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THIOSULFATE REDUCTION IN *HALANAEROBIUM*: A GENOMIC AND PHYSIOLOGICAL INVESTIGATION

A THESIS APPROVED FOR THE
DEPARTMENT OF MICROBIOLOGY AND PLANT BIOLOGY

BY

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I would like to dedicate this work to my family, who have supported me in every endeavor I have decided to undertake, and who always encourage me to try my best. And to my wife Jackie, who has endured with me this struggle to achieve everything I want. She truly is a wonderful and supportive woman who gives me the motivation to achieve my dreams.

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Abstract

Bacteria that produce sulfide have been investigated for their role in corrosion of steel surfaces. Attention has mainly focused on bacteria able to reduce sulfate to sulfide (SRB). However, bacteria that produce sulfide by reducing thiosulfate (TRB) but can't reduce sulfate, have recently become of interest for their role in causing corrosion. Current molecular detection methods fail to detect these TRB, which may use a different pathway than SRB for thiosulfate and sulfite reduction. This study investigates the genetic potential and physiology of a TRB genus, *Halanaerobium*, to produce sulfide from thiosulfate reduction and sulfite reduction. Six *Halanaerobium* genomes were interrogated for genes involved in thiosulfate and sulfite reduction, using the BLAST algorithm. The genomic investigation revealed that five of the six contain sequences of a gene coding for a rhodanese-like protein, *rdlA*, which can reduce thiosulfate to sulfite and subunits for anaerobic sulfite reductase, *asrABC*, which reduces sulfite to sulfide. Three of the strains, two which contained *rdlA* and *asr* sequences, and one which did not contain these sequences were then used in an experiment to confirm reduction of thiosulfate and reduction of sulfite. The strains were grown for 12 days under three treatments; medium amended with thiosulfate alone, with sulfite alone, and amended with both thiosulfate and sulfite. The concentration of thiosulfate, sulfite, sulfide and rhodanese activity was measured over the course of the experiment. The two strains which did contain *rdlA* and *asr* sequences, lost thiosulfate and gained sulfite and sulfide in the treatments amended with thiosulfate alone or with both thiosulfate and sulfite. Rhodanese activity was also detected in these treatments with the same two strains. The same two strains lost sulfite and gained sulfide in the

treatment amended only with sulfite, and rhodanese activity was not observed. The strain without *rdlA* and *asr* sequences did not lose thiosulfate or sulfite nor did it produce sulfide in any of the treatments. Therefore, detection of *rdlA* and *asr* sequences in a strain corresponded with whether a strain reduced thiosulfate and sulfite and produced sulfide. The genomic and physiological evidence presented here suggests that *Halanaerobium* use a rhodanese-like protein to reduce thiosulfate to sulfite and sulfide and then anaerobic sulfite reductase to reduce sulfite to sulfide.

Chapter 1: Literature Review

Biocorrosion in the Oil and Gas Industry

Corrosion in the oil and gas industry has many causes. The focus of this brief overview is biocorrosion from bacteria. Microbially induced corrosion (MIC), or biocorrosion is the influence of microorganisms on corrosion. Microbial activity, such as creating corrosive compounds, can lead to damage of steel surfaces. These corrosive products can include acetate and sulfides (Little and Lee, 2009). The National Association of Corrosion Engineers reports that the annual cost of corrosion in the oil and gas industry is $1.372 billion (https://www.nace.org/Corrosion-Central/Industries/Oil---Gas-Production). It is estimated that about 20% of this cost is related to MIC (Almahamedh et al., 2011). Due to the need to mitigate this MIC many studies have sought to characterize bacterial communities and their mechanisms for corrosion (Beech and Sunner, 2004; Dowing et al., 1991). Bacterial communities of oil and gas facilities, including drill sites, have been investigated for their inhabitants and genetic potential to produce corrosive compounds (Marks et al., 2016; Booker et al., 2017; Liang et al., 2014). These studies have also provided methods of detection for best practices which include both culture dependent (Bhagobaty, 2014) and molecular detection (Muyzer and Marty, 2014; Nagarajan and Loh, 2014). Sulfate reducing bacteria (SRB) have been a main focus of research for their ability to produce sulfide (Enning and Garrelfs, 2014). SRB such as *Desulfovibrio* are capable of sulfide production from reducing sulfate, but also can reduce thiosulfate to sulfite and sulfide (Haschke et al., 1971). However, thiosulfate reducing bacteria that cannot reduce sulfate have been gaining attention for their ability to produce sulfide (Booker et al., 2017;

Lipus et al., 2017; Liang et al., 2014; Liang et al., 2016). Recent studies have shown that a non-sulfate reducing genus *Halanaerobium* is capable of thiosulfate reduction which produces sulfide (Ravot et al., 2005; Booker et al., 2017). This study uses *Halanaerobium* as a model for thiosulfate reducing bacteria (TRB) that do not reduce sulfate.

Hydraulic Fracturing Fluids as a Microbial Habitat

The drilling environment consists of a drilling platform, where a bore hole extends down into the ground. The average depth of a well drilled in 2008 was 1.81 km for exploratory and development wells (U.S Energy Information Administration, EIA.gov). A study by Itavaara et al. (2011) investigated the bacterial communities at different depths of a well in the Outokumpo formation in eastern Finland. The results of these studies showed that Firmicutes dominated the community at 1.4-1.5km with a Na^+ concentration of 1070-1330mg/L and temperature of 35^o- 40^{o}C. Investigations of the bacterial community of some oil and gas production facilities have found sulfide producing bacteria as dominant organisms (Booker et al., 2017; Lipus et al., 2017; Marks et al., 2016). Sulfide producing bacterial genera isolated from oil and gas facilities include *Desulfovibrio* (Haschke et al., 1971; Nakatsukasa and Akagi, 1969; Hatchikian, 1975), *Thermotoga* (Ravot et al., 1995), *Thermoanaerobacter* (Fardeau et al., 1993), *Desulfotomaculum* (Nilsen et al., 1996), *Halanaerobium* (Booker et al. 2017; Ravot et al., 1997; Bhupathiraju et al., 1994; Bhupathiraju et al., 1999) and many others. The growth temperature range of *Halanaerobium* species range from 15^o-51^o C. Along with enjoying a high temperature members of the genera *Halanaerobium* also prefer high salt conditions (about 10%) (Bhupathiraju et al., 1994; Bhupathiraju et al.,

1999; Ravot et al., 1997; Zeikus et al., 1983). This makes high temperature and high salt produced fluids from oil and gas drilling ideal growth environments for *Halanaerobium*. Also, various chemicals added during drilling can be used by microbes. A *Halanaerobium* strain degraded guar gum, which is a polysaccharide commonly used in drilling (Liang et al., 2016).

Halanaerobium

The genus *Halanaerobium* is part of the family Halanaerobiaceae, Order Halanaerobiales, Class Clostridia, Phylum Firmicutes. They are non-spore forming, non-motile, Gram-negative rods, obligate anaerobes, which ferment sugars like fructose, glucose and cellulose (Zeikus et al., 1983). *Halanaerobium* strains have been isolated from a variety of environments around the world which include oil fields, high saline lakes, and oil and gas produced fluids. Their optimal salt concentration is about 10% (Bhupathiraju et al., 1994; Bhupathiraju et al., 1999; Ravot et al., 1997), but, *H. praevalens* can tolerate up to 30% salt (Zeikus et al., 1983). Optimal temperatures are about 37^{o}-40^{o}C, but *H. salsuginis* and *H. praevalens* can tolerate up to 50^{o}C (Bhupathiraju et al., 1994; Bhupathiraju et al., 1999; Ravot et al., 1997; Zeikus et al., 1983). Recent studies have investigated the microbial community of highly saline produced fluids and found *Halanaerobium* to be predominant member of the community (Booker et al., 2017). An interesting feature of the genus is that some species are reported thiosulfate reducers (Bhupathiraju et al., 1999; Ravot et al., 1997; Zeikus et al., 1983) while others are not (Brown, 2012, Bhupathiraju 1994). This ability to reduce thiosulfate to sulfide is of concern, due to the corrosive properties of sulfides.

Thiosulfate Disproportionation

Thiosulfate disproportionation can be classified into four pathways which reduce thiosulfate to sulfite and hydrogen sulfide, presented below (Caspi et al., 2014).

Thiosulfate Sulfurtransferase (Rhodanese, EC 2.8.1.1)

Sulfurtransferases (EC 2.8.1.X) are classified as catalyzing the transfer of sulfur atoms from a sulfur donor to a sulfur acceptor. The sulfurtransferase known as "rhodanese" (EC 2.8.1.1) has been described in many prokaryotes and eukaryotes (Westley et al., 1983). The reaction is described as thiosulfate + hydrogen cyanide → sulfite + thiocyanate + 2H (KEGG enzyme 2.8.1.1). Rhodanese proteins are widespread enzymes which are thought to be used for cyanide detoxification (Cipollone et al., 2005; Raybuck et al., 1992). Rhodanese can be classified into 4 groups (Cipollone et al., 2007). Group I are single domain proteins that contain only one catalytic site. This class of rhodanese has been observed in *E. coli* as a thiosulfate sulfurtransferase (GlpE), which is able to produce thiocyanate from thiosulfate in the presence of thiosulfate and cyanide (Ray et al., 2000). Group II are tandem-domain proteins, where each domain has a rhodanese module and an active cysteine or inactive aspartate residue (Bordo and Bork, 2002). A tandem-domain rhodanese-like protein has been investigated in *Pseudomonas,* coded by rhdA, and shown to be used for cyanide detoxification (Cipallone et al., 2008). An interesting Group II tandem-domain rhodanese-like protein is found in *Halanaerobium congolense DSM11287,* rdlA. The protein coded by rdlA was predicted as a thiosulfate sulfurtransferase and has two domains with active cysteine residues. While the role of RdlA is not certain, it is thought to be involved in reducing thiosulfate (Ravot et al., 2005). Group III rhodanese are multi-domain

enzymes where the rhodanese domain is found with other active domains (Cipollone et al., 2007). An example of this group can be found in *Arabidopsis thaliana*, which has a protein with an inactive rhodanese homology domain, is described as an extracellular calcium sensing receptor, suggesting it has a signaling-like function (Han et al., 2003). Group IV rhodaneses are elongated active-site loop proteins. In Cdc25A (*Homo sapiens*), the rhodanese domain is an elongated stretch of the active-site loop (McCain et al., 2002).

