INTRODUCTION

Soap Lake is a meromictic, alkaline (~pH 9.8) and saline (~14–140 g liter⁻¹) lake located in the semiarid area of eastern Washington State. Of note is the length of time it has been meromictic (at least 2000 years) and the extremely high sulfide level (~140 mM) in its monimolimnion. As expected, the microbial ecology of this lake is greatly influenced by these conditions. A bacterium, *Halanaerobium hydrogeniformans*, was isolated from the mixolimnion region of this lake. *Halanaerobium hydrogeniformans* is a haloalkaliphilic bacterium capable of forming hydrogen from 5- and 6-carbon sugars derived from hemicellulose and cellulose. Due to its ability to produce hydrogen under saline and alkaline conditions, in amounts that rival genetically modified organisms, its genome was sequenced. This sequence data provides an opportunity to explore the unique metabolic capabilities of this organism, including the mechanisms for tolerating the extreme conditions of both high salinity and alkalinity of its environment.

*Keywords: Soap Lake, Halanaerobium hydrogeniformans, alkaliphile, halotolerant, biohydrogen, genome analysis*

Soap Lake is a meromictic, alkaline (~pH 9.8) and saline (~14–140 g liter⁻¹) lake located in the semiarid area of eastern Washington State. Of note is the length of time it has been meromictic (at least 2000 years) and the extremely high sulfide level (~140 mM) in its monimolimnion. As expected, the microbial ecology of this lake is greatly influenced by these conditions. A bacterium, *Halanaerobium hydrogeniformans*, was isolated from the mixolimnion region of this lake. *Halanaerobium hydrogeniformans* is a haloalkaliphilic bacterium capable of forming hydrogen from 5- and 6-carbon sugars derived from hemicellulose and cellulose. Due to its ability to produce hydrogen under saline and alkaline conditions, in amounts that rival genetically modified organisms, its genome was sequenced. This sequence data provides an opportunity to explore the unique metabolic capabilities of this organism, including the mechanisms for tolerating the extreme conditions of both high salinity and alkalinity of its environment.

*Keywords: Soap Lake, Halanaerobium hydrogeniformans, alkaliphile, halotolerant, biohydrogen, genome analysis*
availability of the genome sequence and annotation data of *Halanaerobium hydrogeniformans* enables the determination of the adaptations this organism possesses that facilitates it to thrive under the haloalkaline conditions found in Soap Lake.

**MATERIALS AND METHODS**

*Halanaerobium hydrogeniformans*’ genome data (Brown et al., 2011) was interrogated to gain information on the function of this bacterium’s genome. Information on candidate protein-encoding genes and RNA genes were obtained by using the integrated microbial genomes (IMG) system (Markowitz et al., 2012). Biocyc databases and pathway tools were also used (Caspi et al., 2010). Another sequenced *Halanaerobium, Halanaerobium praevalens* GSL³ (Ivanova et al., 2011) a non-alkaliphilic bacterium, was used as a comparator organism. *Halanaerobium praevalens* GSL³ was first isolated from the sediments of the Great Salt Lake in Utah (Zeikus et al., 1983). Similar amino acid sequences were determined by performing protein BLAST searches (Altschul et al., 1997). The complete genome of *Halanaerobium hydrogeniformans* has been deposited in NCBI Genomes with accession number NC_014654.

**RESULTS AND DISCUSSION**

**GENOME PROPERTIES**

Of the 2391 candidate protein-encoding genes, there are 1867 with function predictions in the genome (Table 1). Four 5S rRNA, 16S rRNA, and 23S rRNA genes each are present as are 57 tRNA genes. There are 2082 genes assigned to clusters of orthologous groups (COGs). Interestingly, approximately 25% of the protein-encoding genes are for transmembrane proteins. The distribution of the genes into COG functional categories is provided in Figure 1 and Table 2. The gene count for the different Kyoto Encyclopedia of Genes and Genomes (KEGG) categories is similar between *Halanaerobium hydrogeniformans* and *Halanaerobium praevalens* GSL³ except for a few categories (Table 2). *Halanaerobium praevalens* GSL³ only has a gene count of 85 for amino acid metabolism while *Halanaerobium hydrogeniformans* has 138. *Halanaerobium praevalens* GSL³ also has lower gene counts for the KEGG categories of metabolism and metabolism of cofactors and vitamins. On the other hand, *Halanaerobium hydrogeniformans* has a much lower gene count for KEGG category cell motility. Though both of these organisms are not considered to be motile, there are strains of *Halanaerobium praevalens* GSL³ that are (Kobayashi et al., 2000 and Eder et al., 2001).

**METABOLIC CAPABILITIES**

*Halanaerobium hydrogeniformans* has 20% of its genes in the COG category of metabolism and 7% of its genes in the carbohydrate category. Thus, it is not surprising that *Halanaerobium hydrogeniformans* is capable of growth on a number of sugars derived from cellulose and hemicellulose (Begemann et al., 2012). When grown on cellobiose, biomass is produced along with fermentative products, such as formate, acetate, and hydrogen (Begemann et al., 2012). By considering the annotated genome, it should be possible to determine the putative pathway from cellobiose to hydrogen. Cellobiose can be brought into the cell by a putative phosphotransferase system (PTS) lactose/cellobiose-specific transporter subunit IIB (gene designated as Halsa_0653). Once inside the cell, cellobiose would be cleaved and enter the Embden-Meyerhof pathway of glycolysis with the formation of pyruvate. The enzymes for this pathway are present in *Halanaerobium Hydrogeniformans*¹. Once formed, there are a number of possible fates for pyruvate. A putative pyruvate formate-lyase catalyzes pyruvate and coenzyme A to form matom and acetyl Co A (Halsa_0723). A possible fate for formate is to be broken down into CO₂ and H₂ by formate-hydrogen lyase (Figure 2). However, there was no gene identified that would code for the enzyme, formate-hydrogen lyase. As reported earlier, *Halanaerobium hydrogeniformans* does accumulate formate (Begemann et al., 2012). Thus, it is unlikely that this organism is forming hydrogen from formate. *Halanaerobium praevalens* GSL³ does not appear to possess this enzyme either. However, formate that is released by these fermentative organisms can be used by sulfate-reducing prokaryotes present in Soap Lake (Dimitriu et al., 2008).

