Recycling of archaeal biomass as a new strategy for extreme life in Dead Sea deep sediments

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ABSTRACT

Archaea and Bacteria that inhabit the deep subsurface (known as the deep biosphere) play a prevalent role in the recycling of sedimentary organic carbon. In such environments, this process can occur over millions of years and requires microbial communities to cope with extremely limited sources of energy. Because of this scarcity, metabolic processes come at a high energetic cost, but the ways heterotrophic microbial communities develop to minimize energy expenses for a maximized yield remain unclear. Here, we report molecular biomarker evidence for the recycling of archaeal cell wall constituents in extreme evaporitic facies of Dead Sea deep sediments. Wax esters derived from the recombination of hydrolyzed products of archaeal membrane lipids were observed in gypsum and/or halite sedimentary deposits down to 243 meters below the lake floor, implying the reutilization of archaeal necromass possibly by deep subsurface Bacteria. By recycling the building blocks of putatively better adapted Archaea, heterotrophic Bacteria may build up intracellular carbon stocks and mitigate osmotic stress in this energy-deprived environment. This mechanism illustrates a new pathway of carbon transformation in the subsurface and demonstrates how life can be maintained in extreme environments characterized by long-term isolation and minimal energetic resources.

INTRODUCTION

In extreme environments, microbial metabolic processes that lower the energetic cost of maintaining life are favored (Hoehler and Jørgensen, 2013). Such settings are characterized by low growth rates (Lomstein et al., 2012) and most energy is diverted to maintenance functions (Van Bodegom, 2007) such as osmotic equilibration, O_2 stress defense, motility, and more sustainable metabolic pathways. These selective conditions for life promote the dominance of prokaryotes and generally favor Archaea relative to Bacteria (Hoehler and Jørgensen, 2013). This is mostly due to the reduced membrane permeability of Archaea, which requires less maintenance energy with respect to bacterial membranes (Valentine, 2007). This advantage is particularly striking in environments characterized by high osmotic stress such as hypersaline environments. There, Bacteria may use alternative strategies that allow competition with the putatively better adapted Archaea, for example by recycling available organic molecules as osmotic solutes (Oren, 1999a). The intracellular accumulation of ambient organic carbon is a common way of economizing energy in harsh environments. For example, under stressed growth conditions, some Bacteria are known to accumulate intracellular lipid droplets (Alvarez et al., 1997; Wältermann and Steinbüchel, 2005) in the form of polyhydroxyalkanoates, triglycerides or wax esters (WEs). However, the presence of such mechanisms in the deep biosphere has not yet been documented, suggesting that they may not be sufficiently effective in these low energy settings for Bacteria to survive.

The Dead Sea is the most saline lake on Earth and has deposited evaporitic minerals since the early Quaternary (Stein, 2001). As a result, its subsurface environment constitutes one of the most extreme ecosystem on the planet. Extreme chemistry of the water allows only for the survival of halophilic Archaea in the water column and recent halite sediments (Bodaker et al., 2010; Thomas et al., 2015). Bacteria have rarely been observed in the most extreme sedimentary facies of the Dead Sea (halite or gypsum), suggesting that these harsh conditions limit their growth (Thomas et al., 2015). To further investigate the composition of the microbial

community and its potential metabolic strategies to survive the most arid periods of the Late Quaternary of the Dead Sea basin, we characterized the lipid biomarker composition of deep hypersaline halite and gypsum sediments. The good lipid preservation in these extreme horizons gives clues to metabolic pathways that allow for the survival of a thus far unrecognized deep biosphere.

