

1 **Adaptations of *Atribacteria* to life in methane hydrates: hot traits for cold life**

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17 **Running Title:** *Atribacteria* adaptations in methane hydrate ecosystem

18 **Dedication:** To Katrina Edwards

19

20 **Originality-Significance Statement:** This work provides insights into the metabolism and

21 adaptations of elusive *Atribacteria* (JS-1 clade) that are ubiquitous and abundant in methane-rich

22 ecosystems. We show that JS-1 (Genus 1) from methane hydrate stability zones contain

23 metabolisms and stress survival strategies similar to hyperthermophilic archaea.

24 **Summary:** Gas hydrates harbor gigatons of natural gas, yet their microbiomes remain  
25 mysterious. We bioprospected methane hydrate-bearing sediments from under Hydrate Ridge  
26 (offshore Oregon, USA, ODP Site 1244) using 16S rRNA gene amplicon, metagenomic, and  
27 metaproteomic analysis. *Atribacteria* (JS-1 Genus 1) sequences rose in abundance with increasing  
28 sediment depth. We characterized the most complete JS-1 Genus 1 metagenome-assembled  
29 genomic bin (B2) from the deepest sample, 69 meters below the seafloor (E10-H5), within the  
30 gas hydrate stability zone. B2 harbors functions not previously reported for *Atribacteria*,  
31 including a primitive respiratory complex and myriad capabilities to survive extreme conditions  
32 (e.g. high salt brines, high pressure, and cold temperatures). Several *Atribacteria* traits, such as a  
33 hydrogenase- $\text{Na}^+/\text{H}^+$  antiporter supercomplex (Hun) and di-myo-inositol-phosphate (DIP)  
34 synthesis, were similar to those from hyperthermophilic archaea. Expressed *Atribacteria* proteins  
35 were involved in transport of branched chain amino acids and carboxylic acids. Transporter genes  
36 were downstream from a novel helix-turn-helix transcriptional regulator, AtiR, which was not  
37 present in *Atribacteria* from other sites. Overall, *Atribacteria* appear to be endowed with unique  
38 strategies that may contribute to its dominance in methane-hydrate bearing sediments. Active  
39 microbial transport of amino and carboxylic acids in the gas hydrate stability zone may influence  
40 gas hydrate stability.

41

## 42 **Introduction**

43 Gas hydrates, also known as clathrates, are cages of ice-like water crystals encasing gas  
44 molecules such as methane ( $\text{CH}_4$ ). Because hydrates form under high pressure and low  
45 temperature, their distribution on Earth is limited to permafrost and continental margins (Hester  
46 and Brewer, 2009). These hydrates harbor gigatons of natural gas, which may serve as a potential  
47 energy source for the future (Chong et al., 2016). They are also susceptible to dissociation due to  
48 rising ocean temperatures, which could release massive methane reservoirs to the atmosphere and  
49 exacerbate global warming (Archer et al., 2009; Ruppel and Kessler, 2017).

50 Despite the global importance of gas hydrates, their microbiomes remain mysterious.  
51 Microbial cells are physically associated with hydrates (Lanoil et al., 2001), and the taxonomy of  
52 these hydrate-associated microbiomes is distinct from non-hydrate-bearing sites (Inagaki et al.,  
53 2006). Because salt ions are excluded during hydrate formation (Ussler III and Paull, 2001;  
54 Bohrmann and Torres, 2006), hydrate-associated microbes likely possess adaptations to survive  
55 high salinity and low water activity, as well as low temperatures and high pressures (Honkalas et  
56 al., 2016). However, knowledge of the genetic basis of such adaptations is incomplete, as  
57 genomic data for hydrate communities are sparse and most hydrate microbiomes have been  
58 characterized primarily through single-gene taxonomic surveys.

59 Global 16S rRNA gene surveys show that the JS-1 sub-clade of the uncultivated bacterial  
60 candidate phylum *Atribacteria* is the dominant taxon in gas hydrates (Reed et al., 2002; Inagaki et  
61 al., 2003; Kormas et al., 2003; Newberry et al., 2004; Webster et al., 2004; Inagaki et al., 2006;  
62 Webster et al., 2007; Fry et al., 2008; Kadnikov et al., 2012; Parkes et al., 2014; Chernitsyna et  
63 al., 2016) and in other deep sediment ecosystems with abundant methane (Gies et al., 2014; Carr  
64 et al., 2015; Hu et al., 2016). The other major *Atribacteria* lineage, OP-9, has only been found in  
65 hot springs (Dodsworth et al., 2013; Rinke et al., 2013) and thermal bioreactors (Nobu et al.,  
66 2015). Marine *Atribacteria* are dispersed through ejection from submarine mud volcanoes  
67 (Hoshino et al., 2017; Ruff et al., 2019), and environmental heterogeneity may select for locally  
68 adapted genotypes. Indeed, *Atribacteria* phylogeny is highly diverse, suggesting the potential for  
69 wide functional variation and niche specialization.

70 Genomic evidence for such *Atribacteria* specialization remains limited. To date, near-  
71 complete single-cell and metagenomic sequences from hot springs, wastewater, lake sediments,  
72 and non-hydrate bearing marine sediments have shown that *Atribacteria* lack respiratory  
73 pathways. The high-temperature OP-9 lineage likely ferments sugars (Dodsworth et al., 2013)  
74 whereas the low-temperature JS-1 lineage ferments propionate to hydrogen, acetate, and ethanol  
75 (Nobu et al., 2016). Both JS-1 and OP-9 lineages possess genes encoding bacterial  
76 microcompartment shell proteins that may sequester toxic aldehydes, enabling their condensation

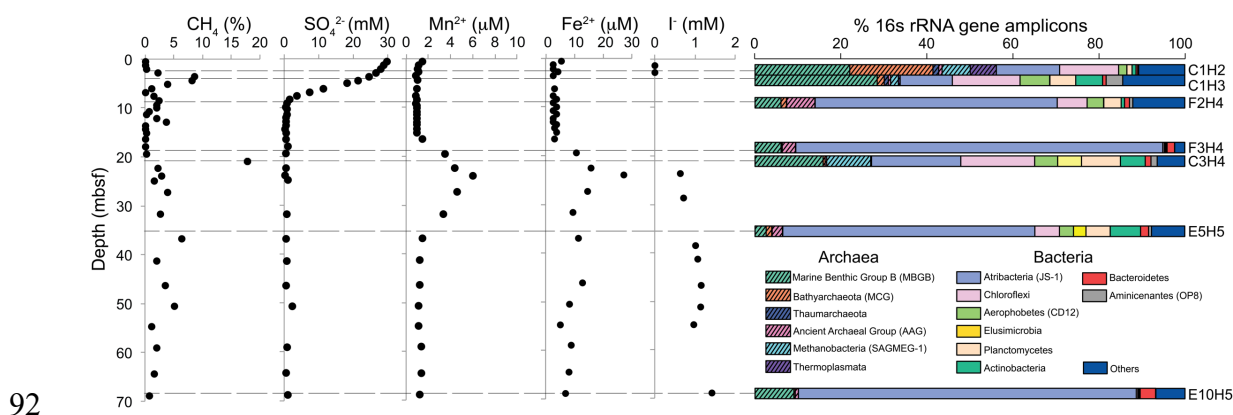
77 to carbohydrates (Nobu et al., 2016). The available data on *Atribacteria* genomes suggest  
 78 diversification linked to organic substrate utilization, although a range of other factors, including  
 79 physical environmental conditions (e.g., temperature and pressure) undoubtedly also play a role.

80 Here we examined the distribution, phylogeny, and metabolic potential of uncultivated  
 81 JS-1 *Atribacteria* in cold, salty, and high-pressure sediments beneath Hydrate Ridge, off the coast  
 82 of Oregon, USA, using a combination of 16S rRNA gene amplicon, metagenomic, and  
 83 metaproteomic analysis. We found that JS-1 Genus-1 are abundant in the gas hydrate stability  
 84 zone (GHSZ) and that they harbor numerous strategies for tolerance of osmotic stress, including  
 85 many biosynthesis pathways for unusual osmolytes similar to those of thermophiles.

86

## 87 Results and Discussion

88 **Geochemical gradients.** Sediment core samples spanned four geochemical zones from 0-69  
 89 meters below seafloor (mbsf) at the ODP Site 1244C,D,E at Hydrate Ridge, off the coast of  
 90 Oregon, USA (Fig. S1; Tréhu et al., 2003): near surface (0-2 mbsf), sulfate-methane transition  
 91 zone (SMTZ; 2-9 mbsf), metal reduction zone (18-36 mbsf), and GHSZ (45-124 mbsf; **Fig. 1**).



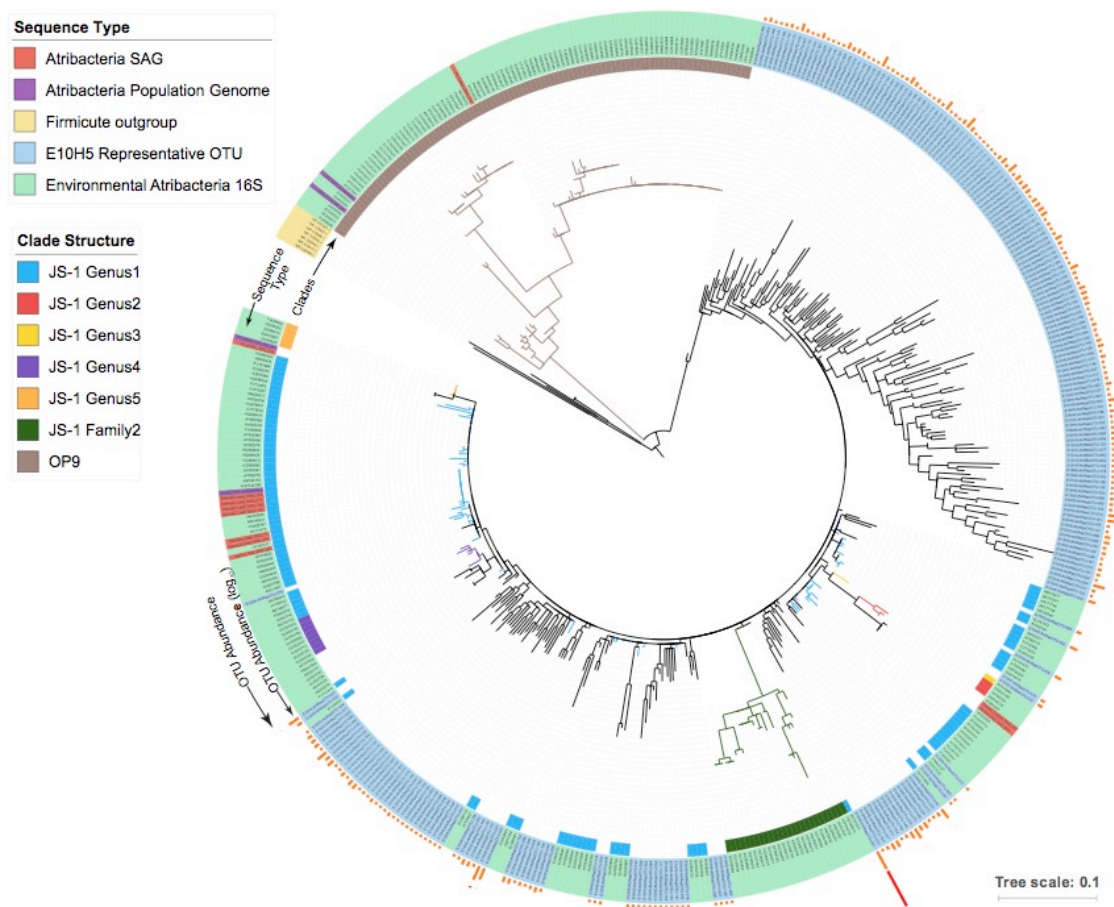
92  
 93 **Figure 1.** Porewater geochemistry (methane, sulfate, manganese, iron, and iodide) and 16S rRNA  
 94 gene composition from sediment depth profiles at ODP 204 Site 1244, Hydrate Ridge, offshore  
 95 Oregon, USA. Hatched and solid bars are archaeal and bacterial 16S rRNA genes, respectively.  
 96 “Others” category represents bacterial and archaeal phyla with <2% of total sequences.

