuscript now suitable for publication in Research in Microbiology.

Best regards,

Uli Stingl

Diversity of Methanogens and Sulfate-Reducing Bacteria in the interfaces of five deep-sea anoxic brines of the Red Sea

Yue Guan[a*], Tyas Hikmawan[a*], André Antunes[b], David Ngugi[a], Ulrich Stingl[a#]

[a]Red Sea Research Center, King Abdullah University of Science and Technology, 23955-6900, Thuwal, Saudi Arabia

[b]Computational Bioscience Research Centre, King Abdullah University of Science and Technology (KAUST), 23955-6900, Thuwal, Saudi Arabia

*Yue Guan and Tyas Hikmawan contributed equally to this work.

[#]Correspondence and requests for reprints to: uli.stingl@kaust.edu.sa

Phone: +966-12-808-2381; Fax: +966-12-8020152

Abstract

Oceanic deep hypersaline anoxic basins (DHABs) are characterized by drastic changes in physico-chemical conditions in the transition from overlaying seawater to brine body. Brine-seawater interfaces (BSIs) of several DHABs across the Mediterranean Sea have been shown to possess methanogenic and sulfate-reducing activities, yet no systematic studies have been conducted to address the potential functional diversity of methanogenic and sulfate-reducing communities in the Red Sea DHABs. Here, we evaluated the relative abundance of Bacteria and Archaea using quantitative PCR and conducted phylogenetic analyses of nearly full-length 16S rRNA genes as well as functional marker genes encoding the alpha subunits of methyl-coenzyme M reductase (*mcrA*) and dissimilatory sulfite reductase (*dsrA*). Bacteria predominated over Archaea in most locations, the majority of which being affiliated with *Deltaproteobacteria*, while *Thaumarchaeota* were the most prevalent Archaea in all sampled locations. The upper convective layers of Atlantis II Deep, which bear increasingly harsh environmental conditions, were dominated by members of the class *Thermoplasmata* (Marine Benthic Group E and Mediterranean Sea Brine Lakes Group 1). Our study revealed unique microbial compositions, the presence of niche-specific groups, and collectively a higher diversity of the sulfate-reducing communities compared to the methanogenic communities in all five studied locations.

Keywords: hypersaline environments; brine pools; biodiversity; methanogens; sulfate reducers

1. Introduction

Hypersaline water bodies at the bottom of the ocean (brine pools) are present in the Mediterranean Sea, the Gulf of Mexico, and the Red Sea [1]. In the Red Sea, a total of 25 such deep-sea hypersaline brine pools have been discovered at depths ranging from 1,193 to 2,850 meters below sea level [1, 2]. These environments are extremely saline (up to 26% salinity), anoxic, rich in heavy metals, and characterized by drastic changes in physicochemical conditions when compared to the overlaying seawater [3].

The interface between the brine pools and the seawater (BSI) represents a highly peculiar environment that harbors a high microbial diversity and biomass [4-6]. The increase in microbial biomass can be explained by the drastic changes in density, which result in an *in situ* particle trap for debris sinking through the water column, thus increasing the concentrations of available nutrients [6, 7]. In addition, the BSI is also characterized by sharp changes in physicochemical parameters including salinity, oxygen concentration, temperature, and redox potential, all of which provide a large variety of environmental niches for different metabolic groups [8, 9]. The microbiology of the BSIs of some of the Red Sea brine pools has been explored with a combination of cultivation-dependent [10, 11] and molecular–based methods [12, 13]. Previous studies based on 16S rRNA gene sequences uncovered novel groups of Archaea and Bacteria inhabiting the BSI of Shaban Deep and Kebrit Deep of the Red Sea [6, 12].

Microbial community studies in the Mediterranean DHABs revealed that diverse biogeochemical processes apparently co-occur in the BSI [8]. Other investigations reported on the importance of methanogenesis and sulfur cycling in these environments [4, 14]. These findings were also corroborated by recent metagenomic studies, where pathways for methanogenesis and/or sulfate reduction were detected in brines from the Red Sea and in

DHABs in the Mediterranean Sea [15-17]. Additionally, unique microbial communities were found to thrive in the sediments of two brine pools in the Red Sea, and many of the reported microorganisms are hypothesized to play a dominant role in the methane and sulfur cycle, based on their phylogenetic affiliations [18].

Taken together, methanogenesis and sulfate reduction could thus be considered very important biogeochemical processes in deep-sea brines [4, 19]. However, the composition of the microbial communities involved in both processes is largely unknown for the Red Sea brine pools. Considering the extreme conditions of these environments and the unique combination of physicochemical features in each individual brine pool [1], we postulated the existence of novel, niche-adapted groups of methanogens and sulfate reducers in the BSIs. Moreover, despite the micro-oxic conditions present in the BSI [20], members of both groups are capable of tolerating minute amounts of oxygen [21-23] and could thus play an important role in these environments. Previous 454 amplicon data [20] uncovered interesting microbial communities in the sampled sites, but as many of the relatively short sequences stem from poorly characterized groups, we decided that nearly full-length 16S rRNA gene sequences would be important to provide better phylogenetic detail and resolution on members of these groups. Therefore, we analyzed the microbial communities in the BSIs of geochemically distinct brine pools of the Red Sea, using the canonical 16S rRNA gene, as well as functional marker genes encoding for the alpha subunits of methyl-coenzyme M reductase (*mcrA*) and dissimilatory sulfite reductase (*dsrA*) to uncover the main methanogenic and sulfate-reducing communities.

2. Materials and methods

2.1. Sample collection

Water samples from the brine-seawater interfaces and the upper convective layers of the deep-sea brines were collected from the R/V *Aegaeo* during the 3rd KAUST Red Sea Expedition in November 2011 using a rosette sampler equipped with 10-l Niskin bottles and a CTD unit for monitoring salinity, temperature, transmission, oxygen, and pressure (Idronaut, Italy). Large volumes (ca. 200 l) of sample were collected from Atlantis II Deep BSI (Ai); first, second, and third upper-convective layer of Atlantis II Deep (labeled as A-UCL1, A-UCL2, A-UCL3, respectively), Discovery Deep BSI (Di), Erba Deep BSI (Ei), Kebrit Deep BSI (Ki), and Nereus Deep BSI (Ni) (Table 1). During sampling, we have avoided mixing between the seawater and the brine samples by carefully controlling the depth of the CTD and sampler when triggering the closure of each Niskin bottle to ensure sampling of desired layers. Furthermore, prior to sample collection on deck, we measured the salinities at the top and bottom of each individual Niskin bottle using a handheld refractometer (Master Refractometer, Atago, Japan) to confirm that the salinities of the samples matched the expected values of the targeted layers. Samples were then concentrated using a Tangential Flow Filtration (TFF) as described elsewhere [20]. Methane and carbon dioxide concentrations in the samples were determined via a commercial service provided by GEOMAR Helmholtz Centre for Ocean Research (Kiel, Germany, http://www.geomar.de).

2.2. DNA extraction, amplification, and sequencing of 16S rRNA genes

Nucleic acids were extracted as previously described [24] and the concentrations of the DNA were measured in a NanoDrop (Thermo Scientific, USA). Partial 16S rRNA genes were amplified by PCR by using combinations of the archaeal-specific primer 4F (5′-

TCCGGTTGATCCTGCCRG-3′) [25], or the bacteria-specific primer 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) paired with the universal primer 1492R (5′-GGTTACCTTGTTACGACTT-3′) [26]. The primers were chosen to produce sequences with maximum length. *In silico* testing using Silva-TestPrime (http://www.arb-silva.de/search/testprime/ [27] with one allowed mismatch indicated a coverage for Bacteria of 65.5% and a coverage for Archaea of 46.6%, while three allowed mismatches indicated a coverage for Bacteria of 71% and a coverage for Archaea of 53.1%. In addition, the above primers have a good coverage of major taxa reported in a previous study using 454 amplicon data [20]. The PCR conditions for archaeal 16S rRNA genes were: an initial denaturation of 5 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1.5 min at 72 °C, and then a final extension step of 7 min at 72 °C. The conditions for bacterial PCR were 3 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 53 °C, and 1.5 min at 72 °C, and then 7 min at 72°C. Purified PCR products were cloned into PCR®2.1 TOPO vectors (Invitrogen) according to the manufacturer's instructions. All clones with inserts from each library (856 and 1040 for archaeal and bacterial libraries, respectively) were selected for plasmid extraction and bi-directional sequencing on an ABI 3730 × l Capillary Sequencer at the Biosciences Core Laboratory at KAUST. Raw 16S rRNA gene sequences were quality checked, trimmed and assembled using Sequencher v.4.9 (Gene Codes Corporation).

2.3. Diversity and phylogenetic analysis

Assembled archaeal and bacterial 16S rRNA sequences were aligned and analyzed using mothur v.1.31, yielding operational taxonomic units (OTUs) grouped at 97% sequence identity level [28]. Potential chimeric sequences were removed using the Uchime 4.2 package [29], and diversity indeces (Shannon) and estimated sample coverage (Good's coverage) were calculated

as implemented in mothur. Sequence alignments of the resulting representative OTUs (62 for Archaea and 281 for Bacteria) and closely related sequences recovered from GenBank using BLASTn [30], were done automatically using the SINA aligner (http://www.arb-silva.de/aligner/) against the SILVA SSU 115 database [31]. The SILVA-aligned sequences were then used to construct phylogenetic trees with a maximum likelihood algorithm using bootstrap analysis (1000 samples) to validate support for clades as implemented in ARB v.5.3 [32].

2.4. Functional gene analysis

Partial *mcrA* and *dsrA* genes were amplified based on previously described methods [33, 34]. Purification of PCR products, clone library construction and sequencing protocols were the same as the ones used for 16S rRNA genes described above. The OTUs of *mcrA* and *dsrA* genes were generated at a 6% distance cutoff using FunGene (http://fungene.cme.msu.edu/) [35]. Deduced amino acid sequences of both genes were aligned with ClustalW [36]. The alignments of *mcrA* and *dsrA* genes were used for phylogenetic analyses with a maximum-likelihood algorithm (amino acids substitution model: LG for *mcrA*, and LG+G for *dsrA* genes) and 1000 bootstraps as implemented in Geneious Pro version 7.1 (Biomatters Ltd.) and MEGA version 6.06 [37], respectively.

