Proteomic stress response by a novel methanogen enriched from the Great Salt Lake

William C Christian[1,2,3], Zackary J Jay[1,2,3], Nikola Tolic[4], Carrie D Nicora[5], Reece Livingstone[1], Stavros Trimmer[1,2,3], Timothy R McDermott[6], Roland Hatzenpichler[1,2,3,7,*]

Affiliations
[1]Department of Chemistry and Biochemistry, Montana State University, Bozeman, MT, USA
[2]Thermal Biology Institute, Montana State University, Bozeman, MT, USA
[3]Center for Biofilm Engineering, Montana State University, Bozeman, MT, USA
[4]Environmental Molecular Sciences Directorate, Pacific Northwest National Laboratory, Richland, WA, USA.
[5]Biological Sciences Directorate, Pacific Northwest National Laboratory, Richland, WA, USA.
[6]Department of Land Resources and Environmental Sciences, Montana State University, Bozeman, MT, USA
[7]Department of Microbiology and Cell Biology, Montana State University, Bozeman, MT, USA.
[*], corresponding author: R.H., rolandhatzenpichler@gmail.com

ORCID numbers
WC, 0000-0003-1682-8083. ZJ, 0000-0003-3062-4933. CDN, 0000-0003-2461-9548. NT, 0000-0003-3950-9130. ST, 0009-0003-3873-4456. TM, 0000-0001-8293-5106. RH, 0000-0002-5489-3444.

Running title: Proteomic response by a methanogen to stress

Abstract

Methanogenic archaea affect the climate through their production of the greenhouse gas, methane. However, it is unclear how a changing climate and other anthropogenic influences impact methanogen physiology and consequent methane flux. The Great Salt Lake (GSL) is an environment that has been heavily impacted by human activity; more than doubling its salt concentration since the last methanogen was cultured from it in 1985. In this study, we enriched a novel methanogen, for which we propose the name *Candidatus* Methanohalophilus hillemani, from the GSL at a time when its salinity reached a historical high. Interestingly, *Ca.* M. hillemani does not increase expression of energy-conservation or osmo-tolerance proteins when challenged with salinity or oxygen. In contrast, *Ca.* M. hillemani prioritizes trace metal uptake and immune functions in response to the presence of the sulfate-reducing bacterium *Desulfovermiculus*. 16S rRNA gene amplicon data from GSL shore soils with extremely high and variable methane flux indicated the presence of *Ca.* M. hillemani. Our results show that *Ca.* M. hillemani is active when challenged with environmental stressors and contributes to the methane flux emanating from the GSL.

Importance

Methanogens are microbes that affect the climate through their production of the greenhouse gas, methane. Changes in climate and land-use patterns are drying up saline lakes, damaging their unique economic and ecological value. As lake levels across the globe fall, it is unclear how methanogens and the amount of methane they produce will concurrently shift. In this study, we measured high methane output from the Great Salt Lake (GSL) across seasons and identified a novel methanogen as part of a larger methanogenic community that is responsible for these methane emissions. We cultured this novel methanogen from GSL sediments and determined that its methane production was largely unaffected by stress conditions. Our findings indicate that methanogens in saline environments, including a novel cultivated species, may be important sources of methane and will continue to produce methane as salinity increases.

Introduction

Methane is a potent greenhouse gas that has contributed 0.3-0.8 °C to global warming since the start of the industrial revolution [1, 2]. Approximately 60% of methane emitted to the atmosphere is produced by methanogens, archaea that couple the production of methane to energy conservation [2, 3]. Due to the oxygen sensitivity of key enzymes in their metabolism, as well as the comparatively low free-energy yield of methanogenesis, it was long assumed that methanogens are restricted to anoxic environments with large stores of organic carbon. Specifically, wetlands, melting permafrosts, rice paddies, and animal digestive tracts are known for their high methane fluxes [2] and have been extensively studied, together with methanogens therein, due to their influence on climate change. However, many additional ecosystems contribute to the methane cycle. Environments experiencing fast changes due to anthropogenic influence are of particular interest because the magnitude and direction (i.e. emission vs. uptake) of methane flux can change on annual to decadal scales [4-7].

The Great Salt Lake (GSL) and other major terminal lakes around the globe are being altered by human activity [8-10]. Increased upstream water consumption and evaporation due to higher temperatures are causing lake levels to plummet [11-13]. In terminal lakes, a decrease in water volume is accompanied by an increase in salinity. It is well known that halophilic methanogens produce methane under hypersaline conditions [14], however, how they respond to increasing salinity, and how this impacts methane flux, is not well known. Recent studies suggest that increasing salinity in freshwater systems decreases methane emissions [15], but also that droughts will increase methane emissions from once-inundated sediments [16]. Studies specifically examining salinity gradients have shown mixed results, with some reporting no correlation between salinity and methane emissions [17] and others finding a negative relationship [18, 19]. These results have contradictory implications for methane emissions from drying terminal lakes, including the GSL. Unfortunately, data examining emissions from these unique environments is rare and to the best of our knowledge, only one study has quantified methane flux from a terminal lake [20], which measured low and spatially homogenous methane emissions from the GSL. Methane production has been observed from GSL sediments [21], and a halophilic methanogen,

