

Isolation and characterization of a novel acidophilic zero-valent sulfur- and ferric iron-respiring Firmicute

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Isolation and characterization of a novel acidophilic zero-valent sulfur- and ferric ironrespiring *Firmicute*

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1 **Isolation and characterization of a novel acidophilic zero-valent**
2 **sulfur- and ferric iron-respiring *Firmicute*.**

3

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Abstract:

A novel, obligately anaerobic, acidophilic bacterium (strain I2511), isolated from sediment in an abandoned copper mine, was shown to couple the oxidation of organic electron donors to the reduction of both zero-valent sulfur and ferric iron in acidic media. The isolate was an obligate heterotroph that used a variety of organic compounds as electron donors and required yeast extract for growth. Alternative electron acceptors (sulfate, tetrathionate, thiosulfate and nitrate) were not used by the novel isolate. The strain grew as motile, endospore-forming rods, and was mesophilic and moderately acidophilic, with a growth rate of 0.01 h^{-1} at optimum pH (3.7) and temperature (35°C). Analysis of its 16S rRNA gene sequence placed strain I2511 within the phylum *Firmicutes*, distantly related to validated species. Phylogenetic analysis and physiological traits indicate that the novel strain represents a species of a candidate novel genus. Strain I2511 was included in a microbial consortium in a low pH “hybrid” sulfidogenic bioreactor designed to remove chalcophilic metals from metal-contaminated liquors and was present in >50 % relative abundance when bioreactor was operated at pH ~ 2.0. Results indicate that the novel isolate could be applied in biotechnologies to treat acidic and neutral pH, metal-rich effluents.

Keywords: Acidophile, Iron reduction, *Firmicutes*, Obligate anaerobe, Sulfidogenesis, Sulfur reduction

1. Introduction

Elemental (zero-valent) sulfur (ZVS) and several sulfur oxyanions can be used as terminal electron acceptors by some prokaryotes, generating hydrogen sulfide. While there are a number of reports describing microbially-catalyzed sulfidogenesis in very acidic natural

40 and engineered eco-systems (reviewed in [1]), there have been relatively few reports of pure
41 cultures of bacteria that are capable of generating H₂S at extremely low pH (< 3).
42 *Thermodesulfobium narugense*, isolated from a hot spring in Japan, was the first acidtolerant
43 sulfate-reducing bacterium to be validated [2]; it grows between pH 4 and 6.5 and is a
44 moderate thermophile. *Desulfosporosinus (D.) acidiphilus* and *Desulfosporosinus acididurans*
45 are moderately acidophilic sulfate-reducing bacteria (aSRB) that are able to grow at lower pH
46 (3.6 and above) and can also reduce ZVS [3 - 5]. *D. acididurans* was reported to represent ~
47 6 % of the bacterial population in a sulfidogenic bioreactor maintained at pH 2.4, which was
48 dominated by currently non-validated SRB [6]. A novel *Deltaproteobacteria*, *Desulfurella (Ds.)*
49 *amilsii*, was shown to reduce ZVS at low pH, growing between pH 3 and 7 and optimally at pH
50 6 - 6.5 [7]. More recently, the aSRB *Thermodesulfobium acidiphilum* [8] and
51 *Thermodesulfobium* sp. strain 3baa [9] have been described as thermophiles which can grow
52 at pH range of 3.7 - 6.5 and 2.6 – 6.6, respectively. Currently, there are no reports of ZVS-
53 reducing bacteria that grow optimally at pH < 4. However, dissimilatory reduction of ZVS at
54 extremely low pH has been reported for some, mostly hyperthermophilic, archaea, such as
55 *Acidianus* spp., *Stygiolobus azoricus* and *Sulfurisphaera ohwakuensis*. Species of the genus
56 *Acidianus* (e.g. *A. brierleyi*, *A. infernus*, *A. ambivalens* and *A. sulfidivorans*), isolated from
57 solfatara environments and acidic thermal springs, are able to grow at pH as low as 1, and the
58 minimum growth pH reported for *A. sulfidivorans* is 0.35 [10].

59 Many early attempts to isolate sulfidogens in low pH were unsuccessful because
60 organic acids (e.g. lactate) were used as the main carbon/energy source in enrichment
61 cultures [1]. At pH below their pK_a values, organic acids exist mainly in non-dissociated forms.
62 These tend to be lipophilic, and can diffuse into the cells lowering intracellular pH, ultimately
63 causing cell death. Non-ionic substrates such as glycerol, hydrogen and methanol are more
64 suitable electron donors for sulfidogenesis at low pH [5, 6]. Another challenge with cultivating
65 sulfidogens in acidic media, also related to the toxicity of organic acids, is that some species
66 are known to be “incomplete substrate oxidizers,” and produce and excrete small molecular

67 weight organic acids when metabolizing larger molecular weight substrates, again inducing
68 toxicity (e.g. *D. acididurans* produces acetic acid in stoichiometric equivalents to glycerol
69 oxidized).

70 Depending on pH, hydrogen sulfide can react with chalcophilic metals, such as copper
71 and zinc, precipitating them as sulfide phases. This is the principle behind sulfidogenic
72 bioreactors, which have been widely studied to remove and recover metals from mine-
73 impacted effluents [6, 11]. Industrial-scale bioreactors currently used for this purpose utilize
74 species of neutrophilic sulfidogens that tend to be highly sensitive to both acidity and soluble
75 transition metals that have to be shielded from direct contact with contaminated mine waters
76 [12].

77 The present study reports the characteristics of a novel obligately anaerobic,
78 acidophilic bacterium (*Firmicute* strain I2511) which was found to couple the oxidation of
79 organic electron donors to the reduction of ZVS and ferric iron. The novel sulfidogen was
80 included in a microbial consortium in a low pH "hybrid" sulfidogenic bioreactor (HSB), designed
81 to target the removal of chalcophilic metals from metal-contaminated liquors.