METHODS

The material used in this study originates from the ICDP Dead Sea Deep Drilling Project (DSDDP) core 5017-1 retrieved from the center of the Dead Sea in winter 2010-2011 (Fig. 1). Sediments were sampled on site from core catchers using sterile tools, and kept in the freezer at -20°C until further processing. The main characteristics of samples analyzed for lipid biomarkers are given in Table S1. Samples were freeze-dried, ground and extracted using multiple sonication cycles (methanol 2×, methanol/DCM (1:1 v/v, 2×) and DCM 3×). Elemental sulfur was removed with activated copper. Lipid extracts were filtered and separated using a deactivated silica gel column (5% H_2O) into five fractions of increasing polarity (hexane/DCM (9:1 v/v), hexane/DCM (1:1 v/v), DCM, ethyl acetate and methanol). Fractions 3 and 4 were silylated with pyridine/BSTFA 2:1 (v/v). Fraction 5 was trans-esterified by overnight incubation with 0.5 mL toluene and 2 mL of 2% H_2SO_4 in methanol at 60°C. Following the addition of NaCl (5%), the organic phase was extracted with hexane:DCM (4:1 v/v, 3×), washed with $NaHCO_3$ (2%) and dried with sodium sulfate before silylation.

All fractions were analyzed by gas chromatography mass spectrometry (GC-MS) on a HP 6890 Series Plus gas chromatograph equipped with a cool on-column injector, and coupled to an Agilent 5975C (VL MSD) mass spectrometer. The GC was equipped with a HP5 column (30 m × 0.25 mm × 0.25 µm, RESTEK). Samples were injected at 60°C (held for 30 sec), and the oven temperature was increased to 130°C at a rate of 20°/min, then to 250°C (5°/min) and 300°C (2°/min), and finally held isothermal for 45 min. Stepwise dilution of external standards

allowed quantification. Isoprenoid fatty acids are less polar than linear acids and partly eluted in fraction F4. A ratio based on specific bacterial fatty acids was calculated using the sum of methyl-branched C_{15} and C_{17} fatty acids over the sum of linear C_{15} and C_{17} fatty acids. Changes in this ratio indicate a shift in bacterial community.

Compound-specific carbon isotope ($\delta^{13}C$) analyses were done using a HP7890B GC coupled to an Isoprime visION isotope ratio mass spectrometer via a GC-5 combustion interface operating at 870°C. The GC was equipped with a BPX5 column (30 m × 0.25 mm × 0.10 µm, SGE) and a cool on-column injector, and the oven temperature was programmed as for GC-MS analyses. The B4 standard mixture (Arndt Schimmelmann, Indiana University) was used to externally calibrate the $\delta^{13}C$ values, and the known BSTFA derivatizing agent $\delta^{13}C$ value was corrected for alcohols and fatty acids. After samples were decalcified, Total Organic Carbon (TOC) was measured and data were normalized to a standard sediment (IVA Analysentechnik, Germany) containing 9.15% of C, measured every ten samples.

RESULTS AND DISCUSSION

We found significant amounts of isoprenoid WE (up to 0.2 $\mu g.g^{-1}$ TOC) in halite and gypsum samples retrieved between 90 and 250 meters below lake floor (mblf ; Fig. 2 and Table S1). WEs are an important type of energy storage molecules that can be produced by Eukarya and Bacteria, particularly under conditions of stress, but they have not been reported in the archaeal domain so far (Wang et al., 2019). WEs are formed by condensation of fatty acids and alcohols available in the environment and provide easily accessible (intracellular) sources of carbon (Wältermann and Steinbüchel, 2005). The major WE detected was phytanyl phytanate (3,7,11,15-tetramethylhexadecyl-3,7,11,15-tetramethylhexadecanoate; iC_{20}-iC_{20}, Fig. 3) which was accompanied by a series of other isoprenoid WEs composed of a C_{20} or C_{25} isoprenoid alcohol esterified to a linear, methyl-branched or isoprenoid acyl chain (Fig. 3a). The isoprenoid WEs were accompanied by significant amounts of membrane core lipids of halophilic Archaea