Methanohalophilus mahii, was isolated from the GSL in 1985 [22-24]. Despite these observations,
many microbial community analyses of the GSL did not find or mention methanogens [25-27].
M. mahii is a methyl-dismutating methanogen that thrives at 7-12% NaCl [23]. The
Methanohalophilus genus includes six described species and several strains [28, 29]. Besides the
GSL, these species were isolated from deep sea salt brines, deep shale reservoirs, hypersaline bays,
and salt pans [29-33]. Known members of the *Methanohalophilus* genus all share methyl-
dismutating methanogenesis from MMA, DMA, TMA (with one exception [34]) and methanol,
but are incapable of growing via acetoclastic or CO_2-reducing hydrogenotrophic methanogenesis.
The salinity range varies by organism, but they have been shown to grow with NaCl concentrations
from 1% to 20% [28]. They tolerate osmotic stress through both 'salt-in' strategies with many ion
symporters and antiporters, and via the biosynthesis of the compatible solute, glycine betaine [34].
All members of this genus also universally encode genes for the detoxification of O_2, including
thioredoxins, peroxiredoxins, alkylhydroperoxidases, and superoxide reductase [28].
In this study, we cultured a novel species of the genus *Methanohalophilus* from the GSL
for which we propose the name *Candidatus* Methanohalophilus hillemani. Using metagenomics
and metaproteomics, we describe its unique features and explore how this organism responds to
environmental stressors, including salinity. Finally, we quantify methane flux from the GSL and
identify the microbes responsible. We combine environmental, microbiological, and meta-omics
techniques to examine how this methanogen responds to changes experienced in the GSL.
99
Materials and methods
Collection of samples and cultivation
Microbialite material was collected in July of 2022 from Bridger Bay in the South arm of
the GSL (Supplementary Figures 1, 2). Methanogens were enriched by adding 10 mM MMA to
0.2 µm filter-sterilized GSL water. Lake water was eventually replaced by media designed by
Paterek and Smith [22], originally used to isolate *Methanohalophilus mahii*, but adjusted to the
salinity of the current GSL (16%).
Methanohalophilus enrichment cultures were grown in Paterek and Smith 1985 media [22],
with differing concentrations of salts and TMA, and without the addition of volatile fatty acid
solution and sodium sulfide. The headspace consisted of a 50/45/5 ratio of $H_2/N_2/CO_2$ at ambient
pressure (~90 kPa in Bozeman, MT). The anoxic media included the following salts to reflect the
salinity of GSL water from April 13th 2023: 100 g/L NaCl, 45 g/L $MgCl_2$, 5.5 g/L KCl, 1.5 g/L
$CaCl_2$, 0.13 g/L LiCl, 19 g/L Na_2SO_4, 0.5 g/L NH_4Cl. Additionally, the media contained (per L):
500 µL of 1 g/L sodium resazurin solution, 10 mL of DSMZ 141 trace element solution, 23.81 mL
of 1 M sodium bicarbonate, 18.87 mL 1 M sodium carbonate, 5 mL of 40 g/L 2-mercaptoethane
sulfonic acid sodium salt (Coenzyme M), and a small spatula tip (1-2 mg) of sodium dithionite as
reductant. The pH was adjusted to ~7.5 and cultures were grown in 9.5 mL of medium in 70 mL
serum bottles. Vancomycin and streptomycin (10 µL of 50 mg/mL), 200 µL of 1 M TMA, and 300
µL of carbon source mix [22] were added to each bottle. Bottles were inoculated with 100 µL from
the previous culture and grown at 30 ºC for approximately one month prior to transfer.

Methane measurements

Methane was measured via GC-FID as previously described [35] with the following modifications: (i) 1 mL of sample was injected into autosampler vials instead of 250 µL, (ii) a 3 mL plastic BD syringe equipped with a sample lock was used in place of a Hamilton syringe, and (iii) an Rtx-1 GC Capillary column was used instead of a GS-CarbonPLOT column.

Gene amplicon analysis

16S rRNA gene analysis was performed as described in Krukenberg et al. [36] using Earth Microbiome Project primers 515F and 806R [37, 38]. The mlas-mod-F and *mcrA*-rev-R primers and the touchdown amplification program from Angel et al. 2012 were used to amplify the alpha-subunit of methyl-coenzyme M reductase (*mcrA*) [39]. Further details can be found in the supplementary methods section.

Metagenomics

A combination of Illumina short-read and Nanopore long read sequencing, following an established workflow [35] with some modifications, was used to obtain the circular genome of *Ca. M. hillemani* str. BB. Fragmented but complete (>99.5 %) genomes were also generated for *Halanaerobium* and *Desulfovermiculus* spp.. The genomes can be found with the following accession numbers for *Ca.* M. hillemani closed genome, *Desulfovermiculus* sp.*,* and *Halanaerobium* sp.*,* respectively: SAMN54119603, SAMN54119604, SAMN54119605. Details on sequencing, assembly, annotation, and genome comparisons are described in the supplementary methods section.

Salt stress experiment

For methane production and metaproteomics experiments, *Methanohalophilus* enrichment cultures were grown (i) as the control (50/45/5 of $H_2/N_2/CO_2$ headspace, 176 g/L total salts), (ii) with a 100% N_2 headspace, (iii) in low salt media (114 g/L salts), (iv) high salt media (227 g/L salts), and (v) micro-oxic conditions (1 mL of filter-sterilized room air injected into the headspace every four days, achieving ca. 0.35% O_2 upon first injection). Cultures grown under control conditions were transferred (1:100) into bottles with these conditions. To acclimate the test cultures to stress conditions, they were grown under these test conditions for three transfers before methane data was recorded during the fourth generation. That culture was then used to inoculate a fifth generation, which was harvested in mid-log phase (based on methane production) for the metaproteomics experiment and the 16S rRNA gene community analysis (n=3 replicates for each condition).

Metaproteomics sample preparation and data analysis

The harvested cultures were sent to the Pacific Northwest National Laboratory (Richland, WA, USA) where 200 µg of protein was used for digestion. MS analysis of the peptides was

performed using an Orbitrap Exploris 480 mass spectrometer (Thermo Scientific, Waltham, MA,
USA). Peptide matching from the acquired datasets was performed using MSGF+ software [40]
against target meta-proteome composed from sequenced genomes (see Supplementary Methods).
Peptide abundances, integrated as area under the peptide elution peaks, were produced from raw
spectra using in-house developed software MASIC [41]. MASIC peptide abundances were log2
transformed to remove skewness in the distribution of measured abundances. Peptide abundances
were corrected for the relative abundance of organisms using the summed area under the peptide
curves mapping to each organism. Transformed abundance values of peptides, and later proteins,
were normalized using mean central tendency method implemented in InfernoR software [42].
Unless otherwise indicated, all proteomic data presented in this study represents normalized
protein abundances. Raw proteomics data has been deposited at massIVE under accession number:
MSV000100495. Further details can be found in the supplementary methods section.
172
Methane flux measurements
Methane flux data was collected in transects, perpendicular to the shoreline, at Bridger Bay
in the south arm of the GSL using A Li-COR 7810 trace gas analyzer (Li-COR, Lincoln, NE,
USA). Terrestrial flux measurements were made as previously described [43], but with a one-
minute measurement period and a 10 second deadband time. To generate depth-resolved 16S
rRNA gene amplicon data, soil cores were collected from within the boundary of the terrestrial
collar which registered the highest methane flux. Aquatic flux measurements were done using a
custom-made floating flux chamber to take duplicate, one minute, flux measurements. Aquatic
fluxes were calculated from the linear change in methane concentration over time, as previously
described [44].
183
Results and discussion
Enrichment of a novel methanogen
Microbialite and water samples from Bridger Bay in the South arm of GSL were collected
in July 2022, when the water level was at its lowest in recorded history (Supplementary Figure 3).
Methanogens were enriched using the growth medium used to isolate *Methanohalophilus mahii*
[22]. We increased the salinity of the medium to match GSL water from April 13th 2023, provided
a headspace consisting of 50% H_2, 45% N_2, and 5% CO_2, and used trimethylamine (TMA) as the
methanogenic substrate. The culture also produced methane from MMA, DMA and methanol, but
not from glycine betaine or hydrogen and carbon dioxide (Supplementary Figure 4).
After 21 months of enrichment, 16S rRNA gene amplicon sequencing indicated that the
culture consisted of 63% *Methanohalophilus*, 20% *Desulfovermiculus* (a sulfate reducing
bacterium - SRB), and 16% *Halanaerobium* (an anaerobic fermenter). An additional 25 taxa
contributed to the remaining <1% of the 16S rRNA gene reads. Metagenomic sequencing of this
sample with both Illumina and Oxford Nanopore enabled assembly of a circular
Methanohalophilus genome (2.09 Mb). Sequence coverage indicated that the culture consisted of
80% *Methanohalophilus*, 7% *Desulfovermiculus* sp., 4% *Halanaerobium* sp., with 9% of