82 2. Materials and methods

83 2.1. *Enrichment, isolation and cultivation of Firmicute I2511*

84 Black sediments collected from an acidic stream draining an abandoned copper mine,
85 Mynydd Parys, North Wales, UK (53° 22' 59.9988" N; 4° 20' 60" W) that had a distinct
86 hydrogen sulfide odor, were used to enrich acidophilic ZVS-reducers. Ten grams of sediment
87 was added to 100 mL of liquid medium containing 5 mM glycerol, 0.01% w/v, yeast extract;
88 chloride salts medium (g L⁻¹: 0.36 NH₄Cl, 0.05 KCl, 0.04 MgCl₂·6H₂O, 0.006 Na₂HPO₄, 0.05
89 KH₂PO₄, 0.014 Ca(NO₃)₂·4H₂O) and 1%, w/v sterile hydrophilic ZVS. To prepare hydrophilic
90 ZVS, sulfur powder (Sigma-Aldrich, UK), which is hydrophobic, was added (10%, w/v) to a
91 liquid culture inoculated with the sulfur-oxidizing acidophilic bacterium *Acidithiobacillus* (*At.*)
92 *thiooxidans*, and incubated at 30°C, shaken at 50 rpm, for 7 days, a technique widely reported

93 to “wet” ZVS, causing it to become hydrophilic. The ZVS was then allowed to settle, removed
94 from the culture, and sterilized at 110°C for 60 min.

95 The enrichment culture was placed in sealed jars under anaerobic atmosphere (Oxoid™
96 AnaeroJar™, Thermo Fisher Scientific; USA) and incubated at 30°C, shaken at 50 rpm.

97 Hydrogen sulfide production was confirmed by the appearance of CuS precipitates inside a
98 “copper trap”, which consisted of an open universal bottle containing 10 mL of 20 mM CuSO₄
99 which was placed inside the sealed jar (Supplementary Fig. 1). Following this, the enrichment
100 cultures were streaked onto aSRB plates [13] and incubated under anaerobic atmospheres at
101 30°C. Single colonies were differentiated by their morphologies and purified by repeated re-
102 streaking onto fresh solid media. From these, isolates were cultivated in 5 mM glycerol, 0.01%
103 (w/v) yeast extract, chloride basal salts, trace elements [13] and 1% (w/v) sterile ZVS pH 3,
104 incubated anaerobically at 30 °C and 50 rpm.

105 2.2. *Growth Characteristics of isolate I2511*

106 2.2.1. *Standard cultivation conditions*

107 Several attempts to cultivate isolate I2511 in aSRB liquid medium [13] in the absence
108 of ZVS, did not result in increased cell numbers or H₂S production. Therefore, it was assumed
109 that the isolate was not able to catalyze the dissimilatory reduction of sulfate. Hence, unless
110 otherwise indicated, the chloride-based basal salts used in the enrichment cultures medium
111 was subsequently replaced by sulfate salts (acidophile basal salts (ABS); [13]). The “standard
112 medium” used to cultivate isolate I2511 contained 5 mM glycerol, 1% (w/v) ZVS, 0.01% (w/v)
113 yeast extract, ABS and trace elements [13], pH adjusted to 3.0 with sulfuric acid. ZVS-
114 containing and ZVS-free liquid media were heat-sterilized at 110 °C for 60 min, and 120 °C
115 for 30 min, respectively. The standard incubation conditions used were anaerobic (generated
116 in sealable jars containing Oxoid™ AnaeroJar™ sachets, Thermo Fisher Scientific; USA), 30
117 °C, shaken at 50 rpm. Growth was monitored by enumerating cells using a Helber counting

118 chamber marked with Thoma ruling (Hawksley, UK) and viewed with a Leitz Labolux phase-
119 contrast microscope at a magnification of 400x.

120 2.2.2. Growth under aerobic and micro-aerobic conditions

121 Growth under aerobic conditions was tested in liquid and on solid media and for micro-
122 aerobic conditions on solid media only. Micro-aerobic atmosphere was generated in sealable
123 jars containing CampyGen™ CN25 sachets (Thermo Fisher Scientific; USA). The selected
124 solid media used (all overlay) were: YE3_g and FeSo_g [14]. Liquid media tested were: (a) ZVS
125 (1%, w/v), 5 mM glycerol and yeast extract (0.01%, w/v), pH 3.0 and (b) 10 mM Fe²⁺ and yeast
126 extract (0.02%, w/v), pH 2.0. Solid and liquid media were inoculated with culture grown in liquid
127 medium containing ZVS (1%, w/v), 5 mM glycerol and yeast extract (0.01%, w/v) pH 3.5,
128 incubated anaerobically. To evaluate growth in liquid media, planktonic bacterial cells were
129 counted and pH measured after 10 and 20 days.

130 2.2.3. Carbon metabolism

131 To investigate whether isolate I2511 required yeast extract for growth, isolate I2511
132 was grown in standard liquid medium with or without yeast extract. Planktonic cells were
133 enumerated after 11 and 18 days. The effect of different concentrations (0.005 or 0.05%, w/v)
134 of yeast extract or peptone on cell yields was tested in replicate liquid cultures. Two other liquid
135 culture variants were set up: (a) 0.05% (w/v) yeast extract or peptone but no ZVS; (b) standard
136 medium with 0.005% (w/v) yeast extract. Planktonic cells were enumerated after 18 days.

137 The ability of isolate I2511 to grow on a range of defined small molecular weight organic
138 compounds was tested in replicate liquid cultures containing ABS and trace elements, 1%
139 (w/v) ZVS, 0.01% (w/v) yeast extract pH 3.0 supplemented with the following compounds:
140 glucose, fructose, 1,3-propanediol, glycerol, lactic acid and citric acid (all at 5 mM); ethanol and
141 methanol (both at 10 mM). In parallel, control cultures were set up containing ZVS and 0.01%
142 (w/v) yeast extract with no additional organic electron donor. To assess growth by
143 fermentation, replicate cultures were supplemented with glucose, glycerol, L-malate or

144 fumarate (all at 5 mM) in the absence of ZVS at pH 4. Growth was assessed from counts of
145 planktonic cells after incubating for 12 - 18 days.