2.5. Quantification of gene copy numbers by real-time PCR

Copy numbers of total bacterial and archaeal 16S rRNA genes from each sample location were determined by quantitative real-time PCR (qPCR) using EXPRESS qPCR SuperMix (Invitrogen) and a two-step qPCR cycling program on an ABI 7900HT Fast Real-Time PCR System instrument (Applied Biosystem). The primers Bac518F and Bac786R for Bacteria and A519F and A727R for Archaea were used as described by Park et al. [38]. Standards were made from plasmids containing inserts of archaeal or bacterial 16S rRNA gene sequences. The

efficiency for archaeal 16S rRNA primers was 99.8% and that for bacterial 16S rRNA primers was 99.5%, as estimated based on the slope of the standard curve. To allow for better comparisons among the different samples, copy numbers of genomic DNA were normalized based on ng of genomic DNA.

2.6. Nucleotide sequence accession numbers

The 16S rRNA gene sequences from this study were deposited in GenBank under accession numbers KJ881441–KJ882283 (Archaea), and KM018335–KM019141, KP083299–KP083370 (Bacteria), while *mcrA* and *dsrA* gene sequences were deposited under accession numbers KJ880100–KJ880274 and KM241874–KM242055, respectively.

3. Results and discussion

3.1. General microbial community structure

The ratios of the copy numbers of bacterial to archaeal 16S rRNA genes as estimated using qPCR were used as a proxy for their abundances in each sample. These ratios ranged in our samples from 0.15 to 179.12 (Table 1), and Bacteria were more abundant than Archaea in six out of eight samples. The predominance of Bacteria over Archaea thus seems to be a general trend in the BSI (and brine bodies) of brine pools from the Red Sea ([5, 17]; this study) and the Mediterranean Sea [8, 19, 39, 40]; the exceptions being the BSIs of Kebrit Deep and Atlantis II Deep (this study) and the brine layer of the Urania DHAB in the Mediterranean Sea [19]. Physico-chemical differences that might be either the reason for, or a result of, the high archaeal abundances are 1) they are highly sulfidic (in the case of Kebrit Deep and Urania DHAB; ~150 μM and 10 mM H_2S, respectively [9]), and 2) the BSIs of Kebrit and Atlantis II Deep have

dissolved oxygen concentrations (DO_2) that are 4–9 times higher than those at the other locations, where DO_2 is close to depletion (Table 1).

Phylogenetic analysis based on 16S rRNA gene sequences revealed a high microbial diversity in the brine interfaces of geochemically distinct brine pools of the Red Sea (Fig. 1). A total of 843 archaeal (>1000 bp length) and 960 bacterial (>1400 bp length) non-chimeric 16S rRNA gene sequences were obtained from the BSIs of the five brines and from the subsequent three upper convective layers of Atlantis II Deep (A-UCL1, A-UCL2, and A-UCL3). These sequences were clustered into 62 and 281 OTUs (at 97% sequences identity level) for archaeal and bacterial genes, respectively. Archaeal sequences were primarily affiliated with the phyla *Thaumarchaeota* (60%) and *Euryarchaeota* (37%), while the bacterial sequences encompassed diverse lineages. Our findings are in general consistent with previous studies [5, 20].

Hierarchical cluster analysis of the archaeal and bacterial 16S rRNA gene based on the Jaccard similarity index indicated similarities and differences among microbial communities of each location (Fig. 1A and 1B). The archaeal community in Kebrit Deep was clearly distinctive from those in the other locations (Fig. 1A). Archaeal communities in the three upper convective layers of Atlantis II Deep, BSIs of Nereus Deep and Erba Deep, and BSIs of Atlantis II Deep and Discovery Deep formed three separate clusters. In contrast with the results of the archaeal communities, we found no apparent clustering of the bacterial communities (Fig. 1B). Though, a highly stratified bacterial community profile was observed in the multi-layered Atlantis II Deep, concurrent with previous reports [5].

This is the first detailed phylogenetic study of nearly full-length prokaryotic 16S rRNA gene sequences from BSIs of Erba and Nereus Deep. Surprisingly, despite their salinity differences (9.8% vs. 15.4 %), they both harbor very similar microbial communities (Fig. 1). The

primary archaeal taxon was Marine Group I *Thaumarchaeota* (77%), while members of the class *Deltaproteobacteria* dominated their bacterial clone libraries (35.5% in Erba, and 27.9% in Nereus). In general, the microbial community compositions based on clone libraries were consistent with previous findings using an amplicon sequencing approach [20]. Additionally, six bacterial OTUs accounting for ~8% of all sequences were present in both brine pools, and are affiliated mostly with sulfate-reducing or sulfur-oxidizing taxa (e.g., *Deltaproteobacteria* and SAR324; Table S1).

3.2. Detailed analysis of archaeal communities

Both *Thaumarchaeota* and *Thermoplasmata* were ubiquitous in all five brine-seawater interfaces (BSI) and the three upper convective layers of Atlantis II Deep (A-UCL1, A-UCL2, and A-UCL3). This suggests that they are important components of deep-sea brine environments, and presumably possess adaptations to thrive in these gradient environments. *Thaumarchaeota* was the predominant group in archaeal clone libraries of all the five BSI samples (73–87%). The archaeal community composition in the Atlantis II BSI was different from the communities found in the subsequent convective layers. Clone libraries showed that the most abundant members in those convective layers belonged to the class *Thermoplasmata* (46-81%). Class *Methanomicrobia*-related sequences were also present (six out of eight sampled locations), but constituted only a small proportion of the archaeal communities (2.5%–11.4%; Fig. 1A). Additionally, a variety of archaeal lineages were found in the investigated Red Sea brine pool BSIs, albeit at low abundances, such as *Archaeoglobi*, *Halobacteria*, Marine Benthic Group A and D, Marine Group III, MSP41, CCA47 cluster and VC2.1 Arc6, Terrestrial Miscellaneous Group, SM1K20 group, South African Goldmine Euryarchaeotal Group (SAGMEG), Deep-Sea Euryarchaeotic Group, Miscellaneous Euryarchaeotic Group (MEG) and Terrestrial Hot Spring

Crenarchaeotic Group (THSCG) (Supplementary Figure S2). Many of the phylogenetic lineages of Archaea retrieved in this study are uncultured at present. A few of these lineages have been previously detected from the sediment samples of Atlantis II Deep and Discovery Deep of the Red Sea in a pyrosequencing approach using 16S rRNA genes [18].

Although we cannot rule out the possibility of reporting DNA sequences of non-metabolically active cells due to long-term preservation of DNA and cells in the deep-sea hypersaline environments, our recently published results [20] have shown that most of the thaumarchaeal sequences retrieved from the exact same sampled locations (BSIs) belonged to a Marine Group I phylotype that is absent in the overlaying water column. Still, this might not apply to all retrieved sequences. The cultivated species in the phylum *Thaumarchaeota* are autotrophic ammonia-oxidizing archaea [41], but a mixotrophic lifestyle in the dark ocean has also been suggested [42]. Considering their abundance and the halotolerant genomic features of thaumarchaeal single-cells from BSIs of Red Sea brines [20], we reconfirm previous findings that the BSI populations might play significant roles in the nitrogen and carbon cycles in the deep-sea brine interfaces with special adaptation to the hypersaline environments.

Detected members of the *Thermoplasmata* were mostly associated with the Candidate division MSBL1 (or Mediterranean Sea Brine Lakes Group 1) and MBGE (Marine Benthic Group E). Considerable proportions (5%–37%) of sequences related to MSBL1 were found in most clone libraries (six out of eight). In the Mediterranean DHABs, MSBL1 communities were commonly found in the lower interfaces and in the brines, and they have been assumed to be methanogenic due to a positive correlation between their abundances and the *in situ* methane concentrations [16, 43].

MBGE sequences were more abundant in the clone libraries of the upper convective layers of Atlantis II Deep than in other samples. This is consistent with previous reports indicating that they frequently occur at locations with higher temperatures (50 °C – 63 °C), correlating with the high G+C content of their 16S rRNA gene sequences [44]. They were often retrieved from deep-sea sediment, hydrothermal environments, chimney samples [45], and iron-rich habitats [46]. Therefore, we speculate that their increased abundance in the upper convective layers of Atlantis II is related to the increased temperature (52 – 65 °C) and iron concentration (24.5–70.5 µM). As no members of the MBGE clade and MSBL1 clade have been cultivated so far, their physiology and ecological roles in hypersaline deep-sea environments require future investigation.

3.3. Detailed analysis of bacterial communities

A wide diversity of Bacteria was observed in the BSIs of Atlantis II, Discovery, Kebrit, Nereus, and Erba Deep, corroborating previous reports on the microbial communities in the Red Sea brine pools [5, 6, 12]. The majority of bacterial 16S rRNA sequences were affiliated with *Proteobacteria* (relative abundance 37.5–89.4%), *Bacteroidetes* (0.8–13.2%), *Deferribacteres* (0.8–13.9%), and *Chloroflexi* (1.1–4.2%) (Fig. 1B). The remaining bacterial sequences belong to uncultured bacterial groups with unknown physiology such as Candidate division KB1, MSBL 2, and ST12-K34 (Fig S3). These uncultivated groups were previously reported from hypersaline brines in the Red Sea and DHABs in the Mediterranean Sea [6, 12, 43].

Deltaproteobacteria was the predominant bacterial class in the BSIs of colder brines such as Kebrit, Erba, and Nereus Deep (Fig. 1B). The high abundance of *Deltaproteobacteria* in the BSIs of these three brines is consistent with previous studies from the Mediterranean DHABs [4, 40]. This group is also one of the most prominent metabolically active microbial groups thriving

in the chemocline of $MgCl_2$-rich Discovery Basin [47], hypersaline Lake Kryos [39], and hydrothermal mud fluids of Urania DHAB, Mediterranean Sea [48]. These findings affirm the argument that sulfate reduction is one of the main metabolic processes occurring in the chemoclines of brine pools, which might be primarily performed by members of the *Deltaproteobacteria* [4, 19].

