Acidithiobacillus

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Genus **Acidithiobacillus**

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

Abstract:

Cells are short, motile rods with a single polar flagellum. Some strains have an obvious glycocalyx. Gram-stain-negative. Endospores, exospores and cysts are not produced. Obligate chemolithoautotrophs, with electron donors including reduced inorganic sulfur species such as thiosulfate, tetrathionate, elementary sulfur (*viz.* α-S_8 and μ-S_∞). Some species can also use molecular hydrogen, ferrous iron or metal sulfides such as pyrite (FeS_2) as electron donors. Some species are diazotrophic. Heterotrophy, methylotrophy and the so-called "C_1 autotrophy" are not observed. Carbon assimilated from CO_2 *via* the transaldolase-variant of the Calvin-Benson-Bassham cycle. Carboxysomes are used for CO_2 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 ($C_{16:0}$), vaccenic acid ($C_{18:1}$), *cis*-11-cyclopropyl-nonadecanoic acid ($C_{19:0}$ *cyclo* ω_8c), palmitoleic acid ($C_{16:1}$), myristic acid ($C_{14:0}$) and lauric acid ($C_{12:0}$). 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_3 and *bd*-I ubiqunol oxidases and, in the iron-oxidizing species, the aa_3-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_3-type ubiquinol oxidase and the non-translocating *bd*-I ubiquinol oxidase are used by all species, with the proton-translocating aa_3-type cytochrome *c* oxidase found only in the iron oxidizing taxa.

The dominant fatty acids are palmitic acid ($C_{16:0}$), vaccenic acid ($C_{18:1}$), *cis*-11-cyclopropyl-nonadecanoic acid ($C_{19:0}$ *cyclo* $\omega_8 c$), palmitoleic acid ($C_{16:1}$), myristic acid ($C_{14:0}$) and lauric acid ($C_{12:0}$). The dominant polar lipids are cardiolipin, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylethanol and aminolipids.

Type species: **Acidithiobacillus thiooxidans** (Waksman and Joffe 1922) Kelly and Wood 2000, 513^{VP} (*Thiobacillus thiooxidans* Waksman and Joffe 1922, 239)

Number of species with validly published names: 7.

Family classification:

Acidithiobacillaceae (fbm00213)

Further Descriptive Information

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

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

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

Growth physiology. To maintain pH homeostasis and a cytosol of pH *c*.6-7, acidophiles must use active transport to remove protons from the cell against a concentration gradient. One could thus assume that the maintenance coefficients (m_S, *cf*. Pirt (1975)) of *Acidithiobacillus* spp. are somewhat higher than neutrophilic species, given the elevated cost of living at low pH, where ATP is used continually for H^+ export and both ATP and NAD(P)H are consumed for repair; however, this does not appear to be the case. In the following examples, growth pH is given in parentheses after the binomial. For *Acidithiobacillus ferrooxidans* (pH 2.5) in thiosulfate-limited chemostats, m_S is 770 µmol $S_2O_3^{2-}$/g dry biomass/h, and under tetrathionate limitation, m_S is 1,030 µmol $S_4O_6^{2-}$/g dry biomass/h. For *Acidithiobacillus thiooxidans* (pH 2.5) under tetrathionate limitation (pH 2.5), m_S is 40 µmol $S_4O_6^{2-}$/g dry biomass/h. By comparison, for neutrophilic (pH 7.6) species under thiosulfate limitation, m_S values are broadly similar, or if anything, higher, *viz*. *Paracoccus versutus* (850 µmol $S_2O_3^{2-}$/g dry biomass/h) and *Annwoodia aquaesulis* (1,270 µmol $S_2O_3^{2-}$/g dry biomass/h) [values determined by hyperbolic fit of data curated by Kelly *et al.* (1997), using the method of Boden and Hutt (2018)]. For comparison, Pirt (1975) reported *Enterobacter cloacae* subsp. *cloacae* grown under glucose-limitation at pH 7.2 under air had m_S of 522 µmol glucose/g dry biomass/h, which he considered fairly typical for heterotrophs. These data suggest that for *Acidithiobacillus* spp., growth at low pH either incurs no greater maintenance costs, or that these costs have been overcome by their 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 (Δp) generated through electron transport as heavily as other organisms (Ingledew, 1982) – they thus have three potential routes of ATP generation: i) substrate-level phosphorylation; ii) Δp formed through proton extrusion to the periplasm during electron transport, fueling ATP synthase, and/or iii) Δp 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 are 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 ($\Delta G°$) 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:

$$\mathbf{S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2SO_4^{2-} + 2H^+}$$

$$\mathbf{\Delta G° = -733.28\ kJ/mol\ thiosulfate}$$

$$S_4O_6^{2-} + 3½O_2 + 3H_2O \rightarrow 4SO_4^{2-} + 6H^+$$

$\Delta G°$ = -1,244.78 kJ/mol tetrathionate

$$S_8 + 12O_2 + 8H_2O \rightarrow 8H_2SO_4$$

$\Delta G°$ = -3,623.36 kJ/mol orthorhombic cyclooctasulfur

$$2FeS_2 + 3O_2 + 6H_2O \rightarrow Fe_2(SO_4)_3 + 12H^+$$

$\Delta G°$ = -243.06 kJ/mol pyrite

$$4FeSO_4 + O_2 + 2H_2SO_4 \rightarrow 2Fe_2(SO_4)_3 + 2H_2O$$

$\Delta G°$ = -71.55 kJ/mol ferrous sulfate

$$4Fe^{2+} + O_2 + 10H_2O \rightarrow 4Fe(OH)_3 + 8H^+$$

$\Delta G°$ = -24.78 kJ/mol ferrous iron

$$2H_2 + O_2 \rightarrow 2H_2O$$

$\Delta G°$ = -474.36 kJ/mol molecular hydrogen

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

$$S_8 + 48Fe^{3+} + 32H_2O \rightarrow 8SO_4^{2-} + 48Fe^{2+} + 64H^+$$

$\Delta G°$ = -1,932.24 kJ/mol orthorhombic cyclooctasulfur

$$H_2 + 2Fe^{3+} \rightarrow 2H^+ + 2Fe^{2+}$$

$\Delta G°$ = -148.54 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 S_8, about 113 mol Fe(II) must be oxidized *versus* one of 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