146 *2.2.4. The use of alternative electron acceptors by isolate I2511*

147 Dissimilatory reduction of ferric iron was tested by growing strain I2511 in liquid medium
148 containing 5 mM glycerol and 0.005% (w/v) yeast extract and ~15 mM $\text{Fe}_2(\text{SO}_4)$. The pH was
149 adjusted to 2.6 with sterile 1 M NaOH, which caused partial precipitation of the ferric iron.
150 Cultures were incubated in sealed jars under anaerobic atmospheres. Replicate cultures were
151 inoculated with an active culture of strain I2511, grown previously in the presence of ferric iron.
152 Replicated non-inoculated controls were set up in parallel. Ferrous iron concentrations and
153 culture pH were determined after 17, 25 and 30 days.

154 To determine whether strain I2511 could catalyze the dissimilatory reduction of
155 tetrathionate, thiosulfate, cysteine or nitrate, replicate cultures were incubated anaerobically
156 at 30°C in aSRB medium supplemented with either 5 mM potassium tetrathionate, 10 mM
157 sodium thiosulfate, 0.02% (w/v) L-cysteine hydrochloride monohydrate or 10 mM sodium
158 nitrate. Positive (ZVS-containing) and negative (no electron acceptor) controls were set up in
159 parallel. Growth was evaluated by enumerating planktonic cells.

160

161 *2.2.5. Effect of pH and temperature on the growth rate of isolate I2511*

162 Isolate I2511 was grown in a pH- and temperature-controlled bioreactor (FerMac 310/60
163 unit, Electrolab Biotech, UK) fitted with a 2.2 L glass vessel and stirred at 150 rpm. The
164 standard liquid medium was slightly modified by including less yeast extract (0.005%, w/v). A
165 continuous stream of oxygen-free nitrogen (OFN, at ~ 200 mL min⁻¹) was used to maintain
166 anaerobic conditions and to deliver H₂S generated inside the bioreactor vessel to an off-line
167 glass vessel that contained 500 mL of 20 mM CuSO₄. To determine the effect of pH on the
168 growth rate of isolate I2511, this was varied between 2.8 and 4.5 and the bioreactor maintained
169 at 30 °C. To determine the effect of temperature on the growth rate of isolate I2511, this was

170 varied between 23°C and 35°C and the bioreactor maintained at pH 3.7. Samples from the
171 bioreactor vessel and the off-line vessel were removed regularly to determine concentrations
172 of glycerol, acetic acid and copper. Semi-logarithmic plots of glycerol oxidized against time
173 were used to determine growth rates. Rates of H₂S production were determined by changes
174 in concentrations of copper in the off-line vessel. Additional tests were carried out to assess
175 the minimum pH (at 30°C) and maximum temperature (at pH 3.7) at which isolate I2511 could
176 grow. Growth was evaluated by counting planktonic bacterial cells after 11 and 18 days of
177 incubation.

178 *2.2.6. Dissimilatory reduction of zero-valent sulfur on solid medium*

179 Reduction of ZVS on solid media was assessed by streaking liquid culture of strain
180 I2511 onto an overlay ZVS (S⁰) plates, developed jointly with Ivan Ñancucheo (Facultad de
181 Ingeniería y Tecnología, Universidad San Sebastian, Chile). The S⁰ plate contained 5 mM
182 glycerol, 0.01% (w/v) yeast extract, chloride basal salts, 0.5 % (w/v) agarose, adjusted to pH
183 3.0 with hydrochloric acid, to which, after setting, 100 µL of a suspension of ZVS (40%, m/v)
184 was spread on the top. Inoculated plates were placed in sealed jars, incubated under an
185 anaerobic atmosphere together with a universal bottle containing 20 mM copper sulfate to
186 indicate production of H₂S, incubated at 30 °C.

187 *2.2.7. Tolerance to copper, sodium chloride and acetic acid*

188 Replicate cultures of I2511 in standard medium (pH 2.5) were supplemented with
189 copper sulfate (at 0.1, 1.0, 5.0 and 10 mM) to assess tolerance to Cu²⁺. Copper-free control
190 cultures were set up in parallel. Growth was assessed by enumerating planktonic cells and
191 from formation of CuS (as a result of production of H₂S). A similar approach was used to
192 determine the tolerance to sodium chloride (50, 100 and 500 mM). To investigate tolerance of
193 strain I2511 to acetic acid, replicate cultures containing 0.5, 1.0 and 3.0 mM acetic acid were
194 set up in standard medium at pH 2.5, together with acetic acid-free controls.

195 2.3. *Relative abundance of isolate I2511 in a low pH sulfidogenic bioreactor*

196 Isolate I2511 was included in a microbial consortium in a low pH “hybrid” sulfidogenic
197 bioreactor (HSB). The design of the HSB, and its effectiveness in removing contaminant
198 transition metals from neutral pH mine waters, are detailed in [15]. The HSB contained both
199 ZVS and sulfate as potential electron acceptors, and glycerol and yeast extract were provided
200 as electron donors/carbon sources. The bioreactor (an upflow biofilm bed sulfidogenic
201 bioreactor of the 2.2 L working volume) was operated at 30 °C, agitated at 50 rpm in continuous
202 flow mode with a feed solution containing 2 mM glycerol, 0.01% (w/v) yeast extract, ABS with
203 pH adjusted to either 2.0 or 2.5 with sulfuric acid. The composition of microbial populations in
204 the HSB, both planktonic and sulfur-attached cells, were analyzed. Samples were taken from
205 the surface liquor of the bioreactor and from the ZVS layer, and treated as previously described
206 [15]. DNA from the biomass was extracted using PowerSoil UltraClean microbial DNA isolation
207 Kits (QIAGEN, Denmark), following manufacturer's instructions. Bacterial and archaeal 16S
208 rRNA genes were amplified and analyzed by terminal restriction enzyme fragment length
209 polymorphism (T-RFLP; [16]), a semi-quantitative approach used to calculate relative
210 abundances in microbial communities.

