Title: Deep Subsurface Pressure Stimulates Metabolic Plasticity in Shale-Colonizing *Halanaerobium*

Running Title: *Halanaerobium* pressure growth

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Abstract

Bacterial *Halanaerobium* strains become the dominant persisting microbial community member in produced fluids across geographically distinct hydraulically fractured shales. *Halanaerobium* is believed to be inadvertently introduced into this environment during the drilling and fracturing process and must therefore tolerate large changes in pressure, temperature, and salinity. Here, we used a *Halanaerobium* strain isolated from a natural gas well in the Utica Point Pleasant formation to investigate metabolic and physiological responses to growth under high-pressure subsurface conditions. Laboratory incubations confirmed the ability of *H. congolense* strain WG8 to grow under pressures representative of deep shale formations (21-48 MPa). Under these conditions, broad metabolic and physiological shifts were identified, including higher abundances of proteins associated with the production of extracellular polymeric substances. Confocal laser scanning microscopy indicated that EPS production was associated with greater cell aggregation when biomass was cultured at high-pressure. Changes in *Halanaerobium* central carbon metabolism under the same conditions were inferred from NMR and gas chromatography measurements, revealing large per-cell increases in production of ethanol, acetate and propanol and cessation of hydrogen production. These metabolic shifts were associated with carbon flux through 1,2 propanediol in response to slower fluxes of carbon through stage 3 of glycolysis. Together, these results reveal the potential for bio-clogging and corrosion (via organic acid fermentation products) associated with persistent *Halanaerobium* growth in deep, hydraulically fractured shale ecosystems, and offer new insights into cellular mechanisms that enable these strains to dominate deep shale microbiomes.

Importance
The hydraulic fracturing of deep shale formations for hydrocarbon recovery accounts for approximately 60% of US natural gas production. Microbial activity associated with this process is generally considered deleterious due to issues associated sulfide production, microbially-induced corrosion, and bio-clogging in the subsurface. Here we demonstrate that a representative Halanaerobium species, frequently the dominant microbial taxa in hydraulically fractured shales, responds to pressures characteristic of the deep subsurface by shifting its metabolism to generate more corrosive organic acids and produce more polymeric substances that cause ‘clumping’ of biomass. While the potential for increased corrosion of steel infrastructure and clogging of pores and fractures in the subsurface may significantly impact hydrocarbon recovery, these data also offer new insights for microbial control in these ecosystems.

Introduction

The hydraulic fracturing (HF) of subsurface formations to release economically important hydrocarbons generates extensive fracture networks in these deep subsurface ecosystems. Shales are thought to be almost sterile prior to HF due to a range of factors, including prior ‘paleo-pasturization’ coupled with extremely low levels of permeability and nanometer-sized pores that physically preclude the development of microbial ecosystems (1). However, microorganisms present in fluids that are injected into newly developed fracture networks during HF are able to colonize the system and persist over extended periods of time (2–4). Thus, microorganisms existing under ambient surface conditions are suddenly exposed to (and must respond to) dramatically different physicochemical conditions in deep shale fracture networks characterized by anoxia, high temperatures, increasing salinity, and elevated pressures.

Prior work by our research group and others has demonstrated that microorganisms associated with the genus Halanaerobium are dominant persisting members of shale communities across geographically distinct shale plays (2–6). Halanaerobium are frequently low-abundance community members (~ 1%) in fracturing fluids, but out-compete other taxa to become enriched in later produced fluid samples (2).
These gram-positive microorganisms have also been observed in other saline environments, including conventional oil and gas reservoirs, and are able to grow on a range of carbon substrates including sugars and guar gum (7). Importantly, these microorganisms occupy key roles in inferred metabolic networks that sustain microbial life in shale ecosystems, centered on the cycling of osmoprotectants and methylene compounds (2, 8). The growth of such microorganisms in fractured shales is commonly viewed as deleterious, due to studies indicating that *Halanaerobium* are able to catalyze thiosulfate-dependent sulfidogenesis (5), grow on additive chemicals present in input fluids (2, 7, 8), and potentially form biofilms in the subsurface. These processes could directly contribute to biofouling in the fracture network, leading to significant decreases in reservoir permeability and associated hydrocarbon recovery. While such processes are undesirable where hydrocarbons are being extracted, reductions in permeability in other geologic systems (e.g., sealing cap rock in geologic CO₂ sequestration reservoirs) may be beneficial (9).

Studies have shown that the salinity of fluids within the shale fracture network rapidly increases over the first ~ 75 days following the HF process, due to the dissolution of solid phase salt minerals in the deep subsurface (8). Microorganisms in the fracture networks may protect themselves against high-salinity conditions through the import of ions (e.g., K⁺) or the utilization of osmoprotectant compounds (e.g., glycine betaine) that maintain intracellular cell turgor pressure (2, 10). While the mechanisms of microbial tolerance to high-pressure are less well understood, recent experiments have indicated that utilization of osmoprotectants and intracellular tolerance to high salt concentrations may stabilize protein structure due to increases in hydrophobic interactions and reduced water activity (11, 12).

Here, we used a *Halanaerobium congolense* strain (WG8) isolated from produced waters from a hydraulically fractured well in the Utica Point Pleasant formation, OH, USA, to investigate cellular responses to high-pressure conditions characteristic of the deep terrestrial subsurface. Using high-pressure growth reactors, shotgun proteomic measurements and ¹H-NMR metabolomics analyses, we identified the
potential for increased production of extracellular polymeric substances (EPS) and altered central metabolism under pressurized growth conditions. Given that these changes resulted in increasing cell clumping and production of potentially corrosive organic acids, the results have implications for maintenance of fracture permeability and well integrity in unconventional systems in the presence of active microbial populations.

