This is a pre-print of an article published in *Microbial Ecology*. The final authenticated version is available online at: 10.1007/s00248-017-1104-x

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Methanogenic and Sulfate-Reducing Activities in a Hypersaline Microbial Mat and Associated Microbial Diversity

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Received: 25 May 2017 / Accepted: 30 October 2017

Abstract

Methanogenesis and sulfate reduction are important microbial processes in hypersaline environments. However, key aspects determining substrate competition between these microbial processes have not been well documented. We evaluated competitive and non-competitive substrates for stimulation of both processes through microcosm experiments of hypersaline microbial mat samples from Guerrero Negro, Baja California Sur, Mexico, and we assessed the effect of these substrates on the microbial community composition. Methylotrophic methanogenesis evidenced by sequences belonging to methanogens of the family *Methanosarcinaceae* was found as the dominant methanogenic pathway in the studied hypersaline microbial mat. Nevertheless, our results showed that incubations supplemented with acetate and lactate, performed in absence of sulfate, also produced methane after 40 days of incubation, apparently driven by

hydrogenotrophic methanogens affiliated to the family *Methanomicrobiaceae*. Sulfate reduction was mainly stimulated by addition of acetate and lactate; however, after 40 days of incubation, an increase of the H_2S concentrations in microcosms amended with trimethylamine and methanol was also observed, suggesting that these substrates are putatively used for sulfate reduction. Moreover, 16S rRNA gene sequencing analysis showed remarkable differences in the microbial community composition among experimental treatments. In the analyzed sample amended with acetate, sulfate-reducing bacteria (SRB) belonging to the family *Desulfobacteraceae* were dominant, while members of *Desulfohalobiaceae*, *Desulfomicrobiaceae*, and *Desulfovibrionaceae* were found in the incubation with lactate. Additionally, we detected an unexpected high abundance of unclassified *Hydrogenedentes* (near 25%) in almost all the experimental treatments. This study contributes to better understand methanogenic and sulfate-reducing activities, which play an important role in the functioning of hypersaline environments.

Keywords

Methanogenesis
Sulfate reduction
Hypersaline environment
Microbial mat

Electronic supplementary material

The online version of this article (https://doi.org/10.1007/s00248-017-1104-x) contains supplementary material, which is available to authorized users.

Introduction

Methanogenic microorganisms can obtain energy for growth from the

oxidation of a limited number of substrates, which lead to the formation of methane gas [4]. In most environments, methanogens are in competition with sulfate-reducing bacteria (SRB) for fermentation products, particularly for the key intermediates, hydrogen and acetate [25, 26, 41, 44]. In hypersaline environments, characterized by high sulfate concentrations, sulfate reduction is the dominant microbial respiratory process, owing to the high affinity of SRB for those competitive substrates [23, 25, 26, 37]. However, based on stable carbon isotopic analyses, incubation experiments and molecular evidence, it has been demonstrated that methylotrophic methanogens predominate in hypersaline environments and therefore, it is recognized that methylated compounds, such as methanol, monomethylamine, dimethylamine, trimethylamine (TMA), and dimethylsulfide, fuel methane production in these ecosystems [22, 25, 37].

Methylated amines and methanol have been identified as the main sources of methane in hypersaline environments [14, 37]. Kelley et al. [22] estimated individual substrate use, determined by ^{13}C-labeling from both North and South America hypersaline ecosystems, and reported that methylamines, principally TMA, contributed with 55–92% of produced methane, while methanol was responsible of 8–40%. Currently, TMA and methanol are regarded as “non-competitive” substrates between methanogens and SRB [37, 44]; however, there is evidence that these compounds can also be used by SRB. Methanol can be an electron donor for sulfate reduction [35, 57] and several SRB strains capable of growth with methanol as sole energy source have been isolated [35, 42]. In fact, SRB would outcompete methanogens for methanol at temperatures above 65 °C [58, 59]. In contrast, the unique evidence suggesting the use of TMA by SRB was reported by King [24], through the use of marine sediments from Lowes Cove, Maine, amended with TMA. However, no further information is available regarding the microbial groups involved in this process and the mechanisms remain unknown. Thus, previous studies have shown that the so-called “non-competitive” substrates could also be used by SRB across different environments; consequently, it is

clear that the competition for methylated compounds between methanogens and SRB is still poorly understood.

The aim of this study was to test the use of competitive (hydrogen and acetate) and non-competitive (TMA and methanol) substrates by methanogens and sulfate reducers, using amended microcosm experiments of a hypersaline microbial mat from Guerrero Negro, Baja California Sur, Mexico, and to elucidate microbial populations favored when these substrates are present in the system.

Materials and Methods

Microbial Mats Sampling

Cores of superficial (1 cm depth) microbial mats were collected in February 2015 from south-west of Area 4, known as Pound 4 near Pound 1 (P4n1) (27°36′49.99″N, 113°54′12.63″W), at “Exportadora de Sal S.A.” (ESSA) in Guerrero Negro, Baja California Sur, Mexico. The salt works and the microbial mats growing in them have been studied over many years [10], but this specific area has been reported only by Orphan et al. [46]. Microbial mat samples were transported in the darkness in large plastic trays containing site water. Upon arrival to the lab, samples of microbial mats were stored at 4 °C for further microcosm experiments. An additional set of microbial mats samples were frozen at −80 °C for molecular analysis.