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

The specific molar growth yields (*Y*) of *Acidithiobacillus* spp. relate not only to the energy output of substrate oxidation but also the energy demands of CO_2 fixation, *via* the transaldolase-variant Calvin-Benson-Bassham (CBB) cycle, this is much higher than *via* Krebs' cycle *etc* in a generalist (Kelly, 1990):

$$\mathbf{12CO_2 + 61ATP + 25.5NADPH + 3NH_3 \rightarrow C_{12}H_{24}O_6N_3 + 18H_2O}$$

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

Metabolism. Although recent advances have been made, the sulfur-oxidation pathways have still not been fully elucidated and probably vary between species, but recent advances have been made. Recent studies have indicated that monosulfanemonosulfonates play key roles in the oxidation of sulfur from pyrite (FeS_2), based on studies of an *A. ferrooxidans* strain isolated from a mine-drainage pond in China (Tu *et al*., 2017). These somewhat arcane, unstable oxyanions have the general formula $S_nO_3^{2-}$, the simplest of which is monosulfanemonosulfonate, better known as thiosulfate, $S_2O_3^{2-}$. Whilst thiosulfate is very stable, the remainder of the series are very unstable, but may be conjugated to protein-carriers *in vivo* to afford stability. It is not yet known if the monosulfanemonosulfonates have roles in the oxidation of other sulfur species. Similarly, it is also not known if their role during ferric iron respiration of sulfur in anoxia (in which they are more stable) is more significant than in the aerobic oxidation of sulfur.

The oxidation of S_8 in *A. ferroxidans* has recently been elucidated in more detail by Wang *et al*. (2019). Briefly, S_8 rings react with thiol groups on cysteine residues on an outer membrane protein, forming H_2S

and sulfite in the periplasm. The former is oxidized by sulfide:quinone oxidoreductase (Sqr, EC 1.8.5.4) on the inner membrane, yielding polysulfane (S_n^{2-}) which reacts with sulfite present to form thiosulfate (polysulfanes are sometimes called "polysulfides" in the literature, but the latter strictly refers to organosulfur compounds of structure R_1-S_n-R_2). Thiosulfate is oxidized by the quinone-linked thiosulfate dehydrogenase (Tqo, EC 1.8.5.2), forming tetrathionate and donating electrons to ubiquinone (this could potentially also be catalyzed by the cytochrome *c*-linked thiosulfate dehydrogenase (TsdA, EC 1.8.2.2), activity of which has been recorded in this genus (Silver and Lundgren, 1968), but no gene homologs are found). Tetrathionate is cleaved into sulfate and polysulfane (S_n^{2-}) by tetrathionate hydrolase (TetH, EC 3.12.1.B1). Polysulfane chains react with glutathione (GSH), forming GSSH, which is oxidized in the cytoplasm by a series of proteins comprising the sulfur transporters TusA and DsrE, thiosulfate sulfurtransferase (rhodanese, Rhd, EC 2.8.1.1), persulfide dioxygenase (Sdo, EC 1.13.11.18) and a heterodisulfide reductase-like complex (Hdr), forming GSH and sulfite, and donating electrons to ubiquinone. This sulfite is conjugated to adenosine 5′-monophosphate (AMP) forming adenosine 5′-phosphosulfate (APS), catalyzed by APS reductase (Apr/Aps, EC 1.8.99.2). APS is then lysed by sulfate adenylyltransferase (Sat, EC 2.7.7.4), forming sulfate, ATP and protons. These terminal reactions from sulfite to sulfate *via* APS reductase *etc* provide ATP by substrate-level phosphorylation: this is distinct from *Thermithiobacillus* spp. in which substrate-level phosphorylation does not occur (Wood and Kelly, 1986). Ubiquinol formed during the earlier steps donates electrons to the bo_3-type ubiquinol oxidase (or, if respiration is 'backed-up' owing to a paucity of ADP, and Δp is very high, to the *bd*-I type ubiquinol oxidase). Electrons from ubiquinol can also be transported in the reverse direction at the expense of Δp, generating NADH.

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

bisulfide (HS^-) and sulfur from S_8 can then bind to the pendent sulfane moiety of SoxYZ and be oxidized to sulfate. Another fate of sulfite is by reaction with S^o, forming thiosulfate (the Suzuki and Silver reaction). The latter is oxidized to tetrathionate by Tqo per *A. ferrooxidans*. From this point onwards, a multiply branched set of pathways occurs, the precise regulation of which is not fully understood. The ultimate fate of tetrathionate-sulfur is to sulfite, which is used in substrate-level phosphorylation reactions per *A. ferrooxidans*. Why the organism needs both the Lu-Kelly cycle *and* APS reductase pathways for sulfite oxidation is unclear, but since the former generates Δp (used to synthesise ATP from ADP, or NADH from NAD^+) and the latter generates ATP without Δp *or* ADP, it would seem that the redox balance of the respiratory chain, the size of the cellular ADP-pool and the NADH demand of the cell would be likely contributors to regulation of which pathway is used at any given time.