sequences remaining unbinned. In addition to these three assembled genomes, the metagenome
contained 16S rRNA gene sequences belonging to *Vibrio cholerae*, a *Desulfohalobium* sp., and an
Izimaplasma sp.. Attempts to isolate the methanogen using dilution to extinction, colony-picking
from solid media, and additional antibiotics were not successful.
204
Characterization of the novel methanogen as *Ca.* Methanohalophilus hillemani
This newly assembled *Methanohalophilus* genome shares less than 94% ANI with all other
genomes within its genus, indicating a novel species [45] and only 91.2% with *M. mahii* (Figure
1). The 16S rRNA gene is less than 99.3% identical to other species, noticeably lower than other
Methanohalophilus species to each other (Supplementary Figure 5). We propose the name
Candidatus Methanohalophilus hillemani strain BB for this archaeon (see etymology section in
the supplementary online materials).
212

Figure 1: Phylogeny of *Ca.* Methanohalophilus hillemani. (A) Pairwise average nucleotide
identity (ANI) and average amino acid identity (AAI) on bottom left and top right, respectively
demonstrating *Ca.* M. hillemani represents a new species within the *Methanohalophilus* genus.
(B) Maximum likelihood tree of 65 aligned and concatenated single-copy *Methanohalophilus*
proteins constructed with FastTree v2.1.11 (Price et. al 2010) and 1,000 bootstraps. Circles
represent bootstrap values. Nodes with <50 support were collapsed.

Comparing protein-coding sequences encoded by *Ca.* M. hillemani to all other
Methanohalophilus spp. showed it shares the major characteristics of the genus, including methyl-
dismutating methanogenesis from MMA, DMA, TMA, and methanol [23, 28, 34]. Other
conserved functions across the genus include glycine betaine transport and synthesis, synthesis
and usage of the 22nd amino acid, pyrrolysine (Pyl), and O_2 tolerance via thioredoxins, peroxidases,

superoxide reductases, and peroxiredoxin. Unique genetic features of *Ca.* M. hillemani as
compared to other members of the genus *Methanohalophilus* are discussed in the Supplementary
Results section.
229
Increasing salinity in the Great Salt Lake
The biomass that led to culturing *Ca.* M. hillemani was collected from the GSL in July
2022, when the lake was at its lowest recorded level (Supplementary Figure 3). Additionally the
sample came from Bridger Bay, which was cut off from the main lake by low water levels, leading
to a salinity much higher (~24%) than the rest of the south arm of the GSL (~17%). Na^+ and Cl^-
concentrations at this time were 3.6 and 4.2 M, respectively, more than twice the concentrations
of these ions measured by Post et al. in 1977 (1.6 and 1.8 M) [46]. In contrast, *Methanohalophilus*
mahii was isolated in 1985 [22], right before GSL reached recorded high water levels
(Supplementary Figure 5). This led us to the hypothesis that *Ca.* M. hillemani may be adapted to
higher osmotic stress levels as compared to *M. mahii*.
240
Methane production under salt stress
To understand how stressors in the GSL ecosystem might impact *Ca.* M. hillemani, control
cultures were compared to those incubated under low salt, high salt, 100% N_2, and micro-oxic
conditions (see methods, above). Proportions of major salt ions in all conditions matched the GSL,
which contrasts previous work on the genus that had only modulated NaCl concentration [22, 29,
47]. Despite differing stressors and relative abundances, methane yield for *Ca.* M. hillemani
cultures across all conditions was consistent (Figure 2a). Mass balance of the methyl groups
provided via TMA shows that after 16 days 68 - 77% was converted to methane, consistent with
the 75% value for methyl-dismutating methanogenesis that has been reported for some
euryarchaeotal methanogens [48, 49].
16S rRNA gene amplicon data showed that *Ca.* M. hillemani was the most abundant
member in all conditions (Figure 2b); however, the community composition of these cultures
differed significantly from the 3-species enrichment indicated by the metagenome. The control,
low salt, and high salt conditions contained primarily (>68%) *Ca.* M. hillemani, while
D*esulfovermiculus* was the only other organism with a relative abundance over 0.5%. Over 96%
of amplicons in the O_2 condition belonged to *Ca.* M. hillemani. By contrast, in the N_2 condition
40% of amplicons belonged to *Ca.* M. hillemani and less than 0.1% to *Desulfovermiculus*. An
Izimaplasma species that was absent from all other samples represented 24% of the community,
and the *Halanaerobium* species that made up <0.1% of amplicons in all other conditions,
constituted 30% in the N_2 condition. Despite differences in culturing conditions and community
composition, especially in the N_2 cultures, all conditions yielded the same amount of methane.
262

Figure 2: *Ca.* M. hillemani produces similar amounts of methane under a variety of conditions. (A) Methane production of the Methanohalophilus enrichment culture grown under various stress conditions. Error bars (where visible) represent the standard deviation of triplicate cultures. (B) Community composition of same samples that were processed for metaproteomics, as assessed by 16S rRNA gene amplicon sequencing. Amplicon sequence variants that constituted less than 0.5% of the total community were clustered into the "Other" category.

Metaproteomics

To understand the biological basis for *Ca.* M. hillemani's consistent methane production across stressful conditions, we performed metaproteomics. Triplicate cultures were harvested in mid-log phase and peptides from cultures of each condition were mapped to predicted proteins from the assembled genomes. Because no bins could be retrieved from *Izimaplasma* and *Desulfohalobium* from the N_2 condition, we were not able to map peptides to these organisms.

Stress conditions do not impact methanogenesis protein expression

For all conditions, the proteins involved in the methyl-dismutating methanogenesis pathway were among the top 10% of *Methanohalophilus* proteins expressed (Supplementary Figure 6). The proteins responsible for the transfer of a methyl group to coenzyme M and its subsequent reduction to methane were all among the top 1%, with one exception (methylcobamide:CoM methyltransferase – in the top 1.1% of the N_2 condition). The only methanogenesis-related protein that was differentially expressed was an MMA-methyltransferase protein found in the control condition but not in the O_2 condition (Figure 3). However, *Ca.* M. hillemani encodes two copies of this protein, and the other copy was in the top 1% of proteins expressed in the O_2 condition, suggesting that the methanogenic pathway was not impacted.