Results

**Halanaerobium growth under pressurized conditions**

*H. congoense* WG8 was able to grow under both atmospheric pressure (0.1 MPa) and elevated pressure conditions characteristic of deep subsurface shales (21 – 48 MPa). Both the highest growth rate (0.104 h⁻¹), and the highest biomass yield were measured under atmospheric pressure incubation conditions, with growth rate and biomass yield decreasing with increasing pressure (growth rate = 0.071, 0.070, and 0.030 h⁻¹ at 21, 35 and 48 MPa, respectively) (Figure 1).

**Fermentation product profiles change under pressurized growth conditions**

In the deep shale environment, it is hypothesized that the degradation and fermentation of chemical additives, such as guar gum, support at least some *Halanaerobium* growth (7). Given that this substrate is metabolized through central glycolysis, we provided glucose as a representative carbon source in the experiments described here. Proton nuclear magnetic resonance (¹H-NMR) and gas chromatography were used to analyze *Halanaerobium* glucose fermentation product profiles following growth at atmospheric pressure (0.1 MPa) and 35 MPa. From these data, a fermentation balance using the oxidized and reduced products yielded a balanced ratio, indicating that all the major fermentation products were accounted for (Table S1). Across the two growth conditions, acetate, formate, ethanol, propanol, acetone, isopropanol, lactate, hydrogen gas, and carbon dioxide were all identified as excreted fermentation products. Due to differences in cell yields under different growth conditions, the per-cell concentration of each product was calculated by dividing the concentration of each fermentation product by cell density. Although the major
fermentation products excreted under both pressure conditions were acetate, ethanol, and formate, pressurized growth led to increases in the per-cell concentration of all aqueous fermentative compounds (Figure 2). Coupled to this increase in aqueous fermentation products, there was a concomitant 2-fold (30%) decrease in per-cell evolved hydrogen concentrations at 35 MPa. Low concentrations of propionate, propanol, isopropanol, and lactate that were identified under atmospheric pressure also showed differential changes in cultures grown at 35 MPa. Although propionate was no longer detected at 35 MPa, per-cell concentrations of propanol, isopropanol, and lactate increased 7-, 10-, and 5-fold, respectively (Figure 2).

From proteomic data collected at 0.1, 21, 35, and 48 MPa, we infer that H. congolense WG8 uses the Embden-Meyerhof-Parnas pathway for pyruvate synthesis, with the major fermentation products (lactate, formate, ethanol, acetate, and CO₂) generated via the mixed-acid fermentation pathway. While lactate dehydrogenase (EC 1.1.1.27 WG8-102157), pyruvate formate lyase (EC 2.3.1.54 WG8-1397), acetate kinase (WG8-10940, 101139), and alcohol dehydrogenase (WG8-10941, 11552, 11320, 11934, 1491, 1513) were identified across all four pressure incubation conditions, no close match to formate dehydrogenase or formate:hydrogen lyase was found in either the H. congolense WG8 proteomic dataset or the genome sequence. Instead, we infer that activity of a pyruvate-ferredoxin oxidoreductase (WG8-11648) is responsible for the similar per-cell CO₂ concentrations detected using gas chromatography at both 0.1 and 35 MPa (13). Pyruvate-ferredoxin oxidoreductase uses pyruvate, CoA, and oxidized ferredoxin to produce acetyl-CoA, CO₂, reduced ferredoxin, and H⁺, and was detected in the proteomic data across all four pressure growth conditions. The decrease in per-cell H₂ production at 35 MPa growth conditions was coupled with a decrease in hydrogenase protein abundances at increased pressures (21-48 MPa) (Table S2, Figure S1).

Global proteome profiles indicate shifts in Halanaerobium physiology and metabolism at pressure
Label-free shotgun proteomic analyses were subsequently used to infer metabolic and physiological changes associated with the observed *Halanaerobium* growth and metabolite profiles across all four growth conditions. There are 2,547 predicted protein coding genes within the *H. congolense* WG8 genome, and 1,826 of these proteins were identified within the proteomic dataset. Only a subset of 255 proteins were found at statistically significant higher abundances when *Halanaerobium* was grown under pressure (student t-test, p value < 0.05 in two of three high-pressure conditions). Of these 255 proteins, 77 were only identified under high-pressure growth conditions and 85 were present in higher abundance in all three pressurized growth conditions.

Decreasing *H. congolense* WG8 growth rates and cell yields (Figure 1) suggested that these cultures were stressed when incubated under high-pressure conditions. No novel bacterial growth mechanism for high-pressure survival has been established, but other high-pressure studies have identified similar stress-induced proteins expressed across many environmental stresses such as temperature, salt, and pH (14). Supporting this inference, proteins associated with diverse stress responses were solely present when strain WG8 was grown at high-pressure, including a universal stress response protein (UspA; WG8-10868), and enzymes that regulate intracellular redox conditions (thioredoxin; WG8-12911). Other proteins including alkaline shock proteins (WG8-1014, 1015) and heat shock proteins (WG8-1189, 11129, 10522) were measured in all conditions, but at higher abundances under high-pressure. Heat shock proteins have been associated with high-pressure growth in *Escherichia coli* and act to maintain the native composition of proteins, making them indicative of piezotolerant organisms responding to increased pressure (14, 15). Additionally, alkaline shock proteins have been shown to be more abundant in *Staphylococcus aureus* biofilms, relative to planktonic cells (16). F-type ATPases (WG8-10748, 10751, 10752, 10753) were also present at higher abundances in biomass incubated at high-pressure, and are believed to aid in high-pressure adaption by maintaining the cellular energy supply when under stress (14). Lastly, deep-sea piezophiles accumulate osmolytes that help protect against oxidants (e.g., free radicals) that are generated under stresses such as high-pressure or salinity (14). *Halanaerobium* WG8
utilizes osmolytes when it is grown at low- and high-pressure with a sodium/hydrogen antiporter detected during both growth conditions (WG8-10764, 11350, 101143). Certain amino acids such as glutamate and glycine may also act as osmolytes, and proteins involved in their synthesis were in higher abundance when *Halanaerobium* WG8 was grown at pressure (14). These proteins include glutamate synthase (WG8-11426), glycine hydroxymethyltransferase (WG8-105103), and glycine dehydrogenase (WG8-1167).