Thiosulfate-thiol Sulfurtransferase (EC 2.8.1.3)

Thiosulfate-thiol sulfurtransferase (EC 2.8.1.3) uses a thiol like L-cysteine, glutathione, or L-homocysteine to reduce thiosulfate to sulfite and sulfide (Chauncey et al., 1983). The reaction is: thiosulfate + 2 glutathione = sulfite + glutathione disulfide + sulfide (KEGG Enzyme 2.8.1.3). The reaction is characterized as transferring the sulfane sulfur (the sulfur atom without oxygen attached) to the thiol, which forms sulfite and a persulfide. Chauncey et al. (1987) showed this reaction with glutathione and concluded S-sulfanylglutathione was an intermediate which reacted with excess glutathione to produce hydrogen sulfide. Species that have been reported using this enzyme include *Thiobacillus thiooxidans* (Suzuki and Werkman, 1958) and *Thiobacillus thioparus* (Peck and Fisher, 1961).

Thiosulfate Reductase (Cytochrome) (EC 1.8.2.5)

A thiosulfate reductase which uses reduced cytochrome as a sulfur group acceptor can be found in species like *Desulfovibrio vulgaris (*Haschke et al., 1971*) D. gigas* (Hatchikian, 1975*),* and *D. nigrificans* (Nakatsukasa and Akagi, 1969). The reaction is thiosulfate + 2 ferrocytochrome c3 ←→ sulfite + hydrogen sulfide + 2

ferrocytochrome c3 (KEGG Enzyme 1.8.2.5, http://www.genome.jp). In *D. desulfuricans* the enzyme has been partially purified and described as using cytochrome c to move electrons between the thiosulfate reductase and the hydrogenase (Ishimoto and Koyama, 1955; Ishimoto and Koyama, 1957). The thiosulfate reductase of *D. vulgaris Hildenborough* was purified and described as reducing thiosulfate in the presence of hydrogen to sulfite and sulfide (Haschke et al., 1971). Using this thiosulfate disproportionation path, *D. sulfodismutans* was shown to grow when acetate was also present as a carbon source (Bak and Cypionka, 1987).

Thiosulfate Reductase (Quinone) (EC 1.8.5.5)

Thiosulfate reductases are characterized as reducing thiosulfate to sulfite and sulfide and are encoded by the locus *phs* (Clark and Barrett, 1987). Thiosulfate reductases have been reported in enteric bacteria such as *Salmonella typhimurium* to reduce thiosulfate to sulfite and sulfide (Stoffels et al., 2012). This Phs enzyme is characterized as a molybdopterin oxidoreductase consisting of three subunits (ABC), with subunit A functioning as the catalytic subunit (Heinzinger et al., 1995). The reaction is: thiosulfate + a quinol = sulfite + hydrogen sulfide (KEGG 1.8.5.5).

Dissimilatory Sulfite Reductases

There are two known enzymes for sulfite reduction; the anaerobic sulfite reductase (Huang & Barrett, 1990) and the "dissimilatory" sulfite reductase (Lee et al., 1973; E.C. 1.8.99.5). Dissimilatory sulfite reductases catalyze the reduction of sulfite to sulfide.

Dissimilatory Sulfite Reductase (EC 1.8.99.5)

Sulfate-reducing bacteria (SRB) include diverse groups such as Proteobacteria (Beeder et al., 1995) and Fimicutes (Daumas et al., 1988). These SRB have been found to

contain a dissimilatory sulfite reductase coded for by *dsrAB* (Muller et al., 2014). The first described dissimilatory sulfite reductase was purified from *Desulfovibrio vulgaris* and described as a tetramer with two types of subunits: DsrA, DsrB (Lee, 1973). A recent study has shown a third subunit, coded by *dsrC,* interacts with the DsrAB to produce hydrogen sulfide. The authors suggest that DsrC interacts with DsrAB by extending its C-terminus and inserting it into a cleft between the DsrA and DsrB proteins (Oliveira et al., 2008). A more recent study supported this work and showed in *D. vulgaris* cell extracts DsrC was mostly not associated with DsrAB, and can be available to interact with other proteins (Venceslau et al., 2013).

Anaerobic Sulfite Reductase

Anaerobic sulfite reductase (Asr, no EC number assigned) has been investigated in *Salmonella enterica typhimurium* for its function and found to reduce sulfite to sulfide via a three-subunit complex. The gene sequences coding for the three subunit proteins (asrABC) are located in an operon, which are induced by sulfite in anaerobic conditions (Huang and Barrett, 1990; Huang and Barrett, 1991). Like other dissimilatory sulfite reductases, the enzyme is composed of a flavoprotein (AsrB) and a hemoprotein containing a siroheme in the active site (AsrC) (Huang & Barrett, 1990). Currently the enzyme has not been purified. Information for Asr is limited to mostly *Salmonella enterica typhimurium,* although two operons coding for anaerobic sulfite reductase, *asrABC1* and *asrABC2*, were identified in *Clostridium perfringens* (Andre et al. 2010). However, metagenomic studies have shown oil and gas drilling fluids, from the Utica Shale and Marcellus Shale, contain genes for the Asr sulfite reductase and not the dissimilatory sulfite reductase (Booker et al., 2017; Lipus et al., 2017). These fluids are

dominated by *Halanaerobium*, and the authors have suggested that Asr is the sulfite reductase used by *Halanaerobium*.

Chapter 2: Thiosulfate Reduction in *Halanaerobium*

Introduction

Developing techniques to detect bacteria involved in corrosion of steel in the oil and gas drilling industry has long been a target of research. Early studies focused on the detection of sulfate reducing bacteria (SRB), which reduce sulfate to sulfite then to sulfide, as a major group responsible for sulfide production in oil production (Cord-Ruwisch et al., 1988). The main molecular detection targets for these SRB are the genes coding for dissimilatory sulfite reductase (DsrAB, EC 1.8.99.5), which have been shown to be effective at quantification of SRB populations (Ben-Dov et al., 2007; Agrawal & Lal, 2009). However, the Dsr is not the only enzyme know to reduce sulfite to sulfide; Huang et al. (1990) demonstrated the anaerobic sulfite reductase in *Salmonella enterica typhimurium LT2* (Asr, EC not assigned) can also produce sulfide from the reduction of sulfite. The Asr has been detected in other genera associated with oil production such as *Clostridium* (Harrison et al., 1984; Andre et al., 2012), but is not currently a target for monitoring sulfide producing bacteria. In addition, sulfide can be produced not only by the reduction of sulfate to sulfite and then sulfide, but also by the reduction of thiosulfate. Thiosulfate is added to drilling fluids and is available to microorganisms for reduction to sulfide (Liang et al., 2014). Thiosulfate reducing bacteria should therefore be of interest in biocorrosion mitigation, especially as the reduction of thiosulfate is suggested to hasten growth of *Halanaerobium* (Ravot et al., 1997).

Thiosulfate reducing bacteria that reduce sulfite to sulfide are not as well studied as SRB, especially thiosulfate reducing bacteria associated with oil and gas production.

However, Ravot et al. (1995) isolated a thiosulfate reducing strain of *Halanaerobium* (*H. congolense DSM11287*) from a corroding oil pipeline and other strains of *Halanaerobium* have been shown to produce sulfide from reducing thiosulfate (*H. kushneri*: Bhupathiraju et al., 1994; *Halanaerobium* strain DL-01: Liang et al.,2016).

Halanaerobium is a genus (Phylum: Firmicutes, Class: Clostridia, Order: Halanaerobiales, Family: Halanaerobiaceae) of obligatory anaerobic bacteria whose strains grow at a NaCl concentration of 3%-25%, pH 5-11, temperature range 15^{o}-51^{o}C and ferment sugars like glucose, fructose, and sucrose (Ravot et al., 1997; Bhupathiraju et al., 1994; Bhupathiraju et al. 1999; Brown et al., 2012; Zeikus et al., 1983). They have been found in locations around the world, including drilling sites (Lipus et al., 2017), drilling fluids (Ravot et al., 1997; Bhupathiraju et al., 1994; Bhupathiraju et al. 1999; Liang et al., 2016) and even highly saline lakes (Brown et al., 2012; Zeikus et al., 1983). Finding *Halanaerobium* in various locations should be of concern for their sulfide production that could lead to corrosion. *H. congolense DSM11287* is a well know thiosulfate reducing strain of *Halanaerobium* described as producing sulfide from thiosulfate reduction. The first report of this species was also the second report of a species in the order *Halanaerobiales* able to reduce thiosulfate to sulfide (Ravot et al., 1997).

