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Thermithiobacillus

Boden, Rich

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Genus **Thermithiobacillus**

Defining publication: Kelly and Wood 2000, 515[VP]

Authors: Rich Boden[1,2*] and Lee P. Hutt[1]

[1] *School of Biological and Marine Sciences, Faculty of Science and Engineering, University of Plymouth, Plymouth, Devon, United Kingdom, PL4 8AA.*

[2] *Sustainable Earth Institute, Faculty of Science and Engineering, University of Plymouth, Plymouth, Devon, United Kingdom, PL4 8AA.*

* Author for correspondence (rich.boden@plymouth.ac.uk)

Etymology: Therm.i.thi.o.ba.cil′lus. L. fem. n. *therma*, a warm or hot bath; 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. *Thermithiobacillus*, sulfur-rodlet from a warm bath, or warm sulfur rodlet.

Abstract:

Cells are short, rapidly motile rods with a single polar flagellum, which can be 3-6 times longer than the cell. Gram-stain-negative. Endospores, exospores and cysts are not produced. Obligate chemolithoautotrophs, using reduced inorganic sulfur species such as thiosulfate, polythionates, elementary sulfur (*viz.* α-S_8) and sometimes hydrogen sulfide, bisulfide or d- or p-block sulfide minerals such as galena (PbS). 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. No vitamins required for growth. Obligately respiratory, with molecular oxygen as the only known terminal

electron acceptor, though nitrate is reduced to nitrite under air. Usually isolated from environments with high Ca^{2+} and/or Mg^{2+} ions. Usually has a high tolerance to Mg^{2+} ions. Most strains grow in the temperature range of 20-52 °C, though some have a narrower range, and from pH 4.0-5.5 to pH 8.0-8.7. Most strains grow best without NaCl, but for one strain the optimum is 350 mM (2 % w/v). The major respiratory quinone is ubiquinone-8 (UQ-8). Dominant fatty acids in thiosulfate-grown cells are usually palmitic acid ($C_{16:0}$), palmitoleic acid ($C_{16:1}$), vaccenic acid ($C_{18:1}$) and *ω*-cyclohexylmargaric acid ($C_{17:0}$ *cyclo*). The dominant polar lipids in the same cells are cardiolipin, phosphatidylethanolamine, phosphatidylglycerol and aminoglycolipids. The G+C fraction of genomic DNA is around 58.5-67.0 mol%. Has Form IAc (carboxysomal) and Form IC (cytoplasmic) D-ribulose 1,5-bisphosphate carboxylase/oxygenase, and forms aa_3 and cbb_3 cytochrome *c* oxidase and the *bd*-I ubiquinol oxidase.

Keywords: chemolithoautotroph, thermophile, sulfur oxidizer, metal sulfide, galena

Description:

Cells are slender, often short rods 0.2-1.0 × 0.6-2.2 µm. **Rapidly motile by means of single polar flagella that is 3-6 times the length of the cell.** Motility may be so rapid that the addition of sodium azide to wet mounts is needed to poison the cells and slow them down such that they are visible. **Gram-stain-negative**. **Endospores, exospores and cysts are not produced**. **Volutin granules accumulated**. No vitamins are required for growth. Colonies on thiosulfate agar are round and entire and usually off-white in colour, turning white and/or yellow with age. **Sulfur-oxidizing obligate autotrophs. Thiosulfate, polythionates ($S_3O_6^{2-}$ to $S_7O_6^{2-}$) support growth, but growth is weaker on $S_5O_6^{2-}$. Elementary sulfur and sulfide support growth in some strains. In one species, synthetic galena (PbS) supports growth, but pyrite (FeS_2) does not, nor do molecular hydrogen, dithionate, thiocyanate or sulfite. Heterotrophic growth and methylotrophic growth do not occur, nor does "C_1 autotrophy" on methylated sulfur species. Obligate aerobes**, but **reduce nitrate to nitrite under air**. **Diazotrophy is not observed**. EDTA is sometimes a nitrogen source. **Assimilates carbon *via* the transaldolase variant of the Calvin-Benson-Bassham (CBB) cycle, using form IC (cytoplasmic) or form IAc (carboxysomal) D-**

ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO). Has both *aa*$_3$ and *cbb*$_3$-type cytochrome *c* oxidases and the *bd*-I type ubiquinol oxidase.

Type species: **Thermithiobacillus tepidarius** (Wood and Kelly 1985) Kelly and Wood 2000, 515^{VP} (*Thiobacillus thioparus* Wood and Kelly 1985, 436)

Number of species with validly published names: 2.

Family classification:

Thermithiobacillaceae (fbm00214)

Further Descriptive Information

Nutrition and growth conditions. Sulfur-oxidizing obligate chemolithoautotrophs, which have relatively high specific molar growth yields (*Y*) compared to other sulfur-oxidizing *Bacteria*. For example, during growth on tetrathionate ($S_4O_6^{2-}$) *Y* in g dry biomass/mol substrate is 20.5 g for *Thermithiobacillus tepidarius*, *versus* 15.9 for *Halothiobacillus* spp. and 11.9-12.1 in *Acidithiobacillus* spp. – data given here are from those curated by Kelly *et al.* (1987). *Thermithiobacillus tepidarius* DSM 3134^T is one of the best studied *Bacteria* with respect to polythionate metabolism (Wood and Kelly, 1986), with chemostat kinetics and enzymology studied from trithionate ($S_3O_6^{2-}$) to heptathionate ($S_7O_6^{2-}$). Polythionates are not readily available and, excepting potassium tetrathionate, must be synthesized by the worker – details of which are summarized in Wood and Kelly (1994), Roy and Trudinger (1970) and Boden *et al*. (2010). Sulfur-oxidation pathways have not been fully elucidated, but thiosulfate dehydrogenase (cytochrome *c*-linked, EC 1.8.2.2) has been purified, and a putative trithionate hydrolase partially purified from *T. tepidarius* DSM 3134^T cells grown on thiosulfate (Lu and Kelly, 1988*a*). Cultures grown on thiosulfate initially convert all of it to

