Microbial metabolisms in a 2.5-km-deep ecosystem created by hydraulic fracturing in shales

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Hydraulic fracturing is the industry standard for extracting hydrocarbons from shale formations. Attention has been paid to the economic benefits and environmental impacts of this process, yet the biogeochemical changes induced in the deep subsurface are poorly understood. Recent single-genome investigations revealed that halotolerant microbial communities were enriched after hydraulic fracturing. Here, the reconstruction of 31 unique genomes coupled to metabolite data from the Marcellus and Utica shales revealed that many of the persisting organisms play roles in methylamine cycling, ultimately supporting methanogenesis in the deep biosphere. Fermentation of injected chemical additives also sustains long-term microbial persistence, while thiosulfate reduction could produce sulfide, contributing to reservoir souring and infrastructure corrosion. Extensive links between viruses and microbial hosts demonstrate active viral predation, which may contribute to the release of labile cellular constituents into the extracellular environment. Our analyses show that hydraulic fracturing provides the organisinal and chemical inputs for colonization and persistence in the deep terrestrial subsurface.

Shale gas accounts for one-third of natural gas energy resources worldwide. It has been estimated that shale gas will provide half of the natural gas in the USA, annually, by 2040, with the Marcellus shale in the Appalachian Basin projected to produce three times more than any other formation¹. Recovery of these hydrocarbons is dependent on hydraulic fracturing technologies, where the high-pressure injection of water and chemical additives generates extensive fractures in the shale matrix. Hydrocarbons trapped in tiny pore spaces are subsequently released and collected at the wellpad surface, together with a portion of the injected fluids that have reacted with the shale formation. The mixture of injected fluids and hydrocarbons collected is referred to as ‘produced fluids’.

Microbial metabolism and growth in hydrocarbon reservoirs has both positive and negative impacts on energy recovery. Whereas stimulation of methanogens in coal beds enhances energy recovery⁶, bacterial hydrogen sulfide production (‘reservoir souring’) decreases profits and contributes to corrosion and the risk of environmental contamination⁷. Additionally, biomass accumulation within newly generated fractures may reduce their permeability, decreasing natural gas recovery. Despite these potential microbial impacts, little is known about the function and activity of microorganisms in hydraulically fractured shale.

Initial work by our group and others⁴⁻⁹ used single marker gene analyses to identify microorganisms from several geographically distinct shale formations. These analyses showed similar halotolerant taxa in produced fluids several months after hydraulic fracturing. To assign functional roles to these organisms, we conducted metagenomic and metabolite analyses on input and produced fluids up to a year after hydraulic fracturing (HF) from two Appalachian basin shales, the Marcellus and Utica/Point Pleasant (Utica) formations. Although an earlier metagenomic study examined shale-produced fluids⁵, the microbial communities were only sampled for nine days after HF. Here, we have reconstructed the first genomes from fractured shale, examining the microbial metabolisms sustained in these engineered, deep subsurface habitats over a period of 328 days. We provide evidence for metabolic interdependencies, and describe chemical and viral factors that control life in these economically important ecosystems. Our results show microbial degradation of chemical additives, the potential for microbially induced corrosion and the formation of biogenic methane, all of which have implications for the sustainability of energy extraction.

Reconstruction of persisting shale genomes

Our earlier study surveyed microbial community structure in fluids from three hydraulically fractured Marcellus shale wells⁵. Five fluid samples from a single well were chosen for paired metagenomic and metabolite analyses, as these samples represented three phases of the energy extraction process. Fluid samples were collected from input materials, and at early (7 and 13 days) and late (82 and 328 days) time points (Fig. 1a) following HF. Microbial community changes during these phases corresponded to increasing salinity (Fig. 2 and Supplementary Table 1). From these Marcellus shale fluid samples we recovered 34 genomic bins, composed of 29 unique genomes (Fig. 1b and Supplementary Table 2). A high percentage of sequenced reads mapped to the assemblies (89–99%) (Supplementary Table 3), signifying that the underlying data were well represented. We also validated that the assembled genomes

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reflected the microbial identities and abundances in the unassembled reads, by comparing genome bin relative abundance to reconstructed near-full-length 16S rRNA genes11 (Supplementary Data File 1).