Ferrous iron oxidation is also variable within the genus. In *A. ferrooxidans* ATCC 23270^T and *A. ferridurans* ATCC 33020^T, an iron:rusticyanan reductase (EC 1.16.9.1), a form of cytochrome *c*, on the outer membrane oxidizes Fe(II) to Fe(III) and transfers the electron to rusticyanin A (RusA) in the periplasm. Rusticyanins are small copper proteins (*c*. 16 kDa), which transfer electrons to a further cytochrome *c*, from which they are transferred to the aa_3-type cytochrome *c*-oxidase and molecular oxygen. For reverse electron transport to generate NADH, electrons are transferred from RusA to a cytochrome c_4, then on to the bc_1 complex, the quinone pool and the NADH dehydrogenase (quinone, EC 1.6.5.11). In *Acidithiobacillus ferriphilus* strains, the iron oxidation enzyme (Iro, EC 1.16.3.*x*, a high-potential iron-sulfur protein, HiPIP) oxidises Fe(II) to Fe(III) in the periplasm, transferring electrons to cytochrome *c*, which are then transferred either directly to the aa_3 cytochrome *c* oxidase per the above, or *via* rusticyanin B (RusB). Although rusticyanins have been assumed for many years to be critical to iron oxidation, *A. ferrivorans* CF27 apparently lacks both *rusA* and *rusB* genes but still grows on ferrous iron (Hedrich *et al*., 2011).

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

Johnson, University of Bangor, UK, *personal communication*). Oxidation of molecular hydrogen can be coupled to molecular oxygen or ferric iron reduction. A respiratory [NiFe]-hydrogenase (EC 1.12.99.6), catalyzes the oxidation of H_2 to H^+, with transfer of electrons to cytochrome *b* and then the quinone pool. Electron transport from ubiquinol can be forward or reverse, as described above. This should not be confused with the cytoplasmic uptake [NiFe]-hydrogenase of *A. ferrooxidans* used during diazotrophic growth, for scavenging molecular hydrogen generated therein (Valdés *et al*., 2008; Vignais *et al*., 2001).

Whilst the original authors reported that *Acidithiobacillus caldus* (Hallberg and Lindström, 1995) was capable of mixotrophic growth (*viz*. simultaneous autotrophic growth at the expense of a sulfur oxyanion and heterotrophic growth on a sugar or complex medium), later workers have not been able to replicate this (D. Barrie Johnson, *personal communication*). Thus, it may have been owing to contamination. No reported growth on organic carbon compounds has been observed for any *Acidithiobacillus* spp.

It is usual to cultivate *Acidithiobacillus* spp. on flowers-of-sulfur or roll sulfur (former comprises orthorhombic cyclooctasulfur (α-S_8) with 'significant' amounts of polymeric (*catena*-S_∞ or μ-S) sulfur (Steudel and Eckert, 2003), whereas the latter is 99 % α-S_8, 0.6 % cycloheptasulfur (S_7), Lesté-Lasserre (2001)). Growth on *catena*-S_∞ has been observed in *A. ferrooxidans* ATCC 23270^T, and this allotrope interestingly produces less growth per unit biomass than α-S_8. This property makes it a potentially interesting additive in biohydrometallurgy, where excess biomass can compromise the mineral surface, but strong acid production is needed (He *et al*., 2011). *A. thiooxidans* growth on elementary sulfur is stimulated by low concentrations of surfactants such as sodium 2-ethylhexyl sulfate (Tergitol 08) or polysorbate 80 (TWEEN® 80) – increasing the wetting of the surface of the sulfur particles (Starkey *et al*., 1956). The dipolar sudanophilic granules in *A. thiooxidans* containing polyhydroxybutyrate were once believed to play a role in the initial wetting of sulfur (Umbreit *et al*., 1942), though it was later found that phosphatidylinositol (PI) was a wetting agent in this species (Schaeffer and Umbreit, 1963), though Jones and Benson (1965) also demonstrated phosphatidylglycerol, phosphatidic acid and phosphatidylcholine in culture supernates, which were not found as major polar lipids cell extracts, and that PI was only present to a comparatively minor degree compared to these compounds.

In vitro, growth of *Acidithiobacillus* strains can be difficult if organic contaminants are present, such as detergents (Onysko *et al*., 1984), carboxylic acids, or some contaminating metals. As with almost all acidophiles, the uncoupling agent-type effect of carboxylate salts is of course inhibitory to growth and must be avoided. At pH below the pK_a, carboxylates revert to carboxylic acids and can thus cross a cell membrane. Upon entry into the cytoplasm, they are at a pH greater than their pK_a and liberate protons, returning to the conjugate base, lowering the intracellular pH, eventually killing the cell – fumarate, succinate, formate, acetate, oxalate, trichloroacetate and many others have been shown to be toxic at 1 mM or below (Tuttle and Dugan, 1976). Sulfamate ($NH_2SO_3^-$) is also toxic to *A. ferrooxidans* but apparently *via* a different mechanism as it has a pK_a of about 0.1 at room temperature, thus would only be inhibitory at very low pH values *via* this uncoupling mechanism but is toxic to cultures at pH 4.5 (Lusty *et al*., 2006).

