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Isolation and characterisation of mineral-oxidizing

"*Acidibacillus*" spp. from mine sites and geothermal environments

in different global locations

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Abstract

Eight strains of acidophilic bacteria, isolated from mine-impacted and geothermal sites from different parts of the world, were shown to form a distinct clade (proposed genus "*Acidibacillus*") within the phylum *Firmicutes*, well separated from the acidophilic genera, *Sulfobacillus* and *Alicyclobacillus*. Two of the strains (both isolated from sites in Yellowstone National Park, USA) were moderate thermophiles that oxidized both ferrous iron and elemental sulfur, while the other six were mesophiles that also oxidized ferrous iron, but not sulfur. All eight isolates reduced ferric iron to varying degrees. The two groups shared <95% similarity of their 16S rRNA genes and were therefore considered to be distinct species: "*A. sulfuroxidans*" (moderately thermophilic isolates) and "*A. ferrooxidans*" (mesophilic isolates). Both species were obligate heterotrophs; none of the eight strains grew in the absence of organic carbon. "*Acidibacillus*" spp. were generally highly tolerant of elevated concentrations of cationic transition metals, though "*A. sulfuroxidans*" strains were more sensitive to some (e.g. nickel and zinc) than those of "*A. ferrooxidans*". Initial annotation of the genomes of two strains of "*A. ferrooxidans*" have revealed the presence of genes (*cbbL*) involved in the RuBisCO pathway for CO_2 assimilation and iron oxidation (*rus*) though with relatively low sequence identities.

1. Introduction

Acidophilic microorganisms comprise a large variety of different species that are widely distributed in all three domains of known life-forms [1]. While the greatest number (and earliest isolates) of known extremely acidophilic bacteria are members of the phylum Proteobacteria, other phyla, including the Firmicutes, Nitrospirae, Actinobacteria and Aquificae, all include species that grow optimally at pH <3. Currently, the Firmicutes (endospore-forming eubacteria with low G+C contents) include two genera of extreme acidophiles, *Sulfobacillus* and *Alicyclobacillus*, most species of which are moderate thermophiles (growth temperature optima of 40 – 60ºC) though some are mesophilic. *Sulfobacillus* spp. are typically found in mineral- and sulfur-rich acidic environments, such as solfatara fields and biomining operations, and are characterised by having far greater metabolic versatilities than many of the more specialised bacteria (such as *Leptospirillum* and *Acidithiobacillus* spp.) with which they frequently cohabit. Species such as *Sb. thermosulfidooxidans, Sb. acidophilus* and *Sb. beneficiens* can grow autotrophically by oxidizing inorganic electron donors (sulfur, ferrous iron and hydrogen) and fixing carbon dioxide, heterotrophically using organic carbon as both energy and carbon source, and chemolitho-heterotrophically whereby they obtain most of their energy from oxidizing inorganic electron donors but use organic materials, such as yeast extract, as carbon sources. *Sulfobacillus* spp. are facultative anaerobes that use either molecular oxygen or ferric iron as terminal electron acceptors. In contrast, the genus *Alicyclobacillus* includes species of moderate (pH growth optima 3 - 5) as well as extreme acidophiles. The earliest isolates were obligately heterotrophic moderate thermophiles that were isolated from pasteurised fruit juices which they had contaminated. Later isolates (e.g. *Alb. disulfidooxidans* and *Alb. ferrooxydans*) more resembled *Sulfobacillus* spp., both in terms of the environments they inhabited and in their metabolic capabilities (e.g. in catalysing the dissimilatory oxidation of sulfur and iron). *Alicyclobacillus* spp. tend, however, to be generally more "heterotrophically inclined" than *Sulfobacillus* spp., and grow more successfully using defined organic compounds such as glucose (e.g. [2].

3

79 For a number of years, some acidophilic Firmicutes isolated from mineral-rich terrestrial or

80 acidic aquatic environments have been found, from sequencing of their 16S rRNA genes, to

81 be affiliated to neither *Sulfobacillus* nor *Alicyclobacillus* (e.g. [3-5]), though these have not been

82 fully characterized. Clones related to these isolates have also been found in diverse locations

83 (e.g. [6-8]). In this paper, we describe the characteristics of eight such strains, isolated from a

84 variety of low pH environments from different global locations, and show that they comprise

85 two distinct species of a proposed novel genus of Firmicutes, "*Acidibacillus*".

86

87 **2. Materials and Methods**

88

89 *2.1. Bacterial origins, isolation and cultivation*

90 The eight bacterial strains studied were isolated from a variety of mine-impacted and

91 geothermal sites from different global locations over a 20 year period and maintained within

92 the *Acidophile Culture Collection* at Bangor University (U.K.; Table 1). Bacteria were isolated

93 on solid media that select for the growth of different physiological groups of acidophilic bacteria

94 [9], either directly by streaking water samples (or mine waste slurries) onto plates, or (in the

95 case of isolates Y0010 and ITV01) following enrichment in acidic liquid media. Plates were

96 incubated at either 30º or 45ºC and isolates purified by repeated re-streaking of single colonies

97 onto fresh solid media. These were then transferred into a liquid medium containing 10 mM

98 ferrous sulfate, 0.02% (w/v) yeast extract and acidophile basal salts (ABS), adjusted to pH 2.0

99 with sulfuric acid. ABS contained (g/L) 0.15 $Na_2SO_4 \cdot 10H_2O$, 0.45 $(NH_4)_2SO_4$, 0.05 KCl, 0.5

100 $MgSO_4 \cdot 7H_2O$, 0.05 KH_2PO_4, and 0.015 $Ca(NO_3)_2 \cdot 4H_2O$.

101

102 *2.2. Phylogenetic analysis*
103

104 DNA was extracted from bacteria grown in 5 mL of ferrous iron/yeast extract liquid

105 medium using the FastDNA *Spin Kit for Soil* (MP Biomedicals) using a modified

4

106   protocol [10]. The 16S rRNA genes were amplified from DNA extracts by PCR using

107   DreamTaq PCR Master Mix (Thermo Fisher) and primers 27F (5´-AGT GTT TGA

108   TCC TGG GTC AG-3´; [11]) and GM4R (5´- TAC CTT GTT ACG ACT T-3´; [12]).

109   PCR products were purified, and overlapping sequencing from both sides of the gene

110   was performed by Seqlab (Germany). Contigs were constructed with the software

111   Geneious Pro 5.4, and the resulting gene sequences were analyzed using BLAST at

112   the NCBI database (http://ncbi.nlm.nih.gov/BLAST) and added to the database.

113   Alignment of the sequences obtained, together with those of related strains, was

114   carried out using Mega 6.0 [13], followed by manual editing to remove gaps and

115   positions of ambiguous nucleotides. Phylogenetic trees were constructed by

116   neighbour-joining analyses. Reliability of the tree topologies was confirmed by

117   bootstrap analysis using 1,000 replicate alignments.

118

119   *2.3. Growth characteristics*

120

121   Growth rates and optimum pH values and temperatures for growth of the two proposed type

122   strains, SLC66^T ("*A. ferrooxidans*") and Y002^T ("*A. sulfuroxidans*") were determined by growing

123   the bacteria in a pH- and temperature-controlled 2 L bioreactor (Electrolab, UK), as described

124   elsewhere [14]. The liquid medium contained 0.1 mM (SLC66^T) or 1 mM (Y002^T) ferrous

125   sulfate, 0.02% (w/v) yeast extract and ABS, and the bioreactor was stirred at 100 rpm and

126   aerated with ~1.5 L of sterile atmospheric air/minute. Since preliminary experiments had

127   confirmed that growth of both isolates was coupled to ferrous iron oxidation, growth rates were

128   routinely determined from semi-logarithmic plots of ferrous iron oxidized against time.

129

130   *2.4. Dissimilatory redox transformations of inorganic electron donors and acceptors*

131

132   Dissimilatory oxidation of ferrous iron was determined by monitoring changes in ferrous iron

133   concentrations and cell numbers in acidic (pH 1.5 to 2.5) liquid medium containing 10 – 25 mM

5

134 Fe^{2+}, amended (or not) with yeast extract. To determine whether bacteria were able to utilize

135 the energy available from oxidizing iron, strains SLC66[T] and Y002[T] were grown in replicate

136 flasks containing 0.005% (w/v) yeast extract and different concentrations of ferrous iron (1, 10

137 and 25 mM, at an initial pH of 1.9 for SLC66[T], and 1, 25 and 50 mM, at an initial pH of 1.7 for

138 the more acidophilic isolate Y002[T]). Culture media were designed to maximize the amount of

139 ferrous iron oxidation without causing hydrolysis (and precipitation) of the ferric iron generated,

140 which would have impaired the accuracy of cell counts. Cultures were incubated (at 30ºC for

141 SLC66[T] and 45ºC for Y002[T]), shaken at 100 rpm, and residual ferrous iron and cell numbers

142 determined daily for up to 6 days.

