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

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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.

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.

120
121 **Methane measurements**
122 Methane was measured via GC-FID as previously described [35] with the following
123 modifications: (i) 1 mL of sample was injected into autosampler vials instead of 250 µL, (ii) a 3
124 mL plastic BD syringe equipped with a sample lock was used in place of a Hamilton syringe, and
125 (iii) an Rtx-1 GC Capillary column was used instead of a GS-CarbonPLOT column.
126
127 **Gene amplicon analysis**
128 16S rRNA gene analysis was performed as described in Krukenberg et al. [36] using Earth
129 Microbiome Project primers 515F and 806R [37, 38]. The mlas-mod-F and *mcrA*-rev-R primers
130 and the touchdown amplification program from Angel et al. 2012 were used to amplify the alpha-
131 subunit of methyl-coenzyme M reductase (*mcrA*) [39]. Further details can be found in the
132 supplementary methods section.
133
134 **Metagenomics**
135 A combination of Illumina short-read and Nanopore long read sequencing, following an
136 established workflow [35] with some modifications, was used to obtain the circular genome of *Ca.*
137 M. hillemani str. BB. Fragmented but complete (>99.5 %) genomes were also generated for
138 *Halanaerobium* and *Desulfovermiculus* spp.. The genomes can be found with the following
139 accession numbers for *Ca.* M. hillemani closed genome, *Desulfovermiculus* sp., and
140 *Halanaerobium* sp., respectively: SAMN54119603, SAMN54119604, SAMN54119605. Details
141 on sequencing, assembly, annotation, and genome comparisons are described in the supplementary
142 methods section.
143
144 **Salt stress experiment**
145 For methane production and metaproteomics experiments, *Methanohalophilus* enrichment
146 cultures were grown (i) as the control (50/45/5 of $H_2/N_2/CO_2$ headspace, 176 g/L total salts), (ii)
147 with a 100% N_2 headspace, (iii) in low salt media (114 g/L salts), (iv) high salt media (227 g/L
148 salts), and (v) micro-oxic conditions (1 mL of filter-sterilized room air injected into the headspace
149 every four days, achieving ca. 0.35% O_2 upon first injection). Cultures grown under control
150 conditions were transferred (1:100) into bottles with these conditions. To acclimate the test
151 cultures to stress conditions, they were grown under these test conditions for three transfers before
152 methane data was recorded during the fourth generation. That culture was then used to inoculate a
153 fifth generation, which was harvested in mid-log phase (based on methane production) for the
154 metaproteomics experiment and the 16S rRNA gene community analysis (n=3 replicates for each
155 condition).
156
157 **Metaproteomics sample preparation and data analysis**
158 The harvested cultures were sent to the Pacific Northwest National Laboratory (Richland,
159 WA, USA) where 200 µg of protein was used for digestion. MS analysis of the peptides was