Proteomic data analyses revealed a strong signal for the utilization of 1,2-propanediol by *H. congolense* WG8 under high-pressure growth conditions. In model organisms (e.g., *Salmonella*) this compound is used as a carbon substrate via the formation of propionyl-CoA that eventually feeds into the TCA cycle as pyruvate (17). The utilization of 1,2-propanediol typically occurs in intracellular compartments known as carboxysomes or bacterial microcompartments. In *H. congolense* WG8, the genes for 1,2-propanediol utilization and microcompartment synthesis are present in a single operon (WG8-10936 – 10958). All proteins encoded by these genes were both detected and present at higher abundances under pressurized growth conditions (Figure 3). These included all three subunits of the propanediol dehydratase (PduCDE; WG8-10954-0956) that catalyzes the formation of propionaldehyde from 1,2-propanediol, and a propionaldehyde dehydrogenase (PduP; WG8-10944) that converts propionaldehyde to propionyl-CoA. Seven proteins involved in microcompartment generation were additionally more abundant at pressure, and likely play a critical role in protecting *H. congolense* WG8 from intracellular toxicity associated with propionaldehyde formation.

Cofactor B12 (adenosylcobalamin) is required by propanediol dehydratase for the aforementioned conversion of 1,2-propanediol to propionaldehyde. Consequently, all proteins involved in uroporphyrinogen synthesis (WG8-10961-10963), and the conversion of precorrin to adenosylcobalamin were present at higher abundances in high-pressure samples (Figure 3, Figure S1). Reflecting the presence of a cobalt active site in adenosylcobalamin, three cobalt transporters (WG8-10976, 10979, 11250) were
present at higher abundances in pressure grown cells, as were outer membrane TonB-dependent transporters (WG8-102136, 10244, 10246, 10245, 102135, 102134) involved in B12 and iron intracellular transport.

Cell clumping was observed when *Halanaerobium* biomass was incubated under pressurized conditions. Approximately 39 proteins previously implicated in biofilm formation and extracellular polymeric substance (EPS) synthesis in other microorganisms were present at higher abundances in cell cultures grown at pressure (Figure 4) (18, 19, 28, 29, 20–27). These proteins were associated with membrane transport (TonB (30), WG8-10244, 102136; ferritin (31), WG8-101121, 10243), sugar biosynthesis (epimerases (32) WG8-10872; isomerases (33) WG8-10873), sugar transport (TamB (34), WG8-10736; TolC (35), WG8-10534), and glycogen formation (pullulanase (36), WG8-10553), and could contribute to increase EPS production and surface attachment under pressurized growth (Figure 5). The potential role of cyclic di-GMP in stimulating biofilm formation was inferred by the presence of three diguanylate cyclase domain-containing proteins (37) (WG8-11421, 1302, 10361), two of which were solely present in cells from high-pressure incubations (Figure 4).

**Halanaerobium** exhibits cell clumping behavior at pressure

To quantify EPS formation and cell clumping by *Halanaerobium*, cultures incubated under atmospheric and high pressure conditions were imaged using confocal laser scanning microscopy (CLSM). Cells incubated at 35 MPa generated approximately 6 times more EPS than those incubated at 0.1 MPa (Figure 5). Floating clusters of biomass were more common when *Halanaerobium* was grown at high pressure (Figure 5).

Phospholipid fatty acid profiles change across pressure incubation conditions

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Phospholipid fatty acid (PLFA) data was obtained from silicic acid chromatography via esterification and gas chromatography-mass spectrometry (GC-MS) for cultures grown under atmospheric (0.1 MPa) to high pressures (up to 48 MPa). PLFA profiles were compared based on both the relative abundance of identified biomarkers and normalized to cell density (pmol/cell⁻¹). Cells grown under elevated pressures showed distinct changes in the abundance and structure of identified fatty acids. As pressure increased from atmospheric to 35 MPa, the relative abundance of both saturated and monosaturated PLFAs increased. However, during growth at the highest pressure (48 MPa), the relative abundance of saturated PLFAs (30%) was similar to cells grown under a surface atmosphere. Despite this similar abundance of saturated PLFAs at the highest and lowest pressures and a relatively even weighted chain length across all pressures (15.9 to 16.3), *Halanaerobium* cells grown at 48 MPa reduced their synthesis of monounsaturated PLFAs by more than half (14.6%) relative to cells grown at atmospheric pressure (35.2%) (Table S3). PLFAs absent at high pressure included iso- and anteiso- monounsaturated fatty acids (iso-C15:1o5t and anteiso-C15:1o5t) as well as C14:1o5c and C18:1o9t. This decrease in the degree of unsaturation at higher pressure has been observed in *Bacillus cereus* isolated from a deep-sea environment in cultures grown under anaerobic, low temperature conditions. (38, 39). A more saturated phospholipid bilayer allows for tighter packing of the membrane, increased thickness of the lipid bilayer, and a decrease in membrane fluidity for higher rigidity (38–42). We also observed an increase in three monounsaturated fatty acids (C18:1o9c-ep, C18:1o9t-ep, and C18:1-OH9,10), the saturated palmitic acid (C16:0), one oxirane (C18:0-OX9), and two cyclopropanes (C17:0Δ 9,10c and C17:0Δ 9,10t) for cells grown under pressure. Increased degree of cyclization and increased straight chain fatty acids composition is associated with membrane bulking and decreased membrane permeability (41, 43), adaptations which could be important for membrane integrity under elevated pressure (42). Indeed, increasing amounts of monounsaturated fatty acids C18:1 under pressurized growth conditions have previously been reported in the piezophilic deep-sea bacterium *Photobacterium profundum* SS9, where they are thought to create local regions of fluidity around membrane bound proteins that prevent the...
rigidity from inhibiting their activity (41). While a similar study in another model bacterium – *Shewanella piezotolerans* WP3 – identified monounsaturated fatty acids, their role in high-pressure adaptation was less clear (42). In conclusion, we infer that although the lipid bilayer as a whole becomes more rigid to decrease permeability at high-pressure, regionalized pockets of fluidity are maintained via increases in monounsaturated fatty acids to allow continued function of membrane proteins.