97  
 98 Sediment porewater methane concentrations rose from negligible at the seafloor to 8% by volume  
 99 at 3-5 mbsf, and remained <5% below 5 mbsf, with the exception of one sample at 21 mbsf.

100 Sulfate rapidly dropped from 28 to <1 mM from 0-9 mbsf and remained <1 mM below 9 mbsf,  
101 with the exception of one sample at 50.7 mbsf (2.3 mM sulfate). Outside of the metal reduction  
102 zone, dissolved Mn was ~1  $\mu$ M and dissolved Fe was 3-10  $\mu$ M. Dissolved Mn and Fe peaked at 6  
103 and 27  $\mu$ M, respectively, coincident with a single layer of disseminated gas hydrate in the metal  
104 reduction zone. Dithionite-extractable Fe and Mn increased slightly from 2 to 21 mbsf (0.4 to  
105 1.1% and 0.002 to 0.005%, respectively; **Table S1**). Iodide concentrations were highest in the  
106 GHSZ (1.4 mM), where liquid brines form as a result of methane hydrate formation. Estimated *in*  
107 *situ* salinity ranged from seawater salinity (35 g kg<sup>-1</sup>) to >100 g kg<sup>-1</sup> (Milkov et al., 2004). Total  
108 organic carbon concentrations in sediment varied between 1-2%. *In situ* temperature ranged from  
109 ~4°C at the seafloor to ~6-11°C in the GHSZ.

110

111 **Phylogenetic diversity.** Phylogenetic diversity and species richness in 16S rRNA gene amplicons  
112 were highest in the SMTZ and decreased with depth except in the metal reduction zone (**Fig. S2**).  
113 The relative abundance of *Atribacteria* (JS-1)-affiliated amplicons increased with depth, from  
114 15% in the near surface to 86% in the GHSZ (**Table S1**). GHSZ sediment (sample E10-H5 from  
115 69 mbsf) contained 230 *Atribacteria* OTUs (89-92% ANI) that spanned a wide diversity of clades  
116 within JS-1 Genus 1 (Yarza et al. 2014) (**Fig. 2**). A single OTU matching GenBank AB804573.1,  
117 from an ocean drilling core from offshore Shimokita Peninsula, Japan, comprised 69% of  
118 *Atribacteria* 16S rRNA sequences in the GHSZ (**Table S2**). Other *Atribacteria* 16S rRNA  
119 sequences also matched marine samples from shallower Hydrate Ridge sediments (Marlow et al.,  
120 2014) and methane hydrate sediment off Taiwan (Lin et al., 2014) (**Table S2**). 16S rRNA  
121 sequences from amplicons and metagenomes generally showed consistent trends (**Fig. S3**).  
122 *Atribacteria* OTU abundance and composition varied significantly with sediment depth (**Fig. S4**).

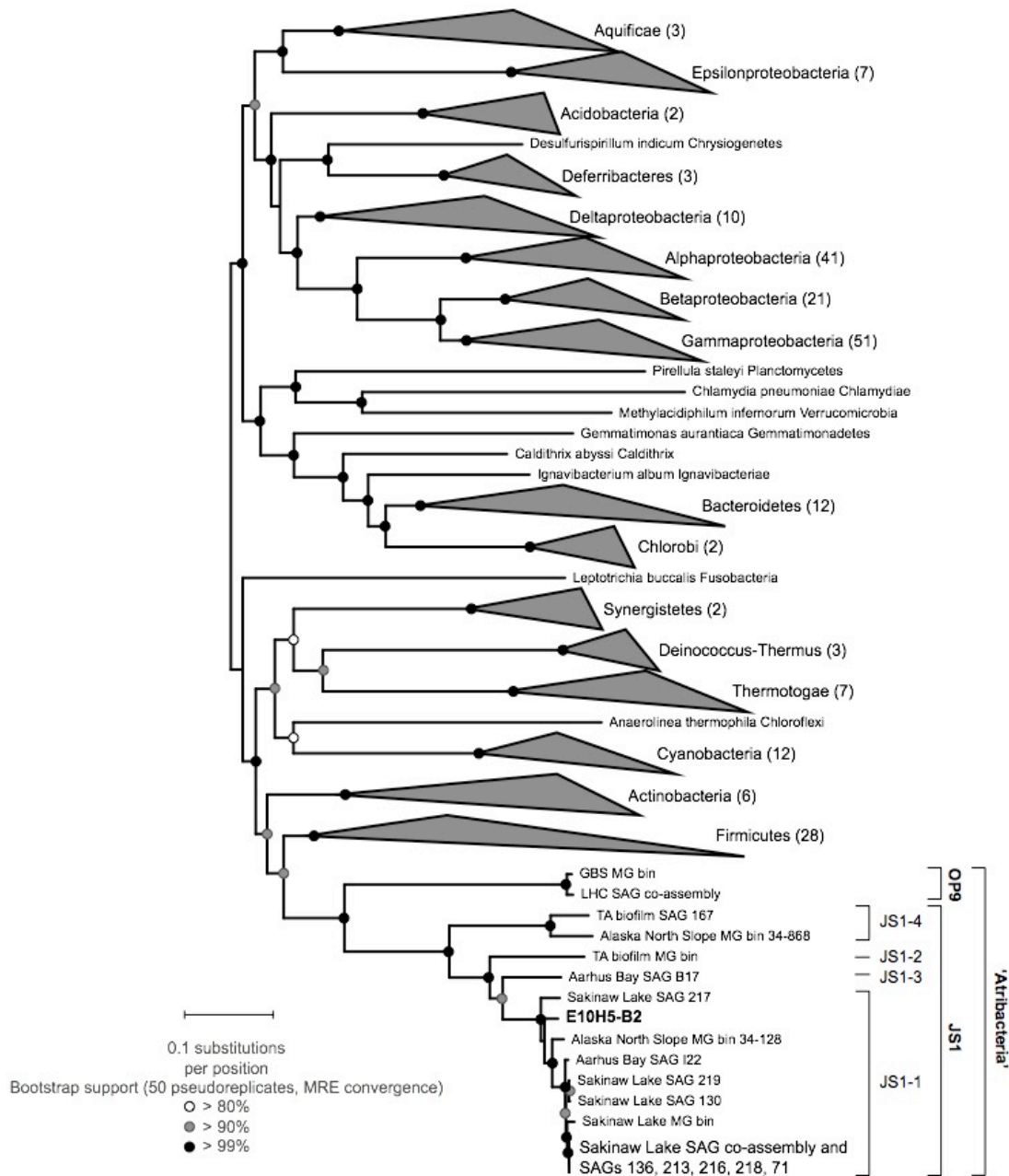


123

124 **Figure 2:** Phylogenetic reconstruction of *Atribacteria* 16S rRNA gene sequences from sample E10-  
125 H5 (69 mbsf). The tree includes the 230 *Atribacteria* OTUs with two or more sequences as well as  
126 reference sequences from environmental clones, SAGs, and MAGs, with *Firmicutes* as the  
127 outgroup. Reconstruction was performed in RAxML with 275 positions spanning the V3-V4 region  
128 of the 16S rRNA gene using a GAMMA model of rate heterogeneity, a GTR model of substitution,  
129 and 500 bootstraps followed by a thorough Maximum Likelihood search. The relative abundances  
130 of recovered amplicons from diverse lineages/OTUs is shown in the outermost circle. Additional  
131 information on the most abundant JS-1 OTUs from E10-H5 is provided in **Table S2**.  
132

133 **JS-1 Genus-1 partial genome.** To gain insight into the function of JS-1 *Atribacteria* in the  
134 GHSZ, we analyzed a 4-Mbp metagenome-assembled genome (MAG) from sample E10-H5  
135 (**Table S3**). This MAG, hereafter designated “B2”, was chosen for its relatively high  
136 completeness (69%) and low contamination (2%). B2 lacked a 16S rRNA gene, but contained a  
137 *rpoB* gene with 94% similarity to *Atribacteria* bacterium 34\_128 from an oil reservoir (Hu et al.,  
138 2016). B2 had 35% GC content, similar to other *Atribacteria* (Carr et al., 2015). Phylogenetic  
139 placement based on 69 concatenated single-copy genes confirmed that B2 belonged to JS1-Genus

140 1 and was most closely related to JS1-Genus 1 genomes from a sediment-hosted aquifer at Rifle,  
 141 Colorado (RBG\_COMBO\_35; Anantharaman et al., 2016), cold CO<sub>2</sub>-rich fluids at Crystal  
 142 Geysir, Utah (CG2\_30\_33\_13; Probst et al., 2017), and hydrothermal vent sediments at Guaymas  
 143 Basin, Gulf of California (4572\_76; Dombrowski et al., 2017) (**Fig. 3**).