When growing these organisms on natural minerals, contaminating metals can pose an issue, in particular hexavalent chromium (CrO_4^{2-}/$Cr_2O_7^{2-}$) is toxic to many strains growing on iron, but on elementary sulfur, the formation of intermediary sulfite and thiosulfate permits some *A. ferrooxidans* to 'resist' $Cr_2O_7^{2-}$ (reducing it to Cr^{3+}) at up to 2 mM. This is *sensu stricto* chemical reduction of $Cr_2O_7^{2-}$ by thiosulfate/sulfite, not biological reduction or true resistance (Sisti *et al*., 1996). *A. ferrooxidans* growth on Fe(II) or FeS_2 is inhibited by Ag^+ at 1 µM (Tuovinen *et al*., 1985; Hoffman and Hendrix, 1976), but the addition of yeast extract to cultures (which presumably binds Ag^+) can alleviate this. Cu^{2+}, Zn^{2+}, Cd^{2+} and Cr^{3+} ions did not inhibit *A. ferrooxidans* at 1-10 mM, but Pb^{2+}, Sn^{2+}, MoO_4^{2-}, Hg^+, Hg^{2+} and Ag^+ were fully inhibitory at 1 mM (Imai *et al*., 1975). Additionally, SeO_3^{2-}, TeO_3^{2-}, AsO_3^{2-} and MoO_4^{2-} were toxic, the latter at concentrations as low as 30 µM, in *A. ferrooxidans* ATCC 13661 (Tuovinen *et al*., 1971; N.B. strain no longer available). In a range of *A. ferrooxidans* isolates from Brazilian uranium and coal mines, resistance to Ag^+, Hg^{2+}, Co^{2+} and Cu^{2+} was found to be variable at strain-level, which could relate to plasmid-mediated resistance (Garcia Jr and da Silva, 1991). *A. ferrooxidans* ATCC 23270^T is sensitive to UO_2^{2-} ions, but adding 5 mM EDTA to cultures was sufficient to overcome this inhibition (Mahapatra and Mishra, 1984). *A. thiooxidans* is inhibited by WO_4^{2-}, a common ingredient in trace-element solutions, which inhibits the enzymes of sulfur oxidation (Negishi *et al*., 2005). In *A. thiooxidans* ATCC 8085, growth on elementary sulfur is inhibited by VO_4^{3-}, MoO_4^{2-} and CrO_4^{2-} at 0.2-0.5 mM (Jack *et al*., 1980).

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

Genomic and biochemical features, and their relation to ecology. The genome sequences of many *Acidithiobacillus* spp. have been completed and made publically available *via* the Integrated Microbial Genomes (IMG) database. Properties of those from species with validly published names are curated in Table I. They are usually about 3 Mbp with 2,500-3,000 protein coding genes. All of the genomes sequenced thus far show the presence of Form IAc ('green') D-ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO, EC 4.1.1.39), which is founded exclusively within carboxysomes ('polyhedral bodies'). These intracellular compartments are consistently found in *Acidithiobacillus* spp., and are involved in the concentration of CO_2. This is hardly surprising since in low pH environments bi/carbonate has a very low availability, and effectively trapping dissolved inorganic carbon (DIC) is a challenge. Form IAc is optimized for low pCO_2 and low to high pO_2. The cytosolic Form II RuBisCO is also found, and is optimized for medium to high pCO_2 with low pO_2. It is probably used when pO_2 is low, or in organisms growing at the higher end of their pH range, or in environments with otherwise high pCO_2. The bo_3-type ubiquinol oxidase (proton translocating, EC 1.10.3.10) is the respiratory terminal oxidase in all *Acidithiobacillus* spp. and is used during growth on sulfur species, oxidation of which is coupled entirely to ubiquinone reduction. The *bd*-I-type ubiquinol oxidase (non-translocating, EC 1.10.3.14) is also found, which is used to restore redox balance when the quinone pool becomes dominated with quinol, usually because of a paucity of ADP and thus a high Δp. 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_3 type is found, which is optimized for atmospheric pO_2, and does not perform well at lower pO_2.

Cultivation, Enrichment and Isolation Procedures

General cultivation: Deionized water that has then been glass-distilled (ddH_2O) 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 Phytagel™ 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 Phytagel™ is autoclaved in the other 50 % of ddH_2O, 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_2O 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 Ňancucheo *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

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

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

General *Acidithiobacillus* basal salts (GABS, pH 4.0-4.5, ionic strength 0.049 M) for cultivation in general on thiosulfate or tetrathionate comprises (g/L in ddH_2O): $(NH_4)_2SO_4$ (0.60), KH_2PO_4 (0.60), $MgSO_4 \cdot 7H_2O$ (0.25), $CaCl_2 \cdot 2H_2O$ (0.05). For growth on thiosulfate or tetrathionate, these are added to 20 or 10 mM, respectively, prior to autoclaving, by directly dissolving the sodium or potassium salts in GABS. After autoclaving, 50 mL aliquots are dispensed into sterile 250-mL glass wide-mouth Erlenmeyer flasks and 0.2 mL 3.6 mM $FeSO_4 \cdot 7H_2O$ in 0.1 N HCl (filter sterilized) is added to each flask as a trace metal.

For growth of *Acidithiobacillus caldus* on tetrathionate we use CA-basal salts (CABS, pH 2.50, ionic strength 0.501 M), which comprises (g/L ddH_2O): Na_2SO_4 (1.41), $(NH_4)_2SO_4$ (3.00), $MgSO_4 \cdot 7H_2O$ (0.50), KCl (0.10), K_2HPO_4 (0.05), trace metals solution CA (10 mL), $K_2S_4O_6$ (5.00). All ingredients except for the potassium tetrathionate and trace metals solution are dissolved in 850 mL ddH_2O. This solution is then adjusted to pH 1.75 using 1 N H_2SO_4, and diluted to 970 mL before autoclaving. Trace metals solution and tetrathionate (in 20 mL ddH_2O) are separately filter sterilized and then added to the cooled, autoclaved soltuion. Trace metal solution CA comprises (g/L ddH_2O): $FeCl_3 \cdot 6H_2O$ (1.10), $Ca(NO_3)_2 \cdot 4H_2O$ (1.00), H_3BO_3 (0.20), $MnSO_4 \cdot H_2O$ (0.20), $ZnSO_4 \cdot 7H_2O$ (0.09), $Na_2MoO_4 \cdot 2H_2O$ (0.08), $CoCl_2 \cdot 6H_2O$ (0.06), $CuSO_4 \cdot 5H_2O$ (0.05), and should be stored at room temperature in non-actinic glass and should not be autoclaved neat.