Consistent with our earlier taxonomic study, six halotolerant bacterial and archaeal members became enriched at later time points (82 and 328 days). We recovered six *Halanaerobium*, two Halomonadaceae, four *Marinobacter*, one *Methanohalophilus*, one *Methanolobus* and two bins from Halobacteroidaceae (Supplementary Table 2 and Supplementary Data File 2). Each of these taxa contain halotolerant and thermotolerant members. Environmental sequences closely related to 16S rRNA genes recovered here were similar to those also recovered from other hydrocarbon reservoirs or hypersaline environments (Supplementary Data File 3). For each of these persisting taxa (Fig. 1b) we obtained a representative genome that was at least 90% complete and, with the exception of *Marinobacter* (which contained several low-abundance *Marinobacter* strains), had less than 1% estimated contamination (Supplementary Table 4). The Halobacteroidaceae genus lacked closely related 16S rRNA genes (∼94% identity) and genomes (76% average nucleotide identity, ANI), suggesting this organism may be unique to shales (Supplementary Fig. 1). Following the naming convention for near-complete (>95% sampling) genomes from metagenomic data sets12, we propose the genus name *Candidatus* Frackibacter based on the colloquial name for hydraulic fracturing, or ‘fracking’. We infer that changes in membership at these later time points were due to growth of these specific taxa rather than DNA persistence in the environment, as cellular biomass increased in this well (Fig. 1b).

Emphasizing the persistence of specific taxa during energy extraction, members of the terminal community were identified from the input fluid through either identical genomes or closely related 16S rRNA genes (Fig. 1c). For instance, a *Halanaerobium* genome detected at both days 82 and 328 had an identical genome in the input fluid (ANI ∼99% with gene synteny) (Supplementary Table 5). Conversely, we did not detect lower-abundance members of the terminal community (for example, methanogens and *Candidatus* Frackibacter, <2% 16S rRNA gene

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**Figure 1 | Genomic sampling from hydraulically fractured Marcellus shale fluids over time.** a. Non-metric multidimensional scaling ordination of 16S rRNA gene data from our previous study5 show similar community trajectories across wells. The Marcellus fluid samples analysed here for metagenomic and metabolite analyses are indicated by green stars. b. Genome bin relative abundance at each time point, with taxa coloured according to the legend and persisting members outlined in a black box. For each time point, cell count data are overlaid with error bars representing the mean ± s.d. of technical replicates (n = 3). c. 16S rRNA gene relative abundance of persisting taxa present at low abundance in the input fluids. The star in the *Halanaerobium* orange bars (b,c) denotes an identical genome recovered from input, day 82 and day 328 samples.
Figure 2 | Quantification of metabolites identified by 1H NMR and chloride in fluids from Marcellus and Utica hydraulically fractured shales (for additional data see Supplementary Table 1). Time 0 on the x axis denotes input HF fluids, with the time the Utica well was shut in shown as grey shading. Marcellus fluids are denoted by solid lines and Utica fluids by dashed lines. For the Utica, initial chloride values were estimated based on the freshwater source (grey dashed line). MMA concentrations are shown with two axes, with the left axis for Marcellus fluids (solid lines) and the right axis for Utica fluids (dashed lines).

abundance) in the input fluids, probably because they were below our detection limit (Supplementary Data File 1b). This finding is the first to demonstrate that HF creates a habitat where low-abundance microorganisms are injected into the deep subsurface, bloom, and persist despite biocide addition14, elevated temperatures (65 °C) and pressures (at least 25 MPa), and salinities that ultimately become briny.

**Glycine betaine and chemical additives fuel methanogenesis**

Halite dissolution from the shale matrix drives large salinity increases in the produced fluids5, so organisms must have adaptations for tolerating a broad salinity range (Fig. 2). We recovered multiple osmoregulation strategies from all genomes (Supplementary Table 6 and Supplementary Discussion). Our metabolite data show that, of the known osmoprotectants43, glycine betaine (GB) was present in the fluids, but mannitol, sorbitol, ecotaine and trehalose were not detected (Supplementary Table 1). Consistent as a response to salinity, GB was below detection in the input and early Marcellus shale fluids, but reached a maximum concentration at day 82 that was maintained at day 328 (Fig. 2).

Uptake and de novo synthesis of GB were features encoded in all near-complete genomes recovered over the last two time points. GB synthesis is encoded in the Methanolphilus genome by a glycine pathway (via sarcosine and dimethylglycine intermediates) and in the Halomonadaceae and Marinobacter genomes by a pathway from choline (Supplementary Discussion). Choline, a common chemical additive in fracturing fluids, was exogenously provided in the input fluids and consumed by day 13 when Marinobacter became abundant (Fig. 1b and Supplementary Table 1). Collectively, our paired metagenomic and metabolite findings show the production and uptake of GB is a halotolerance mechanism widely used by organisms in fractured shales.