Metagenomic studies of produced water from hydraulic fracturing have shown that while sulfate reducing bacteria are present, thiosulfate reducing bacteria also inhabit the water and in samples with high salt and high temperature can be dominant organisms (Lipus et al., 2017). A study of a Mid-East oil field production facility experiencing accelerated biocorrosion found *Halanaerobium* was a dominant genus at

multiple sampling points in the facility (Marks et al., 2016). In high salt produced water from a hydraulically fractured well the bacterial community was found to be dominated by halophilic, sufidogenic bacteria from the *Halanaerobiales* order (Liang et al., 2016). and a thiosulfate-reducing *Halanaerobium* (strain DL-01) was isolated and shown to corrode mild steel (Liang et al., 2016). Therefore, determining the abundance and activity of *Halanaerobium* in oil and gas producing facilities should be of concern. Investigation of the genetic basis of production of sulfide by *Halanaerobium* could result in molecular targets for monitoring

Genetic and Enzymatic Basis of Thiosulfate Reduction

There are several different pathways that produce sulfide from reducing thiosulfate, and are classified under thiosulfate disproportionation (Capsi et al., 2013). Thiosulfate disproportionation I which uses a thiosulfate-thiol sulfurtransferase (E. C. 2.8.1.3; Chauncey et al., 1983), is a thiol dependent reaction which reduces thiosulfate to sulfite and hydrogen sulfide, and was first observed in *Saccharomyces cerevisiae* (Kaji et al., 1959). Thiosulfate disproportionation II uses a cytochrome to complete the reduction of thiosulfate. A cytochrome dependent thiosulfate reductase (EC 1.8.2.5) was purified from *Desulfovibrio vulgaris Hildenborough* (Haschke et al., 1971). The third option, thiosulfate disproportionation III, is a quinone dependent membrane bound complex with the thiosulfate reductase subunit coded for by phsABC. This complex has mostly been studied in *Salmonella enterica typhimurium* where the Phs is well described as an molybdoterin oxidoreductase (EC 1.8.5.5; Clark & Barrett, 1987; Heinzinger et al., 1995). The final reduction path which can produce sulfide is thiosulfate disproportionation IV. In this reaction, a rhodanese-like protein reduces

thiosulfate to sulfite and thiocyanate in the presence of cyanide (Cippollone et al., 2005). This type of rhodanese protein is described as a thiosulfate sulfurtransferase (EC 2.8.1.1). A tandem domain rhodanese-like protein, coded for by *rdlA*, was identified in *H. congolense DSM11287*, and suggested as the enzyme responsible for thiosulfate reduction in this organism (Ravot et al., 2005).

Sulfite reduction can be accomplished using a sulfite reductase. The dissimilatory sulfite reductase (Dsr, EC 1.8.99.5), encoded by the *dsrA* and *dsrB* genes, is used by many sulfate reducing bacteria to reduce sulfite generated from sulfate reduction. DsrAB is well studied in sulfate reducing bacteria, due to their role in the geochemical cycling of sulfur and their global presence (Pester et al. 2012; Muyzer and Stams, 2008). In *D. vulgaris Hildenborough* the enzyme has been purified, which revealed the DsrA binds two sirohydrochlorin and two 4Fe4S centers while the DsrB binds two siroheme 4Fe-4S cofactors (Oliveira et al., 2008).

Another sulfite reductase, anaerobic sulfite reductase (Asr) enzyme is able to reduce sulfite to sulfide. It is well described in *S. enterica typhimurium LT2* as a 3-subunit enzyme, AsrA, AsrB, and AsrC (Huang and Barrett, 1990; Huang & Barrett, 1991). The AsrA and AsrC subunits have 4Fe-4S ferredoxins and the AsrB has an oxidoreductase domain (Huang et al., 1991). Recent studies have shown the Asr subunits: asrA, asrB, and asrC, are expressed at higher levels when thiosulfate is present (Booker et al., 2017).

Genetic Basis of Thiosulfate reduction in Halanaerobium

A thiosulfate sulfur-transferase rhodanese-like protein with potential active sites, encoded by *rdlA*, has been described in *H. congolense DSM11287,* a thiosulfate

reducing species (Ravot 1997; Ravot 2005). As mentioned previously, the rhodanese catalyzes the reduction of thiosulfate to sulfite and thiocyanate when cyanide is present. The rhodanese-like protein encoded by *rdlA*, was suggested to be responsible for thiosulfate reduction in *H. congolense DSM11287* (Ravot et al., 2005). The detection of this enzyme in a thiosulfate reducing *Halanaerobium* species makes it likely to find it in other thiosulfate reducing *Halanaerobium*, but their genomes have not been searched for genes coding for other thiosulfate disproportionation enzymes. Prior studies have also reported genomes of *Halanaerobium* reconstructed from hydraulically fractured well metagenome samples contain sequences for *asrA*, *asrB*, and *asrC* (Booker et al., 2017). *Halanaerobium* are not detected via the *dsrAB* in qPCR, so the logical choice, supported by other reports (Booker et al., 2017; Lipus et al., 2017), is that anaerobic sulfite reductase is the enzyme used by *Halanaerobium* species to reduce sulfite to sulfide.

The hypothesis of this research was that the presence of various thiosulfate reducing and sulfite reducing genes in the genomes of *Halanaerobium* strains grant those strains the ability to reduce thiosulfate and sulfite and produce sulfide. Previous research identified thiosulfate and sulfite reducing genes in metagenomic data dominated by *Halanaerobium* and correlated sulfide production with loss of thiosulfate (Booker et al., 2017). However, no study has shown direct evidence of thiosulfate reduction and sulfite reduction leading to sulfide production. This study combines genomic and physiological investigation of the same strains to present a scenario for the genetic basis of thiosulfate and sulfite reduction by *Halanaerobium*. In the physiological investigation, the concentration of thiosulfate, sulfite, sulfide, and

rhodanese activity is measured over time in three different strains of *Halanaerobium* grown in media containing thiosulfate or sulfite. The genomes of the three strains chosen had been interrogated for the presence of thiosulfate reductases and sulfite reductases, and the strains varied in whether or not they contain sequences for a thiosulfate reductase and/or a sulfite reductase. In addition, this research reports the novel finding of production of sulfide from sulfite by *Halanaerobium*.

Materials & Methods

Origin of Halanaerobium Strains

H. congolense DSM11287 was purchased from the DSZM (German Collection of Microorganisms and Cell Cultures). *H. salsuginis (ATCC51327)* and *H. kushnerii (ATCC7000103)* were purchased from the ATCC (American Type Culture Collection). *H. hydrogeniformans DSM 2228* (Brown et al., 2012) was acquired from Dr. Melanie Mormile. It was originally isolated from a haloalkaline lake, Soap Lake, in Washington State. *H. vreelandii ZB2A* was collected from Annette De Capite, and is a novel species isolated from a middle east oil field (De Capite, 2015).

Cultivation of Halanaerobium

The defined medium base contained the following per liter: NH_4Cl, 1.0g; KCl, 0.1g; KH_2PO_4, 0.1g; $MgSO_4 \cdot 7H_2O$; 0.2g; $CaCl_2 \cdot 2H_2O$, 0.04g; PIPES (piperazine-N,N'-bis[2-ethanesulfonic acid]) dipotassium salt, 1.5g; yeast extract, 1.0g; glucose, 5g; resazurin, 0.0005g; L-cysteine, 0.005g; and $Na_2S \cdot 9H_2O$, 0.005g, as well as 5 mL of trace metal solution (Tanner, 2007), 10 mL of vitamin solution (Tanner, 2007), and a pure N_2 gas phase. The medium was adjusted to culture each strain under optimum

conditions of pH, temperature, and salt content as shown in Table 1. All 3 cultures were grown anaerobically and transferred to fresh media every 7 days.

Whole Genome Sequencing and Contiguous Sequence Assembly

H. vreelandii ZB2A, H. kushnerii ATCC700103, and *H. congolense DSM11287* were grown in defined medium containing 15 mM/L sodium thiosulfate and then collected in early stationary phase by centrifuging 6 mL of culture at 6,000Xg for 15 minutes. Pellets were washed twice in an appropriate brine solution and then resuspended in 1 mL of Tris EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Resuspended samples were treated with DNAzol Direct (DN131, Molecular Research Center) and proteinase-K (Thermoscientific EO0491) prior to DNA extraction with an automated nucleic acid extraction instrument (Maxwell® 16 Cell LEV Total RNA Purification Kit AS1222 by Promega), as described by Oldham et al. (2012). The genomic DNA of *H. congolense DSM11287*, *H. vreelandii ZB2A*, and *H. kushnerii ATCC700103* were sequenced at Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma using an Illumina Miseq (250 base pair paired ends) and then assembled into contiguous sequences (contigs). This was done by first removing Illumina sequence adapters from the reads using Cutadapt 1.14 (Martin et al. 2011). Biopieces was then used to trim reads to a quality score of 30 (BIOpieces, github.com/maasha/biopieces). Trimm-o-matic (Bolger et al., 2014) was used to remove reads less than 50 or greater than 250 base pairs, and then pairs were mated. Quality trimmed mated reads were *de novo* assembled by Velvet (Zerbino et al., 2008) into contigs 1500-1750 base pairs long. Genomic contigs for *H. praevalens DSM 2228* (CP002175.1), *H. salsuginis ATCC51327* (NZ_FOTI01000001.1), and *H.*

hydrogeniformans DSM 6643 (NC_014654.1) were obtained from the Joint Genome Institute database (genome.jgi.doe.gov).

Gene Detection using Custom Workflow

To explore the genomic potential of the *Halanaerobium* species a workflow was designed to locate target genes for thiosulfate (*qrcB, bisC, rdlA*, and *phsABC*) and sulfite reduction (*dsrA, dsrB, asrA, asrB, asrC*). Genomic contigs of *H. congolense DSM11287, H. kushnerii ATCC700103, H. vreelandii ZB2A, H. salsuginis ATCC51327* (NZ_FOTI01000001.1), *H. hydrogeniformans DSM6643* (NC_014654.1), and *H. praevalens DSM2228* (CP002175.1) were examined with Prodigal (Hyatt, 2010) and open reading frames (ORF) were predicted using default parameters. The ORFs were translated into amino acids to be used for gene detection.

The Pfam database (Finn et al., 2016) was used to determine the protein family for each Asr subunit. For this search *H. congolense* Asr amino acid sequences for AsrA (WP_073157054.1), AsrB (WP_073157057.1), and AsrC (WP_073157060) were used. Amino acid sequences from *S. typhimurium LT2* for AsrA (NP_461483.1), AsrB (AAA99276.1), and AsrC (AAL21444.1) were also submitted to determine if the subunits contain the same protein families. If the subunits for the two species contain the same protein families, then they are assumed to have the same function.

