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

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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 the expression of energy-conservation or osmotolerance 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 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 and continued sources of methane as salinity increases.

KEYWORDS archaea, methanogen, salt stress, halophile, environmental stress, metaproteomics, metagenomics, cultivation, extremophiles

Methane is a potent greenhouse gas that has contributed 0.3°C–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

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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 are not well understood. Recent studies suggest that increasing salinity in freshwater systems decreases methane emissions (15) but also suggest 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 are 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 homogeneous 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 methanol and from mono-, di-, and trimethylamines (MMA, DMA, TMA), with one exception (34). Conversely, they are all incapable of growing via acetoclastic or CO₂-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 O₂, 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 2022 from Bridger Bay in the South arm of the GSL (Fig. S1 and S2). 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 13, 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 , and 0.5 g/L NH_4Cl . Additionally, the media contained the following (per L): 500 μ L 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 of 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 a reductant. The pH was adjusted to ~7.5, and the cultures were grown in 9.5 mL of medium in 70 mL serum bottles. Vancomycin and streptomycin (10 μ L of 50 mg/mL), 200 μ L of 1 M TMA, and 300 μ L of carbon source mix (22) were added to each bottle. Bottles were inoculated with 100 μ L from the previous culture and grown at 30°C for approximately 1 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 μ L, (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-Carbon-PLOT 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 *mls-mod-F* and *mcrA*-rev-R primers and the touchdown amplification program from a study by Angel et al. were used to amplify the alpha-subunit of methyl-coenzyme M reductase (*mcrA*) (39). Further details can be found in the Supplemental methods.

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](#), and [SAMN54119605](#). Details on sequencing, assembly, annotation, and genome comparisons are described in the Supplemental methods.

Salt stress experiment

For the methane production and metaproteomics experiments, *Methanohalophilus* enrichment cultures were grown (i) as the control (176 g/L total salts; 50/45/5 of $H_2/N_2/CO_2$ headspace), (ii) with N_2 headspace (176 g/L total salts; 100% N_2 headspace), (iii) in low salt media (114 g/L salts; 50/45/5 of $H_2/N_2/CO_2$ headspace), (iv) high salt media (227 g/L salts; 50/45/5 of $H_2/N_2/CO_2$ headspace), and (v) micro-oxic conditions (176 g/L total salts; 50/45/5 of $H_2/N_2/CO_2$ headspace plus 1 mL of filter-sterilized room air injected into the headspace every 4 days, achieving ca. 0.35% O_2 upon first injection). The ratio of the different salts for all conditions was the same as described for the media above, with each salt being decreased or increased proportionally to achieve the final concentrations. 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 were recorded during the fourth generation. Those fourth generation cultures were 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 data sets was performed using MSGF+ software (40) against the target meta-proteome composed from sequenced genomes (Supplemental 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 the mean central tendency method implemented in InferoR software (42). Unless otherwise indicated, all proteomic data presented in this study represent normalized protein abundances. Raw proteomics data have been deposited at massIVE under accession number: [MSV000100495](#). Further details can be found in the Supplemental methods.

Methane flux measurements

Methane flux data were 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 1-min measurement period and a 10 s 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, 1 min flux measurements. Aquatic fluxes were calculated from the linear change in methane concentration over time, as previously described (44).

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 (Fig. S3). Methanogens were enriched using the growth medium used to isolate *M. mahii* (22). We increased the salinity of the medium to match GSL water from April 13, 2023, provided a headspace consisting of 50% H_2 , 45% N_2 , and 5% CO_2 , and used 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 (Fig. S4).

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.

Characterization of the novel methanogen as *Ca. Methanohalophilus hillemani*

This newly assembled *Methanohalophilus* genome shares less than 95% average nucleotide identity with all other genomes within its genus, indicating a novel species (45) and only 91.2% with *M. mahii* (Fig. 1A). The 16S rRNA gene is less than 99.3% identical to other species, noticeably lower than other *Methanohalophilus* species to each other (Fig. S5). We propose the name *Candidatus Methanohalophilus hillemani* strain BB for this archaeon (see etymology section in the Supplemental text).

