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# Acidithiobacillus

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1 Gbm01079

2 *Genus Acidithiobacillus*

3  
4 **Defining publication:** Kelly and Wood 2000, 513<sup>VP</sup>.

5  
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12  
13 **Etymology:** A.ci.di'thi.o.ba.cil'lus. L. masc. adj. *acidus*, sour, tart; Gr. neut. n. *theîon*, sulfur, brimstone  
14 (transliterated to L. neut. n. *thium*); L. masc. n. *bacillus*, a short rod, a short wand; N.L. masc. n.  
15 *Acidithiobacillus*, acid-loving sulfur rodlet.

16  
17 **Abstract:**

18 Cells are short, motile rods with a single polar flagellum. Some strains have an obvious glycocalyx. Gram-  
19 stain-negative. Endospores, exospores and cysts are not produced. Obligate chemolithoautotrophs, with  
20 electron donors including reduced inorganic sulfur species such as thiosulfate, tetrathionate, elementary  
21 sulfur (*viz.*  $\alpha$ -S<sub>8</sub> and  $\mu$ -S<sub>∞</sub>). Some species can also use molecular hydrogen, ferrous iron or metal sulfides  
22 such as pyrite (FeS<sub>2</sub>) as electron donors. Some species are diazotrophic. Heterotrophy, methylotrophy and  
23 the so-called “C<sub>1</sub> autotrophy” are not observed. Carbon assimilated from CO<sub>2</sub> *via* the transaldolase-variant  
24 of the Calvin-Benson-Bassham cycle. Carboxysomes are used for CO<sub>2</sub> concentration. Obligately respiratory,

with molecular oxygen, ferric iron or elementary sulfur as terminal electron acceptors, varying by species. Most strains grow in the range of 20-37 °C, though some have a narrower range and one species is thermophilic. Optimal growth from pH 2.0-5.8 and an overall range of pH -0.6-6.0. The major respiratory quinone is ubiquinone-8 (UQ-8), and some traces of ubiquinone-9 (UQ-9), ubiquinone-7 (UQ-7) and menaquinones (MK) are also found in some species. The dominant fatty acids are palmitic acid (C<sub>16:0</sub>), vaccenic acid (C<sub>18:1</sub>), *cis*-11-cyclopropyl-nonadecanoic acid (C<sub>19:0</sub> *cyclo ω*<sub>8c</sub>), palmitoleic acid (C<sub>16:1</sub>), myristic acid (C<sub>14:0</sub>) and lauric acid (C<sub>12:0</sub>). The dominant polar lipids are cardiolipin, aminolipids, phospholipid, phosphatidylglycerol, phosphatidylethanolamine. The G+C fraction of genomic DNA is around 52.0-63.9 mol%. Form IAc (carboxysomal) and Form II (cytoplasmic) D-ribulose 1,5-bisphosphate carboxylase/oxygenase are used, as are forms *bo*<sub>3</sub> and *bd*-I ubiquinol oxidases and, in the iron-oxidizing species, the *aa*<sub>3</sub>-type cytochrome *c* oxidase. A description of *Acidithiobacillus concretivorus* comb. nov. is also given.

**Keywords:** chemolithoautotroph, thermophile, acidophile, sulfur oxidizer, iron oxidizer

**Description:**

Cells are slender, often short rods 0.4-0.8 × 1.4-1.8 μm. Rapidly motile and usually monotrichous but some taxa are lophotrichous. Gram-stain-negative. Endospores, exospores and cysts are not produced. Volutin granules accumulated in some species. Sulfur-oxidising obligate autotrophs, with some species also using ferrous iron, sulfide minerals and/or molecular hydrogen as electron donors. Iron-oxidizing species are facultative anaerobes, and use ferric iron as terminal electron when elementary sulfur serves as the electron donor, though the remainder of species are obligate aerobes. Diazotrophy is observed in some species. Assimilates carbon *via* the transaldolase variant of the Calvin-Benson-Bassham (CBB) cycle, using form IAq (cytoplasmic), form II (cytoplasmic) or form IAc (carboxysomal) D-ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO). The proton-translocating *bo*<sub>3</sub>-type ubiquinol oxidase and the non-translocating *bd*-I ubiquinol oxidase are used by all species, with the proton-translocating *aa*<sub>3</sub>-type cytochrome *c* oxidase found only in the iron oxidizing taxa.

51 The dominant fatty acids are palmitic acid (C<sub>16:0</sub>), vaccenic acid (C<sub>18:1</sub>), *cis*-11-cyclopropyl-nonadecanoic  
52 acid (C<sub>19:0 cyclo ω<sub>8c</sub></sub>), palmitoleic acid (C<sub>16:1</sub>), myristic acid (C<sub>14:0</sub>) and lauric acid (C<sub>12:0</sub>). The dominant  
53 polar lipids are cardiolipin, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylethanol and  
54 aminolipids.

55  
56 *Type species: Acidithiobacillus thiooxidans* (Waksman and Joffe 1922) Kelly and Wood 2000, 513<sup>VP</sup>  
57 (*Thiobacillus thiooxidans* Waksman and Joffe 1922, 239)

58  
59 Number of species with validly published names: 7.

#### 61 **Family classification:**

62 *Acidithiobacillaceae* (fbm00213)

#### 64 Further Descriptive Information

65 **Nutrition and growth conditions.** All species are obligate acidophiles, with growth optima of pH 2.0–3.4.  
66 Most are mesophilic, with one species (*Acidithiobacillus caldus*) thermophilic. All species grow under air on  
67 tetrathionate or elementary sulfur as electron donors, and a clade of four species (*A. ferrooxidans*, *A.*  
68 *ferrivorans*, *A. ferridurans* and *A. ferriphilus*) can additionally use ferrous iron or metal sulfide minerals as  
69 electron donors. Molecular hydrogen use varies by species, and in some cases by strain. The “iron clade”  
70 can grow anaerobically at the expense of ferric iron if elementary sulfur is the electron donor. The sulfur-  
71 oxidizing species generally tolerate lower pH values (as low as pH -0.6) than the “iron clade” and usually  
72 also tolerate higher pH values, too. This is owing to the pH effects on Fe(II) and Fe(III) redox chemistry and  
73 their solubility: as the pH rises towards neutrality, the Gibbs energy change for ferrous iron oxidation yields  
74 very little energy (see section on **Growth Physiology**), and the ferric iron produced tends to form ferric

75 hydroxide, which reduced oxygen transfer through the medium: as such, growth towards neutrality on iron  
76 is generally very weak, for the few species that tolerate pH so high.

77 An imperative point when cultivating *Acidithiobacillus* spp. is that they must have access to an electron  
78 donor at all times: when this runs out, they will be unable to extrude protons from the cell, and will be killed  
79 as a result. As such, when washing, suspending or storing cells in low pH buffers (*e.g.* 20 mM glycine-HCl,  
80 pH 2.2-3.6), some cell lysis will occur, even at low temperatures. Some workers omit buffers and just use a  
81 dilute sulfuric acid solution of the appropriate pH for washing and resuspending cells, and then transfer them  
82 to *e.g.* 20 mM PIPES-HCl, pH 7.2 immediately prior to lysing for enzyme assays or proteomic work.  
83 Harvesting procedures that avoid loss of biomass in this way are discussed by Silverman and Lundgren  
84 (1959).

85 **Growth physiology.** To maintain pH homeostasis and a cytosol of pH *c.* 6-7, acidophiles must use active  
86 transport to remove protons from the cell against a concentration gradient. One could thus assume that the  
87 maintenance coefficients ( $m_s$ , *cf.* Pirt (1975)) of *Acidithiobacillus* spp. are somewhat higher than  
88 neutrophilic species, given the elevated cost of living at low pH, where ATP is used continually for H<sup>+</sup>  
89 export and both ATP and NAD(P)H are consumed for repair; however, this does not appear to be the case.  
90 In the following examples, growth pH is given in parentheses after the binomial. For *Acidithiobacillus*  
91 *ferrooxidans* (pH 2.5) in thiosulfate-limited chemostats,  $m_s$  is 770  $\mu\text{mol S}_2\text{O}_3^{2-}/\text{g dry biomass/h}$ , and under  
92 tetrathionate limitation,  $m_s$  is 1,030  $\mu\text{mol S}_4\text{O}_6^{2-}/\text{g dry biomass/h}$ . For *Acidithiobacillus thiooxidans* (pH 2.5)  
93 under tetrathionate limitation (pH 2.5),  $m_s$  is 40  $\mu\text{mol S}_4\text{O}_6^{2-}/\text{g dry biomass/h}$ . By comparison, for  
94 neutrophilic (pH 7.6) species under thiosulfate limitation,  $m_s$  values are broadly similar, or if anything,  
95 higher, *viz.* *Paracoccus versutus* (850  $\mu\text{mol S}_2\text{O}_3^{2-}/\text{g dry biomass/h}$ ) and *Annwoodia aquaesulis* (1,270  $\mu\text{mol}$   
96  $\text{S}_2\text{O}_3^{2-}/\text{g dry biomass/h}$ ) [values determined by hyperbolic fit of data curated by Kelly *et al.* (1997), using the  
97 method of Boden and Hutt (2018)]. For comparison, Pirt (1975) reported *Enterobacter cloacae* subsp.  
98 *cloacae* grown under glucose-limitation at pH 7.2 under air had  $m_s$  of 522  $\mu\text{mol glucose/g dry biomass/h}$ ,  
99 which he considered fairly typical for heterotrophs. These data suggest that for *Acidithiobacillus* spp.,  
100 growth at low pH either incurs no greater maintenance costs, or that these costs have been overcome by their  
101 evolutionary adaptations to low pH. For example, substrate-level phosphorylation may account for a fraction

of ATP biosynthesis, whereas the neutrophilic chemolithoautotrophs given as examples above use only oxidative phosphorylation. *Acidithiobacillus* spp. can also make use of the high extracellular proton concentration to form a proton gradient for ATP biosynthesis, and thus probably don't need to rely on proton motive force ( $\Delta p$ ) generated through electron transport as heavily as other organisms (Ingledeew, 1982) – they thus have three potential routes of ATP generation: i) substrate-level phosphorylation; ii)  $\Delta p$  formed through proton extrusion to the periplasm during electron transport, fueling ATP synthase, and/or iii)  $\Delta p$  formed by external proton concentrations, fueling ATP synthase.

Proteomic work on *Acidithiobacillus caldus* at pH 1.1, 2.5 and 4.0 has shown that some acid resistance systems such as peptidyl-glutamate 4-carboxylase (EC 4.1.1.90) are only expressed at pH 1.1 (Mangold *et al.*, 2013), thus the chemostat data given above at pH 2.5 may not represent the organism needing to make use of the full gamut of acid resistance systems and thus  $m_S$  may be much higher at pH 1.1 and below. Maximum specific molar growth yields ( $Y_{MAX}$ ) are about 12.1 g dry biomass/mol  $S_4O_6^{2-}$  for *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans*, indicating a similar means of energy conservation from tetrathionate. Similar yields are found in the neutrophilic *Halothiobacillus* spp. (gbm01133) grown at pH 7. The  $Y_{MAX}$  for *A. ferrooxidans* on thiosulfate is 8.2 g dry biomass/mol  $S_2O_3^{2-}$ , which is, again, broadly similar to that of *Halothiobacillus neapolitanus* (gbm01133), but much lower than those of *Thiobacillus* spp. (gbm00969) or *Annwoodia* spp. (gbm0161), for instance (data from Kelly *et al.*, 1987). It is difficult to draw conclusions from these data as there are so many gaps in our understanding of the fundamental physiology of these organisms, but it would indicate a great variation in energy conservation or anabolic costs.