tetrathionate in the first 24-48 h of growth, with the latter building up in the medium. In batch culture, the pH rises during this stage, owing to extrusion of hydroxyl ions:

$$\mathbf{2S_2O_3^{2-} + ½O_2 + H_2O \rightarrow S_4O_6^{2-} + 2OH^-}$$

After this stage, the accumulated tetrathionate is oxidized to sulfate, with a concomitant pH drop owing to proton extrusion:

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

The energy yield from the oxidation of thiosulfate *versus* tetrathionate can be summarized *via* the change in Gibbs energy (values given were determined *de novo* by one of us (RB) based on Gibbs energy of formation ($\Delta G_f°$) data from Zhdanov (1985) and Hoare (1985)) :

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

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

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

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

Kelly (1990) has previously used the potential production of ATP from these oxidations as a measure of 'value' of an electron donor. Given the $\Delta G°$ for the formation of ATP from ADP and orthophosphate is +46.1 kJ/mol ATP formed, 16 or 27 mol ATP could potentially be formed from 1 mol thiosulfate or tetrathionate, respectively, assuming perfect coupling, perfect substrate oxidation, no side-product formation, zero maintenance costs *etc*. Given CO_2 is assimilated *via* the transaldolase-variant of the CBB cycle, the formation of 1 mol biomass from 12 mol CO_2 requires:

$$\mathbf{12CO_2 + 61ATP + 25½NADPH + 3NH_3 \rightarrow C_{12}H_{24}O_6N_3 + 18H_2O}$$

Even assuming zero maintenance costs, significant electron donor oxidation is required to produce enough ATP to support the *Y* values observed in chemostat cultures. Since NADH and NADPH are formed by reverse electron transport (Wood and Kelly, 1986), not all electrons that enter the respiratory chain are used to generate proton motive force (Δp) and ATP. Indeed, reverse electron transport *consumes* Δp. Nonetheless, the *Y* of *T. tepidarius* per mole of thiosulfate (20.5 g/mol) is relatively high. Assuming *c*.5 % of Δp is

consumed for NAD(P)H generation and that thiosulfate transport into the cell costs 1 mol ATP/mol, the maximum theoretically possible yield is about 70 g/mol. This indicates a growth efficiency of about 30 %, which is much higher than almost all other sulfur oxidizing *Bacteria* studied. Moreover, the organism has an unusually high P/O ratio of 2, further explaining the high growth yields (Lu and Kelly, 1988*b*). Further details of respiratory chains in *T. tepidarius* DSM 3134^T, their coupling to sulfur oxidation and role in maintaining high biomass yields are found in Kelly *et al*., (1993) and Beffa *et al*. (1992).

In the laboratory, growth of *Thermithiobacillus* strains is usually very rapid, providing the water used is of high quality. Our laboratory uses deionized water that has then been glass-distilled (ddH_2O) instead of water purified by reverse osmosis. Unused water and is discarded within 10 days of distillation. Media are made using analytical or higher grade reagents. Lag phases are usually not very long, excepting growth on elementary sulfur, in which there is a 'wetting' phase, whilst the organism attaches to the sulfur particles. If turbidity, *i.e.* optical density at 440 nm, is monitored, very little is observed for some time on elementary sulfur, since the cells are attached to the solid material. In this case, measurement of pH fall with a sensitive combination electrode is a better measure of growth. See Boden and Hutt (2018a) for calibration details for conversion of optical density to amount of dry biomass. On soluble substrates, turbidity is usually very evident; however, on thiosulfate in batch cultures, some elementary sulfur will form from the acid-lysis of thiosulfate:

$$\mathbf{S_2O_3^{2-} + 2H^+ \rightarrow S^o + H_2O + SO_2}$$

The elementary sulfur and bisulfite ions (formed by reaction of SO_2 with water) are then consumed by the organism. In a pH-controlled, very well-oxygenated batch culture or in the chemostat, no elementary sulfur is produced. Any sulfur that has adhered to glassware can be washed off with benzene or carbon disulfide.

Thermithiobacillus strains published thus far (*viz. T. tepidarius* DSM 3134^T, *T. plumbiphilus* DSM 101799^T and *Thermithiobacillus* sp. NCIMB 8349 [=ParkerM]) all grow well in pH 7.2 E basal salts (EBS), which is described in detail in the chapter on *Thiobacillus* (gbm00969), and has an ionic strength of 0.314 M. This basal salt medium can be supplemented with any electron donor required, and gives reliable growth in the chemostat, with up to 20 mM thiosulfate as the limiting substrate. For phosphate-limited chemostats, reduce

both of the phosphate salts to 0.1 of their original concentration (*cf*. Boden and Hutt, 2018*a* for further details).