Comparing protein-coding sequences encoded by *Ca. M. hillemani* to all other *Methanohalophilus* spp. showed that it shares the major characteristics of the genus, including methyl-dismutating methanogenesis from MMA, DMA, TMA, and methanol (23, 28, 34). Like the other *Methanohalophilus* species, *Ca. M. hillemani* lacks hydrogenases that would be required for hydrogenotrophic methyl-reducing methanogenesis (23, 28). Other conserved functions across the genus include glycine betaine transport and synthesis, synthesis and usage of the 22nd amino acid pyrrolysine (Pyl), and O₂ tolerance via thioredoxins, peroxidases, superoxide reductases, and peroxiredoxins. Unique genetic features of *Ca. M. hillemani*, as compared to other members of the *Methanohalophilus* genus, are discussed in the Supplemental results.

Increasing salinity in the GSL

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 (Fig. S3). Additionally, the sample came from Bridger Bay, which was cutoff 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) (47). In contrast, *M. mahii* was isolated in 1985 (22), right before GSL reached recorded high

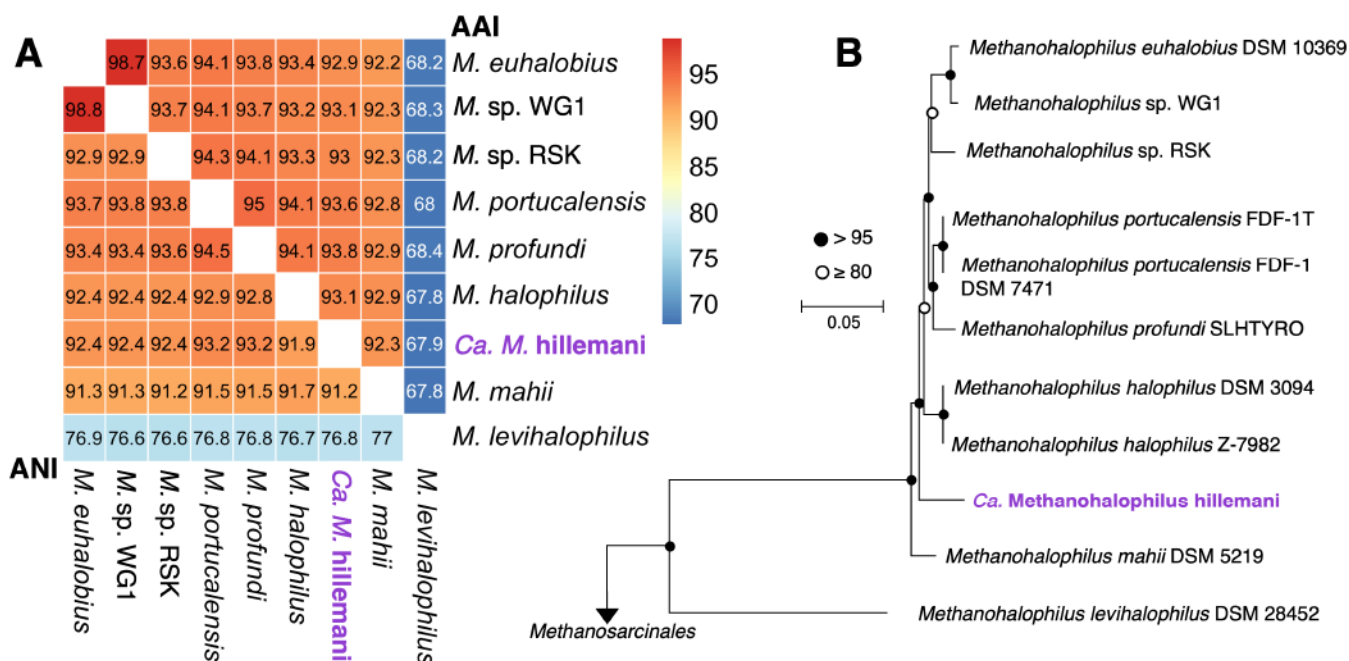


FIG 1 *Ca. M. hillemani* represents a novel species within the genus *Methanohalophilus*. (A) Pairwise average nucleotide identity (ANI) and average amino acid identity (AAI) on the bottom left and top right, respectively, demonstrating that *Ca. M. hillemani* represents a new species within the *Methanohalophilus* genus. (B) Maximum likelihood tree of 65 aligned and concatenated single-copy *Methanohalophilus* proteins constructed with FastTree v2.1.11 (46) and 1,000 bootstraps. Circles represent bootstrap values. Nodes with <50 support were collapsed. Accession numbers for the other *Methanohalophilus* species can be found in Table S1.