Microbially synthesized GB, available extracellularly in the fluids, may be degraded by both of the recovered obligate fermenters (Halanaerobium, Candidatus Frackibacter) (Supplementary Discussion). Candidatus Frackibacter has two mechanisms for reducing GB. The first demethylates GB and oxidizes the methyl group via the Wood–Ljungdahl pathway, producing trimethylamine (TMA; Supplementary Fig. 2)15. The second pathway, also present in some shale Halanaerobium genomes, uses a GB reductase (gdHII), producing TMA and acetate via a Stickland reaction16 (Supplementary Fig. 3). Notably, GB reduction is not widely encoded in isolated Halanaerobium genomes, being present in only 36% of the published genomes (5 of 14, http://img.jgi.doe.gov/, April 2016). In the Marcellus shale, a persisting Halanaerobium (Halan-2; T82, T328) genome is the only one of our three capable of GB reduction. GB fermentation using microbially produced metabolites, rather than a dependency on input fluid chemicals, may sustain life in shales long after hydraulic fracturing.

GB fermentation yields TMA, which we infer is rapidly consumed by methanogens present at the last two time points. Each of the recovered shale methanogen genomes (Methanobus and Methanolphilus) has pathways for utilizing TMA, dimethylamine (DMA), monomethylamine (MMA) and methanol, but cannot use GB, hydrogen/carbon dioxide or acetate (Supplementary Discussion). In addition to microbial synthesis, HF input fluids also contain high concentrations of methylotrophic substrates (1.2 mM MMA and 331 µM methanol) that could support methanogenesis (Fig. 2). It is possible that these compounds are also assimilated as a nitrogen source (MMA) or are oxidized by Pseudomonas and Marinobacter (methanol) at earlier time points57. Although Methanolphilus 16S rRNA genes have been reported from Antrim59 and Burket/Geneseo1 fractured shales, our genomic and metabolite findings identify the endogenous and exogenous sources of methylotrophic substrates, show their co-occurrence with methanogens, and confirm the metabolic pathways for methanogenesis.

**Metabolisms impacting energy extraction**

In addition to containing substrates that could support biogenic methane production, HF input fluids contain high concentrations
of organic substrates such as sucrose (0.3 mM) and ethylene glycol (3.6 mM) (Fig. 2 and Supplementary Table 1). The capacity to respire sucrose is widely encoded in our genomes (for example, *Pseudomonas* and *Marinobacter*), consistent with its consumption by day 7. Ethylene glycol is consumed over time, and is not detected at the last time point. This substrate is probably aerobically oxidized by *Marinobacter* and *Vibrio* at the early time points (alcohol dehydrogenase), and fermented by *Halanaerobium* (propanediol dehydratase, acetaldehyde dehydrogenase) later to yield ethanol, hydrogen and acetate. *Candidatus Frackibacter* also has the capacity to produce acetate via GB fermentation, homoacetogenesis (*H*₂/*CO₂) and sugar respire sucrose is widely encoded in our genomes (for example, *Arcobacter* and *Halanaerobium* acetate increased at day 82, when *Halanaerobium* fermentation at later time points, ethanol and sulfate-reducing metabolic potential. However, we did not identify any sulfate-reducing genes in the Marcellus data set, confirming the presence of dissimilatory sulfate-reducing genes (using three copies of the rhodanese-like thiosulfate:cyanide sulfur-transferase gene⁹), implicating a role for shale *Halanaerobium* in steel corrosion and reservoir souring. Additionally, the near-complete Halomonadaceae genome also encodes multiple thiosulfate sulfur-transferring enzymes, which while not previously reported in these taxa, are implicated in thiosulfate disproportionation, producing sulfide and sulfite. Current microbial corrosion diagnostic practices often rely on detecting the presence of dissimilatory sulfate-reducing genes or measuring sulfate-reducing metabolic potential. However, we did not identify any sulfate-reducing genes in the Marcellus data set, suggesting the need to include alternative biological mechanisms of sulfide production for characterizing microbial corrosion potential.

Owing to the economic importance of hydrocarbons, we analysed shale-produced fluids for degradation pathways, and confirmed the presence of benzene, toluene, ethylbenzene, xylene and naphthalene (BTEX-N) in all fluid samples, while decane was detected in all but the input. We failed to recover any genes for anaerobic hydrocarbon degradation, but the near-complete *Marinobacter*, Halomonadaceae, *Pseudomonas* and *Vibrio* genomes (input or early samples) had the capacity for aerobic hydrocarbon oxidation (Supplementary Discussion).

Figure 3 | Genomic links between viruses and their microbial hosts. Network of viral-host CRISPR links with host genomes represented by ovals, viral contigs by diamonds and closed viral genomes by circles. Colours represent sampling times. Clouds of colour indicate host taxonomy (Fig. 1b). Each edge represents a perfect match between a host CRISPR loci spacer sequence and a viral contig. Numbers in diamonds and circles denote the contig numbers.
including Barnett\(^6\) and Marcellus\(^7\) shale-produced fluids. Of our recovered genomes, these two encode the greatest metabolic versatility, enabling the use of a wide range of carbon sources (for example, acetate, lactate and hexose sugars) with O\(_2\) or nitrate as possible electron acceptors. However, given the lack of detectable nitrate (Supplementary Table 1), we postulate that these facultative anaerobes utilize fermentative metabolisms once dissolved O\(_2\) associated with GB has been depleted\(^24,25\).