**Discussion**

*Halanaerobium* is detected as a dominant microbial community member across geographically distinct deep fractured shale ecosystems (8). Other studies have shown that *Halanaerobium* relative abundance increases within hydraulically fractured shale microbial communities as salinity increases above 10% total dissolved solutes (2). While *Halanaerobium* is able to grow across a broad salinity range, its metabolic flexibility also likely plays a key role in its ability to colonize and persist within these ecosystems. Here the effects of subsurface pressure on the growth rate of *Halanaerobium congolense* WG8 were calculated from laboratory incubations, and suggest that *H. congolense* WG8 is piezotolerant, rather than piezophilic (44). We hypothesize that while this microorganism may effectively grow in a currently unidentified surface ecosystem associated with the hydraulic fracturing process (e.g., water tanks, drill muds), it is also able to grow at pressures characteristic of the deep subsurface, albeit at slower rates.

Metabolite profiles suggest that pressurized growth was associated with broad-scale changes in central metabolism and production of fermentation end products. Under atmospheric pressure, *H. congolense* WG8 disposes of reductant via the generation of gaseous (H₂) and aqueous fermentation products (ethanol, acetate, formate). Under high pressure, *H. congolense* WG8 reduced the per-cell generation of H₂ but increased the production of lactate and alcohols as a mechanism for continued removal of reducing equivalents. While additional small shifts in metabolite profiles could be attributed to pressure-induced
pH changes, we anticipated that the pH in the buffered growth media would not vary significantly under the different pressure growth conditions. The oxidation/reduction potentials of these fermentation products were successfully balanced for high (35 MPa) and low (0.1 MPa) pressure growth (1.14 and 0.91, respectively) indicating that the production of increased lactate and alcohols compensated for the loss of gaseous H\textsubscript{2} (Figure 2, Table S2). Hydrogenases act as electron sinks for fermentative organisms, and their inactivity leads to an increased electron pool available for alcohol and organic acid production (45). Higher alcohol production has been demonstrated in fermenting Clostridium thermocellum mutants with inactivated hydrogenases (45), while lower activity of hydrogenases under supra-optimal pressurized growth conditions has previously been observed in the piezophilic microorganism Pyrococcus yayanosii CH1 (46). While the exact physiological or metabolic driver for these trends are not completely understood, it has been suggested that decreasing abundances of hydrogenase enzymes and associated H\textsubscript{2} production may be associated with adaptation of cell membrane-embedded proteins to changing membrane fluidity (46, 47). The increased production of potentially corrosive organic acids in response to decreasing hydrogenase activity may have implications for steel infrastructure in the subsurface. Acetate can drive corrosion of carbon steel in high salinity environments (48), and therefore metabolic shifts that favor organic acid production under high-pressure may represent another potential issue associated with persistence of fermentative microorganisms such as Halanaerobium in fractured shale networks.

Exposure to high-pressure conditions characteristic of deep shale ecosystems induced a strong proteomic signal for 1,2-propanediol processing, despite the addition of glucose as the sole carbon substrate in culture media. During glycolysis, fructose bisphosphate is converted to both dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (G3P). Under atmospheric pressure, the activity of triose phosphate isomerase immediately converts DHAP to G3P, which is subsequently processed through stage 3 of glycolysis to pyruvate. However, elevated pressures have been shown to reduce the activity of glyceraldehyde 3-phosphate dehydrogenase, which converts G3P to 1,3-bisphosphoglycerate (49). Under...
such conditions, DHAP formation from fructose bisphosphate is favored, and is potentially processed to D-lactate and 1,2 propanediol through the methylglyoxal bypass (Figure 3). All proteins required for the methylglyoxal bypass (methylglyoxal synthase, methylglyoxal reductase, 1,2-propanediol dehydrogenase, and glyoxalase) were observed in proteomic datasets, and were present at higher abundances under pressure.

In *H. congolense* WG8, we hypothesize that the methylglyoxal bypass is used for the removal of DHAP, and the disposal of reducing equivalents through oxidation of NADH to NAD+ via methylglyoxal reductase and 1,2-propanediol dehydrogenase activity. The removal of reductant in this pathway may be important given the inferred decreases in activity of hydrogenase enzymes during high-pressure growth. Additional removal of DHAP may occur through the conversion of DHAP to dihydroxyacetone via dihydroxyacetone kinase (39). Dihydroxyacetone have been shown to accumulate in deep-sea microbial communities and is believed to aid in high-pressure adaption (39). While dihydroxyacetone was not directly measured in this study, dihydroxyacetone kinase was more abundant at pressure and could have utilized some of the DHAP pool. For DHAP incorporated into the methylglyoxal bypass, resulting 1,2-propanediol is shuttled through the propanediol utilization pathway, consisting of 21 proteins that were all present at statistically significant higher abundances when *H. congolense* WG8 was grown under high-pressure. This pathway converts 1,2-propanediol to propanol and propionate, but key intermediates are propionaldehyde and propionyl-CoA. The formation of propionaldehyde and propionyl-CoA takes place within a synthesized microcompartment, due to both the toxicity of propionaldehyde and the requirement for close proximity between cofactor B12 and the propanediol dehydratase active site (50). The microcompartment also protects the radical intermediate formed in the active site of diol dehydratase from escaping or being quenched by undesirable side reactions, which would make the enzyme permanently inactive (51, 52). The diol dehydratase reactivation enzymes, cobalamin reductase, and adenosyltransferase (WG8-10952/10953, 10943, 10945) reactivate the dehydratase active site via replacement of the cofactor B12 molecules.
The presence of propanol at higher per-cell concentrations under high-pressure growth conditions provides additional evidence for the activity of this pathway in *H. congo*ense WG8. The cell is able to dispose of additional reducing equivalents via the oxidation of NADH to NAD⁺ coupled to the conversion of propionaldehyde to 1-propanol. If propionaldehyde is instead converted to propionyl-CoA – the precursor to propionate – NADH is generated (Figure 3). We hypothesize that the requirement to recycle reducing equivalents favors formation of 1-propanol over propionate, and can account for the near absence of propionate in extracellular media under high-pressure growth conditions.