144 **Figure 3: Maximum likelihood phylogeny for B2 with 220 representative and 20 previously**  
 145 **found Atribacteria SAGs and population genomes using multiple (minimum 6, maximum**  
 146 **69) core single copy genes.** Tree made in RAxML with GAMMA model, 1000 rapid bootstraps,  
 147 MRE convergence bootstop (50 replicates) followed by a thorough ML search.  
 148  
 149

150 Despite the relatively cool *in situ* temperature of the E10-H5 sediment (7-8°C  
151 (ShipboardScientificParty, 2003)), the most closely related genomes from cultured isolates were  
152 thermophilic gram-positive Firmicutes: halophilic *Halothermothrix orenii* spp. (Mavromatis et  
153 al., 2009) and metal-respiring *Therminocola potens* strain JR (Byrne and Nicholas, 1986). Below  
154 we highlight features of the B2 genome and proteome potentially relevant to life in the unique  
155 environment of methane clathrates, with particular focus on a putative respiratory complex and  
156 genes involved in stress response and environmental homeostasis.

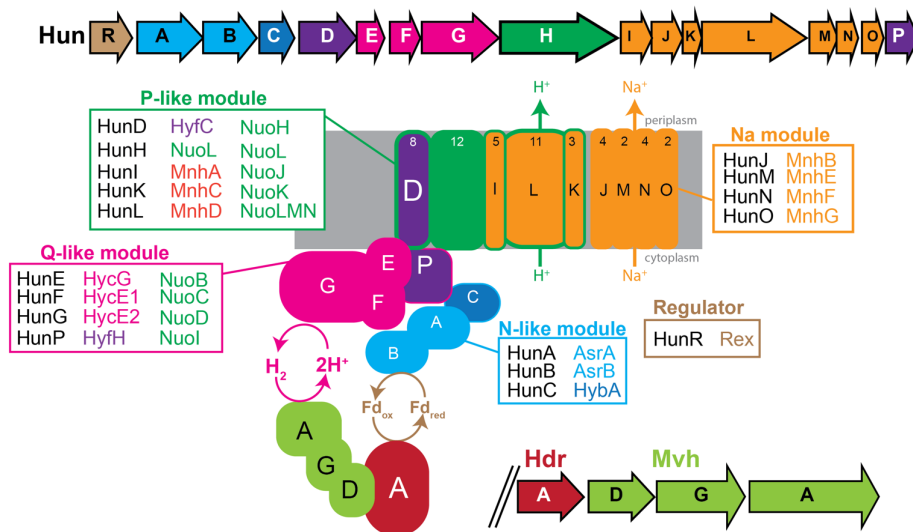
157

158 **Predicted respiratory function of the Hun supercomplex.** B2 contained genes for a putative  
159 operon encoding a 16-subunit respiratory complex, hereafter designated Hun. The *hun* operon  
160 was also present in two other MAGs from ODP Site 1244 (*Planctomycetes* C1H3-B36 and  
161 *Firmicutes* E5H5-B3) and in *Atribacteria*, *Actinobacteria*, and *Omnitrophica* MAGs from other  
162 deep subsurface ecosystems (Rinke et al., 2013; Baker et al., 2015; Anantharaman et al., 2016;  
163 Probst et al., 2017) (**Table S4**). The gene arrangement and predicted function of the putative Hun  
164 complex are similar to those of an ancient Mrp-Mbh-type membrane-bound [NiFe] hydrogenase-  
165 Na<sup>+</sup>/H<sup>+</sup> antiporter respiratory complex in hyperthermophilic archaea (Yu et al., 2018), which is  
166 thought to be the ancestor of Complex I, also known as NADH:ubiquinone oxidoreductase (Nuo)  
167 (Friedrich and Scheide, 2000; Moparthy and Hägerhäll, 2011; Schut et al., 2013). Complex I's  
168 modules likely had separate origins: the ubiquinone-reducing subunits NuoBCD (“Q-module”)  
169 evolved from an ancient membrane-bound [NiFe] hydrogenase, while its proton-pumping  
170 subunits NuoLMN (“P module”) evolved from an ancient Na<sup>+</sup>/H<sup>+</sup> antiporter (Mathiesen and  
171 Hägerhäll, 2002; Moparthy et al., 2014; Spero et al., 2015).

172 *Atribacteria hun* genes likely encode a complex of four protein modules that couple H<sup>+</sup>  
173 and Na<sup>+</sup> translocation to H<sub>2</sub> production, similar to Mrp-Mbh-type complexes in hyperthermophilic  
174 archaea (**Fig. 4**). Based on the similarity of HunAB to anaerobic sulfite reductase (Asr) subunits  
175 A and B, which transfer electrons from ferredoxin to the active site in AsrC (missing in the *hun*



176 operon), we inferred that N module-like subunits HunABC likely accept electrons from  
 177 ferredoxin and pass them through iron-sulfur clusters to Q-module-like subunit HunEFGP.  
 178 Instead of accepting electrons from NADPH and passing them to ubiquinone as in Complex I,  
 179 HunABC likely accepts electrons from ferredoxin and passes them to  $2\text{H}^+$  for reduction to  $\text{H}_2$  at  
 180 HunEFGP's Ni-Fe active site (Table 1; Fig. 4).



181

182 **Figure 4: Predicted structure and function of a multi-subunit respiratory complex,**  
 183 **hereafter “Hun”, found in B2 and other deep subsurface genomes.** *Top:* conserved gene  
 184 cluster arrangement, with each color representing a different predicted protein. *Below:* predicted  
 185 cellular locations and functions based on homologs of the genes of the same colors encoded by  
 186 the putative *hun* operon, and predicted regeneration of substrates by the heterodisulfide reductase  
 187 (HdrA)-methyl viologen hydrogenase (MvhAGD) complex. Predicted functions of *hun* genes are  
 188 based on Mrp-Mbh complexes in thermophilic archaea (Schut et al., 2013; Yu et al., 2018). See  
 189 **Table S4** for accession numbers.

190

191 P-module-like subunits HunDHILK are predicted to be proton-pumping transmembrane proteins  
 192 and Na-module-like subunits HunIJKLMNO are homologs of the  $\text{Na}^+/\text{H}^+$  antiporter  
 193 MnhABCDEFGH in Mrp-Mbh-type complexes. The presence of F0F1-type and V-type ATPases  
 194 suggest that  $\text{H}^+$  and  $\text{Na}^+$  ions pumped outward by HunIJKLMNO are pumped back in to make  
 195 ATP. Electrons from  $\text{H}_2$  could be transferred back to ferredoxin by the activity of the  
 196 heterodisulfide reductase (HdrA)-methyl viologen hydrogenase (MvhAGD) complex (Fig. 4). A  
 197 redox-sensing transcriptional repressor gene (*hunR*) immediately upstream of the *hun* operon

198 suggests that the hydrogenase may not be used strictly for energy conservation, but could also be  
199 for balancing reducing equivalent by disposing of extra electrons (McLaughlin et al., 2010).

200

201 **Osmotic stress survival.** Any life that can persist in brine pockets within methane hydrate must  
202 contend with high salinity (up to ~3x that of seawater) and low water potential. B2 contained  
203 numerous genes for the “salt out” survival strategy, in which osmotic pressure is maintained by  
204 exporting cations (Wood, 2015). B2’s cation export systems included efflux systems,  
205 mechanosensitive ion channels, and Na<sup>+</sup>-H<sup>+</sup> antiporters (**Table 1**).

206 A second salt survival strategy is import and/or biosynthesis of osmolytes, most often  
207 polar, water-soluble, and uncharged organic compounds and/or extracellular polymers. For  
208 example, glycine betaine is abundant in saline fluids from deep sediment basins (Daly et al.,  
209 2016). B2 contained genes for transport of trehalose and biosynthesis of the common osmolytes  
210 glutamine, glutamate, and poly-gamma-glutamate, all of which had homologs in other  
211 *Atribacteria* MAGs (**Table 1**). B2 also encoded genes for glycine betaine and dihydroxyacetone  
212 biosynthesis without homologs in other *Atribacteria*. Surprisingly, B2 also encoded biosynthetic  
213 genes (myo-inositol-1 phosphate synthase (MIPS)/bifunctional IPC transferase and DIPP  
214 synthase (IPCT-DIPPS)) for the unusual solute di-myo-inositol-phosphate (DIP) made by  
215 hyperthermophiles (Santos and Da Costa, 2002). The MIPS gene had closest similarity to  
216 halophilic and psychrophilic *Euryarchaeota*, without homologs in other *Atribacteria*. The IPCT-  
217 DIPPS gene was also present in *Atribacteria* HGW-1 from subsurface Japan (Hernsdorf et al.,  
218 2017) and *Atribacteria* 4572\_76 from Guaymas Basin (Dombrowski et al., 2017).

219 Immediately upstream from B2’s MIPS/IPCT-DIPPS genes was an acyl carrier protein  
220 (*acpP*) gene, commonly involved in fatty acid and polyketide biosynthesis. Sixteen additional  
221 *acpP* copies were present in B2, often flanked by transposon scars, suggestive of recent  
222 horizontal gene transfer (**Table S5**). Other *Atribacteria* MAGs had only 1-2 copies of *acpP*,  
223 usually near fatty acid biosynthesis genes.

224 **Table 1. Putative osmotic stress-related genes in B2.** *Atribacteria* homologs all had >80%  
 225 AAI. AAI to other taxa (56-76%) are provided. \*indicates multiple copies.

