

Hyperthermophilic endospores germinate and metabolise organic carbon in sediments heated to 80°C

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10 **Running title:** Thermophilies produce acetate in heated sediment

11 **Originality-Significance Statement**

12 Roughly one quarter of marine subsurface sediments worldwide (by volume) are estimated to be at
13 temperatures $\geq 80^{\circ}\text{C}$. Whether microbial cells in deep, hot sediments can survive and obtain energy
14 amidst a depleting pool of organic matter is questioned. Endospore formation presents a mechanism
15 for populations adapted to warmer temperatures to persist during burial through cooler, shallower
16 intervals, and exit dormancy at warmer depths. Using sediment incubation experiments, we show that
17 endospores deposited in cool surface sediments germinate and transform organic carbon upon heating
18 to 80°C . Exploration of their genomes suggests endospores of thermophilic bacteria buried in
19 accumulating sediments could germinate and metabolise sedimentary organic carbon and microbial
20 necromass in deeply buried hot sediments.

21 **Abstract**

22 Cold surface sediments host a seedbank of functionally diverse thermophilic bacteria. These
23 thermophiles are present as endospores which are widely dispersed in aquatic environments. Here we
24 investigated the functional potential of endospore populations in cold surface sediments heated to 80°C .
25 Microbial production of acetate was observed at 80°C , and could be enhanced by supplying additional
26 organic carbon substrates. Comparison of 16S rRNA gene amplicon libraries from 80°C enrichments
27 to sediments heated to lower temperatures ($50\text{--}70^{\circ}\text{C}$) showed that temperature selects for distinct
28 populations of endospore-forming bacteria. Whereas sulfate-reducing thermophiles were enriched in
29 $50\text{--}70^{\circ}\text{C}$ incubations, 80°C exceeds their thermal tolerance and selects for hyperthermophilic
30 organotrophic bacteria that are similarly detected in amplicon libraries from sediments heated to 90°C .
31 Genome-resolved metagenomics revealed novel carbon cycling members of *Symbiobacteriales*,
32 *Thermosediminibacteraceae*, *Thermanaeromonas* and *Calditerricola* with the genomic potential for the
33 degradation of carbohydrates, sugars, amino acids and nucleotides. Endospores of thermophilic
34 bacteria are deposited on seabed sediments worldwide where they remain dormant as they are buried
35 in the accumulating sediments. Our results suggest that endospore populations could be activated by
36 temperature increases encountered during burial and show the potential for organotrophic metabolic
37 activity contributing to acetate generation in deep hot sediments.

38

39 Introduction

40 Over one-third of marine sediments globally are heated above 60°C, and one-quarter are above 80°C
41 (LaRowe *et al.*, 2017), yet little is known about microbial populations that reside in deep, hot sediments.
42 As sediment temperature increases with depth, microbial cell numbers are in decline, consistent with
43 biomass generally diminishing with depth owing to decreasing energy availability over time (Kallmeyer
44 *et al.*, 2012). Low biomass in deep, hot sediments makes direct sequencing of microbial DNA
45 challenging (Dombrowski *et al.*, 2018; Heuer *et al.*, 2020), thereby limiting understanding of extant deep
46 subsurface populations.

47 Experiments that employ heating of surface sediments provide an opportunity to explore temperature-
48 dependent effects on sediment chemistry associated with resident microbial populations. In
49 experiments designed to mimic heating-during-burial using both surface and seafloor sediments,
50 acetate production is observed (Wellsbury *et al.*, 1997, 2002; Parkes *et al.*, 2007; Roussel *et al.*, 2015).
51 This mirrors observations of deep, hot sediments *in situ* where acetate accumulation has also been
52 reported (Wellsbury *et al.*, 1997; Egeberg and Barth, 1998; Heuer *et al.*, 2020) that suggest acetate is
53 an important energy source in the deep hot biosphere.

54 Sediment heating studies have also shown that endospores of thermophilic bacteria are widespread in
55 cold surface environments where they cannot grow (Hubert *et al.*, 2009; Müller *et al.*, 2014). These
56 dormant endospore populations are deposited on the seafloor from the water column, following a period
57 of passive dispersal in ocean currents (de Rezende *et al.*, 2013; Nielsen *et al.*, 2017; Volpi *et al.*, 2017).
58 Once deposited, thermophilic endospores remain dormant as they are buried in the accumulating
59 sediments. Dormancy offers a survival strategy for populations adapted to warmer temperatures to
60 persist during burial through cooler, shallower intervals. As such, thermophilic endospores have been
61 recovered from seafloor sediments ranging from 4,500 years old (de Rezende *et al.*, 2013) to 20-
62 million years old (Fang *et al.*, 2017), showing that endospores in deeply buried sediments remain viable.

63 Increasing temperature during burial could provide an opportunity for dormant thermophilic endospores
64 to germinate. In agreement with this, previously reported sediment heating experiments have shown
65 that different temperatures apply selective pressure that results in germination and growth of different
66 dormant endospore populations (de Rezende *et al.*, 2013; Bell *et al.*, 2020). In those studies, incubating
67 sediment at temperatures between 45 and 70°C resulted in the enrichment of distinct populations of

68 sulfate-reducing bacteria, whereas sulfate reduction was not observed at $\geq 80^{\circ}\text{C}$. This apparent cut-off
69 has also been postulated for microorganisms in sedimentary environments, inferred from observations
70 that anaerobic hydrocarbon biodegradation has not occurred in oil reservoir formations buried to depths
71 hotter than 80°C (Wilhelms *et al.*, 2001; Head *et al.*, 2003). On the other hand, the upper temperature
72 limit for life in deeply buried marine sediments has recently been shown to extend up to at least 120°C ,
73 with isotope data (Heuer *et al.*, 2020) and radiotracer experiments (Beulig *et al.*, 2022) suggesting
74 deeply buried hot sediments host acetate-utilising hyperthermophiles. This means that populations in
75 the hyperthermophilic temperature range are not necessarily restricted to hydrothermal systems at mid-
76 oceanic ridges where reduced inorganic compounds support chemoautotrophs with the highest known
77 growth temperatures (Kashefi and Lovley, 2003; Takai *et al.*, 2008).