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Microcosm Experiments

Sterilized serum vials of 120 mL were used to contain the culture media and the anaerobic atmosphere, which was obtained by covering the vials with butyl rubber stoppers and aluminum rivets and displacing the air with abundant N_2 gas. Incubations were performed with 3 g of microbial mat (mat cores 1 cm deep × 1 cm diameter) and 100 mL of artificial brine, prepared simulating the natural conditions of the site, with the

following composition [3] (g L^{-1}): NaCl (72.8), KCl (2), $MgCl{\cdot}6H_2O$ (29.33), $CaCl_2{\cdot}2H_2O$ (3), KBr (0.28), $NaHCO_3$ (0.21), Na_2SO_4 (9.94), and 1 mL L^{-1} trace element solution. The trace element solution contained [7] (mg L^{-1}): $FeCl_2{\cdot}4H_2O$ (2000), H_2BO_3 (50), $ZnCl_2$ (50), $CuCl_2{\cdot}6H_2O$ (90), $CoCl_2{\cdot}6H_2O$ (2000), $NiCl{\cdot}6H_2O$ (920), $Na_2SeO{\cdot}5H_2O$ (162), $(NH_4)6Mo_7O_{24}$ (500), ethylene diamine tetra-acetic acid (EDTA, 1000), $Na_2WO_4{\cdot}H_2O$ (100), and 1 mL L^{-1} of concentrated HCl. The final pH of the medium was 7.5.

To assess the use of competitive and non-competitive substrates for methanogenesis and sulfate reduction, four different substrates were tested: acetate, hydrogen, methanol, and TMA. Additionally, incubations with lactate were performed as a positive control for sulfate reduction. All substrates were supplemented at the final concentration of 0.976 g of chemical oxygen demand (COD) per liter (acetate, 16 mM; hydrogen, 6 mM; lactate, 10 mM; methanol, 20 mM; TMA, 10 mM). Microcosm incubations were placed in the darkness without shaking at 28 °C, for a period of 58 days. Once methanogenesis or sulfate reduction activity decreased, a new pulse of substrate was added to reestablish their initial concentration for three consecutive cycles. Additionally, three different controls were included: control without electron donor (no substrate addition), control without electron acceptor (substrate-without sulfate), and an autoclaved (killed) control. All treatments were performed in triplicate.

Analytical Methods

In order to detect either methane or hydrogen production, headspace of the cultures was monitored over time. Methane and hydrogen were measured using a gas chromatograph (Agilent 6850 Series GC System) equipped with a thermal conductivity detector (TCD), and a packed column (HAYESEP D 100/120) using N_2 as carrier gas. The following temperatures were applied in the method: injector, 250 °C; column, 70 °C; detector, 250 °C. Peak areas of methane and hydrogen were

compared with standards. Gas samples were directly injected (100 µL) with a gas tight sample lock syringe (Hamilton 81056).

As a response of sulfate reduction activity, H_2S was monitored using the copper-sulfate colorimetric method [9]. Methanogenic or sulfate reduction rates were determined on the maximum slope observed on linear regressions, plotting at least three sampling points.

DNA Extraction

Total genomic DNA from a non-manipulated microbial mat sample (inoculum) and from the experimental treatments was obtained. For amended microcosms, based on their high detectable methanogenic or sulfate-reducing activities, the most active samples were selected for further 16S rRNA sequencing. DNA was extracted from 0.1 g of the microbial mat using the commercial kit Power Soil DNA isolation (Mo Bio Laboratories, Carlsbad, CA, USA). Extraction was carried out according to the manufacturer's protocol using a bead beater (Fast DNA prep; MP Biomedicals United States, Solon, OH, USA) at 5.5 speed for 45 s for cell lysis. DNA quality was analyzed by 1% agarose gel.

16S rRNA Amplicon Library Construction for Illumina Sequencing

16S rRNA gene was amplified from extracted DNA using specific primers 341F and 785R, covering the V3 and V4 regions [28]. These primers have been reported to preferentially amplify the 16S rRNA gene region of microorganisms from the bacterial domain; however, in order to assess the archaeal coverage, the primer pair was evaluated in silico using TestPrime tool against Silva rRNA gene database (RefNR SSU release 128) [28], allowing two mismatch per primer, considering the number of degenerate positions. Primers were fused with Illumina adapters overhang nucleotide sequences. The polymerase chain reactions (PCRs) were performed in duplicate for each sample using 50 µl, employing Phusion Taq polymerase (ThermoScientific, USA). The PCR conditions were:

denaturation at 98 °C for 60 s, with 5 cycles of amplification at 98 °C for 60 s, 50 °C for 30 s and 72 °C for 30 s, followed by 25 cycles of amplification at 98 °C for 60 s, 55 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 5 min.

The PCR products were indexed using Nextera XT Index Kit v2 (Illumina, San Diego, CA) according to the Illumina's 16S Metagenomic Sequencing Library Preparation protocol. Libraries were further sequenced by single end with Illumina MiSeq sequencer.