For growth of *Acidithiobacillus ferrooxidans* on tetrathionate, we use high-pH FA-basal salts (FABS4.4, pH 4.4, ionic strength 0.406 M) containing (g/L ddH_2O): KH_2PO_4 (3.00), $MgSO_4 \cdot 7H_2O$ (3.00), $(NH_4)_2SO_4$

(0.50), $CaCl_2 \cdot 2H_2O$ (0.25), $K_2S_4O_6$ (5.00). Ingredients are dissolved in 975 mL ddH_2O and the solution adjusted to pH 4.4 with 1 N H_2SO_4, 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_2SO_4): KH_2PO_4 (0.40), $MgSO_4 \cdot 7H_2O$ (0.04), $(NH_4)_2SO_4$ (0.04), $FeSO_4 \cdot 7H_2O$ (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_2SO_4): K_2HPO_4 (0.50), $MgSO_4 \cdot 7H_2O$ (0.50), $(NH_4)_2SO_4$ (1.00), $FeSO_4 \cdot 7H_2O$ (50.00), $Ca(NO_3)_2$ (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 $NiSO_4 \cdot 6H_2O$ is added to 0.5-1.0 µM (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_2 and 10 % *v/v* CO_2, leaving about 10 % *v/v* O_2 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_2 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 $Na_2S_2O_3 \cdot 5H_2O$ 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 flasks 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_2O and washed in the normal way. They are finally soaked in 2 % *v/v* HNO_3 for 48 h then washed in ddH_2O until the washings are pH neutral.

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

For growth on terminal electron acceptors other than molecular oxygen, ferric sulfate ($Fe_2(SO_4)_3$) at 20 mM (8.00 g/L) is used, with usually elementary sulfur (5.00 g/L) as the electron donor. Cultures are incubated in 'QuickFit' Erlenmeyer flasks sealed with 'SubaSeal' vaccine stoppers – they are flushed thoroughly with argon or oxygen-free nitrogen (including the sterile medium therein) prior to inoculation. Argon is more costly, but as atomic argon is much denser than molecular nitrogen, it is more effective at sweeping air from glassware. It will also form an argon-blanket over the culture held in the vessel, such that any minor air leaks through a vaccine stopper do not reach the culture. A Schlenk manifold for alternate evacuation and argon-gassing can be useful, particularly if large numbers of flasks are to be prepared on a regular basis.

Minerals and elementary sulfur are easily sterilized by Tyndallisation and added to medium at 0.5-5.0 g/L. For details, see the chapter on *Thermithiobacillus* (gbm01080). Sterilization using γ-radiation, where available, is suitable for minerals where steam would cause hydroxide formation. Alternatively, soaking mineral samples in sodium benzoate solution (0.44 M) for 24 h and then *thoroughly* rinsing in sterile ddH_2O and drying can be used – this is useful for mineral packed inside of leaching column reactors (Onysko *et al*., 1984). *A. thiooxidans* and *A. concretivorus* can be removed from natural mineral samples using vapor-phase *p*-chlorocresol or chloroxylenol (7-14 day exposure followed by thorough 'washing' in a stream of sterile air). *A. ferrooxidans* can be removed with the same methods or also with a 1:1 mixture of thymol and *N*,*N*-dicyclohexylamine nitrite. This is also useful for removing *Acidithiobacillus* contaminants from specimen-grade sulfide minerals held in collections, to prevent degradation (Booth and Sefton, 1970).

Analytical methods: During growth on ferrous iron in particular, the amount of biomass present does not appreciably change the optical density of the culture, whereas deposits of jarosite ($KFe^{3+}(OH)_6(SO4)_2$) *do* raise the optical density and this makes it an unsuitable parameter for determination of biomass. In the absence of a cell-counter that tolerates mineral particles and can distinguish jarosite or sulfur or other mineral particles from cells (*e.g.* the CellFacts II Analyzer, CellFacts Analytics Ltd, Coventry, UK), one

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

$$\mathbf{2Ce(SO_4)_2 + 2FeSO_4 \rightarrow Ce_2(SO_4)_3 + Fe_2(SO_4)_3}$$

The ceric sulfate used should be a precise volumetric standard solution, either procured ready-made, or prepared quite economically and standardized oneself. First, 21.00 g ceric hydroxide is dissolved in 100 mL 98 % H_2SO_4 with stirring. Once fully dissolved, 300 mL ddH_2O is cautiously and slowly added over about 30 min. The solution is left overnight, and then filtered through Whatman No. 1 filter paper into a 1-L volumetric flask, and is then diluted to volume with ddH_2O. This solution is then standardized against an arsenic (III) standard, as follows. 0.200 g arsenic (III) oxide (dried at 105 °C for 1-2 h before weighing) is dissolved in 20 mL 2.00 M NaOH, warming gently until absolutely <u>all</u> of the solid material has dissolved. After cooling to room temperature, 25.00 mL 2 M H_2SO_4 is added, followed by 0.6 mL 'osmic acid'. The latter is prepared from 1.26 mL electron microscopy grade 0.4 % (*w/v*) osmium tetroxide solution, diluted to 2 mL in a volumetric flask with 50 mM H_2SO_4 – take great care from this step onwards to work in the fumehood and with eye protection as OsO_4 vapours permanently damage the eye! To this solution, 0.5 mL *N*-phenylanthranilic acid (diphenylamine-2-carboxylic acid) is added as the indicator. This changes from yellow-green to violet at end-point. This solution in its entirety is titrated against the prepared ceric sulfate solution and the precise concentration of the latter is determined from the stoichiometry:

$$\mathbf{2Ce(SO_4)_2 + H_3AsO_3 + H_2O \rightarrow Ce_2(SO_4)_3 + H_3AsO_4 + H_2SO_4}$$

The concentration obtained is then used in all cerimetric titrations of Fe(II) in media. All osmium-containing waste is poured into a large beaker in the fume hood and an equal volume of corn oil or olive oil added, which reduces OsO_4 to safer, insoluble black OsO_2 over about 24 h.