**Active viral predation in the deep subsurface**

Our results indicate that viral-mediated cell lysis is a mechanism to explain how an intracellular osmoosmootectant, like GB, was detected extracellularly in fluids. We recovered 331 viral contigs including 21 closed, circular viral genomes (Supplementary Fig. 5). A comparison of contigs across samples showed that 318 viral contigs were unique, with only 13 viral contigs shared across time points. Of the viral contigs, 86% belonged to members of the Caudovirales, tailed dsDNA viruses, with Myoviridae (44%) and Siphoviridae (26%) families predominating. Previously, only viral reads and prophage genome fragments have been reported\(^10,26,27\).

We mined our microbial genomes for the presence of CRISPR-Cas systems (clustered regularly interspaced short palindromic repeat-CRISPR associated), which act as an acquired immunity to viruses\(^28\) (Supplementary Table 8). CRISPR-Cas frequency estimates range from 81% of archaean and 40% of bacterial genomes in cultivated microbes\(^29\), to 10% of archaeal and bacterial genomes in metagenomic data sets\(^30\). In contrast, 100% of the three archaeal and 84% of the 31 bacterial genome bins of the Marcellus samples had evidence of a CRISPR-Cas system, with type 1 being the most prevalent (Supplementary Table 8). In fact, all microbial genomes at the last time point had a CRISPR-Cas system, signifying that viral immunity may be an important adaption for persistence in hydraulically fractured shales.

Comparing CRISPR arrays in microbial hosts to viral contig sequences allowed us to reconstruct a history of viral encounters, and link 34 viral contigs to 11 microbial genomes (Fig. 3 and Supplementary Fig. 5). Before our findings, the greatest number of reported CRISPR links within a data set was five, from a three-year study in a hypersaline lake\(^11\). Our data showed that viral host specificity varied, with viruses linked to multiple species within a genus (for example, *Halanaerobium, Marinobacter*) and a single viral genome linked to two methanogen genera (Fig. 3). We observed an increase in the number of CRISPR spacers within two *Halanaerobium* genomes between days 82 and 328, demonstrating that adaptive viral resistance probably occurred during this time span (Supplementary Fig. 6 and Supplementary Table 8). Our metagenomic data demonstrate that viral predation and host-acquired immunity are active processes in the deep terrestrial subsurface.

**Strain metabolic diversity across shales**

We examined fluid metabolites collected over 302 days after HF from the Utica shale, a geographically and stratigraphically distinct Appalachian Basin formation. Despite these differences, metabolic trends in the Marcellus and Utica produced fluids were similar. For instance, methanol and ethylene glycol were detected in input fluids and salinity increased over time in both shales (Fig. 2). Unlike the Marcellus, MMA was not detected in the input but was produced over time in the Utica produced fluids, suggestive of ongoing GB production, fermentation and subsequent methanogenesis. However, due to the chemical complexity of the Utica produced fluids, we could not confirm the presence of GB.

To validate that fractured shales harbour microorganisms that produce methane from GB fermentation products, we amended Utica produced fluids with GB. The produced fluid sample was collected 96 days after HF, comparable to our Marcellus sample, where the co-occurrence of GB fermenting and methanogenic organisms was first detected (day 82). Relative to the produced fluids, the addition of GB enriched for *Methanohalophilus* (70%) and three *Halanaerobium* genomes (∼21, 3 and 0.5%) (Supplementary Fig. 7). The presence of a GB reductase system probably explains the changes in relative abundance within these *Halanaerobium*, as the dominant genome in the produced fluids lacked grdl (decreasing from 51 to 3%). This finding demonstrates the power of genome-centric metagenomics to partition local microdiversity, explaining the co-occurrence of strains with distinct functional roles.

In the enrichment, GB fermentation produced TMA, DMA and MMA in low amounts, probably due to active consumption by *Methanohalophilus* (Supplementary Tables 9 and 10). Compared to the unamended control, GB addition produced 6.5 times more methane per day. Collectively, our Marcellus field and Utica laboratory data provide evidence that GB synthesis and subsequent fermentation supports biogenic CH\(_4\), in hydraulically fractured shales.