Annotation	Gene	Accession	Top hit	Top hit
Na <sup>+</sup> /H <sup>+</sup> antiporter	<i>mrpEFGB</i>	RXG65834.1- RXG65838.1	OQY40657.1- OQY40661.1	<i>Atribacteria</i> 4572_76
Na <sup>+</sup> efflux	<i>natB</i>	RXG65900.1	OGD31203.1	<i>Atribacteria</i> RBG....
Threonine efflux	<i>rhtB</i>	RXG66248.1	OGD15641.1	<i>Atribacteria</i> RBG....
Na <sup>+</sup> channel	DUF554	RXG63559.1	KUK55705.1	<i>Atribacteria</i> 34_128
Mechanosensitive ion channel	<i>mscS</i>	RXG63036.1	KUK56353.1	
Trehalose transporter	<i>sugAB</i>	RXG66833.1- RXG66834.1	KUK55397.1 KUK55398.1	
Glutamine synthetase	<i>glnA</i>	RXG65164.1	KUK55578.1	
K <sup>+</sup> transport	<i>trkAH</i> *	RXG63511.1 RXG63512.1	PKP56013.1 PKP56012.1	<i>Atribacteria</i> HGW-1
Aromatic aa exporter	<i>yddG</i> *	RXG63201.1	PKP55084.1	
Glutamate synthase	<i>gltD</i>	RXG66270.1	PKP56573.1	
Proline racemase	<i>prdF</i>	RXG63210.1	PKP58887.1	
Poly-gamma glutamate synthase	<i>pgsCBW</i>	RXG66317.1- RXG66319.1	PKP60458.1- PKP60460.1	
Glycerol uptake	<i>glpF</i>	RXG65629.1	OHV10031.1 (61%)	<i>Kushneria</i> YCWA18
Betaine-aldehyde dehydrogenase	<i>betB</i>	RXG62957.1	KUJ28189.1 (56%)	<i>Catabacter hongkongensis</i>
Dihydroxy-acetone kinase	<i>dhaKLM</i>	RXG65626.1- RXG65628.1	RLC64130.1- (67%) RLC64131.1 (61%)	<i>Chloroflexi</i> bacterium
DIPP synthesis pathway	MIPS/IPCT-DIPPS*	RXG66889.1 RXG66888.1	AAU82306.1 (76%) PKP58414.1	Archaeon GZfos13E1 <i>Atribacteria</i> HGW-1

226

227 Like other *Atribacteria*, B2 contained genes encoding a sugar phosphate-utilizing class of  
 228 proteinaceous bacterial microcompartments that neighbored sugar isomerases, RnfC NADH  
 229 dehydrogenase and an oxidoreductase (Axen et al., 2014; Nobu et al., 2016) (**Table S6**). Further  
 230 exploration of sugar-related genes revealed that B2 and other *Atribacteria* encode the non-  
 231 mevalonate pathway for isoprenoid biosynthesis (*ispDEFGH*), exopolysaccharide synthesis  
 232 proteins, numerous glycosyltransferases for transferring UDP- and GDP-linked sugars to a variety  
 233 of substrates, and several proteins related to N-linked glycosylation (**Table S7**). The capacity for  
 234 glycosylation may be another adaptation for survival of salt stress (Kho and Meredith, 2018).

235

236 **Expression of lipopolysaccharide and transport-related proteins.** Metaproteomic analysis  
 237 identified six expressed peptides affiliated with B2, all associated with assembly or transport  
 238 (**Table 2**). One was an outer member lipopolysaccharide assembly protein (LptD), also known as

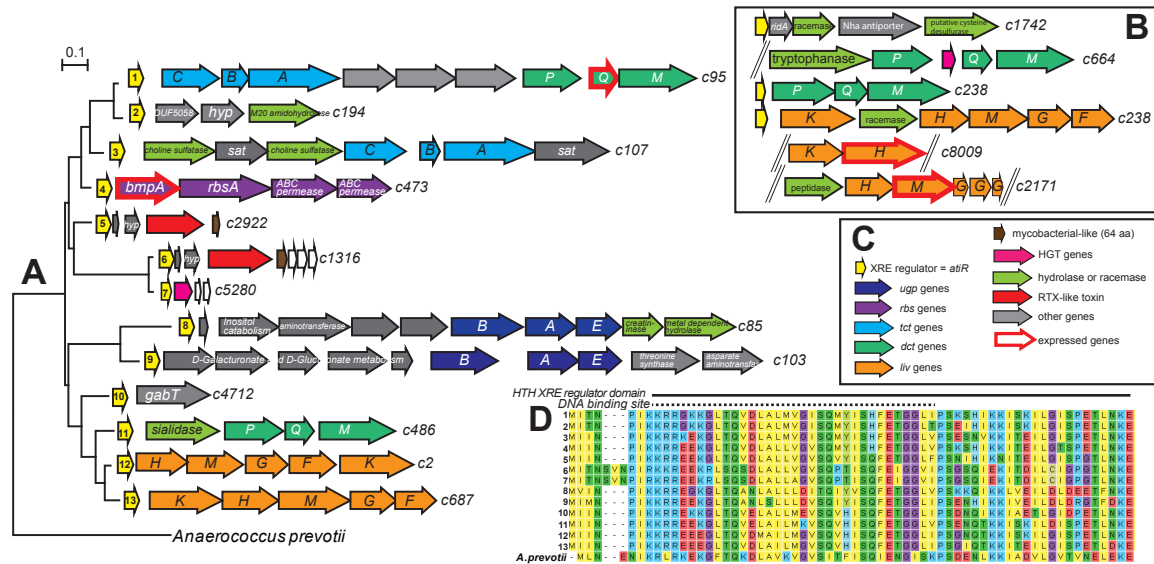
239 Imp/OstA (increased membrane permeability/organic solvent tolerance (Braun and Silhavy,  
 240 2002). Another was a capsular polysaccharide biosynthesis protein (YveK). The other expressed  
 241 peptides were predicted to be transporters of purines (BmpA), branched chain amino acids (LivH,  
 242 LivM), and C4-dicarboxylates (DctQ). All *liv* genes on the operon with the expressed *livH* had  
 243 homologs in other *Atribacteria* genomes (Table S8) with the exception of *livG*, which encodes a  
 244 protein related to the lipopolysaccharide export system ATP-binding protein LptB that may serve  
 245 a specific purpose in methane-hydrate *Atribacteria*. Upstream of *liv* genes we found a *ykkC-ykkD*  
 246 riboswitch implicated in detoxification and efflux control (Barrick et al., 2004), suggesting that  
 247 branched chain amino acids may be involved in environmental stress response, as seen in other  
 248 microbes (Liu et al., 2005).

249 **Table 2. Metaproteomic peptide hits for B2.**

Peptide	Protein	Contig	Gene	Top hit (% identity)	Top hit
EYKPKEDWKMNFSS SYNLNTK	LptD	C10125	33494	OQY39007.1 (90%)	<i>Atribacteria</i> 4572_76
GIILIFLIAVITAVLV SYFVLSPTP	YveK	C456	RXG64813.1	PKP59499.1 (74%)	<i>Atribacteria</i> HGW-1
CSNLIKALLVVLVL SLGITLGIKAP	BmpA	C473	RXG64193.1	PKP58720.1 (94%)	<i>Atribacteria</i> HGW-1
KPFRKSPGLIILLSTV AVGFIIR	LivH	C8009	30420	OQY40503.1 (94%)	<i>Atribacteria</i> 4572_76
LIFLLLLAVAVVVPF LLGLLILRF	LivM	C2171	15004	RKY02958.1 (46%)	<i>Spirochaetes</i> bacterium
NKINLIFSILIIIFLIVL TYEGIILVKVGLNA	DctQ	C95	RXG62936.1	AEG13811.1 (34%)	<i>Desulfofundulus kuznetsovii</i>

250

251 In addition to numerous transporters for branched chain amino acids, B2 encoded abundant  
 252 TRAP (tripartite ATP-independent periplasmic) transporters of dicarboxylic (DctPQM) and  
 253 tricarboxylic (TctCBA) acids (Table S7; Fig. 5). TRAP transporters use an electrochemical  
 254 gradient ( $H^+$  or  $Na^+$ ) and a substrate-binding protein to transport solutes across the membrane  
 255 (Fischer et al., 2010). A conserved arginine residue in the DctP substrate-binding protein confers  
 256 specificity for carboxylate groups (Lecher et al., 2009; Fischer et al., 2015).



257

258 **Figure 5: Phylogeny of HTH-XRE regulators/antitoxins (yellow), hereafter “AtiR”, from B2**  
 259 **and synteny of downstream genes.** Genes highlighted in thick red lines were expressed in the  
 260 metaproteome. **A)** AtiR maximum likelihood phylogeny based on contigs (labeled on the right)  
 261 from E10-H5 B2, with *Anaerococcus prevotii* as the outgroup. **B)** Additional putative operons from  
 262 B2 likely regulated by *atiR*, which is truncated partially or completely on these contigs. **C)** Legend  
 263 for panels A and B; **D)** AtiR amino acid alignment for the 13 AtiR sequences from *Atribacteria*  
 264 E10-H5-B2 shown in panel A. Abbreviations: *bmpA*: basic membrane protein A; *dctPQM*: C4-  
 265 dicarboxylate transporter; *gabT*: 4-aminobutyrate aminotransferase; *livHMGF*: branched chain  
 266 amino acid transporter; *rbs*: ribose transporter; *sat*: sulfate adenylyltransferase; *tctCBA*:  
 267 tricarboxylate transporter; *ugpBAE*: sn-glycerol-3-phosphate transporter. See **Table S7** for  
 268 accession numbers and % identity to closest gene hits in other genomes.

270 **A novel regulator.** Three out of six of the expressed transporter proteins were encoded by genes  
 271 located downstream from a novel gene predicted to encode a helix-turn-helix xenobiotic response  
 272 element transcriptional regulator, which we named “AtiR” (**Table S8; Fig. 5**). AtiR was not  
 273 found in *Atribacteria* MAGs (the top BLAST hit was the skin firmicute *Anaerococcus prevotii*  
 274 (41-49% AAI)), suggesting that it may serve a specific purpose in methane-hydrate *Atribacteria*.  
 275 Genes downstream of *atiR* were dominated by transporters for organic solutes (*tct*, *dct*, *ugp*),  
 276 branched chain amino acids (*liv*), hydrolases (choline sulfatase, sialidase, tryptophanase, cysteine  
 277 desulfurase), peptidases, and racemases (**Table S8; Fig. 5**). In two instances, genes encoding  
 278 RTX-toxin repeats were located on *atiR* contigs (**Table S8**). B2 also contained numerous MazEF  
 279 toxin-antitoxin systems (**Table S9**), which trigger programmed cell death in response to stress  
 280 (Engelberg-Kulka et al., 2005). *Atribacteria* may use AtiR to regulate cellular degradation of

281 peptides and proteins to amino acids, either for nutrients acquisition or for survival under  
282 environmental stress (Bergkessel et al., 2016).

283

284 **Adaptations to life in methane hydrates.** The GHSZ in deep subsurface sediments is dominated  
285 by *Atribacteria* that appear to contain unique adaptations for survival in an extreme system with  
286 high salinity, high pressure, low water activity, and low temperatures. Our analysis of the B2  
287 *Atribacteria* MAG from the GHSZ (69 mbsf at Hydrate Ridge, offshore Oregon, *in situ* sediment  
288 temperature ~6-11°C) revealed multiple survival strategies with similarity to hyperthermophiles.  
289 In B2, these “hot traits in cold life” included genes for an ancient respiratory system (Hun) and an  
290 unusual osmolyte (DIP). Other probable environmental stress adaptations include glycosylation,  
291 membrane modifications, and a novel regulatory mechanism (AtiR) for transport of carboxylic  
292 acids and branched chain amino acids.