143 Specific rates of ferrous iron oxidation by strain Y002[T] grown in ferrous sulfate/yeast extract

144 medium (determined at pH 1.8 and 45ºC) were evaluated as described elsewhere [15]. A

145 modified protocol was used for strain SLC66[T], which involved growing the isolate in a 2 L

146 bioreactor in 25 mM ferrous iron/yeast extract medium (at pH 2.0 and 30ºC) until all of the iron

147 had been oxidized, and then adding a further 3 mM ferrous iron and determining residual Fe^{2+}

148 concentrations over the following 90 minutes. Concentrations of bacterial proteins were

149 measured at the start and end of these experiments to determine whether there had been any

150 significant increase in biomass during the time span of the experiments.

151 Dissimilatory oxidation of elemental sulfur (S^0) was tested by inoculating active cultures into

152 a liquid medium containing ~0.5% (w/v) sterile S^0, with or without 0.02% (w/v) yeast extract,

153 and poised initially at ~pH 3.0. Since the end product of the reaction is sulfuric acid, both

154 changes in pH and sulfate concentrations, as well as increases in cell numbers were used to

155 monitor growth. Oxidation of tetrathionate was assessed by growing isolates Y002[T] and Y0010

156 in pH 3 medium that contained 2.5 mM filter-sterilized potassium tetrathionate, 0% or 0.02%

157 yeast extract, 500 µM ferrous iron and ABS. Growth was monitored by enumerating cells and

158 measuring changes in pH and sulfate concentrations.

159 The oxidative dissolution of pyrite by the novel Firmicutes was tested by inoculating the two

160 proposed type strains (SLC66[T] and Y002[T]) into a liquid medium containing ABS and 1% finely-

161 ground pyrite (Strem Chemicals, USA) supplemented (or not) with yeast extract (0.02%, w/v).

162 Where yeast extract was not included, cultures were supplemented with trace elements.

163 Replicate shake flask cultures were incubated at either 30ºC (SLC66[T]) or 45ºC (Y002[T]). Both

164 non-inoculated cultures and others inoculated with the moderate thermophilic Firmicute, *Sb.*

165 *thermosulfidooxidans*[T] were incubated in parallel, to act as negative and positive controls (*Sb.*

166 *thermosulfidooxidans* cultures contained yeast extract and were incubated at 45ºC). Samples

167 were withdrawn at regular intervals to measure pH, redox potential (E_H values), ferrous iron

168 and total soluble iron.

169 Dissimilatory ferric iron reduction was assessed by growing cultures in 100 mL of liquid

170 medium containing 10 mM ferrous iron, 0.02% yeast extract and ABS, adjusted to either pH

171 2.0 (isolates SLC66[T], SLC40, ITV01, BSH1, GS1 and Gal-G1; incubated at 30ºC) or 1.8

172 (isolates Y002[T] and Y0010; incubated at 45ºC). The shake flask cultures were incubated,

173 aerobically, until ferrous iron concentrations had fallen to <0.5 mM, at which point 20 mL

174 aliquots were withdrawn from each and placed in 25 mL sterile bottles, further yeast extract

175 added (to 0.02% w/v) and the replicate bottles placed in sealed jars under either anaerobic or

176 micro-aerobic environments (using *AnaeroGen* and *CampyGen* systems, Oxoid, U.K.).

177 Samples were withdrawn after 2 and 4 days (moderate thermophiles) and 10 days

178 (mesophiles) and concentrations of ferrous iron determined. Dissimilatory reduction of sulfur

179 was tested in cultures incubated anaerobically in media containing 10 mM ferrous iron, 0.02%

180 yeast extract, 5 mM glucose and 0.5% elemental sulfur. Growth on hydrogen was tested on

181 solid media using protocols described elsewhere [16].

182

183 *2.5. Carbon metabolism*

184

185 Bacteria were grown routinely in liquid medium containing 10 mM ferrous iron and 0.02%

186 (w/v) yeast extract, adjusted to either pH 2.0 (for strains of "*A. ferrooxidans*") or 1.8 (for strains

187 of "*A. sulfuroxidans*"). The effect of adding different concentrations (0, 0.005, 0.02 or 0.5%,

188 w/v) of yeast extract to this medium on cell yields of strains SLC66[T] and Y002[T] was examined.

189 Comparative growth was also assessed in 1 mM ferrous iron/0.005% yeast extract liquid

7

190   medium complimented complex carbon sources (casein hydrolysate and tryptone, both at

191   0.02%, w/v), or defined organic compounds. The latter were: (i) monosaccharides (glucose,

192   fructose, and maltose, all at 5 mM); (ii) alcohols (15 mM ethanol, 10 mM glycerol and 5 mM

193   mannitol); (iii) organic acids (citric acid and lysine, both at 5 mM); (iv) benzyl alcohol (5 mM).

194   Biomass yields were determined from regular counts of bacteria in liquid media over 3 - 5 days

195   incubation period. Growth yields of all isolates (in triplicate cultures) were also compared using

196   the following liquid media: (i) 1 mM ferrous iron; (ii) 20 mM ferrous iron; (iii) 1 mM ferrous iron/5

197   mM glucose; (iv) 20 mM ferrous iron/5 mM glucose; (iv) 1 mM ferrous iron/0.005% yeast

198   extract.

199

200   *2.6. Metal and salt tolerance*

201

202   Standard ferrous iron/yeast extract liquid medium was supplemented with sterile

203   solutions of aluminum, cobalt, copper, ferrous iron, manganese, nickel or zinc sulfates

204   or sodium molybdate, to give final concentrations of 50 -1000 mM (sulfate salts) or

205   0.05 - 0.3 mM (molybdate). The pH of the media was adjusted to 2.0 with sulfuric acid

206   and the cultures incubated, shaken, at 30°C ("*A. ferrooxidans*" strains) or 45°C ("*A.*

207   *sulfuroxidans*" strains) for up to 14 days. Growth was assessed by enumerating

208   bacterial cells. In cultures where ferrous iron tolerance was tested, positive growth was

209   reported by increase in cell numbers determined by SYBR staining [17]. A similar

210   approach to that described above was used to determine salt (sodium chloride)

211   tolerance. Both the highest concentration of metal (or salt) at which growth was

212   observed, and the minimum inhibitory concentrations (MIC) were recorded.

213

214   *2.7. Genome analyses*

215

216   Genomic DNA was extracted from cultures of "*A. ferrooxidans*" SLC66[T] and ITV01,

217   and from "*A. sulfuroxidans*" Y002[T], grown on 10 mM ferrous iron, 0.02% yeast extract

8

218   and 5 mM glucose, using a modified CTAB/high-salt extraction on lysozyme treated

219   cells, followed by alcohol precipitation [18]. Whole-genome sequencing was performed

220   via a combined approach using an Ion Torrent personal genome machine (Life

221   Technologies, Carlsbad, CA), with 400 bp chemistry libraries and 318 semiconductor

222   chip (strain ITV01), and an Illumina MiSeq sequencer with paired-end sequencing kit

223   (strains SLC66^T and Y002^T). Genome assembly was conducted as described

224   elsewhere [19]. Gene sequences coding for proteins in assimilating carbon dioxide and

225   dinitrogen fixation were obtained from the genomes of different acidophilic bacteria:

226   *Acidithiobacillus ferrooxidans* (GCA_000021485.1), *Sulfobacillus acidophilus*

227   (GCA_000219855.1) and *Alicyclobacillus acidocaldarius* (GCA_000024285.1). These

228   were used as query sequences to search the genome scaffolds of the "*Acidibacillus*"

229   spp., employing local Blast with default parameters, using CLC Genomics Workbench

230   7 (https://www.qiagenbioinformatics.com/). The best hits were investigated as putative

231   orthologs and the protein structures were characterized using InterproScan tools [20].