Chemotaxonomic features. Fatty acid and polar lipid data for *T. tepidarius* DSM 3134^T and *Thermithiobacillus* sp. NCIMB 8349 given in this section are from cells obtained from thiosulfate-limited chemostats (20 mM initial concentration), grown at optimal growth temperatures and at dilution rate of 0.1 h^{-1} (Hutt and Boden, *unpublished*). Similar to most *Acidithiobacillales* (obm00092), the dominant fatty acids in most *Thermithiobacillus* spp. are vaccenic acid ($C_{18:1}$), palmitic acid ($C_{16:0}$) and palmitoleic acid ($C_{16:1}$), but *ω*-cyclohexylmargaric acid ($C_{17:0}$ *cyclo*). Elsewhere in the *Acidithiobacillales* (obm00092), the latter is only found in *Acidithiobacillus thiooxidans*; however, the main *ω*-cyclohexyl fatty acid in *Acidithiobacillus* spp. (gbm01079) is *ω*-cyclohexylnonadecylic acid ($C_{19:0}$ *cyclo*). This is not unexpected, *ω*-cyclohexyl fatty acids are common in (acido)thermophiles (Oshima and Ariga, 1975; Boden *et al*., 2017; Da Costa *et al*. 2011), so its presence may be an adaptation to this growth condition. The polar lipids in *T. tepidarius* DSM 3134^T are cardiolipin (diphosphatidylglycerol, CL), phosphatidylethanolamine (PEA), phosphatidylglycerol (PG) and aminoglycolipids. The presence of CL is particularly interesting because it plays a key role in maintaining Δp by acting as a 'proton trap', confining the periplasmic proton pool in a discreet region, thus minimizing loss of protons through the outer membrane or onto other molecules present. By buffering the pH in the periplasm in this way, it may contribute to the high growth efficiency of this genus (Haines and Dencher, 2002). The dominant respiratory quinone is ubiquinone-8 (UQ-8), in common with the rest of the *Acidithiobacillales* (obm00092).

Genomic and biochemical features, and their relation to ecology. The genome sequence of *Thermithiobacillus tepidarius* DSM 3134^T has been completed and published (Boden *et al.*, 2016), and it is publically available *via* the Integrated Microbial Genomes (IMG) database. It is 2.96 Mbp and comprises 2,902 protein-coding genes. We have obtained a number of other *Thermithiobacillus* isolates from the Roman Baths at Bath (UK), namely *Thermithiobacillus* sp. IF and *Thermithiobacillus* sp. GB, which have genome sequences nearly identical to that of the type strain. The Australian strain *Thermithiobacillus* sp. ParkerM (=NCIMB 8349), which is physiologically distinct from *T. tepidarius* DSM 3134^T, has also been sequenced by our group, and the overall genome size is similar to that of *T. tepidarius* (2.96 Mbp). Thus, all

strains sequenced thus far are around the 3 Mbp size range. No sequence has thus far been obtained from *T. plumbiphilus* isolates.

Unsurprisingly, both aa_3 and cbb_3 (low and high oxygen affinity, respectively) types of cytochrome *c* oxidase (EC 1.9.3.1) are encoded in *T. tepidarius*, which is typical for *Bacteria* living in an environment in which oxygen partial pressures (pO_2) change frequently. A non-translocating *bd*-I type ubiquinol oxidase (electrogenic, non-translocating, EC 1.10.3.14) is present. It probably only has a role in 'draining' excess [H] from a quinol pool when the respiratory chain backs-up owing to ADP/NAD^+ deficiency, and it is not involved in generating Δp. Early analyses of the *Thermithiobacillus* sp. ParkerM genome indicate the same arrangement but a ba_3 cytochrome *c* oxidase replaces the aa_3 found in the type strain.

Both the Form IC (cytosolic, "red") and Form IAc (carboxysomal, "green") D-ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO, EC 4.1.1.39) are found in all strains we have examined. Form IC is canonically found mostly in facultative autotrophs (Badger and Bek, 2008), whilst *Thermithiobacillus* spp. appear to all be obligate autotrophs (Hutt, 2016). Form IC is optimized for medium to high pCO_2 with O_2 present, whereas Form IAc is optimized for low pCO_2 and low to high pO_2. Many carboxysome-utilizing autotrophs have Form IAc in their carboxysomes and Form IAq in the cytoplasm – the functionality of the latter roughly overlaps with that of Form IC (Badger and Bek, 2008). It is worth noting that the terminal oxidases encoded in the *T. tepidarius* DSM 3134^T genome are the same forms as those found in *Annwoodia aquaesulis* DSM 4215^T (gbm01601), which was isolated from the exact same location. The latter does not form carboxysomes, and Form IAq (cytosolic, "green") and Form II (cytosolic, medium to high pCO_2 with low pO_2) RuBisCO are present. *A. aquaesulis* appears to have emerged from much deeper in the thermal spring at Bath (UK) than *T. tepidarius* DSM 3134^T (see chapter on *Annwoodia* (gbm01601). Aside from the terminal oxidases, a full respiratory chain, including the ubiquinol-cytochrome *c* oxidoreductase (bc_1 complex, EC 1.20.2.2) essential for reverse electron transport in sulfur autotrophs (from cytochrome *c* to NAD^+) is present in all strains examined.

Searching 16S rRNA gene sequence data from ecological studies in the GenBank database (BLASTn algorithm; query sequence: full length *T. tepidarius* 16S rRNA gene in December 2018), only 10 sequences affiliated to this genus were found. With the exception of culture-collection strains and *T. tepidarius* JNU-2

discussed elsewhere, the unpublished isolate *T. tepidarius* SMMA2, affiliated to geothermal vents in India (LN864468), was found. One clone was found from a malachite green-degrading consortium obtained from benthic waters of the South China Sea (KP183092), suggesting that marine *Thermithiobacillus* spp. may exist. Interestingly, no other *Thermithiobacillus* spp. have shown up in metagenomic databases, suggesting they may are not abundant in the well-sampled surface environments of Earth.