160 performed using an Orbitrap Exploris 480 mass spectrometer (Thermo Scientific, Waltham, MA,
161 USA). Peptide matching from the acquired datasets was performed using MSGF+ software [40]
162 against target meta-proteome composed from sequenced genomes (see Supplementary Methods).
163 Peptide abundances, integrated as area under the peptide elution peaks, were produced from raw
164 spectra using in-house developed software MASIC [41]. MASIC peptide abundances were log2
165 transformed to remove skewness in the distribution of measured abundances. Peptide abundances
166 were corrected for the relative abundance of organisms using the summed area under the peptide
167 curves mapping to each organism. Transformed abundance values of peptides, and later proteins,
168 were normalized using mean central tendency method implemented in InfernoR software [42].
169 Unless otherwise indicated, all proteomic data presented in this study represents normalized
170 protein abundances. Raw proteomics data has been deposited at massIVE under accession number:
171 MSV000100495. Further details can be found in the supplementary methods section.
172
173 **Methane flux measurements**
174 Methane flux data was collected in transects, perpendicular to the shoreline, at Bridger Bay
175 in the south arm of the GSL using A Li-COR 7810 trace gas analyzer (Li-COR, Lincoln, NE,
176 USA). Terrestrial flux measurements were made as previously described [43], but with a one-
177 minute measurement period and a 10 second deadband time. To generate depth-resolved 16S
178 rRNA gene amplicon data, soil cores were collected from within the boundary of the terrestrial
179 collar which registered the highest methane flux. Aquatic flux measurements were done using a
180 custom-made floating flux chamber to take duplicate, one minute, flux measurements. Aquatic
181 fluxes were calculated from the linear change in methane concentration over time, as previously
182 described [44].
183
184 **<u>Results and discussion</u>**
185 **Enrichment of a novel methanogen**
186 Microbialite and water samples from Bridger Bay in the South arm of GSL were collected
187 in July 2022, when the water level was at its lowest in recorded history (Supplementary Figure 3).
188 Methanogens were enriched using the growth medium used to isolate *Methanohalophilus mahii*
189 [22]. We increased the salinity of the medium to match GSL water from April 13th 2023, provided
190 a headspace consisting of 50% H_2, 45% N_2, and 5% CO_2, and used trimethylamine (TMA) as the
191 methanogenic substrate. The culture also produced methane from MMA, DMA and methanol, but
192 not from glycine betaine or hydrogen and carbon dioxide (Supplementary Figure 4).
193 After 21 months of enrichment, 16S rRNA gene amplicon sequencing indicated that the
194 culture consisted of 63% *Methanohalophilus*, 20% *Desulfovermiculus* (a sulfate reducing
195 bacterium - SRB), and 16% *Halanaerobium* (an anaerobic fermenter). An additional 25 taxa
196 contributed to the remaining <1% of the 16S rRNA gene reads. Metagenomic sequencing of this
197 sample with both Illumina and Oxford Nanopore enabled assembly of a circular
198 *Methanohalophilus* genome (2.09 Mb). Sequence coverage indicated that the culture consisted of
199 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.

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).

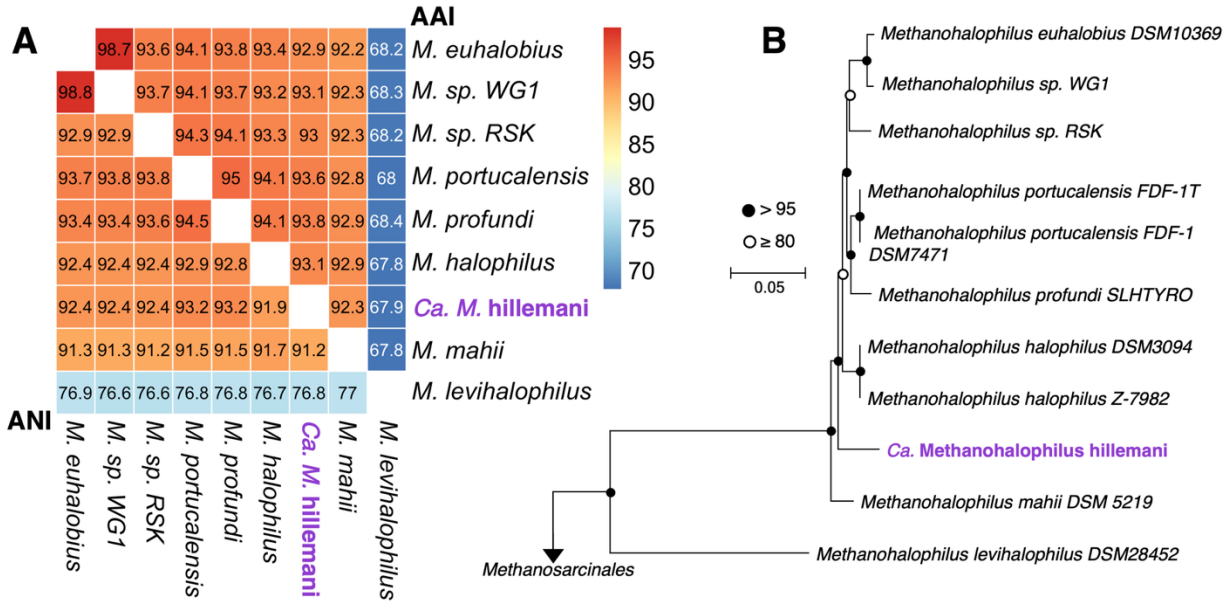


Figure 1: Phylogeny of *Ca.* Methanohalophilus hillemani. (A) Pairwise average nucleotide identity (ANI) and average amino acid identity (AAI) on bottom left and top right, respectively demonstrating *Ca.* M. hillemani represents a new species within the *Methanohalophilus* genus. (B) Maximum likelihood 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,