232

233   *2.8. Electron microscopy*

234

235   Active cultures of strains SLC66^T and Y002^T were fixed in 2.5% glutaraldehyde

236   followed by progressive ethanol dehydration. Fixed cultures were filtered through 0,2

237   µM Nuclepore filters, and the immobilized bacteria critical point-dried and gold-

238   coated, and were visualized using a Zeiss Sigma VP scanning electron microscope.

239

240   *2.9. Analytical methods and reference bacteria*

241

242   Bacteria were enumerated using a Helber counting chamber marked with Thoma

243   ruling (Hawksley, United Kingdom) and viewed with a Leitz Labolux phase-contrast

244   microscope at a magnification of 400X. Ferrous iron was determined using the

245   Ferrozine reagent [21]. Total soluble iron was determined using the same method but

246 following reduction of ferric iron to ferrous by adding ascorbic acid, and ferric iron

247 concentration from differences in the two values. Protein concentrations were

248 measured using the Bradford assay [22]. Concentrations of glucose were determined

249 using a Dionex ICS 3000 ion chromatography system fitted with a Carbo Pac MA1

250 column and ED amperometric detector, and sulfate concentrations using a Dionex

251 IC25 ion chromatograph with an Ion Pac AS-11 column equipped with a conductivity

252 detector. Culture pH was measured using a pHase combination glass electrode, and

253 redox potentials (adjusted to be relative to a standard hydrogen electrode; E_H values)

254 using a combination platinum silver/silver chloride electrode (VWR, UK). Both

255 electrodes were coupled to an Accumet pH/redox meter 50.

256   The type strain of *Sb. thermosulfidooxidans* (DSM 9293) and *Acidiphilium cryptum*

257 strain SJH [9] were used in some experiments

258

259 **3. Results**

260

261 *3.1. Bacterial cells and colonies*

262

263   All eight novel bacteria were isolated on acidic overlay solid media that contained

264 organic carbon (tryptone soya broth or yeast extract) in addition to ferrous iron. They

265 were subsequently subcultured on "FeS<u>o</u>" medium [9] which contains 2.5 mM

266 potassium tetrathionate in addition to ferrous iron and tryptone soya broth. Bacterial

267 colonies on FeS<u>o</u> medium had "fried egg" morphologies (Supplementary Fig. 1) typical

268 of heterotrophic iron-oxidizing acidophiles, the orange coloration of the colony centres

269 resulting from the accumulation of oxidized iron. Cells of "*A. ferrooxidans*" SLC66[T]

270 were motile rods, 1.5 – 1.8 µm long, ~0.4 µm wide, and formed oval endospores which

271 were located at the cell termini. Cells of "*A. sulfuroxidans*" Y002[T] were also motile rods,

272 3 - 4 µm by ~0.5 µm that formed oval endospores located at the cell termini. It was

273 noted that numbers of individual cells of Y002[T] increased during the early phases of

274   incubation (up to 2 days) but declined subsequently; this appeared to be related to

275   cells aggregating as incubation progressed (Supplementary Fig. 2), a feature that was

276   much less apparent in cultures of SLC66^T.

277

278   *3.2. Phylogenetic analysis*

279

280   Analysis and comparison of 16S rRNA gene sequences confirmed that all eight

281   isolates were members of the phylum Firmicutes (order *Bacillales*, family

282   *Alicyclobacillaceae*). Figure 1 shows that they clustered into three closely-related

283   groups, two of which (Groups IA and IB) shared >99% similarity of their 16S rRNA

284   genes and all of these were proposed to be strains of the novel species "*A.*

285   *ferrooxidans*". The two Group II isolates (Y002^T and Y0010) shared >99% 16S rRNA

286   gene similarity but were more distantly related (94% gene similarity) to both Groups IA

287   and IB and considered to be strains of a different species, "*A. sulfuroxidans*". These

288   phylogenetic differences were also reflected in some key physiological traits, described

289   below.

290

291   *3.3. Effects of pH and temperature on growth rates*

292

293   Figure 2 shows the effects of pH and temperature on the culture doubling times

294   (t_d's) of SLC66^T and Y002^T. Both bacteria were confirmed to be extreme acidophiles,

295   though Y002^T was more acidophilic with a pH optimum and minimum for growth of 1.8

296   and 1.6, respectively, while corresponding values for SLC66^T were 2.9 and 1.9. The

297   two isolates also displayed contrasting temperature-related growth: SLC66^T was

298   mesophilic (temperature optimum and maximum of ~30ºC and 37.5ºC) while Y002^T

299   was a moderate thermophile with an optimum growth temperature of ~43ºC and a

300   maximum of 50ºC. When grown at optimum conditions of pH and temperature, SLC66^T

301   had a culture doubling time (t_d) of 6.7 h (corresponding to a growth rate, μ, of 0.10 h$^-$

302　[1]), while the moderate thermophile $Y002^T$ grew much more rapidly (minimum t_d of 2.1

303　h, corresponding to a μ_{max} of 0.33 h^{-1}). Tests carried out in shake flasks confirmed that

304　none of the "Group I" isolates ("*A. ferrooxidans*") grew at pH 1.5, though one Group IA

305　strain (BSH1) and both Group IB strains (ITV01 and Gal-G1) grew at pH 1.75

306　(Supplementary Table 1). None of the four Group IA strains grew at 40°C, in contrast

307　to both Group IB strains (though neither of these grew at 45°C). Like $Y002^T$, the other

308　strain of "*A. sulfuroxidans*" (strain Y0010) grew at 45°C, though this bacterium was less

309　acidophilic than $Y002^T$ and grew at pH 1.75 though not at pH 1.5 (Supplementary Table

310　1).

311

312　*3.4. Dissimilatory redox transformations of inorganic electron donors and acceptors*

313

314　　All eight of the isolates catalysed the oxidation of ferrous iron in acidic media, and

315　this was found to be highly correlated ($r^2 = 0.98 - 0.99$) with growth of the bacteria. In

316　cultures containing very small concentrations (0.005% w/v) of yeast extract, cell yields

317　of both $SLC66^T$ and $Y002^T$ increased in parallel with the amount of ferrous iron oxidized

318　(Fig. 3a). However, this trend was not found in cultures that contained a much higher

319　(0.05%) concentration of yeast extract, and in these cell numbers were also noted to

320　continue to increase beyond the point at which all of the ferrous iron had been oxidized

321　(Supplementary Fig. 3). The specific rates of ferrous iron oxidation were 36.1 +/- 3.4

322　mg min^{-1} mg $protein^{-1}$ ($SLC66^T$, at 30°C and pH 2.0) and 48.5 +/- 1.3 mg min^{-1} mg

323　$protein^{-1}$ ($Y002^T$, at 45°C and pH 1.8).

324　　None of the six Group I ("*A. ferrooxidans*") strains oxidized elemental sulfur. In

325　contrast, pH declined and sulfate concentrations increased as a result of the

326　dissimilatory oxidation of sulfur to sulfuric acid, in yeast extract-containing cultures of

327　both Group II isolates ($Y002^T$ and Y0010; Supplementary Fig. 4). Numbers of $Y002^T$

328　and Y0010 did not, however, correlate with oxidation of sulfur, which was considered

329　to be due to attachment of cells to particulate S^0. In contrast, numbers of both $Y002^T$

12

and Y0010 increased in tetrathionate-containing media, paralleling changes in sulfate

concentrations and culture pH (and was more pronounced in cultures of Y002^T),

confirming that the two strains of "*A. sulfuroxidans*" can oxidize tetrathionate as well

as elemental sulfur.

 "*A. ferrooxidans*" SLC66^T catalysed the oxidative dissolution of pyrite, as

evidenced by increasing concentrations of total soluble iron and cultures developing

more positive E_H values with incubation time, though again this was only observed with

yeast extract-containing cultures (Fig. 4a). Cultures of Y002^T, in contrast, initially failed

to show any evidence of pyrite oxidation in liquid media that had been prepared under

identical conditions. It was also noted that this isolate was unable to oxidize the ferrous

iron released during sterilization of pyrite when the mineral was autoclaved in the

presence of yeast extract. However, when sterile yeast extract solution was added

subsequent to autoclaving pyrite/ABS, oxidative dissolution of pyrite proceeded, as

shown in Fig. 4b. As with SLC66^T, it was found that pyrite oxidation by strain Y002^T

was negligible in yeast extract-free medium, though adding yeast extract to "inorganic"

cultures of Y002^T at day 12 resulted in rapid oxidation of the ferrous iron present, as

indicated by a mean increase in redox potential of >200 mV during the following two

days, and the initiation of pyrite dissolution (Fig. 4b). The addition of further yeast

extract (at day 12) to cultures of Y002^T that had yeast extract added at the start of the

experiment also resulted in more positive E_H and accelerated pyrite oxidation. Pyrite

dissolution by "*A. sulfuroxidans*" Y002^T was noted to be about 40% less extensive than

that observed in cultures of *Sb. thermosulfidooxidans*^{T} grown under identical

conditions.