211 2.4. *Biomolecular Analysis*

212 Genomic DNA was extracted from liquid cultures using modified CTAB/high-salt
213 extraction, followed by alcohol precipitation [17]. The 16S rRNA gene of strain I2511 was
214 amplified using 27F (5'–3' AGAGTT TGATCM TGGCTCAG) and 1387R (5'–3'
215 GGGCGGWGTGTACAAGGC) primers and PCR products were sequenced by Sanger
216 method (Macrogen Inc., South Korea) and analyzed by T-RFLP [16]. The draft genome
217 sequence of strain I2511 (GenBank accession number QXHL00000000) indicated only one
218 16S rRNA gene copy to be present, enabling more accurate calculation of relative abundances
219 using T-RFLP analysis.

225 2.5. *Phylogenetic Analysis*

226 The 16S rRNA gene sequence of strain I2511 was deposited in the GenBank 227 database and compared with those of related species (retrieved from EZBioCloud;[18]).

228 Multiple sequence alignment was performed using SINA [19]. The phylogenetic analysis was
229 performed by MEGAX with the Maximum Likelihood method implemented [20]. Selection of
230 best-fitting substitution models was performed with the Bayesian Information Criterion using
bootstrap analysis using 1000 replicates.

Analytical methods

The pH of liquid cultures were measured using a pHase combination glass electrode coupled to an Accumet 50 pH meter. Concentrations of glycerol, sulfate, and acetic acid were determined by ion chromatography [6]. Concentrations of ferrous iron determined using the Ferrozine assay [22]. Concentrations of soluble copper were measured using a

Isolation and characteristics of isolate I2511

After 12 days of anaerobic incubation, hydrogen sulfide was detected as being

231 Model Selection implemented in iQtree [21]. Tree topology reliabilities were confirmed by 232

233 2.6.

234

235

236

237

238 colorimetric assay [23].

239 **3. Results**

240 3.1.

241

242 generated by liquid enrichment cultures of the black sediments in ZVS-glycerol medium.

243 Single colonies from aSRB plates streak-inoculated from the enrichment cultures were put

244 into standard ZVS medium, and one of these (I2511) was able to generate H₂S. T-RFLP

245 analysis confirmed purity of the culture. Cells were motile rods (3 - 5 µm long and ~0.4 µm

246 wide) that formed oval endospores located at the cell termini. Isolate I2511 produced very

247 small off-white colonies (≤ 1 mm diameter) on aSRB solid medium but these were not

248 encrusted with ZnS, a feature indicative of sulfate-reducers [13]. Strain I2511 also produced

249 small white colonies on S⁰₂ plates, and generated H₂S (confirmed by formation of CuS in a

250 universal bottle containing CuSO₄). ZVS particles disappeared where colonies grew,

251 indicating they were being used to generate H₂S (Supplementary Fig. 2). No growth of

252 isolate was observed under aerobic or micro-aerobic conditions, confirming that it is an
253 obligate anaerobe.

254

255 3.2. *Phylogenetic and genomic analysis*

256 The partial 16S rRNA gene sequence of isolate I2511 (1,388 bp) was deposited in
257 GenBank (accession number KY576736). Analysis of its 16S rRNA gene sequence showed
258 that isolate I2511 was a member of the phylum *Firmicutes* (order Bacillales, family
259 *Alicyclobacillaceae*) and its closest validated relatives were *Alicyclobacillus contaminans*
260 3A191^T and *Alicyclobacillus tolerans* K1^T, which shared 92.9% and 93.1% sequence similarity
261 to isolate I2511, respectively. Isolate IR2 and clone G13 were more closely related bacteria,
262 both sharing 96% identity of their 16S rRNA genes to strain I2511. Strain IR2 was isolated
263 from a sulfidogenic bioreactor inoculated with enrichment culture containing biomass of an
264 acidic stream draining a copper mine [6]. The clone G13 was obtained from glucose
265 enrichment culture inoculated with acidic mine tailings containing high concentrations of
266 methylmercury [24]. Phylogenetic analysis of the 16S rRNA gene of strain I2511 showed that
267 the isolate clustered with strain IR2 and clone G13 in a separate clade from that represented
268 by the closest validated *Alicyclobacillus* species. The phylogenetic relationship of I2511 with
269 the validated species of the genus *Alicyclobacillus* and closely related bacteria is shown in Fig.
270 1.

271 3.3. *Carbon metabolism*

272 There were no increase in cell numbers of isolate I2511 in media where glycerol was
273 the only carbon source provided. However, the isolate grew in glycerol medium that also
274 contained yeast extract, and in medium containing only either yeast extract or peptone as
275 carbon source/electron donor, both of which were also coupled to the reduction of ZVS,
276 generating H₂S. Isolate I2511 grew on a range of organic compounds (Supplementary Fig. 3),
277 all of which were coupled to the dissimilatory reduction of ZVS. Lactic acid was not utilized,

278 and cell numbers were less than in control cultures. The isolate was not able to grow by
279 fermentation of yeast extract or of any of the defined organic substrates tested.

280 3.4. *Alternative electron acceptors*

281 Cultures of I2511 containing glycerol and ferric iron (mostly present as a solid phase)
282 but no ZVS showed increases in both pH and Fe^{2+} concentrations after 17 days of incubation,
283 but beyond this cell number increases were only marginal, and yeast extract (0.005%, v/v)
284 added at day 25, did not induce further iron reduction (Fig. 2). Minor changes in pH and Fe^{2+}
285 concentrations were measured in non-inoculated cultures during the same time.
286 Concentrations of glycerol oxidized and acetic acid produced after 25 days were 0.54 mM and
287 0.51 mM, respectively.