293 Our findings suggest that *Atribacteria* may actively modulate the composition and  
294 concentration of organic compounds in methane hydrate sediments. Active cellular transport of  
295 organics would change environmental concentrations, which in turn could influence hydrate  
296 stability. The hydrophobicity of branched chain amino acids has been shown to influence hydrate  
297 stability; less hydrophobic amino acids like glycine and alanine inhibit hydrate formation by  
298 disrupting the hydrogen bond network, while more hydrophobic amino acids, such as leucine,  
299 valine and isoleucine, promote hydrate growth by strengthening the local water structure (Sa et  
300 al., 2013; Liu et al., 2015; Veluswamy et al., 2017). Gas hydrate growth is also promoted by  
301 anionic surfactants (Kumar et al., 2015), which include carboxylic acids. Thus, we surmise that  
302 bacterial transport of organic compounds may influence hydrate stability. Our results motivate  
303 future studies of methane stability that account for the influence of microbial processes, in  
304 particular those of abundant *Atribacteria*.

305

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318 paper acceptance].

## 319 **Experimental Procedures**

320 **Sample collection.** Sediments were cored at ODP site 1244 (44°35.1784'N; 125°7.1902'W; 895  
321 m water depth; **Fig. S1**) on the eastern flank of Hydrate Ridge ~3 km northeast of the southern  
322 summit on ODP Leg 204 in 2002 (Tréhu et al., 2003) and stored at -80°C at the ODP Gulf Coast  
323 Repository.

324  
325 **Geochemistry.** Data for dissolved methane, sulfate, manganese, iron, and iodide in sediment  
326 porewaters were obtained from (Tréhu et al., 2003). Reactive iron and manganese were extracted  
327 from frozen sediments using the citrate-dithionite method (Roy et al., 2013) and measured by  
328 inductively coupled plasma optical emission spectrometer (Agilent Technologies 700 Series).  
329 Total carbon, total nitrogen and total sulfur were determined by CNS analyzer (Perkin Elmer  
330 2400). Total inorganic carbon was measured by CO<sub>2</sub> coulometer (CM5130) with a CM5130  
331 acidification module. Geochemical metadata are given in **Table S1** and archived in **BCO-DMO**  
332 **project 626690**.

333  
334 **DNA extraction.** DNA was extracted, in duplicate, from 8-20 g of sediment from the following  
335 depths in meters below seafloor (mbsf): 1.95-2.25 (C1-H2); 3.45-3.75 (C1-H3); 8.60 (F2-H4);  
336 18.10 (F3-H4); 20.69 (C3-H4); 35.65 (E5-H5); 68.55 (E10-H5); 138.89 (core E19-H5) using a  
337 MO-BIO PowerSoil total RNA Isolation Kit with the DNA Elution Accessory Kit, following the  
338 manufacturer protocol without beads. Approximately 2 grams of sediments were used per  
339 extraction, and DNA pellets from the two replicates from each depth were pooled together. DNA  
340 concentrations were measured using a Qubit 2.0 fluorometer with dsDNA High Sensitivity  
341 reagents (Invitrogen, Grand Island, NY, USA). DNA yields ranged from 4-15 ng per gram of  
342 sediments. Core E19-H5 (139 mbsf) yielded only 2 ng DNA per gram of sediment and yielded  
343 unreliable data due to contamination with sequences from the enzymes used in the library  
344 preparations. Therefore, this core segment was excluded from further analysis.

345  
346 **16S rRNA gene amplicon sequencing.** Microbial community composition was assessed by  
347 Illumina sequencing of the V3-V4 region of the 16S rRNA gene. The V3-V4 region was PCR-  
348 amplified using primers F515 and R806 (Caporaso et al., 2011), each appended with barcodes  
349 and Illumina-specific adapters according to (Kozich et al., 2013). Reactions consisted of 1-2 µL  
350 DNA template (2 ng), 5 µL of 10x Taq Mutant reaction buffer, 0.4 µL of KlenTaq LA Taq  
351 Polymerase (DNA Polymerase Technology, St. Louis, MO, USA), 2 µL of 10 mM dNTP mix  
352 (Sigma Aldrich, St. Louis, MO, USA), 2 µL of reverse and forward primers (total concentration  
353 0.4 µM), and the remainder DNA-free water to 50 µL (Ambion, Grand Island, NY, USA). PCR  
354 conditions were an initial 5-min denaturation at 94°C, followed by 35 cycles of denaturation at  
355 94°C (40 sec), primer annealing at 55°C (40 sec), and primer extension at 68°C (30 sec).  
356 Amplicon libraries were purified using a QIAquick PCR Purification Kit (Qiagen, Germantown,  
357 MD, USA), quantified by Qubit (Life Technologies), and pooled in equimolar concentration.  
358 Amplicons were sequenced on an Illumina MiSeq across two different runs using the V2 500-  
359 cycle kit with 5% PhiX to increase read diversity. 16S rRNA sequences were deposited into  
360 NCBI SAMN04214977-04214990 (**PRJNA295201**).

361  
362 **16S rRNA gene amplicon analysis.** Sequences were trimmed using Trim Galore (criteria: length  
363 >100 bp length, Phred score >25), and paired reads were merged using FLASH (Magoč &  
364 Salzberg, 2011) with the criteria of a minimum length of 250 bp per input read, minimum length  
365 of 300 bp for merged fragments, and maximum fragment standard deviation of 30 bp. Merged  
366 reads were imported into QIIME1 (Caporaso et al., 2010) and chimeric sequences were detected  
367 by searches using 'identity\_chimeric\_seqs' and then removed. Sequences sharing 97% nucleotide  
368 similarity were clustered into operational taxonomic units (OTUs) using  
369 'pick\_open\_reference\_otus' with taxonomy assigned to OTUs by comparison to the greengenes



370 database (DeSantis et al., 2006). The datasets were rarefied to a uniform depth of 14,391  
371 sequences, and the rarefied OTU table was used for all downstream analyses. A core set of  
372 QIIME diversity analyses was performed using ‘core\_diversity\_analyses’. The phylogenetic  
373 diversity (PD) metric (Faith, 1992) was used to quantify alpha diversity across samples.

374

375 ***Atribacteria* OTU phylogenetic analysis.** We generated a reference alignment of *Atribacteria*  
376 full length 16S rRNA sequences to use as a scaffold for mapping OTU sequences generated in  
377 this study. The reference alignment included *Atribacteria* 16S rRNA gene sequences from  
378 environmental clones (from Nobu et al. (2016), Carr et al. (2015) and Yarza et al. (2014)) and  
379 published SAGs and MAGs available in Prokka at the time of analysis (spring 2018), as well as 8  
380 sequences from *Firmicutes* bacteria for use as an outgroup. The sequences were aligned in  
381 MAFFT with the linsi option, alignment reordering, and reverse complement matching enabled.  
382 We then extracted representative sequences from 230 OTU clusters identified as *Atribacteria* OP-  
383 9 and JS-1 in the E10-H5 amplicon dataset; OTUs represented by only a single sequence were  
384 excluded. These sequences were recruited to the reference alignment via MAFFT using  
385 previously described parameters, without modifying base pair positions in the reference  
386 alignment. The alignment was manually inspected and trimmed to include only the V3-V4 region  
387 spanned by the *Atribacteria* OTU sequences, resulting in a final alignment with 275 bases.

388 This alignment was used for phylogeny reconstruction in RAxML with a GTR model of  
389 base substitution and GAMMA model of rate heterogeneity, and 500 rapid bootstraps followed by  
390 a thorough ML search. The resulting phylogenetic tree was edited for viewing in iTOL. The  
391 relative abundance of each OTU (from which a representative sequence was extracted) was  
392 mapped onto the resulting phylogeny and shown as a proportion of total sequences in the  
393 amplicon dataset.

394 Pairwise distances between all *Atribacteria* sequences in the alignment were calculated  
395 using the p-distance method in MEGA7 and summarized in R as: min 0.0, 1<sup>st</sup> quartile 0.5, median  
396 0.09, mean 0.11, 3<sup>rd</sup> quartile 0.18 and max 0.27. Pairwise distances between only the OTUs  
397 generated in this study were summarized in R as: min 0.004, 1<sup>st</sup> quartile 0.056, median 0.075,  
398 mean 0.076, 3<sup>rd</sup> quartile 0.095 and max 0.194.

399

400 ***Atribacteria* community structure.** OTU abundance from the rarefied *Atribacteria* OTU table  
401 (previously generated during diversity analysis) was used for NMDS analysis after square root  
402 transformation and calculation of Bray-Curtis dissimilarity metrics, all processed via the  
403 metaMDS function from Vegan package in R. After examination of the Shepard plot for scatter  
404 around the regression line, the NMDS plot was created showing individual OTUs and the  
405 midpoint for whole communities. A hierarchical clustering dendrogram was generated using  
406 Bray-Curtis dissimilarities.

407

408 **Multiple displacement amplification, library preparation, and sequencing.** Genomic DNA  
409 was amplified using a REPLI-g Single Cell Kit (Qiagen, Germantown, MD, USA) using UV-  
410 treated sterile plasticware and reverse transcription-PCR grade water (Ambion, Grand Island, NY,  
411 USA). Quantitative PCR showed that the negative control began amplifying after 5 hr of  
412 incubation at 30°C, and therefore, the 30°C incubation step was shortened to 5 hr using a Bio-Rad  
413 C1000 Touch thermal cycler (Bio-Rad, Hercules, CA, USA). DNA concentrations were measured  
414 by Qubit. Two micrograms of MDA-amplified DNA were used to generate genome libraries  
415 using a TruSeq DNA PCR-Free Kit following the manufacturer’s protocol (Illumina, San Diego,  
416 CA, USA). The resulting libraries were sequenced using a Rapid-Run on an Illumina HiSeq 2500  
417 to obtain 100 bp paired-end reads. Sequencing statistics are provided in **Table S3**. Metagenomic  
418 sequences were deposited into NCBI SAMN07256342-07256348 (**PRJNA390944**).

419

420 **Metagenome assembly, binning, and annotation.** Demultiplexed Illumina reads were mapped  
421 to known adapters using Bowtie2 in local mode to remove any reads with adapter contamination.