*Halanaerobium hydrogeniformans*’ genome possesses an idh gene, indicating that lactate dehydrogenase should also be present (Halsa_1287). However, lactate has not been detected as a metabolic product from this organism. It is interesting to note that many fermentative organisms possess idh genes (Carere et al., 2012). However, only a few, such as Bacillus cereus, had been found to produce lactate in high yields.

*Halanaerobium hydrogeniformans* appears to possess three putative pyruvate dehydrogenase genes (Halsa_0164, Halsa_0919, and Halsa_2297; Figure 2). Other genera, *Caldicellulosiruptor, Clostridium*, and *Thermoanaerobacter*, also possess putative pdh genes but there has been no evidence for functional enzyme production (Carere et al., 2012). *Halanaerobium hydrogeniformans* possesses a gene for the formation of pyruvate:ferredoxin oxidoreductase (Halsa_2334) as well as two genes that encode

---

¹http://www.genome.jp/kegg-bin/show_pathway?haa00010

---

### Table 1 | Genome statistics.

|                     | Number         |
|---------------------|----------------|
| Total number of bases| 2,613,117      |
| Number of DNA coding bases| 2,286,541 |
| G+C percentage      | 33.16          |
| Contigs             | 1              |
| Total number of genes| 2463          |
| Number of protein coding genes | 2391 |
| Pseudo genes        | 96             |
| 5S rRNA genes       | 4              |
| 16S rRNA genes      | 4              |
| 23S rRNA genes      | 4              |
| tRNA genes          | 57             |
| Genes with function prediction | 1867 |
| Genes assigned to clusters of orthologous groups (COGs) | 2082 |
| Genes coding transmembrane proteins | 624 |

---

**REFERENCES**

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1997). Basic local alignment search tool. J Mol Biol 215(3):403-10.

Begemann M, Berka RK, Kander N, Kase JS, De long J (2010). Cytochrome c oxidase: a novel enzyme for sulfur metabolism in *Halanaerobium hydrogeniformans* GSL³. FEMS Microbiol Lett 302(1):25-30.

Brown JB, DeLong EF, Rainey PB, Smith AJ, Kamekura T, Sato Y, Nakasone T, Nakasone T, Takahashi M, Watanabe T, et al. (2011). The *Halanaerobium* species: a new type of symbiotic halophiles. Environ Microbiol 13(7):1972-83.

Caspi H, Atreya M, Ziv N, Shemesh A, Ben-Tal N, Shaham E (2010). The *Halanaerobium* species: a new type of symbiotic halophiles. Environ Microbiol 13(7):1972-83.

Carere D, Berka RK, Kander N, Kase JS, De long J (2012). Cytochrome c oxidase: a novel enzyme for sulfur metabolism in *Halanaerobium hydrogeniformans* GSL³. FEMS Microbiol Lett 302(1):25-30.

Dimitriu LG, Britag NA, Khare K, Lachmund D, Kaser F, Brown JB, Brooks JS, DeLong EF, Kamekura T, Sato Y, et al. (2008). The *Halanaerobium* species: a new type of symbiotic halophiles. Environ Microbiol 13(7):1972-83.
a polypeptide pyruvate flavodoxin/ferredoxin oxidoreductase domain-containing protein and subunit beta (Halsa_0798 and Halsa_0799). Furthermore, it possesses two genes, Halsa_1768 and Halsa_1862 that encode for iron hydrogenases. Halsa_1862 is part of a putative operon that includes a NADH dehydrogenase (Halsa_1863), a ferredoxin-like protein (Halsa_1864), a histidine kinase (Halsa_1865), NADH-quinone oxidoreductase subunit E (Halsa_1866), PHP domain-containing protein (Halsa_1867), an iron-sulfur binding hydrogenase (Halsa_1868), an iron-sulfur cluster domain-containing protein (Halsa_1869), an anti-sigma regulatory factor, serine/threonine protein kinase (Halsa_1870), and an unidentified open reading frames (ORF; Halsa_1871; Figure 3). The organism's ability to produce substantial amounts of H₂, 2.3 hydrogen molar yield from cellobiose, (Begemann et al., 2012) is of interest as a possible biofuel-producing organism.

It is likely that fermenters such as *Halanaerobium hydrogeniformans*, has a role in interspecies hydrogen transfer in the Soap Lake ecosystem. For example, sulfate- and iron-reducing bacteria were found in the sediments of Soap Lake (Dimitriu et al., 2008) and these organisms can serve as sinks for the H₂ produced (Jones et al., 1998). However, there have been limited studies on interspecies hydrogen transfer in hypersaline environments. In our own studies, when H₂ and CO₂ were provided as substrates, low numbers of methanogens were detected in the sediments and monimolimnion of Soap Lake while no methanogens were detected in the mixolimnion and chemocline (Dimitriu et al., 2008). Due to thermodynamic constraints (~34 kJ/mol H₂; Oren, 1999), autotrophic methanogenesis is unlikely to occur, especially in environments with large amounts of sulfate present, such as Soap Lake. Sulfate reduction with H₂ is slightly more thermodynamically favorable than methanogenesis in hypersaline environments (Oren, 2010). In fact, hydrogenotrophic sulfate reducers have been reported from the hypersaline soda lakes of the Kulunda Steppe in southeastern Siberia in Russia (Foti et al., 2007). The first report of interspecies hydrogen transfer possible in hypersaline soda lakes involved a hydrogenotrophic sulfate-reducing bacterium, *Desulfophilobium retbaense*, was found to utilize the H₂ produced by two species of *Halanaerobium*, *Halanaerobium saccharolytica* subsp. *Senegalense*, and *Halanaerobium* sp. strain FRIH from glycerol fermentation (Cayol et al.,
Table 2 | Number of genes associated with the general COG functional categories.