288 In contrast, media containing either tetrathionate or thiosulfate as potential alternative
289 electron acceptors to ZVS had similar cell counts to the negative (ZVS-free) control cultures.
290 Similarly, cell numbers did not increase and pH did not change in cultures amended with
291 cysteine. Cell numbers in nitrate-amended cultures were slightly more than in controls but far
292 fewer than those in ZVS-containing cultures. In a repeat of this experiment, no increases in
293 cell numbers were observed. From these experiments, it was concluded that isolate could use
294 ferric iron as an alternative electron acceptor to ZVS, but not tetrathionate, thiosulfate, nitrate
295 or cysteine.

296 3.5. *Effect of pH and temperature on growth rates*

297 Growth experiments showed that hydrogen sulfide production was correlated with both
298 glycerol oxidation ($r = 0.93$) and planktonic cell numbers ($r = 0.91$) (Fig. 3). Concentrations of
299 acetic acid increased with time, and the ratio of acetic acid produced to glycerol oxidized was
300 ~ 0.4 (Fig. 4). When incubated at 30°C, isolate I2511 had an optimum growth at pH ~ 3.7 with
301 a culture doubling time of ~ 62 h (Fig. 5). Under optimum pH (3.7) and 35°C, its culture doubling
302 time was 54 h, corresponding to a μ_{max} of 0.01 h^{-1} . Isolate I2511 did not grow (or generate H_2S)
303 at temperatures $\geq 38^\circ\text{C}$, and below 23°C growth was very slow (doubling time ~ 80 h; at pH
304 3.7 and 23°C). No growth or H_2S production was observed at pH 2.5 and 30°C in bioreactor

305 cultures. However, tests carried out in universal bottles, where pH was not controlled,
306 suggested that the isolate was able to generate H₂S in media poised initially at 1.8
307 (Supplementary Fig 4).

308 3.6. *Tolerance to copper, sodium chloride and acetic acid*

309 Isolate I2511 was highly sensitive to both copper (II) ions and salt (NaCl), with growth
310 being inhibited by the lowest concentrations of these (0.1 and 50 mM, respectively) tested. All
311 cultures supplemented with acetic acid had lower cell counts than the control culture at same
312 pH after 10 days of incubation, though by day 15 cultures containing 0.5 and 1.0 mM acetic
313 acid had similar planktonic cell counts to the control cultures at the same pH (Supplementary
314 Fig. 5). Growth of strain I2511 was completely inhibited by 3 mM acetic acid. Culture pH at
315 day 15 for all tests (including control) were similar at ~ pH 2.0.

316 3.7. *Relative abundance of isolate I2511 in a low pH sulfidogenic bioreactor*

317 While the HSB operated over a wide pH range [15] there was particular interest in the
318 microbial community composition when it was operated at extremely low pH. Prior to the start
319 of experiment, the bioreactor was allowed to adapt to the operating conditions for 20 days for
320 the pH 2.5 feed liquor, and for 10 days for the pH 2.0 feed liquor. With pH 2.5 feed liquor, the
321 pH within the vessel was 2.6, and when using pH 2.0 feed liquor the bioreactor pH ranged
322 from 1.98 - 2.05. Hydrogen sulfide was generated throughout both experiments, but no
323 significant differences in rates were found when using pH 2.0 and pH 2.5 feed liquors (ttest:
324 $t(11) = 0.30$, $p = 0.77$; Supplementary Fig. 6). Changes in rates of sulfate reduction and H₂S
325 production using pH 2.5 and pH 2.0 feed liquors are shown in Supplementary Fig. 7.

326 Some of the sulfate present in the pH 2.5 influent liquor was reduced (these accounted for 77
327 \pm 8% of the H₂S produced), but there was no detectable net sulfate reduction when the
328 bioreactor pH equilibrated at ~ 2.0.

329 The microbial communities that were attached to ZVS particles in the HSB were similar
330 to those of planktonic bacteria at both pH values, though there were major differences in the
331 dominant bacteria present (Fig. 6). At pH 2.6, the sulfate-reducing bacteria *Peptococcaceae*

332 CEB3 [1] and *D. acididurans* were found to be the dominant bacteria, but at pH ~2.0 neither
333 of these was detected, and the most abundant bacterium was strain I2511 (51% relative
334 abundance in the liquid phase and 55% of the attached community), and the facultative
335 anaerobe *At. ferrooxidans* and a putative fermentative *Clostridium* sp. were also

336 detected.

337 4. Discussion

338

339 This study has shown that a novel mesophilic acidophilic isolate of the phylum
340 *Firmicutes*, strain I2511, was able to generate hydrogen sulfide at low pH, *via* the dissimilatory
341 reduction of ZVS. This is only the second non sulfate-reducing acidophile to be demonstrated
342 to have this trait, and the data show that I2511 is far more tolerant of extreme acidity than *Ds.*
343 *amilsii* [7]. The isolate was an obligately anaerobe that (like other *Firmicutes*) formed
344 endospores, and was also an obligate heterotroph. The only other electron acceptor that I2511
345 used (of those tested) was ferric iron. This possibly accounts for its initial growth on solid media
346 that contained iron but not ZVS (at the pH of the aSRB plates there would be some anticipated
347 oxidation of Fe²⁺ to Fe³⁺ during preparation and storage). Rigorous testing confirmed that
348 I2511 was not able to use sulfate, tetrathionate, thiosulfate or nitrate as a terminal electron
349 acceptor, or grow *via* fermentation. Isolate I2511 was able to use a variety of organic
350 compounds but required yeast extract for growth. It oxidized glycerol incompletely, generating
351 acetic acid, but not in stoichiometric amounts. The isolate was mesophilic, with no growth
352 observed at a maximum temperature 38°C. Its pH optimum was 3.7, categorizing it as a
353 moderate, rather than an extreme acidophile [25]. Although it did not grow in the fixed pH
354 bioreactor at pH 2.5, data from the HSB (and to a lesser extent from the batch culture
355 experiment in universal bottles) suggested that it may be capable of growth-decoupled
356 sulfidogenesis at pH values less than 2. This apparent discrepancy might be explained by
357 carryover of acetic acid in the bioreactor when setting up a new growth test (generally ~ 80%
358 of the bioreactor was drained and replaced with fresh medium) which at the HSB in the pH 2.0

359 feed liquor test, acetic acid concentrations were low ($\leq 0,31$ mM). Generating H₂S at low pH
360 is an unusual and relatively rare trait (for acidophilic bacteria, though not for acidophilic
361 archaea) that could be useful in the development of sulfidogenic biotechnologies used to treat
362 acidic, metal-rich effluents.

