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Thiobacillus

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Genus **Thiobacillus**

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Etymology:

Thi.o.ba.cil′lus. Gr. neut. n. *theîon*, sulfur, brimstone; L. masc. n. *bacillus*, a short rod, a short wand; N.L. masc. n. *Thiobacillus*, sulfur rodlet.

Abstract:

Cells are short rods. Cytochrome *c* oxidase-positive and catalase-positive when grown on thiosulfate. Gram-stain-negative. Endospores, exospores and cysts are not produced. Metabolically obligate chemolithoautotrophs, supported by reduced sulfur species and elementary sulfur, and some methylated sulfur compounds. Genes encoding Form IAc, Form IAq and Form II D-ribulose 1,5-bisphosphate carboxylase/oxygenases (RuBisCO) are present in the genomes. Carboxysomes are produced in some species and are repressed at high CO_2 partial

pressures. Volutin (polyphosphate) granules formed in most species. Produce tetrathionate as a detectable intermediate of thiosulfate oxidation. Obligately respiratory, with molecular oxygen and nitrate the only known terminal electron acceptors, with the latter only used in some species. Mesophilic, growing optimally at 25-32 °C, and one psychrophilic species capable of growth down to -2 °C. The major respiratory quinone is ubiquinone-8 (UQ-8). Dominant fatty acids are $C_{16:0}$, $C_{16:1}$, $C_{15:0}$ and $C_{17:1}$. The G+C fraction of genomic DNA is 61.5-66.0 mol%.

Keywords: aerobe, sulfur, chemolithoautotrophs, organosulfur, denitrifier

Description:

Cells are short rods of 0.5-0.8 × 1.0-3.0 µm. **Gram-stain-negative**. **Endospores, exospores and cysts are not produced**. Colonies on thiosulfate agar grown at 30 °C are white or colourless and 1-2 mm diameter, circular, convex, smooth and entire, and become covered in powdery white or yellow elementary sulfur over time. **Obligately chemolithoautotrophic**. **Electron donors for chemolithoautotrophic growth include thiosulfate and tetrathionate. Other polythionates, elementary sulfur, as well as some volatile inorganics such as carbon disulfide and carbonyl sulfide are used in some species, and organosulfur compounds such as dimethylsulfide and dimethyldisulfide are used in some strains of *Thiobacillus thioparus*. Does not grow heterotrophically**. Can grow anaerobically with **denitrification in some species**. Grows optimally at 25-30 °C but growth range varies by species, and from around pH 5 to around pH 9, varying by species, with the optima varying by species. **Assimilates CO_2 *via* the transaldolase-variant Calvin-Benson-Bassham cycle**. **Form II (cytosolic) D-ribulose 1,5-**

bisphosphate carboxylase/oxygenases (RuBisCO) is found in all species. Form IAc (carboxysomal) is found in *T. thioparus* and Form IAq (cytosolic) in all other species.

Type species: **Thiobacillus thioparus** Beijerinck 1904*b*, 153[AL]

Number of species with validly published names: 3.

Family classification:

Thiobacillaceae (fbm00351)

Further Descriptive Information

Morphology. Cells of *Thiobacillus* spp. are short rods (0.5-0.8 × 1.0-3.0 µm) and are Gram-stain-negative. Figure 1 shows transmission electron micrographs of *Thiobacillus thioparus* DSM 505[T] cells obtained from a thiosulfate-limited chemostat. As can be seen from these images, the outer membrane has a 'ruffled' appearance and the periplasm is somewhat dilated as a result – this is visible in both sections shown. The cytoplasm contains numerous carboxysomes ('polyhedral bodies' in the older literature), which usually number *c*.5 per cell when grown under air on thiosulfate. Colonies on thiosulfate agar are circular, entire and rather flat. By reflected light, they are yellowish or pinkish in early growth and then becoming covered in a dense powdery layer of white or yellow elementary sulfur – the colony underneath turns brown with age. By transmitted light, colonies usually look orange or pink, and are darker in color towards the centre. In unshaken batch cultures on thiosulfate, a dense pellicle of cells and elementary

sulfur particles forms – in shaken cultures this is evidenced only as a ring of the same matter on the glass above the level of the culture.