226 superoxide reductases, and peroxiredoxin. Unique genetic features of *Ca.* M. hillemani as
227 compared to other members of the genus *Methanohalophilus* are discussed in the Supplementary
228 Results section.
229
230 **Increasing salinity in the Great Salt Lake**
231 The biomass that led to culturing *Ca.* M. hillemani was collected from the GSL in July
232 2022, when the lake was at its lowest recorded level (Supplementary Figure 3). Additionally the
233 sample came from Bridger Bay, which was cut off from the main lake by low water levels, leading
234 to a salinity much higher (~24%) than the rest of the south arm of the GSL (~17%). Na^+ and Cl^-
235 concentrations at this time were 3.6 and 4.2 M, respectively, more than twice the concentrations
236 of these ions measured by Post et al. in 1977 (1.6 and 1.8 M) [46]. In contrast, *Methanohalophilus*
237 *mahii* was isolated in 1985 [22], right before GSL reached recorded high water levels
238 (Supplementary Figure 5). This led us to the hypothesis that *Ca.* M. hillemani may be adapted to
239 higher osmotic stress levels as compared to *M. mahii*.
240
241 **Methane production under salt stress**
242 To understand how stressors in the GSL ecosystem might impact *Ca.* M. hillemani, control
243 cultures were compared to those incubated under low salt, high salt, 100% N_2, and micro-oxic
244 conditions (see methods, above). Proportions of major salt ions in all conditions matched the GSL,
245 which contrasts previous work on the genus that had only modulated NaCl concentration [22, 29,
246 47]. Despite differing stressors and relative abundances, methane yield for *Ca.* M. hillemani
247 cultures across all conditions was consistent (Figure 2a). Mass balance of the methyl groups
248 provided via TMA shows that after 16 days 68 - 77% was converted to methane, consistent with
249 the 75% value for methyl-dismutating methanogenesis that has been reported for some
250 euryarchaeotal methanogens [48, 49].
251 16S rRNA gene amplicon data showed that *Ca.* M. hillemani was the most abundant
252 member in all conditions (Figure 2b); however, the community composition of these cultures
253 differed significantly from the 3-species enrichment indicated by the metagenome. The control,
254 low salt, and high salt conditions contained primarily (>68%) *Ca.* M. hillemani, while
255 D*esulfovermiculus* was the only other organism with a relative abundance over 0.5%. Over 96%
256 of amplicons in the O_2 condition belonged to *Ca.* M. hillemani. By contrast, in the N_2 condition
257 40% of amplicons belonged to *Ca.* M. hillemani and less than 0.1% to *Desulfovermiculus*. An
258 *Izimaplasma* species that was absent from all other samples represented 24% of the community,
259 and the *Halanaerobium* species that made up <0.1% of amplicons in all other conditions,
260 constituted 30% in the N_2 condition. Despite differences in culturing conditions and community
261 composition, especially in the N_2 cultures, all conditions yielded the same amount of methane.
262