- archaeol (2,3-*O*-diphytanyl-*sn*-glycerol) and extended archaeol (2,3-*O*-phytanyl-*O*-sesterterpanyl-*sn*-glycerol) (Kates, 1997; Dawson et al., 2012) - along with several of their hydrolyzed and eventually oxidized products (Figs 2 and 3b; Table S2). Lipids of halophilic Archaea are composed of C_{20} and C_{25} isoprenoid alkyl chains, which allow for better control of membrane permeability under strong osmotic stress compared to bacterial fatty acid membranes (Valentine, 2007; Koga, 2012). Archaeol was the most abundant isoprenoid alcohol in most of the investigated sediment samples (Figs. 2 and 3). Extended archaeol, a membrane lipid more specific to halophilic Archaea of the Halobacteria class (Dawson et al., 2012) that dominate the Dead Sea halite and gypsum sediments (Thomas et al., 2015), was also found enriched in halite and gypsum facies. In most of the sedimentary intervals where WEs occurred, archaeol and extended archaeol were found in lower abundance than in the other halite/gypsum samples (Fig. 2). All WE-containing intervals also contained hydrolyzed core lipids and some oxidized counterparts (Fig. 2), which consisted of C_{20} and C_{25} isoprenoid alcohols and acids (phytanol, phytanic acid and C_{25} homologues), and of isoprenoid C_{20} and C_{25} monoalkyl glycerol monoethers (Fig 3b and Table S2). The report of WEs with isoprenoid C_{25} carbon chains (iC_{20}-iC_{25} and iC_{25}-iC_{20}) in environmental samples is unprecedented and attests to the incorporation of archaeal core lipid subunits into isoprenoid WEs. The formation of isoprenoid WEs has been previously demonstrated during growth of Bacteria on free isoprenoid compounds such as phytane, phytol or squalene (Rontani et al., 1999; Rontani et al., 2003; Silva et al., 2007). The recycling of phytol derivatives into mixed isoprenoid and non-isoprenoid WEs has also been reported in desiccated mats from modern evaporative alkaline lakes (Finkelstein et al., 2010). However, phytane, phytol and squalene were not observed in the investigated samples from the Dead Sea. As a result, the WEs detected in the Dead Sea halite/gypsum samples mostly consist of lipid subunits derived from the cell walls of Archaea, the dominant organisms in the Dead Sea (Bodaker et al., 2010; Thomas et al., 2015). This recycling is further confirmed by

129 compound-specific stable carbon isotope analyses (Supplementary material Fig. S1), showing
130 that the $\delta^{13}C$ values of WEs agree with those of their building blocks (fatty acids and alcohols).
131
132 Current knowledge suggests that the ability to form and accumulate WEs has only arisen in
133 bacterial and eukaryal domains (Garay et al., 2014; Wang et al., 2019). Eukaryotic life in the
134 Dead Sea has been constrained to humid intervals during glacial periods and occasional blooms
135 triggered by high precipitation during interglacial stages (Oren et al., 1995). Hence, isoprenoid
136 WEs present in the most arid intervals of the Dead Sea sediment (halite/gypsum) cannot
137 originate from eukaryotes. So far, Archaea have never been shown to produce WEs. Given the
138 prevalence of Archaea in the Dead Sea environment, and the fact that ubiquitous groups of
139 Archaea have been shown to possess the genetic machinery for fatty acid synthesis (Iverson et
140 al., 2012; Villanueva et al., 2017), an archaeal origin of the isoprenoid WEs cannot be
141 completely ruled out. However, the recent bioinformatics analysis of presently available
142 archaeal genomes (including some of hyperhalophiles) has failed to identify homologues of
143 genes coding for wax ester synthase (Wang et al., 2019). Abiotic esterification has also been
144 suggested to occur during the transformation of sedimentary lipids (Becker, 2015). But the
145 activity of extracellular enzymes is likely to be inhibited in the hypersaline Dead Sea sediment
146 (Frankenberger Jr and Bingham, 1982; Grant, 2004) and, in the present case, putative abiotic
147 reactions would have yielded a much wider diversity of ester structures (including eukaryotic
148 sterols). The predominance of specific bacterial C_{15} and C_{17} fatty acids (Perry et al., 1979) in
149 WE-containing sedimentary intervals (Fig. 2), instead suggests a bacterial origin of the WEs.
150 Recent work on fluid inclusions allowed the retrieval of bacterial sequences in halite-dominated
151 layers of the deep Dead Sea sediment (Thomas and Ariztegui, 2018). Our data would therefore
152 support the development of Bacteria upon archaeal necromass in the extreme environment of
153 the Dead Sea subsurface.
154