Metabolism. As with most known obligate chemolithoautotrophs, Krebs' cycle is incomplete, with Smith's biosynthetic horseshoe (which lacks 2-oxoglutarate dehydrogenase) taking its place (Hutt *et al.*, 2017). Sulfur compound oxidation occurs by way of the Kelly-Trudinger or so-called "S_4I" pathway in which tetrathionate is produced as a detectable intermediate of thiosulfate catabolism and builds up in the medium during the first 24-48 h of batch culture, before being oxidized to sulfate. Degradation of thiocyanate is catalyzed by thiocyanate hydrolase (EC 3.5.5.8) to carbonyl sulfide and ammonia. The former is hydrolyzed by carbonyl sulfide hydrolase (EC 3.13.1.7), forming sulfide and CO_2 – the former being oxidized to sulfate for energy (Yamanaka *et al*., 2013; Ogawa *et al.* 2013). Proton motive force (Δp) is generated mostly at the level of the terminal oxidase as electrons from thiosulfate oxidation enter the respiratory chain at the level of cytochrome *c*, thus the next coupling site is the terminal oxidase. NAD(P)H is generated by reverse electron transport. Carbon dioxide is fixed by the transaldolase variant of the Calvin-Benson-Bassham cycle with minor contribution from pyruvate carboxylase and phosphoenolpyruvate carboxylase (Hutt *et al.*, 2017).

Nutrition and growth conditions. All species can use thiosulfate or tetrathionate as electron donors for autotrophic growth, and some can also use trithionate, elementary sulfur (*viz.* orthorhombic cyclooctasulfur, α-S_8), thiocyanate and other inorganic sulfur species – as shown in Table 1. Use of volatile inorganics carbon disulfide and carbonyl sulfide and organosulfur species such as dimethylsulfide and dimethyldisulfide is of interest since they are trace gases in the atmosphere of Earth. It must be noted that use of organosulfur species in this genus is still *bona fide* chemolithoautotrophy – the (di)sulfide moieties are oxidized to sulfate with

concomitant building of proton motive force by the respiratory chain, and thus yielding ATP/NAD(P)H. The methyl groups are oxidized to CO_2, which is then assimilated *via* the transaldolase-variant Calvin-Benson-Bassham cycle (Smith and Kelly, 1988). Molecular oxygen and/or nitrate can act as terminal electron acceptors, but the latter is not used in all species. Heterotrophic growth is not observed in this genus. For cultivation, E basal salts (EBS, Boden and Hutt, 2018*a*) supplemented with 20 mM sodium thiosulfate is commonly used. Other electron donors can be used, usually at 10-20 mM. Elementary sulfur is used at about 0.5 % *w/v* and toxic volatiles at 1-5 mM, but these are 're-spiked' at intervals. Growth is performed under a headspace of air – for the generation of biomass for protein or RNA work, it can be useful to use air enriched with 5 % *v/v* CO_2, as this represses carboxysome production in *T. thioparus*, simplifying the proteome and transcriptome somewhat. EBS comprises (g/L unless otherwise stated): KH_2PO_4 (4.0), K_2HPO_4 (4.0), $MgSO_4 \cdot 7H_2O$ (0.8), NH_4Cl (0.4), and trace metal solution T (10 mL). For chemostat work, the addition of a vitamin solution such as solution VJK (Boden and Hutt, 2018*a*) is prudent but none of the characterized strains of *Thiobacillus* are auxotrophic for vitamins. Solution T is detailed in the chapter on *Annwoodia* (gbm01601).

Chemotaxonomic features. Similar to most other *Betaproteobacteria* (cbm00042) and indeed other members of the *Thiobacillaceae* (fbm00351), the dominant fatty acids in *Thiobacillus* spp. are palmitic acid ($C_{16:0}$), palmitoleic acid ($C_{16:1}$), pentadecylic acid ($C_{15:0}$) and heptadecenoic acid ($C_{17:1}$) when grown autotrophically on thiosulfate (Boden et al. 2017) – the latter two fatty acids are distinct from the dominant fatty acids found in the genera *Sulfuritortus* (gbm01835) and *Annwoodia* (gbm01601). The dominant respiratory quinone is ubiquinone-8, per almost all *Betaproteobacteria* (cbm00042).