| KEGG Category                        | Halanaerobium hydrogeniformans gene count | Halanaerobium praevalens gene count |
|---------------------------------------|------------------------------------------|-------------------------------------|
| Amino acid metabolism                 | 138                                      | 85                                  |
| Biosynthesis of other secondary metabolites | 15                                      | 8                                   |
| Carbohydrate metabolism               | 171                                      | 137                                 |
| Cell motility                         | 5                                        | 59                                  |
| Energy metabolism                     | 93                                       | 82                                  |
| Folding, sorting, and degradation     | 32                                       | 30                                  |
| Glycan biosynthesis and metabolism    | 25                                       | 28                                  |
| Lipid metabolism                      | 51                                       | 39                                  |
| Membrane transport                    | 95                                       | 83                                  |
| Metabolism                            | 486                                      | 375                                 |
| Metabolism of cofactors and vitamins  | 110                                      | 83                                  |
| Metabolism of other amino acids       | 24                                       | 32                                  |
| Metabolism of terpenoids and polyketides | 20                                      | 17                                  |
| Nucleotide metabolism                 | 77                                       | 81                                  |
| Replication and repair                | 41                                       | 39                                  |
| Signal transduction                   | 39                                       | 53                                  |
| Transcription                         | 4                                        | 4                                   |
| Translation                           | 77                                       | 80                                  |
| Transport and catabolism              | 2                                        | 1                                   |
| Xenobiotics biodegradation and metabolism | 25                                      | 27                                  |

2002). When Desulfohalobium retbaense was present as an H₂-scavenger, glycerol consumption increased and H₂ concentrations approached or were at undetectable amounts.

From early on, it was recognized that glycerol was a major carbon source in saline lakes (Borowitzka, 1981). Glycerol is produced as an osmoregulatory solute by organisms such as green alga, Dunaliella salina (Oren, 1993). Not only can glycerol be released from lysed cells but can also leak from healthy cells (Bardavid et al., 2008). This source of carbon can be used by halophilic aerobic prokaryotes, such as Haloquadratum and Salinibacter. These aerobic bacteria oxidize glycerol incompletely with excretion of products such as acetic acid, lactic acid, and pyruvic acid (Oren, 2008). Other microorganisms present in these hypersaline environments can subsequently use these products. When a cell takes up glycerol, the glycerol can be converted into dihydroxyacetone and then integrated into pyruvate metabolism, resulting in the products listed above. Glycerol can also be converted into 1,3-propanediol to replenish NAD⁺ from NADH₂ resulting when glycerol is oxidized to dihydroxyacetone and dihydroxyacetone phosphate is oxidized to phosphoenolpyruvate. Much of the NADH₂ produced is recycled to NAD⁺ through the formation of fermentation end products, such as ethanol, acetate, and butyrate. However, some NAD⁺ must be replenished through an alternate pathway (Zeng, 1996). Excess glycerol can be shunted into the 1,3-propanediol production pathway where NADH₂ is re-oxidized to form 1,3-propanediol. This metabolism is present in Halanaerobium hydrogeniformans (Roush et al., 2014).

The metabolism of glycerol is of interest not only for its ecological role as a source of carbon in saline lakes but also for the formation of commodity compounds, such as 1,3-propanediol. Glycerol is formed as a byproduct during biodiesel production (Thompson and He, 2006). The first step in the conversion of glycerol to 1,3-propanediol is the removal of a water molecule from glycerol by the enzyme glycerol dehydratase. This step creates the intermediate 3-hydroxypropanal. Next, the enzyme 1,3-propanediol dehydrogenase, oxidizes NADH₂ to form 1,3-propanediol, replenishing the NAD⁺ needed by the cell for normal metabolism (Zeng, 1996). The genome of Halanaerobium hydrogeniformans revealed that it possessed the possibility of this metabolism². The genes that it possesses that can possibly contribute to this pathway are Halsa_0984 (a putative glycerol dehydratase), Halsa_0672 (a putative 1,3-propanediol dehydrogenase), and Halsa_2285 (another putative 1,3-propanediol dehydrogenase). It was determined experimentally that Halanaerobium hydrogeniformans is capable of forming 1,3-propanediol from glycerol. After a 5-day incubation with 30 mM glycerol and pH 11 and 7% NaCl conditions, Halanaerobium hydrogeniformans was able to convert 31.5% of the glycerol to 1,3-propanediol. When B12 was provided at concentrations from 25 to 100 μg/L, glycerol to 1,3-propanediol conversion ranged from 59.1 to 60.3% (Roush, 2013).

Glycine betaine is another osmoregulatory compound found in hypersaline environments (Welsh, 2000). Halanaerobium
hydrogeniformans possesses an ATP-binding cassette (ABC) transporter, Halsa_1783, that can possibly bring this compound into the cell. Not only can this compound be used as an osmoregulatory compound but can be a potential source of energy and carbon for the cell. Glycine betaine could possibly be used in the Stickland reaction with the amino acid, serine, as observed in Halanaerobacter salinarius (Mounté et al., 1999).

MOBILE DNA

Halanaerobium hydrogeniformans’ genome was interrogated by using IMG to determine the most abundant COGs genes present. The most abundant COG genes in this genome were found to be transposases (Table 3). This should not come as a surprise as Aziz et al. (2010) found that transposases are both ubiquitous and abundant in both genomes and metagenome libraries. They determined the average number of transposases possessed across known genomes to be 38 per genome. Halanaerobium hydrogeniformans contains 72 annotated transposase genes (Table 3).

In comparison, Halanaerobium praevalens GSLT was found to possess 20 annotated transposase genes. Transposase enzymes are responsible for the excision and movement of DNA segments within a chromosome. Transposase-encoding genes are flanked with insertion sequences (IS). These ISs are short, inverted terminal repeats. Previously, it was thought that IS segments of DNA were selfish or parasitic (Orgel and Crick, 1980). However, it is now thought that transposable elements convey selective advantages to their hosts. These advantages can include the mobilization and/or activation of beneficial genes (Nowacki et al., 2009) or to generate phenotypic diversity (Brazelton and Baross, 2009). However, there are costs, such as transposon-induced mutations, that need to be balanced by the organisms (Aziz et al., 2010).

A further breakdown of the transposases in Halanaerobium hydrogeniformans reveals that eight IS families are present in this genome (Table 4). IS families are based upon similarities and differences in structure, organization, and the nucleotide and protein sequence relationships (Mahillon and Chandler, 1998). For example, the IS3 family is characterized by having lengths between 1,200 and 1,550 base pairs (bp) and inverted terminal repeats of 20 to 40 bp (Mahillon and Chandler, 1998). Interestingly, these sequences generally have two consecutive and partially overlapping ORF, orfA and orfB. These mobile segments of DNA transposes through a circular intermediate. Of the IS families identified in Halanaerobium hydrogeniformans’ genome, the only other IS family present that possesses more than one orf is IS21. The IS21 family has two orfs, a long upstream frame, istA, and a shorter downstream frame, istB. These two proteins carry several blocks of highly conserved residues (Mahillon and Chandler, 1998). Work is currently being done by Ron Frank, Missouri S&T, to determine if the putative transposases are active in Halanaerobium hydrogeniformans. If so, it is suspected that these genes can become mobile and potentially activate beneficial genes to increase the fitness of this organism to tolerate environmental pressures (Aziz et al., 2010) that are present in Soap Lake.