All strains of "*Acidibacillus*" tested were able to catalyse the dissimilatory reduction

of ferric iron under anaerobic conditions (Supplementary Fig. 5), though no reduction

was observed in parallel cultures incubated under micro-aerobic conditions (data not

shown). The two strains of "*A. sulfuroxidans*" displayed the greatest propensity for iron

reduction, and the two Group IB strains the least. None of the isolates was found to

358 reduce elemental sulfur or catalyse the dissimilatory oxidation of elemental hydrogen.

359 Cultures on solid media grown in H_2-enriched or H_2-free atmospheres were identical in

360 size and morphology after protracted incubation, in contrast to those of the positive

361 control acidophile, *Sb. thermosulfidooxidans*.

362

363 *3.5. Utilization of organic carbon*

364

365 All of the novel isolates required a source of organic carbon for growth in liquid

366 media, and yeast extract appeared to be superior to all others tested for this purpose.

367 Biomass yields of both SLC66^T and Y002^T correlated with concentrations of yeast

368 extract (Fig. 3b), though cell numbers of SLC66^T were mostly much greater than those

369 in the equivalent cultures of Y002^T, though this was at least partially due to more

370 pronounced cell aggregation of the latter, as noted previously.

371 Addition of some complex and defined organic compounds to ferrous iron/yeast

372 extract liquid media resulted in increased cell numbers of all of the "*Acidibacillus*"

373 strains, though this was limited in scale (Supplementary Table 2). Comparative data

374 for the mesophilic acidophiles SLC66^T and *Acidiphilium* SJH (Fig. 5) show that

375 numbers of the former were far fewer than those of *Acidiphilium* SJH, grown in identical

376 glucose-containing liquid medium. Also, while all of the glucose provided was utilized

377 in the *Acidiphilium* SJH cultures within 3 days, only ~12% of the glucose in cultures of

378 SLC66^T was metabolized. In the case of Y002^T, numbers were >50% greater in cultures

379 containing glucose than in glucose-free controls, but only ~6% of the available glucose

380 was utilized (data not shown). In the case of strain Y002^T, cell numbers were also

381 significantly greater (by ~55% on day 3 and ~500% on day 7) in iron/yeast extract

382 cultures that contained glucose compared to those that did not, though again the

383 amount of glucose consumed was relatively small (5.5% of that provided;

384 concentrations, compensated for evaporative water loss, falling from 4.90 +/- 0.02 mM

385 on day 0 to 4.62 +/- 0.06 on day 7).

386   Figure 6 compares numbers of all eight isolates grown in different inorganic and

387   organic-amended liquid media. While there were some differences displayed between

388   the "*Acidibacillus*" strains, there were some interesting general trends, including the

389   observation that greater amounts of ferrous iron did not generally result in enhanced

390   cell yields in organic carbon-free media, though they did in most cases where glucose

391   was also present. Cell numbers of all of the strains were also much greater in 1 mM

392   ferrous iron medium containing 0.005% yeast extract than in those containing 5 mM

393   glucose, even though the amount of organic carbon present (~ 25 mg/L, compared to

394   360 mg/L) was much less in the former.

395

396   *3.6. Tolerance of "Acidibacillus" spp. to some transition metals, aluminium and sodium*

397   *chloride*

398

399   The tolerance of the six strains of "*A. ferrooxidans*" and the two strains of "*A.*

400   *sulfuroxidans*" to aluminum and selected transition metals are shown in Table 2.

401   Strains belonging to "*A. ferrooxidans*" had in general a higher tolerance towards most

402   of the metals tested than the two "*A. sulfuroxidans*" isolates. The proposed type stain

403   of "*A. ferrooxidans*" (SLC66) had a lower MIC for copper than the other strains

404   belonging to the same species. Strain BSH1 displayed less tolerance of copper but

405   had by far a higher tolerance threshold for cobalt than other strains of this species.

406   Strains of "*A. sulfuroxidans*" were far more sensitive to copper and cobalt than the two

407   "*A. ferrooxidans*" strains None of the isolates were halotolerant, though both strains of

408   "*A. sulfuroxidans*" were able to grow in liquid media containing higher concentrations

409   of sodium chloride than the six strains of "*A. ferrooxidans*" tested (Table 2).

410

411   *3.7. Genome compositions, and carbon- and nitrogen-fixation genes*

412

15

413   Data from the preliminary annotation of the genomes of the three strains of

414   "*Acidibacillus*" (SLC66[T], ITV01 and Y002[T]) showed that they had GC contents of 52%,

415   50% and 46%, respectively. The current assemblies contain 3.03 Mbp for SLC66[T],

416   3.23 Mbp for ITV01 and 2.70 Mbp for Y002[T]. The genomes of strains SLC66[T] and

417   ITV01 contained genes with relatively low (34% and 36%, respectively) similarity to

418   the *cbbL* gene (which encodes the large subunit of RuBisCO form IA, involved in CO_2

419   assimilation) but not the *cbbM* gene (which encodes the large subunit for type II

420   RuBisCO). No gene identified as being necessary for nitrogen fixation were identified

421   in the three genomes, but BLAST searches revealed a low sequence identity (36%)

422   for the gene encoding for rusticyanin (a protein involved in ferrous iron oxidation in the

423   iron-oxidizing acidithiobacilli and some other acidophiles) in the genomes of the three

424   "*Acidibacillus*" strains.

425

426

427   **4. Discussion**

428

429   The bacteria described in this report were isolated from geothermal and mine-

430   impacted sites from different parts of the world. The fact that other closely related

431   acidophiles have also been isolated from sites in Germany [4, 5] and clones identified

432   in samples in China [6], Japan [7] and Argentina [8] suggests that "*Acidibacillus*" spp.

433   are very widely distributed in extremely acidic environments. The first reported strains

434   ("SLC series") were all described as obligately heterotrophic, mesophilic iron-

435   oxidizing acidophiles, and were noted to be only distantly phylogenetically related to

436   other Firmicutes [3]. Six other phylogenetically-related isolates that have since then

437   been added to the *Acidophile Culture Collection* at Bangor University since then were

438   studied alongside two of the original "SLC series" strains in the present study. While

439   the eight strains shared a number of physiological traits, there were also some

440   significant differences.

16

441   Comparison of 16S rRNA gene sequences clearly separated the eight strains

442   studied, at the genus level, from currently classified acidophilic Firmicutes. While they

443   formed a distinct clade, the fact that two of the isolates (Y002^T and Y0010) shared

444   only 94% gene similarity with the other six confirmed that the isolates comprised two

445   distinct species. Subsequent laboratory tests showed that these phylogenetic

446   relationships were reflected in some marked differences in some key physiological

447   characteristics, with the larger group being mesophilic iron-oxidizers ("*A.*

448   *ferrooxidans*") and the smaller group moderately thermophilic iron- and sulfur-

449   oxidizers that were more tolerant of extreme acidity ("*A. sulfuroxidans*"). Interestingly,

450   phylogenetic analysis separated two strains of the larger group (strains ITV01 and

451   Gal-G1) from the other four strains, even though the six strains shared ~99% gene

452   similarity, and this was also reflected in some minor differences in their physiologies.

453   For example, strains ITV01 and Gal-G1 grew at 40⁰C, while the other four strains of

454   "*A. ferrooxidans*" did not, both grew at pH 1.75 while only one Group IA strain (BSH1)

455   grew at this pH value, and strains ITV01 and Gal-G1 were also the least effective of

456   all eight strains at reducing ferric iron.