On the contrary, *Deltaproteobacteria* were less abundant in the clone libraries of the hot brines such as Discovery and Atlantis II Deep. The bacterial clone libraries of Atlantis II Deep shifted from being dominated by *Nitrospinae*-like bacteria in the BSI to being dominated by *Gammaproteobacteria* in the convective layers underneath (Fig. 1B). Similar patterns of gradually changing microbial communities were observed in a previous study based using pyrosequencing of the 16S rRNA genes in these two locations [5]. According to the same study, the combination of high temperature and salinity was presumed to shape the communities in both brines.

The majority of the OTUs in the class *Deltaproteobacteria* fell into four orders: *Desulfobacterales*, *Desulfurellales*, *Desulfovibrionales*, and *Syntrophobacterales* (Fig. 2). The remaining OTUs were assigned into four lineages with no cultured representatives, namely 10bav-F6, DTB120, *Candidatus* Entotheonella, and SAR 324. A large fraction of *Deltaproteobacteria* (around 33.3%) in A-UCL1 could not be classified to any known family, based on the ARB-SILVA SSU 115 database.

Certain bacterial groups seemed specifically adapted to high salinities. For instance, the bacterial community of the deepest convective layer of Atlantis II (A-UCL3) was dominated by Candidate division KB1, a group with no cultured representatives. This group branches between the *Aquificales* and the *Thermotogales*, and is restricted to hypersaline conditions [49]. Members

of KB1 were initially retrieved from sediments in Kebrit Deep [12] and later obtained in enrichments of BSI with high salinities obtained from Lake Medee, the Mediterranean Sea [43]. In this study, Candidate division KB1 was detected in interfaces with higher salinity such as A-UCL3, Di, and Ki (Fig. S3). The metabolic preferences of Candidate division KB1 remain unsolved although they seem to partially rely on reductive cleavage of the osmoprotectant glycine betaine, resulting in the formation of trimethylamine (TMA) and acetate [43].

3.4. Molecular diversity of *mcrA* genes

Up to now, all described methanogenic archaea fall into the seven orders in the phylum *Euryarchaeota*: *Methanococcales*, *Methanopyrales*, *Methanobacteriales*, *Methanosarcinales*, *Methanomicrobiales*, *Methanocellales*, and the recently proposed 7th order, *Methanomassiliicoccales* [50]. The reduction of CO_2, the fermentation of acetate, and the dismutation of methanol or methylamines encompass the three major methanogenic pathways [51]. Although methanogens play an important role in the global carbon cycling in various environments, very little is known about the methanogenic players in the Red Sea brine pools. Methyl-coenzyme M reductase is unique to methanogens and catalyzes the last step in methane formation. Genes encoding the α subunit of this enzyme (*mcrA*) have been employed as a specific marker to detect and differentiate methanogenic and anaerobic methanotrophic communities [52].

Figure 3 summarizes the diversity and phylogenetic analysis based on 199 *mcrA* gene sequences retrieved from the upper convective layers of Atlantis II Deep and the BSIs of Erba and Kebrit Deep. These *mcrA gene* sequences clustered into four OTUs at a 6% amino acid sequence distance cutoff. Two *mcrA* OTUs (OTU1 and OTU2, representing ~99% of the retrieved *mcrA* sequences) grouped together with cultured species of the genera

315 *Methanohalophilus* and *Methanococcoides*, along with clones from various hypersaline
316 environments including the deep-sea hypersaline Lake Thetis [16], Lake Medee [43], Discovery
317 Basin in the Mediterranean Sea [47], and Discovery Deep in the Red Sea [17]. The cultivated
318 representatives of these genera utilize methylated compounds as methanogenic substrates and
319 can produce methane in media with salinities of up to 4M NaCl [53]. This implies that the
320 dismutation of methanol and methylamines is potentially the main methanogenic pathway in
321 diverse hypersaline deep-sea basins. Previous studies have suggested that both methanogenesis
322 and sulfate reduction are major energy-generating processes in the deep-sea hypersaline
323 environment [4, 8]. However, members of methanogens are in competition with sulfate-reducing
324 bacteria for their mutual substrates (H_2 and acetate). They are also more negatively affected by
325 increased redox potential (e.g. the increase in DO_2) and by the availability of other terminal
326 electron acceptors (e.g., nitrate, iron, and sulfate); these conditions do exist in the brine pools.
327 Thus, hydrogenotrophic or acetoclastic methanogens tend to be less common in hypersaline
328 environments due to thermodynamic constraints [54]. Instead, methanogens in hypersaline
329 habitats are thought to be restricted to non-competitive substrates such as methylated amines,
330 which occur as derivatives of compatible solutes [55]. Our results are consistent with this notion.

331 The remaining two rare OTUs were present only in Kebrit BSI clone library. OTU3
332 grouped with a previously unidentified cluster formed by clones recovered from rice field soil
333 [56] and could not be assigned to any of the known methanogens. Interestingly, we found that
334 OTU3 is related to *Candidatus* 'Methanoperedens nitroreducens' (78% similarity) and thus could
335 represent an anaerobic methane-oxidizing microorganism. OTU4 clustered with cultivable
336 species of *Methanomassiliicoccales* [50], but with low amino acid sequences similarities (63%–
337 79%) [57]. *Methanomassiliicoccales* are rarely associated with deep-sea hypersaline locations,

and to our best knowledge, this is the first report of this phylotype from Red Sea brine pools, after their detection from Lake Kyros in the Mediterranean Sea [39]. Previously reported sequences associated with this order stemmed from various environments such as rumen, feces, hindguts, sludge, rice field soil, sediments, and anaerobic digesters. To date, *Methanomassiliicoccus luminyensis* is the only described species in this order [58]. Based on the physiology and the genome content of this isolate, this new order utilizes an H_2-dependent methylotrophic pathway for methanogenesis.

High concentrations of methane gas seem to be a common trait of most DHABs that have been studied to-date, including those studied here (Table 1). However, the link between these geochemical data and the major methane-producing taxa in such locations is still obscure. Our study showed a low diversity of methanogens in our samples and a distribution seemingly restricted to certain layers. Several previous studies have hypothesized that members of the Candidate division MSBL1 could be the enigmatic methanogens in brine pools, given their numerical predominance among Archaea and occurrence in methane-containing layers [4, 43]. So far, however, there is no conclusive evidence to support this hypothesis based on the following observations. In this study, we retrieved abundant MSBL1-related 16S rRNA genes from multiple samples, but except for a single phylotype from Kebrit BSI, no novel clusters of *mcrA* genes were detected. Thus, we assume that the Candidate division MSBL1 does not possess *mcrA* genes. Still, our study does not conclusively rule out the possibility that novel *mcrA* genes have been missed due to primer bias or undersampling of the present clone libraries. Additionally, the fact that the abundance of MSBL1 16S rRNA genes seems to be correlated with locations that possess high methane concentrations might be misleading, as the methane could either be produced abiotically [59] or biotically in deeper layers of the brine pool.

3.5. Molecular diversity of *dsrA* genes

Sulfate-reducing prokaryotes are a phylogenetically diverse group of anaerobes, with the majority of them belonging to the *Deltaproteobacteria* [60]. To yield energy, this group oxidizes hydrogen or small organic compounds and reduces sulfate to sulfide. The dissimilatory sulfite reductase (*dsr*), which contains two subunits (*dsrA* and *dsrB*), is the key enzyme in this process and is widely used as molecular marker to study the diversity of sulfate-reducing communities [61].

In the present study, *dsrA* clone libraries consisting of 220 sequences were constructed from the interfaces of five different brine pools in the Red Sea (Table 1). The *dsrA* gene sequences were clustered into 27 OTUs with a 6 % distance cutoff (Table S2). This is the first report on the diversity of sulfate-reducing bacteria (SRB) communities inhabiting the Red Sea brine pools. Phylogenetic analysis based on deduced amino acid sequences of *dsrA* clones revealed a high diversity of sulfate-reducing communities throughout the BSIs. Compared with the other brines in Mediterranean Sea [39], the Red Sea brine pools seem to possess a relatively higher diversity of SRB.

The majority of *dsrA* gene sequences in Erba Deep, Kebrit Deep, and Nereus Deep were affiliated with *Desulfohalobiaceae* and the members of *Desulfobacteraceae* such as *Desulfatiglans*, *Desulfosalsimonas*, *Desulfobacterium*, and *Desulfobacula* (Fig. 4). In contrast, in the warmer brines of Atlantis II and Discovery Deep, most of the OTUs formed distinct phylogenetic lineages clustering together with environmental sequences (Fig. 4). This result indicated a different composition of sulfate-reducing communities for each geochemically distinct brine pool. In addition, the diversity of sulfate-reducing communities based on *dsrA*

383 genes is in good agreement with the presence of sequences related to the members of
384 *Desulfobacteraceae* and *Desulfohalobiaceae* in the 16S rRNA gene data (Fig. 2).

385 As shown in Figure 4, six different phylogenetic clades of *dsrA* gene sequences were
386 found in the brine-seawater interfaces of the Red Sea brine pools. In Clade BSI I, two OTUs
387 from Kebrit Deep BSI with a relative abundance of more than 30% were affiliated with genus
388 *Desulfobacula.* They formed clusters with the *dsrA* clones from the interface of L'Atalante Deep
389 and Lake Kryos in the Mediterranean Sea, suggesting that the sulfate-reducing groups in these
390 environments are specifically adapted to the conditions in the BSIs [14, 47]. In Clade BSI II, an
391 OTU from Erba and one from Kebrit Deep BSI were affiliated with *Desulfosalsimonas*
392 *propionicica,* a species of the *Desulfobacteraceae*. The sequences from Kebrit Deep in this clade
393 were closely related to a clone obtained from the lower BSI of Lake Kryos [39]. The
394 predominance of *Desulfobacteraceae* in the BSI of different brine pools might be explained by
395 their wide range in nutritional diversity, oxygen tolerance, and metabolic plasticity [62].

396 Clade BSI III is constituted of an OTU recovered from Kebrit Deep BSI (18.2% salinity)
397 that was distantly related to two isolated halophilic SRB species, *Desulfohalobium retbaense*
398 [63] and *Desulfohalobium utahense* (both growing at salinities of up to 24%) [64]. This OTU
399 was only present in Kebrit Deep, suggesting a preference for higher salinity environments. This
400 finding is in a good agreement with previous analyses of *dsrAB* mRNA in the Discovery Basin of
401 the Mediterranean Sea, which revealed that *Desulfohalobiaceae* dominated in the saltier section
402 of the interface (from 1.60 up to 2.23 M $MgCl_2$) [47].