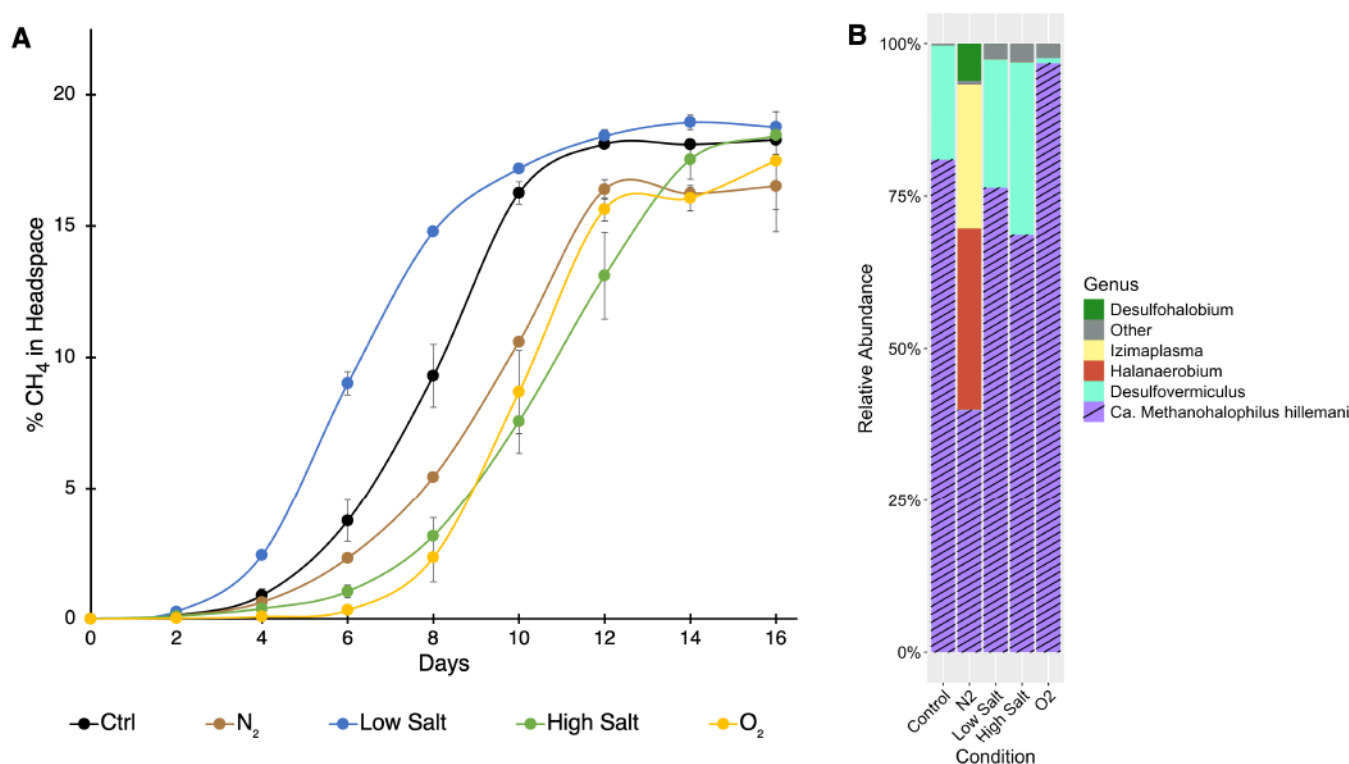


FIG 2 *Ca. M. hillemani* produces similar amounts of methane under a variety of conditions. (A) Methane production of the *Methanohalophilus* enrichment culture grown under various stress conditions. Error bars (where visible) represent the standard deviation of triplicate cultures. (B) Community composition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

water levels (Fig. S3). This led us to the hypothesis that *Ca. M. hillemani* may be adapted to higher osmotic stress levels as compared to *M. mahii*.