457   The three major physiological traits shared by all of the isolates studied were: (i)

458   optimum growth at extremely low (<3) pH, (ii) the ability to catalyse the dissimilatory

459   oxidation of ferrous iron, and (iii) a requirement of organic carbon for growth. In

460   contrast to *Sulfobacillus* spp. [16], none of the isolates used molecular hydrogen as

461   an energy source. All of the isolates also catalysed the dissimilatory reduction of

462   ferric iron under anoxic conditions though, as noted, this was limited in the case of

463   the two Group IB strains, and it was not ascertained whether the bacteria could grow

464   by ferric iron respiration. Ferrous iron is a widely used electron donor among

465   acidophilic prokaryotes, due to it often being present in large concentrations in low

466   pH environments, and also chemically stable at pH < 3 [1]. The ability to oxidize

467   ferrous iron does not necessarily imply that microorganisms are able to conserve the

468   energy from this reaction. However, the observation that cell numbers of both

469 SLC66^T and Y002^T increased in parallel with the amount of iron oxidized (in organic-

470 lean media) strongly suggests that this is the case with "*Acidibacillus*" spp.. The

471 specific rates of ferrous iron oxidation recorded for "*Acidibacillus*" spp. (36.1 +/- 3.4

472 mg min^{-1} mg protein^{-1} for SLC66^T, and 48.5 +/- 1.3 mg min^{-1} mg protein^{-1} for Y002^T)

473 were much lower than those reported for other oxidizing acidophiles (192 – 484 mg

474 min^{-1} mg protein^{-1} for chemolithotrophic *Leptospirillum* and *Acidithiobacillus* spp., and

475 236 – 449 mg min^{-1} mg protein^{-1} for *Sulfobacillus* spp. [15]).

476 　　The two "*A. sulfuroxidans*" strains (Y002^T and Y0010) also catalysed the

477 dissimilatory oxidation of both elemental sulfur and tetrathionate, and it was assumed

478 (though not confirmed) that they also conserved the energy from these reactions.

479 The ability to oxidize both ferrous iron and sulfur is not uncommon among

480 chemolitho-autotrophic and chemolitho-heterotrophic acidophiles, and has been

481 reported for some *Acidithiobacillus* spp. (*At. ferrooxidans, At. ferridurans, At.*

482 *ferrivorans* and *At ferriphilus.* [26]), *Acidihalobacter prosperus* [27], *Acidiferribacter*

483 *thiooxydans* [28] and "*Acidithiomicrobium*" [29]. Among the acidophilic Firmicutes, all

484 classified *Sulfobacillus* spp. (*Sb. thermosulfidooxidans, Sb. acidophilus, Sb.*

485 *thermotolerans, Sb. benefaciens,* and *Sb. sibiricus*) [1], as well as *Alicyclobacillus*

486 *tolerans* and *Alb. aeris* [30] can oxidize both ferrous iron and reduced sulfur. Other

487 species of acidophilic bacteria (e.g. *Leptospirillum ferrooxidans, Ferrimicrobium*

488 *acidiphilum, Acidimicrobium ferrooxidans, Acidithrix ferrooxidans* and "*Ferrovum*

489 *myxofaciens*") catalyse the dissimilatory oxidation of ferrous iron but not sulfur [1] as

490 was the case with the six strains of "*A. ferrooxidans*".

491 　　Yeast extract acted as both an energy and carbon source for these bacteria, as

492 evidenced by: (i) growth continuing in cultures well after all of the ferrous iron had

493 been depleted, (ii) growth yields correlating with concentrations of yeast extract

494 provided (in cultures containing relatively little ferrous iron) and (iii) active growth in

495 yeast extract/ferric iron media. "*Acidibacillus*" spp. can therefore be classified as

496 facultative chemolitho-heterotrophs (i.e. they can obtain energy from both inorganic

497 and organic electron donors but require an organic carbon source). It was noted that

498 cell yields of "*A. ferrooxidans*" SLC66^T tended to be greater than those of "*A.*

499 *sulfuroxidans*" Y002^T in liquid media that contained the same concentrations of yeast

500 extract. This was thought to be due, at least in part, to strain SLC66^T being able to

501 utilize a wider range of organic compounds present in this complex material, as both

502 carbon and energy sources, than strain Y002^T. Cell yields of both SLC66^T and Y002^T

503 were significantly greater when glucose was added to ferrous iron/yeast medium,

504 suggesting that it was metabolized to some extent. In contrast to those of the

505 heterotrophic acidophile, *Acidiphilium* SJH, only small amounts (5.5 - 6%) of the

506 available glucose was utilized in these cultures, suggesting that this compound

507 served as a carbon source, but not an energy source, for "*Acidibacillus*" spp., and

508 that growth was ultimately limited by the energy source available (ferrous iron, and

509 that fraction of yeast extract that could be broken down to generate ATP) in these

510 cultures. Glucose could also act as a carbon source for "*Acidibacillus*", but was far

511 less effective than yeast extract. For most strains, growth yields in glucose-containing

512 media was limited by the availability of ferrous iron, suggesting again that the later

513 served as the sole or main energy source, and glucose as the carbon source for

514 these bacteria. Further annotation of the genomes of these bacteria will undoubtedly

515 throw more light on the biochemical constraints that restrict glucose utilization by

516 these novel acidophiles. The information available so far confirms that "*Acidibacillus*"

517 spp. are not diazotrophic. Genes with relatively low sequence identity to *ccbL* (though

518 not *cbbM*) gene were found in the genomes of two of the three sequenced bacteria,

519 though extensive laboratory tests confirmed that none of the strains could grow in the

520 absence of an organic form of carbon. Intriguing is the fact that all three bacteria

521 appear to contain a gene that is related to that which encodes for rusticyanin, a

522 protein known to be involved in iron oxidation in *Acidithiobacillus* spp. but not, so far,

523 in the iron-oxidizing Firmicutes.

In theory, any acidophilic bacterium that catalyses the dissimilatory oxidation of ferrous iron should accelerate the oxidative dissolution of pyrite, as ferric iron is the primary oxidant of this mineral in acidic liquors [31]. This was the case with both "*Acidibacillus*" type species, though it was not immediately apparent for strain Y002[T]. Autoclaving pyrite in the presence of yeast extract (as is common practice in the Bangor research laboratories, and has not previously proven problematic) generated some, currently unidentified, by-product that inhibited growth and iron oxidation by strain Y002[T], though not by strain SLC66[T] and the positive control Firmicute, *Sb. thermosulfidooxidans*. Adding sterile yeast extract after heat-sterilization of pyrite eliminated this impediment, though pyrite leaching by Y002[T] was far less effective, and appeared to require more yeast extract, than that by *Sb. thermosulfidooxidans*.

The major industrial use of iron- and sulfur-oxidizing acidophilic bacteria is in the commercial bio-processing of sulfide mineral ores to extract and recover base and precious metals ("biomining" [32]). Whether or not "*Acidibacillus*" spp. have a potential role in mineral bioleaching consortia has yet to be evaluated. Both species could, in theory, carry out two critical roles (those of regenerating ferric iron and removing potentially inhibitory organic carbon) and "*A. sulfuroxidans*" strains could also contribute to the process by generating sulfuric acid. Another important required characteristic – that of being able to tolerate highly elevated concentrations of transition and other metals – also appears to be adequate, as the data obtained showed that metal tolerance is similar to that of most of the iron-oxidizing *Acidithiobacillus* spp.. A more significant constraint, however, may be their tolerance to extreme acidity, as many biomining practices operate at pH values <2, and often (in stirred tanks) at ~ pH 1.5. Mesophilic "*A. ferrooxidans*" may, however, play a more important role in the natural attenuation of acidic (pH >2) ferruginous mine waters by catalysing the oxidation of ferrous iron and thereby facilitating the hydrolysis and precipitation of ferric iron [33].