Cultivation, Enrichment and Isolation Procedures

General cultivation: *Thermithiobacillus* spp. are all readily grown on E-basal salts (EBS, *cf*. *Thiobacillus* (gbm00969)) supplemented with a suitable electron donor such as thiosulfate (20 mM), tetrathionate (10 mM), sulfide (5 mM) or elementary sulfur (0.5 % *w/v*). For the latter, we use flowers-of-sulfur or a crystallography-grade zone-refined sulfur (α-S_8) for growth experiments; however, for general maintenance of strains, we use roll sulfur, which has much larger pieces and thus lasts longer. Sterilization of elementary sulfur is covered in the chapter on *Annwoodia* (gbm01601). Thiosulfate is stable during autoclaving but polythionates should be sterilized by filtration and used quite rapidly thereafter, particularly pentathionate and the higher polythionates, as they are not stable. Sulfide must be added to cultures as a neutral sulfide stock solution, to avoid a dramatic increase in pH. This solution is prepared most easily by rapidly washing crystals of sodium sulfide nonahydrate, obtained ideally from a brand new jar, on filter paper in a Büchner funnel that is set up for filtration at the pump. Ice-cold ddH_2O is *rapidly* poured over the crystals and drawn away by the pump to remove polythionates and other sulfur oxyanions that have formed on the outside of the sulfide crystals. The washed crystals are immediately dissolved in a glass serum bottle containing a suitable volume of ddH_2O, a *glass*-coated stirring ‘flea’, and a few drops of a saturated aqueous solution of neutral red. A vaccine stopper and crimp seal are applied and the headspace is flushed with argon to remove air. The crystals are dissolved with vigorous stirring, whereupon 1 N sulfuric acid is injected dropwise until the pH indicator turns from yellow to *just* red (pH 6.8). At this point, the solution can be stored at room temperature for a few months. To use, one draws the solution into a syringe and injects it into culture vessels *via* a syringe filter. Polythionates are conveniently prepared as their sodium salts according to Kelly and Wood (1994), Roy and Trudinger (1970) and Boden *et al*. (2010) – these are then used in cultures from stock solutions. Dithionate ($S_2O_6^{2-}$, not technically a polythionate, and much more stable in solution) is very

hard to procure as most large-scale manufacture has declined since the 1990s. Our laboratory has significant stocks of high purity sodium dithionate dihydrate and we will provide them *gratis* on request for taxonomic and physiological work. Alternatively, sodium dithionate dihydrate can be synthesized readily from the oxidation of sulfur dioxide with manganic oxide (Pfanstiel *et al*., 1946).

EBS-based media can be solidified into plates using 20 g/L Noble or any other very high purity agar, *e.g.* Granulated High Gel Strength Agar (Melford Laboratories Ltd, Chelsworth, Suffolk, United Kingdom). Agar powder is added before autoclaving – agarose is also a suitable alternative. Low-grades of agar tend to give weak growth of *Thermithiobacillus* spp. Whilst low-grade agar can be washed before use, the growth is still not as good as starting with high-purity agar. It can be convenient to add a pH indicator to help identify areas of growth on agar plates. For acidogenic substrates such as sulfur species, bromocresol purple can be used at 0.002 g/L or 5 mL/L of the 0.04 % *w/v* pharmaceutical grade solution from Sigma-Aldrich, which contains fewer organic contaminants than most commercial preparations of the solid dye. It will change from violet to yellow *via* grey at or below pH 5.2.

Liquid cultures are easily grown in 50 mL volumes in 250-mL Erlenmeyer flasks stoppered with a foam or cotton bung – wide-mouth flasks are preferred for autotrophs. For volatile compounds or gases, serum bottles can be used. ‘QuickFit’ Erlenmeyers (250-mL) stoppered with red-rubber ‘SubaSeal’ vaccine stoppers can also be used. The ‘inner’ surfaces of vaccine stoppers are sprayed with 2-3 coats of a PTFE dry lubricant spray before autoclaving, to prevent adsorption or dissolution of substrates into the rubber. Both serum bottles and flasks with vaccine stoppers allow easy withdrawal of samples for quantification of toxic substrates such as sulfide, so that the culture can be ‘re-spiked’ when the substrate depletes (see Boden *et al.* (2010) for analytical methods). For methodologies pertaining to continuous culture (chemostat culture) of *Thermithiobacillus* spp. with specific focus on energy metabolism, *cf.* Wood and Kelly (1986) and Boden and Hutt (2018*a*). For proteomic work and protein purification, chemostat culture using potassium carbonate as the base-feed and/or 5 % CO_2 in air as the sparge-gas will repress carboxysome production and thus greatly simplify the proteome, allowing greater resolution of low abundance proteins, or easier protein purification. Importantly, cultures growing on sulfur oxyanions or elementary sulfur must not be incubated in the same incubators as organisms growing on complex media. The polyamines and ammonia produced

will be drawn rapidly from the air inside the incubator into *Thermithiobacillus* cultures once they have reached exponential phase and the pH is falling rapidly. This is a common cause for culture failure. Whilst no strains in culture are auxotrophic for any B vitamins, we use the vitamin solution VJK (*cf*. Boden and Hutt, 2018*a*) at 1 mL/L for chemostat cultures to ensure limitation is by the intended substrate.

Growth on plates can be done in Tupperware boxes or gas jars containing CO_2-supplemented air (5 % *v/v* from a cylinder – or, at a pinch, a crushed *Alka-Seltzer* tablet in a beaker of water or a *small* piece of dry-ice can be sealed in the box) for more rapid growth. Liquid cultures grown ≥ 48 °C should have tightly fitting lids to avoid evaporation – 120-mL serum bottles are a convenient option for 25 mL cultures, and they can be over-pressured by the injection of 1-5 mL of CO_2 for more rapid growth and/or to repress carboxysome production. Note that elevating the pCO_2 in the air does not alter the growth yield (Wood and Kelly, 1986).