It is also informative to compare the relative 'nutritional value' of various electron donors with respect to ATP formation. The standard Gibbs energy changes ( $\Delta G^\circ$ ) for their oxidations by molecular oxygen (determined *de novo* by RB) are as follows. The oxidation of ferrous iron to ferric hydroxide ( $Fe(OH)_3$ ) is given for comparison as this is the dominant reaction at higher pH values, *versus* the oxidation to ferric sulfate at low pH:



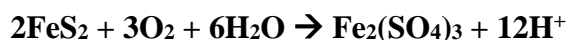
$$\Delta G^\circ = -733.28 \text{ kJ/mol thiosulfate}$$



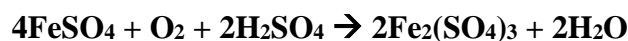
$$\Delta G^\circ = -1,244.78 \text{ kJ/mol tetrathionate}$$



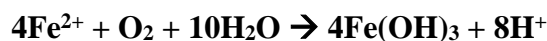
$$\Delta G^\circ = -3,623.36 \text{ kJ/mol orthorhombic cyclooctasulfur}$$



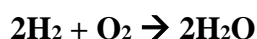
$$\Delta G^\circ = -243.06 \text{ kJ/mol pyrite}$$



$$\Delta G^\circ = -71.55 \text{ kJ/mol ferrous sulfate}$$

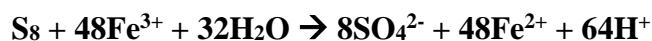


$$\Delta G^\circ = -24.78 \text{ kJ/mol ferrous iron}$$

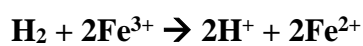


$$\Delta G^\circ = -474.36 \text{ kJ/mol molecular hydrogen}$$

For anaerobic respiration, the  $\Delta G^\circ$  values for the oxidation of elementary sulfur or molecular hydrogen by ferric iron are lower than their aerobic counterparts, as one would expect:



$$\Delta G^\circ = -1,932.24 \text{ kJ/mol orthorhombic cyclooctasulfur}$$



$$\Delta G^\circ = -148.54 \text{ kJ/mol molecular hydrogen}$$

The method of Kelly (1990), elaborated on in the *Thermithiobacillus* (gbm01080) chapter, allows a comparison of the thermodynamic maxima for ATP formation from these data. As such, we can see that on a molar basis and for aerobic growth, elementary sulfur is in theory the best electron donor, yielding about 79 mol ATP/mol, followed by tetrathionate at 27 mol/mol, assuming only forward electron transport and perfect coupling *etc* – however, if we normalize the sulfur species to “per sulfur atom”, the ATP yields are about the same for thiosulfate, tetrathionate and elementary sulfur (7.9, 6.7 and 9.8 mol/mol, respectively). In contrast, ferrous iron presents as a particularly weak electron donor at pH 2.0 (at which these organisms grow), yielding about 0.7 mol ATP/mol. Thus, for an organism on Fe(II) to obtain as much ATP as on  $\text{S}_8$ , about 113 mol Fe(II) must be oxidized *versus* one of  $\text{S}_8$ . For comparison, the growth of a generalist on glucose by oxidation (-2,880 kJ/mol, about 62 mol ATP/mol) or fermentation (-218 kJ/mol, about 4.7 mol ATP/mol), it can be seen that most sulfur compounds could in theory yield broadly similar amounts of ATP to glucose oxidation, but iron gives much less than even glucose fermentation. For anaerobic oxidation of

161 elementary sulfur, the maximum amount of ATP formed (43 mol ATP/mol) is about half of that for the  
 162 aerobic oxidation (80 mol ATP/mol), which correlates to the much lower  $Y$  for ferric iron respiration.

163 The specific molar growth yields ( $Y$ ) of *Acidithiobacillus* spp. relate not only to the energy output of  
 164 substrate oxidation but also the energy demands of CO<sub>2</sub> fixation, *via* the transaldolase-variant Calvin-  
 165 Benson-Bassham (CBB) cycle, this is much higher than *via* Krebs' cycle *etc* in a generalist (Kelly, 1990):



167 The NADPH requirement is particularly difficult to overcome for an obligate autotroph growing on electron  
 168 donors other than molecular hydrogen, since all of this NADPH must be formed from NADH made *via*  
 169 reverse electron transport. The fraction of  $\Delta p$  and thus substrate consumed for NADH production has not  
 170 been estimated in a wide range of organisms but figures in the range of 3-5 % of substrate being oxidized for  
 171 NADH production are probably typical based on current understanding, but this will vary by substrate,  
 172 position of coupling of its oxidation to the respiratory chain *etc*. Electrons from substrates that couple at  
 173 cytochrome *c* have to pass two translocation sites to yield NADH, whereas those that couple at the quinone  
 174 pool only have to pass one – if all translocation sites were equal, we would assume substrates of the latter  
 175 group would not be oxidized as much in order to provide NADH as those in the former.

176 **Metabolism.** Although recent advances have been made, the sulfur-oxidation pathways have still not been  
 177 fully elucidated and probably vary between species, but recent advances have been made. Recent studies  
 178 have indicated that monosulfanemonosulfonates play key roles in the oxidation of sulfur from pyrite (FeS<sub>2</sub>),  
 179 based on studies of an *A. ferrooxidans* strain isolated from a mine-drainage pond in China (Tu *et al.*, 2017).  
 180 These somewhat arcane, unstable oxyanions have the general formula S<sub>*n*</sub>O<sub>3</sub><sup>2-</sup>, the simplest of which is  
 181 monosulfanemonosulfonate, better known as thiosulfate, S<sub>2</sub>O<sub>3</sub><sup>2-</sup>. Whilst thiosulfate is very stable, the  
 182 remainder of the series are very unstable, but may be conjugated to protein-carriers *in vivo* to afford  
 183 stability. It is not yet known if the monosulfanemonosulfonates have roles in the oxidation of other sulfur  
 184 species. Similarly, it is also not known if their role during ferric iron respiration of sulfur in anoxia (in which  
 185 they are more stable) is more significant than in the aerobic oxidation of sulfur.

186 The oxidation of S<sub>8</sub> in *A. ferrooxidans* has recently been elucidated in more detail by Wang *et al.* (2019).

187 Briefly, S<sub>8</sub> rings react with thiol groups on cysteine residues on an outer membrane protein, forming H<sub>2</sub>S



188 and sulfite in the periplasm. The former is oxidized by sulfide:quinone oxidoreductase (Sqr, EC 1.8.5.4) on  
189 the inner membrane, yielding polysulfane ( $S_n^{2-}$ ) which reacts with sulfite present to form thiosulfate  
190 (polysulfanes are sometimes called “polysulfides” in the literature, but the latter strictly refers to  
191 organosulfur compounds of structure  $R_1-S_n-R_2$ ). Thiosulfate is oxidized by the quinone-linked thiosulfate  
192 dehydrogenase (Tqo, EC 1.8.5.2), forming tetrathionate and donating electrons to ubiquinone (this could  
193 potentially also be catalyzed by the cytochrome *c*-linked thiosulfate dehydrogenase (TsdA, EC 1.8.2.2),  
194 activity of which has been recorded in this genus (Silver and Lundgren, 1968), but no gene homologs are  
195 found). Tetrathionate is cleaved into sulfate and polysulfane ( $S_n^{2-}$ ) by tetrathionate hydrolase (TetH, EC  
196 3.12.1.B1). Polysulfane chains react with glutathione (GSH), forming GSSH, which is oxidized in the  
197 cytoplasm by a series of proteins comprising the sulfur transporters TusA and DsrE, thiosulfate  
198 sulfurtransferase (rhodanese, Rhd, EC 2.8.1.1), persulfide dioxygenase (Sdo, EC 1.13.11.18) and a  
199 heterodisulfide reductase-like complex (Hdr), forming GSH and sulfite, and donating electrons to  
200 ubiquinone. This sulfite is conjugated to adenosine 5'-monophosphate (AMP) forming adenosine 5'-  
201 phosphosulfate (APS), catalyzed by APS reductase (Apr/Aps, EC 1.8.99.2). APS is then lysed by sulfate  
202 adenylyltransferase (Sat, EC 2.7.7.4), forming sulfate, ATP and protons. These terminal reactions from  
203 sulfite to sulfate *via* APS reductase *etc* provide ATP by substrate-level phosphorylation: this is distinct from  
204 *Thermithiobacillus* spp. in which substrate-level phosphorylation does not occur (Wood and Kelly, 1986).  
205 Ubiquinol formed during the earlier steps donates electrons to the *bo*<sub>3</sub>-type ubiquinol oxidase (or, if  
206 respiration is ‘backed-up’ owing to a paucity of ADP, and  $\Delta p$  is very high, to the *bd*-I type ubiquinol  
207 oxidase). Electrons from ubiquinol can also be transported in the reverse direction at the expense of  $\Delta p$ ,  
208 generating NADH.

209 Elementary sulfur oxidation in *A. caldus* (Wang *et al.*, 2019) is quite different, after the initial opening of the  
210  $S_8$  ring.  $H_2S$  is oxidised by Sqo to form zero-valence sulfur ( $S^0$ ), which is then converted into polysulfane  
211 ( $S_n^{2-}$ ) in a separate step. Some of the sulfite formed in the ring-opening steps reacts with the pendent  
212 cysteine of the SoxYZ protein of the Lu-Kelly complex, which is catalyzed by SoxAX. The Lu-Kelly cycle  
213 then oxidizes the pendent sulfonate ( $-SO_3^-$ ) moiety with SoxB, which cleaves it as sulfate. Thiosulfate then  
214 in turn binds to the pendent sulfane ( $-S^-$ ) moiety, and is then oxidized by SoxB in turn, forming sulfate. Both

215 bisulfide ( $\text{HS}^-$ ) and sulfur from  $\text{S}_8$  can then bind to the pendent sulfane moiety of SoxYZ and be oxidized to  
216 sulfate. Another fate of sulfite is by reaction with  $\text{S}^0$ , forming thiosulfate (the Suzuki and Silver reaction).  
217 The latter is oxidized to tetrathionate by Tqo per *A. ferrooxidans*. From this point onwards, a multiply  
218 branched set of pathways occurs, the precise regulation of which is not fully understood. The ultimate fate of  
219 tetrathionate-sulfur is to sulfite, which is used in substrate-level phosphorylation reactions per *A.*  
220 *ferrooxidans*. Why the organism needs both the Lu-Kelly cycle *and* APS reductase pathways for sulfite  
221 oxidation is unclear, but since the former generates  $\Delta p$  (used to synthesise ATP from ADP, or NADH from  
222  $\text{NAD}^+$ ) and the latter generates ATP without  $\Delta p$  or ADP, it would seem that the redox balance of the  
223 respiratory chain, the size of the cellular ADP-pool and the NADH demand of the cell would be likely  
224 contributors to regulation of which pathway is used at any given time.

225 Ferrous iron oxidation is also variable within the genus. In *A. ferrooxidans* ATCC 23270<sup>T</sup> and *A. ferridurans*  
226 ATCC 33020<sup>T</sup>, an iron:rusticyanan reductase (EC 1.16.9.1), a form of cytochrome *c*, on the outer membrane  
227 oxidizes Fe(II) to Fe(III) and transfers the electron to rusticyanin A (RusA) in the periplasm. Rusticyanins  
228 are small copper proteins (*c.* 16 kDa), which transfer electrons to a further cytochrome *c*, from which they  
229 are transferred to the *aa*<sub>3</sub>-type cytochrome *c*-oxidase and molecular oxygen. For reverse electron transport to  
230 generate NADH, electrons are transferred from RusA to a cytochrome *c*<sub>4</sub>, then on to the *bc*<sub>1</sub> complex, the  
231 quinone pool and the NADH dehydrogenase (quinone, EC 1.6.5.11). In *Acidithiobacillus ferriphilus* strains,  
232 the iron oxidation enzyme (Iro, EC 1.16.3.*x*, a high-potential iron-sulfur protein, HiPIP) oxidises Fe(II) to  
233 Fe(III) in the periplasm, transferring electrons to cytochrome *c*, which are then transferred either directly to  
234 the *aa*<sub>3</sub> cytochrome *c* oxidase per the above, or *via* rusticyanin B (RusB). Although rusticyanins have been  
235 assumed for many years to be critical to iron oxidation, *A. ferrivorans* CF27 apparently lacks both *rusA* and  
236 *rusB* genes but still grows on ferrous iron (Hedrich *et al.*, 2011).