Specific Gene Detection Using BLAST

To determine specific genes from *Halanaerobium* species in the investigated genomes BLAST (Altschul et al., 1990) was used. For thiosulfate reduction to sulfite amino acid sequences from a thiosulfate-thiol sulfurtransferase (from *Thiobacillus denitrificans*, WP_059759455.1), thiosulfate reductase (from *Desulfovibrio vulgaris Hildenborough*,

YP_009398.1), PhsB (from *S. enterica subsp. enterica serovar typhimurium*, WP_070789800.1), and RdlA (from *H. congolense*, AAS68581.1) were used as a query against the genome contigs of each species using BLASTp. The AsrA, AsrB, and AsrC subunit amino acid sequences from *H. congolense* (WP_073157054.1, WP_073157057.1, WP_073157060) and *H. salsuginis* (WP_089861877.1, WP_089861878.1, WP_089861879) and DsrA and DsrB subunit amino sequences from *Desulfovibrio vulgaris str. Hildenborough* (AAA70107.1, AAA70107.1) were queried using BLASTp against the predicted open reading frames from prodigal for the genome contigs for each species. The default BLASTp parameters were used e.g.: expect threshold: 10, word size: 6, maximum matches in a query range: 0, matrix: blossum62, gap cost: existence,11, extension, 1, and conditional composition score matrix adjustment. For all of these BLASTp queries a percent Grade cutoff of 35% was used to confirm a hit; Grade is a weighted score that combines the E-value, % pairwise identity and query coverage. This search was done for *H. congolense* DSM11287, *H. vreelandii ZB2A*, *H. kushnerii ATCC700103*, *H. salsuginis ATCC51327*, *H. praevalens DSM2228*, and *H. hydrogeniformans DSM6643*.

To confirm a BLAST hit the sequence is used to search the Pfam database and determine protein families found in the sequence. The protein families returned must match the protein families for the previously queried sequences from *H. congolense* (WP_073157054.1, WP_073157057.1, WP_073157060) and *H. salsuginis* (WP_089861877.1, WP_089861878.1, WP_089861879). The Pfam protein families for each sequence are listed in Appendix A.

Thiosulfate and Sulfite Utilization

To determine if *Halanaerobium* species are able to reduce thiosulfate to sulfite and then reduce sulfite to sulfide an experiment was designed to measure thiosulfate loss, sulfite increase/decrease and sulfide increase.

Three treatments were setup using 160 ml serum bottles containing 90 ml of *Halanaerobium* base broth. The first treatment had broth amended with 10mM/L final concentration of sodium thiosulfate (Sigma, S-7143), the second was amended with 2mM/L final concentration of sodium sulfite (Fisher Scientific, BP355-500), and the third was amended with both 10mM/L final concentration of sodium thiosulfate and 2mM/L sodium sulfite. Each treatment had three replicates inoculated with a live culture and one bottle inoculated with a culture killed by autoclaving ("heat-killed control"). An inoculum of each species was prepared by incubating a 200 ml culture for seven days. *H. congolense DSM11287* and *H. salsuginis ATCC51327* were incubated at 45^{o} C and *H. hydrogeniformans DSM6643* was incubated at 31^{o} C. From the inoculum 50 ml was transferred to a separate anoxic bottle and then autoclaved to use as the heat killed inoculum. Once bottles were amended with thiosulfate, sulfite, or both thiosulfate and sulfite, 10 ml of inoculum was added to the bottle. Day 0 (T_0) was defined as the day the inoculum was added to bottles. Measurements taken during the experiment included optical density (OD) at 600 nM using a spectrophotometer (Biochrom WPA Biowave II). Thiosulfate and sulfite were sampled from the liquid phase and their concentration determined from colorimetric titration kits from CHEMetrics (thiosulfate K-9705, range = 5-50ppm, min detection = 0.05mmol/L; sulfite K-9650, range = 50-500ppm, min. detection= 0.6 mmol/L). The thiosulfate and sulfite kits use the

iodometric method (USEPA Method 377.1, 1983) for detection. Sulfide sampled from the liquid phase was measured using the methylene blue assay (EPA Method 376, APHA Standard Methods, Method 4500-S D-2000) from CHEMetrics (Sulfite K9501-D, Range = 0-300ppm, min detection = 0.1mmol/L). For pH strips were used (Range 7.5-14 (109532) & 2-9 (109450), Millipore Sigma). Measurements taken at T_0 included optical density, thiosulfate concentration, sulfite concentration, sulfide concentration, and pH. Measurements of optical density, sulfide concentration and pH were taken on day 4, 6, 8, 10 and 12. For the day 12 sulfide concentration reading 10N NaOH was added to the bottles until the pH was 12 to move any gaseous sulfide (e.g. H_2S) into the aqueous phase as HS^- and S^2 for measurement. As sulfide is a weak acid with a pKa of 6.9, it is only slightly soluble in water at that pH. Sulfide is much more soluble in water at pH 12 (Suleimenov and Krupp, 1994).

Rhodanese Assay for Enzyme Activity

Rhodanese activity was measured by following the protocol described by Singleton and Smith (2005). A 100μl sample from each treatment bottle was taken and was added to a 1.5ml microfuge tube containing 380 ul of phosphate buffer (pH 8.1, 27mM), 10 μl NaS_2O_3 (1M), and 10 μl KCN (1M). The tube was incubated for 20 minutes at 37^0 C, after which 37% formaldehyde was added to stop the reaction. Ferric reagent (500 μL of 15g ferric nitrate in 100 ml 0.1M HNO_3) was then added and the tube centrifuged at 15,000Xg for 5 minutes. The supernatant was removed and optical density of the iron-thiocyanate complex measured with a spectrophotometer (Biochrom WPA Biowave II) at 470nm. The optical density measurement corresponds to the thiocyanate concentration in the sample. To determine if there is increased activity in

the live cultures the treatments are compared to the value of their respective heat killed control as there is a level of spontaneous production of thiocyanate by the reagents (Ravot et al., 2005). The assay was used to measure activity on day 4 and day 12 of the thiosulfate and sulfite utilization assay.

Results.

Gene Detection (Assembly, Prodigal, Pfam)

Table 2 details the assembly of the genomes sequenced, which include number of contigs assembled and the average length of the contigs. Approximately 2,000 contigs were assembled for each sequenced *Halanaerobium* genome, and the approximate average length of the contigs was 1,200 nucleotides. Over 2,000 open reading frames were predicted for each *Halanaerobium* genome, and Table 2 shows the specific number of open reading frames for each species. Table 2 also provides the assembled genome information for genomes collected from National Center for Biotechnology Information, which were complete genomes. Note that similar numbers of open reading frames were found for genomes retrieved from JGI or sequenced and assembled in this study.

The search results for Asr protein families returned the same protein families for both species, *H. congolense* and *S. enterica*. The AsrA subunit belonged to the 4Fe-4S dicluster domain (PF17179). For AsrB there were two protein families, oxidoreductase NAD-binding domain (PF00175) and iron-sulfur cluster binding domain of dihydroorotate dehydrogenase B (PF10418). The AsrC subunit returned three protein families: Nitrite/Sulfite reductase ferredoxin-linked half domain (PF03460), Nitrite and

Sulfite reductase 4Fe-4S domain (PF01077), and 4Fe-4S dicluster domain (PF12838) (Appendix A).

Specific Gene Detection Using BLAST

Table 3 describes the percent identity, length of the match, and Grade score for BLAST matches in the *Halanaerobium* genomes. *H. congolense* DSM11287, *H. praevalens DSM2228, H. vreelandii ZB2A, H. kushnerii ATCC700103*, and *H. salsuginis ATCC51327* had hits for *rdlA*, coding for a rhodanese-like protein, thiosulfate disproportionation IV. *H. hydrogeniformans DSM6643* did not have a hit for *rdlA*. BLAST results for the sulfite reductase revealed hits for *asrB* in *H. congolense DSM11287, H. praevalens DSM2228, H. vreelandii ZB2A, H. kushnerii ATCC700103*, and *H. salsuginis ATCC51327. H. hydrogeniformans DSM6643* had a hit for *asrB*, but upon searching the BLAST hit in the Pfam database, AsrB was found to not contain the iron-sulfur cluster binding domain of dihydroorotate dehydrogenase B (PF10418) that was found in *H. congolense* (WP_073157057.1) and *S. enterica typhimurium* (AAA99276.1), but did have the oxidoreductase NAD-binding domain (PF00175). Hits were not found for *asrA* or *asrC* in any of the genomes except *H. salsuginis ATCC51327*, which had hits for all three Asr subunits. In summary, *H. congolense* DSM11287, *H. praevalens, H. vreelandii ZB2A, H. kushnerii ATCC700103*, and *H. salsuginis ATCC51327* had hits for *rdlA* and *asrB*. No hits were found for thiosulfate-thiol sulfurtransferase, thiosulfate reductase, *phsB* or *dsrAB* in any of the genomes.

Thiosulfate and Sulfite Utilization

H. congolense DSM11287 was observed to grow best in the thiosulfate amended bottles, with an average maximum optical density of 0.481 (std. dev. 0.002) on day 8,

nearly as well in medium amended with both thiosulfate and sulfite, and poorly in medium amended only with sulfite (Fig. 1). Sulfide production was first observed on day 4 for thiosulfate amended bottles, on day 6 thiosulfate plus sulfite amended bottles and only at day 12 for sulfite amended bottles. At the final measurement (day 12) both thiosulfate and thiosulfate plus sulfite amended bottles had the same concentration of sulfide (17.6 mM), while sulfite amended bottles showed a small amount of sulfide (0.25 mM) produced (Table 4). Thiosulfate was lost in both treatments containing thiosulfate, with thiosulfate plus sulfite losing 8.6 mM and thiosulfate amended losing 8 mM. Sulfite increased in bottles amended with thiosulfate and thiosulfate plus sulfite, to a final concentration of 3.1mM and 2.4 mM respectively. In sulfite amended bottles a loss of 0.9 mM sulfite was measured. To summarize, *H. congolense DSM11287* grew well in both conditions where thiosulfate was present but not when amended only with sulfite. Growth was poor in the sulfite only amended bottles, but sulfide was produced in all three treatments.