CYCLIC-di-GMP

The second most numerous group of identified genes in the Halanaerobium hydrogeniformans’ genome are the HD-GYP domain genes of COGs 2206 and 3437 (Table 3). In addition, there are eight genes identified as belonging in COG 2199 of the FOG: GGDEF domain. The GGDEF domain encodes for enzymes that produce cyclic-di-GMP, a ubiquitous second messenger in bacteria (Jenal and Malone, 2006). It is involved in cell signaling, exopolysaccharide formation, attachment, and biofilm production. The HD-GYP domain genes encode for diguanylate cyclase and metal dependent phosphohydrolase, an enzyme responsible for producing cyclic-di-GMP and it requires the presence of divalent cations, most likely Mg$^{2+}$ or Mn$^{2+}$ (Castiglione et al., 2011). Previous analysis performed indicates that both of these metals, Mg$^{2+}$ and Mn$^{2+}$, 8,170.0 and 404.0 mg/kg dry weight, respectively, are present in the sediment of Soap Lake (Sigrid Penrod, personal communication). In comparing Halanaerobium hydrogeniformans’ genome with Halanaerobium praevalens GSLT’s, only Halanaerobium hydrogeniformans’ genome possesses genes for diguanylate cyclase with metal dependent phosphohydrolase. Thus far, only a few environmental signals have been identified that regulate cyclic di-GMP-mediated signaling pathways (Römling et al., 2013), and none are know for Halanaerobium. Halanaerobium hydrogeniformans forms mucous-like mats in cultures that are not vigorously shaken (Begemann et al., 2012). One possible
Table 3 | Most abundant COG genes identified in Halanaerobium hydrogeniformans\(^ {\prime}\)’s genome.

| COG ID | COG Name | Halanaerobium hydrogeniformans | Halanaerobium praevaelens GSL\(^ {T}\) |
|--------|----------|--------------------------------|-------------------------------------|
| 2801   | Transposase and inactivated derivatives | 22                             | 6                                   |
| 2963   | Transposase and inactivated derivatives | 20                             | 6                                   |
| 2206   | HD-GYP domain | 14                             | 9                                   |
| 2826   | Transposase and inactivated derivatives, IS30 family | 12                             | 2                                   |
| 0438   | Glycosyltransferase | 11                             | 9                                   |
| 3328   | Transposase and inactivated derivatives | 10                             | 0                                   |
| 1028   | Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases) | 9                              | 2                                   |
| 3437   | Response regulator containing a CheY-like receiver domain and an HD-GYP domain | 9                              | 3                                   |
| 0747   | Transposase and inactivated derivatives | 8                              | 6                                   |
| 2199   | FOG: GGDEF domain | 8                              | 7                                   |

Halanaerobium praevaelens GSL\(^ {T}\) number of genes for each COG ID is also provided.

Table 4 | Number of annotated genes in insertion sequence families present in Halanaerobium hydrogeniformans.

| Insertion sequence family | Number of genes present |
|---------------------------|-------------------------|
| IS3                       | 22                      |
| IS30                      | 11                      |
| IS260/IS605               | 8                       |
| IS296                     | 3                       |
| IS4                       | 3                       |
| IS6                       | 3                       |
| IS21                      | 2                       |
| IS91                      | 1                       |

role this set of putative genes may play is the formation of these mats.

GLYCOSYLTRANSFERASES
There is evidence for the occurrence of glycosyltransferases, COG 0438 (Table 3). Nine of the 11 putative genes in Halanaerobium hydrogeniformans\(^ {\prime}\)’s genome encode for glycosyl transferase group 1 enzymes. There is one putative sucrose-phosphate synthase (Halsa\(_{0772}\)) and one hypothetical protein (Halsa\(_{0632}\)). These enzymes are defined by the utilization of an activated donor sugar group substrate that contains a phosphate leaving group (Lairson et al., 2008). They are involved in the biosynthesis of cell walls, membranes, and envelop biogenesis. Specifically, these enzymes catalyze the first step in the sucrose synthesis pathway and are thought to play a role in osmotic stress protection (Chua et al., 2008). Halanaerobium hydrogeniformans\(^ {\prime}\) Halsa\(_{0772}\) gene has a 74% identity to a sucrose-phosphate synthase that is present in Halanaerobium praevaelens GSL\(^ {T}\)\(^ {T}\) indicating a common mechanism for osmotic stress protection.

SHORT-CHAIN DEHYDROGENASES/REDUCTASES (SDRs)
Nine putative genes in COG 1028 were found in Halanaerobium hydrogeniformans\(^ {\prime}\)’s genome. Only two were found in Halanaerobium praevaelens GSL\(^ {T}\)\(^ {T}\)’s genome. These genes encode for short-chain dehydrogenases/reductases (SDRs) with different specificities. This super family of enzymes catalyze a variety of NAD(P)(H) oxidation/reduction reactions (Kallberg et al., 2002). These enzymes are also recognized to catalyze the metabolism of steroids, cofactors, carbohydrates, lipids, aromatic compounds, and amino acids, and act in redox sensing. They are also associated with biotin metabolism and fatty acid biosynthesis and metabolism. There hasn’t been much research performed on this family of enzymes in extremophilic bacteria. The research that has been focused on characterizing these enzymes from extremophilic organisms has been on thermophilic prokaryotes such as, Thermus thermophiles HB8 (Asada et al., 2009), Sulfolobus acidocaldarius (Pennacchio et al., 2010), and Thermococcus sibiricus (Stekhanova et al., 2010).