Comparative genomics showed that the dominant *Methanohalophilus* and *Halanaerobium* near-complete genomes (Supplementary Table 2) in the Utica enrichment were closely related strains\(^32\) to the Marcellus genomes (∼99% ANI) (Supplementary Table 5 and Supplementary Data File 2). In contrast, ANI values between the Marcellus and Utica *Methanohalophilus* and other sequenced species (*M. mahii* and *M. halophilus*, both isolated from surface waters), were ∼91 and 92%, respectively. *Methanohalophilus* CRISPR array comparisons identified a single spacer sequence shared between the Marcellus produced fluids and Utica GB enrichment genomes; the two other non-shale-derived *Methanohalophilus* genomes lacked CRISPR-Cas systems. Two viral contigs also had high sequence identity (>95%), showing that these shales share genetically similar viruses. Together, our data demonstrate that environmental filtering results in populations, metabolisms and viral processes shared between these two geographically distinct fractured shale ecosystems.

**Conclusion**

Resolving genomes from Marcellus and Utica produced fluids unveiled microbial metabolisms, adaptations and viral predation resistance mechanisms in fractured shales. From 16S rRNA gene analyses we could not have predicted the role *Halanaerobium* strains play in fermenting GB and HF chemical additives such as ethylene glycol, nor would we have associated the Halomonadaceae with detrimental sulphide production. Our genomic analyses show that closely related strains are niche differentiated. For instance, GB addition selected for the only *Halanaerobium* genome with GB reduction capacity. We identified the metabolic capabilities of *Candidatus Frackibacter*, unique to fractured shales, which can also ferment GB. Our metagenomic data revealed a possible role for viruses in the top-down (predation and lysis) and bottom-up (release of cellular contents; for example, GB) control of microbial communities in fractured shale. Notably, unlike earlier studies, all host genomes recovered at the last time point contained a CRISPR-Cas system. We also identified active host responses to viral predation, including new spacer incorporation over time. Together, our viral findings demonstrate the probable importance of CRISPR-Cas-mediated immunity for microbial persistence in fractured shales.

Here, we show that hydraulic fracturing provides the organisms, chemistry and physical space to support microbial ecosystems in ∼2,500-m-deep shales (Fig. 4). Ultimately, our metagenomic and metabolite results indicate that adaptation to high salinity, metabolism in the absence of oxidized electron acceptors, and viral predation are potential controlling factors mediating long-term microbial metabolism during energy extraction from fractured shales. This study highlights the resilience of microbial life to adapt to, and colonize, a habitat structured by physical and chemical features very different from their origin.
H3C
Methylamines and methanol in the input fluids from other shale produced Halanaerobium from other shale produced Frackibacter and Halomonadaceae have the potential to aerobically oxidize hydrocarbons and respire sugars using nitrate and oxygen as electron acceptors.

Figure 4 | Interconnected metabolisms catalysed by persisting microorganisms in hydraulically fractured shales. a, HF input fluids from both Marcellus and Utica shales contain substrates that sustain microbial metabolism. Parentheses indicate metabolites detected in one shale. b, Microorganisms in shales adapt to high salinities by producing and using osmoprotectants such as GB (red circles), which can be released into fluids by viral lysis. c, Marinobacter and Halomonadaceae have the potential to aerobically oxidize hydrocarbons and respire sugars using nitrate and oxygen as electron acceptors. d, Candidatus Frackibacter and Halanorobium ferment GB, yielding trimethylamine, which supports methanogenesis by Methanohalophilus and Methanolobus (blue box). Methylamines and methanol in the input fluids can also support methanogenesis (yellow box).

Methods
Sample collection and fluid chemistry. Our earlier study characterized some of the geochemistry and conducted 16S rRNA gene surveys from fluids collected (June 2012 to May 2013) from three Marcellus gas wells located in Pennsylvania, USA\(^2\). Hydraulic fracturing (input, noted as T0) and shale-produced fluids were collected from well heads (days 3–14) and gas–fluid separators (49, 82 and 328 days), with fluids from well 1 used for more detailed metagenomics and NMR metabolite analyses here. For our Utica samples, injected fluids and produced fluids from gas–fluid separators\(^6,8,9\) were collected between July 2014 and May 2015 from an oil–gas well in Ohio, USA. The gas–fluid separators at the Marcellus and Utica sites had a capacity of ∼5,560 l, approximately half gas and half produced fluids for the input samples. Technical duplicates for NMR analyses were highly similar, and a discrepancy for the input samples is probably due to unmeasured cations (for example, Al, Fe, Mg, Ca, Na, K, Ba, NH\(_4\), NO\(_3\), SO\(_4\)) were acidified immediately after filtration to ∼0.5% with nitric acid and then analysed using a Perkin Elmer Optima 4300DV inductively coupled plasma optical emission spectrometer. The charge balance discrepancy for the input samples is probably due to unmeasured cations (for example, ammonium and organic additives in the fracturing fluids), and has been documented in other studies\(^8\).