Genomic features. The genome sequences of *Thiobacillus thioparus* DSM 505^T (Hutt *et al*. 2017) and *Thiobacillus denitrificans* ATCC 25259^T (Beller *et al*. 2006) have been completed and reports of their properties published. We (Boden and Hutt) have recently completed the genome sequence of *Thiobacillus thiophilus* DSM 19892^T (IMG ID: 168939) and anticipate publication of a report of its properties in the near future. We herein curate some findings from these genomes. All species have the *cbb*$_3$-type cytochrome *c* oxidase, and *T. thioparus* and *T. denitrificans* additionally have the *aa*$_3$-type, as shown in Table 1. These indicate (on the basis of Badger and Bek (2008)) that growth is optimized for life at low O_2 partial pressures in all species, but all except *T. thiophilus* have oxidases optimized for higher O_2 partial pressures as well. Form II (CbbM) RuBisCO is found in all species, optimized for growth at medium to high CO_2 partial pressures and low O_2 partial pressures. The non-carboxysome-utilizing *T. denitrificans* and *T. thiophilus* also contain Form IAq RuBisCO, optimized for growth at medium to low CO_2 partial pressures, when O_2 is present. *T. thioparus* has carboxysomes (Figure 1) usually numbering *c*.5 per cell when grown on thiosulfate under air, which contain Form IAc RuBisCO, optimized for growth at low CO_2 partial pressures in the presence of low-to-high O_2 partial pressures. Across the genus, this indicates that all species are adapted to life under variable gas regimes and all have capacity for life at high CO_2 availability, plus medium-to-low CO_2 availability in *T. denitrificans* and *T. thiophilus*, whereas *T. thioparus* can survive at much lower CO_2 availability. [Ni,Fe]-hydrogenases (EC 1.12.99.6) are found in this genus as well as in the other *Thiobacillaceae* (fbm00351), which could indicate potential for growth on molecular hydrogen as an electron donor, although direct evidence is scant to date. Beller *et al.* (2006) note that molecular hydrogen-dependent reduction of uranium and other metals occurs in *T. denitrificans*, which could indicate that electrons from molecular hydrogen are used for 'detox'

reductases rather than for generation of Δ*p*. Thiosulfate-oxidizing multienzyme system (TOMES) genes are present, arranged *soxXYZAB,* with *soxCD* apparently not present. As discussed in Hutt *et al.* (2017), there is some evidence of the reductase operons associated with denitrification in *T. thioparus* DSM 505^{T}, even though this strain does not denitrify in our hands or those of previous authors – we have tried under both air and under microxia.

Ecology. *Thiobacillus* spp. can be isolated easily from soils, canal water, river water, reservoir water and from sediments. It is of interest that *T. thiophilus* was isolated from a BTEX-contaminated aquifer (Kellermann and Griebler, 2009), and clone libraries containing 16S rRNA genes with close (>98 % identity) match to *Thiobacillus* spp. have been obtained from tar oil contaminated aquifers (Winderl *et al*. 2008) – whether *Thiobacillus* spp. have any metabolic interaction with BTEX compounds and other hydrocarbons is not known, but they may simply not be killed by them. It is also interesting that the other known species of the *Thiobacillaceae* (fbm00351) *viz*. *Sulfuritortus calidifontis* and *Annwoodia aquaesulis* were isolated from thermal springs that flow from deep aquifers, much like *T. thiophilus*, this observation may indicate populations in the deep subsurface and that the sewage (*T. denitrificans*) and soil (*T. thioparus*) isolates represent descendants of a chthonic lineage.

Cultivation, Enrichment and Isolation Procedures

General cultivation: *Thiobacillus* spp. are grown using thiosulfate, tetrathionate, sulfide or elementary sulfur as the electron donor in a high-phosphate, well-buffered basal salts (E-basal salts, EBS, is described under ***Nutrition and Growth Conditions***). EBS has an ionic strength of 0.314 M and is one of the higher ionic strength media for sulfur oxidizing *Bacteria* – this is discussed in more detail in the chapter on *Annwoodia* (gbm01601). The two phosphate salts of EBS are autoclaved separately, in 20 % of the liquid volume. All other ingredients (plus electron