In continuous culture, high dilution rates (for an autotroph!) of up to 0.44 h^{-1} can be achieved by the type species on tetrathionate. For comparison, the organism was isolated from a natural chemostat fed by a thermal sulfur spring: the Great Bath at the Roman Baths, UK. The Great Bath has a volume of about 562,000 L and is fed by the spring at a flowrate of 46,800 L h^{-1}. The hydrodynamic dilution rate (*D*) therein is therefore about 0.08 h^{-1}, which is well within the growth range of *T. tepidarius*. Whilst growth of *T. plumbiphilus* on galena (PbS) has been demonstrated (Watanabe *et al*., 2016), that of *T. tepidarius* has not; however, it is worth noting that the base of the Great Bath is covered with thick sheets of lead (Southern, 2012), which may interact with the dissolved sulfur species from the spring. This has not been investigated thus far, but studies of the inflowing water (Andrews *et al*., 1982) and the yellow mineral deposits in the Great Bath (Riley, 1961) show negligible dissolved or deposited lead, respectively.

Maintenance: *Thermithiobacillus* spp. can be stored short-term by streaking on EBS-thiosulfate agar slants in glass Universal bottles. After a week or so, a film of biomass (rather than distinct colonies, as the organism tends to grow in the water of syneresis, rather than on the agar directly) will be evident. These slants will keep (as long as tightly sealed) at 4 °C for 1-2 months. To use biomass from these cultures, it can be washed off of the slant using sterile 0.9 % (*w/v*) saline (1-2 mL), which avoids substrate carryover. Frozen stocks can be made by growing liquid cultures on thiosulfate. Absolutely pure DMSO (*cf*. Boden and

Hutt, 2018*b*) is added to 20 % *v/v*, and the cultures buried in ice for 1 h to enable the cryoprotectant to penetrate cells. Cultures are then aliquotted into 2-mL Cryovials half-filled with sterile Ballotini® glass beads (1 mm diameter). Sealed vials are dropped into liquid nitrogen to freeze and are stored at -80 °C. To revive cultures, a bead is broken off and rolled on an agar plate and the remaining stock is returned to the freezer.

A medium-term storage option is to grow cultures on roll sulfur (in reasonably large chunks) added to EBS at about 0.1-0.5 % (*w/v*). Once the culture reaches about pH 5.2, a few tyndallized marble chips are added, which will slowly buffer the pH back to circumneutrality if left on the bench overnight, whilst also providing CO_2. These cultures then be stored on the bench or at 4 °C for several months or until a dense white $CaSO_4$ precipitate becomes evident in the flasks.

For longer-term storage, lyophilisation can be difficult with obligate autotrophs, but it is worth attempting with new strains. The method given for *Methylophaga* spp. (gbm01218) can be used with 4-6 slants used in place of 2 slants per batch.

Enrichment and isolation: 50 mL of the appropriate basal salts, supplemented with the required electron donor, are dispensed into a sterile wide-mouth Erlenmeyer flask. 1-2 g solid material (*e.g.* soil, corroded concrete/limestone, minerals) is added directly. For water samples (*e.g.* thermal spring water), the concentration of biomass can be very low, so passage of 250-1,000 mL through a 45 mm 0.2 µm pore size nitrocellulose filter helps concentrate the cells. The filter is added to the medium as the inoculum. If using water from a thermal spring that has been exposed to sunlight, pre-filtration (0.44 µm pore size) can be helpful to remove *Eukarya* that will annoyingly take over the culture. If elementary sulfur is used as the electron donor, the appropriate concentration is 0.5-1.0 % (*w/v*); for galena it is 0.05-0.10 % (*w/v*). Both can act as an electron donor *and* inoculum if not sterilized before use, which can be advantageous in some situations. Flasks are stoppered *loosely* with sterile cotton wool and are incubated with rapid shaking at 30-45 °C. Once turbidity and/or pH change are evident, they 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.

The long-term incubation of solid materials in a moist atmosphere of flowing air streams supplemented with gaseous ammonia and hydrogen sulfide has also been employed for the isolation of *Thermithiobacillus* spp. (Parker, 1945*a*,*b*).

It is worth adding that we have found that *T. tepidarius* DSM 3134^{T} tolerates over 0.35 M Mg^{2+} ions, which is much higher than either *Thiobacillus* spp. (gbm00969) or *Annwoodia* spp. (gbm01601) (Boden and Hutt, *unpublished*). As such, Mg^{2+} ion additions might selectively enrich *Thermithiobacillus* spp., though we have not tested this any further.

Taxonomic comments

Where three-letter abbreviations are required for clarity, we recommend "*Ttb.*" be used. A 16S rRNA gene tree is given in Figure 1, which shows the position of *Thermithiobacillus* spp. *versus* other members of the *Acidithiobacillales* and the separation of the genera therein. The genus *Thermithiobacillus* was first proposed by Kelly and Wood (2000), accommodating a single species, which was a reclassification of the organism that Wood and Kelly (1985) named *Thiobacillus tepidarius*, isolated from the thermal spring at the Roman Baths, Bath, UK. This was not, however, the first *Thermithiobacillus* sp. in public service collections – *Thiobacillus* sp. NCIMB 8349 (= ParkerM = M79 = DSM 103443) was isolated by Parker (1945*a*,*b*; 1947) from decomposing concrete obtained from the sewer outfall of Melbourne Australia and was deposited into the (then) NCIB in 1959 by Butlin and Postgate (Dr Peter Green, formerly of the NCIMB, *personal communication*). Boden *et al.* (2012) examined this strain and found it closely related to the type species of *Thermithiobacillus*. A phylogenetic and physiological study has since been completed (Hutt, 2016) along with the genome sequence of this strain (Boden, *unpublished*). A third *Thermithiobacillus* strain in public service collections represents a second species with a validly published name. *T. plumbiphilus* was isolated from synthetic galena (PbS) in Japan (Watanabe *et al.*, 2016). Additionally, we have isolated a number of *Thermithiobacillus* strains from the Roman Baths at Bath, UK. A 16S rRNA gene library therefrom indicated that at least 6 operational taxonomic units (OTUs, 97 % cut-off, Boden and Hutt, *unpublished*) associated to the genus, though all isolates thus far are closely affiliated to the type species.