403 The OTUs in Clade BSI IV were widely distributed in the Red Sea brine pools (Fig. 4,
404 Table S2), especially in the ecosystems with higher sulfate concentration (Table 1). They were
405 related to *Desulfatiglans anilini,* a sulfate-reducing bacterium that is capable of degrading a

406 variety of aromatic compounds including phenol [65]. Almost all halophilic and halotolerant
407 strains of SRB isolated so far are incomplete oxidizers, which oxidize organic substrates to
408 acetate [66]. Interestingly, sequences related to these groups are also found in Kebrit Deep
409 (salinity 18%) and formed a cluster with a clone from the brine of L'Atalante Deep (salinity
410 27%), which exceeds the maximum salt limit predicted for complete oxidizers (approximately
411 13%) [67].

412 In Clade BSI V (Fig. 4), *dsrA* gene sequences from Erba and Nereus Deep were distantly
413 related to *Thermodesulfatator atlanticus*, a chemolithoautotrophic SRB species within the family
414 *Thermodesulfobacteriaceae* that was isolated from a hydrothermal vent [68]. Some of the OTUs
415 in the hot brines Atlantis II and Discovery Deep formed three deeply branching evolutionary
416 lineages (Clade BSI VI) that were different from any isolated sulfate-reducing bacteria (Fig. 4).
417 As there are no cultivated representatives, the metabolism and physiology of this clade remains
418 obscure. However, phylogenetic analysis indicates that they are related to organisms retrieved
419 from similar environments in the Mediterranean Sea [14], implying that these phylotypes are
420 specifically adapted to the DHABs. Considering the high abundance of deeply branching
421 sequences in the Clade BSI VI (Fig. 4), we assume that the interfaces of the Atlantis II and
422 Discovery Deep harbor specific sulfate-reducing communities that are quite different to known
423 SRB.

424 In conclusion, the bacterial communities were very diverse and, based on 16S rRNA gene
425 copy numbers, dominated over archaea in the majority of our samples. In the multi-layered
426 Atlantis II Deep, archaeal and bacterial communities were stratified. Marine Group I
427 *Thaumarchaeota*, MBGE and Candidate division MSBL1 *Thermoplasmata*, halophilic
428 methanogens, and members of class *Deltaproteobacteria* were the most common microbial

groups associated with the chemoclines of the Red Sea brine pools. Methanogens were restricted to a few taxa in all studied locations, reiterating the harshness of these habitats. The sulfate-reducing communities were collectively diverse based on *dsr*A gene sequences, with the majority of the OTUs in Erba, Kebrit, and Nereus Deep being affiliated with genus *Desulfatiglans*. Additionally, the high-temperature Atlantis II and Discovery Deep harbor deeply branched lineage of *dsrA* gene sequences, which suggest that novel lineages of SRB reside in these environments. In the broader sense, these findings provide more insights on the ubiquity of methanogenic archaea and sulfate-reducers in hypersaline habitats, and should increase the impetus for future cultivation-attempts of the novel halophilic microorganisms.

Conflict of interest

All authors declare no conflict of interest.

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References

[1] Antunes A, Ngugi DK, Stingl U. Microbiology of the Red Sea (and other) deep‐sea anoxic brine lakes. Environ Microbiol Rep 2011;3:416-33.

[2] Backer H, Schoell M. New deeps with brines and metalliferous sediments in the Red Sea. Nat Phys Sci 1972;240:153-8.

[3] Hartmann M, Scholten JC, Stoffers P, Wehner F. Hydrographic structure of brine-filled deeps in the Red Sea - new results from the Shaban, Kebrit, Atlantis II, and Discovery Deep. Mar Geol 1998;144:311-30.

[4] Borin S, Brusetti L, Mapelli F, D'Auria G, Brusa T, Marzorati M, et al. Sulfur cycling and methanogenesis primarily drive microbial colonization of the highly sulfidic Urania deep hypersaline basin. Proc Nati Acad of Sci 2009;106:9151-6.

[5] Bougouffa S, Yang JK, Lee OO, Wang Y, Batang Z, Al-Suwailem A, et al. Distinctive microbial community structure in highly stratified deep-sea brine water columns. Appl Environ Microbiol 2013;79:3425-37.

[6] Eder W, Schmidt M, Koch M, Garbe-Schönberg D, Huber R. Prokaryotic phylogenetic diversity and corresponding geochemical data of the brine-seawater interface of the Shaban Deep, Red Sea. Environ Microbiol 2002;4:758-63.

[7] Larock PA, Lauer RD, Schwarz JR, Watanabe KK, Wiesenburg DA. Microbial biomass and activity distribution in an anoxic, hypersaline basin. Appl Environ Microbiol 1979;37:466-70.

[8] Daffonchio D, Borin S, Brusa T, Brusetti L, van der Wielen PWJJ, Bolhuis H, et al. Stratified prokaryote network in the oxic–anoxic transition of a deep-sea halocline. Nature 2006;440:203-7.

[9] Sass AM, Sass H, Coolen MJ, Cypionka H, Overmann J. Microbial communities in the chemocline of a hypersaline deep-sea basin (Urania basin, Mediterranean Sea). Appl Environ Microbiol 2001;67:5392-402.

[10] Antunes A, Eder W, Fareleira P, Santos H, Huber R. *Salinisphaera shabanensis* gen. nov., sp. nov., a novel, moderately halophilic bacterium from the brine-seawater interface of the Shaban Deep, Red Sea. Extremophiles 2003;7:29-34.

[11] Antunes A, Franca L, Rainey FA, Huber R, Nobre MF, Edwards KJ, et al. *Marinobacter salsuginis* sp. nov., isolated from the brine-seawater interface of the Shaban Deep, Red Sea. Int J Syst Evol Microbiol 2007;57:1035-40.

[12] Eder W, Jahnke LL, Schmidt M, Huber R. Microbial diversity of the brine-seawater interface of the Kebrit Deep, Red Sea, studied via 16S rRNA gene sequences and cultivation methods. Appl Environ Microbiol 2001;67:3077-85.

[13] Abdallah RZ, Adel M, Ouf A, Sayed A, Ghazy MA, Alam I, et al. Aerobic methanotrophic communities at the Red Sea brine-seawater interface. Front Microbiol 2014;5:487.

[14] van der Wielen PWJJ, Heijs SK. Sulfate-reducing prokaryotic communities in two deep hypersaline anoxic basins in the Eastern Mediterranean deep sea. Environ Microbiol 2007;9:1335-40.

491 [15] Wang Y, Yang J, Lee OO, Dash S, Lau SCK, Al-Suwailem A, et al. Hydrothermally
492 generated aromatic compounds are consumed by bacteria colonizing in Atlantis II Deep of the
493 Red Sea. ISME J 2011;5:1652-9.

494 [16] La Cono V, Smedile F, Bortoluzzi G, Arcadi E, Maimone G, Messina E, et al. Unveiling
495 microbial life in new deep-sea hypersaline Lake Thetis. Part I: Prokaryotes and environmental
496 settings. Environ Microbiol 2011;13:2250-68.

497 [17] Wang Y, Cao H, Zhang G, Bougouffa S, Lee OO, Al-Suwailem A, et al. Autotrophic
498 microbe metagenomes and metabolic pathways differentiate adjacent Red Sea brine pools. Sci
499 Rep 2013;3.

500 [18] Siam R, Mustafa GA, Sharaf H, Moustafa A. Unique prokaryotic consortia in
501 geochemically distinct sediments from Red Sea Atlantis II and Discovery Deep brine pools. Plos
502 One 2012;7:e42872.

503 [19] van der Wielen PWJJ, Bolhuis H, Borin S, Daffonchio D, Corselli C, Giuliano L, et al. The
504 enigma of prokaryotic life in deep hypersaline anoxic basins. Science 2005;307:121-3.

505 [20] Ngugi DK, Blom J, Alam I, Rashid M, Ba-Alawi W, Zhang G, et al. Comparative genomics
506 reveals adaptations of a halotolerant thaumarchaeon in the interfaces of brine pools in the Red
507 Sea. ISME J 2014;9:396-411.

508 [21] Angel R, Matthies D, Conrad R. Activation of methanogenesis in arid biological soil crusts
509 despite the presence of oxygen. Plos One 2011;6:e20453.

510 [22] Brune A, Frenzel P, Cypionka H. Life at the oxic-anoxic interface: microbial activities and
511 adaptations. Fems Microbiol Rev 2000;24:691-710.

512 [23] Marschall C, Frenzel P, Cypionka H. Influence of oxygen on sulfate reduction and growth
513 of sulfate-reducing bacteria. Arch Microbiol 1993;159:168-73.

514 [24] Ngugi DK, Stingl U. Combined Analyses of the ITS Loci and the Corresponding 16S rRNA
515 Genes Reveal High Micro- and Macrodiversity of SAR11 Populations in the Red Sea. Plos One
516 2012;7:e50274.

517 [25] Kendall MM, Wardlaw GD, Tang CF, Bonin AS, Liu Y, Valentine DL. Diversity of archaea
518 in marine sediments from Skan Bay, Alaska, including cultivated methanogens, and description
519 of *Methanogenium boonei* sp. nov. Appl Environ Microbiol 2007;73:407-14.

520 [26] Lane D. 16S/23S rRNA sequencing. in: E Stackebrandt and M Goodfellow (Eds.), Nucleic
521 acid techniques in bacterial systematics, John Wiley and Sons Ltd., New York, 1991, pp. 115-75.

522 [27] Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, et al. Evaluation of
523 general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-
524 based diversity studies. Nucleic Acids Res 2012;41:e1.

525 [28] Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing
526 mothur: Open-source, platform-independent, community-supported software for describing and
527 comparing microbial communities. Appl Environ Microb 2009;75:7537-41.

528 [29] Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and
529 speed of chimera detection. Bioinformatics 2011;27:2194-200.

530 [30] Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z, Miller W, et al. Gapped
531 BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids
532 Res 1997;25:3389-402.