422 Demultiplexed Illumina read pairs were quality trimmed with Trim Galore (Babraham  
423 Bioinformatics) using a base Phred33 score threshold of Q25 and a minimum length cutoff of 80  
424 bp. Paired-end reads were then assembled into contigs using SPAdes assembler with --meta  
425 option for assembling metagenomes, iterating over a range of k-mer values  
426 (21,27,33,37,43,47,51,55,61,65,71,75,81,85,91,95). Assemblies were assessed with reports  
427 generated with QUAST. Features on contigs were predicted through the Prokka pipeline with  
428 RNAmmer for rRNA, Aragorn for tRNA, Infernal and Rfam for other non-coding RNA and  
429 Prodigal for protein coding genes. Metagenomic 16S rRNA sequences were analyzed by  
430 BLASTN analysis against the Greengenes reference database. Matches with a bit score above 50  
431 and reads matching multiple reference genes with the highest bit score were retained for  
432 comparison with 16S rRNA amplicons (**Fig. S3**). Annotation of protein-coding genes was  
433 performed as follows: 1) BLASTP search against the default set of core genomes, followed by  
434 HMM search against a set of default core HMM profiles available in Prokka, 2) use of the  
435 BLAST Descriptor Annotator algorithm in BLAST2GO, which conducts BLAST against the  
436 NCBI nr database, 3) KEGG orthology assignment using GhostKoala and 4) InterProScan  
437 analysis, which involves cross-reference HMM searches across multiple databases to find Pfam  
438 families with close homology.

439 Metagenome contigs were partitioned through MetaBAT (Kang et al., 2015) into  
440 metagenome-assembled genomes (MAGs) using tetranucleotide frequency and sequencing depth.  
441 Sequencing depth was estimated by mapping reads on to assembled contigs using Bowtie2 and  
442 Samtools. Completeness, contamination and strain level heterogeneity were assessed using single  
443 copy marker genes in CheckM (Parks et al., 2015). Gene features and their functional annotations  
444 for genome bins were extracted from the metagenome for the contigs that belong to the bins.  
445 Initial taxonomic affiliation for bins was inferred via the least common ancestor (LCA) algorithm  
446 in MEGAN6 and by the top BLAST matches to the marker gene *rpoB*. The B2 MAG was  
447 deposited into Genbank as “Candidatus *Atribacteria* bacterium 1244-E10-H5-B2”  
448 (SAMN07342547; **NMQN0000000.1**).

449  
450 **Phylogeny reconstruction for MAGS.** Coding sequences from whole genomes were  
451 downloaded from the NCBI representative genomes collection using NCBI e-utilities, comprising  
452 405 genomes in total, spanning all bacterial lineages. Only one candidate per genus with more  
453 than 1000 genes and maximum isolate information available was selected for this purpose.  
454 Sequence duplication (100% identity, unlikely to be biological duplication) within genomes was  
455 removed using CD-HIT. Available reference *Atribacteria* genomes, 24 in total, as either single-  
456 cell amplified genomes (SAGs) or MAGs, were downloaded and annotated using the Prokka  
457 pipeline. A list of 139 core single copy genes (CSCG) as HMM profiles was obtained from Rinke  
458 et al. (2013). B2 and representative reference *Atribacteria* genomes were then scanned for the  
459 presence of these HMM profiles using HMMer with the recommended score threshold for each  
460 profile as provided in Rinke et al. (2013). In a series of manual subsampling steps, 69 CSCG  
461 clusters were selected in 220 representative genomes and 20 *Atribacteria* genomes where 1) 69  
462 clusters were present in only a single copy, 2) all 69 clusters were present in 220 representative  
463 genomes and 3) the minimum number of clusters present in any *Atribacteria* genome was 6. All  
464 69 CSCG clusters were aligned individually using the L-INS-i mode in MAFFT. Alignments  
465 were then concatenated using a custom script *Aln.cat.rb* from the Enveomics collection (link)  
466 with invariable sites removed. Phylogeny reconstruction was performed in RAXML using a  
467 GAMMA model of rate heterogeneity, iterating over all models of protein substitution to choose  
468 the one with best log likelihood. The analysis was performed with 1000 rapid bootstraps with the  
469 MRE convergence bootstrap criterion (50 bootstrap replicates performed), followed by a  
470 thorough ML search. The resulting phylogenetic tree was modified for optimal viewing in iTOL  
471 with a full view including all lineages and a pruned view confirming placement of MAG B2 in  
472 the *Atribacteria* phylogeny. *Atribacteria* taxonomic classifications were based on Yarza et al.  
473 (2014). To examine gene orthology between B2 and other reference *Atribacteria*, 23 reference

474 *Atribacteria* (MAGs and SAGs) genomes were annotated using Prokka. The predicted genes were  
475 analyzed by BLAST best hit (BBH) clustering for orthologous group identification through  
476 Proteinortho5. In B2, 55% of genes (2333/4254) lacked orthologs in other *Atribacteria* genomes.

477

478 **Metaproteomic sample preparation, mass spectrometry, and data analyses.** Proteins from  
479 E10-H5 were extracted from a 10 g of frozen sediment using a protocol adapted from Nicora et  
480 al. (2013). Briefly, 2.5 mL of desorption buffer (0.5 M NaCl, 0.1 M glycerol, 0.2% SDS, 6 M  
481 urea, 1 mM EDTA, 100 mM ammonium bicarbonate) and 2 mL of a pH-buffered amino acid  
482 solution (containing equimolar histidine, lysine, and arginine, all 83 g l<sup>-1</sup> in ultra-pure water,  
483 pH 7.0) was added to the sample on ice. The goal of the pH-buffered amino acid solution is to fill  
484 the electronegative mineral sites in the sample with positively charged amino acids to reduce  
485 absorption of proteins to the particles. Samples were vortexed 4x, alternating 5 minutes vortexing  
486 and 5 min ice. The sediment slurry was then sonicated with Bronson probe sonicator (4 x 30 s) to  
487 lyse cells and heated at 95°C for 5 min. The sediment was pelleted by centrifugation (10,000 x g,  
488 30 min, 4°C), and the supernatant was collected and stored on ice. The sediment pellet was  
489 washed 2 more times with 3 mL desorption buffer and supernatants were combined. In order to  
490 remove the SDS prior to protein digestion and mass spectrometry analysis, the filter aided sample  
491 preparation (FASP) method was used (Ostasiewicz et al., 2010). Millipore Amicon 10 kDa filter  
492 units were used and cleaned following manufacturer's directions. Samples were loaded on top of  
493 filters (~9 mL) and centrifuged (3000 rpm, 90 min, 4°C). In order to remove all SDS, proteins  
494 retained on the filter were rinsed 3 times by adding 5 mL of 8 M urea in 50 mM ammonium  
495 bicarbonate and repeating the prior centrifugation step. Iodoacetamide (3 mL, 15 mM) was added  
496 to samples, incubated in the dark at room temperature for 30 minutes, and then centrifuged (3000  
497 rpm, 90 min, 4°C). Proteins were then rinsed two times with 10 mL of 100 mM ammonium  
498 bicarbonate and centrifuged to remove liquid (3000 rpm, 90 min, 4°C). To digest protein on the  
499 filter, 0.5 µg of trypsin (modified, sequencing grade, Promega) was added to the filter, topped  
500 with 2.5 mL of 25 mM ammonium bicarbonate, vortexed, and incubated 12 hr at room  
501 temperature. Filtrate was collected by centrifugation (3000 rpm, 90 min, 4°C), and SpeedVaced to  
502 near dryness at 4°C. Peptides were then resuspended in 50 µL of 2% acetonitrile and 0.1% formic  
503 acid and desalted using Nest Group C18 Proto centrifugal macro columns following  
504 manufacturer's instructions. Each 10 µL sample was separated on a NanoAquity UPLC with a 60  
505 min gradient (2-35% acetonitrile) and analyzed on a Thermo Scientific Orbitrap Fusion Tribrid  
506 Mass Spectrometer operated in top20 data dependent acquisition mode.

507 A protein database for identifying the collected fragmentation spectra was generated from  
508 *Atribacteria* MAGs (C1H2\_C3H4ab\_E10H5\_contam.fasta). These databases were concatenated  
509 with 50 common contaminants, yielding a protein database of 10,325 proteins. To assign spectra  
510 to peptide sequences, correlative database searches were completed using Comet v. 2015.01 rev.  
511 2 (Eng et al., 2013; Eng et al., 2015). Comet parameters included: trypsin enzyme specificity,  
512 semi-digested, allowance of 1 missed cleavage, 10 ppm mass tolerance, cysteine modification of  
513 57 Da (resulting from the iodoacetamide) and modifications on methionine of 15.999 Da  
514 (oxidation). Minimum protein and peptide thresholds were set at  $P > 0.95$  on Protein and Peptide  
515 Prophet (Nesvizhskii et al., 2003). Protein inferences from the whole-cell lysates were accepted  
516 by ProteinProphet if the thresholds noted above were passed, two or more peptides were  
517 identified, and at least one terminus was tryptic (Keller et al., 2002; Nesvizhskii et al., 2003;  
518 Pedrioli, 2010). For each peptide discussed in the manuscript, manual inspection of the spectral  
519 identification was completed. The mass spectrometry proteomics data have been deposited to the  
520 ProteomeXchange Consortium via the PRIDE partner repository (Vizcaíno et al., 2015) with the  
521 dataset identifier **PXD01247** (<https://www.ebi.ac.uk/pride/archive/> Login:  
522 reviewer08969@ebi.ac.uk Password: BP2V3yGA).

523

524

525 **References**

- 526 Anantharaman, K., Brown, C.T., Hug, L.A., Sharon, I., Castelle, C.J., Probst, A.J. et al.  
527 (2016) Thousands of microbial genomes shed light on interconnected biogeochemical  
528 processes in an aquifer system. *Nat Commun* **7**: 13219.
- 529 Archer, D., Buffett, B., and Brovkin, V. (2009) Ocean methane hydrates as a slow tipping  
530 point in the global carbon cycle. *Proc Natl Acad Sci* **106**: 20596-20601.
- 531 Axen, S.D., Erbilgin, O., and Kerfeld, C.A. (2014) A taxonomy of bacterial  
532 microcompartment loci constructed by a novel scoring method. *PLoS Comput Biol* **10**:  
533 e1003898.
- 534 Baker, B.J., Lazar, C.S., Teske, A.P., and Dick, G.J. (2015) Genomic resolution of linkages  
535 in carbon, nitrogen, and sulfur cycling among widespread estuary sediment bacteria.  
536 *Microbiome* **3**: 14.
- 537 Barrick, J.E., Corbino, K.A., Winkler, W.C., Nahvi, A., Mandal, M., Collins, J. et al. (2004)  
538 New RNA motifs suggest an expanded scope for riboswitches in bacterial genetic control.  
539 *Proc Natl Acad Sci* **101**: 6421-6426.
- 540 Bergkessel, M., Basta, D.W., and Newman, D.K. (2016) The physiology of growth arrest:  
541 uniting molecular and environmental microbiology. *Nature Reviews Microbiology* **14**: 549.
- 542 Bohrmann, G., and Torres, M.E. (2006) Gas Hydrates in Marine Sediments. In *Marine*  
543 *Geochemistry*. Schulz, H.D., and Zabel, M. (eds). Berlin, Heidelberg: Springer, pp. 481-  
544 512.
- 545 Braun, M., and Silhavy, T.J. (2002) Imp/OstA is required for cell envelope biogenesis in  
546 *Escherichia coli*. *Mol Microbiol* **45**: 1289-1302.
- 547 Byrne, M.D., and Nicholas, D. (1986) Multiple-phase equilibration headspace analysis for  
548 the determination of N<sub>2</sub>O and N<sub>2</sub> during bacterial denitrification. *Anal Biochem* **154**: 470-  
549 475.
- 550 Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh,  
551 P.J. et al. (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences  
552 per sample. *Proc Natl Acad Sci* **108**: 4516-4522.
- 553 Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K.  
554 et al. (2010) QIIME allows analysis of high-throughput community sequencing data.  
555 *Nature Methods* **7**: 335-336.
- 556 Carr, S.A., Orcutt, B.N., Mandernack, K.W., and Spear, J.R. (2015) Abundant Atribacteria  
557 in deep marine sediment from the Adelie Basin, Antarctica. *Front Microbiol* **6**: 872.
- 558 Chernitsyna, S., Mamaeva, E., Lomakina, A., Pogodaeva, T., Galach'yants, Y.P., Bukin,  
559 S. et al. (2016) Phylogenetic diversity of microbial communities of the Posolsk Bank  
560 bottom sediments, Lake Baikal. *Microbiology* **85**: 672-680.