552 **Conflict of interest**

553

554   There are no conflicts of interest.

555

556 **Acknowledgements**

557

560

561 **Appendix A. Supplementary data**

562

563   Supplementary data related to this article can be found at http://xxxx

564

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659

660

661

662 Table 1

663 Sites of origin of bacteria identified as *Acidibacillus* spp..

Isolate	Origins and dates of isolation	Reference
SLC66[T] & SLC40	Experimental system used to accelerate the oxidation of mine waste (pH 2.9, 25°C); Utah (1994)	[3]
Gal-G1	Geothermal area (pH 3.0, 80°C); Soufriere Hills, Montserrat, W.I. (1996)	[23]
Y002[T] & Y0010	Geothermal area (pH 2.7, 30-60°C) Yellowstone National Park, Wyoming (2000)	[24]
ITV01	Stream draining waste rock at a copper mine (pH 4.9, 32°C); Brazil (2013)	I. Ñancucheo et al. (unpublished)
BSH1	Constructed wetland receiving coal mine drainage (pH 7.0, 14°C); England (2014)	C. Falagan et al. (unpublished)
GS1	Sediment in a pit lake at an abandoned copper mine (pH 3.8, 23°C); Spain (2015)	[25]

664

665

Table 2

Tolerance of strains of "*Acidibacillus*" to selected metals and chloride. The values (in mM) shown are minimum

inhibitory concentrations (MICs) and those in parentheses are the highest concentrations of that metals/chloride

where growth was observed.