It was previously reported that in response to long-term high and low-salt stress, *Methanohalophilus portucalensis* transcripts for methylamine methyltransferases and corrinoid proteins were upregulated [47]. Conversely, we show that the *Ca.* M. hillemani methanogenic proteome is highly and uniformly expressed, regardless of high or low salt concentrations. One possible explanation for the lack of observed methanogenic protein expression response, is the high basal expression of energy-conservation proteins (Supplementary Figure 6). This may be advantageous in an environment with constant stressors that require large amounts of energy to tolerate (*i.e.* osmotic and oxidative stress). This strategy could be especially feasible in the GSL, where methylated amines are likely always in high abundance due to the production of a metabolic precursor, glycine betaine [50], as a compatible solute.

Another possibility is that methanogenic protein activity, as opposed to abundance, is post-translationally regulated in response to stress. An investigation of protein phosphorylation in *Methanohalophilus portucalensis* found that 26% of the phosphorylated proteins were involved in methanogenesis, energy conservation, and osmolyte biosynthesis [51]. That study found that a mutated glycine sarcosine N-methyltransferase (responsible for glycine betaine synthesis), modified to act like a phosphorylated version, had reduced activity that resulted in lower salinity tolerance when expressed in *E. coli*. While they did not have analogous data for phosphorylated methanogenic proteins, they demonstrate that *Methanohalophilus* proteins can be regulated beyond the level of protein expression. The transcriptomic and proteomic findings discussed here, together with the lack of differential expression of methanogenic proteins, suggests that future work on the *Methanohalophilus* genus should examine forms of regulation downstream of protein expression.

Limited number of proteins differentially expressed in response to changing salinity

Contrary to our expectations, low and high salt conditions resulted in relatively few proteins that were both highly and differentially expressed, compared to the control (Figure 3). Both high and low salt conditions showed two upregulated proteins from an iron transport system, FeoA and FeoB, of which FeoA was not detected in the control. Of the many DNA repair proteins encoded by *Ca.* M. hillemani, none were both upregulated and expressed above median protein expression in the high salt condition. The lack of DNA-repair protein expression in response to high salinity was unexpected [52], though basal expression of several of these proteins was observed in the control. Alternatively, the need for DNA repair may be mitigated by osmo-tolerance via high expression of compatible solute. pathways. Indeed, glycine betaine synthesis and import genes are in the top 10% of expressed proteins in the low-salt, high-salt, and control conditions. As discussed above, post-translational regulation could also play a role in osmo-tolerance without requiring differential expression of proteins [51].

Protein expression was more affected by the O_2 growth condition. Two proteins associated with protein modification and two DNA-repair proteins were up-regulated and expressed above the median (Figure 3). An additional three DNA-repair proteins were up regulated but not expressed above the median. Surprisingly, none of the oxygen detoxification genes were

upcoming. Constitutive expression could also explain this lack of response to oxygen—superoxide reductase, peroxiredoxin, and catalase-peroxidase are all in the top 10% of expressed proteins in the control.

Figure 3: *Ca.* Methanohalophilus hillemani differential protein expression between control and test conditions. Maximum differentially expressed proteins occurred between the control and N_2 conditions, all of which were more highly expressed in the control. Down-regulated proteins in the N_2 condition included proteins involved in immune response, metal metabolism, and the cell-surface. Proteins shown in this figure meet three criteria: (i) above median normalized protein expression for that condition; (ii) statistically significantly differentially expressed ($p < 0.05$); and (iii) function could be predicted (*i.e.* hypotheticals not included). A negative log fold change indicates the protein was more abundant in the control, positive indicates more abundant in the test condition. Asterisks in the boxes indicate that the protein was not detected in either that test condition or the control and a log2 fold change of 10 or -10 was imputed. Bolded proteins indicate that the coding sequence for this protein was unique to *Ca.* M. hillemani and no homologs were identified in other *Methanohalophilus* genomes. Proteins with grey boxes do not indicate absence

in a specific condition, but that the protein did not meet all three criteria listed above. See
Supplementary Tables 1-5 for raw values.

Unique *Ca.* M. hillemani proteins are differentially expressed in the N_2 condition

When comparing the number of differentially expressed proteins between the control and
test conditions, the N_2 condition resulted in twice as many differentially expressed proteins
(Supplementary Table 6). This was consistent when filtering for only the proteins that were
expressed above the median for each condition or control (Supplementary Table 7). Notably, all
26 differentially and highly expressed *Ca.* M. hillemani proteins in the N_2 condition were less
abundant than in the control (Figure 3). Additionally, eight of these 26 differentially and highly
expressed proteins are unique proteins to *Ca.* M. hillemani, among the *Methanohalophilus* genus
(see Supplementary Results). However, none of these unique and highly expressed proteins were
detected in the N_2 condition; in contrast, all eight were found in every other condition, with seven
expressed above the median. We hypothesize that these distinct protein expression patterns in the
N_2 condition are due to the differences in community composition (Figure 2B).

Metal uptake, DNA repair, and immunity down-regulated in the N_2 condition

Down-regulated functions in the N_2 condition included immune response and DNA repair,
cell-surface features, and metal metabolism (Figure 3). A cobaltochelatase and a
cobalt/magnesium transporter were not detected and down-regulated 19-fold, respectively, in the
N_2 condition (Figure 3). Additionally, one iron transporter was down regulated 29-fold and two
other iron transporters, detected in all other conditions, were absent from the N_2 condition.

Highly expressed immune-response and DNA-repair proteins were noticeably absent from
the N_2 condition. Seven proteins in this category (Figure 3) were undetectable in the N_2 condition
yet were expressed above the median in all other conditions. Eleven additional proteins involved
in immune response or DNA repair were also detected in every condition except for N_2. These
included two CRISPR-related proteins, two proteins involved in the sulfur-modification of DNA
[53], four DNA helicases, two DNA repair proteins, and an additional copy of the type 1
restriction-modification system protein, HsdS.

Ca.* M. hillemani is adapted to sulfide stress and competition with *Desulfovermiculus

The genomic and proteomic data do not support the hypothesis that osmotic tolerance
distinguishes *Ca.* M. hillemani and *M. mahii*. Both species are genetically capable of synthesizing
and taking up the compatible solute, glycine betaine, and both encode ion transporters to maintain
osmotic balance. While *Ca.* M. hillemani encodes unique proteins involved in DNA repair and
immune response, it encodes no unique proteins predicted to specifically promote osmotolerance
(Supplementary Table 8). In fact, differing salt concentrations resulted in a relatively small
proteomic change in this halophilic methanogen.