ABC TRANSPORTERS
There are a number of ATP-binding cassettes (ABC) transporters represented in Halanaerobium hydrogeniformans\(^ {\prime}\)’s genome. Of these, seven COG 0747 putative genes have been identified, Halsa\(_{0302}\), Halsa\(_{0968}\), Halsa\(_{1628}\), Halsa\(_{1745}\), Halsa\(_{2053}\), Halsa\(_{2146}\), and Halsa\(_{2227}\) (Table 3). These genes encode for ABC-type nickel/dipeptide/oligopeptide periplasmic transport systems (Tam and Saier, 1993). Nickel is required for five types of enzymes; urease, hydrogenase, carbon monoxide dehydrogenase, methyl-S-coenzyme M reductase, and one class of superoxide dismutase (Hausinger, 1997). Halanaerobium hydrogeniformans does not appear to possess any of these enzymes. However, there are 524 genes that have been identified as hypothetical proteins and have no assigned functions. Thus far, only two possible hydrogenases, Halsa\(_{1768}\) and Halsa\(_{1862}\), have been identified. These are both Fe-only hydrogenases. It will be interesting to determine...
the concentration of nickel that is required by the organism as well as to determine if there are nickel-requiring enzymes present.

The protein-coding genes that were connected to membrane transport KEGG pathways were explored through IMG. These genes can indicate what is needed and utilized by the bacterium. For example, there are numerous genes that encode for iron uptake proteins. Iron III can possibly be taken up by proteins encoded by AfuA (Halsa_2074), AfuB (Halsa_2073), and AfuC (Halsa_2072). Siderophore-mediated transport of iron complexes are likely in this bacterium. These proteins can possibly be encoded by FhuD (Halsa_2140, Halsa_2186, Halsa_2212, and Halsa_2233), FhuB (Halsa_1986, Halsa_2185, Halsa_2211, and Halsa_2232), and FhuC (Halsa_1985, Halsa_2184, Halsa_2210, and Halsa_2231). FhuD is a periplasmic protein and FhuB and FhuC are cytoplasmic membrane-associated proteins responsible for siderophore-mediated iron transport (Katoh et al., 2001). It appears that Halanaerobium hydrogeniformans also possesses genes for proteins responsible to taking up another metal, tungstate. TupA (Halsa_2175), TupB (Halsa_2174), and TupC (Halsa_2173) were each found to be present. These genes do not appear to be present in Halanaerobium praevalens GSL². Zinc is another metal that is possibly taken up by Halanaerobium hydrogeniformans. ZnuA (Halsa_0273), ZnuB (Halsa_0275), and ZnuC (Halsa_0274) were found in the bacterium's genome.

Halanaerobium hydrogeniformans' ability to utilize various carbon sources can be inferred by the transporters that it contains. Halsa_1981 was identified as possibly being involved with uptake of glucose/mannose (MalK), maltose/maltodextrin (MalK), galactose oligomer/maltotriosasccharide (MsmX), arabinoogalasscharide (MsmX), raffinose/stachyose/melibiose (MsmK), sorbitol/manitol (SnoK), α-glucoside (AgkK), cellobiose (MskK), and chitobiose (MskK). In addition to Halsa_1981, other genes are present that could encode for other carbon-intake ABC transporters. A total of 10 putative genes for ABC transporters for ribose/autoinducer 2/D-xylene, RhsB, RhsC, and RhsA, were identified. The genes, UgPB, UgPA, and UgPE, responsible for n-glyceral 3-phosphate uptake were also found. Currently, the range of sources of carbon is unknown for Halanaerobium hydrogeniformans. Previous studies have demonstrated that the bacterium can use glucose, cellobiose, ribose, xylose, arabinose, galactose, and mannose (Begemann et al., 2012).

Glycerol can be used as either a carbon source or as an osmoprotectant (Oren, 1993). Halanaerobium hydrogeniformans possesses the genes, OpuBB and OpuBA, that are putative osmo-protectant ABC transport genes. In addition, it has putative trehalose/maltose ABC transport genes, ThuE, ThuF, and ThuG. Trehalose is considered a universal stress molecule and can serve as an osmoprotectant and in Chromohalobacter salexigens, it can serve to protect against temperature extremes (Reina-Bueno et al., 2012). However, trehalase was not confirmed to protect against desiccation. Halanaerobium hydrogeniformans does appear to have a mechanism to protect itself against desiccation. When grown with little or no agitation, it grows in an opaque mass (Begemann et al., 2012). It possesses an operon that contains a capsular exopolysaccharide family protein (Halsa_0553), a lipopolysaccharide biosynthesis protein (Halsa_0554), a polysaccharide export protein (Halsa_0555), and a PHP domain-containing protein (Halsa_0556). Thus, Halanaerobium hydrogeniformans appears to be capable of protecting itself against osmotic and desiccation pressures.

PHOSPHOTRANFERASE SYSTEMS (PTSs)

In addition to the ABC transport systems, Halanaerobium hydrogeniformans has numerous PTSs to bring in sources of carbon. Glucose, maltose, arabinit/salicin, N-acetyl muramic acid, and trehalose can be brought into a cell with the Crr kinase protein (Halsa_0150 and Halsa_1861). N-acetyl-D-glucosamine can possibly be brought into the cell with NagE (Halsa_0149). Proteins encoded by CelA (Halsa_0141), CelB (Halsa_0142), and CelC (Halsa_0143), could bring cellobiose into the cell. Putative genes for mannitol (Mla), sorbitol (SrlA, SrlE, SrlB), galactitol (GatA, GatB, GatC), and fructose (FruA) are also present.

Halanaerobium hydrogeniformans has two putative nitrogen-related PTS genes, Halsa_0019 and Halsa_2283. Nitrogen-related PTS genes are found in Gram-negative bacteria, can regulate carbon and nitrogen metabolism, are required for virulence by some bacteria, and can play a role in potassium homeostasis (Pflüger-Grau and Görke, 2010). Halsa_0019 is likely to be involved with the regulation of fructose metabolism. Halsa_0020 is a putative gene for FruA, a fructose PTS, and Halsa_0018 is a putative 1-phosphofructokinase. In addition, when a BLAST search was performed on the amino acid sequence encoded by Halsa_0019, a 79 and 76% identity was found with a fructose-specific PTS from Halanaerobium saccharolyticum and Halanaerobium praevalens, respectively. The role for Halsa_2283 isn’t as apparent as for Halsa_0019. The gene in the same operon, Halsa_2284, was not identified. In addition, when a BLAST search was performed on the amino acid sequence encoded by Halsa_2283, only a 54% identity was found for a fructose-specific PTS from Halanaerobium saccharolyticum.