Sequence Data Analysis

Mothur open source software (v 1.34.4) was used for analysis of 16S rRNA gene libraries [31]. Clean tags per sample were obtained with a minimum length of 150 bp. Sequences with homopolymer runs of eight or more bases, those with more than one mismatch to the sequencing primer and Q-value average below 30 were discarded. The potential occurrence of chimeric sequences was analyzed using UCHIME algorithm. Group membership was determined prior to trimming of the barcode and primer sequence. Total read numbers were normalized to equal abundance of the sample with the least sequencing efficiency. Sequences were aligned against the SILVA 123 16S/18S rRNA gene database, using the nearest alignment space termination (NAST) algorithm, and trimmed for the optimal alignment region. With the non-redundant sequences, a pairwise distance matrix was calculated and reads were clustered into operational taxonomic units (OTUs) at 3% distance using the furthest neighbor method. Mothur's Bayesian classifier and the SILVA 123 reference set were used to categorize taxonomically the sequences and OTUs. Additionally, a heatmap was constructed using the heatmap tool (heatmap.2) in the plots package within the statistical program R (http://www.r-project.org/).

The nucleotide sequence data reported are available in the GenBank database under the BioSample accessions: SAMN06857862,

SAMN06857863, SAMN06857864, SAMN06857865, SAMN06857866, SAMN06857867, SAMN06857868, SAMN06857869, SAMN06857870.

Results

Methanogenic and Sulfate Reduction Activities

Addition of TMA and methanol as electron donors to microbial mats incubations remarkably stimulated methane production under both conditions of presence and absence of sulfate (Fig. 1). In fact, the maximum methanogenic rates were observed in these treatments (Table 1). Significant increases on methane concentration occurred between the first 20 days of incubation with lower production rates for the following days, when TMA and methanol were used as substrates. At the end of the experiments, the observed methane productions accounted for 17.6 and 23% of added substrates, for TMA and methanol, respectively (Table 2). In contrast, methanogenesis was not stimulated in experiments amended with acetate and hydrogen; however, a small amount of methane was quantified after a long lag phase (40 days) in incubations performed in the absence of sulfate, with acetate and lactate as electron donors (Fig. 1). Moreover, traces of methane were found in the experiments supplemented with lactate, and hydrogen traces were also detected in microbial mats incubations amended with lactate in the absence of sulfate (data not shown). Methanogenic and sulfate-reducing activities were not observed in killed controls.

Fig. 1

Methane production from microbial mat samples amended with different electron donors. Substrates were supplemented at the same level of chemical oxygen demand (0.976 g L^{-1}; acetate, 16 mM; hydrogen, 6 mM; lactate, 10 mM; methanol, 20 mM; TMA, 10 mM) at three different times on days 0, 20, and 40. All experimental treatments were placed in the darkness at 28 °C, for a period of 58 days. Incubations labeled with W were incubated without sulfate. Results are means of triplicate incubations and error bars indicate the standard deviation

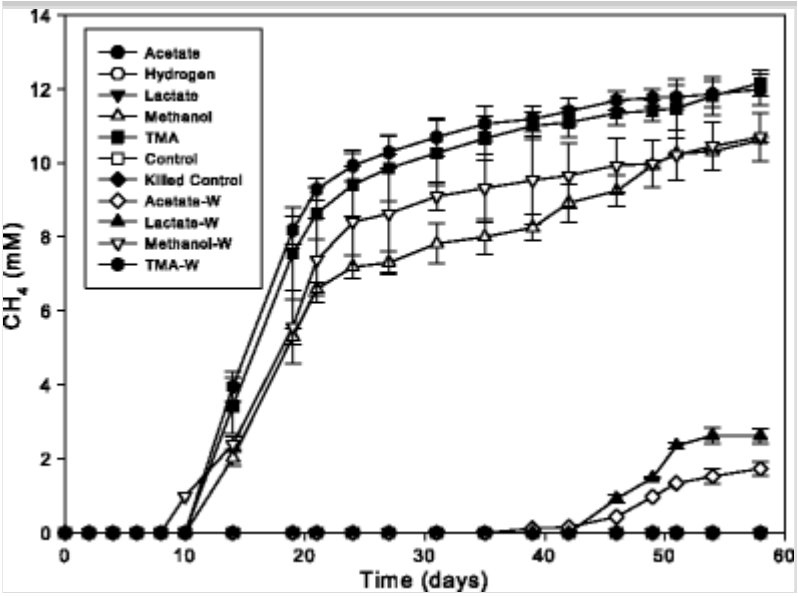


Table 1

Maximum methanogenic and sulfate-reducing activities observed in microcosm experiments of hypersaline microbial mats amended different substrates. Activities were measured in mmol of CH_4 or H_2S produced per gram of sediment per day (mmol/g sed –d)

Substrate/Process	Methanogenic activity (mmol/g sed –d)	Sulfate-reducing activity (mmol/g sed –d)

	Experiments without sulfate	Experiments with sulfate	
Hydrogen	ND	ND	0.94 (±0.02)
Acetate	0.16 (±0.01)	ND	5.88 (±0.85)
Lactate	0.19 (±0.01)	ND	2.57 (±0.37)
Methanol	0.88 (±0.02)	0.92 (±0.16)	0.28 (±0.01)
Trimethylamine	0.97 (±0.26)	1.09 (±0.38)	0.53 (±0.01)
Control incubated without substrate added did not present measurable activity. *ND* not detected			

Table 2

Balance (in mg COD L^{-1}) for the oxidation of different substrates in amended microcosm experiments of hypersaline microbial mats after 58 days of incubation