237 Molecular hydrogen oxidation coupled to growth has been observed in *A. caldus*, *A. ferrooxidans* and *A.*  
238 *ferridurans*, but not *A. thiooxidans* (Hedrich and Johnson, 2013*b*). Growth on molecular hydrogen typically  
239 gives very good specific molar growth yields (two or more orders of magnitude higher than on ferrous iron),  
240 and the culture pH remains circumneutral, whereas on Fe(II) or sulfur compounds, it drops during growth.  
241 This has proven to be a useful means of generating high growth yields for genomic work (Prof D. Barrie

242 Johnson, University of Bangor, UK, *personal communication*). Oxidation of molecular hydrogen can be  
243 coupled to molecular oxygen or ferric iron reduction. A respiratory [NiFe]-hydrogenase (EC 1.12.99.6),  
244 catalyzes the oxidation of H<sub>2</sub> to H<sup>+</sup>, with transfer of electrons to cytochrome *b* and then the quinone pool.  
245 Electron transport from ubiquinol can be forward or reverse, as described above. This should not be  
246 confused with the cytoplasmic uptake [NiFe]-hydrogenase of *A. ferrooxidans* used during diazotrophic  
247 growth, for scavenging molecular hydrogen generated therein (Valdés *et al.*, 2008; Vignais *et al.*, 2001).  
248 Whilst the original authors reported that *Acidithiobacillus caldus* (Hallberg and Lindström, 1995) was  
249 capable of mixotrophic growth (*viz.* simultaneous autotrophic growth at the expense of a sulfur oxyanion  
250 and heterotrophic growth on a sugar or complex medium), later workers have not been able to replicate this  
251 (D. Barrie Johnson, *personal communication*). Thus, it may have been owing to contamination. No reported  
252 growth on organic carbon compounds has been observed for any *Acidithiobacillus* spp.

253 It is usual to cultivate *Acidithiobacillus* spp. on flowers-of-sulfur or roll sulfur (former comprises  
254 orthorhombic cyclooctasulfur ( $\alpha$ -S<sub>8</sub>) with ‘significant’ amounts of polymeric (*catena*-S<sub>∞</sub> or  $\mu$ -S) sulfur  
255 (Steudel and Eckert, 2003), whereas the latter is 99 %  $\alpha$ -S<sub>8</sub>, 0.6 % cycloheptasulfur (S<sub>7</sub>), Lesté-Lasserre  
256 (2001)). Growth on *catena*-S<sub>∞</sub> has been observed in *A. ferrooxidans* ATCC 23270<sup>T</sup>, and this allotrope  
257 interestingly produces less growth per unit biomass than  $\alpha$ -S<sub>8</sub>. This property makes it a potentially  
258 interesting additive in biohydrometallurgy, where excess biomass can compromise the mineral surface, but  
259 strong acid production is needed (He *et al.*, 2011). *A. thiooxidans* growth on elementary sulfur is stimulated  
260 by low concentrations of surfactants such as sodium 2-ethylhexyl sulfate (Tergitol 08) or polysorbate 80  
261 (TWEEN<sup>®</sup> 80) – increasing the wetting of the surface of the sulfur particles (Starkey *et al.*, 1956). The  
262 dipolar sudanophilic granules in *A. thiooxidans* containing polyhydroxybutyrate were once believed to play  
263 a role in the initial wetting of sulfur (Umbreit *et al.*, 1942), though it was later found that  
264 phosphatidylinositol (PI) was a wetting agent in this species (Schaeffer and Umbreit, 1963), though Jones  
265 and Benson (1965) also demonstrated phosphatidylglycerol, phosphatidic acid and phosphatidylcholine in  
266 culture supernates, which were not found as major polar lipids cell extracts, and that PI was only present to a  
267 comparatively minor degree compared to these compounds.

268 *In vitro*, growth of *Acidithiobacillus* strains can be difficult if organic contaminants are present, such as  
269 detergents (Onysko *et al.*, 1984), carboxylic acids, or some contaminating metals. As with almost all  
270 acidophiles, the uncoupling agent-type effect of carboxylate salts is of course inhibitory to growth and must  
271 be avoided. At pH below the  $pK_a$ , carboxylates revert to carboxylic acids and can thus cross a cell  
272 membrane. Upon entry into the cytoplasm, they are at a pH greater than their  $pK_a$  and liberate protons,  
273 returning to the conjugate base, lowering the intracellular pH, eventually killing the cell – fumarate,  
274 succinate, formate, acetate, oxalate, trichloroacetate and many others have been shown to be toxic at 1 mM  
275 or below (Tuttle and Dugan, 1976). Sulfamate ( $\text{NH}_2\text{SO}_3^-$ ) is also toxic to *A. ferrooxidans* but apparently *via*  
276 a different mechanism as it has a  $pK_a$  of about 0.1 at room temperature, thus would only be inhibitory at very  
277 low pH values *via* this uncoupling mechanism but is toxic to cultures at pH 4.5 (Lusty *et al.*, 2006).

278 When growing these organisms on natural minerals, contaminating metals can pose an issue, in particular  
279 hexavalent chromium ( $\text{CrO}_4^{2-}/\text{Cr}_2\text{O}_7^{2-}$ ) is toxic to many strains growing on iron, but on elementary sulfur,  
280 the formation of intermediary sulfite and thiosulfate permits some *A. ferrooxidans* to ‘resist’  $\text{Cr}_2\text{O}_7^{2-}$   
281 (reducing it to  $\text{Cr}^{3+}$ ) at up to 2 mM. This is *sensu stricto* chemical reduction of  $\text{Cr}_2\text{O}_7^{2-}$  by thiosulfate/sulfite,  
282 not biological reduction or true resistance (Sisti *et al.*, 1996). *A. ferrooxidans* growth on Fe(II) or  $\text{FeS}_2$  is  
283 inhibited by  $\text{Ag}^+$  at 1  $\mu\text{M}$  (Tuovinen *et al.*, 1985; Hoffman and Hendrix, 1976), but the addition of yeast  
284 extract to cultures (which presumably binds  $\text{Ag}^+$ ) can alleviate this.  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$  and  $\text{Cr}^{3+}$  ions did not  
285 inhibit *A. ferrooxidans* at 1-10 mM, but  $\text{Pb}^{2+}$ ,  $\text{Sn}^{2+}$ ,  $\text{MoO}_4^{2-}$ ,  $\text{Hg}^+$ ,  $\text{Hg}^{2+}$  and  $\text{Ag}^+$  were fully inhibitory at 1  
286 mM (Imai *et al.*, 1975). Additionally,  $\text{SeO}_3^{2-}$ ,  $\text{TeO}_3^{2-}$ ,  $\text{AsO}_3^{2-}$  and  $\text{MoO}_4^{2-}$  were toxic, the latter at  
287 concentrations as low as 30  $\mu\text{M}$ , in *A. ferrooxidans* ATCC 13661 (Tuovinen *et al.*, 1971; N.B. strain no  
288 longer available). In a range of *A. ferrooxidans* isolates from Brazilian uranium and coal mines, resistance to  
289  $\text{Ag}^+$ ,  $\text{Hg}^{2+}$ ,  $\text{Co}^{2+}$  and  $\text{Cu}^{2+}$  was found to be variable at strain-level, which could relate to plasmid-mediated  
290 resistance (Garcia Jr and da Silva, 1991). *A. ferrooxidans* ATCC 23270<sup>T</sup> is sensitive to  $\text{UO}_2^{2-}$  ions, but  
291 adding 5 mM EDTA to cultures was sufficient to overcome this inhibition (Mahapatra and Mishra, 1984). *A.*  
292 *thiooxidans* is inhibited by  $\text{WO}_4^{2-}$ , a common ingredient in trace-element solutions, which inhibits the  
293 enzymes of sulfur oxidation (Negishi *et al.*, 2005). In *A. thiooxidans* ATCC 8085, growth on elementary  
294 sulfur is inhibited by  $\text{VO}_4^{3-}$ ,  $\text{MoO}_4^{2-}$  and  $\text{CrO}_4^{2-}$  at 0.2-0.5 mM (Jack *et al.*, 1980).

295 **Chemotaxonomic features.** Fatty acid and polar lipid data for *Acidithiobacillus* spp. are given in Table I  
296 and are broadly similar to those of *Thermithiobacillus* spp. (gbm01080), but with a greater proportion of  
297 cyclopropyl and  $\omega$ -cyclohexyl fatty acids, which is probably owing to their acidophilic nature (Da Costa *et*  
298 *al.* 2011). The polar lipids in *Acidithiobacillus* spp. include cardiolipin (diphosphatidylglycerol, CL), which  
299 has a role in maintaining  $\Delta p$  by acting as a ‘proton trap’. This confines the periplasmic proton pool in a  
300 discreet region, minimizing proton loss through the membrane. In acidophiles, this role is expanded to  
301 include trapping ingressing protons to prevent damage (Haines and Dencher, 2002). The dominant  
302 respiratory quinone is ubiquinone-8 (UQ-8), in common with the rest of the *Acidithiobacillales* (obm00092),  
303 but minor amounts of ubiquinone-9 (UQ-9), ubiquinone-7 (UQ-7) are found in some species, and an  
304 unidentified menaquinone (MK) was also found in *A. caldus*.

305 **Genomic and biochemical features, and their relation to ecology.** The genome sequences of many  
306 *Acidithiobacillus* spp. have been completed and made publically available *via* the Integrated Microbial  
307 Genomes (IMG) database. Properties of those from species with validly published names are curated in  
308 Table I. They are usually about 3 Mbp with 2,500-3,000 protein coding genes. All of the genomes  
309 sequenced thus far show the presence of Form IAc (‘green’) D-ribulose 1,5-bisphosphate  
310 carboxylase/oxygenase (RuBisCO, EC 4.1.1.39), which is founded exclusively within carboxysomes  
311 (‘polyhedral bodies’). These intracellular compartments are consistently found in *Acidithiobacillus* spp., and  
312 are involved in the concentration of CO<sub>2</sub>. This is hardly surprising since in low pH environments  
313 bi/carbonate has a very low availability, and effectively trapping dissolved inorganic carbon (DIC) is a  
314 challenge. Form IAc is optimized for low pCO<sub>2</sub> and low to high pO<sub>2</sub>. The cytosolic Form II RuBisCO is also  
315 found, and is optimized for medium to high pCO<sub>2</sub> with low pO<sub>2</sub>. It is probably used when pO<sub>2</sub> is low, or in  
316 organisms growing at the higher end of their pH range, or in environments with otherwise high pCO<sub>2</sub>. The  
317 *bo*<sub>3</sub>-type ubiquinol oxidase (proton translocating, EC 1.10.3.10) is the respiratory terminal oxidase in all  
318 *Acidithiobacillus* spp. and is used during growth on sulfur species, oxidation of which is coupled entirely to  
319 ubiquinone reduction. The *bd*-I-type ubiquinol oxidase (non-translocating, EC 1.10.3.14) is also found,  
320 which is used to restore redox balance when the quinone pool becomes dominated with quinol, usually  
321 because of a paucity of ADP and thus a high  $\Delta p$ . It effectively acts as a [H] relief valve. In iron-oxidizing

species, in which Fe(II) oxidation is cytochrome *c* mediated, a cytochrome *c* oxidase (EC 1.9.3.1) of the *aa<sub>3</sub>* type is found, which is optimized for atmospheric pO<sub>2</sub>, and does not perform well at lower pO<sub>2</sub>.