OTHER TRANSPORT SYSTEMS

Being bacterial and not archaeal, one of the intriguing aspects of the Halanaerobiales order is that they use a “salting in” mechanism to protect themselves against osmotic shock (Dektova and Boltynskaya, 2007). Halanaerobium hydrogeniformans possesses putative genes that possibly encode for TrkA-C domain containing proteins (Halsa_0281, Halsa_0709, and Halsa_1061) and TrkA-N domain containing proteins (Halsa_0737, Halsa_1057, Halsa_1056, Halsa_1352, and Halsa_1257). These genes are responsible for potassium ion transport into the cell. In addition, there are a number of putative symporters for the cell. These include a putative sodium/dicarboxylate symporter (Halsa_0959), sodium/sulfate symporter (Halsa_1097), and sodium/proline symporter (Halsa_1726). It is interesting to note that these symporters would bring sodium into the cell. There are also putative Na⁺/H⁺ antiporters present. These antiporters would remove sodium from the cell while bringing in protons and contributing to the pH homeostasis of the cell (Janto et al., 2011). Halsa_0468, Halsa_1158, Halsa_1560, and Halsa_2086 possibly encode for putative Na⁺/H⁺ antiporter NaC-like proteins. In addition, Halsa_0689 and Halsa_0691 possibly code for cation/proton antiporters. The gene that is present between these two, Halsa_0690, is a putative multiple resistance and
pH regulation protein F gene. The two genes after Halsa_0691, (Halsa_0692 and Halsa_0693) possibly encode for subunits of a multicomponent Na\(^+\)/H\(^+\) antiporter. Thus, many of these genes are likely involved with the maintenance of osmotic pressure and Halsa_0690 might be involved with pH regulation of the cell.

Besides potassium and sodium, other cations need to be transported into the cell. There are three copies, (Halsa_0666, Halsa_1667, and Halsa_2286) of a magnesium transporter for Halanaerobium hydrogeniformans. There is one putative gene for a cobalt transport protein (Halsa_1351). Halsa_1241 is a putative gene for a chromate transporter. Two putative zinc/iron permease genes are next to each other on the genome (Halsa_2161 and Halsa_2162). These cations, along with iron, would need to be taken up into the cell to serve as co-factors for enzymatic activity. Furthermore, one possible way that ammonium can enter the cell is through putative cation transporter Halsa_1351.

Another aspect that needs to be balanced between the cell and its haloalkaline environment is the anions, especially chloride. For example, Halobacillus halophilus, a low G+C, Gram-positive, moderately halophilic bacterium, has an absolute requirement for chloride (Saum et al., 2013). Halanaerobium hydrogeniformans possesses a putative Cl\(^-\) channel voltage-gated family protein (Halsa_0736) and an anion transporter (Halsa_0628) that can possibly transport chloride into the cell and help to achieve an anionic balance.

### SUMMARY

Halanaerobium hydrogeniformans is a unique bacterium that is ideally adapted to its haloalkaliphilic lake environment. It is capable of utilizing a variety of carbon sources and appears to possess the cell membrane transport systems to bring them into the cell. Once inside the cell, there is a complete Embden-Meyerhof pathway of glycolysis. However, the Kreb’s cycle is not complete. The organism relies on a number of fermentative metabolisms. It has been found to form acetate, formate, and hydrogen as fermentation products from simple sugars. It can also ferment glycerol, a widespread carbon source in saline environments. The bacterium also possesses transporters to bring in required metals and other ions. In addition to the metals required for enzymatic activity, the organism also possesses a variety of transporters that can bring in potassium and remove sodium to help to regulate the osmotic pressure. The Na\(^+\)/H\(^+\) antiporters are important for both maintaining osmotic pressure and the pH of the cell. The organism also possesses a number of transposases. The transposases enable the organism to mobilize genes and affect gene regulation.

Halanaerobium hydrogeniformans has a number of similarities to Halanaerobium praevalens GSL\(^T\). Both organisms do not appear to possess formate-hydrogen lyase while they do appear to possess glycosyl transferases and fructose-specific phosphotransferase. On the other hand, the two organisms have a number of differences that are likely related to the environments, hypersaline vs. haloalkaline, where they were isolated from. Halanaerobium praevalens GSL\(^T\) possesses fewer genes for metabolism, such as the genes required for amino acid metabolism and cofactor and vitamin production. Halanaerobium praevalens GSL\(^T\) does not possess diguanylate cyclase with metal dependent phosphohydrolase genes or many of the metal-uptake proteins that Halanaerobium hydrogeniformans possesses. Furthermore, Halanaerobium praevalens GSL\(^T\) possesses less than a third of the number of transposase genes that H. hydrogeniformans does. The presence of these genes in Halanaerobium hydrogeniformans likely enables the organism to better tolerate the alkaline conditions, in addition to the saline conditions, and the metal content present in the sediments of Soap Lake. Furthermore, the transposases could provide genetic diversity that can lead to adaptive advantages for Halanaerobium hydrogeniformans.

### ACKNOWLEDGMENTS

These sequence data were produced by the US Department of Energy Joint Genome Institute http://www.jgi.doe.gov/ in collaboration with the user community. I thank my former undergraduate students, Jill Wildhaber and Sarah Rommelfanger, and my former graduate student, Daniel Roush, who helped to look through the genome data with me.

### REFERENCES

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402. doi: 10.1093/nar/25.17.3389

Anderson, G. C. (1958). Seasonal characteristics of two saline lakes in Washington. *Limnol. Oceanogr.* 3, 51–68. doi: 10.4319/lo.1958.3.1.0051

Asada, Y., Endo, S., Inoue, Y., Mamiya, H., Hara, A., Kunishima, N., et al. (2009). Biochemical and structural characterization of a short-chain dehydrogenase/reductase of Thermus thermophilus HB8 a hyperthermophilic aldose-1-dehydrogenase with broad substrate specificity. *Chem. Biol. Interact.* 178, 117–126. doi: 10.1016/j.chembioint.2008.09.018

Asao, M., Takaichi, S., and Madigan, M. T. (2007). *Thioacapsa imhoffii*, sp. nov., an alkalophilic purple sulfur bacterium of the family Chromatiaceae from Soap Lake, Washington (USA). *Arch. Microbiol.* 188, 665–675. doi: 10.1007/s00203-007-0287-9