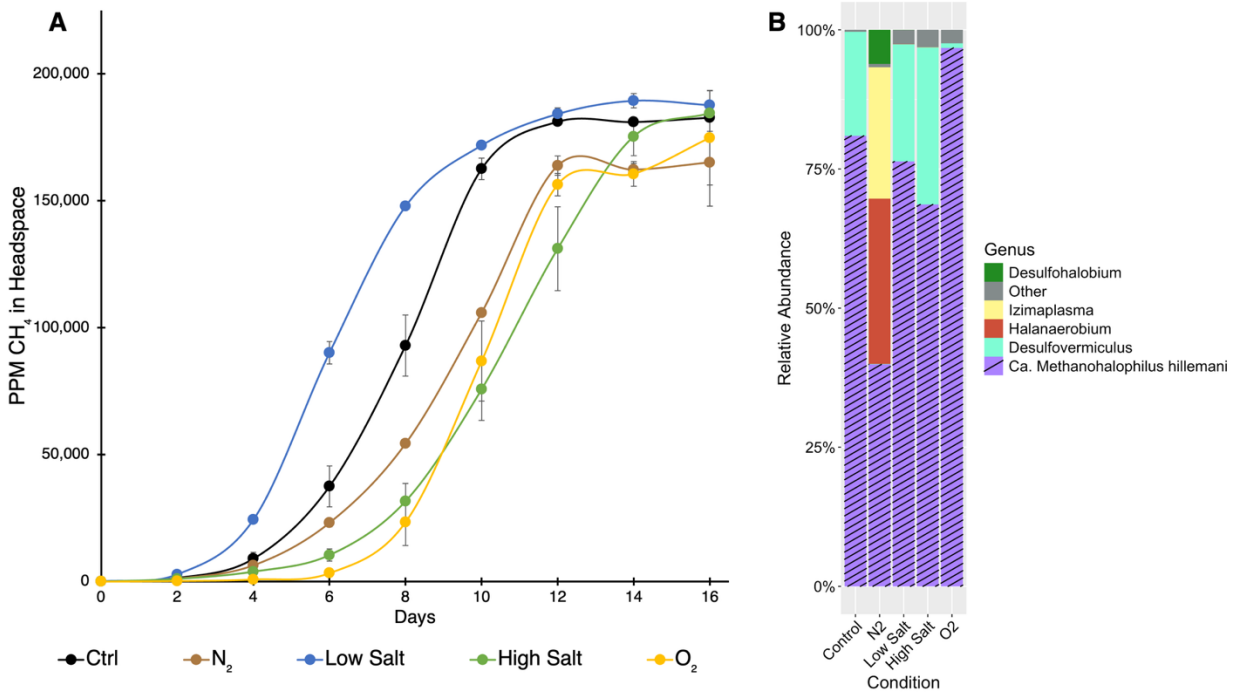


Figure 2: *Ca.* M. hillemani produces similar amounts of methane under a variety of conditions. (A) Methane production of the Methanohalophilus enrichment culture grown under various stress conditions. Error bars (where visible) represent the standard deviation of triplicate cultures. (B) Community composition 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

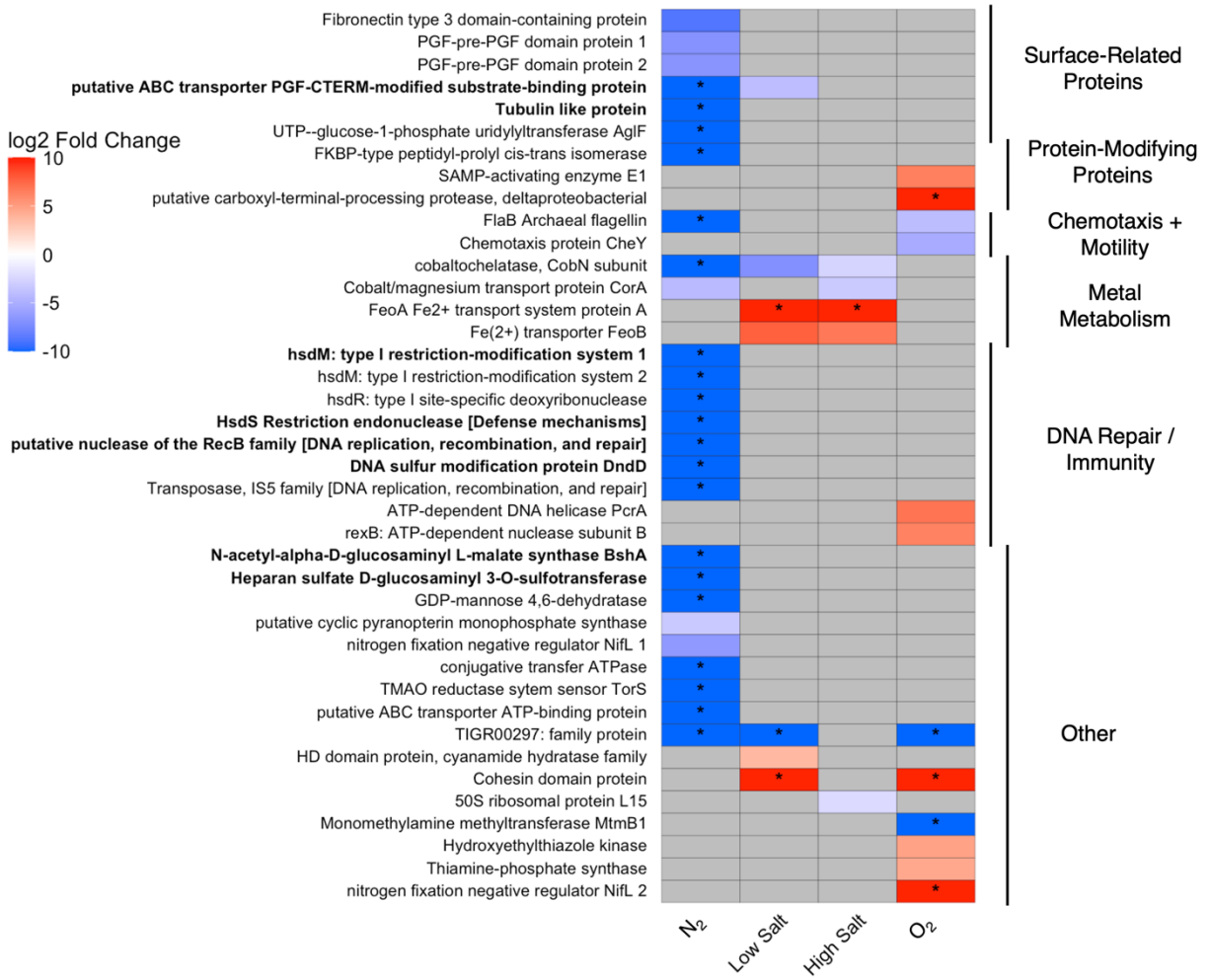
To understand the biological basis for *Ca.* M. hillemani's consistent methane production across stressful conditions, we performed metaproteomics. Triplicate cultures were harvested in mid-log phase and peptides from cultures of each condition were mapped to predicted proteins from the assembled genomes. 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.