One further isolate has been published, though it is not deposited in any culture collections – *T. tepidarius* JNU-2 (Yang *et al.*, 2015). It was isolated from municipal wastewater sludge (Wuxi, Jiangsu, China) following enrichment using EBS supplemented with an alternative trace-metals solution and 40 mM thiosulfate at 37 °C. It was applied to the removal of sulfide following treatment of sulfate waste, using an internal airlift loop reactor, and it may have biotechnological potential.

List of species in the genus **Thermithiobacillus**

1. ***Thermithiobacillus plumbiphilus*** Watanabe, Miura, Shinohara, Kojima, Fukui 2016, 1988^{VP}

   plum.bi.phi′lus. L. neut. n. *plumbum*, lead; N.L., masc. adj. *philus* (from Gr. masc. adj. *phílos*, that which is dearly loved, that which is belovèd), loving; N.L. masc. adj. *plumbiphilus*, lead-loving.

   Cells 0.5-1.0 × 0.7-2.2 µm. Grows on synthetic galena (PbS), thiosulfate, and tetrathionate but not sulfite, sulfide, elementary sulfur or molecular hydrogen as electron donors. Dominant fatty acids when grown on thiosulfate are palmitoleic acid ($C_{16:1}$), palmitic acid ($C_{16:0}$) and vaccenic acid ($C_{18:1}$). Grows optimally without NaCl but tolerates up to 452.5 mM. Grows optimally at pH 6.4-7.1 and within a range of pH 5.8-8.7. Grows from 5-37 °C and optimally 28-32 °C.

   Type strain isolated from and industrially synthesized galena (plumbous sulfide), Japan.

   DNA G+C content (mol%): 58.5 (HPLC)
   Type strain: wk12 = NBRC 111508 = DSM 101799
   GenBank accession (16S rRNA gene): LC088006

2. ***Thermithiobacillus tepidarius*** (Wood and Kelly 1985) Kelly and Wood 2000, 515^{VP} (*Thiobacillus tepidarius* Wood and Kelly 1985, 436)

   te.pi.dar′i.us. L. neut. n. *tepidarium*, warm room in a Roman bathhouse, containing a bath fed by natural thermal waters; N.L. masc. adj. *tepidarius*, warm-bathing, bathing in a tepidarium.

Cells 0.2-0.4 × 0.6-1.0 µm. Grows on thiosulfate, trithionate, tetrathionate, pentathionate (weakly), hexathionate, heptathionate, octathionate, elementary sulfur or sulfide but not sulfite, dithionate, thiocyanate, pyrite (FeS_2) or methylated sulfur species as electron donors. All strains use ammonium as nitrogen source and EDTA is used by some strains. Volutin (polyphosphate) granules formed. Nitrate is reduced under air but not used as a terminal electron acceptor. Tetrathionate builds up in cultures grown on thiosulfate and is then oxidized to sulfate. Optimal growth occurs without NaCl, at 43-45 °C (range: 20-52 °C) and from pH 6.0 – 7.5 (range: pH 5.5 – 8.0). Dominant fatty acids in thiosulfate-grown cells are palmitic acid ($C_{16:0}$), palmitoleic acid ($C_{16:1}$) and *ω*-cyclohexylmargaric acid ($C_{17:0}$ *cyclo*). Dominant polar lipids are cardiolipin, phosphatidylethanolamine, phosphatidylglycerol and aminoglycolipids.

Type strain isolated from the thermal waters of the tepidarium at the Roman Baths, Bath, UK.

DNA G+C content (mol%): 66.60 (*Bd*), 66.84 (sequence).
Type strain: DSM 3134 = ATCC 43215
GenBank accession (16S rRNA gene): AJ459801
IMG accession (genome sequence): 2523533554