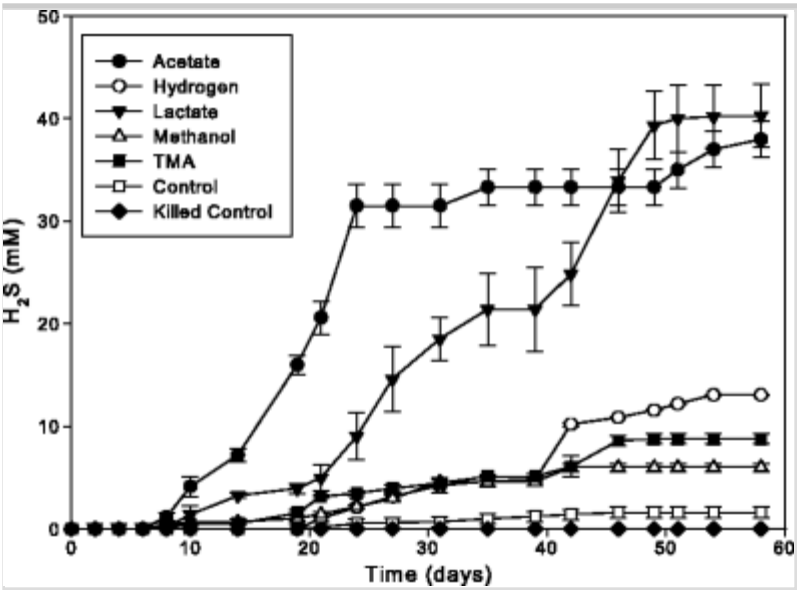
TreaTreatment/product	Methane/conversion (%)	H_2S/conversion (%)	Final conversion (%)
Acetate	110/3.7	2082.8/71.1	74.8
Hydrogen	ND	733.7/25	25.0
Lactate	160/5.4	2476/84.5	89.9
Methanol	674/9.6	281.6/23	32.6
Trimethylamine	500/17	458.2/15.6	32.6
Control	ND	102.4	00.0
version = (identified products-endogenous generation)/(input COD)			
ND not detected, *COD* chemical oxygen demand			

All substrates supplemented in microbial mats incubations promoted

sulfate-reducing activities (Fig. 2). Addition of acetate and lactate notably stimulated sulfate reduction in experiments of hypersaline microbial mats, followed by hydrogen, TMA, and methanol (Fig. 2). Lactate was the principal substrate utilized as evidenced by the extent of conversion (Table 2), with sulfate-reducing activity appearing since the first 10 days of incubation. However, the maximum respiratory rate for sulfate reduction was observed in experiments amended with acetate (Table 1). It is important to highlight that in acetate incubations, the most prominent activity occurred within the first 21 days of cultivation and then, an inhibition of the process occurred. Hydrogenotrophic sulfate reduction had a somewhat longer lag phase of 20 days, with a conversion of 25% of added substrate at the end of the experiment. Moreover, TMA and methanol additions resulted in H_2S production with a substrate conversion of 15.6 and 9.6%, respectively, linked to this process.

Fig. 2

Concomitant sulfate-reducing activities (measured as H_2S production) in incubations supplemented with competitive and non-competitive substrates (acetate, 16 mM; hydrogen, 6 mM; lactate, 10 mM; methanol, 20 mM; TMA, 10 mM). Substrates were supplemented at three different times during the 58 days (days 0, 20, and 40). All treatments were performed in the darkness, at 28 °C. Standard deviation is indicated by the error bars

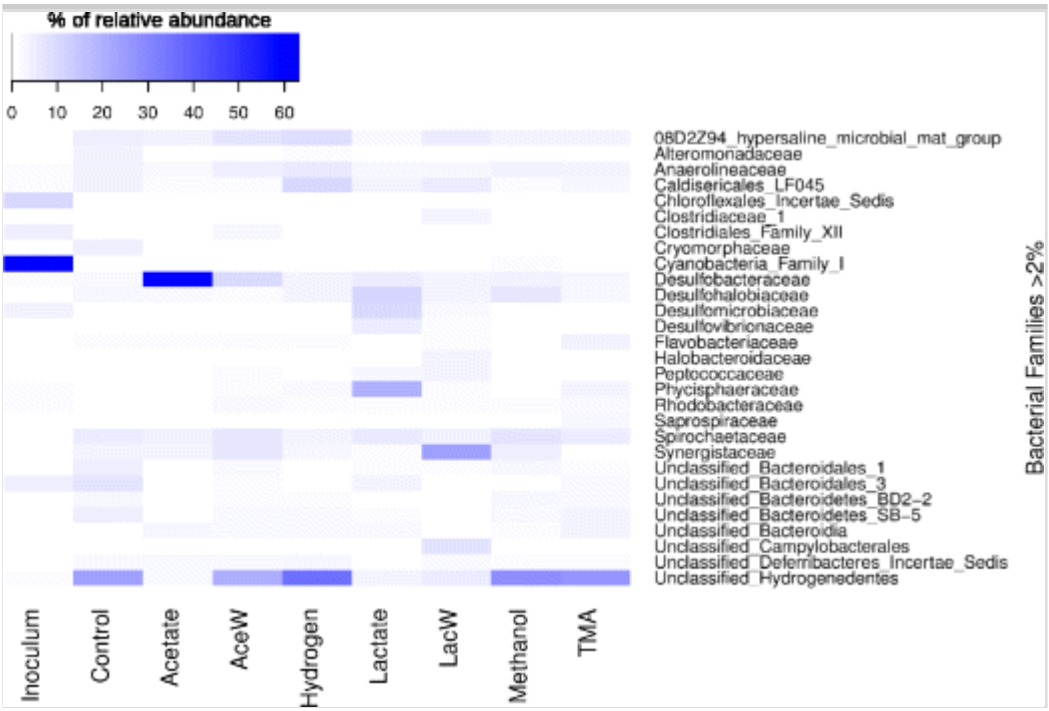


Microbial Community Composition in Non-manipulated Microbial Mats and Microcosms Experiments