donor and agar, if needed, see below) dissolved in the remaining 80 % of the liquid. After autoclaving at 15 psi for 15 min and cooling completely, the two solutions are mixed and vitamins are added, if using. Thiosulfate and tetrathionate can be autoclaved, and elementary sulfur must be Tyndallized, but all other electron donors should be sterilized by filtration and added from stock solutions after mixing the cooled components. During growth on thiosulfate, the pH of the medium will drop, with growth usually terminating at about pH 5.2 in EBS, but in more weakly buffered media, it will drop to pH 4.5. Elementary sulfur is thrown down during batch cultivation on thiosulfate under air, or at lower oxygen partial pressures, but it can be prevented by growing the organism in the chemostat (Boden and Hutt, 2018*a*). The most convenient means of growth is to use 50 mL EBS supplemented with the electron donor in 250-mL Erlenmeyer flasks stoppered lightly with foam or cotton stoppers. Whilst some sources state that shaking cultures of autotrophs will remove CO_2, but in our hands, very good growth can be obtained by shaking the flasks really rather violently. Baffled flasks (or, in a pinch, the addition of a handful of glass microscope slides or a surgical steel spring to the flask before autoclaving) will produce better growth. It is important to note that in *T. thioparus*, carboxysomes are formed and their proteins represent a very large fraction of the proteome – it may thus be prudent to use an elevated CO_2 partial pressure for some work in order to repress this.

Maintenance: EBS can be solidified by the addition of 1-2 % (*w/v*) Noble agar (Sigma or Difco) or other high purity agar – we use a high purity Granulated Agar by Mellford as well, which seems to be equally good for *Thiobacillus* spp. but some *Thiobacillus* strains can be ‘fussy’ and grow weakly on agar – we have found LUDOX® colloidal silica (W. R. Grace and Co.) to be a suitable alternative to agar – it is used to replace some of the water of EBS which is then dispensed into small glass Petri dishes and autoclaved: it solidifies during this process into firm

white plates (for full details, refer to the chapter on *Acidithiobacillus* (gbm01079)). Whilst 'nice' colonies are often formed on thiosulfate agar, some *Thiobacillus* spp. grow in the water of syneresis that forms on the top of the agar over time. This gives growth as a white or yellow 'smear' rather than proper colonies. The addition of a pH indicator can make distinguishing of growth on agar plates much easier - bromocresol purple is best in our hands as it changes at pH 5.2 from violet to yellow – *via* grey – and does not inhibit growth – we use 5 mL/L of medium of the 0.04 % *w/v* pharmaceutical grade stock sold by Sigma-Aldrich as it does not contain appreciable organic contaminants. For short-term storage, agar slants or plates can be stored at 4 °C for up to a month or so. For medium-term storage, liquid cultures can be grown on *e.g.* 20 mM thiosulfate and then, at late exponential phase, are brought back to pH 7.2 by the dropwise addition of sterile bicarbonate solution – the cultures are then supplemented with thiosulfate to 10 mM and stored in the fridge where they will slowly lower the pH once more but remain viable for 6-8 weeks (sometimes longer) before subculture is needed – which will be indicated by the pH indicator turning yellow once more. Longer term, many strains are not lyophilizable or do not freeze well, and thus the service collections provide them as a live culture. We store *Thiobacillus thioparus* strains at -80 °C by adding glycerol to cultures to 10 % *v/v* and resting in ice for 1 h before freezing in liquid nitrogen and slowly 'warming' to -80 °C in dry ice, prior to storing them at this temperature, where they last at least a few years – very high purity dimethylsulfoxide (DMSO) is also a useful cryoprotectant, but some strains do not tolerate it. If in doubt, consultation with the staff at a service collection that holds *Thiobacillus* isolates will usually provide useful 'tricks' – at the time of writing, the staff at the DSMZ in Germany are particularly knowledgeable and helpful in this area.