Other excreted fermentation products (isopropanol, ethanol, and acetate) were also present at higher per-cell concentrations under high-pressure growth conditions. The most studied pathway for fermentative isopropanol formation is the isopropanol-butanol-ethanol pathway which requires the enzymes acetoacetyl-CoA:acetate/butyrate:CoA transferase and acetoacetate decarboxylase, neither of which are present in the *H. congo*ense WG8 genome (53). We hypothesize that isopropanol may instead be synthesized through the 1,2-propanediol utilization pathway, via acetone or propionaldehyde intermediates. Such a reaction requires re-arrangement of either alcohol on the terminal and middle carbons in 1,2-propanediol (52); while alcohol re-arrangement to the terminal carbon resulting in propanol formation is the most common route, high pressure conditions could alter this process to generate increased concentrations of isopropanol (Figure 6).

In addition to 1,2-propanediol, diol dehydratase and associated proteins can act on other 1,2 diols such as ethylene glycol and 2,3-butanediol, forming acetate and ethanol (52, 54, 55). Ethylene glycol is frequently present in chemical additives used in the hydraulic fracturing process, where it serves a multi-functional purpose as a crosslinker, friction reducer, gelling agent, and non-emulsifier. The activity of the diol dehydratase and associated proteins within *Halanaerobium* microcompartments suggests that these
chemical additives could be degraded by microbial activity, with implications for the effectiveness of the compounds added into the shale formation.

High-pressure cultivation also induced a series of putative stress responses in *H. congo**lense* WG8. Previous studies have shown that antioxidant enzymes catalase, DNA-binding protein (Dps), alkyl hydroperoxide reductase, and DNA recombination protein RecA offer protection to the deep-sea bacterial strain *Shewanella piezotolerans* WP3 against oxidative stress induced by high pressure (56). Proteomic evidence revealed increased abundances of alkyl hydroperoxide reductase and DNA recombination protein RecA in *Halanaerobium* WG8 cultures grown at the highest pressure treatment of 48 MPa. These proteins could be acting to defend the cell against oxidative stress induced by high pressure through signaling and DNA repair (56). Both heat and alkaline shock proteins that were more abundant in high-pressure incubations have been detected in *Escherichia coli* cultured under similar conditions, and are indicative of organisms responding to pressure and oxidative stress (47, 56). These proteins are believed to stabilize protein quaternary structure, thus maintaining membrane integrity, translation processes, and stability of macromolecules at high pressure (47). Under the same conditions we also identified greater abundances of multiple proteins associated with EPS production and biofilm formation. These proteins included nucleoside diphosphate sugar epimerases (WG8-12747/10461) that are involved in the glycosylation of the cell surface and were previously detected in a proteomic study of *Halorubrum lacusprofundi* biofilm formation (32). Both tagaturonate epimerase (WG8-10872) and glucuronate isomerase (WG8-10873) are involved in converting hexuronic acids to fructuronate, and subsequently fructuronate to glucuronate, which is a known substrate in biofilm exopolymer synthesis in *Lactobacillus casei*, *Streptococcus thermophilus*, and *Pseudomonas aeruginosa* (33). Translocation and assembly module TamB (WG8-10736) is a membrane protein involved in the secretion of adhesion proteins that promote biofilm formation in *Escherichia coli* (34). Multiple outer membrane TonB-associated proteins (WG8-102136, 10246, 10245, 10244, 10243, 102135, 102134, 101121) involved in large molecule movement across the membrane were also present at higher abundances in pressure-grown cells.
proteins can transport molecules including carbohydrates, metals, and quorum sensing signaling molecules (30, 31, 57), and have been implicated in stimulating biofilm formation in *P. aeruginosa*, *Thermotoga maritima*, and *Staphylococcus aureus* (29–31). Glucose-1-phosphate adenyllyltransferase (WG8-10557/10556) and 1,4-alpha-glucan branching enzyme (WG8-10558) were both present in greater abundances under pressurized growth conditions and are involved in cellular glycogen synthesis. Studies with known biofilm-forming microorganisms (e.g., *Salmonella enteritidis*) have demonstrated that these microorganisms accumulate intracellular glycogen to help in EPS production (26). Supporting our inference that *Halanaerobium* may utilize a similar mechanism for EPS generation, a pullulanase enzyme (WG8-10553) needed to hydrolyze starch linkages for EPS biosynthesis was more abundant at pressure (36) (Figure S1).

Other proteins associated with WG8 growth at pressure that may play roles in EPS formation include mannonate dehydratase (WG8-11618/10874), which was upregulated in *Enterococcus faecium* biofilms (20) and maltose phosphorylase (WG8-103108), which is involved in the conversion of maltose to glucose. This maltose to glucose conversion is of interest because glucose has been found to enhance biofilm formation in *Staphylococcus epidermidis* (27). Other high-pressure associated proteins include phosphofructokinase (WG8-11516/10552) which has been detected at higher abundances in *Streptococcus mutans* biofilms (24) and phosphomannomutase (WG8-11441/11253), which is associated with exopolysaccharide biosynthesis in *Pseudomonas aeruginosa* (23). The L-fucose isomerase enzyme (WG8-10560) plays a key role in production of L-fucose, a known component of a tetrasaccharide repeat in *Klebsiella pneumoniae* and *Enterobacter aerogenes* biofilms (58). N-acetylglucosamine-6-phosphate deacetylase (WG8-103102) is involved in the synthesis of the alginate precursor fructose-6-phosphate, an adhesive component of *P. aeruginosa* biofilms (59).