78 During experiments testing the thermal tolerance of endospore-forming sulfate-reducing bacteria, we
79 observed acetate production in sediments heated to 80°C . It was expected that sulfate reducing
80 bacteria would produce acetate from the incomplete oxidation of organic electron donors in cooler
81 incubations, but 80°C exceeded the thermal tolerance of sulfate reducing bacteria (Bell *et al.*, 2020).
82 This study therefore tests the hypothesis that sediments host populations of hyperthermophilic
83 endospores that can be stimulated to catalyse organic matter degradation upon heating to 80°C .

84 **Results**

85 **Acetate production in heated sediments**

86 Surface sediments were mixed with anoxic medium and the resulting slurries incubated at 80°C .
87 Sediments were either heated without additional carbon amendment or were supplemented with
88 organic carbon compounds. Heating the sediment resulted in acetate and propionate production in both
89 sets of incubations (Fig. 1 A & B), with the addition of supplemental organic carbon resulting in higher
90 acetate concentrations being indicative of microbial acetate generation in these incubations.

91 At high temperature, sediment is increasingly subject to abiotic, thermal transformations of organic
92 matter (Lin *et al.*, 2017; Otte *et al.*, 2018). To confirm that acetate production observed at 80°C was
93 derived from microbial degradation of organic carbon, sediments were also chemically sterilised with
94 zinc chloride prior to incubation. In these sterile controls, acetate production was significantly lower
95 (0.01 mM acetate production) than in unsterilised sediment that was unamended (2.5 mM acetate

96 production) or supplemented with organic substrates (6.9 mM acetate production). Propionate was
97 produced to a similar concentration in both sterilised (Fig. 1C) and unsterilised sediments (Fig. 1A & B),
98 suggesting propionate results from abiotic, thermal reactions at 80°C. Accumulation of formate (1.0
99 mM) and lactate (0.6 mM) was also observed. The sterilised control shows that while some acetate can
100 be produced by abiotic, thermal reactions, much higher levels observed in unsterilised sediment is
101 indicative of microbial activity being stimulated at high temperature.

102 **Temperature-dependent germination of endospore populations**

103 16S rRNA gene amplicon libraries were generated from sediments incubated at 50, 60, 70, 80 and
104 90°C and compared with each other and to amplicon libraries prepared from unheated sediment (Fig.
105 2). The read abundance of endospore forming *Firmicutes* increased following heating in all sediment
106 incubations (Fig. 2A), indicating that dormant endospore populations in the sediment germinated,
107 resulting in their genomic DNA being extractable from vegetative cells when using standard protocols.

108 Different populations of endospore-forming bacteria were detected at 80°C compared to lower
109 temperature incubations. At 80°C, reads from *Firmicutes* were assigned to the classes
110 *Thermovenabulia*, *Symbiobacteriia*, *Thermoanaerobacteria* and *Clostridia* (Fig. 2B). Pearson
111 correlation of each operational taxonomic unit (OTU) with acetate accumulation showed high correlation
112 (>0.6) for OTUs from *Thermovenabulia* and *Thermoanaerobacteria* (Fig. S1). OTUs from lineages
113 enriched at 80°C were present in lower relative abundances during incubation at 70°C and were not
114 detected at 60 or 50°C (Fig. 2C). On the other hand, *Thermosediminibacter* and *Symbiobacteriales*
115 OTUs were also detected at 90°C, suggestive of a hyperthermophilic temperature physiology for these
116 lineages which are not normally considered to include hyperthermophiles able to grow at temperatures
117 $\geq 80^\circ\text{C}$.

118 **Growth temperature prediction from genomes supports thermophilic activity**

119 Two metagenomic datasets were created from sediment slurries incubated at 80°C. Assembled
120 metagenomic contigs were binned and dereplicated resulting in 13 metagenome-assembled genomes
121 (MAGs) from four phyla (*Firmicutes* (× 4), *Proteobacteria* (× 4), *Actinobacteriota* (× 4) and
122 *Campylobacterota* (× 1); genome completeness is provided in Dataset S1). The optimum growth
123 temperature for each of the thirteen MAGs was predicted based on inferred amino acid composition (Li

124 *et al.*, 2019). The four *Firmicutes* MAGs were predicted to have growth temperature optima ranging
125 from 69–75°C (Fig. 3). *Proteobacteria*, *Actinobacteriota* and *Campylobacterota* were predicted to be
126 mesophiles with temperature optima ranging from 24–29°C (Dataset S1). MAGs from microorganisms
127 predicted to be mesophiles were likely binned from relic DNA (Lennon *et al.*, 2018) arising from
128 organisms present *in situ* that died at elevated temperature, and were excluded from further analysis.