- 561 Chong, Z.R., Yang, S.H.B., Babu, P., Linga, P., and Li, X.-S. (2016) Review of natural gas  
562 hydrates as an energy resource: Prospects and challenges. *Appl Energ* **162**: 1633-1652.
- 563 Daly, R.A., Borton, M.A., Wilkins, M.J., Hoyt, D.W., Kountz, D.J., Wolfe, R.A. et al.  
564 (2016) Microbial metabolisms in a 2.5-km-deep ecosystem created by hydraulic fracturing  
565 in shales. *Nat Microbiol* **1**: 16146.
- 566 Dodsworth, J.A., Blainey, P.C., Murugapiran, S.K., Swingley, W.D., Ross, C.A., Tringe,  
567 S.G. et al. (2013) Single-cell and metagenomic analyses indicate a fermentative and  
568 saccharolytic lifestyle for members of the OP9 lineage. *Nat Commun* **4**: 1854.
- 569 Dombrowski, N., Seitz, K.W., Teske, A.P., and Baker, B.J. (2017) Genomic insights into  
570 potential interdependencies in microbial hydrocarbon and nutrient cycling in hydrothermal  
571 sediments. *Microbiome* **5**: 106.
- 572 Eng, J.K., Jahan, T.A., and Hoopmann, M.R. (2013) Comet: an open - source MS/MS  
573 sequence database search tool. *Proteomics* **13**: 22-24.
- 574 Eng, J.K., Hoopmann, M.R., Jahan, T.A., Egertson, J.D., Noble, W.S., and MacCoss, M.J.  
575 (2015) A deeper look into Comet—implementation and features. *J Am Soc Mass Spec* **26**:  
576 1865-1874.
- 577 Engelberg-Kulka, H., Hazan, R., and Amitai, S. (2005) mazEF: a chromosomal toxin-  
578 antitoxin module that triggers programmed cell death in bacteria. *J Cell Sci* **118**: 4327-  
579 4332.
- 580 Faith, D.P. (1992) Conservation evaluation and phylogenetic diversity. *Biol Conserv* **61**:  
581 1-10.
- 582 Fischer, M., Zhang, Q.Y., Hubbard, R.E., and Thomas, G.H. (2010) Caught in a TRAP:  
583 substrate-binding proteins in secondary transport. *Trends Microbiol* **18**: 471-478.
- 584 Fischer, M., Hopkins, A.P., Severi, E., Hawkhead, J., Bawdon, D., Watts, A.G. et al. (2015)  
585 Tripartite ATP-independent periplasmic (TRAP) transporters use an arginine-mediated  
586 selectivity filter for high affinity substrate binding. *J Biol Chem* **290**: 27113-27123.
- 587 Friedrich, T., and Scheide, D. (2000) The respiratory complex I of bacteria, archaea and  
588 eukarya and its module common with membrane - bound multisubunit hydrogenases.  
589 *FEBS Lett* **479**: 1-5.
- 590 Fry, J.C., Parkes, R.J., Cragg, B.A., Weightman, A.J., and Webster, G. (2008) Prokaryotic  
591 biodiversity and activity in the deep seafloor biosphere. *FEMS Microbiol Ecol* **66**: 181-  
592 196.
- 593 Gies, E.A., Konwar, K.M., Beatty, J.T., and Hallam, S.J. (2014) Illuminating microbial  
594 dark matter in meromictic Sakinaw Lake. *Appl Environ Microbiol* **80**: 6807-6818.

- 595 Hernsdorf, A.W., Amano, Y., Miyakawa, K., Ise, K., Suzuki, Y., Anantharaman, K. et al.  
596 (2017) Potential for microbial H<sub>2</sub> and metal transformations associated with novel bacteria  
597 and archaea in deep terrestrial subsurface sediments. *ISME J* **11**: 1915-1929.
- 598 Hester, K.C., and Brewer, P.G. (2009) Clathrate hydrates in nature. *Ann Rev Mar Sci* **1**:  
599 303-327.
- 600 Honkalas, V., Dabir, A., and Dhakephalkar, P.K. (2016) Life in the Anoxic Sub-Seafloor  
601 Environment: Linking Microbial Metabolism and Mega Reserves of Methane Hydrate. In  
602 *Anaerobes in Biotechnology*. Hatti-Kaul, R., Mamo, G., and Mattiasson, B. (eds). Cham:  
603 Springer, pp. 235-262.
- 604 Hoshino, T., Toki, T., Ijiri, A., Morono, Y., Machiyama, H., Ashi, J. et al. (2017)  
605 Atribacteria from the subseafloor sedimentary biosphere disperse to the hydrosphere  
606 through submarine mud volcanoes. *Front Microbiol* **8**: 1135.
- 607 Hu, P., Tom, L., Singh, A., Thomas, B.C., Baker, B.J., Piceno, Y.M. et al. (2016) Genome-  
608 resolved metagenomic analysis reveals roles for candidate phyla and other microbial  
609 community members in biogeochemical transformations in oil reservoirs. *mBio* **7**: e01669-  
610 01615.
- 611 Inagaki, F., Suzuki, M., Takai, K., Oida, H., Sakamoto, T., Aoki, K. et al. (2003) Microbial  
612 communities associated with geological horizons in coastal subseafloor sediments from the  
613 Sea of Okhotsk. *Appl Environ Microbiol* **69**: 7224-7235.
- 614 Inagaki, F., Nunoura, T., Nakagawa, T., Teske, A., Lever, M.A., Lauer, A. et al. (2006)  
615 Biogeographical distribution and diversity of microbes in methane hydrate-bearing deep  
616 marine sediments on the Pacific Ocean Margin. *Proc Natl Acad Sci* **103**: 2815–2820.
- 617 Kadnikov, V.V., Mardanov, A.V., Beletsky, A.V., Shubenkova, O.V., Pogodaeva, T.V.,  
618 Zemskaya, T.I. et al. (2012) Microbial community structure in methane hydrate-bearing  
619 sediments of freshwater Lake Baikal. *FEMS Microbiol Ecol* **79**: 348-358.
- 620 Kang, D.D., Froula, J., Egan, R., and Wang, Z. (2015) MetaBAT, an efficient tool for  
621 accurately reconstructing single genomes from complex microbial communities. *PeerJ* **3**:  
622 e1165.
- 623 Keller, A., Purvine, S., Nesvizhskii, A.I., Stolyar, S., Goodlett, D.R., and Kolker, E. (2002)  
624 Experimental protein mixture for validating tandem mass spectral analysis. *OMICS* **6**: 207-  
625 212.
- 626 Kho, K., and Meredith, T.C. (2018) Salt-induced stress stimulates a lipoteichoic acid-  
627 specific three component glycosylation system in *Staphylococcus aureus*. *J Bacteriol* **200**:  
628 e00017-00018.
- 629 Kormas, K.A., Smith, D.C., Edgcomb, V., and Teske, A. (2003) Molecular analysis of deep  
630 subsurface microbial communities in Nankai Trough sediments (ODP Leg 190, Site 1176).  
631 *FEMS Microbiol Ecol* **45**: 115-125.

- 632 Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., and Schloss, P.D. (2013)  
633 Development of a dual-index sequencing strategy and curation pipeline for analyzing  
634 amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl Environ*  
635 *Microbiol* **79**: 5112-5120.
- 636 Kumar, A., Bhattacharjee, G., Kulkarni, B., and Kumar, R. (2015) Role of surfactants in  
637 promoting gas hydrate formation. *Ind Eng Chem Res* **54**: 12217-12232.
- 638 Lanoil, B.D., Sassen, R., La Duc, M.T., Sweet, S.T., and Nealson, K.H. (2001) Bacteria  
639 and Archaea physically associated with Gulf of Mexico gas hydrates. *Appl Environ*  
640 *Microbiol* **67**: 5143-5153.
- 641 Lecher, J., Pittelkow, M., Zobel, S., Bursy, J., Bönig, T., Smits, S.H. et al. (2009) The  
642 crystal structure of UehA in complex with ectoine—a comparison with other TRAP-T  
643 binding proteins. *J Mol Biol* **389**: 58-73.
- 644 Lin, L.-H., Wu, L.-W., Cheng, T.-W., Tu, W.-X., Lin, J.-R., Yang, T.F. et al. (2014)  
645 Distributions and assemblages of microbial communities along a sediment core retrieved  
646 from a potential hydrate-bearing region offshore southwestern Taiwan. *Journal of Asian*  
647 *Earth Sciences* **92**: 276-292.
- 648 Liu, Y., Chen, B., Chen, Y., Zhang, S., Guo, W., Cai, Y. et al. (2015) Methane storage in  
649 a hydrated form as promoted by leucines for possible application to natural gas  
650 transportation and storage. *Energy Technol* **3**: 815-819.
- 651 Liu, Y., Gao, W., Wang, Y., Wu, L., Liu, X., Yan, T. et al. (2005) Transcriptome analysis  
652 of *Shewanella oneidensis* MR-1 in response to elevated salt conditions. *J Bacteriol* **187**:  
653 2501-2507.
- 654 Marlow, J.J., Steele, J.A., Case, D.H., Connon, S.A., Levin, L.A., and Orphan, V.J. (2014)  
655 Microbial abundance and diversity patterns associated with sediments and carbonates from  
656 the methane seep environments of Hydrate Ridge, OR. *Front Mar Sci* **1**: 44.
- 657 Mathiesen, C., and Hägerhäll, C. (2002) Transmembrane topology of the NuoL, M and N  
658 subunits of NADH: quinone oxidoreductase and their homologues among membrane-  
659 bound hydrogenases and bona fide antiporters. *BBA-Bioenergetics* **1556**: 121-132.
- 660 Mavromatis, K., Ivanova, N., Anderson, I., Lykidis, A., Hooper, S.D., Sun, H. et al. (2009)  
661 Genome analysis of the anaerobic thermohalophilic bacterium *Halothermothrix orenii*.  
662 *PLoS One* **4**: e4192.
- 663 McLaughlin, K.J., Strain-Damerell, C.M., Xie, K., Brekasis, D., Soares, A.S., Paget, M.S.,  
664 and Kielkopf, C.L. (2010) Structural basis for NADH/NAD<sup>+</sup> redox sensing by a Rex  
665 family repressor. *Mol Cell* **38**: 563-575.
- 666 Milkov, A.V., Dickens, G.R., Claypool, G.E., Lee, Y.-J., Borowski, W.S., Torres, M.E. et  
667 al. (2004) Co-existence of gas hydrate, free gas, and brine within the regional gas hydrate  
668 stability zone at Hydrate Ridge (Oregon margin): evidence from prolonged degassing of a  
669 pressurized core. *Earth Planet Sci Lett* **222**: 829-843.