288 It was previously reported that in response to long-term high and low-salt stress,
289 *Methanohalophilus portucalensis* transcripts for methylamine methyltransferases and corrinoid
290 proteins were upregulated [47]. Conversely, we show that the *Ca.* M. hillemani methanogenic
291 proteome is highly and uniformly expressed, regardless of high or low salt concentrations. One
292 possible explanation for the lack of observed methanogenic protein expression response, is the
293 high basal expression of energy-conservation proteins (Supplementary Figure 6). This may be
294 advantageous in an environment with constant stressors that require large amounts of energy to
295 tolerate (*i.e.* osmotic and oxidative stress). This strategy could be especially feasible in the GSL,
296 where methylated amines are likely always in high abundance due to the production of a metabolic
297 precursor, glycine betaine [50], as a compatible solute.
298 Another possibility is that methanogenic protein activity, as opposed to abundance, is post-
299 translationally regulated in response to stress. An investigation of protein phosphorylation in
300 *Methanohalophilus portucalensis* found that 26% of the phosphorylated proteins were involved in
301 methanogenesis, energy conservation, and osmolyte biosynthesis [51]. That study found that a
302 mutated glycine sarcosine N-methyltransferase (responsible for glycine betaine synthesis),
303 modified to act like a phosphorylated version, had reduced activity that resulted in lower salinity
304 tolerance when expressed in *E. coli*. While they did not have analogous data for phosphorylated
305 methanogenic proteins, they demonstrate that *Methanohalophilus* proteins can be regulated
306 beyond the level of protein expression. The transcriptomic and proteomic findings discussed here,
307 together with the lack of differential expression of methanogenic proteins, suggests that future
308 work on the *Methanohalophilus* genus should examine forms of regulation downstream of protein
309 expression.
310
311 **Limited number of proteins differentially expressed in response to changing salinity**
312 Contrary to our expectations, low and high salt conditions resulted in relatively few
313 proteins that were both highly and differentially expressed, compared to the control (Figure 3).
314 Both high and low salt conditions showed two upregulated proteins from an iron transport system,
315 FeoA and FeoB, of which FeoA was not detected in the control. Of the many DNA repair proteins
316 encoded by *Ca.* M. hillemani, none were both upregulated and expressed above median protein
317 expression in the high salt condition. The lack of DNA-repair protein expression in response to
318 high salinity was unexpected [52], though basal expression of several of these proteins was
319 observed in the control. Alternatively, the need for DNA repair may be mitigated by osmo-
320 tolerance via high expression of compatible solute. pathways. Indeed, glycine betaine synthesis
321 and import genes are in the top 10% of expressed proteins in the low-salt, high-salt, and control
322 conditions. As discussed above, post-translational regulation could also play a role in osmo-
323 tolerance without requiring differential expression of proteins [51].
324 Protein expression was more affected by the O_2 growth condition. Two proteins associated
325 with protein modification and two DNA-repair proteins were up-regulated and expressed above
326 the median (Figure 3). An additional three DNA-repair proteins were up regulated but not
327 expressed above the median. Surprisingly, none of the oxygen detoxification genes were