363 Phylogenetic analysis of the 16S rRNA gene of strain I2511 showed that the isolate
364 clustered in a separate clade from that represented by the closest validated *Alicyclobacillus*
365 species. Some characteristics of strain I2511 (i.e. a mesophilic obligate anaerobe, which
366 catalyzes the dissimilatory reduction of both ZVS and ferric iron and has relatively low
367 tolerance to copper and sodium chloride) are distinct from its closest known relatives *Alb.*
368 *contaminans*^T and *Alb. tolerans*^T. For example, *Alb. contaminans*^T is a moderately thermophilic
369 strict aerobe, and tolerates up to ~350 mM NaCl. *Alb. tolerans*^T is a moderately thermophilic
370 facultative anaerobe and grows as a heterotroph and also autotrophically using Fe²⁺ and ZVS
371 as electron donors. The acidophilic genera of the family *Alicyclobacillaceae* (*Alicyclobacillus*,
372 *Sulfobacillus* and “*Acidibacillus*”) share several characteristics. Many species are strict
373 aerobes, others are facultative anaerobes and use either molecular oxygen or ferric iron as
374 electron acceptors, many are thermo-tolerant or moderately thermophilic, several are obligate
375 heterotrophs, some are facultative autotrophs, some species can use ferrous iron or reduced
376 sulfur as electron donors, and display elevated tolerance to transition metals. However, none
377 of the species described to date can grow anaerobically by sulfur respiration, which clearly
378 delineates isolate I2511 (which is a strict anaerobe) from other species of the family
379 *Alicyclobacillaceae*.

380 While more research is required in order to validate isolate I2511, the data suggest that
381 it is the first cultivated species of a novel genus within the phylum *Firmicutes*. Questions about
382 how it interacts with other microorganisms in the wider environment need also to be
383 addressed, along with more work on examining its potential for bioremediation of polluted
384 environments.

385

386 **Conflict of interest**

387

388 The authors confirm that there are no conflicts of interest.

389

390 **Acknowledgements**

391

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394

395 **Appendix A. Supplementary data**

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397 Supplementary data related to this article can be found at <http://xxxx>

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Figure 1. Phylogenetic analysis by the Maximum Likelihood method showing the relationship
of isolate I2511 16S rRNA gene sequences to closely related bacteria and validated species
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470 Biol 2020;39:63-76.

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473 **Legends to figures**

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477 of the genus *Alicyclobacillus*. The support in bootstrap analysis (1000 replicates) with values
478 $\geq 50\%$ are indicated by “●”. The bar represents 0.05 substitutions per site. The 16S rRNA
479 gene sequence of *Sulfobacillus thermosulfidooxidans*^T (NR040945) was used as the 480
outgroup.

481 Figure 2. Reductive dissolution of solid phase ferric iron by isolate I2511 (solid lines) and 482 non-
inoculated cultures (broken lines) after 17, 25 and 30 days of incubation at 30°C. Key:

483 (▲) ferrous iron concentrations (mM) and (■) pH. Bars show mean values and error bars

484 indicate data ranges (n=2).

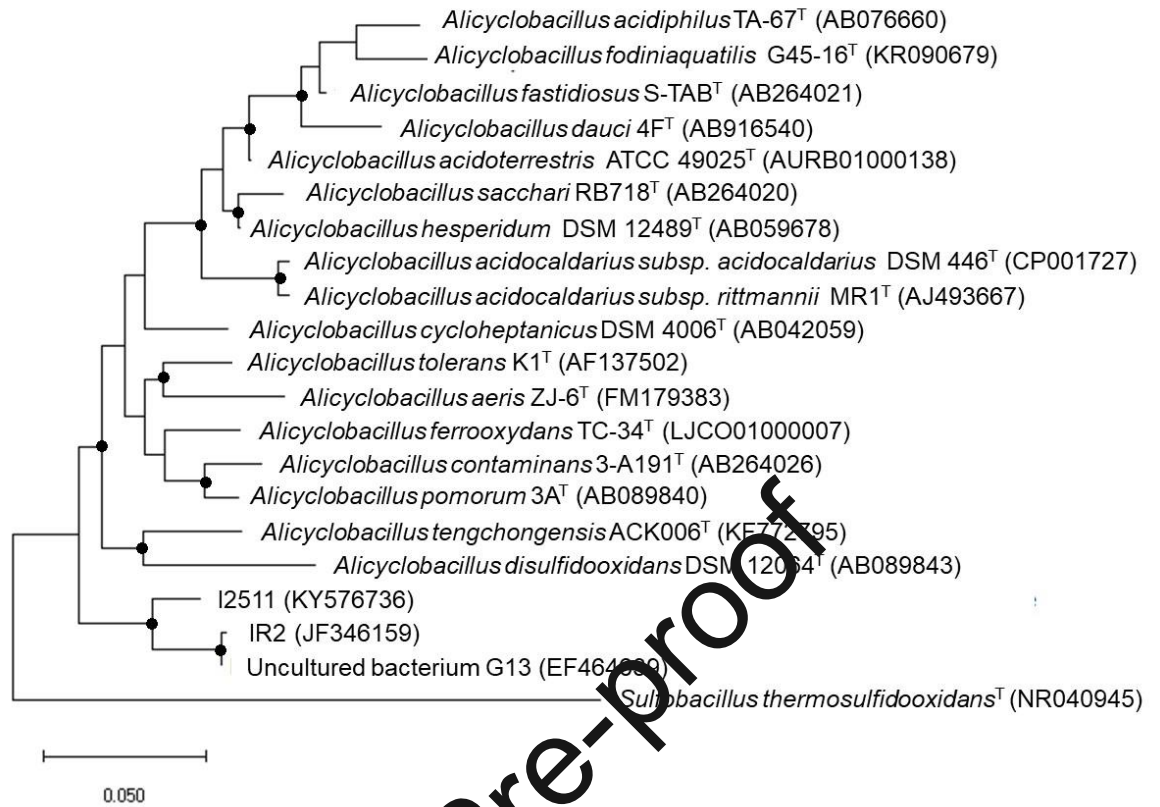
485 Figure 3. Relationship between hydrogen sulfide production and glycerol oxidation (\blacktriangle ; $r =$
486 0.93) and hydrogen sulfide production and planktonic cell numbers (\bullet ; $r = 0.91$) when isolate
487 I2511 was grown in a bioreactor at pH 3.2 and 30°C.