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, 48). The lag time for methane production increased slightly with increasing salinity (Fig. 2A). Nevertheless, the overall methane yield for *Ca. M. hillemani* cultures was consistent across all conditions tested (Fig. 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 (49, 50).

16S rRNA gene amplicon data showed that *Ca. M. hillemani* was the most abundant member in all conditions (Fig. 2B); however, the community composition of these cultures differed significantly from the three-species enrichment indicated by the metagenome. The control, low salt, and high salt conditions contained primarily (>68%) *Ca. M. hillemani*, while *Desulfovermiculus* 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 N₂ cultures, all conditions yielded the same amount of methane.

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 had been retrieved for the *Izimaplasma* and *Desulfohalobium* spp. found in the N₂ 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 (Fig. S6). 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 N₂ condition). The only methanogenesis-related protein that was differentially expressed was an MMA-methyltransferase protein found in the control condition, but not in the O₂ condition (Fig. 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 O₂ 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 (48). 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 (Fig. S6). 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 (51), 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 *M. portucalensis* found that 26% of the phosphorylated proteins were involved in methanogenesis, energy conservation, and osmolyte biosynthesis (52). 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 *Escherichia 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 by *Ca. M. hillemani*, suggest that future work on the *Methanohalophilus* genus should examine forms of regulation downstream of protein expression.

Limited number of proteins are 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 (Fig. 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