129 Phylogenomic analysis of the four thermophilic *Firmicutes* MAGs showed taxonomic affiliations with the
130 families ZC4RG38 (class *Symbiobacteriales*), *Calditerricolaceae*, *Thermosediminibacteraceae*, and
131 *Moorellaceae* (Fig. 3). Comparing these four MAGs with the Genome Taxonomy Database (GTDB) did
132 not uncover any close relatives with >95% amino acid identity (AAI). Closest relatives ranged between
133 79.9–93.3% AAI indicating that these four MAGs represent new genera or species within their
134 respective phylogenetic groups. Genome-based predictions of optimal growth temperature among
135 related microorganisms included in the phylogenomic tree suggests that the nearest relatives are
136 thermophiles (Fig. 3). Accordingly, these populations have been discovered in geothermal subsurface
137 aquifers (Mori *et al.*, 2002; Ogg and Patel, 2009) and high temperature compost (Moriya *et al.*, 2011;
138 Martins *et al.*, 2013).

139 *Symbiobacteriales* and *Thermosediminibacteraceae* MAGs contained 16S rRNA gene sequences that
140 were ≥99% identical to OTUs detected by amplicon sequencing. Based on nucleotide identity, the
141 *Symbiobacteriales* MAG corresponds to OTU2 and the *Thermosediminibacteraceae* MAG corresponds
142 to OTU5 (see OTUs detected at 80 and 90°C in Fig. 2C). The 16S rRNA gene sequence from the
143 *Thermanaeromonas* MAG shared greatest nucleotide identity (95%) with OTU36 (Fig. 2C). The
144 *Calditerricola* MAG did not include a 16S rRNA gene, but an unbinned *Calditerricola* 16S rRNA gene
145 sequence shared 99.7% nucleotide identity with *Calditerricola* OTU1256 present at only 0.02% read
146 abundance.

147 **Genomic evidence for sporulation and dormancy**

148 Thermophilic *Firmicutes* detected in ≥80°C sediment incubations are predicted to be endospore-formers
149 based on their viable persistence in an environment much below their temperature requirement for
150 growth. The potential for sporulation was confirmed by the presence of core sporulation genes that are
151 conserved in well-known spore-forming *Bacilli* and *Clostridia* (Galperin *et al.*, 2012). These include

152 genes required for pre-septation (Stage 0), post-septation (Stage II), post-engulfment (Stages III-VI),
153 spore coat assembly and germination, and were present in all *Firmicutes* MAGs (Dataset S2).

154 **Endospore populations have the metabolic potential for acetate metabolism**

155 *Symbiobacteriales* can produce acetate via ADP-forming acetyl-CoA synthetase (*acdAB*) while
156 *Thermosediminibacteraceae* can produce acetate in a two-step conversion via phosphate
157 acetyltransferase (*pta*) and acetate kinase (*ackA*). Genes from multiple pathways for organic carbon
158 degradation to acetate were present (Fig. 4). Both *Symbiobacteriales* and *Thermosediminibacteraceae*
159 possess the phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS) for uptake
160 and concomitant phosphorylation of carbohydrates (Deutscher *et al.*, 2006). This includes enzyme I
161 (encoded by the *ptsI* gene) and HPr (encoded by the *ptsH* gene) as well as a sugar-specific enzyme II
162 (EII) permease. Multiple EII permeases and ABC transporters were found (Fig. 4) including those for
163 the uptake of plant and algal derived saccharides (e.g., cellobiose and mannibiose). Both genomes
164 contained multiple glycoside hydrolases (Fig. 4) for the breakdown the glycosidic bonds in
165 carbohydrates producing free fermentable glucose and other monosaccharides.

166 *Thermanaeromonas* and *Calditerricola* do not harbour genes for acetate production and are therefore
167 unlikely contributors to the acetate production observed in heated sediment incubations. Instead, the
168 presence of AMP-forming acetyl-CoA synthetase (*acs*) in both *Calditerricola* and *Thermanaeromonas*
169 genomes suggest these organisms can assimilate acetate.

170 **Genomic potential to degrade necromass and spore lysates in heated sediment**

171 Cellular necromass contains mostly proteins and amino acids as well as DNA, RNA and membrane
172 sugars. *Thermosediminibacteraceae* has the genomic potential to metabolise amino acids by co-
173 fermentation using Stickland reactions, with isoleucine and/or arginine as electron donor and proline
174 and/or glycine as electron acceptor (Fig. 4). *Thermosediminibacteraceae*, *Symbiobacteriales* and
175 *Thermoanaeromonas* genomes all contain extracellular nuclease for degradation of DNA polymers, and
176 all MAGs contained nucleoside and nucleobase transporters that may be used for uptake of
177 extracellular DNA (Pérez Castro *et al.*, 2021). *Thermanaeromonas* further has the potential to degrade
178 xanthine, a nitrogen-rich organic compound from nucleic acids that is widespread in aquatic
179 environments (Cunliffe, 2016).

180 Both *Thermosediminibacterace* and *Symbiobacteriales* have EII permeases for the uptake of
181 membrane sugars (e.g., *N*-acetyl glucosamine and glucosamine) (Fig. 4). Amino sugars phosphorylated
182 by the PTS system then enter glycolysis following the removal of acetyl and amino groups by the
183 enzymes *N*-acetylglucosamine-6-phosphate deacetylase (*nagA*) and glucosamine-6-phosphate
184 deaminase (*nagB*). The *Thermosediminibacteraceae* MAG also contains genes for degradation of
185 ethanolamine, a phospholipid readily available from the degradation of cell membranes.