Nine representative treatments were selected for sequencing, based on their high detectable activities during incubation. A total of 1,440,854 high quality reads of 16S rRNA gene sequences were obtained. For a better assessment, libraries were normalized to the same numbers of reads, 119,913 reads per sample. Rarefaction curves suggest that addition of different substrates to the original inoculum reduced the microbial diversity by selecting particular taxonomic groups of microorganisms (Online Resource 1). Non-manipulated microbial mat sample (inoculum) from P4n1 was dominated by *Cyanobacteria*, *Chloroflexales*, and unclassified *Bacteroidales* with relative abundances of 63.2, 9.9, and 4.1%, respectively (Fig. 3). However, the microbial community composition was modified under microcosm incubation without substrate addition (control), being unclassified *Bacteroidales* (24.8%) and unclassified *Hydrogenedentes* (22.9%) the most abundant populations after 58 days of incubation under anaerobic conditions (Fig. 3).

Fig. 3

Heatmap representation of the relative abundance (< 2%) of predominant bacterial families, detected in microbial mat samples amended with several electron donors, after 58 days of incubation. Color intensity indicate the relative percentages



Microbial community composition from the analyzed sample amended with acetate, performed in presence of sulfate, was mainly dominated by *Proteobacteria*, exclusively belonging to *Desulfobacteraceae* (59.8%). Meanwhile, in the incubation supplied with acetate, but performed in the absence of sulfate, a broader microbial diversity was present, displaying groups related to unclassified *Hydrogenedentes* (19.9%), *Desulfobacteraceae* (9.3%), *08D2Z94 hypersaline microbial mat group* (6.9%), and *Synergistaceae* (5.8%). 16S rRNA gene data from the sequenced incubation spiked with hydrogen revealed the presence of unclassified *Hydrogenedentes* (34.9%), *Caldisericales LF045* (9.5%) and *08D2Z94 hypersaline microbial mat group* (8.7%). In the experimental treatment amended with both lactate and sulfate, the most abundant

groups observed were *Phycisphaeraceae* (19.1%) and SRB belonging to *Desulfohalobiaceae* (10.7%), *Desulfomicrobiaceae* (9.1%) and *Desulfovibrionaceae* (5%). In the incubation with lactate where sulfate was excluded from the artificial brine, the microbial community composition changed to *Synergistaceae* (23.2%), unclassified *Campylobacterales* (7.3%), and *Caldisericales LF045* (4.8%). The microbial community in the examined methanol enrichment was composed of unclassified *Hydrogenedentes* (27.8%), *Spirochaetaceae* (6.8%), and *Desulfohalobiaceae* (5.8%). Furthermore, in the experimental treatment amended with TMA, the main microbial groups found were unclassified *Hydrogenedentes* (26%), *Spirochaetaceae* (4.6%), *Flavobacteriaceae* (3.5%), and *Rhodobacteraceae* (2.1%). Detailed community analysis at family level is presented in Online Resource 2.

Archaeal sequences were not detected in the non-manipulated sample from P4n1 possibly due to the universal primers used in this study, which mainly recovered a high proportion of bacterial sequences [28]. However, it was possible to retrieve 37,687 representatives' sequences of Archaea found in the experimental treatments. In analyzed microbial mats amended with methanol and TMA, a high proportion of sequences related to *Methanosarcinaceae* was detected, representing the 11.9 and 12.7% of the total relative abundance, respectively. Furthermore, concomitant to the production of methane found in experiments supplemented with both acetate and lactate, performed in absence of sulfate, methanogens belonging to the family *Methanomicrobiaceae* (0.1%) were detected in those treatments. Other archaeal groups were found among the experimental conditions, such as *Marine Benthic Group D*, *Woesearchaeota* (DHVEG-6), and unclassified *Bathyarchaeota*, but these groups represented less than 1% of the relative abundance (Online Resource 3).

Discussion

Methanogenic Activities

Several studies have shown that TMA and methanol are significant sources of methane produced in hypersaline ecosystems [14, 22, 25]. TMA is the breakdown product of glycine betaine, an abundant osmolyte present in hypersaline environments [45]. Methanol is derived for the degradation of pectin, which is produced by vascular plants, diatoms, and cyanobacteria [38]. In this study, it was found that addition of TMA and methanol remarkably stimulates methanogenesis in our amended microcosm experiments of hypersaline microbial mats (Fig. 1). These results agree with previous reports on methane production in hypersaline environments in the Napoli mud volcano [32], in microbial mats from Baja California Sur, Mexico [14], and in the Orca Basin, Gulf of Mexico [61]. Furthermore, with the addition of methylated substrates, methane production occurred both in the absence and in the presence of sulfate. Similar results have been recorded in incubations under natural conditions as well as with modified hypersaline microbial mats from Baja California Sur, Mexico [3, 14]. In contrast, experimental control without external substrate supply did not produce methane. In this sense, stable isotope measurements of produced methane in hypersaline environments have suggested that methanogens are operating under conditions of substrate limitation, and when higher concentrations of substrate are added to samples, methanogenesis is further stimulated [20, 21]. These geochemical data from natural and manipulated hypersaline microbial mats have extended the traditional boundaries for the characteristics of isotopic methane biologically produced [53].