Asao, M., Takaichi, S., and Madigan, M. T. (2012). Amino acid-assimilating photosynthetic heliobacteria from soda lake environments: *Heloresistis acididaniminovar*. sp. nov. and *Candidatus Helonomas lunata.* *Extremophiles* 16, 585–595. doi: 10.1007/s00792-012-0458-8

Aziz, R. K., Breitbart, M., and Edwards, R. A. (2010). Transposases are the most abundant, most ubiquitous genes in nature. *Nucleic Acids Res.* 38, 4207–4217. doi: 10.1093/nar/gkq140

Bardavid, R. E., Khristo, P., and Oren, A. (2008). Interrelationships between Dunaliella and halophilic prokaryotes in saltern crystallizer ponds. *Extremophiles* 12, 5–14. doi: 10.1007/s00792-006-0053-y

Begemann, M. B., Mormile, M. R., Sitton, O. C., Wall, J. D., and Elias, D. A. (2012). A streamlined strategy for biohydrogen production with Halanaerobium hydrogeniformans, an alkaliphilic bacterium. *Front. Microbiol.* 3:93. doi: 10.3389/fmicb.2012.00093

Borowitzka, L. J. (1981). The microflora. Adaptation to life in extremely saline lakes. *Hydrobiologia* 81, 33–46. doi: 10.1007/BF0048704

Brazelton, W. J., and Baross, J. A. (2009). Abundant transposases encoded by the metagenome of a hydrothermal chimney biofilm. *ISME J.* 3, 1420–1424. doi: 10.1038/ismej.2009.79

Brown, S. D., Begemann, M. B., Mormile, M. R., Wall, J. D., Han, C. S., Goodwin, L. A., et al. (2011). Complete genome sequence of the haloalkaliphilic, hydrogen-producing bacterium *Halanaerobium hydrogeniformans*. *J. Bacteriol.* 193, 3682–3683. doi: 10.1128/JB.05209-11

Carere, C. R., Rydzak, T., Verbeke, T. J., Cicak, N., Levine, D. B., and Sparling, R. (2012). Linking genome content to biofuel production yields: a meta-analysis of major catabolic pathways among select H2 and ethanol-producing bacteria. *BMC Microbiol.* 12:295. doi: 10.1186/1471-2180-12-295

Casp, R., Altman, T., Dale, J. M., Dreher, K., Fulcher, C. A., Gilham, F., et al. (2010). The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. *Nucleic Acids Res.* 38, D473–D479. doi: 10.1093/nar/gkp875
Castiglione, N., Stelitano, V., Rinaldo, S., Giardina, G., Caruso, M., and Cutruzzola, F. (2011). Metabolism of cyclic-di-GMP in bacterial biofilms: from a general overview to biotechnological applications. Indian J. Biotechnol. 10, 423–431.

Cayol, J. L., Fardeau, M. L., Garcia, J. L., and Ollivier, B. (2002). Evidence of interspecies hydrogen transfer from glycerol in saline environments. Extremophiles 6, 131–134. doi:10.1007/s007920100229

Chadwick, L. J., and Irngens, R. L. (1991). Hydrogen gas production by an Ectothiorhodospira vacuolata strain. Appl. Environ. Microbiol. 57, 594–596.

Chua, T. K., Bujnicki, J. M., Tan, T.-C., Huynh, F., Patel, B. K., and Sivaraman, J. (2011). Molecular aspects of bacterial pH sensing and homeostasis. Nat. Rev. Microbiol. 9, 330–343. doi:10.1038/nrmicro2549

Krulwich, T. A., Sachs, G., and Padan, E. (2011). Molecular aspects of bacterial pH sensing and homeostasis. Indian J. Biotechnol. 10, 423–431.

Lairson, L. H., Henriass, B., Davies, G. J., and Withers, S. G. (2008). Glycolysistransferase: structures, functions, and mechanisms. Annu. Rev. Biochem. 77, 521–555. doi:10.1146/annurev.biochem.76.061005.092322

Mahillon, J., and Chandler, M. (1998). Insertion sequences. Microbiol. Mol. Biol. Rev. 62, 725–774.

Markowitz, V. M., Chen, I.-M. A., Palaniappan, K., Chu, K., Szeto, E., Grechkin, Y., et al. (2012). IMG: the integrated microbial genomes database and comparative analysis system. Nucleic Acids Res. 40, D115–D122. doi:10.1093/nar/gkr1044

Mormile, M. R., Romine, M. F., Garcia, M. T., Ventosa, A., Bailey, T. J., and Peyton, B. M. (1999). Halonomonas campsisps sp. nov., a denitrifying, moderately haloalkaliphilic bacterium. Syst. Appl. Microbiol. 22, 551–558. doi:10.1016/S0378-2720(99)80008-3

Mounté, S., Manac’h N., Hirschier, A., Caumette, P., Willison, J. C., and Matheron, R. (1999). Halopenaerobacter salinarius sp. nov., a novel halophilic fermentative bacterium that reduces glycine-betaine to trimethylamine with hydrogen or serine as electron donors: emendation of the genus Halopenaerobacter. Int. J. Syst. Bacteriol. 49, 103–112. doi:10.1099/00262617-000713-94-103

Nowacki, M., Higgins, B. P., Maquilan, G. M., Swart, E. C., Doak, T. G., and Landweber, L. F. (2009). A functional role for transposases in a large eukaryotic genome. Science 324, 935–938. doi:10.1126/science.1170023

Oren, A. (1993). Availability, uptake and turnover of glycerol in hypersaline environments. FEMS Microbiol. 12, 15–23. doi:10.1111/j.1574-6976.1993.tb00122.x

Oren, A. (1999). Bioenergetic aspects of halophilism. Microbiol. Mol. Biol. Rev. 63, 334–348.