533 [31] Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, et al. SILVA: a
534 comprehensive online resource for quality checked and aligned ribosomal RNA sequence data
535 compatible with ARB. Nucleic Acids Res 2007;35:7188-96.

536 [32] Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, et al. ARB: a software
537 environment for sequence data. Nucleic Acids Res 2004;32:1363-71.

538 [33] Springer E, Sachs MS, Woese CR, Boone DR. Partial gene sequences for the A subunit of
539 methyl-coenzyme M reductase (mcrI) as a phylogenetic tool for the family *Methanosarcinaceae*.
540 Int J Syst Bacteriol 1995;45:554-9.

541 [34] Pester M, Bittner N, Deevong P, Wagner M, Loy A. A ‘rare biosphere’ microorganism
542 contributes to sulfate reduction in a peatland. ISME J 2010;4:1591-602.

543 [35] Fish JA, Chai B, Wang Q, Sun Y, Brown CT, Tiedje JM, et al. FunGene: the functional
544 gene pipeline and repository. Front Microbio 2013;4:291.

545 [36] Thompson JD, Higgins DG, Gibson TJ. Clustal-W - Improving the sensitivity of progressive
546 multiple sequence alignment through sequence weighting, position-specific gap penalties and
547 weight matrix choice. Nucleic Acids Res 1994;22:4673-80.

548 [37] Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular
549 evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum
550 parsimony methods. Mol Biol Evol 2011;28:2731-9.

551 [38] Park BJ, Park SJ, Yoon DN, Schouten S, Damste JSS, Rhee SK. Cultivation of autotrophic
552 ammonia-oxidizing archaea from marine sediments in coculture with sulfur-oxidizing bacteria.
553 iol Environ Microb 2010;76:7575-87.

554 [39] Yakimov MM, La Cono V, Spada GL, Bortoluzzi G, Messina E, Smedile F, et al. Microbial
555 community of the deep-sea brine Lake Kryos seawater-brine interface is active below the
556 chaotropicity limit of life as revealed by recovery of mRNA. Environ Microbiol 2014;17:364–
557 82.

558 [40] Yakimov MM, La Cono V, Denaro R, D'Auria G, Decembrini F, Timmis KN, et al. Primary
559 producing prokaryotic communities of brine, interface and seawater above the halocline of deep
560 anoxic lake L'Atalante, Eastern Mediterranean Sea. ISME J 2007;1:743-55.

561 [41] Konneke M, Bernhard AE, de la Torre JR, Walker CB, Waterbury JB, Stahl DA. Isolation
562 of an autotrophic ammonia-oxidizing marine archaeon. Nature 2005;437:543-6.

563 [42] Swan BK, Chaffin MD, Martinez-Garcia M, Morrison HG, Field EK, Poulton NJ, et al.
564 Genomic and metabolic diversity of Marine Group I Thaumarchaeota in the mesopelagic of two
565 subtropical gyres. Plos One 2014;9.

566 [43] Yakimov MM, La Cono V, Slepak VZ, La Spada G, Arcadi E, Messina E, et al. Microbial
567 life in the Lake Medee, the largest deep-sea salt-saturated formation. Sci Rep 2013;3.

568 [44] Kato S, Kobayashi C, Kakegawa T, Yamagishi A. Microbial communities in iron-silica-rich
569 microbial mats at deep-sea hydrothermal fields of the Southern Mariana Trough. Environ
570 Microbiol 2009;11:2094-111.

571 [45] Kato S, Takano Y, Kakegawa T, Oba H, Inoue K, Kobayashi C, et al. Biogeography and
572 biodiversity in sulfide structures of active and inactive vents at deep-sea hydrothermal fields of
573 the Southern Mariana Trough. Appl Environ Microbiol 2010;76:2968-79.

574 [46] Takai K, Nunoura T, Ishibashi J-i, Lupton J, Suzuki R, Hamasaki H, et al. Variability in the
575 microbial communities and hydrothermal fluid chemistry at the newly discovered Mariner
576 hydrothermal field, southern Lau Basin. J Geophys Res 2008;113:G02031.

577 [47] Hallsworth JE, Yakimov MM, Golyshin PN, Gillion JLM, D'Auria G, de Lima Alves F, et
578 al. Limits of life in MgCl2-containing environments: chaotropicity defines the window. Environ
579 Microbiol 2007;9:801-13.

580 [48] Yakimov MM, Giuliano L, Cappello S, Denaro R, Golyshin PN. Microbial community of a
581 hydrothermal mud vent underneath the deep-sea anoxic brine lake Urania (eastern
582 Mediterranean). Orig Life Evol Biosph 2007;37:177-88.

583 [49] Eder W, Ludwig W, Huber R. Novel 16S rRNA gene sequences retrieved from highly saline
584 brine sediments of Kebrit Deep, Red Sea. Arch Microbiol 1999;172:213-8.

585 [50] Borrel G, O'Toole PW, Harris HMB, Peyret P, Brugere JF, Gribaldo S. Phylogenomic data
586 support a seventh order of methylotrophic methanogens and provide insights into the evolution
587 of methanogenesis. Genome Biol Evol 2013;5:1769-80.

588 [51] Ferry JG. Methanogenesis Biochemistry. eLS, John Wiley & Sons, Ltd, 2001.

589 [52] Friedrich MW. Methyl-coenzyme M reductase genes: Unique functional markers for
590 methanogenic and anaerobic methane-oxidizing Archaea. in: JR Leadbetter (Ed.), Methods in
591 Enzymology, Academic Press, 2005, pp. 428-42.

592 [53] Boone DR, Mathrani IM, Liu Y, Menaia JAGF, Mah RA, Boone JE. Isolation and
593 characterization of *Methanohalophilus portucalensis* sp. nov. and DNA reassociation study of
594 the genus *Methanohalophilus*. Int J Syst Bacteriol 1993;43:430-7.

595 [54] Scholten JCM, Joye SB, Hollibaugh JT, Murrell JC. Molecular analysis of the sulfate
596 reducing and archaeal community in a meromictic soda lake (Mono Lake, California) by
597 targeting 16S rRNA, *mcrA*, *apsA*, and *dsrAB* genes. Microb Ecol 2005;50:29-39.

598 [55] Ollivier B, Caumette P, Garcia JL, Mah RA. Anaerobic bacteria from hypersaline
599 environments. Microbiol Rev 1994;58:27-38.

600 [56] Conrad R, Klose M, Noll M, Kemnitz D, Bodelier PLE. Soil type links microbial
601 colonization of rice roots to methane emission. Glob Change Biol 2008;14:657-69.

602 [57] Paul K, Nonoh JO, Mikulski L, Brune A. "*Methanoplasmatales*," *Thermoplasmatales*-
603 related archaea in termite guts and other environments, are the seventh order of methanogens.
604 Appl Environ Microbiol 2012;78:8245-53.

605 [58] Dridi B, Fardeau ML, Ollivier B, Raoult D, Drancourt M. *Methanomassiliicoccus*
606 *luminyensis* gen. nov., sp nov., a methanogenic archaeon isolated from human faeces. Int J Syst
607 Evol Microbiol 2012;62:1902-7.

608 [59] Schmidt M, Botz R, Faber E, Schmitt M, Poggenburg J, Garbe-Schonberg D, et al. High-
609 resolution methane profiles across anoxic brine-seawater boundaries in the Atlantis-II,
610 Discovery, and Kebrit deeps (Red Sea). Chem Geol 2003;200:359 - 75.

611 [60] Muyzer G, Stams AJ. The ecology and biotechnology of sulphate-reducing bacteria. Nat
612 Rev Microbiol 2008;6:441-54.

613 [61] Wagner M, Loy A, Klein M, Lee N, Ramsing NB, Stahl DA, et al. Functional marker genes
614 for identification of sulfate‐reducing prokaryotes. in: JR Leadbetter (Ed.), Methods in
615 Enzymology, Academic Press, 2005, pp. 469-89.

616 [62] Rabus R, Hansen TA, Widdel F. Dissimilatory sulfate- and sulfur-reducing prokaryotes. in:
617 M Dworkin, S Falkow, E Rosenberg, KH Schleifer and E Stackebrandt (Eds.), Prokaryotes
618 Springer New York, Singapore, 2006, pp. 659-768.

619 [63] Ollivier B, Hatchikian CE, Prensier G, Guezennec J, Garcia JL. *Desulfohalobium retbaense*
620 gen. nov., sp. nov., a halophilic sulfate-reducing bacterium from sediments of a hypersaline lake
621 in Senegal. Int J Syst Bacteriol 1991;41:74-81.

622 [64] Jakobsen TF, Kjeldsen KU, Ingvorsen K. *Desulfohalobium utahense* sp. nov., a moderately
623 halophilic, sulfate-reducing bacterium isolated from Great Salt Lake. Int J Syst Evol Microbiol
624 2006;56:2063-9.

625 [65] Ahn YB, Chae JC, Zylstra GJ, Haggblom MM. Degradation of phenol via phenylphosphate
626 and carboxylation to 4-hydroxybenzoate by a newly isolated strain of the sulfate-reducing
627 bacterium *Desulfobacterium anilini*. Appl Environ Microbiol 2009;75:4248-53.

[66] Oren A. Diversity of halophiles. in: K Horikoshi (Ed.), Extremophiles handbook, Springer Japan, 2011, pp. 309-25.

[67] Oren A. Bioenergetic aspects of halophilism. Microbiol Mol Biol Rev 1999;63:334-48.

[68] Alain K, Postec A, Grinsard E, Lesongeur F, Prieur D, Godfroy A. *Thermodesulfatator atlanticus* sp nov., a thermophilic, chemolithoautotrophic, sulfate-reducing bacterium isolated from a Mid-Atlantic Ridge hydrothermal vent. Int J Syst Evol Microbiol 2010;60:33-8.