Marcellus and Utica fluid samples (paired to our metagenomic samples) were sent to the Pacific Northwest National Laboratory for NMR metabolite analysis. For the Utica input samples, technical duplicates for NMR analyses were highly similar, with mean concentrations reported (Supplementary Table 1). Samples were diluted by 10% (vol/vol) with 5 mM 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal standard.
internal standard. All NMR spectra were collected using a Varian Direct Drive 600 MHz NMR spectrometer equipped with a 5 mm triple resonance, salt-tolerant cold probe. The 1D 1H NMR spectra of all samples were processed, assigned, and analysed using Chenomx NMR Suite 8.1 with quantitation based on spectral intensities relative to the internal standard. Candidate metabolites present in each of the complex mixtures were identified by matching the chemical shift, J-coupling and intensity information of experimental NMR signals against the NMR signals of standard metabolites in the Chenomx library. The 1D 1H spectra were collected following standard Chenomx data collection guidelines, using a 1D nuclear Overhauser effect spectroscopy (NOEYS) presaturation experiment with 65,536 complex points and at least 512 scans at 298 K. Additionally, 2D NMR spectra (including 1H-15N HSQC, 1H-13C HSQC, 1H-1H total correlation spectroscopy (TOCSY)) were acquired on most of the fluid samples, aiding in the 1D 1H assignments of acetate, ethanolamine, methanol and MMA.

Because of its significance in this work as an intermediate linking GB fermentation to methanogenesis, MMA was confirmed in a series of 1H NOSY and 2D 1H–31P HSQC NMR spectra, where ‘spiking’ of several different samples was made using an MMA standard. Two additions of ~25 mM MMA were made to fluid samples, and only the assigned MMA peak (1H chemical shift ∼2.62 ppm and 31P chemical shift ∼27.7 ppm) increased in intensity. GB concentrations were too low for confirmation with 2D NMR experiments in the produced fluids. The GB in the Marcellus produced fluid sample series was resolvable and quantified by only the ∼3.30 ppm 1H resonance but not at ∼3.92 ppm due to spectral overlap with ethanolamine. This was confirmed by spiking using a GB standard. In the Utica produced fluids, both resonances (∼3.27 and ∼3.92 ppm 1H) were identifiable. GB enrichment could then be resolved and quantified in enrichment cultures with Utica fluids supplemented with GB (10 mM), and in controls not amended with GB, largely due to the dilution of ethanolamine and other confounding compounds. To compare temporal trends in the key metabolites between the Marcellus and Utica input and produced fluids in Fig. 2, each metabolite concentration was graphed in R over time for both wells with the same x axis (time, days).

**Genetic analysis using Illumina sequencing.** For genomic sample collection, 300–1,000 ml samples were concentrated to 0.22-μm pore size polyethersulfone (PES) filters (Millipore, Fisher Scientific). Viruses were probably contaminated on filters by flocculation with iron, which precipitated during the filtering process when the samples were first exposed to oxygen. Total nucleic acids were extracted from the filter using the PowerSolv DNA Isolation kit (MoBio) for Marcellus fluids and a modified phenol chloroform nucleic acid extraction for Utica fluids and enrichment cultures. Total cells with intact membranes were enumerated from unfiltered fluid samples for calibrated gate ranges on a Guava EasyCyte flow cytometer (EMD Millipore). Briefly, samples were fixed with 1% glutaraldehyde and stained with 0.1% SYBR Gold (Life Technologies), and quantified via flow-cytometry. For each time point, technical triplicates were measured and the data reported in Fig. 1 represent the mean ± s.d. (n = 3).

For the Marcellus input and produced fluids, Illumina HiSeq 2000 libraries were prepared using the Nugen Ovation Ultralow Library System following the manufacturer’s instructions. Genomic DNA was sheared by sonication, and fragments were end-repaired. Sequencing adapters were ligated and library fragments were amplified with ∼8–10 cycles of PCR before Pippin Prep size selection. After size selection, quantification and validation (Illumina HiSeq platform for paired-end reads 113 cycles were collected). Fastq files were generated using CASSAVA 1.8.2. Similar protocols were used for the Utica fluid GB enrichment culture, where sequencing was conducted on an Illumina HiSeq 2500 platform using a Kapa Hyper Prep library system with five cycles of PCR before solid-phase reversible immobilization (SPRI) size selection.

All metagenomics methods and scripts contributing to analyses in this manuscript are included in Supplementary Data File 4. Briefly, Illumina sequences from each of the five samples (input, T7, T13, T82 and T328) were first trimmed from both the 5′ and 3′ ends using Sickle (https://github.com/najoshi/sickle), then each sample was demultiplexed and uniquely assigned to samples using default parameters. Scaffold coverage was calculated by mapping reads back to the assemblies using Bowtie2 (ref. 37). Given the dominance and high strain variation in some samples, highly abundant genomes (>400×) often failed to assemble. Using an approach outlined in ref. 38, subsambases were performed to reconstruct the dominant genomes in the day 13 and day 82 samples using high-quality reads (≥1 kb) and paired reads, respectively. Results from the subassemblies are included (Supplementary Table 2).