488 Figure 4. Cumulated amounts of hydrogen sulfide produced (\blacksquare), glycerol oxidised (\blacktriangle) and
489 acetic acid produced (\bullet) of isolate at pH 2.8 and 30°C.

490 Figure 5. Effect of pH on the culture doubling times (t_d) of isolate I2511 at fixed temperature
491 (30°C).

Figure 6. T-RFLP profiles of bacterial 16S rRNA genes amplified from DNA extracts and digested with the restriction enzyme HaeIII during experiments operating the HSB at low pH in continuous flow mode. "Sulfur phase" corresponds to DNA extracted from sulfur-attached bacterial communities. "Liquid phase" corresponds to DNA extracted from planktonic cells.

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gene sequences to validated species of the genus *Alicyclobacillus*. The analysis (1000 replicates) with values $\geq 50\%$ are indicated by “•”. The bar represents 0.05 substitutions per site. The 16S rRNA gene sequence of Figure 1. Phylogenetic analysis by the Maximum Likelihood method showing the relationship of isolate I2511 16S rRNA closely related bacteria

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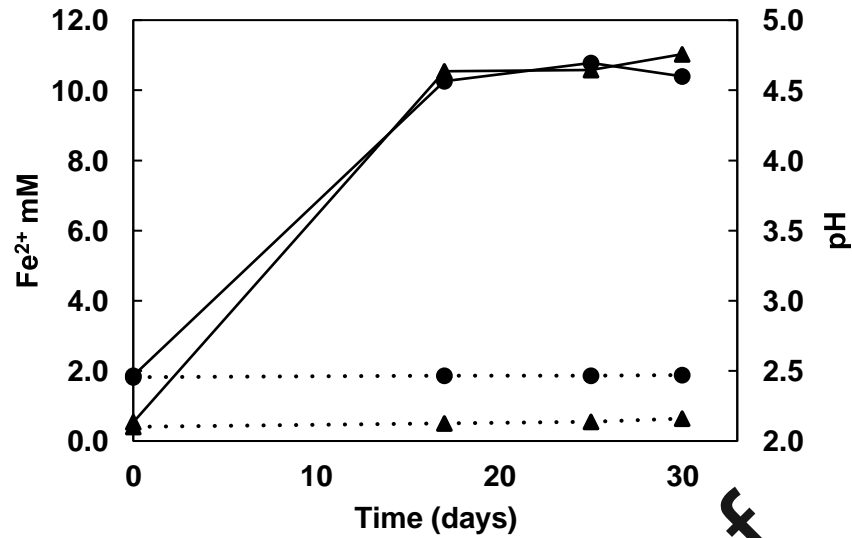


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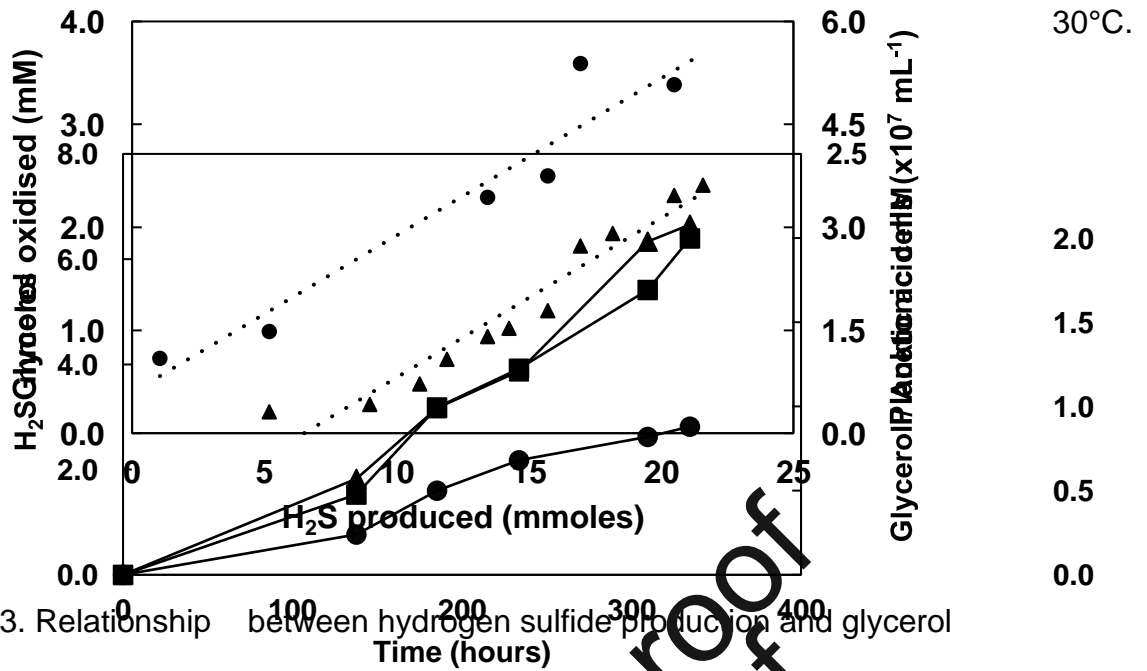


Figure 3. Relationship between hydrogen sulfide production and glycerol oxidation (\blacktriangle ; $r = 0.93$) and hydrogen sulfide production and planktonic cell numbers (\bullet ; $r = 0.91$) when isolate 12511 was grown in a 50 mL reactor at pH 3.2 and 30°C.