186 Genes for dissimilatory sulfite reductase (*dsrAB*), the sulfur relay protein *dsrC* and the electron transport
187 complex *dsrMKJOP* are present in the *Thermanaeromonas* MAG. The absence of sulfate
188 adenylyltransferase (*sat*) and adenylylsulfate reductase (*aprAB*) and presence of thiosulfate reductase
189 (*phsA*) suggests that this organism has the potential to use thiosulfate and/or sulfite. Given that
190 thiosulfate and sulfite are not expected to be present in these experiments, *Thermanaeromonas*
191 presumably grows fermentatively, as reported for the closest related isolate, *T. toyohensis*, in the
192 absence of sulfur compounds (Mori *et al.*, 2002). Alternatively, *Thermanaeromonas* could yield sulfite
193 from the desulfonation of sulfolactate by sulfolactate sulfo-lyase (*syuAB*), that cleaves *R*-sulfolactate
194 into pyruvate and sulfite (Denger and Cook, 2010). Sulfolactate is a widespread natural product in
195 plants, algae and prokaryotes, and is also a component of bacterial endospores that gets released upon
196 germination (Bonsen *et al.*, 1969; Rein *et al.*, 2005). Sulfolactate produced by germinating endospores
197 could therefore provide a source of organosulfate in heated sediments.

198 **Discussion**

199 Distinct populations of thermophilic endospore forming bacteria germinate when surface sediments are
200 heated (Fig. 2). Thermophilic sulfate reducing bacteria prevail at temperatures $\leq 70^{\circ}\text{C}$, whereas
201 hyperthermophilic organotrophs with the potential to metabolise different pools of organic carbon were
202 selected by heating to 80 and 90°C . When bacteria are provided with multiple carbon sources, easily
203 accessible compounds are selectively metabolised (Deutscher, 2008; Görke and Stülke, 2008). Acetate
204 production in heated sediments supplemented with organic carbon is therefore likely derived from the
205 metabolism of glucose and other easily accessible components. However, acetate was also produced
206 at 80°C without any organic carbon supplement, indicating that components of sedimentary organic
207 matter are accessible to microbial biodegradation by thermophiles and hyperthermophiles.

208 Organotrophic bacteria enriched here at $\geq 80^{\circ}\text{C}$ have the genomic potential to degrade multiple
209 components of sedimentary organic matter. In surface sediments organic matter typically consists of
210 10–20% carbohydrates, 10% nitrogenous compounds (mostly amino acids) and 5–15% lipids, with the
211 remaining fractions consisting of unidentified organic compounds considered to be recalcitrant (Arndt
212 *et al.*, 2013). Genomes of hyperthermophilic endospores contained genes for the degradation of plant
213 and algal derived carbohydrates and components of cellular necromass. In this study, sediment was
214 heated to 80°C , which is commonly used as a pasteurisation step designed to kill vegetative cells of
215 psychrophilic and mesophilic microorganisms (Hubert *et al.*, 2009). Dead mesophiles formerly prevalent
216 at ambient in situ temperature may therefore offer a source of amino acids, nucleic acids, phospholipids
217 and membrane sugars that can be metabolised by thermophilic organotrophs in surface sediment
218 heating experiments.

219 Compounds released by germinating endospores provide another potential source of energy.
220 Germinating endospores release the biodegradable compounds sulfolactate (5% dry weight) and
221 dipicolinic acid (5–15% dry weight) (Bonsen *et al.*, 1969; Setlow, 2006; McClintock *et al.*, 2018).
222 *Thermanaeromonas* was the only spore-former in this study with the potential to metabolise
223 sulfolactate, whereas all *Firmicutes* MAGs had genes for dipicolinic acid uptake. While anaerobic
224 transformation of dipicolinic acid to acetate, propionate, ammonia and CO_2 has been shown for a
225 coculture of marine microorganisms (Seyfried and Schink, 1990), an exact enzymatic pathway for
226 dipicolinic acid fermentation has not been elucidated.

227 Earlier studies that report acetate production upon heating surface or deep sediments have attributed
228 this observation to the temperature activation of organic carbon increasing its bioavailability, thus
229 providing a continuous supply of energy for bacteria and archaea during burial in the deep biosphere
230 (Wellsbury *et al.*, 1997; Parkes *et al.*, 2007). Evidence of microbial acetate production presented here
231 suggests that activation and metabolism of dormant thermophilic endospore populations could also
232 contribute to acetate generation in deep hot sediments. Surface sediments worldwide contain
233 thermophilic endospores (Müller *et al.*, 2014) that via burial seed deeper sediments (Inagaki *et al.*,
234 2015), where community assembly is driven by selection mechanisms that favour populations adapted
235 to energy limitation (Petro *et al.*, 2017; Starnawski *et al.*, 2017). The resilience of endospores makes
236 these populations well-suited to not being filtered out, possibly explaining estimates that endospores
237 account for a significant proportion of microbial biomass in deeper sediments where they have been

238 proposed to outnumber vegetative cells (Lomstein *et al.*, 2012; Wörmer *et al.*, 2019). Buried endospores
239 remain viable (Lee *et al.*, 2005; de Rezende *et al.*, 2013; Fang *et al.*, 2017) and have the potential to
240 germinate *in situ* when the ambient temperature is high enough, facilitating thermophilic or
241 hyperthermophilic metabolism in deeply buried sediments.

242 Surface sediments used here and in similar experiments (Wellsbury *et al.*, 1997; Parkes *et al.*, 2007)
243 contain more organic carbon than would be likely encountered in deep, hot marine sediment layers.
244 Degradation of organic matter by shallow organotrophic communities means organic matter availability
245 generally diminishes with depth, such that amino acids from microbial necromass have been proposed
246 as quantitatively the most important organic substrates in deep sediments (Lomstein *et al.*, 2012; Lever
247 *et al.*, 2015; Orsi *et al.*, 2020). Thermophilic and hyperthermophilic spores detected here have the
248 genomic potential to gain energy from microbial necromass and experimental evidence suggests that
249 necromass oxidation occurs widely in the marine environment (Langerhuus *et al.*, 2012; Lomstein *et*
250 *al.*, 2012; Tully *et al.*, 2016; Pérez Castro *et al.*, 2021; Wasmund *et al.*, 2021). However, available
251 energy from necromass declines with depth such that microorganisms in deep sediments suffer from
252 extreme energy limitation in the absence of residual organic carbon or other energy sources (Bradley
253 *et al.*, 2018; Orsi *et al.*, 2020).