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**Genetic analysis using Illumina sequencing.** For genomic sample collection, 300–1,000 ml samples were concentrated to 0.22-μm pore size polyethersulfone (PES) filters (Millipore, Fisher Scientific). Viruses were probably contaminated on filters by flocculation with iron, which precipitated during the filtering process when the samples were first exposed to oxygen. Total nucleic acids were extracted from the filter using the PowerSolv DNA Isolation kit (MoBio) for Marcellus fluids and a modified phenol chloroform nucleic acid extraction for Utica fluids and enrichment cultures. Total cells with intact membranes were enumerated from unfiltered fluid samples for calibrated gate ranges on a Guava EasyCyte flow cytometer (EMD Millipore). Briefly, samples were fixed with 1% glutaraldehyde and stained with 0.1% SYBR Gold (Life Technologies), and quantified via flow-cytometry. For each time point, technical triplicates were measured and the data reported in Fig. 1 represent the mean ± s.d. (n = 3).

For the Marcellus input and produced fluids, Illumina HiSeq 2000 libraries were prepared using the Nugen Ovation Ultralow Library System following the manufacturer’s instructions. Genomic DNA was sheared by sonication, and fragments were end-repaired. Sequencing adapters were ligated and library fragments were amplified with ∼8–10 cycles of PCR before Pippin Prep size selection. After size selection, quantification and validation (Illumina HiSeq platform for paired-end reads 113 cycles were collected). Fastq files were generated using CASSAVA 1.8.2. Similar protocols were used for the Utica fluid GB enrichment culture, where sequencing was conducted on an Illumina HiSeq 2500 platform using a Kapa Hyper Prep library system with five cycles of PCR before solid-phase reversible immobilization (SPRI) size selection.

All metagenomics methods and scripts contributing to analyses in this manuscript are included in Supplementary Data File 4. Briefly, Illumina sequences from each of the five samples (input, T7, T13, T82 and T328) were first trimmed from both the 5′ and 3′ ends using Sickle (https://github.com/najoshi/sickle), then each sample was demultiplexed and uniquely assigned to samples using default parameters. Scaffold coverage was calculated by mapping reads back to the assemblies using Bowtie2 (ref. 37). Given the dominance and high strain variation in some samples, highly abundant genomes (>400×) often failed to assemble. Using an approach outlined in ref. 38, subsambases were performed to reconstruct the dominant genomes in the day 13 and day 82 samples using high-quality reads (≥1 kb) and paired reads, respectively. Results from the subassemblies are included (Supplementary Table 2).
GB enrichment and control cultures were monitored for control, 30:70 produced with 9.7 mM GB and a trace element solution (DSMZ 141). As a no-donor GB pH of 7.1 using 1 mM NaOH. After autoclaving, this medium was supplemented utilizing and methane-producing organisms. The GB enrichment consisted of 30% incubation without amendment, and before and after amendment to stimulate GB functional prediction via modelling, catalytic or structural residues, or homology-based approach to help annotate genes. The latter is important, as numbers and E.C. number with positive records saved to ProtPipeliner, a python script developed in-house for generation of acid sequences were pulled from the Marcellus and Utica GB enrichment genomic data over time were graphed using iTOL. For the S3 protein tree, amino-acid sequences were aligned using MUSCLE version 3.8.31 and run through ProtPipeliner, a python script developed in-house for generation of phylogenetic trees (https://github.com/insolvendi/protpipeliner). A maximum likelihood phylogeny for the alignment of 53 ribosomal proteins was conducted using RAxML version 8.3.1 under the LG+γ model of evolution with 100 bootstrap replicates and visualized in iTOL. The 16S rDNA sequences for the key terminal taxa in Fig. 1 were also blasted to NCBI to show the relationship between key terminal taxa in this study and environmental sequences. Sequences were trimmed using the V3-V4 region, and the top ten non-redundant hits from NCBI were included in the analysis. 16S rDNA gene sequences were aligned in Geneious R8 using MUSCLE, and a phylogenetic tree was constructed with RAxML 7.2.8 (GTR Gamma nucleotide model, 999 bootstrap replicates) (Supplementary Data File 2). Sequences were aligned using MUSCLE version 3.8.31 and run through ProtPipeliner, a python script developed in-house for generation of phylogenetic trees (https://github.com/insolvendi/protpipeliner). A maximum likelihood phylogeny for the alignment of 53 ribosomal proteins was conducted using RAxML version 8.3.1 under the LG+γ model of evolution with 100 bootstrap replicates and visualized in iTOL. The 16S rDNA sequences for the key terminal taxa in Fig. 1 were also blasted to NCBI to show the relationship between key terminal taxa in this study and environmental sequences. Sequences were trimmed using the V3-V4 region, and the top ten non-redundant hits from NCBI were included in the analysis. 16S rDNA gene sequences were aligned in Geneious R8 using MUSCLE, and a phylogenetic tree was constructed with RAxML 7.2.8 (GTR Gamma nucleotide model, 999 bootstrap replicates) (Supplementary Data File 3).

