# 'Omics Analyses of the Hydraulically Fractured Shale Isolate *Halanaerobium* Highlights Membrane Modifications that Underpin Adaptation Under Deep Subsurface Biogeochemical Drivers

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**Project Goals:**

The injection of fluids and proppants to fracture the deep shale introduces microbial cells and substrates to low-permeability rocks. Microorganisms in hydraulically fractured wells govern biogeochemical reactions and often produce acids and sulfides, leading to corrosion and gas souring, and form biofilms, resulting in clogging and fouling events. The overarching goal of this research is to advance our comprehension of the microbial diversity and function in non-sterile hydraulically fractured wells. Our current understanding of microbial growth within fractured hydrocarbon-bearing rock is based primarily on genomic information, we identified three specific objectives that will shed light on in situ physiologies and kinetic rates, governing biogeochemical reactions: (1) characterize variables influencing growth parameters and membrane features of shale taxa, (2) characterize interactions between shale matrices and microorganisms, and (3) elucidate engineered and environmental processes driving biogeochemical signatures at field scale.

**Abstract**

The Gram-positive *Halanaerobium* spp. is a dominant bacterial genus across geographically distinct fractured shale formations, which are increasingly used for natural gas extraction. These bacteria encounter harsh physicochemical conditions in the deep terrestrial biosphere, including high temperatures, brine-level salinities, anoxia, and elevated pressures. Microbial membranes act as the first line of defense against these environmental stressors, and maintaining membrane functionality during changing environmental conditions requires careful regulation of intact lipid composition and membrane-embedded proteins. Here we characterized the physiology of the model organism *Halanaerobium congolense* WG10, grown for the first time under continuous culture (chemostat) conditions. The anaerobe *H. congolense* WG10 was cultivated at 20% salinity under three growth rates (hydraulic retention times (HRTs) of 48, 24, and 19.2 hrs) and two temperatures (25ºC and 40ºC) under complete control of system pH and redox conditions using a 1-L Sartorius Biostat® Q-plus system. We applied an integrative 'omics approach to characterize metabolomic, proteomic, and lipidomic features (MPLEx analysis) and quantify metabolite production (using 1H-NMR and GC-FID) under steady state growth rates.

'Omics analysis of metabolites, proteins and lipids for steady state cells revealed that *Halanaerobium* alters its cell membrane to maintain fluidity in the lipid bilayer while maximizing growth rate.

*Halanaerobium* modulates the ratio of phospholipid headgroups in response to changes in temperature, and increases cardiolipin and phosphatidylethanolamine polar lipid abundance with increasing carbon availability. We also observed higher abundance of neutrally charged simple glycerol lipids at lower temperature and growth rates, while glycerophospholipids with larger polar heads and zwitterionic lipids prevail at warmer temperatures and faster growth rates. The observed accumulation of cardiolipin and phosphatidylethanolamine at higher temperature and growth rates is likely to results in the destabilization *of H. congolense* WG10 membrane, and the formation of localized, reversible HII phases in its membrane. On the other hand, at sub-optimal temperature for growth, *H. congolense* WG10 responds with the stabilization of the cytoplasmic membrane by a better compaction/higher packing of the phospholipid species within the bilayer leaflets.

Proteomics analysis identified a total of 2,227 out of 2,800 predicted protein-coding genes. Among these, 356 proteins were found to be significantly higher in abundance in one or more treatments (Student's t test, $p < 0.05$), including known stress regulators involved in cellular envelope homeostasis such as cold shock proteins (CspA), a typA, bipA GTP binding protein involved in stress response, and a nucleotide-binding universal stress protein (UspA). We also identified lipid-A synthesis proteins at lower temperature or high growth rate, a lipopolysaccharide endotoxin uncommonly found in Gram-positive bacteria.

Both proteomic and metabolic data supported significant activity for the utilization of 1,3-propanediol, especially in warmer temperatures (40ºC) and longer HRTs (24 and 48 hrs). Proteins associated with the methylglyoxal bypass pathway (e.g. glyoxalase) and two subunits of the propanediol dehydratase (PduD, PduE), which catalyzes the formation of propionaldehyde from 1,3-propanediol, were important during lower temperature growth (25ºC), suggesting this pathway is activated under cold stress. The propanediol dehydratase is a cobamide-dependent enzyme that has been shown to also dehydrate ethylene glycol (and other ethoxylate-based surfactants) to acetaldehyde. The formation of both propionaldehyde and acetaldehyde was confirmed with GC-FID analysis. In addition to aldehydes, we identified ketones (acetone), volatile fatty acids (acetate, lactate, formate), alcohols (ethanol, propanol), and amino acids (alanine, valine) metabolites via 1H-NMR and/or GC-FID.

Collectively, our 'omics continuous culture approach sheds new light on the metabolism and membrane features of the halotolerant bacterium *Halanaerobium* under biogeochemical drivers relevant to engineered shale, with implications on membrane charge, permeability, and metabolism.

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