Instead, the main proteomic differences were observed from the N_2 condition. Immune-
response and DNA-repair proteins, including proteins unique to *Ca.* M. hillemani, were highly

expressed in all conditions except the N_2 condition. All other conditions contained 50% H_2 in the
headspace and 15-500x higher relative abundance of the SRB, *Desulfovermiculus* (Figure 2b).
Sulfide produced by SRB damages DNA [54] and can be detrimental to methanogens [55, 56].
Accordingly, sulfide has been shown to change transcription patterns, including genome-wide
shifts in *E. coli* [57] and upregulation of genes involved in DNA-repair in methanogens [58]. Our
proteomic results indicate that either *Desulfovermiculus* specifically, or high levels of sulfide,
induce a distinct proteomic response from *Ca.* M. hillemani. Unique immune-response and DNA-
repair proteins expressed by *Ca.* M. hillemani in the presence of *Desulfovermiculus* potentially
represent novel adaptions to protect or repair DNA from H_2S-related damage.
Additionally, reduced expression of metal uptake and chelation by *Ca.* M. hillemani in the
N_2 condition, compared to all other conditions, suggests competition for metals with
Desulfovermiculus. Cobalt is crucial for methyl-dismutating methanogenesis as it is the cofactor
in corrinoid proteins used to transfer methyl groups to MCR (Supplementary Figure 6) [59].
However, the conversion of sulfate to sulfide by SRB can directly diminish the availability of
cobalt through Co^{2+} - sulfide co-precipitation [60]. Additionally, SRB utilize cobalt as an
enzymatic cofactor for various metabolisms, including dissimilatory sulfate reduction [61, 62],
which could lead to competition. Indeed, *Desulfovermiculus* expresses two cobaltochelatases in
the control condition, one just below median normalized expression and the other well above.
Tolerating sulfide stress and competing against *Desulfovermiculus* for cobalt may be important
factors impacting *Ca.* M. hillemani physiology when H_2 is available.
405
Ca. M. hillemani uses pyrrolysine sparingly
Methylated amine-utilizing methanogens are known to encode the 22nd amino acid,
pyrrolysine (Pyl), which plays an important role in methylamine activation in tri, di, and
monomethylamine methyltransferases (MttB, MtbB, MtmB, respectively) [63-65]. Indeed, *Ca.* M.
hillemani encodes the genes required for Pyl synthesis (PylBC) and uses (Pyl-tRNA ligase). An
mRNA secondary structure element, PYLIS (analogous to SECIS for selenocysteine), has been
suggested to enable Pyl insertion into proteins [66, 67] and PYLIS elements were annotated within
Ca. M. hillemani's methylated-amine methyltransferase genes. Accordingly, Pyl residues were
identified in peptidesmapping to all three types of methylated-amine methyltransferases encoded
by *Ca.* M. hillemani.
Kivenson *et al.* have recently proposed that some methanogens, including
Methanohalophilus mahii, *M. euhalobius*, and *M. levihalophilus,* have reprogrammed the TAG
codon to universally incorporate Pyl, instead of being read as a Stop-codon, across the proteome
[68]. While the authors confirmed their hypothesis for a marine and a human-gut-associated
methanogen, experimental data on halophilic methanogens was not yet available. Moreover, how
and if microbes regulate the incorporation of Pyl at TAG codons is a topic of current debate [68,
69]. Having already identified Pyl residues in peptides mapping to methyltransferase proteins, we
used Kivenson's modification to Prodigal [68], as well as our own modifications to Prokka, to re-
predict and annotate genes for *Ca.* M. hillemani. This resulted in 95 predicted proteins containing

an in-frame TAG codon. In this reanalysis, we found peptide hits from predicted-Pyl-containing
proteins downstream of the predicted-Pyl residue. This could suggest that the TAG codon is
resulting in a Pyl residue, not being read as a Stop-codon. However, for non-methyltransferase
proteins predicted to contain pyrrolysine, only 7 of the 3,463 (0.2%) detected peptides associated
with those proteins contained Pyl. In contrast, 2,920 out of the 27,114 (10.7%) peptides associate
with methyltransferase proteins contained Pyl. The low percentage of non-methyltransferase
peptides identified as containing Pyl could indicate that TAG codons, in contrast to genomic
predictions for the genus [68], are primarily read as Stop in *Ca.* M. hillemani.
433
High methane flux from the Great Salt Lake
To assess methanogen activity *in situ*, we measured methane flux from the GSL. We
focused on Bridger Bay, where the *Ca.* M. hillemani inoculum was obtained (Supplementary
Figure 2). Methane fluxes were very high but also variable, with fluxes reaching 3 mmol
CH_4/m^2/day observed across sampling dates (Figure 4a, Supplementary Figure 7). These fluxes
are comparable to high-methane flux environments such as thawing permafrost [43, 70] and
freshwater wetlands [71]. The highest flux measured was 377 mmol CH_4/m^2/day (Supplementary
Figure 8), which greatly exceeds values reported from either permafrost or wetlands.
Fluxes were measured in transects perpendicular to the water's edge, including both
terrestrial and aquatic measurements. We repeatedly observed a zone of elevated terrestrial
methane flux between 5 and 15 meters from the shoreline (Figure 4a). This was counterintuitive,
as we expected that the most anoxic and methanogen-conducive conditions to be right at the
shoreline. However, this trend was observed across sampling dates and transects (Supplementary
Figure 7).
Our results contrast the only other study that measured methane flux from the GSL [20],
which observed similar aquatic fluxes (0.42 +/- 0.08 mmol CH_4/m^2/day), but much lower terrestrial
fluxes (0.47 +/- 0.1 mmol CH_4/m^2/day). Their findings of similar terrestrial and aquatic fluxes and
minimal flux variability between sites did not corroborate our results. We hypothesize that this
disparity is due to the difference in spatial scales for our two studies. Cobo *et al.* [20] examined
differences between basins, over kilometer scales. Their transects included steep vertical gradients
over a kilometer from the contemporary GSL shoreline. Our measurements were on the meter
scale and centered on the contemporary lake shore. It is only with our higher spatial resolution
(and smaller scope), that we observed extremely high methane fluxes and flux variability.
Together, Cobo's and our results show that the GSL does have extremely high methane flux across
seasons; however, the zone of highest flux is located just meters from the current shoreline.

Figure 4: Methane flux and methanogenic community from Bridger Bay on the Great Salt Lake. (A) Methane fluxes measured from soil within Transect A at Bridger Bay in May and July of 2025 showing a zone of high methane flux 10-15 m from the shoreline. (B) Relative abundance of different methanogens in a sediment core taken from the Transect A 10m flux site from July 15, 2025 based on 16s rRNA gene amplicon sequencing. *Ca.* M. hillemani and other *Methanohalophilus* that could not be classified to the species level are presented. For most taxa, taxonomic classification was done at the genus level. *Ca.* M. hillemani was manually categorized based on a one-nucleotide difference from other *Methanohalophilus* 16s rRNA genes in their amplified region. Sequences that could not be assigned a genus are labeled "Family" or "Order" prior to the name. "Uncultured Methanogens" (possibly members of the order *Methanofastidiosales*, see main text) were the most abundant sequences. Only methanogen

sequences are shown. (C) *mcrA* gene amplicon relative abundance in the same core samples as
(B), corroborating the presence of *Ca.* M. hillemani. Other *Methanohalophilus mcrA* gene
sequences were also present, including some that could not be classified to the species level. For
most taxa, taxonomic classification was done at the species level. For taxa that could not be
assigned to species, "Family" or "Genus" is included in the name.