	Cu	Zn	Ni	Co	Al	Mn	Fe(II)	Mo	Cl
SLC66^T	300 (200)	1000 (800)	400 (200)	300 (200)	800 (600)	>800	800 (600)	<0.05	100 (50)
SLC40	600 (400)	1000 (800)	400 (200)	300 (200)	800 (600)	>800	600 (400)	<0.05	50 (25)
BSH1	400 (200)	1000 (800)	400 (200)	>600	1000 (800)	800 (600)	800 (600)	<0.05	100 (50)
GS1	800 (600)	800 (600)	400 (200)	300 (200)	600 (400)	800 (600)	600 (400)	<0.05	100 (50)
ITV01	600 (400)	1000 (800)	400 (200)	300 (200)	800 (600)	>800	800 (600)	<0.05	100 (50)
G1	800 (600)	600 (400)	400 (200)	300 (200)	800 (600)	600 (400)	800 (600)	<0.05	50 (25)
Y002^T	100 (50)	200 (100)	200 (100)	50 (30)	400(300)	800 (600)	600 (400)	<0.05	250 (100)
Y0010	300 (200)	200 (100)	300 (200)	150 (100)	600 (400)	400 (300)	600 (400)	<0.05	250 (100)
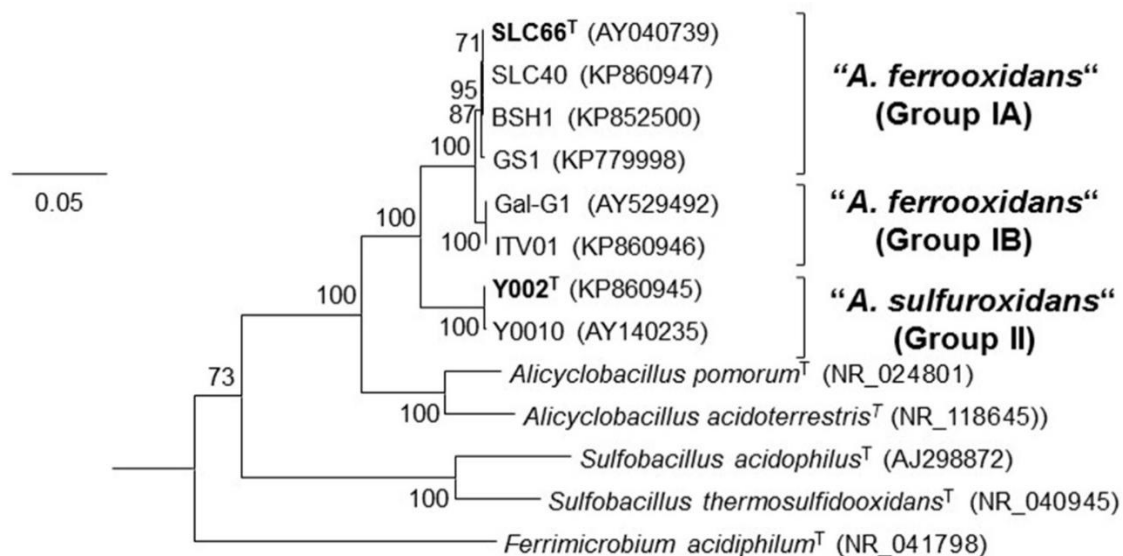
Fig. 1. Neighbour-joining phylogenetic tree derived from 16S rRNA gene sequence data showing the relationship of "*Acidibacillus ferrooxidans*" and "*A. sulfuroxidans*" to other Gram-positive acidophiles. GenBank accession numbers are given in parentheses for each strain. The tree was rooted with *Acidianus brierleyi*[T] (not shown). Bootstrap values are given at the respective nodes.
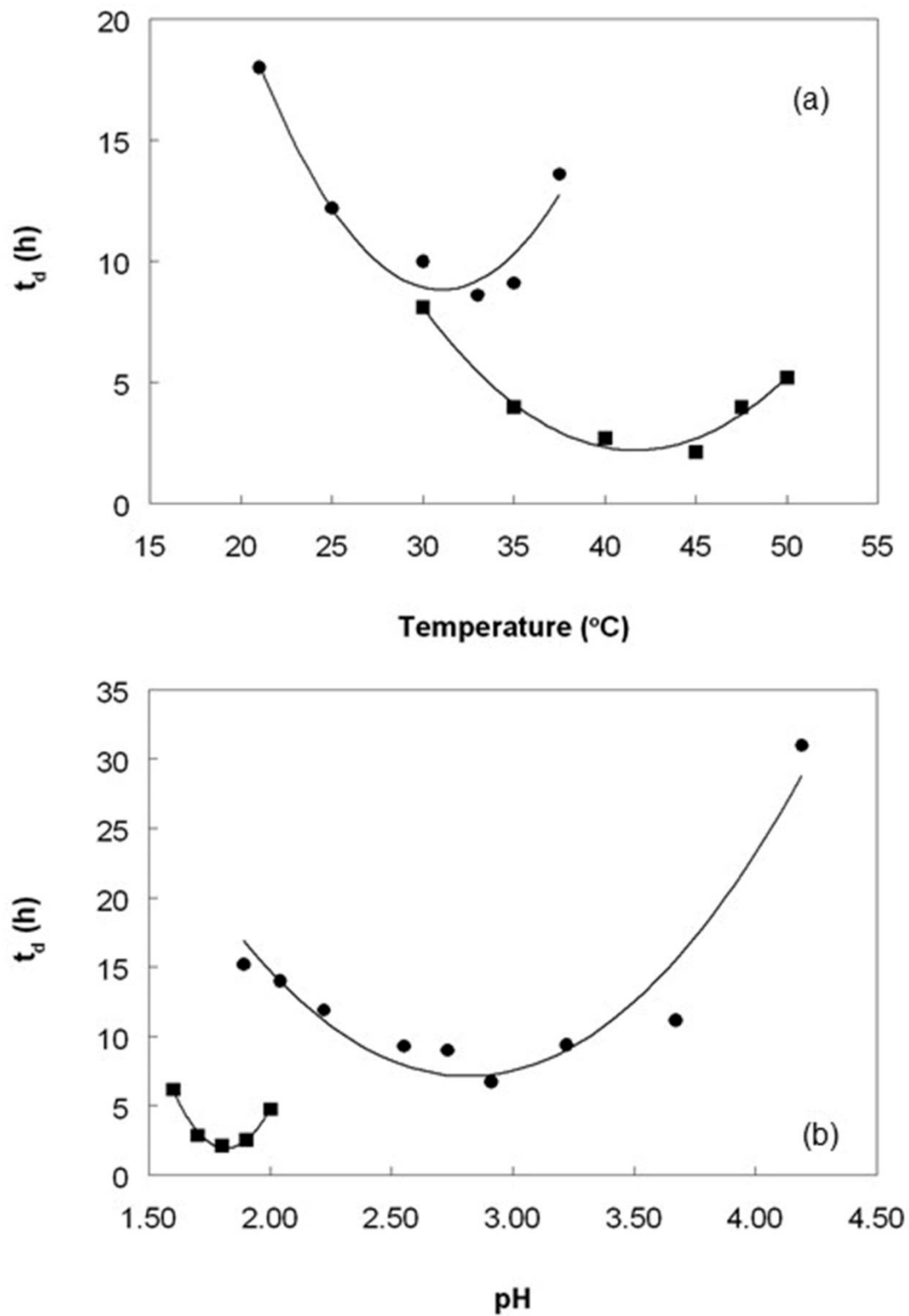
Fig. 2. Effects of (a) temperature and (b) pH on culture doubling times of "*A. ferrooxidans*" SLC66[T] (●) and "*A. sulfuroxidans*" Y002[T] (■).
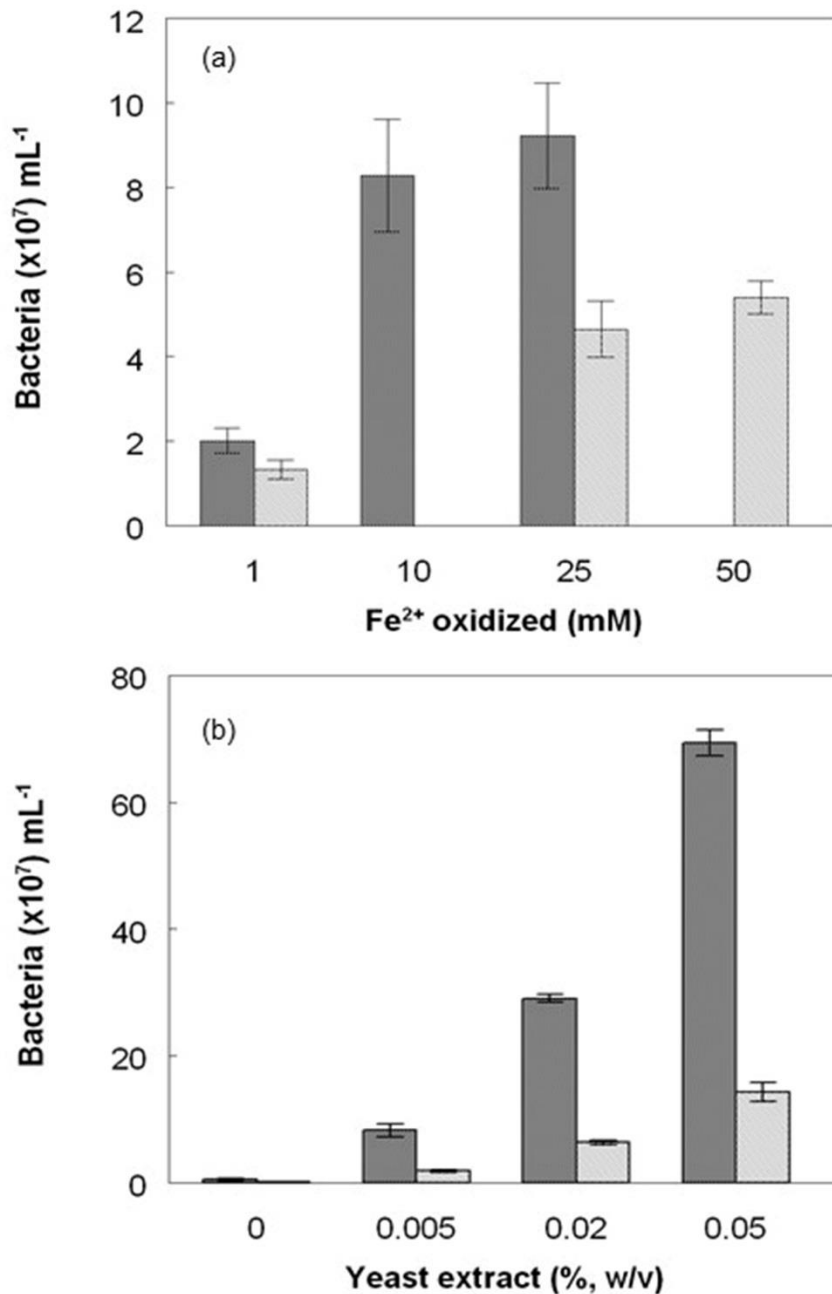
Fig. 3. (a) Effect of ferrous iron concentrations, and (b) yeast extract concentrations on cell numbers of *A. ferrooxidans* SLC66[T] (dark shaded bars) and *A. sulfuroxidans* Y002[T] (light shaded bars). Cultures were grown either at a fixed (0.005%, w/v) yeast extract concentration and variable amounts of ferrous iron and cells counted when all of the iron had been oxidized (a), or at a fixed (10 mM) ferrous iron concentration and variable amounts of yeast extract and maximum cell numbers (found after 2 days incubation in cultures of Y002[T], and 4 days in cultures of SLC66[T]) recorded. Bars indicate mean values and the error bars data range (n = 2).