Finally, proteins associated with the phosphoenolpyruvate phosphotransferase system and other sugar transport systems (WG8-11518, 11087, 102110, 102109) have been shown to play regulatory roles in
biofilm formation in *Vibrio cholerae* and *Thermotoga maritima* (22, 29). Other regulatory proteins include the sigma-54 modulation protein (WG8-1072), which has been found to control biofilm development of *Vibrio fischeri* (25), and host factor-I protein (WG8-102151), which plays a role in sigma factor RpoS regulation, a master regulator of biofilm formation and utilized during high pressure growth in *E. coli* (28, 47).

All the proteins highlighted above provide strong evidence that *H. congoense* WG8 is capable of forming biofilm-like structures under pressures representative of the deep subsurface. Complementing these inferences, CLSM analysis of biomass revealed greater cell aggregation and production of EPS-like material in high-pressure incubations. We hypothesize the increased EPS formation is a *Halanaerobium* WG8 stress response, as indicated by slower growth rates at increased pressures. Other studies have grown *E. coli* in pressurized microfluidic devices and found the mechanical stress associated with living in tightly packed environment induced a biochemical stress response that included EPS generation and biofilm formation (60). Biofilm and EPS-associated structures could potentially impact hydrocarbon recovery from fractured shales; fractures within the shale matrix are nanometers to centimeters in size (1, 61) and bio-clogging associated with EPS production by *Halanaerobium* strains could potentially reduce the permeability of the system. Increased EPS production and formation of biofilm-like structures could also impact the efficacy of biocides that are injected into the target formation. Indeed, prior research has suggested that EPS-type materials can offer protection to microorganisms against a wide range of environmental stresses including host immune defenses (62), ultraviolet radiation, supercritical carbon dioxide (9), and biocides (63, 64). It is possible the stress response induced by high pressure may have the unintended advantage of offering *Halanaerobium* increased protection from added biocides. EPS-related biocide resistance may at least partly explain the observed persistence of microbial consortia, that include *Halanaerobium*, within hydraulically fractured shales for multiple years following the fracturing process (2, 3, 65–67).
Conclusions

*H. congolense* WG8 is a piezotolerant microorganism that is characteristic of *Halanaerobium* strains that dominate microbial ecosystems in hydraulically fractured shales. The metabolic and physiological response to the onset of high-pressure growth conditions include the inferred production of increased EPS that drives cell aggregation, and re-arrangement of central metabolism such that production of organic acids and alcohols are favored over hydrogen. Both of these responses could drive potentially deleterious processes in the subsurface such as bio-clogging of newly-generated fracture networks and pores, and increased rates of corrosion of carbon steel infrastructure associated with hydrocarbon recovery. Additionally, the increased activity of diol dehydratases under high-pressure conditions highlights the metabolic plasticity and versatility of *Halanaerobium* under rapidly changing environmental conditions and may contribute to *in situ* degradation of chemical additives used in the hydraulic fracturing process. Together, these results highlight the importance of studying microbial physiology and metabolism under representative environmental conditions, and stress the importance of microbial control in hydraulically-fractured shales.

Methods

*Halanaerobium growth experiments.* *Halanaerobium congolense* WG8 was isolated from a produced water sample from the Utica shale, as described previously (5), and draft genome sequenced at the Joint Genome Institute using Illumina HiSeq technology. Growth curves were performed in triplicate at 0.1, 21, 35, and 48 MPa with optical density measurements collected every 24 hours. Biomass was incubated at 40 °C in anaerobic Hungate tubes (99% N₂ headspace) containing 9 mL of saltwater liquid media (described in Booker et al., 2017) inoculated with 10% *Halanaerobium* WG8 growing in mid-log phase (5). Tubes were modified per the protocol outlined in Bowles et al. (68) so that they could be pressurized within titanium pressure vessels manufactured by the Marine Science Development Shop at Scripps
Institution of Oceanography. Water was used as a pressurizing phase in these reactors. To generate standard growth curves that related *Halanaerobium* optical density to cells/mL, optical density (600 nm) measurements and 500 µL of culture were collected every 24 hours from each culture tube until stationary growth phase was reached. Each 500 µL of culture collected was vortexed to disperse cells for more accurate optical density measurements. Two 10µL samples were taken from the 500 µL aliquot, and were counted using a hemocytometer. These counts were used to calculate the number of cells present in the growth culture and correlate to optical density (Figure S2). Growth rates were calculated using ln(OD) taken during the period of exponential growth, while cell yield was inferred from the highest optical density reading and corresponding cell counts (69).

**Proton-NMR measurements of fermentation products.** Biological triplicate cell cultures pressurized at 0.1 and 35 MPa were collected during mid-log growth phase. Supernatant was filtered through a 0.22 µm filter, flash frozen, and shipped to the Environmental Molecular Sciences Laboratory (EMSL) for metabolite quantification using $^1$H-NMR. The 1D $^1$H NMR spectra of all samples were collected following standard Chenomx (Edmonton, Alberta, Canada) sample preparation and data collection guidelines (70). Biological triplicate data was acquired on a Varian Direct Drive (VNMR) 600 MHz spectrometer (Agilent Technologies) equipped with a Varian triple resonance salt-tolerant cold probe with a cold carbon preamplifier. A Varian standard one dimensional proton nuclear Overhauser effect spectroscopy (NOESY) with presaturation (TNNOESY) was collected on each sample, using the Chenomx standard data collection protocol (70). Collected spectra were analyzed using Chenomx 8.3 software, with quantifications based on spectral intensities relative to a calibrated reference solution (100% D$_2$O, 0.5 mM 2,2-dimethyl-2-silapentane-5-sulfonate-d6 (DSS)), as previously described (2).