328 upregulated. Constitutive expression could also explain this lack of response to oxygen—
329 superoxide reductase, peroxiredoxin, and catalase-peroxidase are all in the top 10% of expressed
330 proteins in the control.
331



332
333 **Figure 3: *Ca.* Methanohalophilus hillemani differential protein expression between control**
334 **and test conditions.** Maximum differentially expressed proteins occurred between the control and
335 N_2 conditions, all of which were more highly expressed in the control. Down-regulated proteins in
336 the N_2 condition included proteins involved in immune response, metal metabolism, and the cell-
337 surface. Proteins shown in this figure meet three criteria: (i) above median normalized protein
338 expression for that condition; (ii) statistically significantly differentially expressed ($p < 0.05$); and
339 (iii) function could be predicted (*i.e.* hypotheticals not included). A negative log fold change
340 indicates the protein was more abundant in the control, positive indicates more abundant in the test
341 condition. Asterisks in the boxes indicate that the protein was not detected in either that test
342 condition or the control and a log2 fold change of 10 or -10 was imputed. Bolded proteins indicate
343 that the coding sequence for this protein was unique to *Ca.* M. hillemani and no homologs were
344 identified in other *Methanohalophilus* genomes. Proteins with grey boxes do not indicate absence

345 in a specific condition, but that the protein did not meet all three criteria listed above. See
346 Supplementary Tables 1-5 for raw values.
347
348 **Unique *Ca.* M. hillemani proteins are differentially expressed in the N_2 condition**
349 When comparing the number of differentially expressed proteins between the control and
350 test conditions, the N_2 condition resulted in twice as many differentially expressed proteins
351 (Supplementary Table 6). This was consistent when filtering for only the proteins that were
352 expressed above the median for each condition or control (Supplementary Table 7). Notably, all
353 26 differentially and highly expressed *Ca.* M. hillemani proteins in the N_2 condition were less
354 abundant than in the control (Figure 3). Additionally, eight of these 26 differentially and highly
355 expressed proteins are unique proteins to *Ca.* M. hillemani, among the *Methanohalophilus* genus
356 (see Supplementary Results). However, none of these unique and highly expressed proteins were
357 detected in the N_2 condition; in contrast, all eight were found in every other condition, with seven
358 expressed above the median. We hypothesize that these distinct protein expression patterns in the
359 N_2 condition are due to the differences in community composition (Figure 2B).
360
361 **Metal uptake, DNA repair, and immunity down-regulated in the N_2 condition**
362 Down-regulated functions in the N_2 condition included immune response and DNA repair,
363 cell-surface features, and metal metabolism (Figure 3). A cobaltochelatase and a
364 cobalt/magnesium transporter were not detected and down-regulated 19-fold, respectively, in the
365 N_2 condition (Figure 3). Additionally, one iron transporter was down regulated 29-fold and two
366 other iron transporters, detected in all other conditions, were absent from the N_2 condition.
367 Highly expressed immune-response and DNA-repair proteins were noticeably absent from
368 the N_2 condition. Seven proteins in this category (Figure 3) were undetectable in the N_2 condition
369 yet were expressed above the median in all other conditions. Eleven additional proteins involved
370 in immune response or DNA repair were also detected in every condition except for N_2. These
371 included two CRISPR-related proteins, two proteins involved in the sulfur-modification of DNA
372 [53], four DNA helicases, two DNA repair proteins, and an additional copy of the type 1
373 restriction-modification system protein, HsdS.
374
375 ***Ca.* M. hillemani is adapted to sulfide stress and competition with *Desulfovermiculus***
376 The genomic and proteomic data do not support the hypothesis that osmotic tolerance
377 distinguishes *Ca.* M. hillemani and *M. mahii*. Both species are genetically capable of synthesizing
378 and taking up the compatible solute, glycine betaine, and both encode ion transporters to maintain
379 osmotic balance. While *Ca.* M. hillemani encodes unique proteins involved in DNA repair and
380 immune response, it encodes no unique proteins predicted to specifically promote osmotolerance
381 (Supplementary Table 8). In fact, differing salt concentrations resulted in a relatively small
382 proteomic change in this halophilic methanogen.
383 Instead, the main proteomic differences were observed from the N_2 condition. Immune-
384 response and DNA-repair proteins, including proteins unique to *Ca.* M. hillemani, were highly