Archaeal sequences (> 0.1% in abundance) were not detected in the original inoculum, possibly due to their low abundances under environmental conditions or to the universal primers used in this work, since they preferentially amplify the bacterial domain [28]. However, in silico analysis supported a good coverage (~ 95%) of traditional methanogens members of the archaeal phylum *Euryarchaeota*, allowing to retrieve some archaeal reads related to the family *Methanosarcinaceae*, mainly in experiments amended with methanol and TMA

(Online Resource 3). Other studies have shown that halophilic methanogens belonging to the order *Methanosarcinales* are dominant in hypersaline environments [14, 37, 61], which is consistent with the results collected in the present work. Interestingly, incubations amended with acetate and lactate as electron donors, performed in absence of sulfate, promoted methane production, but no methane production was observed in hydrogen-amended incubations (Fig. 1). Molecular data derived from incubations amended with acetate and lactate revealed the presence of methanogenic microorganisms belonging to the family *Methanomicrobiaceae*. This phylogenetic group can be distinguished from other methanogens because all members use H_2 + CO_2 as a substrate for methanogenesis [13]. Hydrogenotrophic methanogenesis in hypersaline microbial mats has barely been investigated. Smith et al. [52] detected potential hydrogenotrophic methanogens related to the order *Methanomicrobiales* in similar microbial mats incubated for 1 year under controlled conditions of low sulfate concentration. García-Maldonado et al. [15] reported novel phylogenetic lineages of putative hydrogenotrophic methanogens from natural as well as from manipulated samples in hypersaline microbial mats from Baja California Sur, Mexico.

The lack of evidence of hydrogenotrophic methanogenesis in experiments amended with hydrogen suggests that hydrogen consumption might have been dominated by other microbial groups, possibly SRB, and that this substrate might have not been available for methanogens. In similar microbial mats, Burrow et al. [6] through microcosms experiments inhibiting SRB, reported a significant increase in H_2 efflux, evidencing that SRB are a major group of hydrogenotrophs in these ecosystems. In addition, absence of hydrogenotrophic methanogenesis in incubations supplemented with hydrogen, possibly occurred because in acetate- and lactate-spiked incubations without sulfate, degradation of these organic compounds supported an increase of fermentative microorganisms (bacterial group *Synergistaceae*, see below), establishing a potential syntrophic interaction between fermentative bacteria and

hydrogenotrophic methanogens. It has been demonstrated that these microbial interactions can accelerate the metabolism of microbes that are not active or in low abundance under natural conditions, and it is well known that these interactions are of particular importance in methanogenic consortia [30].

This study provides information evidencing that hydrogenotrophic methanogenesis would perform in hypersaline environments in the presence of particular organic compounds, such as acetate and lactate. Previous works have reported an increase on hydrogenotrophic methanogenesis by the addition of organic matter to hypersaline microbial mats [21]; however, further studies are clearly needed in order to understand the potential role of hydrogenotrophic methanogens in the decay of organic matter in hypersaline environments and their syntrophic relationship with other fermentative microorganisms, as well as their appearance under less stressing conditions by sulfate or salinity.

Sulfate Reduction Activities

Sulfate reduction is an important process in the mineralization of organic matter under anoxic conditions, especially in marine and hypersaline ecosystems where high concentrations of sulfate prevail [5, 27]. Measurements of H_2S production in our microcosm experiments showed that acetate and lactate stimulated sulfate reduction more rapidly and with shorter lag phase than the other tested substrates (Fig. 2), but an inhibition of sulfate reduction was observed only in incubations amended with acetate as electron donor. Acetogenic SRB have been reported to be more susceptible to sulfide inhibition than other groups of SRB [29]. Additionally, the extent of conversion evidenced that acetate and lactate were preferentially used by SRB (Table 2). These results are in agreement with previous reports suggesting that natural populations of SRB in hypersaline environments are carbon limited [5, 55]. Furthermore, molecular data of SRB in enrichments with acetate and lactate revealed a contrast difference between each substrate. The analyzed incubation

amended with acetate had a dominant abundance of *Proteobacteria* related exclusively to the family *Desulfobacteraceae*. Most members of this family completely oxidize organic substrates to carbon dioxide. On the other hand, in the experiment supplemented with lactate, sequences related to *Desulfohalobiaceae*, *Desulfomicrobiaceae*, and *Desulfovibrionaceae* were observed, which members have been recognized to oxidize organic substrates incompletely to acetate [50]. Thus, SRB community composition in these enrichment cultures is correlated with the type of substrate added. Previous works have been focused on determining the diversity and distribution of SRB in microbial mat systems [48, 55]. *Desulfobacteraceae* have been reported to be restricted to the deepest levels within the mat community, suggesting a role of last consumers in the trophic levels [33, 48]; meanwhile, *Desulfohalobiaceae*, *Desulfomicrobiaceae*, and *Desulfovibrionaceae* have been found within the 0–2 mm depth range, suggesting that these groups are oxygen-tolerant members [39]. Therefore, in addition to the information related to the distribution of SRB in a deep gradient, with the results obtained, it can be distinguished the presence of these groups potentially associated to differences in substrate usage.