H. salsuginis ATCC51327 grew best in thiosulfate amended bottles (average maximum optical density of 0.609 (std. dev. 0)), while bottles with sulfite or thiosulfate plus sulfite grew almost identically (Fig. 2). Sulfide production was observed on day 6 for thiosulfate only amended bottles and observed on day 12 for thiosulfate plus sulfite and sulfite amended bottles (Fig. 2). Thiosulfate amended bottles had the highest sulfide production with 9.1 mM; the final sulfide concentration for thiosulfate plus sulfite was 0.5mM, and sulfite only amended 0.3 mM (Table 4). Thiosulfate loss occurred in both treatments with thiosulfate. Thiosulfate loss was just slightly more in thiosulfate plus sulfite amended bottles with 9 mM lost compared to thiosulfate amended bottles 8 mM

loss. Sulfite increased in bottles amended with thiosulfate and thiosulfate plus sulfite, while a decrease occurred in sulfite only amended bottles (Table 4). In summary, *H. salsuginis ATCC51327* grew best in thiosulfate only amended bottles, which produced sulfide sooner than bottles amended with both thiosulfate and sulfite. The amount of sulfide produced was almost equal at the end of the experiment for sulfite amended and thiosulfate/sulfite amended bottles. Growth was poor in the sulfite amended bottles, but sulfide was produced.

H. hydrogeniformans DSM6643 showed the best growth in thiosulfate amended conditions, with average maximum optical density was 0.260 (std. dev. 0.010) on day 4 (Figure 3). The sulfite amended bottles had a slightly lower average maximum OD than thiosulfate plus sulfite. Thiosulfate loss, sulfite gain, sulfite loss, or sulfide production were not observed in any treatments (Table 4).

Rhodanese Assay for Enzyme Activity

Rhodanese activity was detected in *H. congolense DSM11287* grown in medium amended with thiosulfate or with both thiosulfate and sulfite, but not when amended with sulfite alone (Table 5). On day 4 the activity was higher than on day 12. For *H. salsuginis ATCC51327*, activity was only detected when grown in medium amended with thiosulfate, and activity was higher on day 4 than day 12. Rhodanese activity for any treatment containing *H. hydrogeniformans DSM6643* was not above background levels for either day 4 or day 12.

Mass Balance of Sulfur for Thiosulfate and Sulfite Reduction

A mass balance of sulfur can be calculated to estimate if the loss of thiosulfate in thiosulfate amended incubations matches the gain in sulfite and sulfide. As thiosulfate

contains two sulfur atoms, for every thiosulfate molecule reduced it is possible to form one sulfite molecule plus one hydrogen sulfide molecule or two hydrogen sulfide molecules. Table 6 displays the sulfur in millimoles lost or gained through reducing thiosulfate or sulfite. The table calculates the percent of sulfur atoms in thiosulfate/sulfite converted to sulfide in terms of millimole of sulfur. In *H. congolense DSM11287* thiosulfate amended treatments 129.38% of sulfur from thiosulfate was converted to sulfite-S and sulfide-S. In *H. salsuginis ATCC51327* the sulfite-S and sulfide-S produced was 84.38% of the theoretical yield. The yield for *H. congolense DSM11287* being greater than 100% may be due the measurement error of the detection kits used. The yields support that the sulfur from thiosulfate ended up as sulfite-S or sulfide-S. For sulfite amended treatments, the sulfur yields as sulfide for *H. congolense DSM11287* and *H. salsuginis ATCC51327* were almost the same at 27.77% and 27.27%. This is well below what is theoretically expected, but does support that some sulfite is being reduced to sulfide. In thiosulfate plus sulfite treatments *H. congolense DSM11287* also showed a high yield (119.04% theoretical). *H. salsuginis ATCC51327* had a low yield (16.11%). Even though the yield for *H. salsuginis ATCC51327* is much lower than *H. congolense DSM11287,* Table 6 supports the interpretation that both organisms are reducing thiosulfate to sulfite and sulfide due to a loss of sulfur from thiosulfate. While not all sulfur is accounted for, especially in the sulfite amended treatments, enough is accounted for to support the suggested pathway for thiosulfate reduction. The sulfur not accounted for could be due to error in measurements from the test kits or to the sulfur being in some form not detectable by the test kits.

Discussion

Genomic Potential and Physiological Evidence for Thiosulfate Reduction and Sulfite Reduction in Halanaerobium spp.

Previous work had reported that four of the six strains investigated reduce thiosulfate to sulfide but did not reduce sulfite to sulfide (Ravot et al., 1997; Bhupathiraju et al., 1994; Bhupathiraju et al. 1999; Brown et al., 2012; Zeikus et al., 1983). However, this research shows that five of the six strains have the genomic potential to reduce thiosulfate to sulfite and sulfide and then sulfite to sulfide. The genomic potential of *Halanaerobium* strains containing both *rdlA* and *asr* subunits is supported by the physiology experiments observing utilization of thiosulfate and sulfite by two strains with the genetic potential to reduce thiosulfate and sulfite and no reduction by a strain without the genetic potential.

The *rdlA* sequence detected for the rhodanese like protein (classified as a thiosulfate sulfurtransferase) was the only thiosulfate reductase gene detected, of four tested. That rhodanese activity was detected only in treatments where thiosulfate loss occurred also supports its identification as the thiosulfate disproportionation enzyme for the *Halanaerobium* strains tested. Previous research also found rhodanese activity linked to thiosulfate reduction in *Halanaerobium* strains (Ravot et al., 2005; De Capite, 2015). The detection of Asr subunits in *Halanaerobium* genomes and no detection of Dsr subunits leaves Asr as the plausible choice for sulfite reduction to hydrogen sulfide. The anaerobic sulfite reductase (Asr) enzyme has been observed to reduce sulfite to hydrogen sulfide in *S. enterica typhimurium LT2* (Huang and Barrett, 1990). All of the species with hits except *H. salsuginis ATCC51327* and *H. hydrogeniformans DSM6643*

have been previously described as sulfide producers from thiosulfate (Ravot et al., 1997; Bhupathiraju et al., 1994; Bhupathiraju et al. 1999; Brown et al., 2012; Zeikus et al., 1983). The BLAST hits agree with observed physiology that the two *Halanaerobium* species tested (*H. congolense DSM11287* and *H. salsuginis ATCC51327* have the ability to reduce thiosulfate to sulfite and then to sulfide.

H. hydrogeniformans: Absence of Genomic Potential is Associated with the Inability to Reduce Thiosulfate or Sulfite

H. hydrogeniformans DSM6643 had no sequences matching *rdlA*, did not produce sulfide, lose thiosulfate or have rhodanese activity, so the genomic evidence and physiological evidence support each other. *H. hydrogeniformans DSM6643* was not observed to produce sulfide from sulfite, and the hit detected for AsrB was found to not contain the same two protein families as AsrB *S. enterica LT2* and *H. congolense DSM11287*, and may explain why sulfite reduction is not observed. *H. hydrogeniformans DSM6643* had an iron-sulfur cluster binding domain of dihydroorotate dehydrogenase B (PF10418) that was found in *H. congolense* (WP_073157057.1) and *S. enterica typhimurium* (AAA99276.1), but did not have the oxidoreductase NAD-binding domain (PF00175) (Appendix A). Even though *H. hydrogeniformans DSM6643* did not reach as high of an optical density as *H. congolense DSM11287* and *H. salsuginis ATCC51327*, the maximum average OD was close to the reported maximum optical density when grown in the presence of glucose: 3.01 (Brown et al., 2012). The pH range for *H. hydrogeniformans DSM6643* is reported to be 8-12, so although the pH dropped to 8 at day 4, the culture was still within the growth range (Brown et al., 2012). This suggests that while *H. hydrogeniformans*

DSM6643 indeed grew under the experimental conditions, it did not produce sulfide or reduce thiosulfate.

Other Studies on Genetic Basis of Thiosulfate Reduction in Halanaerobium

Two recent studies have used metagenomic analysis of functional genes to suggest the metabolic pathway used for the reduction of thiosulfate to sulfide in *Halanaerobium* species (Booker et al., 2017; Lipus et al., 2017). Both studies found that sequences coding for a rhodanese-like protein (*rdlA*, EC 2.8.1.1), and anaerobic sulfite reductase (*asr,ABC* (EC 1.8.99.5) were present in *Halanaerobium* genomes recovered from metagenomics sequencing of highly saline production water samples. Sulfide production was observed by Booker et al. (2017) in incubations of samples while thiosulfate was lost. Lipus et al. (2017) noted that 40 of 42 produced water samples taken from hydraulically fractured Marcellus Shale were dominated by the order *Halanaerobiales*, which contained a majority of sequences for *Halanaerobium*. These *Halanaerobium* sequences were investigated for the functional gene potential and found to contain *rdlA* and *asr* sequences.

Importance of Monitoring Halanaerobium

Halanaerobium have been found as dominant organisms in high salt and high temperature produced water samples at different locations around the world with high salt and high temperature environments (Lipus et al., 2017; Booker et al., 2017, Marks et al. 2016; Liang et al, 2014; Liang et al., 2016). These studies suggest that thiosulfate-reducing bacteria such as *Halanaerobium* may be contributing to sulfide production in the produced water, which can cause corrosion, well souring and fouling of gas and oil products.