## Cultivation, Enrichment and Isolation Procedures

**General cultivation:** Deionized water that has then been glass-distilled (ddH<sub>2</sub>O) is what we use in our laboratory and it gives very reliable, reproducible growth. We dispose of it after 10 days and store it in acid-washed Nalgene aspirators. As with all acidophiles, they should not be grown in the vicinity of organisms on ammonium- or protein-rich media, since ammonia or polyamines produced will dissolve readily from the air into low pH media, causing the death of the organisms. The same applies to the autoclaving of these media: it must be done separately, in a clean autoclave that does not contain spills of protein-rich broths.

We use various basal salts for cultivation – all of them can be solidified in several ways, but agar or agarose are generally unable to set firmly at low pH values. Instead, 15.0 g/L Phytigel™ or Gelzan™ CM (previously Gelrite® – all are brands of gellan gum from a *Sphingomonas* sp.) is used. The medium volume is reduced by 50 % and the Phytigel™ is autoclaved in the other 50 % of ddH<sub>2</sub>O, and, after cooling both components to *c.* 60 °C, they are combined and the plates poured. Thick plates must be poured for thermophiles, as they will dry out rapidly during incubation at elevated temperatures. Silica gel media are not usually necessary for most strains but can be useful nonetheless. For 1 L of silica gel-set medium, all ingredients are combined in 250 mL ddH<sub>2</sub>O to which 750 mL LUDOX® HS-40 colloidal silica (W. R. Grace & Co., Columbia, MD) is then added, with stirring. The mixture is then poured into 6-cm diameter glass Petri dishes and autoclaved. After autoclaving is complete, allow the autoclave to cool naturally overnight without releasing the pressure early as this will blow holes in the solidified medium. Note that the use of larger plates tends to result in cracks forming (Kingsbury and Barghoorn, 1954). A clean white, opaque, pearlescent medium is formed using LUDOX® HS-40 in tetrathionate-based media. For especially ‘fussy’ strains, the LUDOX® HS-40 can be deionized by passing through a mixed-bed of Amberlite™ IR-120 and Amberite™ IR-45 immediately before use. Tuovinen and Kelly (1973) cover various other gelling agents and their properties with respect to this genus. See also Johnson (1995) and Nancucheo *et al.* (2016) for useful discussion on solid media design, including useful layered media. These contain heterotrophic acidophilic *Acidiphilium* spp. or “*Acidocella aromatica*” in a lower layer to consume organic acids produced

349 during agar(ose) hydrolysis at very low pH. Such organic acids would otherwise poison *Acidithiobacillus*  
350 spp. growing on the upper layer.

351 Brock (1975) determined that water potential ( $\psi$ ) was a critical parameter for successful growth of *A.*  
352 *ferrooxidans* in particular, and reported that  $\psi$  below -32 bars were inhibitory for growth (ionic strength >  
353 3.2 M). This is an important consideration for growth, particularly in ore-column leaching experiments or  
354 column enrichments where moisture levels are low. Additionally in flask or reactor culture where solute  
355 concentrations can be very high e.g. when high concentration ferrous sulfate is used as the electron donor.  
356 Ionic strengths (determined per Debye and Hückel, 1923) are given for each medium in this section.

357 General *Acidithiobacillus* basal salts (GABS, pH 4.0-4.5, ionic strength 0.049 M) for cultivation in general  
358 on thiosulfate or tetrathionate comprises (g/L in ddH<sub>2</sub>O): (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (0.60), KH<sub>2</sub>PO<sub>4</sub> (0.60), MgSO<sub>4</sub>·7H<sub>2</sub>O  
359 (0.25), CaCl<sub>2</sub>·2H<sub>2</sub>O (0.05). For growth on thiosulfate or tetrathionate, these are added to 20 or 10 mM,  
360 respectively, prior to autoclaving, by directly dissolving the sodium or potassium salts in GABS. After  
361 autoclaving, 50 mL aliquots are dispensed into sterile 250-mL glass wide-mouth Erlenmeyer flasks and 0.2  
362 mL 3.6 mM FeSO<sub>4</sub>·7H<sub>2</sub>O in 0.1 N HCl (filter sterilized) is added to each flask as a trace metal.

363 For growth of *Acidithiobacillus caldus* on tetrathionate we use CA-basal salts (CABS, pH 2.50, ionic  
364 strength 0.501 M), which comprises (g/L ddH<sub>2</sub>O): Na<sub>2</sub>SO<sub>4</sub> (1.41), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (3.00), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.50),  
365 KCl (0.10), K<sub>2</sub>HPO<sub>4</sub> (0.05), trace metals solution CA (10 mL), K<sub>2</sub>S<sub>4</sub>O<sub>6</sub> (5.00). All ingredients except for the  
366 potassium tetrathionate and trace metals solution are dissolved in 850 mL ddH<sub>2</sub>O. This solution is then  
367 adjusted to pH 1.75 using 1 N H<sub>2</sub>SO<sub>4</sub>, and diluted to 970 mL before autoclaving. Trace metals solution and  
368 tetrathionate (in 20 mL ddH<sub>2</sub>O) are separately filter sterilized and then added to the cooled, autoclaved  
369 solution. Trace metal solution CA comprises (g/L ddH<sub>2</sub>O): FeCl<sub>3</sub>·6H<sub>2</sub>O (1.10), Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O (1.00),  
370 H<sub>3</sub>BO<sub>3</sub> (0.20), MnSO<sub>4</sub>·H<sub>2</sub>O (0.20), ZnSO<sub>4</sub>·7H<sub>2</sub>O (0.09), Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O (0.08), CoCl<sub>2</sub>·6H<sub>2</sub>O (0.06),  
371 CuSO<sub>4</sub>·5H<sub>2</sub>O (0.05), and should be stored at room temperature in non-actinic glass and should not be  
372 autoclaved neat.

373 For growth of *Acidithiobacillus ferrooxidans* on tetrathionate, we use high-pH FA-basal salts (FABS4.4, pH  
374 4.4, ionic strength 0.406 M) containing (g/L ddH<sub>2</sub>O): KH<sub>2</sub>PO<sub>4</sub> (3.00), MgSO<sub>4</sub>·7H<sub>2</sub>O (3.00), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>

(0.50),  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (0.25),  $\text{K}_2\text{S}_4\text{O}_6$  (5.00). Ingredients are dissolved in 975 mL ddH<sub>2</sub>O and the solution adjusted to pH 4.4 with 1 N H<sub>2</sub>SO<sub>4</sub>, before distributing into flasks and autoclaving.

For growth of *A. ferrooxidans* on ferrous iron, we use low-pH FA-basal salts (FABS1.4, pH 1.4, ionic strength 0.744 M) containing (g/L 0.1 N H<sub>2</sub>SO<sub>4</sub>):  $\text{KH}_2\text{PO}_4$  (0.40),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.04),  $(\text{NH}_4)_2\text{SO}_4$  (0.04),  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (13.90) – after dissolving all of the ingredients, check the pH and adjust if required before distributing into flasks and autoclaving.

The final medium in most common use for various *Acidithiobacillus* spp. is 9K (pH 3.0, ionic strength 1.35 M) originally by Silverman and Lundgren (1959), which comprises (g/L 5mM H<sub>2</sub>SO<sub>4</sub>):  $\text{K}_2\text{HPO}_4$  (0.50),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.50),  $(\text{NH}_4)_2\text{SO}_4$  (1.00),  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (50.00),  $\text{Ca}(\text{NO}_3)_2$  (0.5), KCl (0.1). If necessary adjust pH to 3.0 after dissolution of ingredients and prior to autoclaving. This medium can also be used with tetrathionate (10 mM) or elementary sulfur/minerals (5 g/L) in place of ferrous sulfate.

For growth on molecular hydrogen, CABS without tetrathionate is used, and  $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$  is added to 0.5-1.0  $\mu\text{M}$  (0.3 mg/L), either from a stock solution or by adding to the trace metals solution CA. A ‘QuickFit’ flask with ‘SubaSeal’ vaccine stopper is used with a headspace containing 40 % v/v H<sub>2</sub> and 10 % v/v CO<sub>2</sub>, leaving about 10 % v/v O<sub>2</sub> from air. An inverted, sterile flask can easily be filled by the downward displacement of air from a hydrogen cylinder, and then sealed with a vaccine stopper. Air and CO<sub>2</sub> are then injected to give the appropriate final concentrations and a slight overpressure.

For growth on thiosulfate, CABS or GABS media can be used with  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$  to 20 mM (5.00 g/L) in place of tetrathionate, but it will chemically break down at low pH.

It is important to note that any flasks previously containing iron salts as electron donors or terminal electron acceptors should be cleaned as follows to avoid damage to glassware or laboratory plumbing. Flasks are emptied of culture without autoclaving them first. They are then filled to the brim with concentrated HCl (37 % w/v) and left overnight in the fumehood. This acid will turn somewhat yellow with iron leached from the glass surface. ‘Dirty’ acid is re-used for this washing procedure until almost black in colour, and is then disposed of as hazardous waste. Flasks are rinsed thoroughly in ddH<sub>2</sub>O and washed in the normal way. They are finally soaked in 2 % v/v HNO<sub>3</sub> for 48 h then washed in ddH<sub>2</sub>O until the washings are pH neutral.



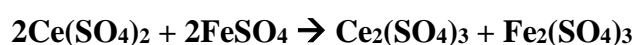
401 If flasks are not acid-washed in this way to remove iron deposits, they will precipitate on contact with  
402 detergents and become irreversibly stuck to the glass. It is important to note that flasks used for iron cultures  
403 should never be washed in the dishwasher (even following HCl washing) as, over time, a ferric phosphate  
404 deposit forms inside of the appliance and associated plumbing, which cannot be removed.

405 For growth on terminal electron acceptors other than molecular oxygen, ferric sulfate ( $\text{Fe}_2(\text{SO}_4)_3$ ) at 20 mM  
406 (8.00 g/L) is used, with usually elementary sulfur (5.00 g/L) as the electron donor. Cultures are incubated in  
407 'QuickFit' Erlenmeyer flasks sealed with 'SubaSeal' vaccine stoppers – they are flushed thoroughly with  
408 argon or oxygen-free nitrogen (including the sterile medium therein) prior to inoculation. Argon is more  
409 costly, but as atomic argon is much denser than molecular nitrogen, it is more effective at sweeping air from  
410 glassware. It will also form an argon-blanket over the culture held in the vessel, such that any minor air  
411 leaks through a vaccine stopper do not reach the culture. A Schlenk manifold for alternate evacuation and  
412 argon-gassing can be useful, particularly if large numbers of flasks are to be prepared on a regular basis.