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

$$\mathbf{Zn(Hg) + 2Fe^{3+} \rightarrow Zn^{2+} + Hg^{o} + 2Fe^{2+}}$$

The Jones reductor comprises a 360 × 20 mm glass tube topped with a *c*. 100 mL reservoir (60 × 45 mm). 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-L Büchner flask set up at the pump. A second flask is set up as a trap, to protect the pump. On the underside of the bung, a further length of glass tubing is connected with silicone tubing so as to reach to 0.5 cm from the bottom of the flask. About 250-300 g high purity zinc turnings in a wide beaker with a glass-covered stirring 'flea' are covered with 70 mM $HgCl_2$ solution and stirred for 10 min. The supernate is decanted off and the amalgamated zinc washed with 3-4 changes of ddH_2O – it should be bright silver in color – and is then packed into the glass tube, 500 mL ddH_2O are slowly drawn through the tube with a gentle vacuum from the pump, leaving the column material covered in water when the tap is closed. It should never be left exposed to the air at any time. To use, 50 mL 2 N H_2SO_4 is added to the reservoir and is drawn into the column with a vacuum until just below the top of the amalgam. This step is repeated twice and a clean, dry 1-L Büchner flask is then put into place. The analyte is diluted 10 fold in 2 N H_2SO_4 and is drawn through the column at about 1 mL/s. Once the reservoir is almost empty, 2 × 100 mL volumes of 0.5 N H_2SO_4 are drawn through, into the same flask, followed by 100 mL ddH_2O. The flask is disconnected and ferrous iron determined therein from a 50.00 mL aliquot by titration as above. If determining ferric iron in this way in leaching experiments in chloride-rich ores, it is necessary to add 25 mL Zimmermann-Reinhardt solution after reduction but prior to titration. This prevents the oxidation of chloride at the expense of Ce(IV), which would overestimate the Fe(II) present. This solution is made by dissolving 50 g manganous sulfate tetrahydrate in 250 mL ddH_2O to which 100 mL 98 % H_2SO_4 in 300 mL ddH_2O is added with stirring, followed by 100 mL 85% H_3PO_4.

Growth on other electron donors other than iron is often stronger, but at times it is still not possible to 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_2O 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 $CuSO_4 \cdot 5H_2O$ 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 $Fe(NO_3)_3 \cdot 9H_2O$ in 0.80 M $HClO_4$ 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^{-1} cm^{-1}, 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 $S_4O_6^{2-}$ yields 1 mol SCN^-, thus $[SCN^-]_A = [S_4O_6^{2-}]_A$

B: 1 mol $S_4O_6^{2-}$ yields 2 mol SCN^-, and 1 mol $S_2O_3^{2-}$ yields 1 mol SCN^-, thus $[SCN^-]_B - [SCN^-]_A = [S_2O_3^{2-}]_B$

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 $LaCl_3$ solution to remove interfering carbonates and phosphates beforehand. A volume of a standard $BaCl_2$ solution is then added and the resulting $BaSO_4$ precipitate allowed to settle. Residual Ba^{2+} in the supernate can then be determined by ICP-OES or AA based on the stoichiometry:

$$\mathbf{BaCl_2 + SO_4^{2-} \rightarrow BaSO_4 + 2Cl^-}$$

From this, the original sulfate concentration is found by the difference between the initial amount added and the amount remaining in solution. Ba^{2+} 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_3 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_2O. $BaCl_2$ (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_4^{2-} 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

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

Taxonomic comments

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

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

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

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

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

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

Motile straight rods 0.5 × 1.5-2.0 µm with square ends. Deeply stained volutin granules (polyphosphate). Motile by single polar flagella 4-6 µm long. Tiny clear colonies on thiosulfate agar, turning white-yellow with age. Strict aerobe. Oxidizes thiosulfate, hydrogen sulfide and elementary 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 ($C_{19:0}$ *cyclo* ω8*c*), ω-cyclohexylmargaric acid ($C_{17:0}$ *cyclo*), palmitic acid ($C_{16:0}$), lauric acid ($C_{12:0}$), 3-hydroxymyristic acid ($C_{14:0}$ 3-OH) and palmitoleic acid ($C_{16:1}$).

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**

1. ***Acidithiobacillus albertensis*** (Bryant, McGroarty, Costerton, Laishley 1988), Kelly and Wood 2000, 514^{VP} (*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

IMG accession number (genome sequence): 275118577

2. *Acidithiobacillus caldus* (Hallberg and Lindström 1995) Kelly and Wood 2000, 514^{VP} (*Thiobacillus caldus* Hallberg and Lindström 1995, 619)

cal′dus. L. masc. adj. *caldus*, warm, hot.