Investigations of produced water show *Halanaerobium* as a predominant and important organism in some gas production pipelines (Liang et al., 2016). A *Halanaerobium* strain isolated from the pipeline fluid was shown to be more resistant to a biocide than *Desulfovibrio alaskensis*, a sulfate reducing bacteria (Liang et al., 2016). This point underscores the need for monitoring of thiosulfate reducing sulfide producers in oil and gas produced water. If only sulfate reducing bacteria are monitored, many *Halanaerobium* or other thiosulfate reducers will be missed and could potentially still be viable after biocide addition.

Thiosulfate reduction and sulfite reduction are not unique to *Halanaerobium*. The ability to reduce thiosulfate to sulfide has been investigated in species like *S. enterica typhimyrium LT2* (Stoffels et al., 2012), members of the *Thermotogales* order (Ravot et al. 1995), *Anaerobaculum* (Maune and Tanner, 2012; Liang et al., 2014) and *Firmicutes* such as *Clostridium thiosulfatireducens* (Hernandez-Eugenio et al., 2002). Members of the Thermotogales, Synergistetes and Clostridales are frequently found in oil and gas production samples (Ravot et al, 1995; Duncan et al., 2017; Maune and Tanner, 2012) and have been implicated in biocorrosion. As stated previously, biocorrosion is expensive, costing the oil and gas industry $1.372 billion of which 20% can be due to MIC (https://www.nace.org/Corrosion-Central/Industries/Oil---Gas-Production; Almahamedh et al., 2011). A detection method that is reliable for non-sulfate reducing sulfide producers is needed. I propose targeting *rdlA* and/or *asr* genes for detection of *Halanaerobium* species capable of sulfide production. Detection based

on *asr* subunits may also help to detect a wider range of the thiosulfate reducing bacteria found in oil and gas production facilities.

Future work based on this research would be to create primers for qPCR to determine functional gene populations for *rdlA* and *asr*. Primers may be easier to create for the *rdlA* than *Asr* based on the high grade % hits returned for *rdlA* versus *asr* (Table 3). This lack of hits could be due to lack of coverage for the genome sequencing or that the sequence varies greatly from species to species. Even though Ravot et al. (2005) showed the *rdlA* sequence varied widely from species to species of different phyla of bacteria, the results of searching the *Halanaerobium* genomes found high grade % hits for the sequence. Primers could be developed solely for *Halanaerobium* detection, which could still potentially identify the *rdlA* of other species within the Firmicutes. These primers could then be used as a way to monitor the *Halanaerobium* and other populations capable of thiosulfate reduction.

In summary, the genetic potential of *Halanaerobium* species to reduce thiosulfate and sulfite was detected in 5 of 6 genomes investigated. Sequences matching the *rdlA* gene coding for the thiosulfate disproportionation enzyme rhodanese like protein were detected in 5 of the genomes while none of the genomes contained *phs* sequences, thiosulfate reductase (cytochrome), or thiosulfate reductase (thiol) corresponding to alternative thiosulfate reductases. Sequences matching the anaerobic sulfite reductase subunit B were found in 5 of the genomes but *dsrAB* was found in none of the 6. Two strains with genetic potential to reduce thiosulfate and sulfite (*H. congolense DSM11287* and *H. salsuginis ATCC51327*) and one with no genetic potential (*H. hydrogeniformans DSM6643)* were cultivated in medium amended with

thiosulfate/sulfite. *H. congolense DSM11287* and *H. salsuginis ATCC51327* cultures were observed to produce sulfide while reducing thiosulfate and sulfite and had rhodanese activity while reducing thiosulfate. *H. hydrogeniformans DSM6643* did not produce sulfide, reduce thiosulfate, reduce sulfite or have rhodanese activity. Conclusion: based on the information I obtained on the genetic potential of the *Halanaerobium* strains together with the findings from the physiological experiments, I propose that *Halanaerobium* use a rhodanese to reduce thiosulfate to sulfite and sulfide, then an anaerobic sulfite reductase to reduce the sulfite to sulfide.

Tables and Figures

Table 1. Halanaerobium Species Physiology.

This study shows selected data on growth conditions and strain origin for three strains used in this study.

Species	Optimal pH (Range)	Optimal NaCl% (Range)	Optimal Temperature (^{o}C)(Range)	Strain Origin
H. congolense DSM11287	7 (6.3-8.5)	10 (4-24)	42 (20-45)	Corroded oil pipeline
H. salsuginis ATCC51327	6.1 (5.6-8)	9 (6-24)	40 (22-51)	Oil brine (oil well)
H. hydrogeniformans DSM6643	11 (7.5-12)	7 (2.5-15)	33 (NA)	Hyper saline lake

[1]Ravot et al. 1997, [2]Bhupathiraju et al. 1994, [3]Brown et al. 2012

Table 2. Assembly Parameters.
The reads for each genome were assembled *de novo* using Velvet. The number of contigs assembled are listed with their average contig length in nucleotides. Prodigal was used to predict the open reading frames of each *Halanaerobium* genome using default parameters. Evidence for thiosulfate reduction as previously reported for each strain and is noted in the table.

Species, Strain	# Contigs Assembled (Nucleotide)	Average Contig Length (Nucleotide)	# Open Reading Frames Predicted by Prodigal	Thiosulfate Reduction Reported?
H. congolense DSM11287	2120	1234	3262	Yes [1]
H. praevalens DSM2228 GCF_000165465.1	Complete	NA	2119	Yes[2]
H. vreelandii ZB2A	1989	1195	2990	Yes[3]
H. kushnerii ATCC700103	2423	1229	3662	Yes[3]
H. salsuginis ATCC51327 GCF_900114545.1	Complete	NA	2818	No[4]
H. hydrogeniformans DSM6643 GCF_000166415.1	Complete	NA	2426	No[5]

NA Not Applicable [1]Ravot et al. 1997, [2]Zikus et al., [3] De Capite et al.,[4]Bhupathiraju et al. 1994, [5]Brown 2012

Table 3. BLASTp Matches to *Halanaerobium* Genomes Open Reading Frames. The protein sequences for rdlA, PhsB, thiosulfate reductase (cytochrome), thiosulfate reductase (quinone), asrA, asrB, asrC, dsrA and dsrB were queried against the open reading frame translations (predicted by prodigal) using BLASTp to determine genetic potential for thiosulfate and sulfite reduction. Hits returned from the BLASTp search had their sequences querried against the Pfam database to confirm they contained the same protein familes (Appendix A) as sequences originally queried against the ORF's. Only BLASTp hits which had a match to those protein families and a grade above 35% were considered a hit. Grade is a weighted score which uses the E-value, % pairwise identity, and % query coverage. The table displays the Sequence query that matched, % query coverage, length of match and Grade. Genomic potential of strains agrees with previous reports other than for *H. salsuginis ATCC51327*, which was described as a non-thiosulfate reducer (Bhupathiraju et al. 1994).No genome hits were found for *H. hydrogeniformans DSM6643*. No hits for PhsB, thiosulfate reductase (cytochrome), thiosulfate reductase (quinone), dsrA, or dsrB were found.

Genome Hit	Best Query Match (Accession#)	Grade (%)	Percent query coverage	Length of Match (Amino Acid)
H. congolense DSM11287				
rdlA	*H. congolense* rdlA (AAS68581.1)	100	100	300
asrB	*H. congolense* asrB (WP_073157057.1)	41.7	83.39	223
H. salsuginis ATCC51327				
rdlA	*H. congolense* rdlA (AAS68581.1)	86.9	92	276
asrA	*H. salsuginis* asrA (WP_089861877.1)	100	100	350
asrB	*H. salsuginis* asrB (WP_089861878.1)	100	100	270
asrC	*H. salsuginis asrC (WP_089861879.1)*	99.9	100	336
H. kushnerii ATCC700103				
rdlA	*H. congolense* rdlA (AAS68581.1)	91.8	99.33	298
asrB	*H. congolense* asrB (WP_073157057.1)	36.5	73.06	198
H. vreelandii ZB2A				
rdlA	*H. congolense* rdlA (AAS68581.1)	56.8	45.67	137
asrB	*H. salsuginis* asrB (WP_089861878.1)	41.7	83.33	225
H. praevalens DSM2228				
rdlA	*H. congolense* rdlA (AAS68581.1)	85	93	279
asrB	*H. salsuginis asrB (WP_089861878.1)*	44.4	88.9	258
H. hydrogeniformans DSM6643				
	No Matches			

Table 4. Results of Thiosulfate and Sulfite Utilization Experiments

Table 4. Results of Thiosulfate and Sulfite Utilization Experiments.
Average values and standard deviations (SD) for live cultures were obtained from three replicates for each treatment. Starting concentrations were measured at Day 0 and final concentrations were measured at Day 12. Thiosulfate was measured using CHEMeterics Thiosulfate titration kit K-9705 and sulfite with CHEMetrics sulfite kit K-9650. Sulfide was measured on day 12 with CHEMetrics sulfide kit K-9510 after adding 10N NaOH to bring the pH to 12. Standard deviation values for thiosulfate, sulfite, and sulfide was 0. The table shows that when thiosulfate was lost sulfite and sulfide increased, when sulfite was lost sulfide was produced. Strains that were found to have *rdlA* and *asr* sequences in their genome (*H. congolense* DSM11287, *H. salsuginis* ATCC51327) display the ability to reduce thiosulfate and sulfite.