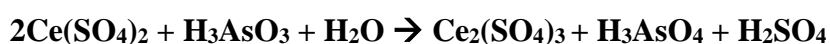
413 Minerals and elementary sulfur are easily sterilized by Tyndallisation and added to medium at 0.5-5.0 g/L.  
414 For details, see the chapter on *Thermithiobacillus* (gbm01080). Sterilization using  $\gamma$ -radiation, where  
415 available, is suitable for minerals where steam would cause hydroxide formation. Alternatively, soaking  
416 mineral samples in sodium benzoate solution (0.44 M) for 24 h and then *thoroughly* rinsing in sterile ddH<sub>2</sub>O  
417 and drying can be used – this is useful for mineral packed inside of leaching column reactors (Onysko *et al.*,  
418 1984). *A. thiooxidans* and *A. concretivorus* can be removed from natural mineral samples using vapor-phase  
419 *p*-chlorocresol or chloroxylenol (7-14 day exposure followed by thorough 'washing' in a stream of sterile  
420 air). *A. ferrooxidans* can be removed with the same methods or also with a 1:1 mixture of thymol and *N,N*-  
421 dicyclohexylamine nitrite. This is also useful for removing *Acidithiobacillus* contaminants from specimen-  
422 grade sulfide minerals held in collections, to prevent degradation (Booth and Sefton, 1970).

423 **Analytical methods:** During growth on ferrous iron in particular, the amount of biomass present does not  
424 appreciably change the optical density of the culture, whereas deposits of jarosite ( $\text{KFe}^{3+}(\text{OH})_6(\text{SO}_4)_2$ ) *do*  
425 raise the optical density and this makes it an unsuitable parameter for determination of biomass. In the  
426 absence of a cell-counter that tolerates mineral particles and can distinguish jarosite or sulfur or other  
427 mineral particles from cells (*e.g.* the CellFacts II Analyzer, CellFacts Analytics Ltd, Coventry, UK), one

428 must use a proxy measurement for growth. Growth on molecular hydrogen or tetrathionate do not pose  
 429 such an issue and the optical density at 440 nm is used against a calibration curve. Cells can be counted in a  
 430 Petroff-Hausser chamber after dilution 1:1 in 5 % (w/v) formalin. Tuovinen and Kelly (1973) report plate-  
 431 count methods that may be useful still for some purposes. Measurement of iron oxidation is also a very  
 432 useful measure of growth – this is easy to do by titration of media. Assuming a starting concentration of 50  
 433 mM Fe(II), 5 mL volumes of culture (no need to remove biomass) are titrated against against 0.100 M ceric  
 434 sulfate in sulfuric acid, using the ferrous sulfate complex of 1,10-phenanthroline (ferroin) as the indicator.  
 435 This changes from red to blue at end-point. This titration follows the stoichiometry:



437 The ceric sulfate used should be a precise volumetric standard solution, either procured ready-made, or  
 438 prepared quite economically and standardized oneself. First, 21.00 g ceric hydroxide is dissolved in 100 mL  
 439 98 % H<sub>2</sub>SO<sub>4</sub> with stirring. Once fully dissolved, 300 mL ddH<sub>2</sub>O is cautiously and slowly added over about  
 440 30 min. The solution is left overnight, and then filtered through Whatman No. 1 filter paper into a 1-L  
 441 volumetric flask, and is then diluted to volume with ddH<sub>2</sub>O. This solution is then standardized against an  
 442 arsenic (III) standard, as follows. 0.200 g arsenic (III) oxide (dried at 105 °C for 1-2 h before weighing) is  
 443 dissolved in 20 mL 2.00 M NaOH, warming gently until absolutely all of the solid material has dissolved.  
 444 After cooling to room temperature, 25.00 mL 2 M H<sub>2</sub>SO<sub>4</sub> is added, followed by 0.6 mL ‘osmic acid’. The  
 445 latter is prepared from 1.26 mL electron microscopy grade 0.4 % (w/v) osmium tetroxide solution, diluted to  
 446 2 mL in a volumetric flask with 50 mM H<sub>2</sub>SO<sub>4</sub> – take great care from this step onwards to work in the  
 447 fumehood and with eye protection as OsO<sub>4</sub> vapours permanently damage the eye! To this solution, 0.5 mL  
 448 *N*-phenylanthranilic acid (diphenylamine-2-carboxylic acid) is added as the indicator. This changes from  
 449 yellow-green to violet at end-point. This solution in its entirety is titrated against the prepared ceric sulfate  
 450 solution and the precise concentration of the latter is determined from the stoichiometry:



452 The concentration obtained is then used in all cerimetric titrations of Fe(II) in media. All osmium-containing  
 453 waste is poured into a large beaker in the fume hood and an equal volume of corn oil or olive oil added,  
 454 which reduces OsO<sub>4</sub> to safer, insoluble black OsO<sub>2</sub> over about 24 h.

455 For total iron (from which ferric iron can then be determined by subtracting the ferrous iron), either ICP-  
456 OES or ICP-MS can be used. For 'real time' determinations, titration following reduction to  $\text{Fe}^{2+}$  is useful.  
457 The medium is passed through a Jones reductor to reduce  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ , and the latter determined by titration  
458 as described above. The Jones reductor uses zinc amalgam to reduce ferric iron:



460 The Jones reductor comprises a  $360 \times 20$  mm glass tube topped with a *c.* 100 mL reservoir ( $60 \times 45$  mm).  
461 There is a sintered glass frit and tap at the distal end of the tube, which is connected *via* a rubber bung to a 1-  
462 L Büchner flask set up at the pump. A second flask is set up as a trap, to protect the pump. On the underside  
463 of the bung, a further length of glass tubing is connected with silicone tubing so as to reach to 0.5 cm from  
464 the bottom of the flask. About 250-300 g high purity zinc turnings in a wide beaker with a glass-covered  
465 stirring 'flea' are covered with 70 mM  $\text{HgCl}_2$  solution and stirred for 10 min. The supernate is decanted off  
466 and the amalgamated zinc washed with 3-4 changes of ddH<sub>2</sub>O – it should be bright silver in color – and is  
467 then packed into the glass tube, 500 mL ddH<sub>2</sub>O are slowly drawn through the tube with a gentle vacuum  
468 from the pump, leaving the column material covered in water when the tap is closed. It should never be left  
469 exposed to the air at any time. To use, 50 mL 2 N  $\text{H}_2\text{SO}_4$  is added to the reservoir and is drawn into the  
470 column with a vacuum until just below the top of the amalgam. This step is repeated twice and a clean, dry  
471 1-L Büchner flask is then put into place. The analyte is diluted 10 fold in 2 N  $\text{H}_2\text{SO}_4$  and is drawn through  
472 the column at about 1 mL/s. Once the reservoir is almost empty,  $2 \times 100$  mL volumes of 0.5 N  $\text{H}_2\text{SO}_4$  are  
473 drawn through, into the same flask, followed by 100 mL ddH<sub>2</sub>O. The flask is disconnected and ferrous iron  
474 determined therein from a 50.00 mL aliquot by titration as above. If determining ferric iron in this way in  
475 leaching experiments in chloride-rich ores, it is necessary to add 25 mL Zimmermann-Reinhardt solution  
476 after reduction but prior to titration. This prevents the oxidation of chloride at the expense of Ce(IV), which  
477 would overestimate the Fe(II) present. This solution is made by dissolving 50 g manganous sulfate  
478 tetrahydrate in 250 mL ddH<sub>2</sub>O to which 100 mL 98 %  $\text{H}_2\text{SO}_4$  in 300 mL ddH<sub>2</sub>O is added with stirring,  
479 followed by 100 mL 85%  $\text{H}_3\text{PO}_4$ .

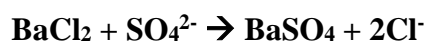
480 Growth on other electron donors other than iron is often stronger, but at times it is still not possible to  
481 measure *via* conventional means. During growth on thiosulfate or tetrathionate, their depletion can easily be

determined. Sulfate is also easily determined, which is the end product of elementary sulfur oxidation. Thiosulfate and tetrathionate can be determined by ion chromatography or HPLC, for which there are many published methods. When not available, a colorimetric assay (based on Kelly *et al.*, 1969) can be used. In this assay, thiosulfate and tetrathionate must be determined together. Culture (0.5-1.0 mL, no need to remove biomass) is added to duplicate 25 mL volumetric flasks. To each flask, 4 mL of 0.2 M phosphate buffer (pH 7.4) and 5.0-5.5 mL ddH<sub>2</sub>O are added. To the first flask ("A"), 5 mL 0.1 M KCN solution is added, and the flask capped and incubated at 4 °C for at least 20 min (they can be incubated for days, if necessary). To the second flask ("B"), 5 mL 0.1 M KCN are added and the flask capped and incubated at 4 °C for 20 min, following which 1.5 mL 0.1 M CuSO<sub>4</sub>·5H<sub>2</sub>O is added and the flask shaken, then incubation continued for at least 15 min (or many days). To both flasks, 3 mL 0.75 M Fe(NO<sub>3</sub>)<sub>3</sub>·9H<sub>2</sub>O in 0.80 M HClO<sub>4</sub> is added, and the solution rapidly shaken to dissolve any precipitates before diluting to volume and measuring absorbance at 460 nm against reagent blanks. This measurement should be done rapidly as the red ferric thiocyanate complex is light labile. The ferric thiocyanate complex has an extinction coefficient of 4.4 mM<sup>-1</sup> cm<sup>-1</sup>, from which the concentrations each in flask can be determined with the Beer-Lambert equation. The reactions in the flasks and concentrations of analytes are given as follows:

**A:** 1 mol S<sub>4</sub>O<sub>6</sub><sup>2-</sup> yields 1 mol SCN<sup>-</sup>, thus [SCN<sup>-</sup>]<sub>A</sub> = [S<sub>4</sub>O<sub>6</sub><sup>2-</sup>]<sub>A</sub>

**B:** 1 mol S<sub>4</sub>O<sub>6</sub><sup>2-</sup> yields 2 mol SCN<sup>-</sup>, and 1 mol S<sub>2</sub>O<sub>3</sub><sup>2-</sup> yields 1 mol SCN<sup>-</sup>, thus [SCN<sup>-</sup>]<sub>B</sub> - [SCN<sup>-</sup>]<sub>A</sub> = [S<sub>2</sub>O<sub>3</sub><sup>2-</sup>]<sub>B</sub>

For determination of sulfate, ion chromatography is also often used. Indirect determination of sulfate with ICP-OES, atomic absorption spectroscopy *etc* are also possible, providing samples are pre-treated with acidified LaCl<sub>3</sub> solution to remove interfering carbonates and phosphates beforehand. A volume of a standard BaCl<sub>2</sub> solution is then added and the resulting BaSO<sub>4</sub> precipitate allowed to settle. Residual Ba<sup>2+</sup> in the supernate can then be determined by ICP-OES or AA based on the stoichiometry:



From this, the original sulfate concentration is found by the difference between the initial amount added and the amount remaining in solution. Ba<sup>2+</sup> can also be determined by titration if the solution is adjusted to pH 11.5-12.7, against a standard EDTA solution, using methylthymol blue in KNO<sub>3</sub> solution as the indicator.

Sulfate can be determined directly by various turbidometric methods that are suitable for rapid-screening. Briefly, analyte (1-5 mL) is added to a 100-mL volumetric flask containing 10.00 mL 4.1 M NaCl in 0.2 M HCl. 20.00 mL 30 % (v/v) glycerol in ethanol is added and the contents of the flask are diluted to volume with ddH<sub>2</sub>O. BaCl<sub>2</sub> (0.30g, finely ground) is added to each flask and flasks are immediately mixed by end-over-end rotation at a rate of 1 rps for 1 min. Flasks are then allowed to stand for *exactly* 3 min. Optical density at 440 nm is determined. We have found that this method is much more reproducible in colorimeters that use round test tubes in place of cuvettes, rather than in digital spectrophotometers, and we get very precise determinations against external standards. This is probably owing to the shape of the sample container (round *vs* square), the morphology of the light beam and the bandwidth of the light being broader in a colorimeter. The concentration of SO<sub>4</sub><sup>2-</sup> must be determined from a calibration curve, and an external standard of defined concentration should be procured and used with each batch of assays.