Oren, A. (2008). Interrelationships between Dunaliella and halophilic prokaryotes in saltern crystallizer ponds. Extremophiles 12, 5–14. doi:10.1007/s00792-006-0053-y

Oren, A. (2010). Thermodynamic limits to microbial life at high salt concentrations. Environ. Microbiol. 13, 1908–1923. doi:10.1111/j.1462-2920.2010.02365.x

Orgel, L. E., and Crick, F. H. (1980). Selfish DNA: the ultimate parasite. Nature 284, 604–607. doi:10.1038/284604a0

Paul, V. G., Minteer, S. D., Treu, B. L., and Mormile, M. R. (2014). Ability of a halophilic bacterium isolated from Soap Lake, Washington to generate electricity at pH 11.0 and 7% salinity. Environ. Technol. 35, 1003–1011. doi:10.1080/09593330.2013.858186

Penna-cchio, A., Giordano, A., Pucci, B., Rossi, M., and Raia, C. A. (2010). Biochemical characterization of a recombinant short-chain NAD(H)-dependent dehydrogenase/reductase from Sulfolobus acidocaldarius. Extremophiles 14, 193–204. doi:10.1007/s00792-009-0298-5

Peyton, B. M., and Yonge, D. R. (2002). Biodegradation of Non-Point Source Pollutants in Soap Lake, Washington. Project Completion Report. State of Washington Water Research Report WRR-11. Pullman, WA: State of Washington Water Research Center.

Pflüger-Grau, K., and Görke, B. (2010). Regulatory roles of the bacterial nitrogen-related phosphotransferase system. Trends Microbiol. 18, 205–214. doi:10.1016/j.tim.2010.02.003

Pollock, J., Weber, K. A., Lack, J., Achenbach, L. A., Mormile, M. R., and Coates, J. D. (2007). Alkaline iron (III) reduction by a novel alkaliphilic, halotolerant, Bacillus sp. isolated from salt flat sediments of Soap Lake. Appl. Microbiol. Biotechnol. 77, 927–934. doi:10.1007/s00253-007-1220-5

Reina-Bueno, M., Argandoña, M., Salvador, M., Rodríguez-Moya, J., Iglesias-Guerra, F., Coonka, L. N., et al. (2012). Role of trehalose in salinity and temperature tolerance in the model halophilic bacterium, Chromohalobacter salexigens. PLoS ONE 7:e33587. doi:10.1371/journal.pone.0033587

Römling, U., Galperin, M. Y., and Gomelsky, M. (2013). Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. Annu. Rev. Biochem. 82, 157–183. doi:10.1146/annurev-biochem-013111-114357

Roush, D. W. (2013). Production of 1,3-propanediol from glycerol under haloalkaline conditions by Halanaerobium hydrogeniformans. Ph.D. thesis, Missouri University of Science and Technology, Rolla, MO.

Roush, D. W., Elias, D. A., and Mormile, M. R. (2014). Metabolic capabilities of the members of the order Halanaerobiales and their potential biotechnological applications. Curr. Biotechnol. 3, 3–9. doi:10.2174/22115501301011403012741

Saum, S. H., Pfeiffer, F., Palm, P., Rampp, M., Schuster, S. C., Müller, V., et al. (2013). Chloride and organic osmoregulators: a hybrid strategy to cope with elevated salinities by the moderately halophilic, chloride-dependent bacterium
**Genomic insights of haloalkaliphilic Halanaerobium hydrogeniformans**

Halobacillus halophilus. *Environ. Microbiol.* 15, 1619–1633. doi: 10.1111/j.1462-2920.2012.02770.x

Sorokin, D., Foti, M., Pinkart, H. C., and Muyzer, G. (2007). Sulfur-oxidizing bacteria in Soap Lake (Washington State), a meromictic, haloalkaline lake with an unprecedented high sulfide content. *Appl. Environ. Microbiol.* 73, 451–455. doi: 10.1128/AEM.02777-06

Stekhanova, T. N., Mardanov, A. V., Bezsudnova, E. Y., Gumerov, V. M., Ravin, N. V., Skryabin, K. G., et al. (2010). Characterization of a thermostable short-chain alcohol dehydrogenase from the hyperthermophilic archaeon *Thermococcus sibiricus*. *Appl. Environ. Microbiol.* 76, 4096–4098. doi: 10.1128/AEM.02797-09

Tam, R., and Saier, M. H. (1993). Structural, functional, and evolutionary relationships among extracellular solute-binding receptors of bacteria. *Microbiol. Rev.* 57, 320–346.

Thompson, J. C., and He, B. B. (2006). Characterization of crude glycerol from biodiesel production from multiple feedstocks. *Appl. Eng. Agric.* 22, 261–265. doi: 10.13031/2013.20272

Welsh, D. T. (2000). Ecological significance of compatible solute accumulation by micro-organisms: from single cells to global climate. *FEMS Microbiol. Rev.* 24, 263–290. doi: 10.1111/j.1574-6976.2000.tb00542.x

Zeikus, J. G., Hegge, P. W., Thompson, T. E., Phelps, T. I., and Langworthy, T. A. (1983). Isolation and description of *Haloanaerobium praevalens* gen. nov. and sp. nov., an obligately anaerobic halophile common to Great Lake sediments. *Curr. Microbiol.* 9, 225–234. doi: 10.1007/BF01356756

Zeng, A.-P. (1996). Pathway and kinetic analysis of 1,3-propanediol production from glycerol fermentation by Clostridium butyricum. *Bioprocess Eng.* 14, 169–175. doi: 10.1007/BF01464731

Zhao, B., and Chen, S. (2012). Alkalitalea saponilacus gen. nov., sp. nov., an obligately anaerobic, alkaliophilic, xylanolytic bacterium from a meromictic soda lake. *Int. J. Syst. Evol. Microbiol.* 62, 2618–2623. doi: 10.1099/ijs.0.038315-0

**Conflict of Interest Statement:** The author holds two patents on biohydrogen production by Halanaerobium hydrogeniformans. Elias, Mormile, Begemann, and Wall. "A combined fossil fuel free process of lignocellulosic pretreatment with biological production", U.S. Patent No. US 8,148,133, Issued: April 3, 2012. Elias, Mormile, Begemann, and Wall. "Fossil Fuel-Free Process of Lignocellulosic Pretreatment with Biological Hydrogen Production", U.S. Patent No. US 8,034,592 B2, Issued October 11, 2011.

Received: 19 August 2014; paper pending published: 10 October 2014; accepted: 03 November 2014; published online: 19 November 2014.

Citation: Mormile MR (2014) Going from microbial ecology to genome data and back: studies on a haloalkaliphilic bacterium isolated from Soap Lake, Washington State. *Front. Microbiol.* 5:628. doi: 10.3389/fmicb.2014.00628

This article was submitted to Extreme Microbiology, a section of the journal Frontiers in Microbiology.

Copyright © 2014 Mormile. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.