**Gas Chromatography.** Biological triplicates of *Halanaerobium* WG8 were grown at 0.1 and 35 MPa until mid-log phase was reached. Samples for gas production were taken at the beginning of lag phase and once mid-log phase was reached. Cultures grown at 35 MPa were transferred into 20 mL vacuum sealed
bottles. All samples were shaken for 1 hour at 170 rpm to allow soluble H$_2$ and CO$_2$ to become gaseous. We acknowledge this method does not guarantee all soluble H$_2$ and CO$_2$ becomes gaseous and therefore our CO$_2$ and H$_2$ measurements likely underestimate the production of these gases by *Halanaerobium congolense* WG8. After shaking, each sample was inverted and stored at 4 °C overnight. To measure the concentration of carbon dioxide and hydrogen gas associated with each sample, 5 mL of head space was sampled and analyzed using a GC-2014 Shimadzu Gas Chromatograph. Measured peak area was converted to moles using the density and molecular weight of each gas.

**Proteomics sample preparation.** Total protein profiles of *Halanaerobium* grown at 0.1 and 35 MPa were determined using shotgun proteomics. Triplicate biomass from 0.1 and 35 MPa growth experiments was harvested at mid-log phase by centrifugation at 10,000 rpm and 4 °C for 10 minutes. The cell pellets were immediately flash frozen in liquid nitrogen to preserve protein signatures. Sample prep for proteomic analysis was previously described in Booker *et al.*, 2017 (5). Briefly, total proteins were extracted from each cell pellet using an extraction kit (Expedeon San Diego, CA) and digested in 0.05 µM trypsin. Resulting peptides were filtered, concentrated, and diluted to 0.3 µg/µL for MS analysis.

**Proteomics measurements.** MS analysis of peptide mixtures was previously described in Booker *et al.*, 2017 (5). Briefly, peptide mixtures were separated using a 2D-LC ACQUITY UPLC M-Class System (Waters, Milford, MA) with a silica hand packed column with 3 µm particle Jupiter C18 derivatized silica beads (Phenomenex, Torrance, CA). Mobile phases consisted of 0 to 100% Acetonitrile/0.1% Formic acid against water/0.1% Formic acid. This LC system was coupled to in-house built nano electrospray apparatus. MS analyses were performed using a ThermoFisher QExactive Pro (Framingham, MA) and measured peptides were searched against predicted peptides derived from the *H. congolense* WG8 genome. Resulting peptide identifications were filtered via MSGF Q-Value <= 0.01, which is ~1% false discovery rate (FDR) at each individual dataset level. There were 7672 reversed identifications out of
813,177 total filter passing IDs for a 0.94% FDR at the PSM (peptide to spectrum match) level. For comparative analyses between triplicate biological replicates, protein spectral counts were normalized using the normalized spectral abundance frequency (NSAF) method (71), and Z-score values were calculated to display differences in protein abundances. Significant differences in protein abundances across incubation conditions were determined using a two-tailed student t-test with unpaired equal variance across triplicate NSAF values, with resulting p-values of 0.05 or below indicating significance.

**Phospholipid Fatty Acid Analysis.** Culture samples were extracted ultrasonically according to the modified Bligh and Dyer procedure (72, 73) after adding an intact polar lipid (Phosphate Buffer + phosphatidylcholine; POPC). Total lipid extracts (TLEs) were transferred into test tubes using three washes of 2 mL of chloroform, after which the solvent was evaporated with N\textsubscript{2} at 37°C. Dried TLEs were re-suspended in 2 mL of chloroform and fractionated using silicic acid chromatography, with PLFAs recovered from methanol. Extracts were next evaporated to dryness before methylation using methanolic potassium hydroxide (73, 74). Fatty acid methyl esters (FAMES) were next dissolved in 200 μL of hexane containing 50 pmol/μL of external injection standard (docosanoic acid methyl ester; Matreya, Inc) and transferred into GC-MS vials containing 500 μL glass inserts. Sample aliquots were injected into an Agilent 6890 series gas chromatograph (GC) interfaced to an Agilent 5973 mass selective detector (MS) equipped with a non-polar cross-linked methyl silicone column (Restek RTX-1 column 60m, 0.25mm I.D. x0.25μm film thickness). GC operating conditions were as follows: 60 °C for 2 minutes then increased at 10 °C/minute to 150 °C, followed by a second ramp at 3 °C/minute to 312 °C for a total run time of 65 minutes (75). The injector temperature was 230°C; the detector temperature was 300 °C, and Helium was the carrier gas. The following methyl ester standards (Matreya LLC, State College, Pennsylvania, USA) were included in each sample run to calibrate retention times and assist with peak identification: Bacterial Acid Methyl Ester CP Mixture (BacFAME [1114]), Polyunsaturated FAME Mixture 2 (PUFA-2 [1081]), and Polyunsaturated FAME Mixture 3 (PUFA-3 [1177]). Identified peaks were confirmed across all samples, with GC-MS spectra validated using Agilent MSD ChemStation Data 21

Downloaded from http://aem.asm.org/ on April 29, 2019 by guest
Analysis Software F.01.00 with the NIST11 compound library. A single-ion monitoring program was also used to scan the base peaks for lipids to validate all identified peaks. Once peaks were identified, lipid concentration was calculated based on external standard peak area. An internal standard curve ranging from 1 to 50 pmol/µL was used to determine detection limit and establish the sample dilution range. Lipid extraction and GC-MS analysis were performed at the Center for Environmental Biotechnology at the University of Tennessee (Pfiffner Lab, Knoxville, TN, USA).