**Maintenance:** Mineral or elementary sulfur oxidizing strains are maintained quite easily following growth in 9K or GEBS under air using 5 g/L sterile pyrite, chalcopyrite, lignite or roll sulfur as the electron donor. After growth is evident, viability is retained for 2-4 months at 4-10 °C (Gupta and Agate, 1986). Many *Acidithiobacillus* spp. do not preserve well by freezing with glycerol or dimethylsulfoxide, but successful recovery of frozen cultures of *A. ferrooxidans* has been reported from cells grown on ferrous iron, washed twice and resuspended in the same medium minus the iron before mixing with Protect-100 beads (Pro-lab Diagnostics Inc., Toronto, Canada). Whilst these beads are no longer manufactured, the new Microbank™ system is based on a similar technology and may be a viable alternative. After mixing vials at room temperature for 45 min, they are frozen at -70 °C. Recovery is by dispensing into 10 mL of the same ferrous iron medium (LaCombe Barron and Lueking, 1990). Lyophilization of *Acidithiobacillus* spp. is not usually very successful (Gupta and Agate, 1986).

**Enrichment and isolation:** For solid samples, 1-2 g soil, corroded concrete, coal or minerals are added directly to 50 mL of the appropriate basal salts, supplemented with the required electron donor in a sterile, wide-mouth Erlenmeyer flask. For water samples such as acid mine drainage, acid lake water *etc*, our practice is to pass 250-1,000 mL through a 45 mm 0.2 µm pore size glass fibre filter to concentrate the biomass – this is then added to the medium. NB: if using water from a lake *etc* that has been exposed to

535 sunlight, pre-filtration (0.44  $\mu\text{m}$  pore size) can be helpful to remove acidophilic *Eukarya*. If elementary  
536 sulfur, pyrite, coals and so on are used as the electron donor, they are used at 0.5-1.0 % (w/v). Because they  
537 frequently contain acidiphilic sulfur- or mineral-oxidizing *Bacteria*, they must be sterilized before use, but if  
538 they are not, they can act as both the electron donor *and* inoculum. Flasks are stoppered *loosely* with sterile  
539 cotton wool and incubated with rapid shaking at 20-50 °C and once turbidity and/or pH change, iron  
540 oxidation *etc* are evident (increase in cell number/optical density or evidence of substrate oxidation),  
541 enrichments are sub-cultured (usually every 7-14 days) into fresh medium (10 % v/v). After 5 or so serial  
542 subcultures, streak plates or serial dilution spread-plates are prepared and colonies purified *etc*.

### 543 Taxonomic comments

544 Where three-letter abbreviations are required for clarity, we recommend “*Atb.*” and not the two-letter  
545 abbreviation “*At.*”. Note Chapter 4 *Advisory Notes* of the *Bacteriological Code* states that **a single letter**  
546 **only** should be used for generic abbreviations, excepting when several taxa are mentioned in the publication  
547 with the same initial letter, in which case the whole names should be used (Parker *et al.*, 2019). Since this  
548 system would be ungainly, Trüper and Madigan (1999) recommended three-letter abbreviations are used in  
549 such circumstances: this genus is not an exception to this and two-letter abbreviations should be avoided,  
550 and in studies of *Acidithiobacillus* spp. only, the single-letter abbreviation is correct.

551 The type species *A. thiooxidans* as well as *A. caldus*, *A. albertensis* and *A. ferrooxidans* were originally  
552 described as members of the genus *Thiobacillus* (gbm00969). It is worth noting that *A. ferrooxidans* was  
553 also referred to by the synonym “*Ferrobacillus ferrooxidans*” in many early publications (*e.g.* Silverman  
554 and Lundgren, 1959), but this name was not included in the Approved Lists of 1980. These species were  
555 reclassified by Kelly and Wood (2000) when the genus *Acidithiobacillus* was created. *Thiobacillus*  
556 *concretivorus* falls within the genus *Acidithiobacillus* on the basis of 16S rRNA gene phylogeny, but Kelly  
557 and Wood (2000) considered it to be a heterotypic synonym of *A. thiooxidans*. During the work of Kelly and  
558 Wood (2000), it was found that *Acidithiobacillus* formed a distinct clade from *Thermithiobacillus*  
559 (gbm01080) on the basis of the 16S rRNA gene, which is still the case (a phylogenetic tree on this basis is  
560 given in the chapter on *Thermithiobacillus* (gbm01080)). We now know that many of the iron-oxidizing  
561 *Acidithiobacillus* spp. share very high 16S rRNA (*rrs*) gene identities (Table I) such that they could be

562 considered strains of the same species if judged by this criterion alone. They are, in fact, *bona fide*  
563 species. As such, we must now reappraise the position of *T. concretivorus*, which is potentially also a *bona*  
564 *fide* species. To remove uncertainty, with *T. concretivorus* still lingering under a generic epithet that relates  
565 to an entirely different class, we herein circumscribe it into *Acidithiobacillus* as *Acidithiobacillus*  
566 *concretivorus* comb. nov. and give a protologue at the end of this section. *A. albertensis* is similarly closely  
567 related to *A. thiooxidans* on this basis but as we have the genome sequence available, whereas we do not for  
568 *A. concretivorus* comb. nov. A concatamer tree of the 53 ribosomal proteins (Figure 1) shows clearly that *A.*  
569 *albertensis* is essentially identical to other *A. thiooxidans* strains, so would either be a subspecies of *A.*  
570 *thiooxidans* or just a heterotypic synonym – this cannot be ascertained without further work.

571 It is also very evident from both 16S rRNA gene studies and the ribosomal protein analysis (Figure 1) that  
572 *Acidithiobacillus caldus* is probably not a *bona fide* species of *Acidithiobacillus*, nor does it affiliate with  
573 *Thermithiobacillus* (gbm01080), thus it probably represents a novel genus within this order. This conclusion  
574 is also supported by other comparisons to *bona fide* *Acidithiobacillus* spp.: it is thermophilic, in common  
575 with most *Thermithiobacillus* strains, whereas *Acidithiobacillus* are usually mesophiles or psychrophiles; it  
576 has a higher G+C fraction of its genomic DNA, and a smaller genome sequence – both are closer to those of  
577 *Thermithiobacillus* spp., however, it clusters within the *Acidithiobacillaceae* (fbm00213) rather than the  
578 *Thermithiobacillaceae* (fbm00214), and thus cannot be considered a close relative of *Thermithiobacillus*  
579 (gbm01080).

580 **Description of *Acidithiobacillus concretivorus* comb. nov. (Parker 1945a, *Thiobacillus***  
581 ***concretivorus*)**

582 con.cre.ti'vo.rus. N.L. neut. n. *concretum* (from L. masc. adj. *concretus*, hardened, condensed), firm  
583 or solid matter, concrete; L. part. adj. *vorans*, devouring, swallowing up, destroying; N.L. part. adj.  
584 *concretivorus*, concrete-devouring, concrete-destroying.

585 Motile straight rods  $0.5 \times 1.5\text{-}2.0 \mu\text{m}$  with square ends. Deeply stained volutin granules  
586 (polyphosphate). Motile by single polar flagella  $4\text{-}6 \mu\text{m}$  long. Tiny clear colonies on thiosulfate agar,  
587 turning white-yellow with age. Strict aerobe. Oxidizes thiosulfate, hydrogen sulfide and elementary  
588 sulfur as electron donors. Thiosulfate is oxidized to tetrathionate and then sulfate. Strict aerobe.

Mesophile. Extreme acidophile. Uses carboxysomes. Type strain has growth optima at pH 2.0-4.0 and 28 °C, but growth observed at pH -0.6 to 6.0. Dominant fatty acids following growth on elementary sulfur at pH 4.4, 30 °C are 10-*trans*-cyclopropylnonadecylic acid (C<sub>19:0</sub> *cyclo ω8c*), *ω*-cyclohexylmargaric acid (C<sub>17:0</sub> *cyclo*), palmitic acid (C<sub>16:0</sub>), lauric acid (C<sub>12:0</sub>), 3-hydroxymyristic acid (C<sub>14:0</sub> 3-OH) and palmitoleic acid (C<sub>16:1</sub>).

Type strain isolated from decomposing concrete in the sewer outfall of Melbourne, Australia.

*DNA G+C content (mol%): N.D.*

*Type strain:* NCIMB 8345 = ATCC 19703

*GenBank accession (16S rRNA gene):* KX894722

### *List of species in the genus Acidithiobacillus*

- Acidithiobacillus albertensis*** (Bryant, McGroarty, Costerton, Laishley 1988), Kelly and Wood 2000, 514<sup>VP</sup> (*Thiobacillus albertis* Bryant, McGroarty, Costerton, Laishley 1988, 221)

al.ber.ten'sis. N.L. masc. adj. *albertensis*, pertaining to Alberta, Canadian province.

Motile straight rods 0.45 × 1.2-1.5 μm with a tuft of flagella at one pole. Small dark green colonies with yellow halos on thiosulfate agar (pH 4.0) containing bromophenol blue, or yellow colonies without indicator. Membrane-bound sulfur granules formed on thiosulfate media. Oxidizes thiosulfate to tetrathionate and then to sulfate. Uses thiosulfate, elementary sulfur and tetrathionate as electron donors. Can use ferric iron as terminal electron acceptor when growing on elementary sulfur as electron donor. Mesophile. Acidophile. Does not produce volutin or poly-β-hydroxybutyrate granules. Uses carboxysomes. Type strain has growth optima at pH 3.5-4.0 and 28-30 °C. Ubiquinol oxidases only. Form IAc (carboxysomal) and Form II (cytoplasmic) RuBisCO. Likely a heterotypic synonym of *A. thiooxidans*.

Type strain isolated from acid soil next to a sulfur stockpile at Fox Creek, Alberta, Canada.

*DNA G+C content (mol%):* 52.5 (sequence), 61.5 (UV absorption)

*Type strain:* DSM 14366 = ATCC 35403

*GenBank accession (16S rRNA gene):* AJ459804



616 *IMG accession number (genome sequence): 275118577*

- 617
- 618 **2. *Acidithiobacillus caldus*** (Hallberg and Lindström 1995) Kelly and Wood 2000, 514<sup>VP</sup> (*Thiobacillus*  
619 *caldus* Hallberg and Lindström 1995, 619)

620 cal'dus. L. masc. adj. *caldus*, warm, hot.

621 Motile straight rods 0.7-0.8 × 1.2-1.8 µm. Motile by single polar flagella. Electron-dense material at  
622 poles of cells. Tiny clear colonies on thiosulfate agar, turning white-yellow with age. Strict aerobe.  
623 Oxidises thiosulfate, sulfide, elementary sulfur, tetrathionate and molecular hydrogen as electron  
624 donors. Does not use ferrous iron or iron sulfides. Heterotrophic growth is not observed. Mixotrophic  
625 growth (tetrathionate/glucose) has been reported in one study, but this needs further work to confirm.  
626 Strict aerobe. Not diazotrophic. Moderate thermophile. Extreme acidophile. Uses carboxysomes.  
627 Type strain has growth optima at pH 2.0-2.5 and 45 °C, but growth observed at pH 1.0-3.5 and 32-  
628 52°C. Ubiquinol oxidases only. Form IAc (carboxysomal) and Form II (cytoplasmic) RuBisCO. In  
629 addition to ubiquinone-8 (common to all *Acidithiobacillia* as the dominant respiratory quinone), a  
630 menaquinone is also present.