Figure 4. Cumulated amounts of hydrogen sulfide produced (\blacksquare), glycerol oxidised numbers (\bullet ; $r = 0.91$) when isolate 12511 was grown in a 50 mL reactor at pH 3.2 and 30°C. (\blacktriangle) and acetic acid produced (\bullet) of isolate at pH 7.8 and 30°C.

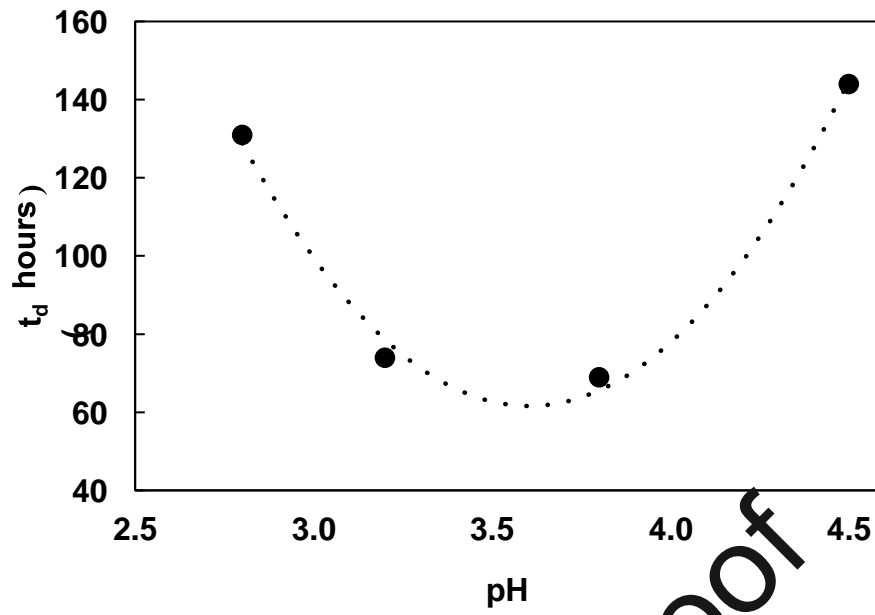


Figure 5. Effect of pH on the culture doubling times (t_d) of isolate I2511 at fixed

temperature (30°C).

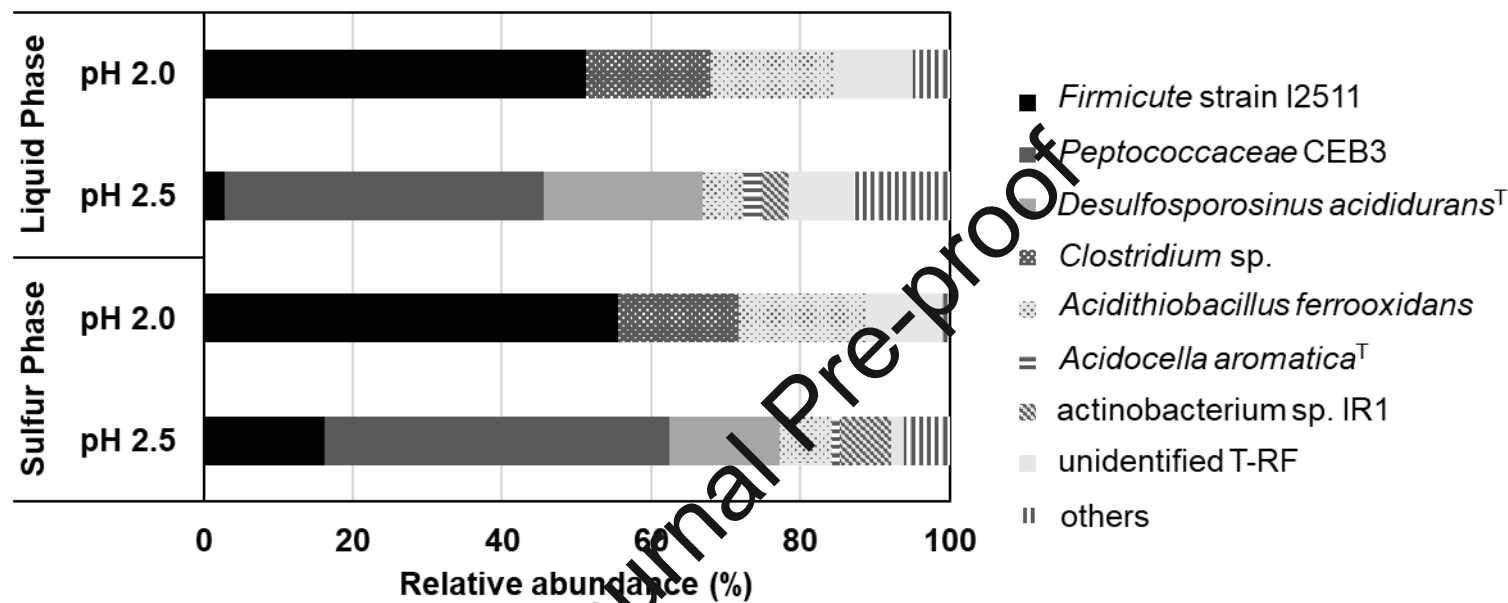


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