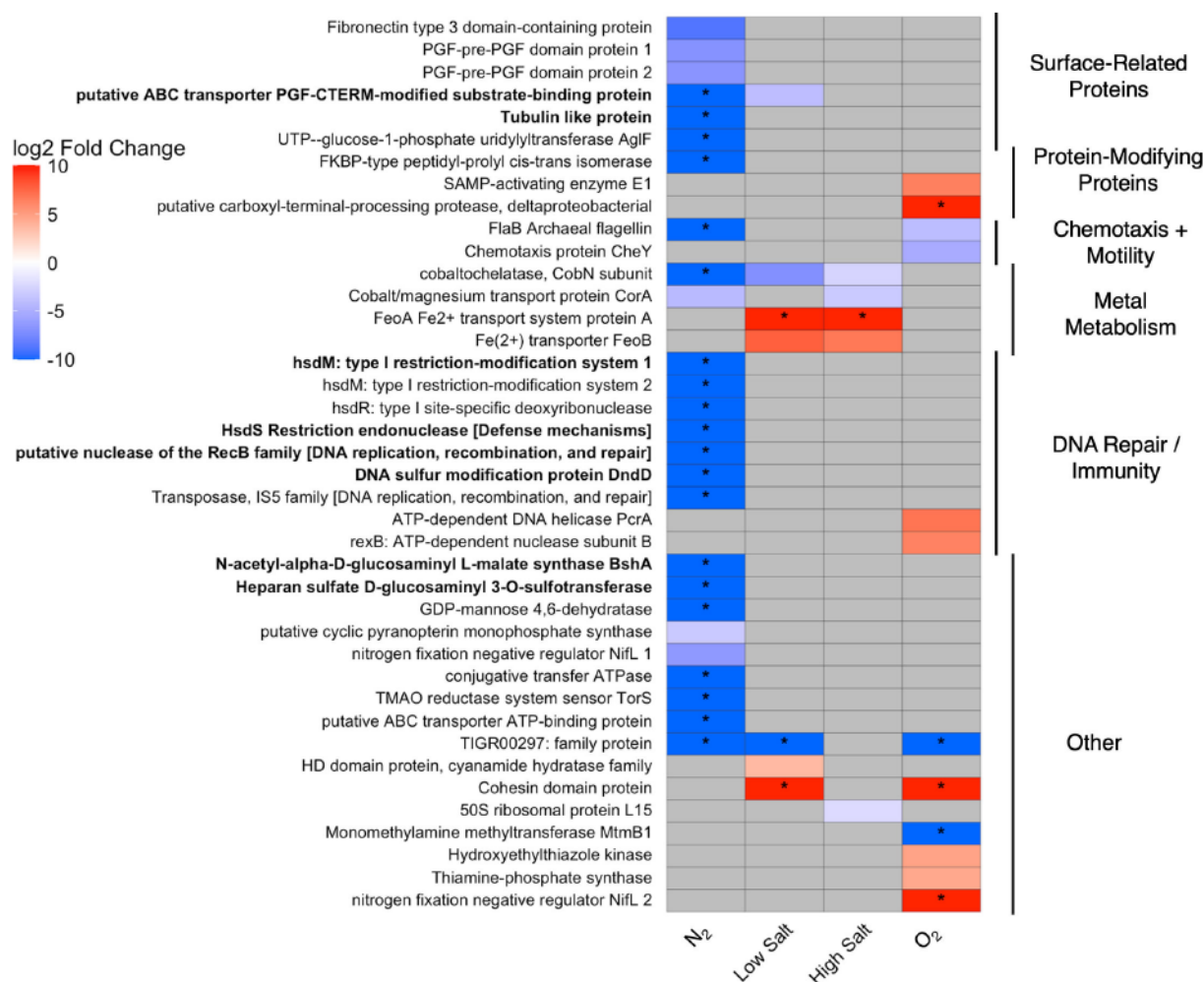


FIG 3 *Ca. M. hillemani* differential protein expression between control and test conditions. The maximum number of differentially expressed proteins occurred between the control and N_2 conditions, and all of these proteins were more highly expressed in the control. Downregulated proteins in the N_2 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 gray boxes do not indicate absence in a specific condition, but that the protein did not meet all three criteria listed above. See Tables S3 to S7 for raw values.

of DNA-repair protein expression in response to high salinity was unexpected (53), although basal expression of several of these proteins was observed in the control. Alternatively, the need for DNA repair may be mitigated by osmotolerance 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 osmotolerance without requiring differential expression of proteins (52).

Protein expression was more affected by the O_2 growth condition. Two proteins associated with protein modification and two DNA-repair proteins were upregulated and expressed above the median. An additional three DNA-repair proteins were upregulated but not expressed above the median. Thiamine is known to confer antioxidant properties (54), and two proteins involved in thiamine biosynthesis, hydroxyethylthiazole kinase and thiamine phosphate synthase, were upregulated in the O_2 condition. Surprisingly,

none of the oxygen detoxification genes were upregulated. 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.

Unique *Ca. M. hillemani* proteins are differentially expressed in the N₂ condition

When comparing the number of differentially expressed proteins between the control and test conditions, the N₂ condition resulted in twice as many differentially expressed proteins (Table S8). This was consistent when filtering for only the proteins that were expressed above the median for each condition or control (Table S9). Notably, all 26 differentially and highly expressed *Ca. M. hillemani* proteins in the N₂ condition were less abundant than in the control (Fig. 3). Additionally, 8 of these 26 differentially and highly expressed proteins are unique proteins to *Ca. M. hillemani*, among the *Methanohalophilus* genus (Supplemental results). However, none of these unique and highly expressed proteins were detected in the N₂ 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 N₂ condition are due to the differences in community composition (Fig. 2B).

DNA repair, immunity, and metal uptake were downregulated in the N₂ condition

Downregulated functions in the N₂ condition included immune response and DNA repair, cell-surface features, and metal metabolism (Fig. 3). A cobaltochelatase and a cobalt/magnesium transporter were not detected and downregulated 19-fold, respectively, in the N₂ condition (Fig. 3). Additionally, one iron transporter was downregulated 29-fold, and two other iron transporters, detected in all other conditions, were absent from the N₂ condition.

Highly expressed immune-response and DNA-repair proteins were noticeably absent from the N₂ condition. Seven proteins in this category (Fig. 3) were undetectable in the N₂ 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 N₂. These included two CRISPR-related proteins, two proteins involved in the sulfur-modification of DNA (55), 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 (Table S10). In fact, differing salt concentrations resulted in a relatively small proteomic change in this halophilic methanogen.