385 expressed in all conditions except the N_2 condition. All other conditions contained 50% H_2 in the
386 headspace and 15-500x higher relative abundance of the SRB, *Desulfovermiculus* (Figure 2b).
387 Sulfide produced by SRB damages DNA [54] and can be detrimental to methanogens [55, 56].
388 Accordingly, sulfide has been shown to change transcription patterns, including genome-wide
389 shifts in *E. coli* [57] and upregulation of genes involved in DNA-repair in methanogens [58]. Our
390 proteomic results indicate that either *Desulfovermiculus* specifically, or high levels of sulfide,
391 induce a distinct proteomic response from *Ca.* M. hillemani. Unique immune-response and DNA-
392 repair proteins expressed by *Ca.* M. hillemani in the presence of *Desulfovermiculus* potentially
393 represent novel adaptions to protect or repair DNA from H_2S-related damage.
394 Additionally, reduced expression of metal uptake and chelation by *Ca.* M. hillemani in the
395 N_2 condition, compared to all other conditions, suggests competition for metals with
396 *Desulfovermiculus*. Cobalt is crucial for methyl-dismutating methanogenesis as it is the cofactor
397 in corrinoid proteins used to transfer methyl groups to MCR (Supplementary Figure 6) [59].
398 However, the conversion of sulfate to sulfide by SRB can directly diminish the availability of
399 cobalt through Co^{2+} - sulfide co-precipitation [60]. Additionally, SRB utilize cobalt as an
400 enzymatic cofactor for various metabolisms, including dissimilatory sulfate reduction [61, 62],
401 which could lead to competition. Indeed, *Desulfovermiculus* expresses two cobaltochelatases in
402 the control condition, one just below median normalized expression and the other well above.
403 Tolerating sulfide stress and competing against *Desulfovermiculus* for cobalt may be important
404 factors impacting *Ca.* M. hillemani physiology when H_2 is available.
405
406 ***Ca.* M. hillemani uses pyrrolysine sparingly**
407 Methylated amine-utilizing methanogens are known to encode the 22nd amino acid,
408 pyrrolysine (Pyl), which plays an important role in methylamine activation in tri, di, and
409 monomethylamine methyltransferases (MttB, MtbB, MtmB, respectively) [63-65]. Indeed, *Ca.* M.
410 hillemani encodes the genes required for Pyl synthesis (PylBC) and uses (Pyl-tRNA ligase). An
411 mRNA secondary structure element, PYLIS (analogous to SECIS for selenocysteine), has been
412 suggested to enable Pyl insertion into proteins [66, 67] and PYLIS elements were annotated within
413 *Ca.* M. hillemani's methylated-amine methyltransferase genes. Accordingly, Pyl residues were
414 identified in peptidesmapping to all three types of methylated-amine methyltransferases encoded
415 by *Ca.* M. hillemani.
416 Kivenson *et al.* have recently proposed that some methanogens, including
417 *Methanohalophilus mahii*, *M. euhalobius*, and *M. levihalophilus,* have reprogrammed the TAG
418 codon to universally incorporate Pyl, instead of being read as a Stop-codon, across the proteome
419 [68]. While the authors confirmed their hypothesis for a marine and a human-gut-associated
420 methanogen, experimental data on halophilic methanogens was not yet available. Moreover, how
421 and if microbes regulate the incorporation of Pyl at TAG codons is a topic of current debate [68,
422 69]. Having already identified Pyl residues in peptides mapping to methyltransferase proteins, we
423 used Kivenson's modification to Prodigal [68], as well as our own modifications to Prokka, to re-
424 predict and annotate genes for *Ca.* M. hillemani. This resulted in 95 predicted proteins containing