Table

Table 1. Physical and geochemical parameters of the sampling locations, bacteria/archaea ratio, number of clones, and alpha diversity

Sampling site[a]		Kebrit	Erba	Nereus	Discovery	Atlantis II			
		Ki	Ei	Ni	Di	Ai	A-UCL1	A-UCL2	A-UCL3
Latitude (N)[b]		24° 43.41'	20° 43.80'	23° 11.53'	21° 16.98'	21° 20.76'	21° 20.76'	21° 20.76'	21° 20.76'
Longitude (E)[b]		36° 16.63'	38° 10.98'	37° 25.09'	38° 3.18'	38° 4.68'	38° 4.68'	38° 4.68'	38° 4.68'
Depth (m)[b]		1467	2381	2432	2038	1998	2006	2025	2048
Thickness (m)		3	10	12	35	4	10	12	20
Salinity (%)[b]		18.2	9.8	15.4	13.8	5.6	8.6	11.2	15.4
H_2S (µmol/l)[b]		149.8	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.
CH_4 (ppmV)		28955.5	15.5	23	53	b.d.l.	85.5	330	977
CO_2 (ppmV)		103731	<400	<400	791.5	7878	18953	19288	12226
16S rRNA gene copy nr. ($x10^4$)	*Bacteria*	6.60	20.60	11.41	457.69	48.05	59.11	1.44	1.27
	Archaea	42.69	1.03	0.08	54.81	77.8	0.33	0.39	0.09
Bacteria/Archaea 16S rRNA gene copy nr. ratio[c]		0.15	20.00	142.63	8.35	0.62	179.12	3.69	14.11
Bacteria/Archaea ratio after normalization[d]		0.07	9.07	64.07	3.79	0.28	81.26	1.67	6.40
Nr. of 16S rRNA gene clones [nr. of OTUs]	*Bacteria*	250 [73]	189 [91]	104 [53]	121 [53]	92 [18]	181 [59]	-[e]	23 [12]
	Archaea	160 [22]	155 [18]	84 [9]	118 [9]	95 [7]	96 [6]	100 [12]	35 [12]
Shannon index	*Bacteria*	3.03	4.03	3.65	3.3	1.84	3.43	-	2.24
	Archaea	1.44	1.45	1.1	1.11	0.64	0.87	1.86	2.36
Good's coverage	*Bacteria*	0.71	0.52	0.5	0.56	0.8	0.67	-	0.48
	Archaea	0.93	0.97	0.96	1	0.97	1	0.99	0.94
Nr. of clones of functional genes [OTUs]	*mcrA*	12 [3]	152 [2]	-	-	-	-	20 [1]	15 [1]
	dsrA	42 [8]	112 [13]	31 [7]	18 [3]	5 [3]	12 [5]	-	-
Good's coverage	*mcrA*	0.75	0.99	-	-	-	-	0.95	0.93
	dsrA	0.81	0.88	0.77	0.83	0.5	0.58		

[a] Abbreviations for sampling sites: Ki, Kebrit Deep BSI; Ei, Erba Deep BSI; Ni, Nereus Deep BSI; Di, Discovery Deep BSI; Ai, Atlantis II Deep BSI; A-UCL1, A-UCL2, and A-UCL3, the first, second, and third upper-convective layer, respectively.
[b] For physicochemical data please refer to Ngugi et al. 2014
[c] To allow for a better comparison among the different samples, copy numbers of genomic DNA were normalized based on ng of genomic DNA.
[d] Archaeal and bacterial abundance ratios were estimated based on the qPCR results and the average 16S rRNA gene copy (4.1 per cell in Bacteria and 1.86 per cell in Achaea (Lee et al. 2009).
[e] The bacterial library of A-UCL2 was not generated because of technical issues.

662

663 **Figure legends**

664 **Figure 1.** Taxonomic classification and relative abundance of archaeal (A) and bacterial (B)
665 communities in the brine-seawater interfaces of five different brine pools of the Red Sea. A total
666 of 843 archaeal and 960 bacterial 16S rRNA gene fragments were classified using mothur based
667 on SILVA database at 97% cutoff. The cluster dendrogram illustrates the linked hierarchical
668 clustering of different environments based on the relative abundance of the OTUs in each
669 sampling location. (**Ai**, Atlantis II Deep BSI; A-UCL1, A-UCL2, and A-UCL3, the first, second,
670 and third upper convective layer, respectively; **Di**, Discovery Deep BSI; **Ei**, Erba Deep BSI; **Ki**,
671 Kebrit Deep BSI; **Ni**, Nereus Deep BSI. MSBL1, Mediterranean Sea Brine Lakes Group 1;
672 SAGMEG, South African Goldmine Euryarchaeotal Group).

673 **Figure 2.** 16S rRNA gene-based phylogenetic tree of the *Deltaproteobacteria* group, including
674 the representative sequences from the Atlantis II, Discovery, Erba, Kebrit, and Nereus Deep. The
675 topology of the tree is based on maximum-likelihood algorithm with 1000 bootstraps. The scale
676 bar represents 0.10 fixed mutation per nucleotide position. Bootstrap values above 50% are
677 shown.

678 **Figure 3.** Phylogenetic tree of *mcrA genes* showing the relationship of representative *mcrA*
679 clones retrieved from the deep-sea brines of the Red Sea to known methanogens and
680 environmental sequences. Taxonomy is based on FunGene (http://fungene.cme.msu.edu).
681 Bootstrap values are based on 1000 replicates and values above 50% are shown. Percentages in
682 parentheses indicate the relative abundance in each sample.

683 **Figure 4.** Phylogenetic tree based on deduced amino acid sequences of the *dsrA* clones from the
684 brine-seawater interfaces of Red Sea brine pools, including sequences from Mediterranean
685 DHABs. The topology of the tree is based maximum-likelihood method using 1000 bootstrap
686 replicates. The scale bar represents 0.10 fixed mutation per nucleotide position and bootstrap
687 values above 50% are shown. Percentages in parentheses indicate the relative abundance in each
688 sample.

689

690 **Supplementary materials**

691 **Table S1.** Shared OTUs among the sampled locations and the relative abundance of archaeal and
692 bacterial 16S rRNA genes.

693 **Table S2.** OTU classification and the relative abundance of *dsrA* genes in each sampled
694 locations.

695

696 **Figure S1.** Sampling locations of five brine pools in the Red Sea.

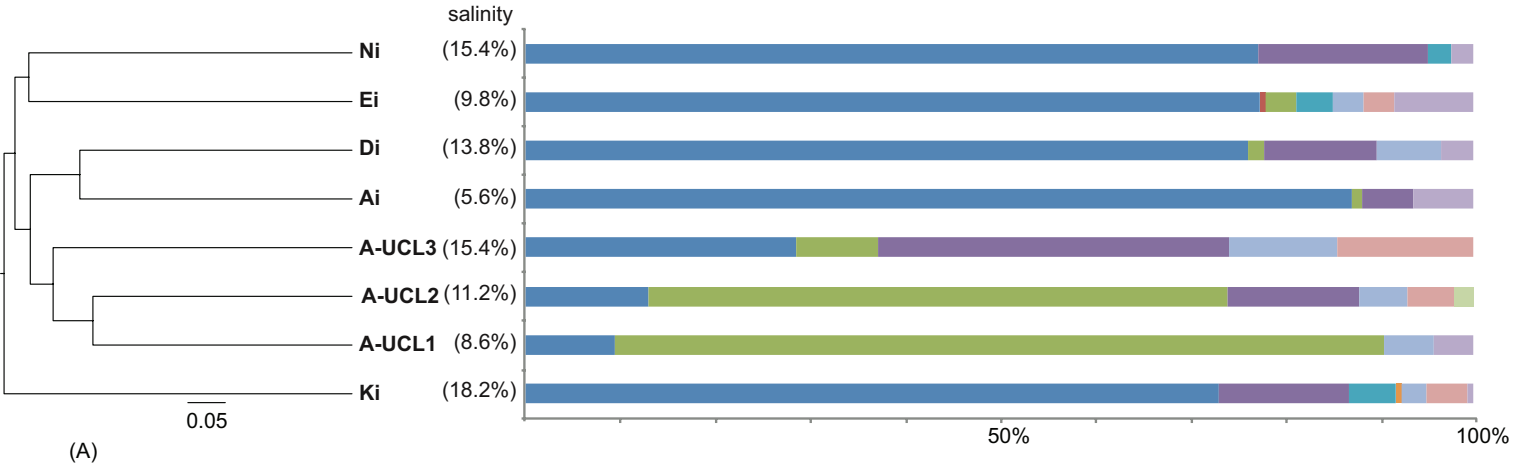
697 **Figure S2.** Phylogenetic tree showing the affiliation of archaeal lineage detected from the
698 interfaces of the Red Sea brine pools. The tree was constructed by maximum likelihood analysis
699 using ARB. Taxonomy is based on SSURef_115_SILVA (http://www.arb-silva.de). Dots at
700 nodes indicate bootstrap values above 50%.

701 **Figure S3.** Major lineages of Bacteria (excluding *Proteobacteria* phylum), harboring
702 representative sequences from the interfaces of the Red Sea brine pools. Taxonomy is based on
703 SSURef_115_SILVA (http://www.arb-silva.de), constructed by maximum likelihood analysis
704 using ARB.

705 **Figure S4.** Rarefaction analysis of *mcrA* clone libraries.

706 **Figure S5.** Rarefaction analysis of *dsrA* clone libraries.