Treatment	Thiosulfate Start (mM/L)	Thiosulfate Final (mM/L)	Sulfite Start (mM/L)	Sulfite Final (mM/L)	Sulfide Start (mM/L)	Sulfide Final (mM/L)
H.congolense DSM11287						
Thiosulfate	9.8	1.8	0	3.1	0	17.6
Sulfite	0	0	2	1.1	0	0.25
Thiosulfate & Sulfite	9.8	1.2	2	4.4	0	17.6
H. salsuginis ATCC51327						
Thiosulfate	9.8	1.8	0	4.4	0	9.1
Sulfite	0	0	2	0.8	0	0.3
Thiosulfate & Sulfite	9.8	0.8	2	4.4	0	0.5
H. hydrogeniformans DSM6643						
Thiosulfate	9.8	9.8	0	0	0	0
Sulfite	0	0	2	2	0	0
Thiosulfate & Sulfite	9.8	9.8	2	2	0	0

Table 5. Rhodanese Activity During Thiosulfate and Sulfite Utilization Experiments.
The thiocyanate concentration (470nm) on days 4 and 12 was used to quantify the level of rhodanese activity (Singleton & Smith, 1988). The concentration in heat killed controls measures the background level of thiocyanate produced abiotically. Average values and standard deviations (SD) for live cultures were obtained from three replicates for each treatment. Average values and SD for the heat-killed control treatment were obtained from combining the heat killed controls values from the 3 treatments for each species. The table supports the confirmation of activity by the rhodanese detected in the genome of the strains. When thiosulfate is present, there is increased rhodanese activity.

Treatment	Thiocyanate in mM Day 4 (SD)	Optical Density at 600nm Day 4 (SD)	Thiocyanate in mM Day 12 (SD)	Optical Density at 600nm Day 12 (SD)
H. congolense DSM11287				
Thiosulfate	1.710 (0.105)	0.240 (0.008)	1.000 (0.064)	0.407 (0.009)
Sulfite	0.170 (0.011)	0.061 (0.003)	0.140 (0.004)	0.010 (0.001)
Thiosulfate & Sulfite	0.380 (0.004)	0.090 (0.002)	1.380 (0.017)	0.396 (0.004)
Heat Killed Average	0.088 (0.004)	0.051 (0.012)	0.067 (0.001)	0.040 (0.036)
H. salsuginis ATCC51327				
Thiosulfate	0.570 (0.007)	0.193 (0.008)	0.400 (0.008)	0.609 (0.000)
Sulfite	0.050 (0.010)	0.028 (0.001)	0.040 (0.000)	0.140 (0.008)
Thiosulfate & Sulfite	0.050 (0.006)	0.029 (0.002)	0.090 (0.001)	0.142 (0.008)
Heat Killed Average	0.079 (0.009)	0.032 (0.004)	0.029 (0.001)	0.016 (0.006)
H. hydrogeniformans DSM6643				
Thiosulfate	0.380 (0.019)	0.260 (0.010)	0.120 (0.001)	0.144 (0.004)
Sulfite	0.390 (0.004)	0.222 (0.004)	0.200 (0.014)	0.051 (0.011)
Thiosulfate & Sulfite	0.390 (0.011)	0.187 (0.001)	0.110 (0.004)	0.064 (0.006)
Heat Killed Average	0.225 (0.008)	0.048 (0.015)	0.120 (0.041)	0.019 (0.012)

Table 5. Rhodanese Activity During Thiosulfate and Sulfite Utilization Experiments

Table 6. Mass Balance of Sulfur.

This Table displays the millimoles of sulfur measured for each treatment at start and final time points. The theoretical yield for thiosulfate amended treatment and thiosulfate plus sulfite was calculated by dividing the millimoles of sulfur gained from sulfite and sulfide by thiosulfate loss measured. For sulfite treatments, the theoretical yield was calculated by dividing the sulfur gained from sulfide by the sulfur loss from sulfite. The table supports the sulfur from the reduction of thiosulfate goes to sulfite and sulfide.

Thiosulfate Amended 1.96mMole of S	**Thiosulfate lost (mMole of Sulfur)**	**Sulfite Gained (mMole of Sulfur)**	**Sulfide (H_2S) Gained (mMole of Sulfur)**	**% Theoretical Thiosulfate Converted to Sulfite/Sulfide**
H. congolense DSM11287	1.6	0.31	1.76	2.07/1.6 = **129.38%**
H. salsuginis ATCC51327	1.6	0.44	0.91	1.35/1.6 = **84.38%**
Sulfite Amended 0.2mMole of S		**Sulfite Lost**	**Sulfide Gained**	**% Theoretical Sulfite Converted to Sulfide**
H. congolense DSM11287	No Data	0.09	0.025	0.025/0.09 = **27.77%**
H. salsuginis ATCC51327	No Data	0.11	0.03	0.03/0.11 = **27.27%**
Thiosulfate and Sulfite Amended 2.16mMole of S	**Thiosulfate Lost**	**Sulfite Gained**	**Sulfide Gained**	**% Theoretical Thiosulfate Converted to Sulfite/Sulfide**
H. congolense DSM11287	1.68	0.24	1.76	(1.76+0.24)/1.68 = **119.04%**
H. salsuginis ATCC51327	1.8	0.24	0.05	(0.24+0.05)/1.8 = **16.11%**

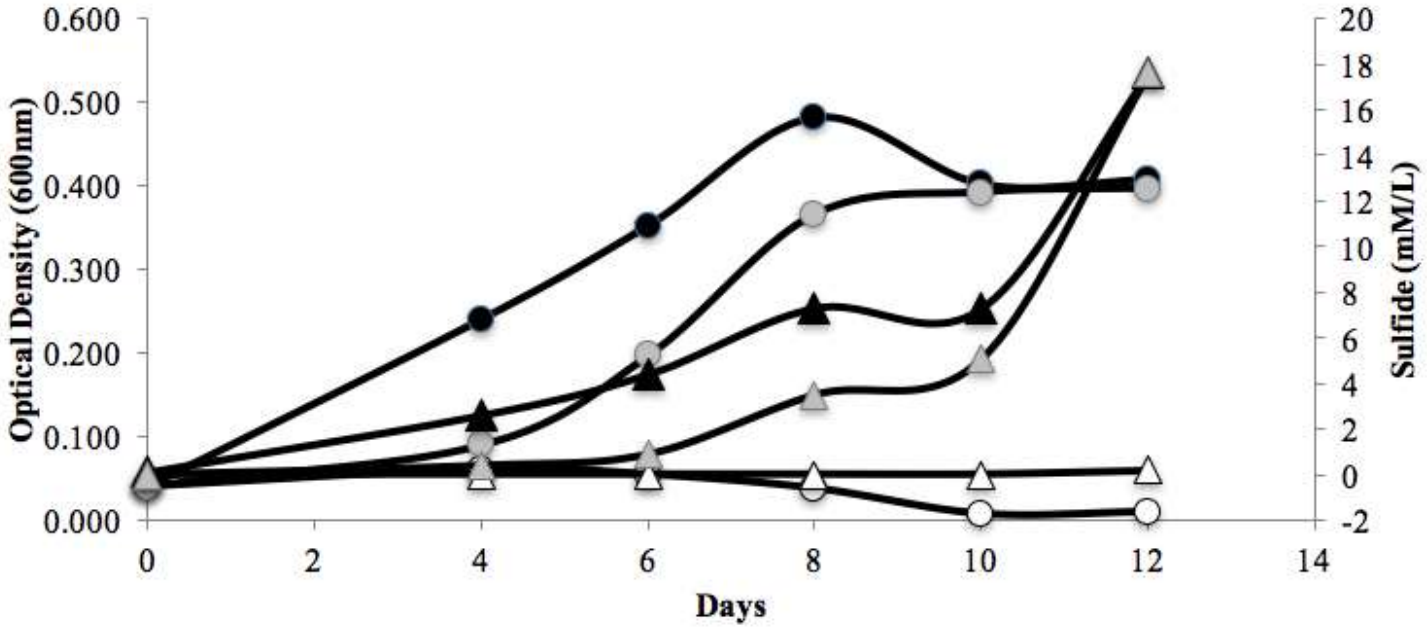

Figure 1. Growth of *Halanaerobium congolense DSM11287*

Growth of *Halanaerobium congolense DSM11287* in media amended with thiosulfate (10 mM/L), sulfite (2 mM/L), or thiosulfate (10 mM/L) and plus sulfite (2 mM/L). The average value of triplicate incubations is plotted, standard deviation values are too small to be seen on these plots. The optical density of thiosulfate-amended incubations is shown as a filled black circle, and the sulfide production is shown as a filled black triangle. The optical density of sulfite amended incubations is shown as an white circle, and sulfide production as an white triangle. The optical density of thiosulfate plus sulfite amended incubations is shown as a gray circle, and the sulfide production as a gray triangle. Optical Density (600 nm) and sulfide were measured on Days 0, 4, 6, 8, and 12. CHEMetrics

sulfide methylene blue test kit (K9510D) was used to measure sulfide. The Day 12 sample was brought to pH 12 before sulfide was measured. The heat killed control for each treatment is not plotted as the optical density and sulfide concentration did not vary during the experiment. *H. congolense DSM11287* grew best in thiosulfate amended bottles, and worst in sulfite amended bottles. Sulfide production was observed in all treatments, with thiosulfate and thiosulfate plus sulfite bottles both ending with the same concentration of sulfide.