Hydrogen addition also promoted sulfate reduction and sequencing data revealed the presence of SRB belonging to *Desulfobacteraceae*. These results agree with previous reports over hydrogenotrophic sulfate reduction in hypersaline environments. Burow et al. [6] combined biogeochemical and molecular data demonstrating that members of *Desulfobacterales* are important hydrogenotrophs in microbial mats from Elkhorn Slough. Therefore, it has been suggested that SRB are primary consumers of hydrogen under dark, anoxic conditions, due to the abundance of sulfate in these ecosystems [33]. Furthermore, our experiments supplemented with methylated compounds stimulated H_2S production, although in small proportion. Molecular data in the analyzed replicate amended with methanol showed an increase of sequences related to *Desulfohalobiaceae* and *Desulfobacteraceae* versus the control treatment. Kjeldsen et al. [27] elaborated selective enrichment cultures for

SRB, using methanol as electron donor, in hypersaline sediments from Great Salt Lake, and through amplification of 16S rRNA and *dsrAB* genes, reported members related to *Desulfobacterales*, in agreement with the results of the present study. Additionally, *Desulfohalobium uthahense*, belonging to the family *Desulfohalobiaceae*, was isolated from an anoxic hypersaline environment (270 g NaCl L^{-1}) and it has been reported as capable of growing with methanol as an electron donor [18]. Unfortunately, sequences with short fragment lengths, as obtained here, lead to uncertainty in assignments at genus level; however, H_2S production in incubations amended with methanol, as well as molecular evidence collected, supports the use of methanol by SRB in hypersaline microbial mats from Guerrero Negro.

In the case of TMA amended microcosms, as a result of sulfate reduction, H_2S production was also observed. Sulfate reduction with TMA as electron donor proceeds according to the following stoichiometry:

$$4(CH_3)_3NH^+ + 9SO_4{}^{2-} + 9H^+ \rightarrow 12CO_2 + 9HS^- + 4NH_4 + 12H_2O = -308.3\ \Delta G^{\circ'}\ mol^{-1}\ TMA$$

King et al. [26] reported in marine sediments from Lowes Cove that SRB can in fact utilize TMA. Contrasting, Tazaz et al. [53] using microcosm experiments amended with ^{13}C-labeled methanol and methylamines in endoevaporites from Baja California Sur, Mexico, reported that the production of ^{13}C labeled carbon dioxide from sulfate reduction was not observed and therefore concluded that SRB were not utilizing these non-competitive substrates at high salt concentrations (> 120 ppt). Molecular data in our experiments supplemented with TMA did not reveal proliferation of a particular group of SRB; however, compared with all the others treatments, the highest increment in the relative abundance of

sequences belonging to the family *Rhodobacteraceae* was observed. This family is comprised by ubiquitous aquatic bacteria thrive in marine environments with a wide diversity of metabolic functions. Recently, the family *Rhodobacteraceae* (marine *Roseobacter* clade) have been reported to use TMA as sole carbon and/or nitrogen source, using flavin-containing monooxygenase for the breakdown of TMA, redefining our understanding over carbon and nitrogen fluxes in marine environments [8, 36]. Proliferation by *Rhodobacteraceae* in the analyzed sample amended with TMA, suggests that TMA consumption by this group would be performed in hypersaline environments as well. Future studies using more sensitive techniques are needed in order to understand the consumption of the so called “non-competitive” substrates in hypersaline environments by different microbial groups, other than methanogens, and their contribution in hypersaline systems.

Others Microbial Groups

Original sample (unaltered) from P4n1 showed a predominance of *Cyanobacteria* and *Chloroflexales* and unclassified *Bacteroidales* (Fig. 3). Similar results have been found in studies conducted at ESSA’s Pound 4 near 5 (P4n5) [16, 34]; however, to our knowledge, this is the first report on the microbial community composition at site P4n1 through next-generation sequencing. Furthermore, a clear difference in the microbial community composition was observed, depending on the substrate supplemented. As a general pattern, an unexpected relative abundance of *Hydrogenedentes* near 25% was detected in almost all treatments, including the experimental control without substrate supplied (the enrichments with acetate and lactate were the exception). *Hydrogenedentes* is a novel phylum previously called as *NKB19*. This group has been detected in low abundances in microbial mats of Buzzard’s Bay [1], Shark Bay [60], and Guerrero Negro [16], as well as in gypsum crust from Salar de Llamara [47]. Nevertheless, previous works have been done during the day, under oxic conditions. To our knowledge, this is the first time in which Illumina massive sequencing is

performed in Guerrero Negro microbial mats, under strictly anaerobic, dark conditions, with the addition of different substrates, which may explain these results. *Hydrogenedentes* have been associated with methanogenic environments, but their ecological role has remained unknown [43, 49]. Transcriptome analyses of this group revealed extracellular hydrolysis of triacylglycerols and also expressed genes for syntrophically oxidizing glycerol to acetate [43]. Further investigations are required for a better understanding of the ecological function of *Hydrogenedentes* and their contribution in hypersaline ecosystems.