Confocal Scanning Laser Microscopy. *Halanaerobium* cultures were incubated at 0.1 and 35 MPa in biological quadruplicates for 72 hours at 40 °C. After incubation, cells were prepped for confocal scanning laser microscopy imaging at the Ohio State University Molecular and Cellular Imaging Center in Wooster, OH. The bottom 2 ml of the cell cultures were fixed by adding an equal volume of 8% paraformaldehyde in 200 mM Tris-HCl pH7.2 buffer and incubated at 4 °C overnight without shaking. Cells were collected by centrifugation at 1000 g for 10 minutes, resuspended in 200 µl of 10 mM Tris-HCl pH 7.2, and then stained. Cells were stained with 50 µg/ml of Concavalin-A AlexaFluor 488 (Invitrogen, cat.# C11252) for 40 minutes to visualize α-mannopyranosyl and α-glucopyranosyl residues (green) within the extracellular polymeric substance matrix and with 1 µM of Syto59 (Invitrogen, cat.# 11341) for 30 minutes to visualize nucleic acids (red). After staining, cells were collected by centrifugation, washed once with 200 µl of 10 mM Tris-HCl pH 7.2, and resuspended in 15 µl of 10 mM Tris-HCl pH 7.2. Samples were immediately mounted on a glass slide and imaged on a Leica TCS-SP6 confocal microscope. For the quantification of the green fluorescence (extracellular polymeric matrix) stacks (average projections) of seven focal planes (z=24 µm) were acquired using a 63Xx1.20 water objective. A total of 20 images (five images from four separate slides) for each growing condition were collected. Grey pixel values for each image were acquired using ImageJ, and the total green fluorescence was calculated from the Integrated Density for each image, adjusted for the background fluorescence.
values. Values from each sample were averaged and total green fluorescence (EPS) was plotted for *Halanaerobium* grown at both 0.1 and 35 MPa.

**Accession number.** The genome of *Halanaerobium congolense* WG8 was sequenced and annotated by the Joint Genome Institute and is publicly available in the JGI Genome Portal database (http://genome.jgi.doe.gov/) under IMG ID number 2642422587.

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**Figure Legends**

Figure 1. *H. congolense* WG8 growth at high pressure. Growth rates (red) and corresponding cell densities (grey) across a pressure gradient (0.1 - 48 MPa). Growth rate and cell densities decreased as incubation pressure increased.

Figure 2. Major fermentation products excreted by *H. congolense* WG8 detected using NMR and Gas Chromatography. (A) The heat map shows the Z score (the number of standard deviations away from the mean) of normalized concentrations. Bolded product names signify differences in concentrations between treatments where *p* < 0.05 (B) Fold changes in per-cell fermentation products. All positive values represent increased production under high pressure growth conditions. Stars represent statistically significant changes in fermentation product formation (*p* < 0.05).

Figure 3. Predicted carbon flux through *H. congolense* WG8 when grown under pressure (21, 35, & 48 MPa) using proteomic and NMR analyses. *H. congolense* WG8 is a strict fermenter, and glucose was the substrate provided during growth experiments. Major fermentation products are acetate, ethanol, formate, lactate, propanol, carbon dioxide, and hydrogen gas (only when grown at 0.1 MPa). The production of 1,2-propanediol is hypothesized to be a result of the methylglyoxal bypass, which may become important during high pressure growth because activity of triose phosphate isomerase (5) decreases under pressure. 1,2-propanediol, and other alcohols converted into aldehydes are processed in a microcompartment to
contain toxic aldehyde intermediates. Arrows size represents the increased abundance of a protein under 1, 2, or 3 high pressure growth conditions. Arrows outlined in black represent statistical significance of changes in protein abundance (p < 0.05). Arrow colors are based on Z-score values calculated from protein abundances. Proteins: (1) Phosphotransferase; (2) Glucose-6-phosphate isomerase; (3) Phosphofructokinase; (4) Fructose bisphosphate aldolase; (5) Triose phosphate isomerase; (6) glyceraldehyde-3-phosphate dehydrogenase; (7) 3-phosphoglycerate kinase; (8) Phosphoglycerate mutase; (9) Enolase; (10) Pyruvate kinase; (11) Lactate dehydrogenase; (12) Pyruvate formate lyase; (13) Pyruvate-ferredoxin oxidoreductase (14) Aldehyde dehydrogenase; (15) alcohol dehydrogenase; (16) Phosphotransacetylase; (17) Acetate kinase. Methyl Glyoxal Bypass (18) Methylglyoxal synthase; (19) Glyoxalase; (20) Methylglyoxal reductase; (21) 1,2-propanediol dehydrogenase. Microcompartm ent (22) Propanediol dehydratase; (23) Alcohol dehydrogenase; (24) propionaldehyde dehydrogenase; (25) Phosphotransacetylase; (26) Propionate kinase; (27) Hydrogenase

Figure 4. Proteins potentially involved in stress response and associated EPS formation in *H. congoense* WG8. Black outlined boxes represent a significant difference (p < 0.05, student t-test) in protein abundances between low and high pressure conditions. No outline represents changes in protein concentration that were not statistically significant (p > 0.05, student t-test).

Figure 5. Confocal scanning laser microscopy analysis of *Halanaerobium* grown at high and low pressure. Bar graph represents the average amount of extracellular polymeric substance produced by the cells in three biological replicates grown at high and low pressure (p < 0.05, student t-test). Confocal image panel shows examples of *Halanaerobium* biofilms. Styo59 (red) was used to stain nucleic acids, while Alexa488-ConA (green) was used to stain α-mannopyranosyl and α-glucopyranosyl residues within the EPS matrix.
Figure 6. Proposed mechanism for diol dehydratase catalyzed reactions. This mechanism involved a free radical induced rearrangement of -OH groups to generate aldehydes and ketones. Reaction (1) is believed to be the most common route to generate propionaldehyde from 1,2 propanediol. Propionaldehyde is converted to 1-propanol. Reaction (2) could be induced under high pressure conditions, leading to the formation of acetone from 1,2 propanediol. Acetone may be an isopropanol precursor. Ado• denotes the 5'-deoxyadenosyl radical supplied by the coenzyme B_{12}. 
Figure 1.

Figure 2.
Figure 4.

Figure 5.
Figure 6.

(1) 1,2 Propanediol → Ado^+ → Ado-H → OH → Ado-H → OH → OH → OH → H2O → Propionaldehyde

(2) 1,2 Propanediol → Ado^+ → Ado-H → OH → OH → OH → OH → H2O → Acetone