254 Energy limitation could provide an obstacle for endospores in buried sediments. While our results
255 suggest that temperature is important for activation among specific endospore populations, germination
256 also requires nutrients (or germinants) such as sugars, amino acids, and nucleosides that interact with
257 specific germination receptors in the spore coat (Kochan *et al.*, 2018). Although metabolism of
258 exogenous nutrients (and generation of ATP) is not needed for germination (Setlow *et al.*, 2017),
259 dormant endospores still need to sense conditions that are conducive for growth. On this basis, for
260 buried organotrophic endospores to become active members of the deep hot biosphere, they must not
261 only encounter a suitable temperature, but the surrounding sediments must also have sufficient organic
262 substrates to ensure that germination does not lead to starvation. In this context, the ability to degrade
263 spore components such as dipicolinic acid or sulfolactate, as well as the presence of different enzymes
264 for carbohydrate metabolism in thermophiles relative to mesophiles (Hubert *et al.* 2010), are features
265 that could contribute to the fitness of spore-forming bacteria in the deep biosphere.

266 Sediment heating experiments offer useful models for making predictions about microbial ecology in
267 the deep hot subsurface, where sampling is difficult and biomass levels are generally too low to facilitate

268 direct metagenomic studies. Sediments harbour a seed bank of endospores with genomic and
269 functional diversity, and that can remain viable for long periods of time to enable their selection when
270 environmental circumstances are appropriate. Our results offer insight into the metabolic potential of
271 thermophilic and hyperthermophilic organotrophs with the potential to germinate in deep, hot
272 environments where organic carbon, necromass or metabolites released during germination could
273 contribute to their organotrophic metabolism as vegetative cells.

274 **Experimental Procedures**

275 **Preparation of heated sediment slurries**

276 Sediment from the Tyne estuary, United Kingdom (54°57'51"N, 1°40'60"W) was used as inoculum of
277 endospores in all sediment heating experiments. Sediment was collected at low tide with a trowel to
278 ~20 cm depth and stored in sealed plastic containers at 4°C. Sediment collected in 2013 and 2017 was
279 used for experiments performed 2014 and 2022. Anoxic sediment slurries were prepared by mixing
280 sediment and anoxic seawater medium at a fixed ratio (1 g sediment to 2 mL medium) under a constant
281 flow of N₂ (Widdel and Bak, 1992; Isaksen *et al.*, 1994). For experiments supplemented with organic
282 carbon, tryptic soy broth (3 g/L), glucose (3 mM) and the carboxylic acids acetate, propionate, butyrate
283 and lactate (3 mM each) were added from sterile stock solutions. Glass serum bottles containing
284 sediment slurries (50 mL) were sealed with a butyl rubber stopper and incubated at 50, 60, 70, 80 and
285 90°C. Sterilised controls were prepared by addition of zinc chloride into the slurries to a final
286 concentration of 10% prior to incubation at 80°C.

287 During the incubation period, heated anoxic sediment slurries were subsampled (1.5 mL) with an N₂
288 flushed syringe. Subsamples were centrifuged (13,000 g, 5 min) with the resulting supernatant used to
289 measure organic acids and the sediment pellet used for DNA extraction.

290 **Organic acid measurements**

291 Sediment pore water organic acids were measured using two methods: ion (exclusion) chromatography
292 (IC) or high performance liquid chromatography (HPLC). Samples measured by IC were filtered through
293 0.45 µm Teflon filters and acidified with 0.1 M octanesulfonic acid (1:1). Acidified samples were
294 sonicated for 30 minutes to remove bicarbonate as CO₂. Acetate, propionate and butyrate were
295 measured in the acidified samples by ion (exclusion) chromatography (IC) using a Dionex ICS-1000
296 with an AS40 auto-sampler equipped with an IonPac ICE-AS1, 4 × 250 mm analytical column. The IC

297 flow rate was 0.16 mL/min, the eluent was 1.0 mM heptafluorobutyric acid and the cation regenerant
298 solution used for the AMMS-ICE II Suppressor was 5 mM tetrabutylammonium hydroxide. Samples
299 measured by HPLC were filtered through 0.20 µm PTFE filters. Formate, acetate, propionate, lactate,
300 butyrate and succinate were measured using UV (210 nm) on an HPLC RSLC Ultimate 3000 following
301 the method previously described by (Volpi *et al.*, 2017) for heated sediment incubations. Briefly, organic
302 acids were separated using an Aminex HPX-87H, 7.8 × 300 mm analytical column using 5 mM H₂SO₄
303 as the isocratic eluent, a flow rate of 0.6 mL/min, and the column oven was heated to 60°C.