Enrichment and isolation: *Thiobacillus* spp. can be enriched on EBS supplemented with a suitable electron donor and incubated under air, or, for the denitrifying species, with the addition of 30 mM potassium nitrate, incubated in completely-filled, sealed bottles of strong glass. Electron donors suitable for isolation include thiosulfate (10-20 mM) or tetrathionate (5-10 mM) but volatile inorganics such as carbon disulfide or carbonyl sulfide or organics such as dimethylsulfide (1-5 mM, monitored and re-spiked 2-3 times between each subculture – *cf.* Boden and Hutt (2018*b*) for analytical methodologies) can also be used. Volatile electron donors must be added to cold EBS after autoclaving – it is most convenient to add them "neat" from the bottle (assuming analytical grade to be effectively sterile!) straight into culture flasks – which should be 'QuickFit' Erlenmeyers sealed with 'SubaSeal' vaccine stoppers that have been coated with PTFE Dry Lubricant Spray on the inside before autoclaving. 45 mL volumes of EBS containing the appropriate electron donor are dispensed into Erlenmeyer flasks (see above for special instructions re: volatile substrates) with loose cotton or foam stoppers – we find 'wide mouth' flasks particularly useful for autotrophs, as they promote better CO_2 exchange with the atmosphere. 5 mL of the inoculum is added (pond water, lake water *etc* or for solid samples, we shake 5-10 g *e.g.* soil with 20 mL sterile water for 1 h then allow to settle and use 5 mL of the supernate as the inoculum) and the flask incubated with shaking at 25-30 °C. For coupling thiosulfate oxidation to nitrate reduction, the same mix is prepared and then poured into *c*.30-mL thick-walled Universal bottles, right up to the top, and the lid screwed on tightly. If denitrification occurs, bubbles will be observed on the walls – nitrate and nitrite concentrations can be rapidly approximated in a 1:10 dilution of the culture using QuantoFix nitrate/nitrite determination dipsticks – this allows the worker to know if toxic nitrite is building up, which may indicate subculture is needed, if above 10 mM or so. Production of elementary sulfur and/or

a pH fall indicate sulfur oxidation is occurring – sometimes the elementary sulfur will disappear. If elementary sulfur (*viz.* flowers-of-sulfur) is used as the electron donor (at 0.5 g/L), it will float at first but then sink once the organism begins to coat it in surfactants and hydrophilic materials – but turbidity is usually not obvious in these cases. Before subculturing cultures grown on sulfur, vigorous vortex mixing can be used to release cells, or, rather than transferring just supernate, transfer some of the sulfur as well. Serial subculture (10 % *v/v* at each step) is conducted 5-6 times – by the 4^{th} or 5^{th}, the time to reach stationary phase is usually about 3-4 days on thiosulfate/polythionates, a little longer on elementary sulfur, but can be as long as 14 days on volatiles as the concentration is lower, they are metabolized more slowly owing to toxicity, and re-spiking is needed. Streak-plates are made on *e.g.* EBS/thiosulfate agar containing bromocresol purple (see above). For denitrifiers, these contain 30 mM potassium nitrate and are incubated under oxygen-free nitrogen or argon (there is no need to use a reducing atmosphere – the molecular hydrogen therein may interfere with some *Thiobacillus* spp. as they contain hydrogenases). For aerobes they are incubated under air, which can be enriched with CO_2 to 5 % *v/v*, or alternatively, a *small* piece of dry-ice can be put into a box with the plates, or even just a small beaker containing sodium carbonate and a weak mineral acid (*Alka-Seltzer* tablets and water are commonly used for this purpose, and are quite convenient) – this additional CO_2 often affords bigger colonies and makes the initial isolation step much easier.

For isolates on volatile substrates, EBS agar without an electron donor is used and plates are incubated in metal or plastic gas-jars or in sealed Tupperware™ boxes and their atmosphere enriched with the substrate in gas-phase, by using either a substrate "bomb" comprising a glass Bijou tube packed with lens tissue into which 0.5 mL of the neat substrate is added and is then left in the corner of the box, or triangles of sterilized filter paper are put into the inside of the lid

of each inverted plate and 1-5 µL of the substrate added thereto. This gives excellent growth of *Thiobacillus thioparus* strains on dimethylsulfide or dimethyldisulfide in our hands. For gases like methanethiol or carbonyl sulfide, a small volume can be added to gas-jars *via* their ports just after sealing. If plates in boxes or clear gas-jars are incubated either in single layers or interlaced with empty plates, it can be easy to see from the outside which plates have turned yellow (indicating oxidation of sulfur to sulfuric acid), which means one can avoid opening them too frequently and releasing the substrate. Dimethylsulfide and dimethyldisulfide *can* be incorporated into molten agar just before pouring plates, however, we *strongly* recommend this approach is never taken as the smell permeates the surrounding area, is nauseatingly strong and does not disappear for some days and above all is quite unnecessary – the aforementioned methods give more than satisfactory growth!