Figure 1


salinity
Ni (15.4%)
Ei (9.8%)
Di (13.8%)
Ai (5.6%)
A-UCL3 (15.4%)
A-UCL2 (11.2%)
A-UCL1 (8.6%)
Ki (18.2%)
0.05
50%
100%
(A)
Marine Group1
Marine Benthic Group A
Marine Benthic Group E
MSBL1/SAGMEG
Thermoplasmatales
Methanomicrobia
Halobacteria
Archaeoglobi
Unclassified Thermoplasmata
Unclassified

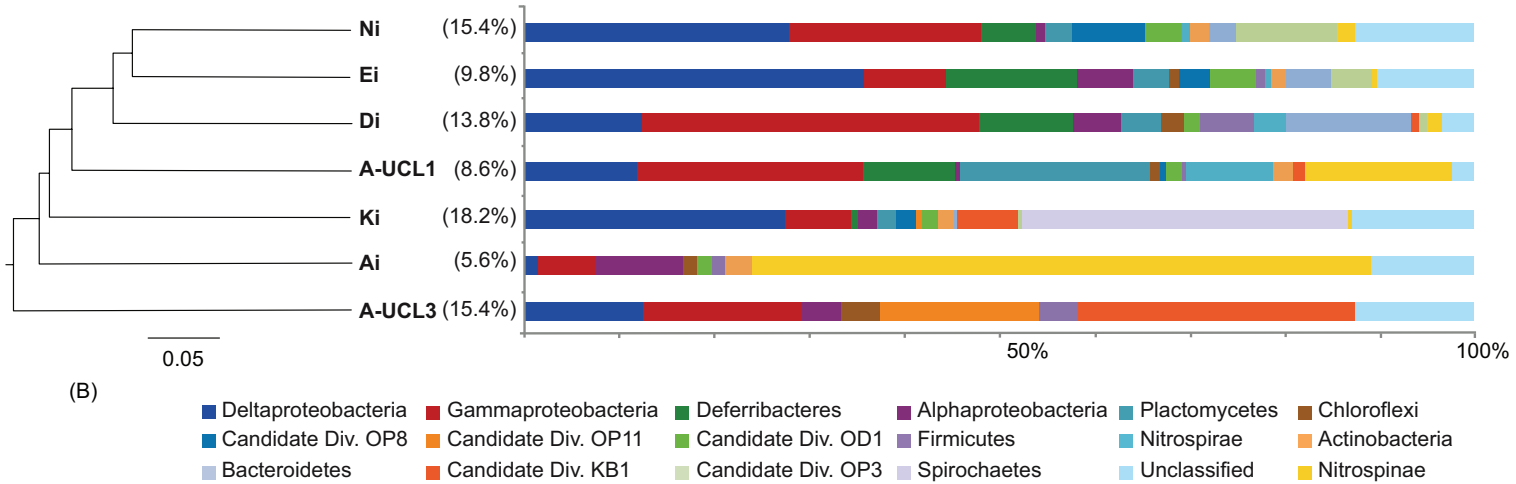

Ni (15.4%)
Ei (9.8%)
Di (13.8%)
A-UCL1 (8.6%)
Ki (18.2%)
Ai (5.6%)
A-UCL3 (15.4%)
0.05
50%
100%
(B)
Deltaproteobacteria
Gammaproteobacteria
Deferribacteres
Alphaproteobacteria
Planctomycetes
Chloroflexi
Candidate Div. OP8
Candidate Div. OP11
Candidate Div. OD1
Firmicutes
Nitrospirae
Actinobacteria
Bacteroidetes
Candidate Div. KB1
Candidate Div. OP3
Spirochaetes
Unclassified
Nitrospinae

Figure 2

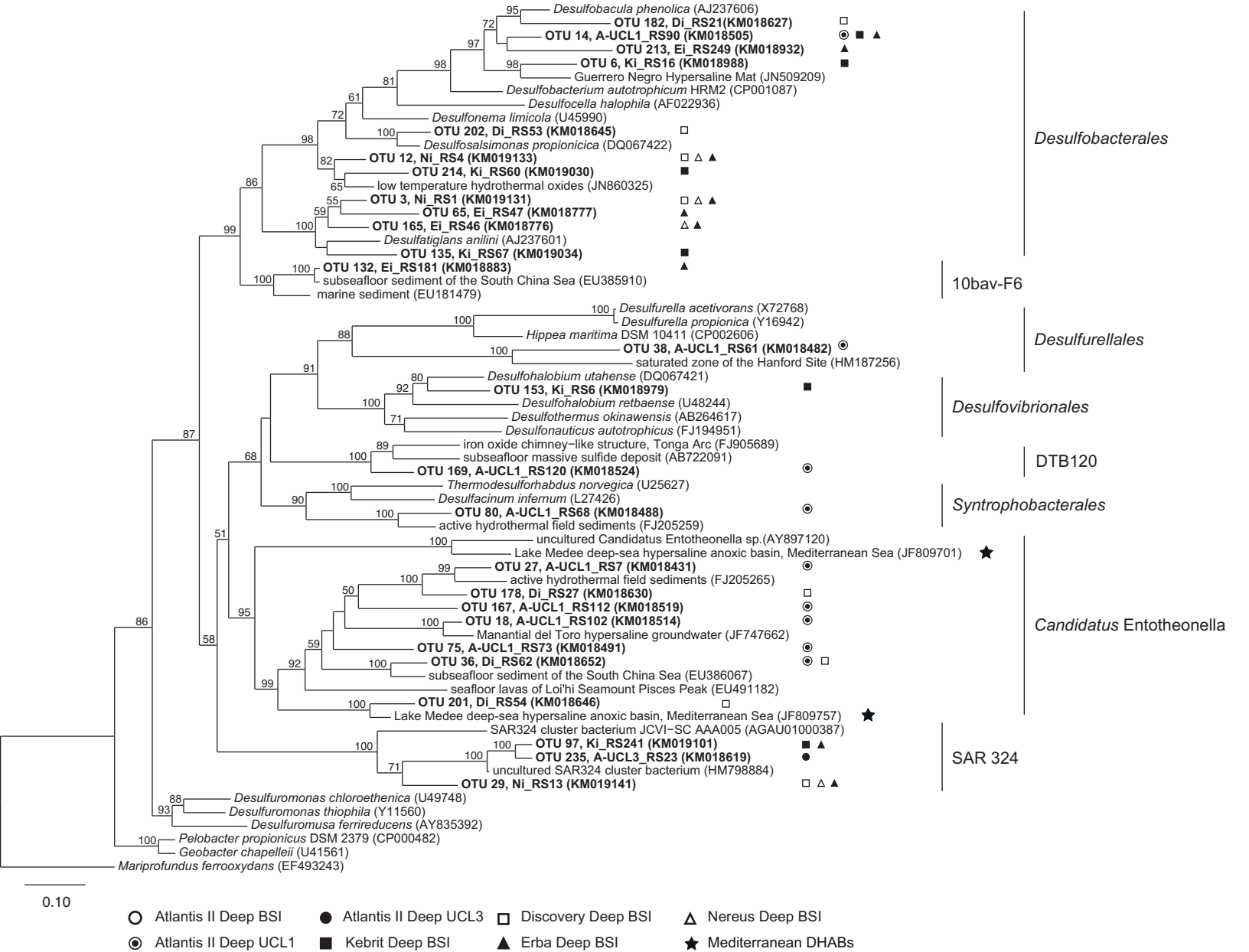
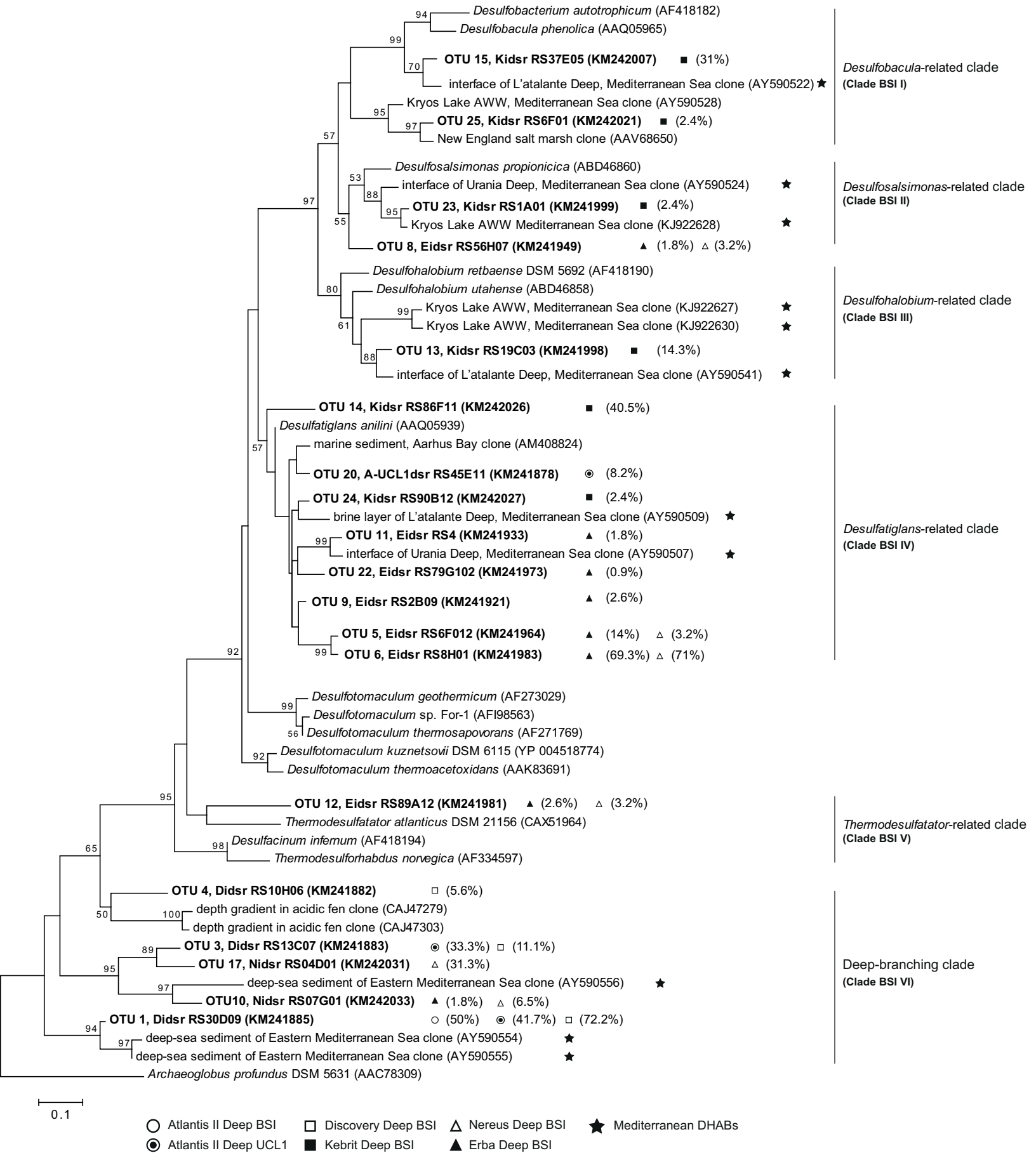