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

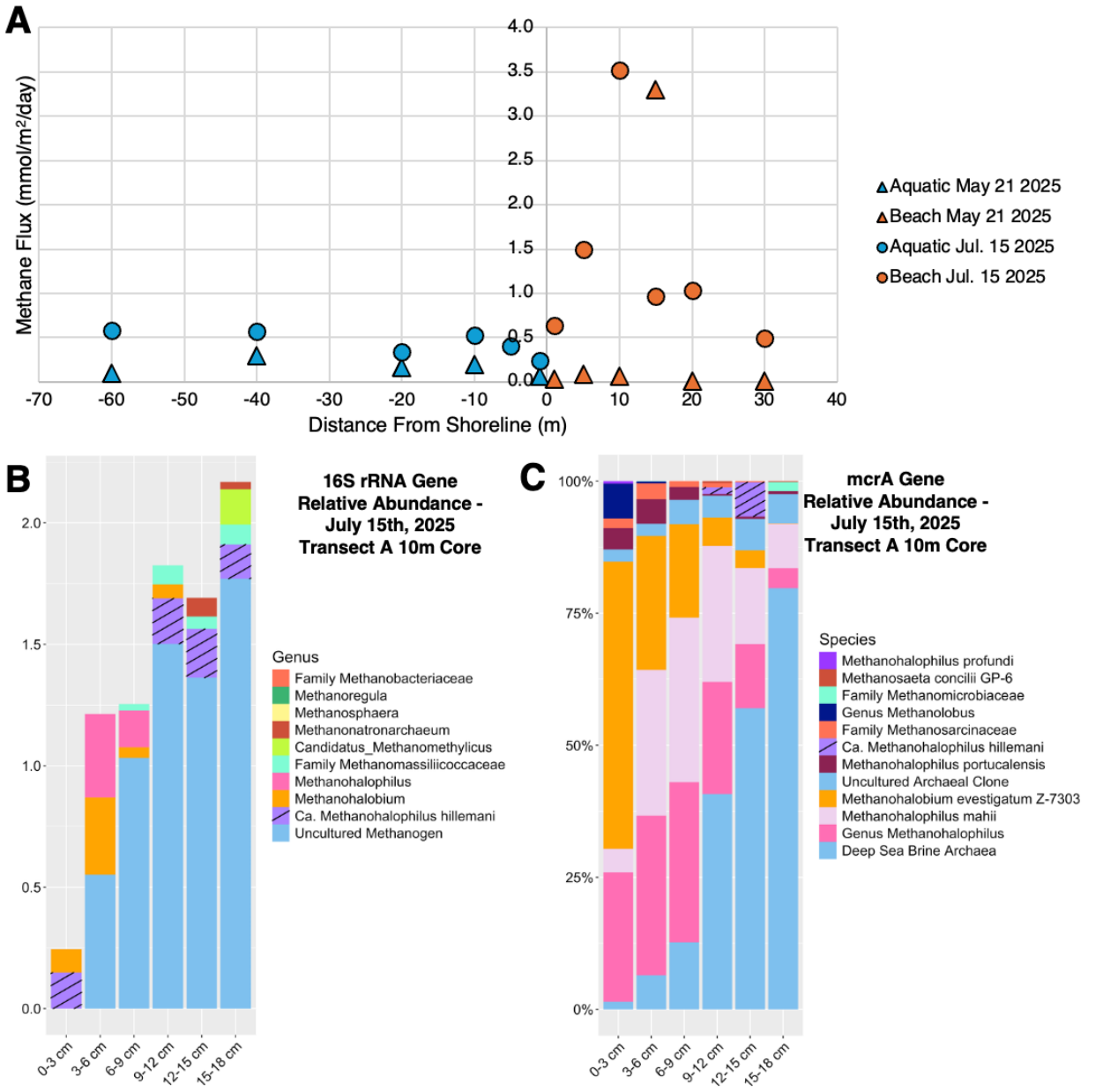


Figure 4: Methane flux and methanogenic community from Bridger Bay on the Great Salt Lake. (A) Methane fluxes measured from soil within Transect A at Bridger Bay in May and July of 2025 showing a zone of high methane flux 10-15 m from the shoreline. (B) Relative abundance of different methanogens in 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.

Conclusion

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.

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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.
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