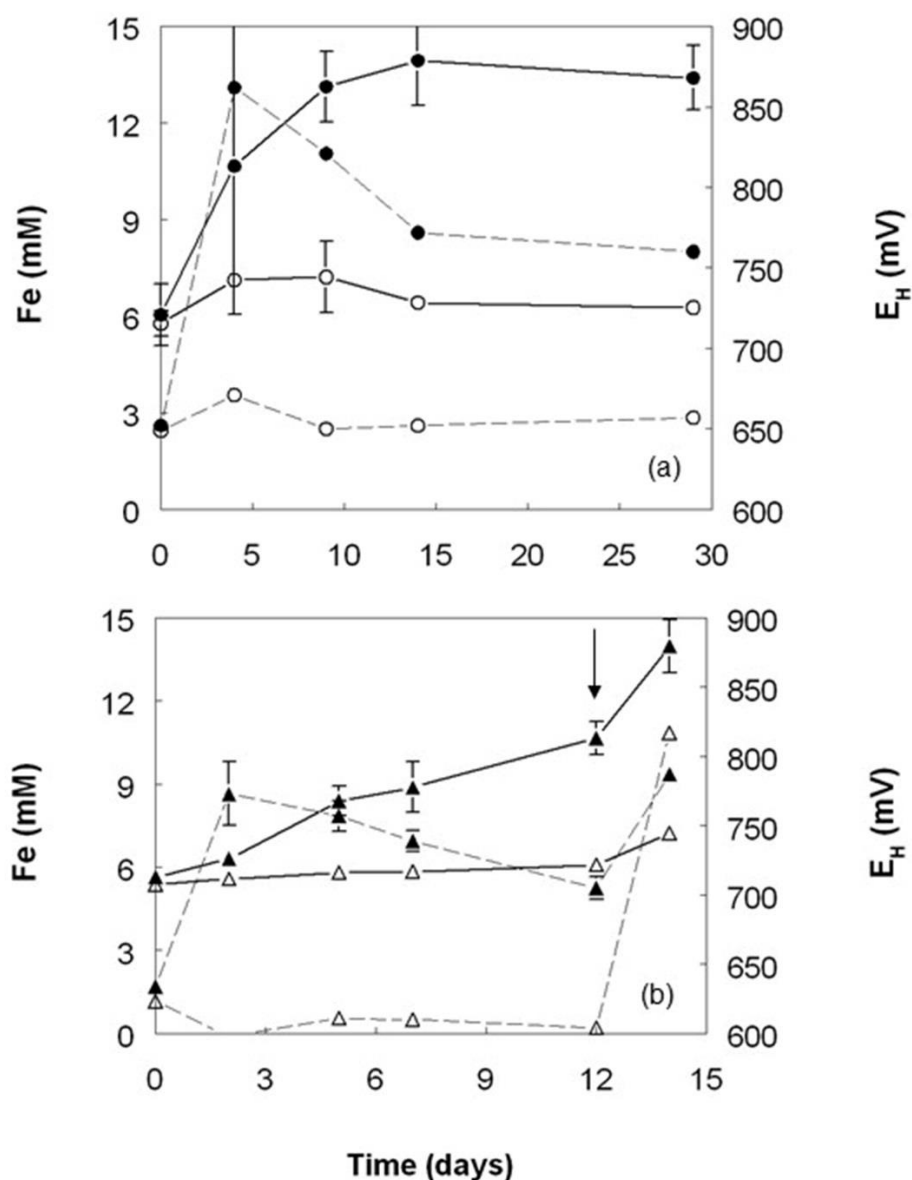
Fig. 4. Oxidative dissolution of pyrite by (a) "*A. ferrooxidans*" SLC66[T] (at 30ºC) and (b) "*A. sulfuroxidans*" Y002[T] (at 45ºC) in the presence and absence of 0.02% yeast extract. Key: SLC66[T] with (●) and without (o) yeast extract; Y002[T] with (▲) and without (Δ) yeast extract. Solid lines show total soluble iron concentrations and broken lines redox potentials (E_H) values (symbols show mean values and error bars range values of replicate cultures). The arrow in (b) shows the point at which (day 12) sterile yeast extract was added to both the yeast extract-free and yeast extract-containing cultures. Non-inoculated control cultures showed little change in total soluble iron and redox potential during the time course of the experiments. Data points indicate mean values and error bars data range (n = 2).
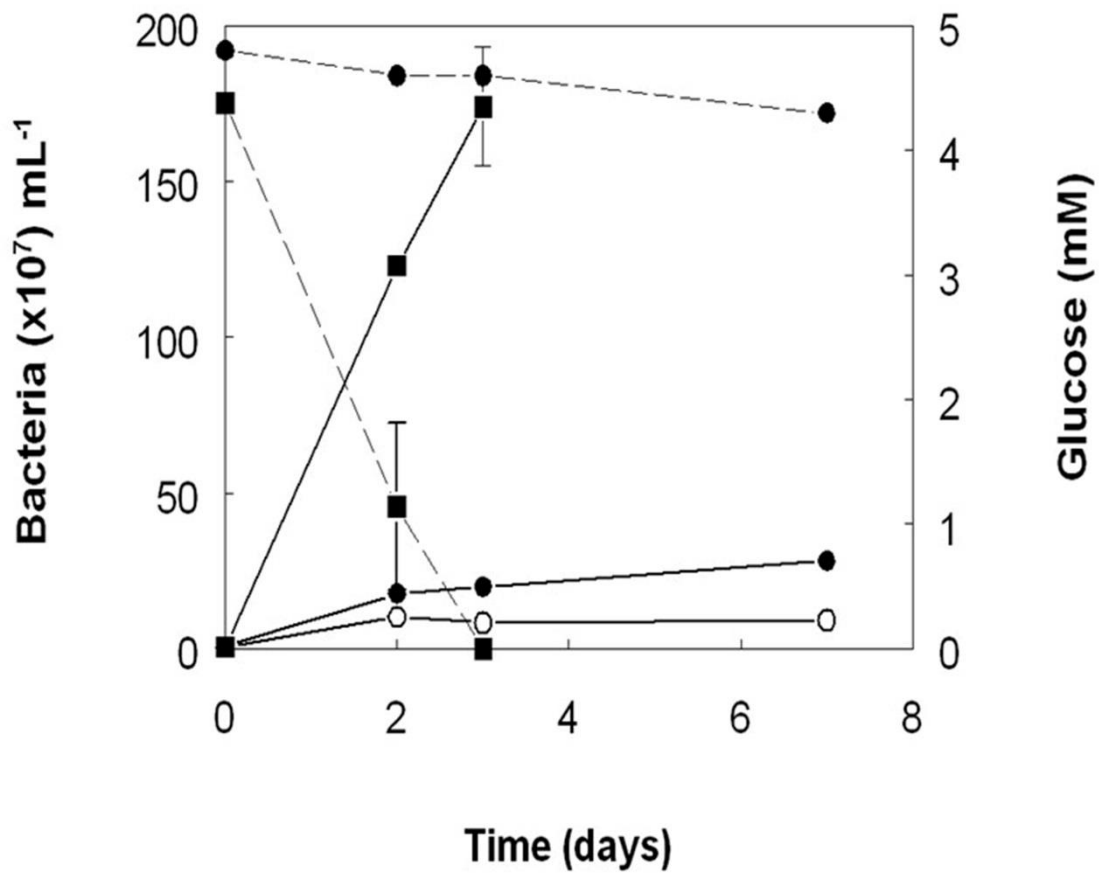
Fig. 5. Comparison of changes in cell numbers (solid lines) and glucose concentrations (broken lines) in cultures of "*A. ferrooxidans*" SLC66[T] and *Acidiphilium* SJH. Key: cell numbers and glucose concentrations in cultures of "*A. ferrooxidans*" SLC66[T] grown with (●) or without (o) 5 mM glucose; cell numbers and glucose concentrations in cultures of *Acidiphilium* SJH grown with 5 mM glucose (■).Data points indicate mean values and error bars data range (n = 2).
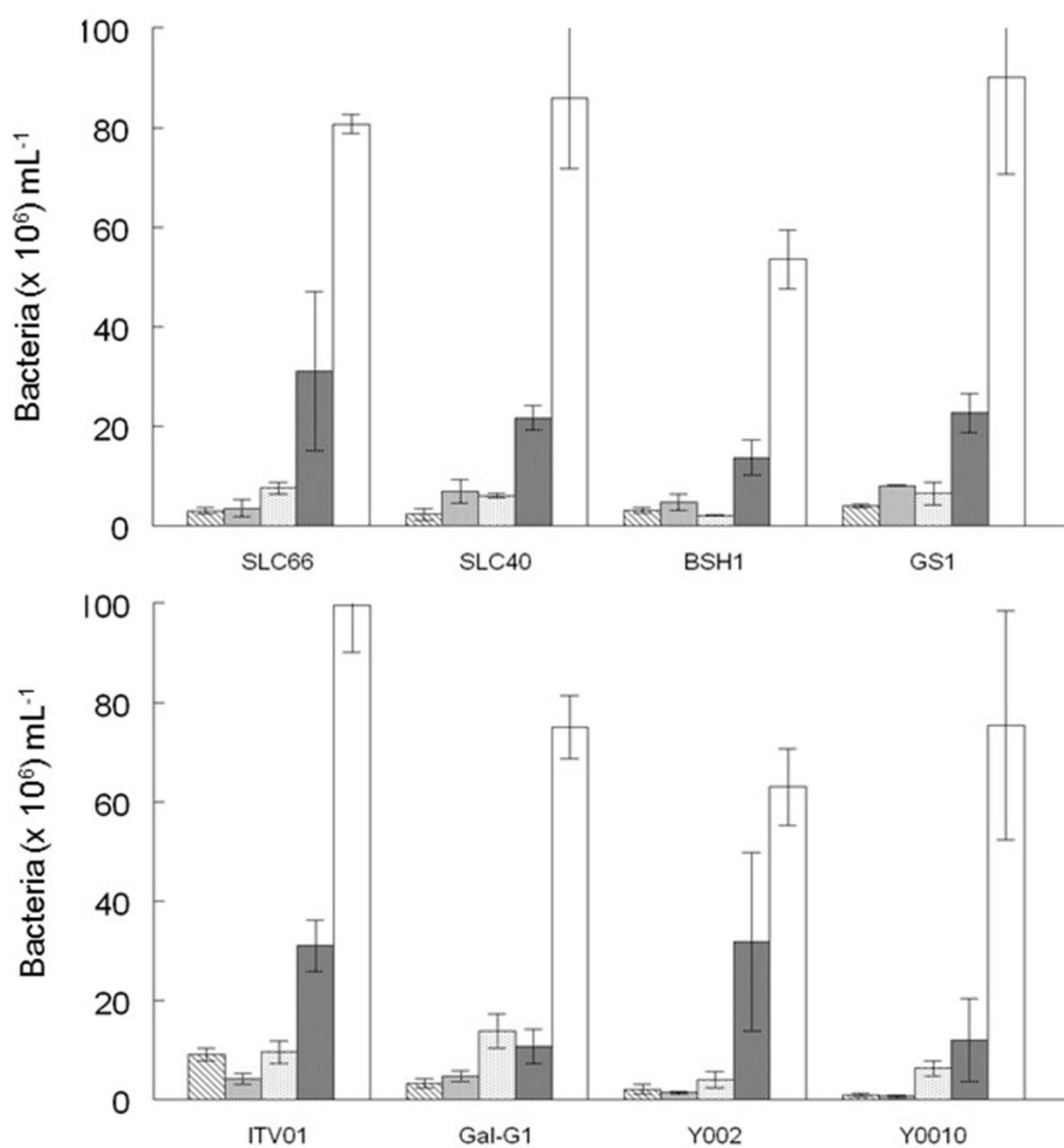
Fig. 6. Cell counts of "*Acidibacillus*" spp. grown in different liquid media; Key: ▨ 1 mM Fe^{2+}; ▨ 20 mM Fe^{2+}; ▨ 1 mM Fe^{2+}/5mM glucose; ▨ 20 mM Fe^{2+}/5 mM glucose; ☐ 1 mM Fe^{2+}/0.005% yeast extract. Bars indicate mean values and error bars standard deviations (n = 3).