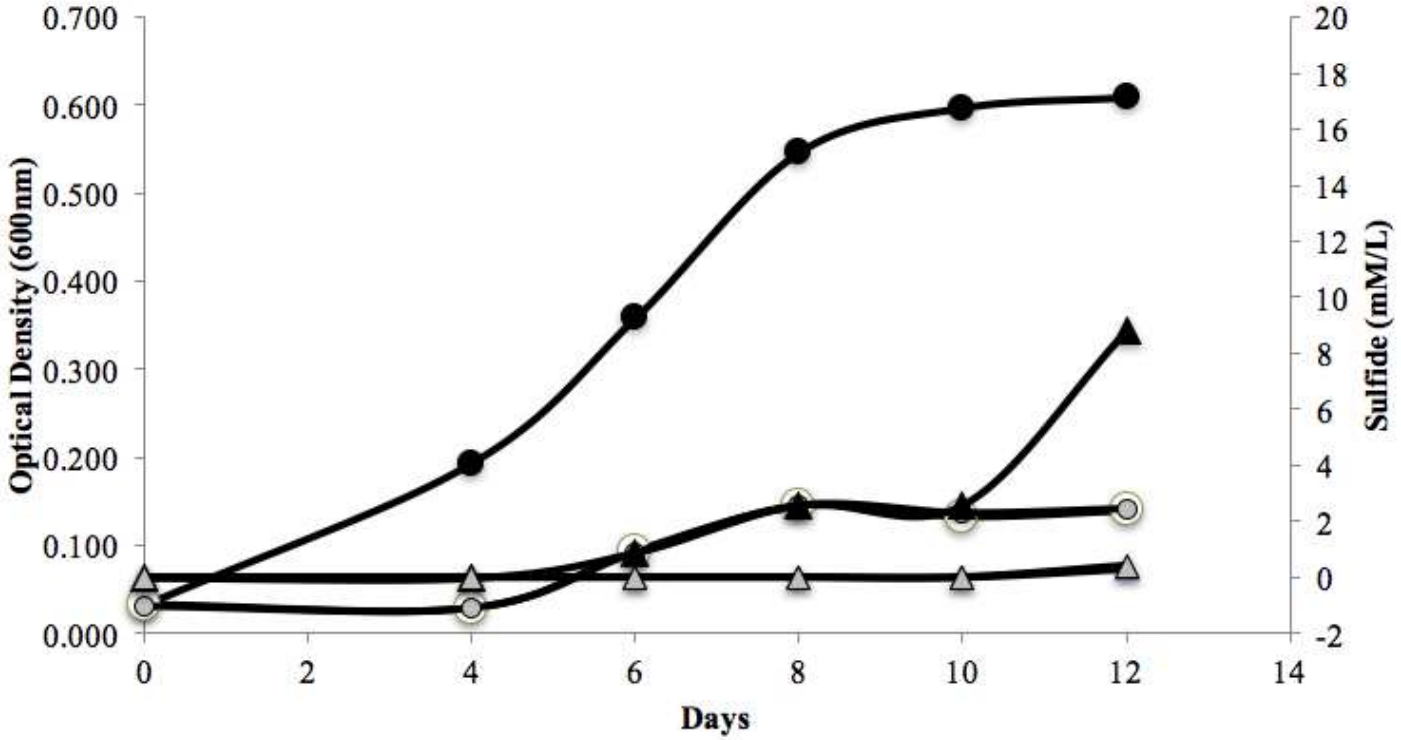

Figure 2. Growth of *Halanaerobium salsuginis ATCC51327*

Growth of *Halanaerobium salsuginis ATCC51327* in media amended with thiosulfate (10 mM/L), sulfite (2 mM/L), or thiosulfate (10 mM/L) plus sulfite (2 mM/L). The average value of triplicate incubations is plotted, standard deviation values are too small to be seen on these plots. The optical density of thiosulfate-amended incubations is shown as a filled black circle, and the sulfide production is

shown as a filled black triangle. The optical density of sulfite amended incubations is shown as an white circle, and sulfide production as an white triangle. The optical density of thiosulfate plus sulfite amended incubations is shown as a gray circle, and the sulfide production as a gray triangle. Optical Density (600 nm) and sulfide were measured on Days 0, 4, 6, 8, and 12. CHEMetrics sulfide methylene blue test kit (K9510D) was used to measure sulfide. The Day 12 sample was brought to pH 12 before sulfide was measured. The heat killed control for each treatment is not plotted as the optical density and sulfide concentration did not vary during the experiment. *H. salsuginis ATCC51327* grew best in thiosulfate bottles, and produced the most sulfide. Sulfite and thiosulfate plus sulfite amended bottles grew almost the same, shown by their plot lines covering each other; sulfide production was low in these treatments too.

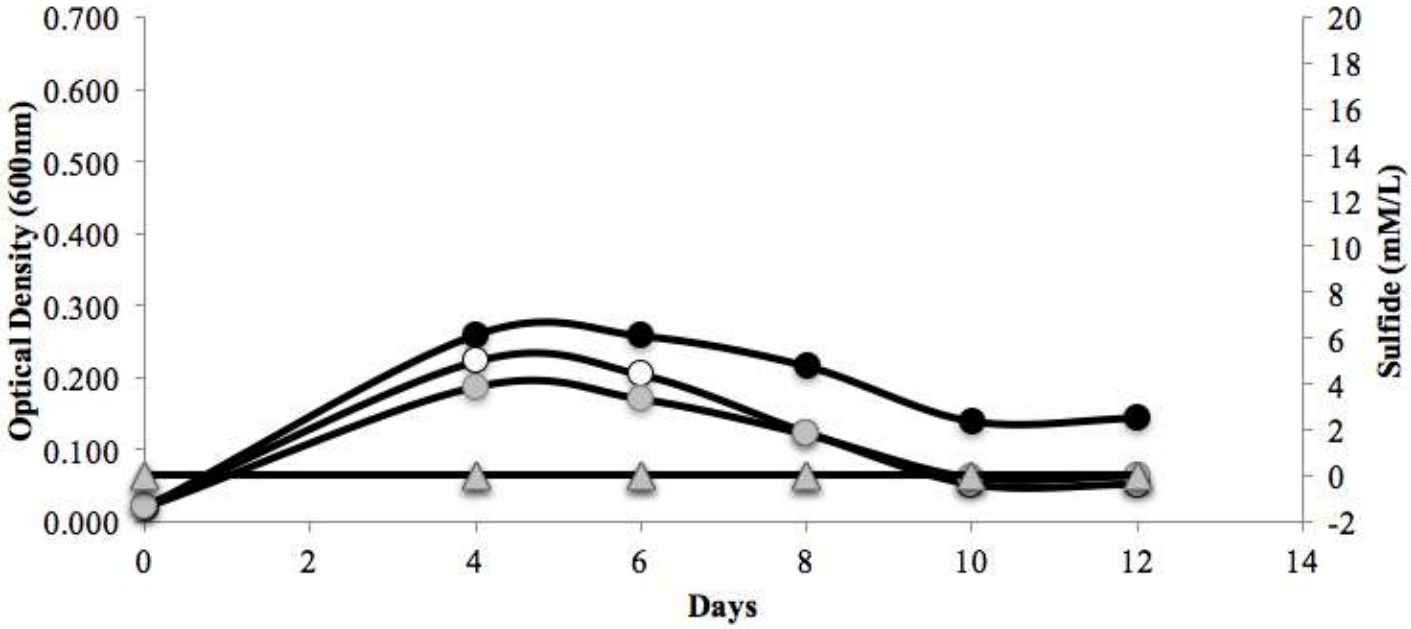

Figure 3. Growth of *Halanaerobium hydrogeniformans DSM6643*

Halanaerobium hydrogeniformans DSM6643 in media amended with thiosulfate (10 mM/L), sulfite (2 mM/L), or thiosulfate (10 mM/L) plus sulfite (2 mM/L). The average value of triplicate incubations is plotted, standard deviation values are too small to be seen on these plots. The optical density of thiosulfate-amended incubations is shown as a filled black circle, and the sulfide production is shown as a filled black triangle. The optical density of sulfite amended incubations is shown as an white circle, and sulfide production as an white triangle. The optical density of thiosulfate plus sulfite amended incubations is shown as a gray circle, and the sulfide production as a gray triangle. Optical Density (600 nm) and sulfide were measured on Days 0, 4, 6, 8, and 12. CHEMetrics sulfide

methylene blue test kit (K9510D) was used to measure sulfide. The Day 12 sample was brought to pH 12 before sulfide was measured. The heat killed control for each treatment is not plotted as the optical density and sulfide concentration did not vary during the experiment. *H. hydrogeniformans DSM6643* grew best in thiosulfate amended bottles, while sulfite amended were the worst. In no treatment was sulfide produced.

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Appendix A: Pfam Domains of Thiosulfate and Sulfite Reductases

Thiosulfate Reductases	Subunits	Pfam domain 1	Pfam domain 2	Pfam domain 3
Rhodanese (tandem-domain) (EC 2.8.1.1)	rdlA AAS68581.1	Rhodanese (PF00581)	Rhodanese (PF00581)	
Quinone (EC 1.8.5.5)	PhsA NP_461010.1	Molybdopterin oxidoreductase Fe4S4 domain (PF04879)	NADH dehydrogenase (ubiquinone) (PF00384)	Molydopterin dinucleotide binding domain (PF01568)
	PhsB NP_461009.1	4Fe-4S dicluster domain (PF13247)		
	PhsC NP_461008.1	Prokaryotic cytochrome b561 (PF01292)		
Cytochrome (EC 1.8.2.5)	qrcB YP_009398.1	NADH dehydrogenase (ubiquinone) (PF00384)	Molydopterin dinucleotide binding domain (PF01568)	
Thiol-dependent (EC 2.8.1.3)	BisC WP_059759455.1	NADH dehydrogenase (ubiquinone) (PF00384)	Molydopterin dinucleotide binding domain (PF01568)	
Sulfite Reductases	Subunits	Pfam domain 1	Pfam domain 2	Pfam domain 3
DSR (EC 1.8.99.5)	DsrA YP_009626.1	Nitrite/Sulfite reductase ferredoxin-like half domain (PF03460)	Nitrite and sulfite reductase 4Fe-4S domain (PF01077)	
	DsrB YP_009627.1	Nitrite/Sulfite reductase ferredoxin-like half domain (PF03460)	Nitrite and sulfite reductase 4Fe-4S domain (PF01077)	
ASR	AsrA WP_073157054.1 AAA99275.1	4Fe-4S dicluster (PF17179)		
	AsrB WP_073157057.1 AAA99276.1	oxidoreductase NAD-binding domain (PF00175)	dihydroorotate dehydrogenase B (PF10418)	
	AsrC WP_073157060.1 AAL21444.1	Nitrite/Sulfite reductase ferredoxin-like half domain (PF03460)	Nitrite and sulfite reductase 4Fe-4S domain (PF01077)	NADH dehydrogenase (ubiquinone) (PF12838)