The sequenced sample supplemented with acetate had an exclusively dominant abundance of *Desulfobacteraceae* (Fig. 3). In agreement with these results, Lee et al. [33] confirmed that *Chloroflexi* and *Desulfobacteraceae* are significant consumers of acetate in hypersaline microbial mats from Guerrero Negro. Sequences related to *Chloroflexi* were not detected in this treatment, possibly due to the selective process originated by the long incubation time. Despite this, our results confirmed the strong competition for acetate between SRB and methanogens in hypersaline environments. The most representative groups in the analyzed microbial mat incubated with hydrogen were *Caldisericales LF045* and *08D2Z94 hypersaline microbial mat group*. Members of *Caldisericales* have been described as obligate anaerobic chemoheterotrophs that reduce sulfurous compounds during respiration [40]; therefore, they would potentially be syntrophic partners of hydrogenotrophic SRB. The *08D2Z94 hypersaline microbial mat group* has been detected in similar microbial mats [16, 34], but their ecological role in hypersaline mats remains enigmatic. In the experiment amended with lactate, it was promoted an increase of members of the family *Phycisphaeraceae*, which have been described as a heterotrophic fermentative group in marine ecosystems [12]. In contrast, in the experiment performed with lactate in the absence of sulfate, the most predominant bacterial group was *Synergistaceae*. This family has been distinguished as degrader of amino acids that has been found in a wide range of anaerobic habitats [17, 19]. Because in this treatment, also hydrogenotrophic methanogenesis was

observed, this bacterial group would be syntrophic partner with methanogens, involved in the degradation of the organic matter in hypersaline environments. *Spirochaetaceae* family was the main group increased in the experiment amended with methanol, compared with all treatments. However, this group has not been reported to grow with methanol. Addition of TMA favored an increment of the family *Flavobacteriaceae*. This is the first study that links TMA enrichment with the proliferation of this group and no current information exist on the growing of *Flavobacteriaceae* utilizing TMA.

Archaeal sequences found under the experimental conditions established were related to unclassified *Bathyarchaeota* and to the *Marine Benthic Group D*. These taxonomic groups have been detected in marine methanogenic environments and it has been proposed that these microorganisms are possibly involved in anaerobic methane oxidation (AMO) [11, 54, 56]. Additionally, molecular data in both experimental conditions, control and amended with methylated compounds, evidenced low abundances of the unclassified bacterial group *Sh765B-TzT-29*. Environmental group *Sh765B-TzT-29* has been recently reported as a clade of microorganisms possible linked to AMO coupled to iron reduction [51]. However, based on our analytical methods, AMO was not possible to quantify. Recently, Beaudoin [2] through biogeochemical evidence in microcosm experiments supplemented with ^{13}C-labeled CH_4 in hypersaline microbial mats and endoevaporites from both North and South America, conclude that very little, if any, methane is biologically oxidized in hypersaline ecosystems by sulfate reduction. Additional studies are clearly needed in order to analyze if AMO coupled to the reduction of novel electrons acceptors other than sulfate, such as nitrate and nitrate or metals (iron and manganese), would be performed in hypersaline ecosystems under natural or controlled laboratory conditions.

As the results showed, molecular data from experimental incubations were potentially related to substrate availability and several sulfate reducers and methanogens were detected consistently with the kinetic

data as reliable patterns. However, because only the most active replicate within treatments was sequenced, we cannot evaluate if other microbial assemblies can perform the same activities (functional redundancy). This limitation should be taken into account when interpreting our molecular results. Some studies over substrate use by methanogens have been done in hypersaline environments, but sulfate reduction activities have been overlooked. This study provides information about both methanogenic and sulfate reduction activities, using competitive and non-competitive substrates in a hypersaline microbial mat and the microbial diversity associated when the substrates are added to the system, under strictly anaerobic, dark conditions, after a long incubation time of 58 days. Complementary studies are needed using labeled substrates or/and functional genes in order to understand more precisely the ecological functions of the microbial diversity reported.

Conclusions

This study reports on the microbial community composition of hypersaline microbial mat samples from ESSA's P4n1 and their change under laboratory conditions of substrate addition for methanogenesis and sulfate reduction activities. It was found that methylotrophic methanogenesis is the dominant methanogenic pathway; however, in incubations performed without sulfate, methane production was also detected in the presence of acetate and lactate, apparently carried out by hydrogenotrophic methanogens. Additionally, acetate, lactate and hydrogen were the preferential substrates for sulfate reduction, which confirms the strong competition for these key fermentation products between methanogens and sulfate reducers. Nevertheless, we observed an increase in the H_2S concentration in TMA and methanol-amended treatments, suggesting that these substrates are not exclusively used by methanogens. Furthermore, molecular data showed that microbial community composition dramatically changed depending on the substrate supplied, suggesting highly sensitivity of microbial assemblages of mats related to substrate availability. Future investigations should address the

ecological function of the microbial diversity reported as well as environmental factors driving competition between methanogens and sulfate reducers.

Acknowledgements

We are grateful to Exportadora de Sal, S.A. de C.V. for access to their facilities in Guerrero Negro to collect microbial mats and particularly to Adrián Villavisencio Cerda and Ignacio Leree Murillo for assistance during field work. We would like to thank A. Aguilar-Aguilar, D. Partida-Gutiérrez, G. Vidriales-Escobar and J.P. Rodas-Ortiz, for technical support.

Funding Information

This research was financially supported by grant 1289 (program Frontiers in Science) from CONACYT and by the Marcos Moshinsky Foundation. Santiago Cadena thanks CONACYT fellowship 570049-2014-2016.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Human and animal rights and informed consent For this type of study formal consent is not required. This article does not contain any studies with human participants or animals performed by any of the authors

Electronic supplementary material

ESM 1	ESM 2	ESM 3	ESM 4
(PPTX 243 kb)	(XLSX 14 kb)	(PPTX 38 kb)	(CSV 157956 kb)

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