631 Type strain isolated from coal spoil heap at the former Kingsbury Colliery, Warwickshire, UK.

632 *DNA G+C content (mol%):* 66.60 (*B.d.*), 66.4 (sequence), 63.9 (*T<sub>m</sub>*)

633 *Type strain:* KU = ATCC 51756 = DSM 8584

634 *GenBank accession (16S rRNA gene):* Z29975

635 *IMG accession (genome sequence):* 2811995294

- 636
- 637 **3. *Acidithiobacillus concretivorus*** (Parker 1945a) comb. nov. (*Thiobacillus concretivorus* Parker 1945,  
638 81<sup>AL</sup>)

639 con.cre.ti'vo.rus. N.L. neut. n. *concretum* (from L. masc. adj. *concretus*, hardened, condensed), firm  
640 or solid matter, concrete; L. part. adj. *vorans*, devouring, swallowing up, destroying; N.L. part. adj.  
641 *concretivorus*, concrete-devouring, concrete-destroying.

642 **See protologue for this comb. nov., given above, for properties.**

643 Type strain isolated from decomposing concrete in the sewer outfall of Melbourne, Australia.

644 *DNA G+C content (mol%): N.D.*

645 *Type strain:* NCIMB 8345 = ATCC 19703

646 *GenBank accession (16S rRNA gene):* KX894722

647  
648 **4. *Acidithiobacillus ferridurans*** Hedrich and Johnson 2013a, 4024<sup>VP</sup>

649 fer.ri.du'rans. L. neut. n. *ferrum*, iron; L. part. adj. *durans*, hardening, enduring; N.L. part. adj.  
650 *ferridurans*, iron-enduring, referring to growth at high Fe(II) and Fe(III) concentrations.

651 Motile straight rods 1-2  $\mu\text{m}$  long. Motility is strain variable. Small iron-stained colonies on acidic  
652 ferrous iron media and large dark-brown colonies grown on molecular hydrogen. Oxidises ferrous  
653 iron, molecular hydrogen, elementary sulfur or tetrathionate as electron donors. Can use ferric iron as  
654 terminal electron acceptor when growing on elementary sulfur as electron donor. Diazotrophic.

655 Mesophile. Extreme acidophile. Uses carboxysomes. Type strain has growth optima at pH 2.1 and 29  
656 °C. Uses rusticyanin A (RusA). Dominant fatty acids following growth on molecular hydrogen at pH  
657 2.0, 30 °C are 10-*trans*-cyclopropylnonadecylic acid (C<sub>19:0</sub> *cyclo*  $\omega$ 8*c*), *cis*-vaccenic acid (C<sub>18:1</sub>  $\omega$ 7*c*),  
658 palmitic acid (C<sub>16:0</sub>), palmitoleic acid (C<sub>16:1</sub>),  $\omega$ -cyclohexylmargaric acid (C<sub>17:0</sub> *cyclo*) and lauric acid  
659 (C<sub>12:0</sub>), and polar lipids are phosphatidylglycerol, phosphatidylethanolamine and aminolipids. Type  
660 strain tolerates up to 0.8 M Zn<sup>2+</sup>, 1 M Mg<sup>2+</sup>, 0.4 M Fe<sup>2+</sup>, 0.2M Fe<sup>3+</sup> and 1 mM UO<sub>2</sub><sup>2+</sup>, but only 40  
661  $\mu\text{M}$  MoO<sub>4</sub><sup>2-</sup>.

662 Type strain isolated from drainage water at a uranium mine, Ningyo-Tohge, Tottori, Japan.

663 *DNA G+C content (mol%):* 56.0-60.0 (*T<sub>m</sub>*)

664 *Type strain:* JCM 18981 = ATCC 33020

665 *GenBank accession (16S rRNA gene):* AJ278719

666  
667 **5. *Acidithiobacillus ferriphilus*** Falagán and Johnson 2016, 210<sup>VP</sup>

668 fer.ri.phi'lus. L. neut. n. *ferrum*, iron; N.L., masc. adj. *philus* (from Gr. masc. adj. *philos*, that which  
669 is dearly loved, that which is beloved), loving; N.L. masc. adj. *ferriphilus*, iron-loving, referring to  
670 growth at high Fe(II) concentrations.

671 Motile straight rods 1-2  $\mu\text{m}$  long. Small ferric-iron-stained colonies on ferrous iron media. Uses  
 672 ferrous iron, elementary sulfur and tetrathionate as electron donors. Can use ferric iron as terminal  
 673 electron acceptor when growing on elementary sulfur as electron donors. Mesophile. Extreme  
 674 acidophile. Some psychrotolerant strains. Type strain has growth optima at pH 2.0 and 30 °C. Some  
 675 strains grow at  $>1 \text{ M Fe}^{2+}$ ,  $0.5 \text{ M Fe}^{3+}$ ,  $0.8 \text{ M Co}^{2+}$ , and  $1.2 \text{ M Mg}^{2+}$ , but all are sensitive to  $\text{MoO}_4^{2-}$  at  
 676  $100 \mu\text{M}$ . When grown on ferrous iron at pH 1.6, 30 °C, the dominant fatty acids are *cis*-vaccenic acid  
 677 ( $\text{C}_{18:1} \omega 7c$ ), palmitoleic acid ( $\text{C}_{16:1}$ ), 2-hydroxyvaccenic acid ( $\text{C}_{18:1} 2\text{-OH}$ ), 3-hydroxymyristic acid  
 678 ( $\text{C}_{14:0} 3\text{-OH}$ ), palmitic acid ( $\text{C}_{16:0}$ ) and lauric acid ( $\text{C}_{12:0}$ ). The polar lipids are aminolipids,  
 679 phospholipids and phosphatidylglycerol.

680 Type strain isolated from Galway's soufrière, an acidic pool in a geothermal region of Montserrat  
 681 (British Overseas Territory, Caribbean).

682 *DNA G+C content (mol%):* 57.4 (*B.d.*), 66.84 (sequence).

683 *Type strain:* M20 = DSM 100412 = JCM 30830

684 *GenBank accession (16S rRNA gene):* KR905751

- 685  
 686 **6. *Acidithiobacillus ferrivorans*** Hallberg, González-Toril and Johnson 2010*b* 469<sup>VP</sup> (Effective  
 687 publication: Hallberg, González-Toril and Johnson 2010*a* 18)

688 *fer.ri.vor'ans*. L. neut. n. *ferrum*, iron; L. part. adj. *vorans*, devouring, swallowing up; N.L. part. adj.  
 689 *ferrivorans*, iron-devouring.

690 Motile straight rods  $0.5 \times 1.6\text{-}2.4 \mu\text{m}$  long. Motile. Small 'fried-egg' colonies, orange with off-white  
 691 margins, on acidic iron/tetrathionate agar, turning opaque white/yellow with age. Uses elementary  
 692 sulfur, thiosulfate, tetrathionate, sulfide, ferrous iron and pyrite as electron donors, with use of  
 693 molecular hydrogen varying by strain. Can use ferric iron as terminal electron acceptor when  
 694 growing on elementary sulfur as electron donor. Mesophile. Extreme acidophile. Some  
 695 psychrotolerant strains. Type strain has growth optima at pH 2.5 and 25-32 °C but grows pH 1.9-3.4  
 696 and 4-37 °C. Uses rusticyanin B (RusB). Has *aa*<sub>3</sub>-type cytochrome *c*-oxidase and *bo*<sub>3</sub>-type ubiquinol  
 697 oxidase but not the *bd*-I-type ubiquinol oxidase common to other *Acidithiobacillus* spp. Form IAc

(carboxysomal) and Form II (cytoplasmic) RuBisCO. Type strain grows at 0.2 M Fe<sup>2+</sup>, <0.1 M Fe<sup>3+</sup>, <0.05 M Cu<sup>2+</sup>, and 0.2 M Zn<sup>2+</sup>, but is sensitive to MoO<sub>4</sub><sup>2-</sup> at 100 μM.

Type strain isolated from drainage water of a spoil heap at disused copper mine in northern Norway.

*DNA G+C content (mol%): 55.5 (T<sub>m</sub>)*

*Type strain:* NO-37 = DSM 22755 = JCM 15606

*GenBank accession (16S rRNA gene):* AF376020

**7. *Acidithiobacillus ferrooxidans*** (Temple and Colmer 1951) Kelly and Wood 2000, 513<sup>VP</sup>

(*Thiobacillus ferrooxidans* Temple and Colmer 1951, 605)

fer.ro.ox'i.dans. L. neut. n. *ferrum*, iron; Gr. masc. adj. *oxús* (L. transliteration *oxys*), sharp, acidic; N.L. v. *oxydo*, to make acid, to oxidize; N.L. part. adj. *ferrooxidans*, iron-oxidizing.

Motile straight rods 0.5 × 1.0 μm. Probably atrichous or at least non-motile. Volutin (polyphosphate) and poly-β-hydroxybutyrate granules. Colonies on thiosulfate agar are thin and small and become white with age. On ferrous iron agar, colonies are amber and become coated with ferric hydroxide with time. Uses elementary sulfur, thiosulfate, tetrathionate, ferrous iron, pyrite and molecular hydrogen as electron donors. Can use ferric iron as terminal electron acceptor when growing on elementary sulfur as electron donor. Diazotrophic. Mesophile. Acidophile. Type strain has growth optima at pH 2.5-5.8 and 30-35 °C and grows pH 2.0-6.0 and 10-37 °C. Some strains grow at 0.4 M Fe<sup>2+</sup>, 0.2 M Fe<sup>3+</sup>, 0.05 M Cu<sup>2+</sup>, and 0.3 M Zn<sup>2+</sup>, but are sensitive to MoO<sub>4</sub><sup>2-</sup> at 100 μM. When grown on ferrous iron under air at pH 2.1, 30 °C, the dominant fatty acids are *cis*-vaccenic acid (C<sub>18:1</sub> ω7c), palmitoleic acid (C<sub>16:1</sub>), palmitic acid (C<sub>16:0</sub>), 10-*trans*-cyclopropylnonadecylic acid (C<sub>19:0</sub> *cyclo* ω8c), myristic acid (C<sub>14:0</sub>) and lauric acid (C<sub>12:0</sub>). Uses rusticyanin A (RusA). Form IA<sub>c</sub> (carboxysomal) and Form II (cytoplasmic) RuBisCO. Has *aa*<sub>3</sub>-type cytochrome *c*-oxidase and *bo*<sub>3</sub>-type and *bd*-I-type ubiquinol oxidases.

Type strain isolated from acid mine drainage at the Pittsburgh coal seam, PA, USA.

*DNA G+C content (mol%): 58.77 (sequence)*

*Type strain:* ATCC 23270 = CIP 104768 = DSM 14882

*GenBank accession (16S rRNA gene):* AF465604

726 *IMG accession (genome sequence): 643348501*

727

728 **8. *Acidithiobacillus thiooxidans*** (Waksman and Joffe 1922) Kelly and Wood 2000, 513<sup>VP</sup>

729 (*Thiobacillus thiooxidans* Waksman and Joffe 1922, 239)

730 thi.o.ox'i.dans. Gr. neut. n. *thêton*, sulfur, brimstone (L. transliteration, *thium*); Gr. masc. adj. *oxús*  
 731 (L. transliteration *oxys*), sharp, acidic; N.L. v. *oxydo*, to make acid, to oxidize; N.L. part. adj.  
 732 *thiooxidans*, sulfur-oxidizing.