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

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

DNA G+C content (mol%): 66.60 (*B.d.*), 66.4 (sequence), 63.9 (T_m)
Type strain: KU = ATCC 51756 = DSM 8584
GenBank accession (16S rRNA gene): Z29975
IMG accession (genome sequence): 2811995294

3. *Acidithiobacillus concretivorus* (Parker 1945*a*) comb. nov. (*Thiobacillus concretivorus* Parker 1945, 81^{AL})

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

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

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 2013*a*, 4024^{VP}

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 µm 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 ($C_{19:0}$ *cyclo* ω8*c*), *cis*-vaccenic acid ($C_{18:1}$ ω7*c*),
658 palmitic acid ($C_{16:0}$), palmitoleic acid ($C_{16:1}$), ω-cyclohexylmargaric acid ($C_{17:0}$ *cyclo*) and lauric acid
659 ($C_{12:0}$), and polar lipids are phosphatidylglycerol, phosphatidylethanolamine and aminolipids. Type
660 strain tolerates up to 0.8 M Zn^{2+}, 1 M Mg^{2+}, 0.4 M Fe^{2+}, 0.2M Fe^{3+} and 1 mM UO_2^{2+}, but only 40
661 µM MoO_4^{2-}.

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_m)
664 *Type strain*: JCM 18981 = ATCC 33020
665 *GenBank accession (16S rRNA gene)*: AJ278719
666
667 **5.** ***Acidithiobacillus ferriphilus*** Falagán and Johnson 2016, 210^{VP}

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

Motile straight rods 1-2 µm long. Small ferric-iron-stained colonies on ferrous iron media. Uses ferrous iron, elementary sulfur and tetrathionate as electron donors. Can use ferric iron as terminal electron acceptor when growing on elementary sulfur as electron donors. Mesophile. Extreme acidophile. Some psychrotolerant strains. Type strain has growth optima at pH 2.0 and 30 °C. Some strains grow at >1 M Fe^{2+}, 0.5 M Fe^{3+}, 0.8 M Co^{2+}, and 1.2 M Mg^{2+}, but all are sensitive to MoO_4^{2-} at 100 µM. When grown on ferrous iron at pH 1.6, 30 °C, the dominant fatty acids are *cis*-vaccenic acid ($C_{18:1}$ *ω7c*), palmitoleic acid ($C_{16:1}$), 2-hydroxyvaccenic acid ($C_{18:1}$ 2-OH), 3-hydroxymyristic acid ($C_{14:0}$ 3-OH), palmitic acid ($C_{16:0}$) and lauric acid ($C_{12:0}$). The polar lipids are aminolipids, phospholipids and phosphatidylglycerol.

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

DNA G+C content (mol%): 57.4 (*B.d.*), 66.84 (sequence).
Type strain: M20 = DSM 100412 = JCM 30830
GenBank accession (16S rRNA gene): KR905751

6. ***Acidithiobacillus ferrivorans*** Hallberg, González-Toril and Johnson 2010*b* 469^{VP} (Effective publication: Hallberg, González-Toril and Johnson 2010*a* 18)

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

Motile straight rods 0.5 × 1.6-2.4 µm long. Motile. Small 'fried-egg' colonies, orange with off-white margins, on acidic iron/tetrathionate agar, turning opaque white/yellow with age. Uses elementary sulfur, thiosulfate, tetrathionate, sulfide, ferrous iron and pyrite as electron donors, with use of molecular hydrogen varying by strain. Can use ferric iron as terminal electron acceptor when growing on elementary sulfur as electron donor. Mesophile. Extreme acidophile. Some psychrotolerant strains. Type strain has growth optima at pH 2.5 and 25-32 °C but grows pH 1.9-3.4 and 4-37 °C. Uses rusticyanin B (RusB). Has aa_3-type cytochrome *c*-oxidase and bo_3-type ubiquinol 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^{2+}, <0.1 M Fe^{3+}, <0.05 M Cu^{2+}, and 0.2 M Zn^{2+}, but is sensitive to MoO_4^{2-} 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_m)
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^{VP}

(*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^{2+}, 0.2 M Fe^{3+}, 0.05 M Cu^{2+}, and 0.3 M Zn^{2+}, but are sensitive to MoO_4^{2-} at 100 µM. When grown on ferrous iron under air at pH 2.1, 30 °C, the dominant fatty acids are *cis*-vaccenic acid ($C_{18:1}$ *ω7c*), palmitoleic acid ($C_{16:1}$), palmitic acid ($C_{16:0}$), 10-*trans*-cyclopropylnonadecylic acid ($C_{19:0}$ *cyclo ω8c*), myristic acid ($C_{14:0}$) and lauric acid ($C_{12:0}$). Uses rusticyanin A (RusA). Form IAc (carboxysomal) and Form II (cytoplasmic) RuBisCO. Has aa_3-type cytochrome *c*-oxidase and bo_3-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

IMG accession (genome sequence): 643348501

8. ***Acidithiobacillus thiooxidans*** (Waksman and Joffe 1922) Kelly and Wood 2000, 513^{VP} (*Thiobacillus thiooxidans* Waksman and Joffe 1922, 239)

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

Motile straight rods 0.5 × 1.0-2.0 µm. Monotrichous. Volutin (polyphosphate) and poly-*β*-hydroxybutyrate granules. Colonies on thiosulfate agar are small and transparent. Uses elementary sulfur, thiosulfate, and sulfide as electron donors. Obligate aerobe. Diazotrophy not observed. Mesophile. Extreme acidophile. Type strain has growth optima at pH 2.0-3.5 and 28-30 °C and grows pH 0.5-6.0 and 18-37 °C. When grown on elementary sulfur under air at pH 4.4, 30 °C, the dominant fatty acids are *ω*-cyclohexylnonadecylic acid ($C_{19:0}$ *cyclo*), *ω*-cyclohexylmargaric acid ($C_{17:0}$ *cyclo*), palmitoleic acid ($C_{16:1}$), vaccenic acid ($C_{18:1}$) and palmitic acid ($C_{16:0}$). Has Form IAc (carboxysomal) and Form II (cytoplasmic) RuBisCO and bo_3-type and *bd*-I-type ubiquinol oxidases.