Taxonomic comments

Where a three-letter abbreviation is needed to avoid confusion with other genera, we recommend "*Tbc*." be used. A phylogenetic tree is given in the chapter on *Annwoodia* (gbm01601), which shows the position of *Thiobacillus* spp. and other related taxa. The genus *Thiobacillus* has been changed somewhat over the last 115 years since Beijerinck (1904*a*, *b*) first defined it. For most of the 20th century, all sulfur-oxidising Gram-stain-negative organisms were allocated to this genus, without regard for most of their properties, though it became clear in the 1980s and 1990s that from the dominant respiratory quinones alone, the organisms in this genus were very diverse. From the early 1990s, studies by Wood and Kelly (1993), Kelly and Wood (2000*a*,*b*), Kelly *et al.* (2000), Katayama *et al*. (1995), Moreira and Amils (1997) reclassified a large number of *Thiobacillus* spp. to *Paracoccus* (gbm00860), *Thiomonas* (gbm00960), *Thiomicrospira* (gbm01221), and the newly created genera *Acidithiobacillus* (gbm01079),

Thermithiobacillus (gbm01080), *Halothiobacillus* (gbm01133) and *Starkeya* (gbm00828). Further work in the last 5 years by Boden *et al.* (2017) has reclassified one further species to the newly created genus *Annwoodia* (gbm01601). As of 2019, the genus *Thiobacillus* comprises only 3 species and we are confident that all 3 are correctly assigned now, with the advent of whole-genome sequencing and more advanced phylogenetic methods. It is worth noting that a number of strains in the public service collections that were assigned to *Thiobacillus* were examined by Boden *et al*. (2012) - in spite of the recent division of the genus with the creation of *Annwoodia* (gbm01601), most of these *Thiobacillus* strains (*viz*. E6, Tk-m, Pankhurst T4 and White 2K) are still *Thiobacillus* isolates, and probably affiliate most strongly with *T. thioparus*.

List of species in the genus **Thiobacillus**

1. ***Thiobacillus denitrificans*** (ex. Beijerinck 1904*b*) Kelly and Harrison, 1989, 1855 emend. Kelly and Wood 2000*a*, 548^{VP}.

   de.ni.tri'fi.cans. N.L. v. *denitrifico*, to denitrify; N.L. part. adj. *denitrificans*, denitrifying.

   Motile rods 0.5 × 1.0-3.0 µm. Colonies are clear, turning white with age. Optimal growth at 28-32 °C and pH 6.8-7.4. Does not produce carboxysomes. Can use thiosulfate, tetrathionate, elementary sulfur, thiocyanate, sulfide and pyrrhotite as electron donors for chemolithoautotrophic growth, but not dithionate, carbon disulfide, carbonyl sulfide, dimethylsulfide, dimethyldisulfide and thiols are not used. Denitrifies, giving vigorous evolution of molecular nitrogen when grown on thiosulfate and nitrate in the absence of air. Uses Form IAq and Form II RuBisCO and type aa_3 and cbb_3 cytochrome *c* oxidases, does not have a ubiquinol oxidase. Type strain isolated from a sewage lagoon, UK.

   DNA G+C fraction (mol%): 66.07 (sequence).

Type strain: AB7 = ATCC 23644 = CIP 104767 = DSM 12475 = JCM 3870 = NCIMB 9548.

GenBank accession (16S rRNA gene): AJ243144

IMG accession (genome sequence): 637000324

2. *Thiobacillus thioparus* Beijerinck 1904*b*, 153[AL]

thi.o'par.us. neut. n. *theîon*, sulfur, brimstone; N.L. masc. adj. *parus* (from L. v. *paro*, to provide, to furnish, to prepare), producing, providing; N.L. masc. adj. *thioparus*, sulfur-producing.