304 **DNA extraction and 16S rRNA gene amplicon sequencing**

305 DNA was extracted from sediment pellets using the PowerSoil DNA isolation Kit (MoBio Laboratories)
306 following the manufacturer's protocol, except for the elution step which was modified by adding 50 µL
307 elution buffer and allowing 30 minutes before eluting by centrifugation. Extracted DNA was used as a
308 template for PCR amplification using Golay barcoded fusion primers targeting the V4-V5 region of the
309 16S rRNA gene (Caporaso *et al.*, 2012). The PCR protocol included denaturation at 95°C for 4 minutes
310 followed by 25 cycles consisting of denaturation (1 min, 95°C), annealing (45s, 55°C) and extension (1
311 min, 72°C) and a final extension for 10 minutes at 72°C. PCR products derived from a common sub-
312 sampling time from triplicate sediment slurries were in most instances pooled prior to clean-up using
313 Agencourt Ampure XP paramagnetic beads resulting in a single pooled amplicon library for a given
314 experimental time point. 16S rRNA gene amplicons were sequenced on an Ion Torrent Personal
315 Genome Machine (School of Natural and Environmental Sciences, Newcastle University, UK) in
316 accordance with the manufacturer's instructions (Life Technologies). Sequencing data were processed
317 by the Torrent Suite Software V4.0. Raw sequence reads were demultiplexed and quality filtered in
318 QIIME version 1.9.1 (Caporaso *et al.*, 2010). All subsequent sequence analysis was performed with
319 USEARCH v11 (Edgar, 2013). Sequences were truncated to 350 bp (*fastx_truncate*) and clustered into
320 operational taxonomic units (OTUs) sharing 97% sequence identity with UPARSE (*cluster_otus*).
321 Taxonomy was predicted with SINTAX (Edgar, 2016) with a USEARCH compatible (Lee, 2020) Silva
322 138 database (Quast *et al.*, 2013). Amplicon data were visualised with the R package Ampvis2
323 (Andersen *et al.*, 2018). Normalised OTU counts were used to calculate the correlations between each
324 OTU and the concentration of organic acids at the corresponding sampling time points using the Python
325 library Pandas (The Pandas Development Team, 2021).

326 **Metagenomic sequencing, assembly, binning and analyses**

327 Metagenomic sequencing was performed on an Illumina NovaSeq 6000 with a S4 300 cycle flow cell.
328 Libraries were prepared by shearing to an insert size of ~200 bp using a Covaris instrument, followed
329 by library construction with the NEB Ultra II DNA library prep kit. Reads were preprocessed with BBDuk
330 (Bushnell *et al.*, 2017) and assembled separately with two assemblers (1) metaSPAdes (Nurk *et al.*,
331 2017) and (2) MEGAHIT (Li *et al.*, 2015) using the *meta-sensitive* option. Raw reads were mapped to
332 each of the assemblies with BMap (Bushnell *et al.*, 2017). Each of the assemblies were binned with
333 both MetaBAT2 (Kang *et al.*, 2019) and CONCOCT (Alneberg *et al.*, 2014). Bins from the same
334 assembler were refined using DAS Tool (Sieber *et al.*, 2018). The best bins from each of the approaches
335 were selected with dRep (Olm *et al.*, 2017) using the parameters; completeness 75%, contamination
336 5%, primary cluster average nucleotide identity (ANI) 90%, secondary cluster ANI 99%. This resulted
337 in a total of 13 representative metagenome assembled genomes (MAGs).

338 Protein-coding genes were predicted with prodigal (Hyatt *et al.*, 2010). Amino acid sequences used to
339 predict the optimum growth temperature of microorganisms using Tome (Li *et al.*, 2019). MAGs from
340 microorganisms predicted to be thermophiles were annotated with METABOLIC v4.0 (Zhou *et al.*,
341 2019), which integrates annotation of protein-coding genes with KOfam (Aramaki *et al.*, 2020), TIGRfam
342 (Selengut *et al.*, 2007), Pfam (Finn *et al.*, 2014), dbCAN2 (Zhang *et al.*, 2018) and MEROPS (Rawlings
343 *et al.*, 2016). 16S rRNA gene sequences in the metagenomic dataset were identified with METAXA2
344 (Bengtsson-Palme *et al.*, 2015) and phyloFlash (Gruber-Vodicka *et al.*, 2020). 16S rRNA sequences
345 from the metagenomic dataset were aligned to 16S rRNA gene amplicon sequences with BLAST
346 (Altschul *et al.*, 1990).

347 MAGs were taxonomically classified with GTDB-Tk v1.5.0 with reference data for GTDB R06-RS202
348 (Chaumeil *et al.*, 2019). To create a phylogenomic tree, representative genomes from the four families
349 *ZC4RG38*, *Calditerricolaceae*, *Thermosediminibacteraceae* and *Moorellaceae* were downloaded from
350 GTDB v202 (Parks *et al.*, 2020) using *gtdb-get-accessions-from-GTDB* in GToTree v1.6.12 (Lee, 2019).
351 A concatenated alignment was created using 119 single-copy genes targeted by the *Firmicutes* HMM
352 profile in GToTree and programs within; (Edgar, 2004; Capella-Gutiérrez *et al.*, 2009; Hyatt *et al.*, 2010;
353 Eddy, 2011; Tange, 2018; Lee, 2019). The phylogenomic tree was created with IQ-tree (Nguyen *et al.*,
354 2015). Substitution models for each partition were selected with ModelFinder (-m MFP)

355 (Kalyaanamoorthy *et al.*, 2017) and support for phylogenetic groups was determined with UFBoot (-bb
356 1000) (Minh *et al.*, 2013). An *Actinobacteriota* MAG from this study was used to root the tree.

357 **Data availability**

358 Organic acid data data from in this study are provided in the Supplementary Information (Dataset S4).
359 Sequence data have been deposited in GenBank under the BioProject PRJNA371432. BioSample
360 metadata for MAGs described in this study are available in the NCBI BioSample database
361 (<http://www.ncbi.nlm.nih.gov/biosample/>) under accession numbers SAMN22252053–
362 SAMN22252056.

363 **Acknowledgments**

364 This work was supported by UK Natural Environment Research Council awards to CRJH
365 (NE/J024325/1) and EB (NE/K501025/1), from research grants to CRJH from the UK Engineering and
366 Physical Sciences Research Council (EP/J002259/1), ExxonMobil Research and Engineering (New
367 Jersey) through the Knowledge Build program, and by a Campus Alberta Innovates Program (CAIP)
368 chair awarded to CRJH.