155 The concentration of intracellular WEs in Bacteria has been shown to arise particularly in
156 situations of nutrient starvation, especially when nitrogen is limiting (Ishige et al., 2003).
157 Additionally, the recycling of archaeal biomass by Archaea themselves was suggested to
158 minimize the energy cost of life in nutrient- and carbon-limited environments (Takano et al.,
159 2010). Here, we propose that the recycling of archaeal necromass into storage lipids like WEs
160 constitutes a way to save and store energy in the nutrient- and energy-demanding hypersaline
161 environment of the Dead Sea. The presence of WEs with mixed linear and isoprenoid acyl
162 chains alongside the predominant isoprenoid WEs indicates that the WE-forming microbial
163 population also recycled bacterial and/or eukaryal fatty acids, in addition to archaeal lipids (Fig.
164 4). As a result, the subsurface biosphere creates easily accessible carbon stocks from necromass,
165 in a lake with rare allochthonous inputs and primary production (Oren, 1999b).
166 The accumulation of WEs has previously been reported in episodically desiccated microbial
167 mats in hypersaline environments (Finkelstein et al., 2010), and was suggested to allow for
168 better microbial cell survival during periods of desiccation, due to the release of H_2O during
169 esterification. The Dead Sea sediments that bear WEs originate from the deepest part of the
170 lake (i.e., 297.5 m below lake level), where neither trace of desiccation (Neugebauer et al.,
171 2016) nor microbial mats have been reported. These sedimentary levels correspond, however,
172 to periods when salinity was the highest in the deep brine, as supported by the bromide
173 concentration curve (Fig. 2), a reliable salinity proxy for the Dead Sea water column during the
174 Quaternary (Levy et al., 2017). The WE-bearing levels generally correspond to the highest
175 concentrations of this element (Fig. 2; Table S1), which are observed during the driest climatic
176 intervals (Marine Isotopic Stages 5E, early 5A and the 2/1 transition). Water availability is a
177 major issue not only in dry environments, but also in hypersaline settings due to high salt
178 concentrations inducing osmotic stress. By recombining hydrolyzed moieties of core lipids
179 originating from the buried archaeal necromass, the WE-forming bacterial population may
180 therefore create accessible water molecules that favor its survival.

Our results illustrate the high adaptability of the subsurface biosphere and its ability to use varied strategies for energy production and preservation under adverse conditions. By studying an environment that pushes life to its limits, we catch a glimpse of the processes that fuel life in the deep subsurface, and advance our understanding of the deep sedimentary carbon cycle.

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295
296 **FIGURE CAPTIONS**
297 Fig. 1: Localization of the Dead Sea cores. The material used in this study originates from
298 drilling site 5017-1.
299
300 Fig. 2: Occurrence of wax esters (WEs) and other lipid biomarkers in the Dead Sea core
301 (gypsum and halite intervals are shown in black, dashed lines indicate the position of the
302 samples analyzed). "Hydrolized core lipids" refer to degradation products of archaeol and
303 extended-archaeol, which are presented as alkyl chains. The formation of the WE building
304 blocks is schematized on the right-hand side of the panel. The bacterial fatty acids ratio (sum
305 of methyl-branched C_{15} and C_{17} over sum of linear C_{15} and C_{17} fatty acids) indicates the
306 presence of bacteria potentially involved in WE-production. The sampled layers are compared
307 to bromide concentration in the sediment pore waters [used as a proxy for lake water
308 dilution/concentration (Levy et al., 2017)] and fitted to ^{14}C ages (Kitagawa et al., 2016) and
309 U/Th ages (Torfstein et al., 2015). Red items indicate an archaeal origin, and blue items a
310 bacterial origin.