Novel methanogens likely contribute to Great Salt Lake methane flux

To examine the ecological relevance of the novel methanogen in the GSL ecosystem, we
performed 16S rRNA and *mcrA* gene amplicon sequencing on samples from a soil core taken from
the transect A 10-meter sample, where extremely high and variable methane flux was observed
(Supplementary Figure 8). The 16S rRNA gene amplicon data revealed that across all depths, *Ca.*
M. hillemani was the second most abundant methanogen, while other *Methanohalophilus*
sequences were the fourth most abundant (Fig. 4C). *Desulfovermiculus* was the most abundant
genus of SRB (Supplementary Figure 9). *Ca.* M. hillemani was similarly abundant across all
depths, and it represented the largest fraction of the methanogenic community near the surface.
This suggests some level of O_2 tolerance, consistent with our culture experiments (Fig. 2B). The
most abundant methanogens, by far, were represented by a group of 11 poorly-characterized
sequences. A classifier trained on the ARB-silva 16S rRNA gene database could only identify
these sequences as members of the order *Methanofastidiosales* [72], while BLAST only retrieved
"uncultured archaeon clone" sequences. The relative abundance of all methanogens increased with
depth, ranging from ~0.25% at the surface to over 2% of the total community at the 15-18 cm
depth.

The most abundant *mcrA* gene amplicon sequences were also poorly characterized – their
top BLAST hits were to sequences recovered from deep sea anoxic brines (Figure 4c).
Methanohalophilus sequences that could not be assigned to a specific species were the next most
abundant. Of the *Methanohalophilus* sequences that were identified to the species level, *M. mahii*
was the most abundant, followed by *M. portucalensis* and *Ca.* M. hillemani. Like the 16S rRNA
gene amplicon data, the poorly characterized sequences increased in relative abundance with
increasing depth. In both the 16S rRNA and *mcrA* gene amplicon datasets, the most abundant
methanogenic sequences were poorly characterized, indicating that novel methanogens may be the
dominant community in the high-methane-flux GSL soil.

<u>Conclusion</u>

Ca. Methanohalophilus hillemani is a novel methanogen, collected from the south arm of
the GSL when its salinity was at an all-time high. Metaproteomic analysis of this organism showed
that methanogenesis and osmo-tolerance proteins had high basal expression. The expression of
these proteins was not impacted by salinity changes, suggesting that future proteomic studies of
the *Methanohalophilus* genus should focus on post-translational modification and other forms of
regulation, for example on protein activity or metabolome level. Large differences in the expressed
proteome were instead observed in the N_2 condition, which lacked a large population of the SRB,

Desulfovermiculus. In conditions under which *Ca.* M. hillemani and *Desulfovermiculus* were both present, the methanogen highly expressed metal transporters and chelatases. We hypothesize that, with sufficient H_2, SRB directly compete for and reduce the availability of trace metals, forcing *Ca.* M. hillemani to invest in uptake of metals vital to its energy metabolism. Coculturing of the two also led to the upregulation of unique immune-response and DNA-repair proteins by *Ca.* M. hillemani, indicating the adaptation of novel strategies to tolerate stress and/or *Desulfovermiculus*. High methane flux was measured from the shore of the GSL, and amplicon data of a soil core identified *Ca.* M. hillemani to be present at significant levels, confirming the ecological relevance of this novel methanogen. In summary, this study cultured a novel methanogen from the GSL, demonstrated that its physiology may not be controlled as much by changes in salinity as by community interactions, and identified it in GSL soils exhibiting high methane flux.

Acknowledgements
This work was supported by the NASA Exobiology award 80NSSC21K0487 (to T.R.M.) and Department of Energy, Office of Science, Biological and Environmental Research award DE-SC0025661 (to R.H.). A portion of this research was performed under FICUS award 509790 (DOI:10.46936/fics.proj.2023.60847/60008905) and used resources at the Environmental Molecular Sciences Laboratory, a DOE Office of Science User Facility sponsored by the Biological and Environmental Research program under contract no. DE-AC05-76RL01830. Battelle operates PNNL for the DOE under contract DE-AC0576RLO01830. We thank Bonnie Baxter (Westminster University) for her guidance on field sampling, Angel Nieto and Ciara Pike for assistance during field work, Veronika Kivenson (UC Berkeley) for helpful discussions on pyrrolysine and alternative codon use, and the laboratory of Samantha Joye (University of Georgia) for help in quantifying major ions in GSL water. Finally, we thank the family of the late Dr. Maurice Hilleman for allowing us to name the newly cultured methanogen after him in recognition of his life-saving contributions to microbiology, vaccine development, and public health (see Supplementary Etymology).

Contributions
Conceptualization: W.C.C., T.R.M. and R.H.
Methodology: W.C.C., T.R.M. and R.H.
Validation: W.C.C., Z.J.J., N.T., T.R.M., and R.H.
Formal analysis: W.C.C., Z.J.J. and N.T.
Investigation: W.C.C., C.D.N., S.T. and R.L.
Resources: T.R.M. and R.H.
Data curation: W.C.C., Z.J.J., and N.T.
Writing–original draft: W.C.C.
Writing–review and editing: W.C.C., Z.J.J., N.T., T.R.M., and R.H.
Visualization: W.C.C. and Z.J.J.
Supervision: T.R.M. and R.H.

Project administration: T.R.M. and R.H.

Funding acquisition: T.R.M. and R.H.

<u>References</u>

1.   *Summary for Policymakers*, in *Climate Change 2021 – The Physical Science Basis: Working*
Group I Contribution to the Sixth Assessment Report of the Intergovernmental Panel on
Climate Change, C. Intergovernmental Panel on Climate, Editor. 2023, Cambridge University
Press: Cambridge. p. 3-32.

2.   Saunois, M., et al., *Global Methane Budget 2000–2020.* Earth Syst. Sci. Data, 2025. **17**(5): p.
1873-1958.

3.   Conrad, R., *The global methane cycle: recent advances in understanding the microbial*
processes involved. Environmental Microbiology Reports, 2009. **1**(5): p. 285-292.

4.   Keuschnig, C., et al., *Reduced methane emissions in former permafrost soils driven by*
vegetation and microbial changes following drainage. Global Change Biology, 2022. **28**(10):
p. 3411-3425.

5.   Wang, N., et al., *Microbial mechanisms for methane source-to-sink transition after wetland*
conversion to cropland. Geoderma, 2023. **429**: p. 116229.

6.   Shoemaker, J.K., et al., *Forest ecosystem changes from annual methane source to sink*
depending on late summer water balance. Geophysical Research Letters, 2014. **41**(2): p. 673-
679.

7.   He, T., et al., *Meta-analysis shows the impacts of ecological restoration on greenhouse gas*
emissions. Nature Communications, 2024. **15**(1): p. 2668.