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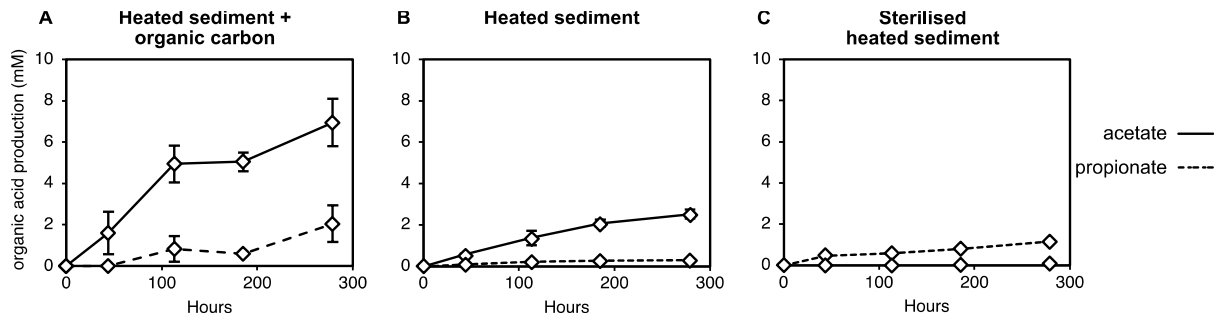
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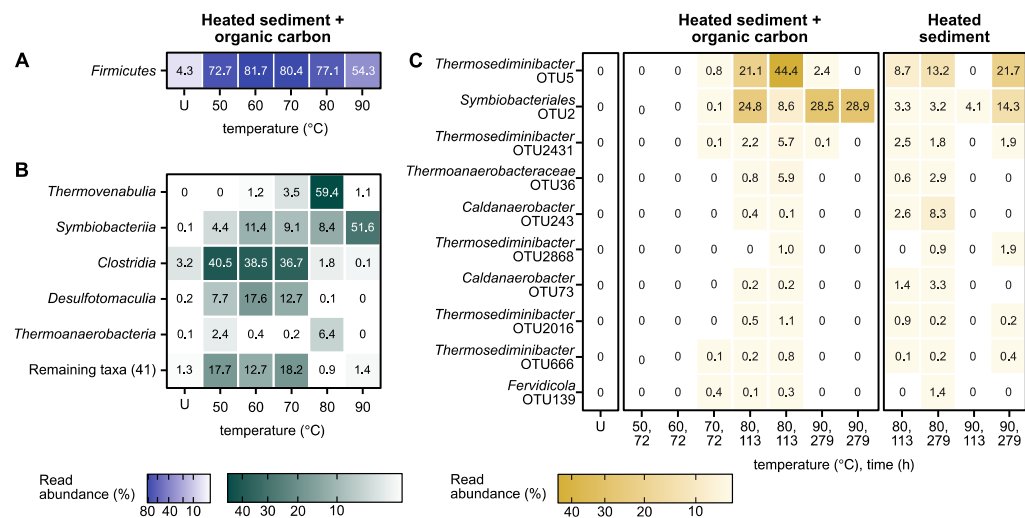
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606 **Figure 1: Acetate and propionate production in sediment heated to 80°C.** Acetate (solid line) and
 607 propionate (dashed line) were measured in anoxic sediment slurries heated to 80°C for 279 h. Sediment
 608 slurries were either (A) supplemented with organic carbon; (B) heated without organic carbon
 609 amendment, or (C) heated and sterilised with zinc chloride. Error bars show standard deviation of three
 610 replicate incubations.

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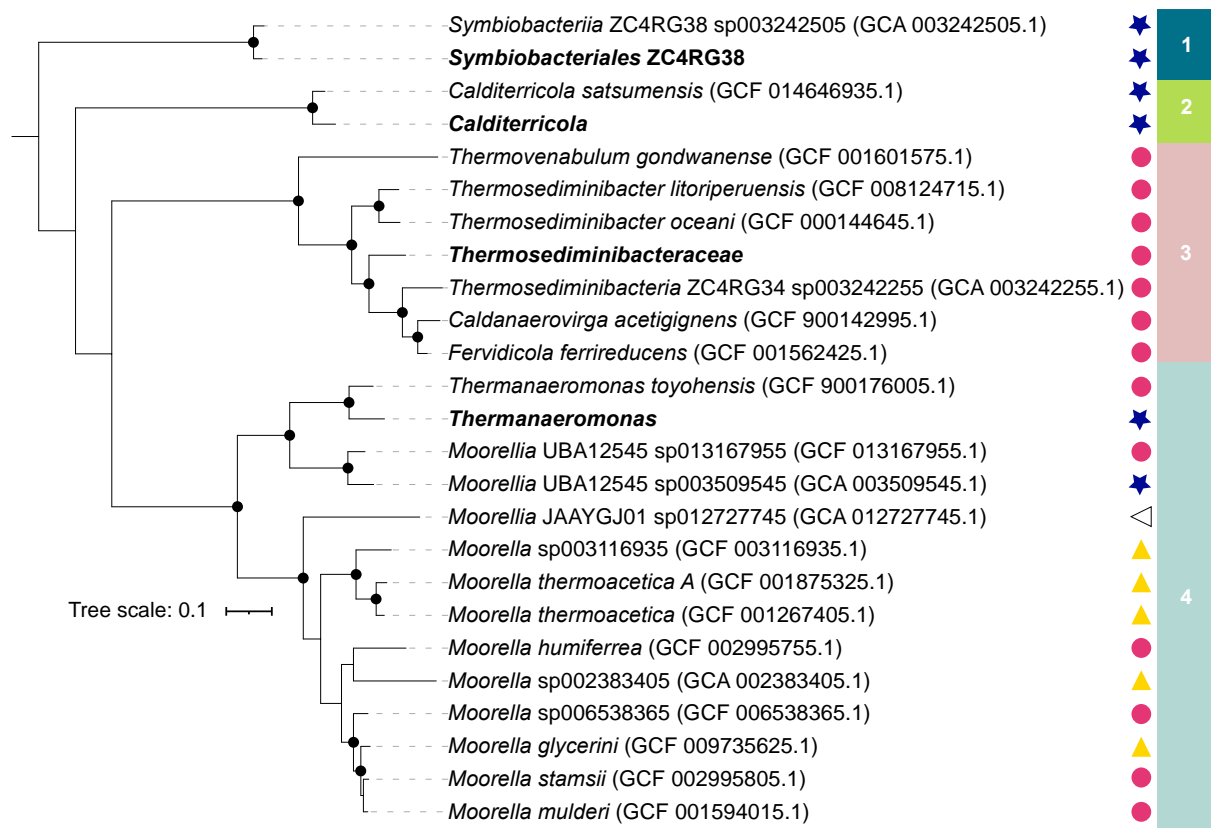
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615 **Figure 2: Enrichment of *Firmicetes* in heated sediment.** 16S rRNA gene amplicon libraries of
 616 sediments heated to 50, 60, 70, 80 and 90°C compared to unheated sediments (“U”). (A) Read
 617 abundance of the endospore forming phylum *Firmicetes*. (B) Classes within the *Firmicetes* with the
 618 greatest read abundance (top five). (C) OTUs within the *Firmicetes* with the greatest read abundance
 619 in sediments heated to ≥80°C (top ten).