733 Motile straight rods 0.5 × 1.0-2.0 µm. Monotrichous. Volutin (polyphosphate) and poly-β-  
 734 hydroxybutyrate granules. Colonies on thiosulfate agar are small and transparent. Uses elementary  
 735 sulfur, thiosulfate, and sulfide as electron donors. Obligate aerobe. Diazotrophy not observed.  
 736 Mesophile. Extreme acidophile. Type strain has growth optima at pH 2.0-3.5 and 28-30 °C and  
 737 grows pH 0.5-6.0 and 18-37 °C. When grown on elementary sulfur under air at pH 4.4, 30 °C, the  
 738 dominant fatty acids are ω-cyclohexylnonadecylic acid (C<sub>19:0</sub> *cyclo*), ω-cyclohexylmargaric acid  
 739 (C<sub>17:0</sub> *cyclo*), palmitoleic acid (C<sub>16:1</sub>), vaccenic acid (C<sub>18:1</sub>) and palmitic acid (C<sub>16:0</sub>). Has Form IAc  
 740 (carboxysomal) and Form II (cytoplasmic) RuBisCO and *bo*<sub>3</sub>-type and *bd*-I-type ubiquinol oxidases.  
 741 Type strain isolated from compost of soil, phosphorite and elementary sulfur, NJ, USA.

742 *DNA G+C content (mol%): 53.16 (sequence)*

743 *Type strain: ATCC 19377 = CIP 104597 = DSM 14887 = JCM 3867 = NCIMB 8343*

744 *GenBank accession (16S rRNA gene): Y11596*

745 *IMG accession (genome sequence): 2510461056*

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904

Character	<i>A. albertensis</i>	<i>A. caldus</i>	<i>A. concretivorus</i> comb. nov.	<i>A. ferridurans</i>	<i>A. ferriphilus</i>	<i>A. ferrivorans</i>	<i>A. ferrooxidans</i>	<i>A. thiooxidans</i>
Origin of type strain	Soil near sulfur stockpile, Fox Creek, Alberta, Canada.	Coal spoil heap, Kingsbury Colliery, UK.	Decomposing sewer concrete, Melbourne, Australia.	Drainage water in uranium mine, Ningyo-Tohge, Tottori, Japan.	Acidic pool, Montserrat	Spoil heap drainage at disused copper mine, Norway.	Acid mine drainage from coal seam, Pittsburgh, PA, USA.	Compost of soil, phosphorite and sulfur, NJ, USA
16S rRNA gene identity to <i>A. thiooxidans</i> ATCC 19377 <sup>T</sup>	99.9	95.1	99.8	98.2	97.8	97.6	98.1	100
Cell size (µm)	0.45 × 1.2-1.5	0.7-0.8 × 1.2-1.8	0.5 × 1.0-2.0	<i>N.D.</i> × 1.0-2.0	<i>N.D.</i> × 1.0-2.0	0.5 × 1.6-2.4	0.5 × 1.0	0.5 × 1.0-2.0
Flagellation	Lophotrichous	Monotrichous	Monotrichous	ν	Monotrichous	Monotrichous	Atrichous?*	Monotrichous
Carboxysomes	+	+	<i>N.D.</i>	<i>N.D.</i>	+	+	+	+
<b>Intracellular inclusions:</b>								
Sulfur	+	<i>N.D.</i>	<i>N.D.</i>	<i>N.D.</i>	<i>N.D.</i>	<i>N.D.</i>	<i>N.D.</i>	-
Volutin	-	<i>N.D.</i>	+	<i>N.D.</i>	<i>N.D.</i>	<i>N.D.</i>	+	+
Poly-β-hydroxybutyrate	-	<i>N.D.</i>	<i>N.D.</i>	<i>N.D.</i>	<i>N.D.</i>	<i>N.D.</i>	+	-
<b>Electron donors:</b>								
Elementary sulfur (S <sub>8</sub> )	+	+	+	+	+	+	+	+
Thiosulfate (S <sub>2</sub> O <sub>3</sub> <sup>2-</sup> )	+	+	+	<i>N.D.</i>	<i>N.D.</i>	+	+	+
Tetrathionate (S <sub>4</sub> O <sub>6</sub> <sup>2-</sup> )	+	+	<i>N.D.</i>	+	+	+	+	<i>N.D.</i>
Sulfide (S <sup>2-</sup> )	-	+	+	<i>N.D.</i>	<i>N.D.</i>	+	<i>N.D.</i>	-
Ferrous iron (Fe <sup>2+</sup> )	-	-	<i>N.D.</i>	+	+	+	+	-
Pyrite (FeS <sub>2</sub> )	-	-	<i>N.D.</i>	+	<i>N.D.</i>	+	+	-
Molecular hydrogen (H <sub>2</sub> )	-	+	<i>N.D.</i>	+	<i>N.D.</i>	ν	+	<i>N.D.</i>
Ferric iron (Fe <sup>3+</sup> ) as terminal electron acceptor	-	-	-	+	+	+	+	-
Diazotrophy	-	-	<i>N.D.</i>	+	<i>N.D.</i>	+	+	-
Temperature range and [optimum] (°C)	[28-30]	32-52 [45]	10-37 [28]	[29]	[30]	4-37 [25-32]	10-37 [30-35]	18-37 [28-30]
pH range and [optimum]	2.0-4.5 [3.5-4.0]	1.0-3.5 [2.0-2.5]	-0.6-6.0 [2.0-4.0]	[2.1]	[2.0]	1.9-3.4 [2.5]	2.5-6.0 [2.5-5.8]	0.5-6.0 [2.0-3.5]
Dominant fatty acids	<i>N.D.</i>	<i>N.D.</i>	[S <sub>8</sub> , pH c.4.4, 30°C]† C <sub>19:0</sub> <i>cyclo</i> , C <sub>17:0</sub> <i>cyclo</i> C <sub>16:0</sub> , C <sub>12:0</sub> C <sub>14:0</sub> 3-OH, C <sub>16:1</sub>	[H <sub>2</sub> , pH 2.0, 30°C] C <sub>19:0</sub> <i>cyclo</i> ω8c, C <sub>18:1</sub> ω7c, C <sub>16:0</sub> , C <sub>16:1</sub> , C <sub>17:0</sub> <i>cyclo</i> , C <sub>12:0</sub>	[Fe <sup>2+</sup> , pH c.1.6, 30°C] C <sub>18:1</sub> ω7c, C <sub>16:1</sub> C <sub>18:1</sub> 2-OH C <sub>14:0</sub> 3-OH C <sub>16:0</sub> , C <sub>12:0</sub>	<i>N.D.</i>	[Fe <sup>2+</sup> , pH 2.1, 30°C] C <sub>18:1</sub> ω7c C <sub>16:1</sub> , C <sub>16:0</sub> C <sub>19:0</sub> <i>cyclo</i> ω8c C <sub>14:0</sub> , C <sub>12:0</sub>	[S <sub>8</sub> , pH c.4.4, 30°C] C <sub>19:0</sub> <i>cyclo</i> C <sub>17:0</sub> <i>cyclo</i> C <sub>16:1</sub> , C <sub>18:1</sub> , C <sub>16:0</sub> C <sub>14:0</sub> 3-OH
Dominant polar lipids	<i>N.D.</i>	<i>N.D.</i>	<i>N.D.</i>	PG, PE, ALs (3×)	AL, PL, PG	<i>N.D.</i>	<i>N.D.</i>	<i>N.D.</i>
<b>Genomic properties:</b>								
G+C fraction (mol%)	52.5 <sup>g</sup>	61.4 <sup>g</sup> , 63.9 <sup>f</sup>	<i>N.D.</i>	56.0-60.0 <sup>f</sup>	57.8-58.2 <sup>f</sup>	55.5 <sup>f</sup>	58.8 <sup>g</sup> , 59.2 <sup>f</sup>	53.2 <sup>g</sup>
Genome size (Mbp)	3.47	2.99	<i>N.D.</i>	<i>N.D.</i>	<i>N.D.</i>	2.98-3.42§	2.98	3.02
Protein coding genes	3,671	2,980	<i>N.D.</i>	<i>N.D.</i>	<i>N.D.</i>	3,125-3,606§	3,147	3,080
D-ribulose 1,5-bisphosphate carboxylase genes	Form IAc ( <i>cbbLS</i> ) Form II ( <i>cbbM</i> )	Form IAc ( <i>cbbLS</i> ) Form II ( <i>cbbM</i> )	<i>N.D.</i>	<i>N.D.</i>	<i>N.D.</i>	Form IAc ( <i>cbbLS</i> )‡ Form II ( <i>cbbM</i> )	Form IAc ( <i>cbbLS</i> ) Form II ( <i>cbbM</i> )	Form IAc ( <i>cbbLS</i> ) Form II ( <i>cbbM</i> )
Rusticyanin genes	-	-	<i>N.D.</i>	<i>rusA</i> ‡	<i>N.D.</i>	<i>rusB</i> ‡	<i>rusA</i>	-
<b>Terminal oxidases:</b>								
Cytochrome <i>c</i> -oxidases	-	-	<i>N.D.</i>	<i>N.D.</i>	<i>N.D.</i>	<i>aa3</i> §	<i>aa3</i>	-
Ubiquinol oxidases	<i>bo3</i> , <i>bd-I</i>	<i>bo3</i> , <i>bd-I</i>	<i>N.D.</i>	<i>N.D.</i>	<i>N.D.</i>	<i>bo3</i> §	<i>bo3</i> , <i>bd-I</i>	<i>bo3</i> , <i>bd-I</i>

**Table I.** Curated properties of *Acidithiobacillus* spp. Data are curated from the original protologues and from the curation of Parker and Temple (1957). For cellular fatty acids, growth conditions are given in brackets, which also apply to polar lipids, if reported. Those reported for *A. concretivorans* comb. nov. and *A. thiooxidans* are from Katayama-Fujimura *et al.* (1982).

Polar lipids: PG – phosphatidylglycerol; PE – phosphatidylethanolamine; AL – aminolipid; PL – phospholipid.

\* Some authors report *A. ferrooxidans* ATCC 23270<sup>T</sup> as motile but the genome lacks flagellar genes and this particular strain has been shown as non-motile by some workers.

† Data for *A. concretivorus* comb. nov. ATCC 15494 rather than type strain.

‡ Data from PCR based work (*A. ferridurans*, Hedrich and Johnson, 2013; *A. ferrivorans*, Hallberg *et al.* 2010)

§ Data based on *A. ferrivorans* YL15 and *A. ferrivorans* CF27 genomes.

Superscripts on G+C fractions are: *g* – from genome sequence; *t* – thermal denaturation ( $T_m$ ).

**Figure 1.** Maximum likelihood tree of the *Acidithiobacillales* on the basis of 53 concatenated ribosomal protein gene sequences translated *in silico* into amino acid sequences, pertaining to *rpsA-rpsU*, *rplA-rplF*, *rplL-rplX*, and *rpmA-rpmJ*. Gene concatamer sequences were downloaded *en bloc* from the ribosomal multilocus sequence typing (rMLST) database (<http://pubmlst.org/rmlst>) and were translated *in silico* and aligned using the MUSCLE algorithm (Edgar, 2004) in MEGA X (Kumar *et al.* 2018), per Boden *et al.* (2017*a,b*). The aligned data were model-tested in MEGA X on the basis of the lowest corrected Aikake information criterion (AICc, Hurvich and Tsai, 1989; Aikake, 1973). The outgroup (not shown) is the equivalent concatamer from *Pseudomonas aeruginosa* DSM 50071<sup>T</sup> (152515). Type strains of species are emboldened. Numbers in parentheses this legend and in the figure refer to genome accession numbers of each strain in the rMLST database. The tree was constructed in MEGA X with partial deletion of gaps (95 % cut-off), and the final analysis involved 5,766 aa. The model of Le and Gascuel (2008) was used with a discrete gamma distribution (5 categories, gamma parameter = 0.8351) with 18.88 % of sites evolutionarily invariant. Tree shown had the highest log likelihood (-47458.98). Branch lengths are proportional to the number of substitutions, the bar representing 0.05 substitutions per site. Bootstrap values at nodes are on the basis of 5,000 replications (values < 70 % are omitted for clarity).

[atb rMLST pretty.tif]