Desulfobacula phenolica (AJ237606)
OTU 182, Di_RS21(KM018627)
OTU 14, A-UCL1_RS90 (KM018505)
OTU 213, Ei_RS249 (KM018932)
OTU 6, Ki_RS16 (KM018988)
Guerrero Negro Hypersaline Mat (JN509209)
Desulfobacterium autotrophicum HRM2 (CP001087)
Desulfocella halophila (AF022936)
Desulfonema limicola (U45990)
OTU 202, Di_RS53 (KM018645)
Desulfosalsimonas propionicica (DQ067422)
OTU 12, Ni_RS4 (KM019133)
OTU 214, Ki_RS60 (KM019030)
low temperature hydrothermal oxides (JN860325)
OTU 3, Ni_RS1 (KM019131)
OTU 65, Ei_RS47 (KM018777)
OTU 165, Ei_RS46 (KM018776)
Desulfatiglans anilini (AJ237601)
OTU 135, Ki_RS67 (KM019034)
OTU 132, Ei_RS181 (KM018883)
subseafloor sediment of the South China Sea (EU385910)
marine sediment (EU181479)
Desulfobacterales
10bav-F6
Desulfurella acetivorans (X72768)
Desulfurella propionica (Y16942)
Hippea maritima DSM 10411 (CP002606)
OTU 38, A-UCL1_RS61 (KM018482)
saturated zone of the Hanford Site (HM187256)
Desulfurellales
Desulfohalobium utahense (DQ067421)
OTU 153, Ki_RS6 (KM018979)
Desulfohalobium retbaense (U48244)
Desulfothermus okinawensis (AB264617)
Desulfonauticus autotrophicus (FJ194951)
Desulfovibrionales
iron oxide chimney−like structure, Tonga Arc (FJ905689)
subseafloor massive sulfide deposit (AB722091)
DTB120
OTU 169, A-UCL1_RS120 (KM018524)
Thermodesulforhabdus norvegica (U25627)
Desulfacinum infernum (L27426)
OTU 80, A-UCL1_RS68 (KM018488)
active hydrothermal field sediments (FJ205259)
Syntrophobacterales
uncultured Candidatus Entotheonella sp.(AY897120)
Lake Medee deep-sea hypersaline anoxic basin, Mediterranean Sea (JF809701)
OTU 27, A-UCL1_RS7 (KM018431)
active hydrothermal field sediments (FJ205265)
OTU 178, Di_RS27 (KM018630)
OTU 167, A-UCL1_RS112 (KM018519)
OTU 18, A-UCL1_RS102 (KM018514)
Manantial del Toro hypersaline groundwater (JF747662)
OTU 75, A-UCL1_RS73 (KM018491)
OTU 36, Di_RS62 (KM018652)
subseafloor sediment of the South China Sea (EU386067)
seafloor lavas of Loi'hi Seamount Pisces Peak (EU491182)
OTU 201, Di_RS54 (KM018646)
Lake Medee deep-sea hypersaline anoxic basin, Mediterranean Sea (JF809757)
Candidatus Entotheonella
SAR324 cluster bacterium JCVI−SC AAA005 (AGAU01000387)
OTU 97, Ki_RS241 (KM019101)
OTU 235, A-UCL3_RS23 (KM018619)
uncultured SAR324 cluster bacterium (HM798884)
OTU 29, Ni_RS13 (KM019141)
SAR 324
Desulfuromonas chloroethenica (U49748)
Desulfuromonas thiophila (Y11560)
Desulfuromusa ferrireducens (AY835392)
Pelobacter propionicus DSM 2379 (CP000482)
Geobacter chapelleii (U41561)
Mariprofundus ferrooxydans (EF493243)
0.10
Atlantis II Deep BSI
Atlantis II Deep UCL3
Discovery Deep BSI
Nereus Deep BSI
Atlantis II Deep UCL1
Kebrit Deep BSI
Erba Deep BSI
Mediterranean DHABs

Figure 3


Lake Thetis clone (AEJ86659)
Lake Medee clone (AGC09905)
Hypersaline microbial mats clone (ACD02050)
Methanohalophilus portucalensis (BAO50256)
Red Sea Discovery Deep clone (AGA20325)
Methanohalophilus mahii (CP001994)
Methanohalophilus halophilus (BAL72744)
Mediterranean Discovery Basin interface clone (ABC73709)
OTU1, Erba_mcrA_37 (KJ880113) (83.3%) (17.8%) (100%) (100%)
Deep-sea hydrothermal vent clone (AEP93544)
Methane seep sediments clone (BAN63117)
OTU2, Erba_mcrA_83 (KJ880182) (82.2%)
Pearl River Estuary clone (ACD65231)
Cascadia Margin clone (BAJ05280)
Methanococcoides methylutens (AAC43410)
Methanohalobium evestigatum (CP002069)
Uncultured Methanohalobium sp. (AGU16959)
Mud Volcano ANME-3 clone (CAL58674)
Mud Volcano ANME-3 clone (CAL58676)
Methanosarcina acetivorans (AE010299)
Methanosarcina barkeri (CP000099)
River sediment clone (AGC54101)
Methane-containing Arctic permafrost clone (AGU01576)
OTU3, Kebrit_mcrA_10 (KJ880102) (8.3%)
Rice field soil clone (CAN90077)
Rice field soil clone (CAN90086)
Methanosaeta harundinacea (CP003117)
Methanosaeta concilii (CP002565)
Methanocella arvoryzae (AM114193)
Methanocella conradii (CP003243)
Methanoculleus bourgensis (HE964772)
Methanoplanus limicola (CM001436)
OTU4, Kebrit_mcrA_7 (KJ880105) (8.3%)
River sediment clone (AGC54146)
Lonar crater lake sediments clone (AET80960)
Cand. Methanomethylophilus alvus (CP004049)
Methanomassiliicoccus luminyensis B10 (AEO13319)
Methanocaldococcus jannaschii (L77117)
Methanotorris igneus (CP002737)
Methanopyrus kandleri (AE009439)
0.03
Atlantis II Deep UCL2
Erba Deep BSI
Kebrit Deep BSI
Atlantis II Deep UCL3
Mediterranean DHABs

Figure 4


Desulfobacterium autotrophicum (AF418182)
Desulfobacula phenolica (AAQ05965)
OTU 15, Kidsr RS37E05 (KM242007) ■ (31%)
interface of L'atalante Deep, Mediterranean Sea clone (AY590522)
Kryos Lake AWW, Mediterranean Sea clone (AY590528)
OTU 25, Kidsr RS6F01 (KM242021) ■ (2.4%)
New England salt marsh clone (AAV68650)
Desulfobacula-related clade
(Clade BSI I)
Desulfosalsimonas propionicica (ABD46860)
interface of Urania Deep, Mediterranean Sea clone (AY590524)
OTU 23, Kidsr RS1A01 (KM241999) ■ (2.4%)
Kryos Lake AWW Mediterranean Sea clone (KJ922628)
OTU 8, Eidsr RS56H07 (KM241949) ▲ (1.8%) △ (3.2%)
Desulfosalsimonas-related clade
(Clade BSI II)
Desulfohalobium retbaense DSM 5692 (AF418190)
Desulfohalobium utahense (ABD46858)
Kryos Lake AWW, Mediterranean Sea clone (KJ922627)
Kryos Lake AWW, Mediterranean Sea clone (KJ922630)
OTU 13, Kidsr RS19C03 (KM241998) ■ (14.3%)
interface of L'atalante Deep, Mediterranean Sea clone (AY590541)
Desulfohalobium-related clade
(Clade BSI III)
OTU 14, Kidsr RS86F11 (KM242026) ■ (40.5%)
Desulfatiglans anilini (AAQ05939)
marine sediment, Aarhus Bay clone (AM408824)
OTU 20, A-UCL1dsr RS45E11 (KM241878) ◉ (8.2%)
OTU 24, Kidsr RS90B12 (KM242027) ■ (2.4%)
brine layer of L'atalante Deep, Mediterranean Sea clone (AY590509)
OTU 11, Eidsr RS4 (KM241933) ▲ (1.8%)
interface of Urania Deep, Mediterranean Sea clone (AY590507)
OTU 22, Eidsr RS79G102 (KM241973) ▲ (0.9%)
OTU 9, Eidsr RS2B09 (KM241921) ▲ (2.6%)
OTU 5, Eidsr RS6F012 (KM241964) ▲ (14%) △ (3.2%)
OTU 6, Eidsr RS8H01 (KM241983) ▲ (69.3%) △ (71%)
Desulfatiglans-related clade
(Clade BSI IV)
Desulfotomaculum geothermicum (AF273029)
Desulfotomaculum sp. For-1 (AFI98563)
Desulfotomaculum thermosapovorans (AF271769)
Desulfotomaculum kuznetsovii DSM 6115 (YP 004518774)
Desulfotomaculum thermoacetoxidans (AAK83691)
OTU 12, Eidsr RS89A12 (KM241981) ▲ (2.6%) △ (3.2%)
Thermodesulfatator atlanticus DSM 21156 (CAX51964)
Desulfacinum infernum (AF418194)
Thermodesulforhabdus norvegica (AF334597)
Thermodesulfatator-related clade
(Clade BSI V)
OTU 4, Didsr RS10H06 (KM241882) □ (5.6%)
depth gradient in acidic fen clone (CAJ47279)
depth gradient in acidic fen clone (CAJ47303)
OTU 3, Didsr RS13C07 (KM241883) ◉ (33.3%) □ (11.1%)
OTU 17, Nidsr RS04D01 (KM242031) △ (31.3%)
deep-sea sediment of Eastern Mediterranean Sea clone (AY590556)
OTU10, Nidsr RS07G01 (KM242033) ▲ (1.8%) △ (6.5%)
OTU 1, Didsr RS30D09 (KM241885) ○ (50%) ◉ (41.7%) □ (72.2%)
deep-sea sediment of Eastern Mediterranean Sea clone (AY590554)
deep-sea sediment of Eastern Mediterranean Sea clone (AY590555)
Deep-branching clade
(Clade BSI VI)
Archaeoglobus profundus DSM 5631 (AAC78309)
0.1
Atlantis II Deep BSI
Atlantis II Deep UCL1
Discovery Deep BSI
Kebrit Deep BSI
Nereus Deep BSI
Erba Deep BSI
Mediterranean DHABs

Supplementary tables

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