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622 **Figure 3: Phylogenomic tree with predicted optimum growth temperature (OGT) of thermophilic**

623 **endospore-forming *Firmicutes*.** Four thermophile MAGs from this study are shown in bold.

624 Representative genomes from the same families (1) ZC4RG38, (2) *Calditerricolaceae*, (3)

625 *Thermosediminibacteraceae*, and (4) *Moorellaceae* were downloaded from GTDB and included in the

626 tree. The tree is based on 119 concatenated single copy genes. Bootstrap support values ≥99% (1000

627 replicates) are indicated by filled circles. The scale bar corresponds to per cent average amino acid

628 substitution over the alignment and supports that all four genomes represent novel lineages within their

629 respective phylogenetic groups. Amino acid based predictions of OGT were made using Tome (Li et

630 al., 2019).

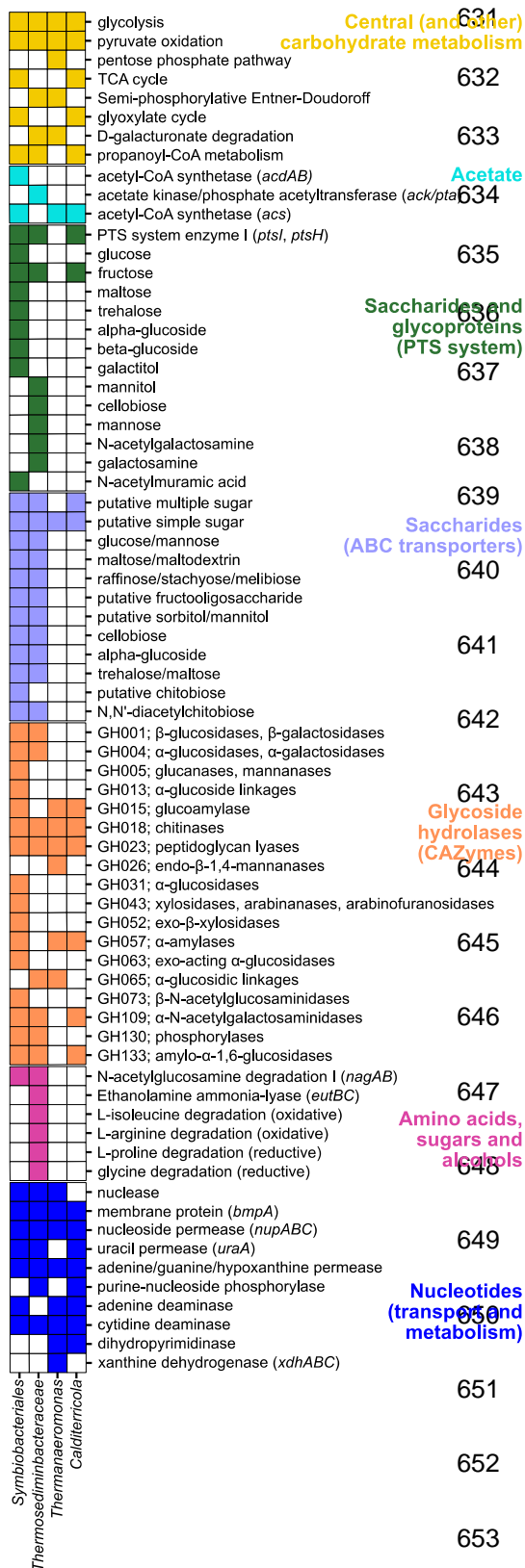


Figure 4: Metabolic potential of thermophilic *Firmicutes*. Presence (filled squares) of select metabolic pathways and genes in four high-quality MAGs of thermophilic endospore-forming *Firmicutes*. Details of KEGG modules, CAZymes and MetaCyc pathways used to create the figure are provided in Dataset S3.

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