Type strain isolated from compost of soil, phosphorite and elementary sulfur, NJ, USA.

DNA G+C content (mol%): 53.16 (sequence)
Type strain: ATCC 19377 = CIP 104597 = DSM 14887 = JCM 3867 = NCIMB 8343
GenBank accession (16S rRNA gene): Y11596
IMG accession (genome sequence): 2510461056

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904

Character	***A. albertensis***	***A. caldus***	***A. concretivorus* comb. nov.**	***A. ferridurans***	***A. ferriphilus***	***A. ferrivorans***	***A. ferrooxidans***	***A. thiooxidans***
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 *A. thiooxidans* ATCC 19377^{T}	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	*N.D.* × 1.0-2.0	*N.D.* × 1.0-2.0	0.5 × 1.6-2.4	0.5 × 1.0	0.5 × 1.0-2.0
Flagellation	Lophotrichous	Monotrichous	Monotrichous	*v*	Monotrichous	Monotrichous	Atrichous?*	Monotrichous
Carboxysomes	+	+	*N.D.*	*N.D.*	+	+	+	+
Intracellular inclusions:								
Sulfur	+	*N.D.*	*N.D.*	*N.D.*	*N.D.*	*N.D.*	*N.D.*	-
Volutin	-	*N.D.*	+	*N.D.*	*N.D.*	*N.D.*	+	+
Poly-*β*-hydroxybutyrate	-	*N.D.*	*N.D.*	*N.D.*	*N.D.*	*N.D.*	+	-
Electron donors:								
Elementary sulfur (S_8)	+	+	+	+	+	+	+	+
Thiosulfate ($S_2O_3^{2-}$)	+	+	+	*N.D.*	*N.D.*	+	+	+
Tetrathionate ($S_4O_6^{2-}$)	+	+	*N.D.*	+	+	+	+	*N.D.*
Sulfide (S^{2-})	-	+	+	*N.D.*	*N.D.*	+	*N.D.*	-
Ferrous iron (Fe^{2+})	-	-	*N.D.*	+	+	+	+	-
Pyrite (FeS_2)	-	-	*N.D.*	+	*N.D.*	+	+	-
Molecular hydrogen (H_2)	-	+	*N.D.*	+	*N.D.*	*v*	+	*N.D.*
Ferric iron (Fe^{3+}) as terminal electron acceptor	-	-	-	+	+	+	+	-
Diazotrophy	-	-	*N.D.*	+	*N.D.*	+	+	-
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	*N.D.*	*N.D.*	[S_8, pH *c*.4.4, 30°C]† $C_{19:0}$ *cyclo*, $C_{17:0}$ *cyclo* $C_{16:0}$, $C_{12:0}$ $C_{14:0}$ 3-OH, $C_{16:1}$	[H_2, pH 2.0, 30°C] $C_{19:0}$ *cyclo* *ω*8*c*, $C_{18:1}$ *ω*7*c*, $C_{16:0}$, $C_{16:1}$, $C_{17:0}$ *cyclo*, $C_{12:0}$	[Fe^{2+}, pH *c*.1.6, 30°C] $C_{18:1}$ *ω*7*c*, $C_{16:1}$ $C_{18:1}$ 2-OH $C_{14:0}$ 3-OH $C_{16:0}$, $C_{12:0}$	*N.D.*	[Fe^{2+}, pH 2.1, 30°C] $C_{18:1}$ *ω*7*c* $C_{16:1}$, $C_{16:0}$ $C_{19:0}$ *cyclo* *ω*8*c* $C_{14:0}$, $C_{12:0}$	[S_8, pH *c*.4.4, 30°C] $C_{19:0}$ *cyclo* $C_{17:0}$ *cyclo* $C_{16:1}$, $C_{18:1}$, $C_{16:0}$ $C_{14:0}$ 3-OH
Dominant polar lipids	*N.D.*	*N.D.*	*N.D.*	PG, PE, ALs (3×)	AL, PL, PG	*N.D.*	*N.D.*	*N.D.*
Genomic properties:								
G+C fraction (mol%)	52.5[g]	61.4[g], 63.9[t]	*N.D.*	56.0-60.0[t]	57.8-58.2[t]	55.5[t]	58.8[g], 59.2[t]	53.2[g]
Genome size (Mbp)	3.47	2.99	*N.D.*	*N.D.*	*N.D.*	2.98-3.42§	2.98	3.02
Protein coding genes	3,671	2,980	*N.D.*	*N.D.*	*N.D.*	3,125-3,606§	3,147	3,080
D-ribulose 1,5-bisphosphate carboxylase genes	Form IAc (*cbbLS*) Form II (*cbbM*)	Form IAc (*cbbLS*) Form II (*cbbM*)	*N.D.*	*N.D.*	*N.D.*	Form IAc (*cbbLS*)‡ Form II (*cbbM*)	Form IAc (*cbbLS*) Form II (*cbbM*)	Form IAc (*cbbLS*) Form II (*cbbM*)
Rusticyanin genes	-	-	*N.D.*	*rusA*‡	*N.D.*	*rusB*‡	*rusA*	-
Terminal oxidases:								
Cytochrome *c*-oxidases	-	-	*N.D.*	*N.D.*	*N.D.*	aa_3§	aa_3	-
Ubiquinol oxidases	bo_3, *bd*-I	bo_3, *bd*-I	*N.D.*	*N.D.*	*N.D.*	bo_3§	bo_3, *bd*-I	bo_3, *bd*-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^{T} 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 acyl 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^{T} (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]