- 670 Moparthi, V.K., and Hägerhäll, C. (2011) The evolution of respiratory chain complex I  
671 from a smaller last common ancestor consisting of 11 protein subunits. *J Mol Evol* **72**: 484-  
672 497.
- 673 Moparthi, V.K., Kumar, B., Al-Eryani, Y., Sperling, E., Górecki, K., Drakenberg, T., and  
674 Hägerhäll, C. (2014) Functional role of the MrpA-and MrpD-homologous protein subunits  
675 in enzyme complexes evolutionary related to respiratory chain complex I. *BBA-  
676 Bioenergetics* **1837**: 178-185.
- 677 Nesvizhskii, A.I., Keller, A., Kolker, E., and Aebersold, R. (2003) A statistical model for  
678 identifying proteins by tandem mass spectrometry. *Anal Chem* **75**: 4646-4658.
- 679 Newberry, C.J., Webster, G., Cragg, B.A., Parkes, R.J., Weightman, A.J., and Fry, J.C.  
680 (2004) Diversity of prokaryotes and methanogenesis in deep subsurface sediments from  
681 the Nankai Trough, Ocean Drilling Program Leg 190. *Environ Microbiol* **6**: 274-287.
- 682 Nicora, C.D., Anderson, B.J., Callister, S.J., Norbeck, A.D., Purvine, S.O., Jansson, J.K.  
683 et al. (2013) Amino acid treatment enhances protein recovery from sediment and soils for  
684 metaproteomic studies. *Proteomics* **13**: 2776-2785.
- 685 Nobu, M.K., Narihiro, T., Rinke, C., Kamagata, Y., Tringe, S.G., Woyke, T., and Liu, W.-  
686 T. (2015) Microbial dark matter ecogenomics reveals complex synergistic networks in a  
687 methanogenic bioreactor. *ISME J* **9**: 1710-1722.
- 688 Nobu, M.K., Dodsworth, J.A., Murugapiran, S.K., Rinke, C., Gies, E.A., Webster, G. et al.  
689 (2016) Phylogeny and physiology of candidate phylum ‘Atribacteria’ (OP9/JS1) inferred  
690 from cultivation-independent genomics. *ISME J* **10**: 273-286.
- 691 Ostasiewicz, P., Zielinska, D.F., Mann, M., and Wiśniewski, J.R. (2010) Proteome,  
692 phosphoproteome, and N-glycoproteome are quantitatively preserved in formalin-fixed  
693 paraffin-embedded tissue and analyzable by high-resolution mass spectrometry. *J Prot Res*  
694 **9**: 3688-3700.
- 695 Parkes, R.J., Cragg, B., Roussel, E., Webster, G., Weightman, A., and Sass, H. (2014) A  
696 review of prokaryotic populations and processes in sub-seafloor sediments, including  
697 biosphere: geosphere interactions. *Mar Geol* **352**: 409-425.
- 698 Parks, D.H., Imelfort, M., Skennerton, C.T., Hugenholtz, P., and Tyson, G.W. (2015)  
699 CheckM: assessing the quality of microbial genomes recovered from isolates, single cells,  
700 and metagenomes. *Genome Res* **25**: 1043-1055.
- 701 Pedrioli, P.G. (2010) Trans-Proteomic Pipeline: A Pipeline for Proteomic Analysis. In  
702 *Proteome Bioinformatics*. Hubbard, S., and Jones, A. (eds): Humana Press, pp. 213-238.
- 703 Probst, A.J., Castelle, C.J., Singh, A., Brown, C.T., Anantharaman, K., Sharon, I. et al.  
704 (2017) Genomic resolution of a cold subsurface aquifer community provides metabolic  
705 insights for novel microbes adapted to high CO<sub>2</sub> concentrations. *Environ Microbiol* **19**:  
706 459-474.



- 707 Reed, D.W., Fujita, Y., Delwiche, M.E., Blackwelder, D.B., Sheridan, P.P., Uchida, T.,  
708 and Colwell, F.S. (2002) Microbial communities from methane hydrate-bearing deep  
709 marine sediments in a forearc basin. *Appl Environ Microbiol* **68**: 3759-3770.
- 710 Rinke, C., Schwientek, P., Sczyrba, A., Ivanova, N.N., Anderson, I.J., Cheng, J.-F. et al.  
711 (2013) Insights into the phylogeny and coding potential of microbial dark matter. *Nature*  
712 **499**: 431-437.
- 713 Roy, M., McManus, J., Goni, M.A., Chase, Z., Borgeld, J.C., Wheatcroft, R.A. et al. (2013)  
714 Reactive iron and manganese distributions in seabed sediments near small mountainous  
715 rivers off Oregon and California (USA). *Cont Shelf Res* **54**: 67-79.
- 716 Ruff, S., Felden, J., Gruber-Vodicka, H., Marcon, Y., Knittel, K., Ramette, A., and Boetius,  
717 A. (2019) In situ development of a methanotrophic microbiome in deep-sea sediments. *The*  
718 *ISME journal* **13**: 197.
- 719 Ruppel, C.D., and Kessler, J.D. (2017) The interaction of climate change and methane  
720 hydrates. *Rev Geophys* **55**: 126-168.
- 721 Sa, J.-H., Kwak, G.-H., Lee, B.R., Park, D.-H., Han, K., and Lee, K.-H. (2013)  
722 Hydrophobic amino acids as a new class of kinetic inhibitors for gas hydrate formation.  
723 *Sci Rep* **3**: 2428.
- 724 Santos, H., and Da Costa, M.S. (2002) Compatible solutes of organisms that live in hot  
725 saline environments. *Environ Microbiol* **4**: 501-509.
- 726 Schut, G.J., Boyd, E.S., Peters, J.W., and Adams, M.W. (2013) The modular respiratory  
727 complexes involved in hydrogen and sulfur metabolism by heterotrophic  
728 hyperthermophilic archaea and their evolutionary implications. *FEMS Microbiol Rev* **37**:  
729 182-203.
- 730 ShipboardScientificParty (2003) Site 1244. In *Proc ODP, Init Repts, 204*. Tréhu, A.,  
731 Bohrmann, G., Rack, F., Torres, M., and al., e. (eds). College Station, TX: Ocean Drilling  
732 Program, pp. 1–132 doi:110.2973/odp.proc.ir.2204.2103.2003.
- 733 Spero, M.A., Aylward, F.O., Currie, C.R., and Donohue, T.J. (2015) Phylogenomic  
734 analysis and predicted physiological role of the proton-translocating NADH: quinone  
735 oxidoreductase (complex I) across bacteria. *mBio* **6**: e00389-00315.
- 736 Tréhu, A., Bohrmann, G., Rack, F., and Torres, M. (2003) Volume 204 Initial Reports. In  
737 *Proc ODP, Initial Reports*, pp. 77845-79547.
- 738 Ussler III, W., and Paull, C.K. (2001) Ion exclusion associated with marine gas hydrate  
739 deposits. In *Natural Gas Hydrates: Occurrence, Distribution, and Detection*. Paull, C.K.,  
740 and Dillion, W.P. (eds): American Geophysical Union.
- 741 Veluswamy, H.P., Lee, P.Y., Premasinghe, K., and Linga, P. (2017) Effect of biofriendly  
742 amino acids on the kinetics of methane hydrate formation and dissociation. *Ind Eng Chem*  
743 *Res* **56**: 6145-6154.

- 744 Vizcaino, J.A., Csordas, A., Del-Toro, N., Dianas, J.A., Griss, J., Lavidas, I. et al. (2015)  
745 2016 update of the PRIDE database and its related tools. *Nuc Acid Res* **44**: D447-D456.
- 746 Webster, G., Parkes, R.J., Fry, J.C., and Weightman, A.J. (2004) Widespread occurrence  
747 of a novel division of bacteria identified by 16S rRNA gene sequences originally found in  
748 deep marine sediments. *Appl Environ Microbiol* **70**: 5708-5713.
- 749 Webster, G., Yarram, L., Freese, E., Koster, J., Sass, H., Parkes, R.J., and Weightman, A.J.  
750 (2007) Distribution of candidate division JS1 and other Bacteria in tidal sediments of the  
751 German Wadden Sea using targeted 16S rRNA gene PCR-DGGE. *FEMS Microbiol Ecol*  
752 **62**: 78-89.
- 753 Wood, J.M. (2015) Bacterial responses to osmotic challenges. *J Gen Physiol* **145**: 381-388.
- 754 Yarza, P., Yilmaz, P., Pruesse, E., Glöckner, F.O., Ludwig, W., Schleifer, K.-H. et al.  
755 (2014) Uniting the classification of cultured and uncultured bacteria and archaea using 16S  
756 rRNA gene sequences. *Nat Rev Microbiol* **12**: 635-645.
- 757 Yu, H., Wu, C.-H., Schut, G.J., Haja, D.K., Zhao, G., Peters, J.W. et al. (2018) Structure  
758 of an ancient respiratory system. *Cell* **173**: 1636-1649.  
759