Fig. 3: Characteristic chromatograms of isoprenoid WEs and related building blocks. (a) Total ion chromatogram (TIC) of the WE fraction (Fraction 2) from the gypsum sample at 91.04 mblf and (b) extracted ion chromatogram (m/z 103+133+159+205+218) of a polar lipid fraction (Fraction 4) from the gypsum sample at 243.22 mblf. These intervals were selected because they show the highest concentrations of isoprenoid WEs and hydrolyzed core lipids, respectively. MGM: monoalkyl glycerol monoethers. Red symbols indicate an archaeal origin and blue symbols a bacterial/eukaryal origin. The numbers above the symbols shown in (b) correspond to the number of carbon atoms in the chain.

Fig. 4: Schematic pathway for isoprenoid WE formation by recycling of membrane lipids in Dead Sea sediments. Intact phospholipids from archaeal and bacterial/eukaryal communities are hydrolyzed and eventually oxidized, and some of these degradation products are transformed into WEs by bacterial communities in the deep halite/gypsum sediment horizons. Symbols refer to the compounds identified in Fig. 3. Moieties in red have an archaeal origin and blue moieties have a bacterial/eukaryal origin.

Figure 1

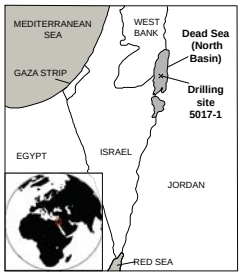
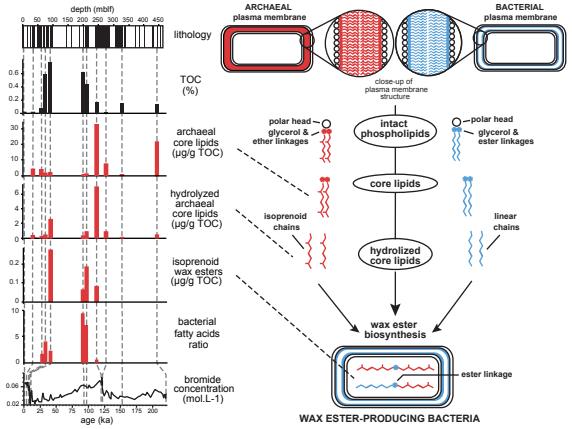
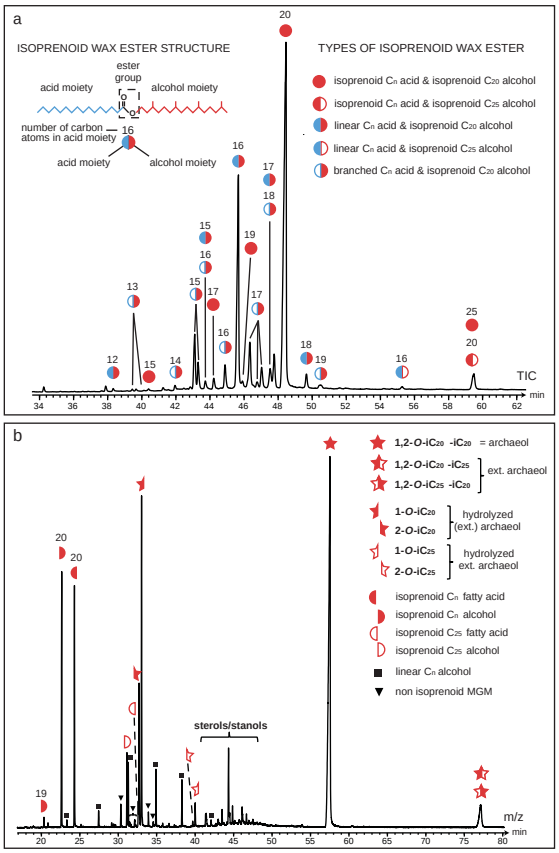