Instead, the main proteomic differences were observed in the N₂ condition. Immune-response and DNA-repair proteins, including proteins unique to *Ca. M. hillemani*, were highly expressed in all conditions except for the N₂ condition. It was previously shown that DNA-repair transcripts were upregulated by methanogens in response to the presence of sulfide (56). Additionally, high levels of sulfide can be detrimental to methanogens (57, 58) and are known to damage DNA (59). Sulfide is produced by SRBs using H₂ as an electron donor, and notably, all conditions in our study, except for the N₂ condition, contained a headspace with 50% H₂. While we did not take sulfide

measurements during the proteomics experiment, conditions with H₂ in the headspace yielded cultures with a 15–500 times higher relative abundance of the SRB *Desulfovermiculus*, compared to the N₂ condition (Fig. 2B). A different SRB, *Desulfohalobium*, was present in the N₂ condition at lower relative abundances. However, since no bin for this genus was retrieved from our metagenome, we were unable to map proteins to this organism. Together, these results indicate that either high levels of sulfide or the presence of the specific SRB *Desulfovermiculus* induced a distinct proteomic response by *Ca. M. hillemani*. Unique immune-response and DNA-repair proteins expressed by *Ca. M. hillemani* in the presence of *Desulfovermiculus* potentially represent novel adaptations to protect or repair DNA from SRB-related damage.

Additionally, reduced expression of metal uptake and chelation by *Ca. M. hillemani* in the N₂ 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 (Fig. S6) (60). However, the conversion of sulfate to sulfide by SRB can directly diminish the availability of cobalt through Co²⁺ - sulfide co-precipitation (61). Additionally, SRB utilize cobalt as an enzymatic cofactor for various metabolisms, including dissimilatory sulfate reduction (62, 63), which could lead to competition. Indeed, *Desulfovermiculus* expresses two cobalto-chelataes 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 H₂ is available.

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 mono-methylamine methyltransferases (MttB, MtbB, and MtmB, respectively) (64–66). Indeed, *Ca. M. hillemani* encodes the genes required for Pyl synthesis (PylBC) and use (Pyl-tRNA ligase). An mRNA secondary structure element, PYLIS (analogous to SECIS for selenocysteine), has been suggested to enable Pyl insertion into proteins (67, 68), and PYLIS elements were annotated within *Ca. M. hillemani*'s methylated-amine methyltransferase genes. Accordingly, Pyl residues were identified in peptides mapping to all three types of methylated-amine methyltransferases encoded by *Ca. M. hillemani*.

Kivenson et al. have recently proposed that some methanogens, including *M. mahii*, *Methanohalophilus euhalobius*, and *Methanohalophilus levihalophilus*, have reprogrammed the TAG codon to universally incorporate Pyl, instead of being read as a Stop-codon, across the proteome (69). While the authors confirmed their hypothesis for a marine and a human gut-associated methanogen, experimental data on halophilic methanogens were not yet available. Moreover, how and if microbes regulate the incorporation of Pyl at TAG codons is a topic of current debate (69, 70). Having already identified Pyl residues in peptides mapping to methyltransferase proteins, we used Kivenson's modification to Prodigal (69), 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 (69), are primarily read as Stop in *Ca. M. hillemani*.

High methane flux from the GSL

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 (Fig. S2). Methane fluxes were very high but also variable, with fluxes reaching 3 mmol CH₄/m²/day observed consistently across sampling dates (Fig. 4A and Fig. S7). These fluxes are comparable to high methane flux environments such as thawing permafrost (43, 71) and freshwater wetlands (72). The highest flux measured was 377 mmol CH₄/m²/day (Fig. S8), 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 m from the shoreline (Fig. 4A). This was counterintuitive, as we expected that the most anoxic and methanogen-conducive conditions would be right at the shoreline. However, this trend was observed across sampling dates and transects (Fig. S7).

Our results contrast with the only other study that measured methane flux from the GSL (20), which observed similar aquatic fluxes (0.42 ± 0.08 mmol CH₄/m²/day), but much lower terrestrial fluxes (0.47 ± 0.1 mmol CH₄/m²/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 the 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.

Novel methanogens likely contribute to the GSL 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-m sample, where extremely high and variable methane flux was observed (Fig. S8). 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. 4B). *Desulfovermiculus* was the most abundant genus of SRB (Fig. S9). *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 O₂ tolerance, consistent with the higher relative abundance of *Ca. M. hillemani* in the O₂ condition of 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 (version 138.2) could only identify these sequences as members of the order *Methanofastidiosales* (73), 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 (Fig. 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 data sets, the most abundant methanogenic sequences were poorly

characterized, indicating that novel methanogens may be the dominant community in the high-methane-flux GSL soil.