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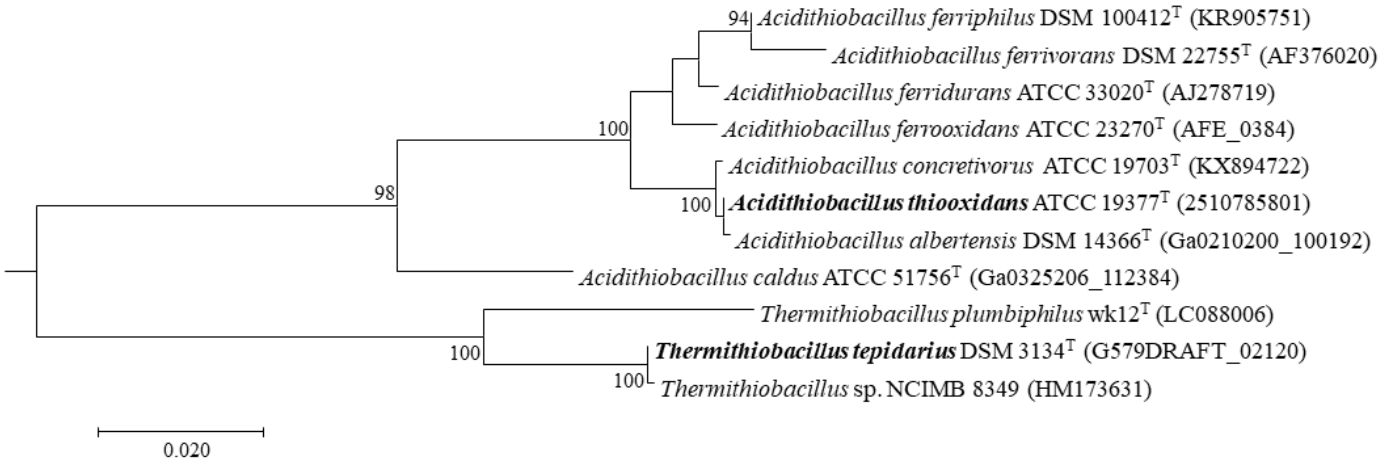
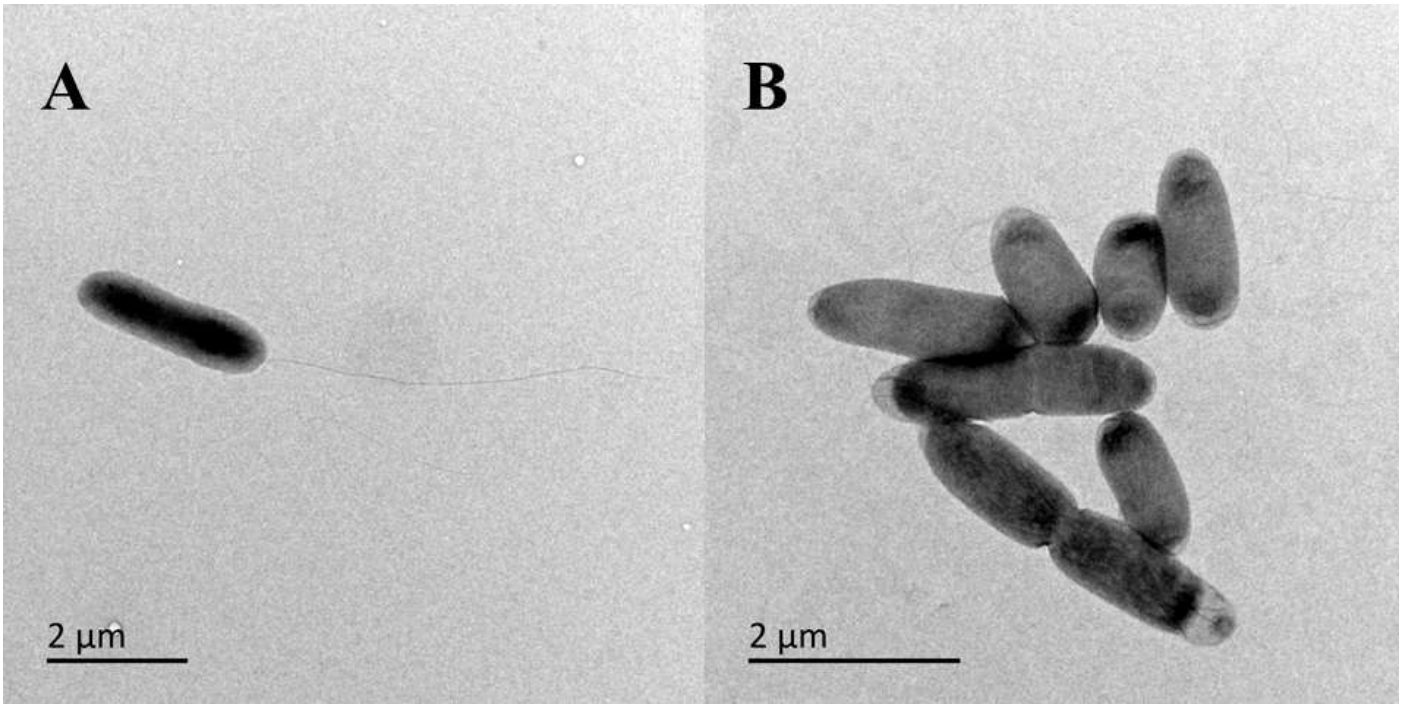


Figure 1. Maximum likelihood tree on the basis of near-full-length 16S rRNA (*rrs*) gene sequences of *Thermithiobacillus* spp. within the *Acidithiobacillales* (obm00092). Type species of each genus are emboldened. The outgroup is the same sequence from *Pseudomonas aeruginosa* DSM 50071^{T} (HE978271). *Acidithiobacillus concretivorus* ATCC 19703^{T} is included, even though it has often been considered a heterotypic synonym of *A. thiooxidans*. This conclusion probably needs reexamination using whole-genome methods, thus we have reverted to considering it as a separate taxon. Accession numbers refer to the GenBank or IMG databases (the latter are numeric-only or contain an underscore ('_'). Tree shown had the highest log-likelihood (-3,696.95) and was constructed in MEGA X (Kumar *et al*. 2018) with partial deletion of gaps (95 % cut-off). The final analysis used 1,298 nt. Sequences were aligned using MUSCLE (Edgar, 2004). Model-testing was undertaken in MEGA X on the basis of the lowest corrected Aikake information criterion (AICc, Hurvich and Tsai, 1989; Aikake, 1973) – the General Time Reversible model of Nei and Kumar (2000) was used with a gamma distribution (5 discreet gamma categories, gamma parameter 0.4750) and 51.12 % of sites evolutionarily invariant. Numbers at nodes represent bootstrap values ≥ 70% on the basis of 5,000 bootstrap replications. Bar = 0.02 nucleotide substitutions per position.