Motile rods 0.5 × 1.7 µm. Colonies are white, turning pink-brown with age. Optimal growth at 25-30 °C and pH 6.0-8.0. Produces carboxysomes when grown under air. Can use thiosulfate, trithionate, tetrathionate, thiocyanate and sulfide as electron donors for chemolithoautotrophic growth. Some strains can use carbon disulfide, carbonyl sulfide, dimethylsulfide, dimethyldisulfide and methanethiol, with the former two substrates usually giving comparatively weak growth. Dithionate and elementary sulfur are not used. Does not denitrify. Uses Form IAc and Form II RuBisCO and type aa_3 and cbb_3 cytochrome *c* oxidases, and *bd*-I ubiquinol oxidase. Type strain isolated from agricultural soil, New Jersey, USA.

DNA G+C fraction (mol%): 62.30 (sequence).

Type strain: Starkey = ATCC 8158 = CIP 104484 = DSM 505 = JCM 3859 = NBRC 103402.

GenBank accession (16S rRNA gene): HM173629

IMG accession (genome sequence): 2765235835

3. *Thiobacillus thiophilus* Kellerman and Griebler 2009, 587[VP].

thi.o'phi.lus. Gr. neut. n. *theîon*, sulfur, brimstone; Gr. masc. adj. *phílos*, dearly loved, beloved; N.L. masc. adj. *thiophilus*, sulfur-loving.

Motile rods 0.5-0.8 × 1.8-2.5 µm. Colonies are white, turning yellow with age. Optimal growth at 25-30 °C and pH 7.5-8.3. Does not produce carboxysomes. Can use thiosulfate or tetrathionate as electron donors for chemolithoautotrophic growth but does not use elementary sulfur, trithionate, thiocyanate, sulfide, pyrrhotite or molecular hydrogen. Denitrifies, giving vigorous evolution of molecular nitrogen when grown on thiosulfate and nitrate in the absence of air. Uses Form IAq and Form II RuBisCO and type cbb_3 and cytochrome *c* oxidase, and *bd*-I ubiquinol oxidase. Type strain isolated from a BTEX contaminated aquifer, Germany.

DNA G+C fraction (mol%): 62.15 (sequence).
Type strain: D25TN = DSM 19892 = JCM 15047.
GenBank accession (16S rRNA gene): EU685841
IMG accession (genome sequence): 2765235835

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Character	***Thiobacillus thioparus***	***Thiobacillus denitrificans***	***Thiobacillus thiophilus***
16S rRNA gene identity of type strain to *T. thioparus* DSM 505^T (%)	100	97.6	97.6
Cell length (µm)	1.7	1.0-3.0	1.8-2.5
Cell diameter (µm)	0.5	0.5	0.5-0.8
Isolation sources of key strains in international service collections (type strain in bold)	**Agricultural soil, USA**; Pond water, UK; Activated sludge, Japan; Gasworks sludge, UK; Soil, UK; Biofilters (various);	**Sewage lagoon, UK**; Pond water, Germany; Brackish mud, Senegal; Carbonization effluent lagoon, UK; Anaerobic digester, UK	**BTEX[a] contaminated aquifer, Germany**
Colony colour (thiosulfate agar, under air)	White, turning pink/brown with age	Clear, turning white with age	White, turning yellow with age
Use of nitrate as a nitrogen source	+	±	*N.D.*
Use of nitrate as a terminal electron acceptor	-	+	+
Temperature range and [optimum] (°C)	*N.D.* [25-30]	*N.D.* [28-32]	-2-30 [25-30]
pH range and [optimum]	5.0-9.0 [6.0-8.0]	*N.D.* [6.8-7.4]	6.3-8.7 [7.5-8.3]
Carboxysomes produced	+	-	-
Electron donors for autotrophic growth:			
Thiosulfate ($S_2O_3^{2-}$)	+	+	+
Dithionate ($S_2O_6^{2-}$)	-	-	*N.D.*
Trithionate ($S_3O_6^{2-}$)	+	*N.D.*	-
Tetrathionate ($S_4O_6^{2-}$)	+	+	+
Elementary sulfur (S_8)	-	+	-
Thiocyanate (SCN^-)	+	+	-
Sulfide (S^{2-})	+	+	-
Carbon disulfide (CS_2)	*w*	-	*N.D.*
Carbonyl sulfide (COS)	*w*	-	*N.D.*
Methanethiol (CH_3SH)	*d*	-	*N.D.*
Dimethylsulfide ($(CH_3)_2S$)	*d*	-	*N.D.*
Dimethyldisulfide ($(CH_3)_2S_2$)	*d*	-	*N.D.*
Pyrrhotite (FeS)	*N.D.*	+	-
Molecular hydrogen (H_2)	*N.D.*	*N.D.*	-
D-ribulose 1,5-bisphosphate carboxylase/oxygenase genes[b]	Form IAc (*cbbLS*) Form II (*cbbM*)	Form IAq (*cbbLS*) Form II (*cbbM*)	Form IAq (*cbbLS*) Form II (*cbbM*)
Terminal oxidases encoded in genome:			
Cytochrome *c* oxidases	*aa*$_3$, *cbb*$_3$	*aa*$_3$, *cbb*$_3$	*cbb*$_3$
Ubiquinol oxidases	*bd*-I	-	*bd*-I
G+C fraction (mol%) from genome	62.30	66.07	62.15