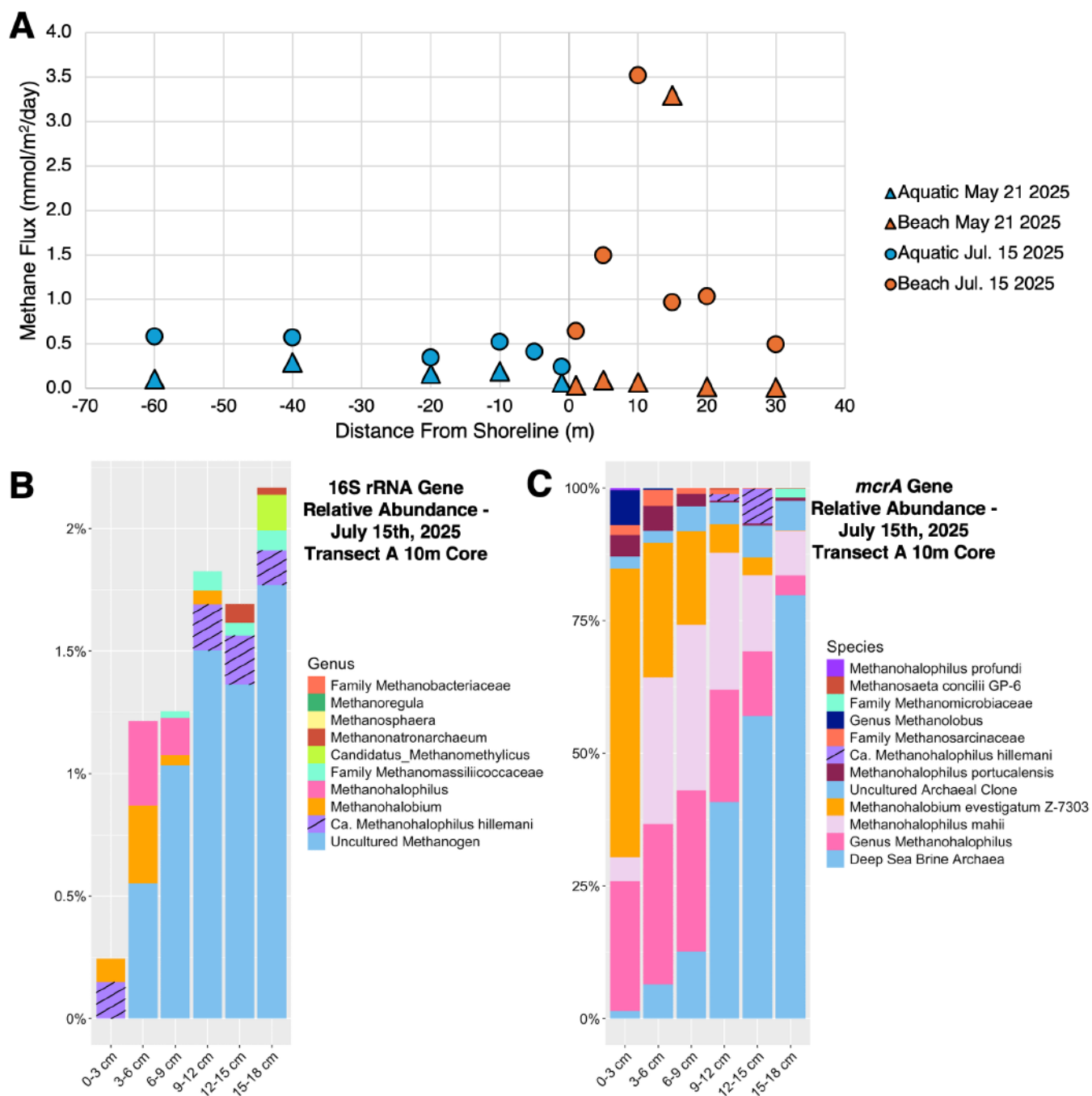


FIG 4 Methane flux and methanogenic community from Bridger Bay on the Great Salt Lake. (A) Methane fluxes measured from Transect A at Bridger Bay in May and July of 2025 show a zone of high terrestrial methane flux 10–15 m from the shoreline. (B) Relative abundance of different methanogens in a soil core taken from the Transect A 10 m flux site from July 15, 2025, based on 16S rRNA gene amplicon sequencing. 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 the 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 soil core samples as (B), corroborating the presence of *Ca. M. hillemani*. Other *Methanohalophilus* 16S rRNA genes 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.