Phylogenetic and metabolic analyses. The 16S rDNA genes recovered from the five nearest neighbours to each EMIRGE sequence from our Marcellus metagenomic samples were obtained from SILVA (release 125)37, anchored with cultivated representatives (Supplementary Data File 1). 16S rDNA gene sequences were aligned in Geneious R8 using MUSCLE, a phylogenetic tree was constructed with RAxML 7.2.8 (GTR Gamma nucleotide model, 999 bootstrap replicates), and relative abundance data over time were graphed using iTOL. For the S3 protein tree, amino-acid sequences were pulled from the Marcellus and Utica GB enrichment genomic bins and were augmented with sequences mined from NCBI and IGI IMG databases (Supplementary Data File 2). Sequences were aligned using MUSCLE version 3.8.31 and run through ProtPipeliner, a python script developed in-house for generation of phylogenetic trees (https://github.com/insolvendi/protpipeliner). A maximum likelihood phylogeny for the alignment of 53 ribosomal proteins was conducted using RAxML version 8.3.1 under the LG+γ model of evolution with 100 bootstrap replicates and visualized in iTOL. The 16S rDNA sequences for the key terminal taxa in Fig. 1 were also blasted to NCBI to show the relationship between key terminal taxa in this study and environmental sequences. Sequences were trimmed using the V3-V4 region, and the top ten non-redundant hits from NCBI were included in the analysis. 16S rDNA gene sequences were aligned in Geneious R8 using MUSCLE, and a phylogenetic tree was constructed with RAxML 7.2.8 (GTR Gamma nucleotide model, 999 bootstrap replicates) (Supplementary Data File 3).

Metabolic profiling was largely conducted by manual analyses. For the osmoprotectants inventory, the annotated gene lists were searched by name, KEGG number and E.C. number with positive records saved to files that were manually inspected to remove misidentified genes. The results were compared to the same functions in available genomes from the same genus in the IMG database on 15 August 2015 (http://img.jgi.doe.gov/). For key functional genes we used both a list and a homology-based approach to help annotate important, as many methylamine cycling genes were incorrectly annotated or not included on scaffolds >5,000 bp. Putative GdeG/PraA were identified from the Utica GB enrichment by blasting known homologues capable of glycine/sarcosine/GB/proline reduction. The MttB and GrdEGI/PrdA trees were constructed similarly to above by aligning manually curated amino sequences in MUSCLE with RAxML 7.2.8 (GTR Gamma nucleotide model, 999 bootstrap replicates) (Supplementary Data File 3).

GB enrichment culture from Utica produced fluids. NMR metabolite analyses were performed on Utica produced fluids that had been filtered and stored at ~80 °C since the time of collection, after 483 days of anoxic (100% N2 headspace) induced incubation without amendment, and before and after amendment to stimulate GB utilization of the producing organisms. GB enrichment culture from Utica produced fluids was used as an example for metabolic profiling. After autoclaving, 30% of the produced fluids were used to recover the dominant Halanaerobium genomic bin. All scaffolds in the assembly, ranging in lengths of 45 kb and 500 kb, were searched for homologues for MttB, Grd, and methanogenesis pathways, CRISPR arrays were only analysed for the binned scaffolds (nucleotide ≥ 1 kb).

Accession codes. Sequencing data have been deposited in the NCBI sequence read archive under project PRJNA38326. The near complete representative genomes from Candidatus Frackbacuter-2, Halanaerobium-1, Balomonadaceae-1, Idiomarina-1, Marinobacter-3 population, Methanohlhalophilus and Methanobobus have been assigned accession numbers SAMN04432553, SAMN04417677, SAMN04432558, SAMN04432559, SAMN04432574, SAMN04432578 and SAMN04432593, respectively, and therefore, the methanogenic genomes recovered from GB enrichment have been assigned accession numbers SAMN05172726 and SAMN05172790. The 16S rDNA 454 pyrotags from our previous study67 can be accessed from the NCBI under Bioproject accession number PRJNA296885, with biosample numbers SAMN02441908 to SAMN02441927. Additionally, genomic information (annotation, nucleotide and amino-acid files for each genome listed above), the FASTA files used in any phylogenetic analyses and viral genomes/contigs, and all EMIRGE 16S rDNA sequences are provided at https://chimerasc.ohio-state.edu/daily_et_al_nature.html.

Received 17 May 2016; accepted 15 July 2016; published 5 September 2016

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