8.   Stone, R., *Saving Iran's great salt lake.* Science, 2015. **349**(6252): p. 1044-1047.

9.   Aladin, N., I. Plotnikov, and B. T, *The Aral Sea: water level, salinity and long-term changes*
in biological communities of an endangered ecosystem - past, present and future. 2008.

10. Patel, K. *Getting Saltier*. 2019.

11. Baxter, B.K. and J.K. Butler, *Climate Change and Great Salt Lake*, in *Great Salt Lake*
Biology: A Terminal Lake in a Time of Change, B.K. Baxter and J.K. Butler, Editors. 2020,
Springer International Publishing: Cham. p. 23-52.

12. Wurtsbaugh, W.A., et al., *Decline of the world's saline lakes.* Nature Geoscience, 2017.
10(11): p. 816-821.

13. Wang, J., et al., *Recent global decline in endorheic basin water storages.* Nature Geoscience,
2018. **11**(12): p. 926-932.

14. McGenity, T.J. and D.Y. Sorokin, *Methanogens and Methanogenesis in Hypersaline*
Environments, in *Biogenesis of Hydrocarbons*, A.J.M. Stams and D.Z. Sousa, Editors. 2019,
Springer International Publishing: Cham. p. 283-309.

15. Soued, C., et al., *Salinity causes widespread restriction of methane emissions from small*
inland waters. Nature Communications, 2024. **15**(1): p. 717.

16. Paranaíba, J.R., et al., *Cross-continental importance of CH(4) emissions from dry inland-*
waters. Sci Total Environ, 2022. **814**: p. 151925.

17. Welti, N., M. Hayes, and D. Lockington, *Seasonal nitrous oxide and methane emissions*
across a subtropical estuarine salinity gradient. Biogeochemistry, 2017. **132**(1): p. 55-69.

18. Patel, L., et al., *Salinity and phosphorus as key regulators of methane emissions in tropical*
aquaculture ponds. Aquaculture International, 2025. **33**(4): p. 302.

19. Hartman Wyatt, H., et al., *Multiple microbial guilds mediate soil methane cycling along a*
wetland salinity gradient. mSystems, 2024. **9**(1): p. e00936-23.

20. Cobo, M., T. Goldhammer, and S. Brothers, *A desiccating saline lake bed is a significant*
source of anthropogenic greenhouse gas emissions. One Earth, 2024. **7**(8): p. 1414-1423.

21. Baxter, B.K. and P. Zalar, *Chapter 4 - The extremophiles of Great Salt Lake: Complex*
microbiology in a dynamic hypersaline ecosystem, in *Model Ecosystems in Extreme*
Environments, J. Seckbach and P. Rampelotto, Editors. 2019, Academic Press. p. 57-99.

22. Paterek, J.R. and P.H. Smith, *Isolation and characterization of a halophilic methanogen from*
great salt lake. Appl Environ Microbiol, 1985. **50**(4): p. 877-81.

23. Spring, S., et al., *The genome sequence of Methanohalophilus mahii SLP(T) reveals*
differences in the energy metabolism among members of the Methanosarcinaceae inhabiting
freshwater and saline environments. Archaea, 2010. **2010**: p. 690737.

24. PATEREK, J.R. and P.H. SMITH, *Methanohalophilus mahii gen. nov., sp. nov., a*
Methylotrophic Halophilic Methanogen†. International Journal of Systematic and
Evolutionary Microbiology, 1988. **38**(1): p. 122-123.

25. Tazi, L., et al., *Life in extreme environments: microbial diversity in Great Salt Lake, Utah.*
Extremophiles, 2014. **18**(3): p. 525-35.

26. Shoemaker, A., et al., *Wood–Ljungdahl pathway encoding anaerobes facilitate low-cost*
primary production in hypersaline sediments at Great Salt Lake, Utah. FEMS Microbiology
Ecology, 2024. **100**(8).

27. Kanik, M., et al., *Unexpected Abundance and Diversity of Phototrophs in Mats from*
Morphologically Variable Microbialites in Great Salt Lake, Utah. Applied and Environmental
Microbiology, 2020. **86**(10): p. e00165-20.

28. Guan, Y., et al., *Comparative Genomics of the Genus Methanohalophilus, Including a Newly*
Isolated Strain From Kebrit Deep in the Red Sea. Frontiers in Microbiology, 2019. **10**.

29. L'Haridon, S., et al., *Methanohalophilus profundi sp. nov., a methylotrophic halophilic*
piezophilic methanogen isolated from a deep hypersaline anoxic basin. Systematic and
Applied Microbiology, 2020. **43**(5): p. 126107.

30. Davidova, I.A., et al., *Taxonomic description of Methanococcoides euhalobius and its transfer*
to the Methanohalophilus genus. Antonie van Leeuwenhoek, 1997. **71**(4): p. 313-318.

31. Katayama, T., et al., *Methanohalophilus levihalophilus sp. nov., a slightly halophilic,*
methylotrophic methanogen isolated from natural gas-bearing deep aquifers, and emended
description of the genus Methanohalophilus. Int J Syst Evol Microbiol, 2014. **64**(Pt 6): p.
2089-2093.

32. Wilharm, T., T.N. Zhilina, and P. Hummel, *DNA-DNA Hybridization of Methylotrophic*
Halophilic Methanogenic Bacteria and Transfer of Methanococcus halophilusvp to the Genus

Methanohalophilus as Methanohalophilus halophilus comb. nov. International Journal of Systematic and Evolutionary Microbiology, 1991. **41**(4): p. 558-562.

33. Boone, D.R., et al., *Isolation and Characterization of Methanohalophilus portucalensis sp. nov. and DNA Reassociation Study of the Genus Methanohalophilus.* International Journal of Systematic and Evolutionary Microbiology, 1993. **43**(3): p. 430-437.

34. Borton, M.A., et al., *Comparative genomics and physiology of the genus Methanohalophilus, a prevalent methanogen in hydraulically fractured shale.* Environmental Microbiology, 2018. **20**(12): p. 4596-4611.

35. Kohtz, A.J., et al., *Cultivation and visualization of a methanogen of the phylum Thermoproteota.* Nature, 2024. **632**(8027): p. 1118-1123.

36. Krukenberg, V., et al., *Microbial Community Response to Polysaccharide Amendment in Anoxic Hydrothermal Sediments of the Guaymas Basin.* Frontiers in Microbiology, 2021. **Volume 12 - 2021**.

37. Parada, A.E., D.M. Needham, and J.A. Fuhrman, *Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples.* Environ Microbiol, 2016. **18**(5): p. 1403-14.

38. Thompson, L.R., et al., *A communal catalogue reveals Earth's multiscale microbial diversity.* Nature, 2017. **551**(7681): p. 457-463.

39. Angel, R., P. Claus, and R. Conrad, *Methanogenic archaea are globally ubiquitous in aerated soils and become active under wet anoxic conditions.* The ISME Journal, 2012. **6**(4): p. 847-862.