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 osmotolerance 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 the protein activity or metabolome levels. Large differences in the expressed proteome were instead observed in the N₂ 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 chelatas. We hypothesize that with sufficient H₂, SRB directly compete for and reduce the availability of trace metals, forcing *Ca. M. hillemani* to invest in the 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 sulfide 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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William C. Christian, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review and editing | Zackary J. Jay, Data curation, Formal analysis, Validation, Visualization, Writing – review and editing | Nikola Tolic, Data curation, Formal analysis, Validation, Writing – review and editing | Carrie D. Nicora, Investigation | Reece Livingstone, Investigation | Stavros Trimmer, Investigation | Timothy R. McDermott, Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – review and editing | Roland Hatzenpichler, Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – review and editing

DATA AVAILABILITY

The metagenomic and amplicon sequencing data for this study are all publicly available and accessible through the NCBI under BioProject number [PRJNA1381644](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1381644). The raw metagenomic sequencing data are available under SRA accession numbers [SRR36460065](https://www.ncbi.nlm.nih.gov/sra/SRR36460065) for Illumina sequencing and [SRR36460064](https://www.ncbi.nlm.nih.gov/sra/SRR36460064) for Nanopore sequencing. The metagenome assembled genomes for *Ca. M. hillemani*, *Desulfovermiculus* sp., and *Halanaerobium* sp. can be found under the following BioSample accession numbers, respectively: [SAMN54119603](https://www.ncbi.nlm.nih.gov/biosample/SAMN54119603), [SAMN54119604](https://www.ncbi.nlm.nih.gov/biosample/SAMN54119604), and [SAMN54119605](https://www.ncbi.nlm.nih.gov/biosample/SAMN54119605). The 16S rRNA gene amplicon data from the *Ca. M. hillemani* enrichment cultures are available under the SRA accession numbers [SRR38289149](https://www.ncbi.nlm.nih.gov/sra/SRR38289149) through [SRR38289163](https://www.ncbi.nlm.nih.gov/sra/SRR38289163). The 16S rRNA gene amplicon data from the 15 July 2025, GSL soil core are under the SRA accession numbers [SRR38293099](https://www.ncbi.nlm.nih.gov/sra/SRR38293099) through [SRR38293104](https://www.ncbi.nlm.nih.gov/sra/SRR38293104). The *mcrA* gene amplicon data from the 15 July 2025, GSL soil core are available under the SRA accession numbers [SRR38293131](https://www.ncbi.nlm.nih.gov/sra/SRR38293131) through [SRR38293136](https://www.ncbi.nlm.nih.gov/sra/SRR38293136). The metaproteomic data from this study are publicly available in the MassIVE database under accession number [MSV000100495](https://massive.ucsb.edu/ViewMain.aspx?id=MSV000100495).

ADDITIONAL FILES

The following material is available [online](#).

Supplemental Material

Supplemental figures (Spectrum00412-26-S0001.docx). Figures S1 to S11.

Supplemental text (Spectrum00412-26-S0002.docx). Supplemental methods, results, and etymology.

Supplemental tables (Spectrum00412-26-S0003.xlsx). Tables S1 to S17, S19, and S22.

Table S18 (Spectrum00412-26-S0004.xlsx). QC 2D plots from the precursor mass spectra; chromatography and mass features.

Table S20 (Spectrum00412-26-S0005.xlsx). Log2 and CLR transformed peptide abundance values.

Table S21 (Spectrum00412-26-S0006.xlsx). De-logged Methnaohalophilus peptide abundances, normalized to the relative abundance of Methnaohalophilus in each sample.

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