406

407 **Table 1**. Comparative properties of *Thiobacillus* spp.

408 *a*. BTEX = benzene, toluene, ethylbenzene and xylene.

409 *b*. RuBisCO form nomenaclature after Badger and Bek (2008).

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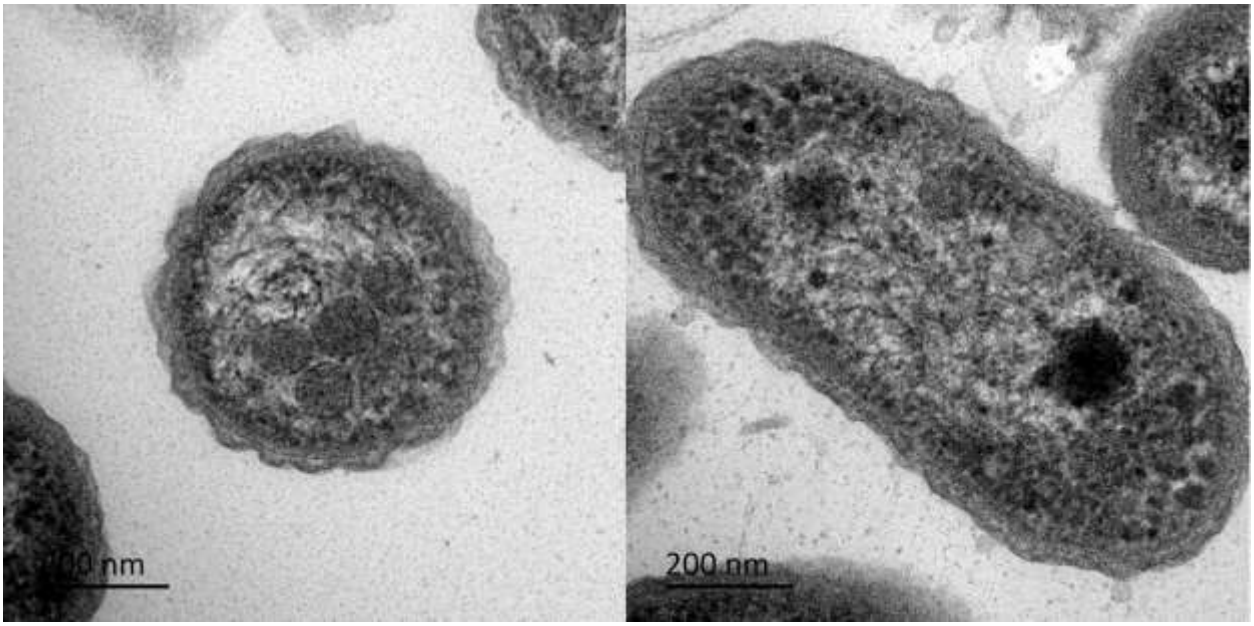

413

414 **Figure 1**. Transmission electron micrographs of *Thiobacillus thioparus* Starkey^{T} cells

415 obtained from a thiosulfate-limited chemostat, in transverse and longitudinal

416 sections, showing presence of multiple carboxysomes (in the transverse section)

417 and showing the ‘puffy’ or ‘ruffled’ periplasm/outer membrane often evident in

418 *Thiobacillus* spp. Full details of preparation are given in Hutt *et al.* (2017). Scale

419 bars indicate 200 nm.

420 **{Figure 1ab.tif}**