[Acidithiobacillia 16S.tif]



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475 **Figure 2.** Scanning electronmicrographs of *Thermithiobacillus tepidarius* DSM 3134^{T} cells obtained from
476 thiosulfate-limited chemostats (EBS+20 mM $S_2O_3^{2-}$, D = 0.02 h^{-1}), grown, harvested, washed and fixed per
477 Hutt (2016), showing **A**: single cell with long flagellum intact (13,500 ×); **B**: cluster of cells in various
478 stages of division (20,000 ×).

479 **[Ttb SEM images.tif]**

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Character	***Thermithiobacillus tepidarius* DSM 3134^T**	***Thermithiobacillus plumbiphilus* DSM 101799^T**	***Thermithiobacillus* sp. NCIMB 8349**
Isolation source	Thermal spring water, United Kingdom	Synthetic galena, Japan	Decomposing concrete, Australia
16S rRNA gene identity to DSM 3134^T (%)	100	95.7	99.9
Cell length × diameter (µm)	0.6-1.0 × 0.2-0.4	0.7-2.2 × 0.5-1.0	1.45 × 0.7
Flagellar length (µm)	3.1	*N.D.*	5.2
Volutin (polyphosphate) granules	+	*N.D.*	+
Nitrate reduction under air	+	*N.D.*	+
Maximum specific growth rates (µ) in batch culture (h^{-1}):			
Thiosulfate ($S_2O_3^{2-}$)	0.055	*N.D.*	0.250
Tetrathionate ($S_4O_6^{2-}$)	0.440	*N.D.*	0.429
Lowest pH after growth on thiosulfate in batch culture	4.5	*N.D.*	3.6
Kinetic parameters in thiosulfate-limited chemostat culture:			
Maximum specific molar growth yield (Y_{max}) (g dry biomass/mol)	10.7	*N.D.*	11.4
Maximum specific molar growth rate (μ_{max}) (h^{-1})	*N.D.*	*N.D.*	0.480
Terminal electron acceptors:			
Molecular oxygen (O_2)	+	+	+
Nitrate (NO_3^-)	-	-	-
Nitrous oxide (N_2O)	-	*N.D.*	*N.D.*
***Electron donors*:**			
Thiosulfate ($S_2O_3^{2-}$)	+	+	+
Sulfite (SO_3^-)	-	-	-
Dithionate ($S_2O_6^{2-}$)	-	*N.D.*	-
Trithionate ($S_3O_6^{2-}$)	+	*N.D.*	+
Tetrathionate ($S_4O_6^{2-}$)	+	+	+
Pentathionate ($S_5O_6^{2-}$)	*w*	*N.D.*	-
Hexathionate ($S_6O_6^{2-}$)	+	*N.D.*	+
Heptathionate ($S_7O_6^{2-}$)	+	*N.D.*	*N.D.*
Thiocyanate (SCN^-)	-	*N.D.*	-
Elementary sulfur (S_8)	+	-	+
Sulfide (S^{2-})	+	-	*N.D.*
Molecular hydrogen (H_2)	*N.D.*	-	*N.D.*
Pyrite (FeS_2)	-	*N.D.*	-
Galena (PbS)	*N.D.*	+	*N.D.*
Nitrogen sources:			
EDTA	+	*N.D.*	-
Resistance to:			
Penicillin G	-	*N.D.*	+
Cephalothin	+	*N.D.*	-
G+C fraction (mol%)	66.6 (*Bd*), 66.8 (sequence)	58.5 (HPLC)	67.0 (sequence)
Dominant fatty acids	$C_{16:0}$, $C_{16:1}$, $C_{17:0}$ *cyclo*	$C_{16:1}$, $C_{16:0}$, $C_{18:1}$	$C_{16:0}$, $C_{16:1}$, $C_{18:1}$
Polar lipids	Cardiolipin Phosphatidylethanolamine Phosphatidylglycerol Aminoglycolipid	*N.D.*	Cardiolipin Phosphatidylethanolamine Phosphatidylglycerol Aminoglycolipid

Thiosulfate dehydrogenase (cytochrome *c*-linked, EC 1.8.2.2) specific activity (µmol/min/(mg protein)$^{-1}$)*	4.41 ± 0.14	*N.D.*	10.07 ± 0.08
NaCl range and [optimum] (mM)	[0]	0-452.5 [0]	[350]
Temperature range and [optimum] (°C)	20-52 [43-45]	5-37 [28-32]	20-54 [42-45]
pH range and [optimum]	5.5-8.0 [6.0-7.5]	5.8-8.7 [6.4-7.1]	4.0-8.5 [6.0-7.0]

Table I. Comparative properties of *Thermithiobacillus* spp., comprising the two species with validly published names and the well-characterised *Thermithiobacillus* sp. NCIBM 8349 (=ParkerM, Parker, 1945*a*,*b*, 1947; Hutt, 2016; Boden *et al.*, 2012).

Fatty acid and polar lipid data (*pace* those from *T. plumbiphilus*) are unpublished data from Boden and Hutt. All other data are from Wood and Kelly (1985, 1986), Parker (1945*a*,*b*, 1947), Hutt (2016), Boden *et al.* (2012), Boden *et al.* (2016) or Watanabe *et al* (2016).

* Data obtained from cells harvested from thiosulfate-limited chemostats (D = 0.02 h^{-1}, 20 mM).