40. Kim, S., N. Gupta, and P.A. Pevzner, *Spectral Probabilities and Generating Functions of Tandem Mass Spectra: A Strike against Decoy Databases.* Journal of Proteome Research, 2008. **7**(8): p. 3354-3363.

41. Monroe, M.E., et al., *Brief Communication: MASIC: A software program for fast quantitation and flexible visualization of chromatographic profiles from detected LC-MS(/MS) features.* Comput. Biol. Chem., 2008. **32**(3): p. 215–217.

42. Polpitiya, A.D., et al., *DAnTE: a statistical tool for quantitative analysis of -omics data.* Bioinformatics, 2008. **24**(13): p. 1556-8.

43. Farina, M., et al., *Methane emission hotspots in a boreal forest-fen mosaic potentially linked to deep taliks.* Environmental Research Letters, 2025. **20**(10): p. 104029.

44. Alowaifeer, A.M., et al., *Aerobic methane synthesis and dynamics in a river water environment.* Limnology and Oceanography, 2023. **68**(8): p. 1762-1774.

45. Jain, C., et al., *High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries.* Nature Communications, 2018. **9**(1): p. 5114.

46. Post, F.J., *The microbial ecology of the Great Salt Lake.* Microb Ecol, 1977. **3**(2): p. 143-65.

47. Shih, C.J. and M.C. Lai, *Differentially expressed genes after hyper- and hypo-salt stress in the halophilic archaeon Methanohalophilus portucalensis.* Can J Microbiol, 2010. **56**(4): p. 295-307.

48. Keltjens, J.T. and G.D. Vogels, *Conversion of Methanol and Methylamines to Methane and Carbon Dioxide*, in *Methanogenesis: Ecology, Physiology, Biochemistry & Genetics*, J.G. Ferry, Editor. 1993, Springer US: Boston, MA. p. 253-303.

49. Hippe, H., et al., *Utilization of trimethylamine and other N-methyl compounds for growth and methane formation by Methanosarcina barkeri.* Proceedings of the National Academy of Sciences, 1979. **76**(1): p. 494-498.

50. Daly, R.A., et al., *Microbial metabolisms in a 2.5-km-deep ecosystem created by hydraulic fracturing in shales.* Nature Microbiology, 2016. **1**(10): p. 16146.

51. Wu, W.-L., et al., *Phosphoproteomic analysis of Methanohalophilus portucalensis FDF1T identified the role of protein phosphorylation in methanogenesis and osmoregulation.* Scientific Reports, 2016. **6**(1): p. 29013.

52. Sepúlveda-Correa, A., et al., *Effect of salinity on genes involved in the stress response in mangrove soils.* Antonie van Leeuwenhoek, 2023. **116**(11): p. 1171-1184.

53. Wang, L., et al., *DNA phosphorothioate modification—a new multi-functional epigenetic system in bacteria.* FEMS Microbiology Reviews, 2019. **43**(2): p. 109-122.

54. Hoffman, M., et al., *Generation of DNA-Damaging Reactive Oxygen Species via the Autoxidation of Hydrogen Sulfide under Physiologically Relevant Conditions: Chemistry Relevant to Both the Genotoxic and Cell Signaling Properties of H2S.* Chemical Research in Toxicology, 2012. **25**(8): p. 1609-1615.

55. Khanal, S.K. and J.C. Huang, *Effect of high influent sulfate on anaerobic wastewater treatment.* Water Environ Res, 2005. **77**(7): p. 3037-46.

56. Gupta, A., et al., *Methanogenesis and sulfate reduction in chemostats—II. Model development and verification.* Water Research, 1994. **28**(4): p. 795-803.

57. Hori, K., R. Balasubramanian, and S. Masuda, *Sulfide-Responsive Transcription Control in Escherichia coli.* Microorganisms, 2025. **13**(2).

58. Bechtold, E.K., et al., *Sulfide stress tolerance as a controller of methane production in temperate wetlands.* The ISME Journal, 2025. **19**(1): p. wraf196.

59. Martens, J.H., et al., *Microbial production of vitamin B12.* Appl Microbiol Biotechnol, 2002. **58**(3): p. 275-85.

60. Krumholz, L., D. Elias, and J. Suflita, *Immobilization of cobalt by sulfate-reducing bacteria in subsurface sediments.* Geomicrobiology Journal, 2003. **20**(1): p. 61-72.

61. Ekstrom, E.B. and F.M.M. Morel, *Cobalt Limitation of Growth and Mercury Methylation in Sulfate-Reducing Bacteria.* Environmental Science & Technology, 2008. **42**(1): p. 93-99.

62. Gavel, O.Y., et al., *ATP Sulfurylases from Sulfate-Reducing Bacteria of the Genus Desulfovibrio. A Novel Metalloprotein Containing Cobalt and Zinc.* Biochemistry, 1998. **37**(46): p. 16225-16232.

63. Mahapatra, A., et al., *Characterization of a Methanosarcina acetivorans mutant unable to translate UAG as pyrrolysine.* Mol Microbiol, 2006. **59**(1): p. 56-66.

64. Hao, B., et al., *A new UAG-encoded residue in the structure of a methanogen methyltransferase.* Science, 2002. **296**(5572): p. 1462-6.

65. O'Donoghue, P., et al., *Reducing the genetic code induces massive rearrangement of the*
proteome. Proceedings of the National Academy of Sciences, 2014. **111**(48): p. 17206-17211.
66. Zhang, Y., et al., *Pyrrolysine and selenocysteine use dissimilar decoding strategies.* J Biol
Chem, 2005. **280**(21): p. 20740-51.
67. Théobald-Dietrich, A., R. Giegé, and J. Rudinger-Thirion, *Evidence for the existence in*
mRNAs of a hairpin element responsible for ribosome dependent pyrrolysine insertion into
proteins. Biochimie, 2005. **87**(9-10): p. 813-7.
68. Kivenson, V., et al., *Sporadic distribution of a new archaeal genetic code with all TAG codons*
as pyrrolysine. bioRxiv, 2024: p. 2024.09.30.615893.
69. Shalvarjian, K.E., et al., *The Ambiguous Genetic Code of Methanogenic Archaea that Grow*
on Methylamines. bioRxiv, 2025: p. 2025.06.11.659114.
70. Walter Anthony, K.M., et al., *Upland Yedoma taliks are an unpredicted source of atmospheric*
methane. Nature Communications, 2024. **15**(1): p. 6056.
71. Cui, S., et al., *Wetland hydrological dynamics and methane emissions.* Communications Earth
& Environment, 2024. **5**(1): p. 470.
72. Nobu, M.K., et al., *Chasing the elusive Euryarchaeota class WSA2: genomes reveal a*
uniquely fastidious methyl-reducing methanogen. The ISME Journal, 2016. **10**(10): p. 2478-
2487.
728