MEDITERRANEAN SEA
WEST BANK
Dead Sea (North Basin)
GAZA STRIP
Drilling site 5017-1
EGYPT
ISRAEL
JORDAN
RED SEA

Figure 2


depth (mblf)
lithology
TOC (%)
archaeal core lipids (µg/g TOC)
hydrolyzed archaeal core lipids (µg/g TOC)
isoprenoid wax esters (µg/g TOC)
bacterial fatty acids ratio
bromide concentration (mol.L-1)
age (ka)

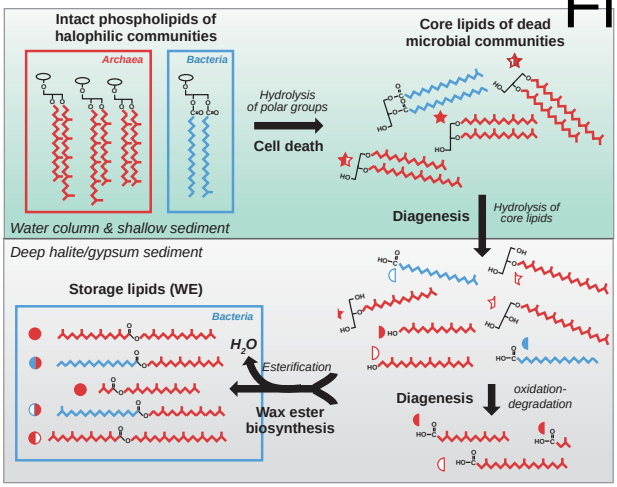
ARCHAEAL plasma membrane
BACTERIAL plasma membrane
close-up of plasma membrane structure
polar head
glycerol & ether linkages
intact phospholipids
polar head
glycerol & ester linkages
core lipids
isoprenoid chains
linear chains
hydrolized core lipids
wax ester biosynthesis
ester linkage
WAX ESTER-PRODUCING BACTERIA

Figure 3


a
ISOPRENOID WAX ESTER STRUCTURE
ester group
acid moiety
alcohol moiety
number of carbon atoms in acid moiety
TYPES OF ISOPRENOID WAX ESTER
isoprenoid Cn acid & isoprenoid C20 alcohol
isoprenoid Cn acid & isoprenoid C25 alcohol
linear Cn acid & isoprenoid C20 alcohol
linear Cn acid & isoprenoid C25 alcohol
branched Cn acid & isoprenoid C20 alcohol
TIC
min

b
1,2-O-iC20 -iC20 = archaeol
1,2-O-iC20 -iC25
1,2-O-iC25 -iC20
ext. archaeol
1-O-iC20
2-O-iC20
hydrolyzed (ext.) archaeol
1-O-iC25
2-O-iC25
hydrolyzed ext. archaeol
isoprenoid Cn fatty acid
isoprenoid Cn alcohol
isoprenoid C25 fatty acid
isoprenoid C25 alcohol
linear Cn alcohol
non isoprenoid MGM
sterols/stanols
m/z
min

Figure 4


Intact phospholipids of halophilic communities
Archaea
Bacteria
Hydrolysis of polar groups
Cell death
Core lipids of dead microbial communities
Water column & shallow sediment
Diagenesis
Hydrolysis of core lipids
Deep halite/gypsum sediment
Storage lipids (WE)
Bacteria
H_2O
Esterification
Wax ester biosynthesis
Diagenesis
oxidation-degradation