# *Thiobacillus thiophilus* sp. nov., a chemolithoautotrophic, thiosulfate-oxidizing bacterium isolated from contaminated aquifer sediments

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Strain D24TN$^T$ was enriched and isolated from sediment collected from a tar oil-contaminated aquifer at a former gasworks site located in Duesseldorf-Flingern, Germany. Cells of strain D24TN$^T$ were rod-shaped, non-spore-forming and stained Gram-negative. Thiosulfate was used as an electron donor. The organism was obligately chemolithoautotrophic and facultatively anaerobic, and grew with either oxygen or nitrate as electron acceptor. Growth was observed at pH values between 6.3 and 8.7 and at temperatures of −2 to 30 °C; optimum growth occurred at pH 7.5–8.3 and 25–30 °C. The DNA G+C content was 61.5 mol%. On the basis of the 16S rRNA gene sequence analysis, strain D24TN$^T$ clustered in the *Betaproteobacteria* and was most closely related to *Thiobacillus denitrificans* (97.6 %) and *Thiobacillus thioparus* (97.5 %). Based on the phenotypic, chemotaxonomic and phylogenetic data, strain D24TN$^T$ represents a novel species of the genus *Thiobacillus*, for which the name *Thiobacillus thiophilus* sp. nov. is proposed. The type strain is D24TN$^T$ (=DSM 19892$^T$=JCM 15047$^T$).

$CO_2$ fixation is one of the world's most important biogeochemical processes. While the importance of $CO_2$ fixation on the terrestrial surface is known, there is little information about autotrophic processes in the subsurface (Kinkle & Kane, 2000). Most biogeochemical transformations in groundwater ecosystems are mediated by bacteria and the subsurface complies with all requirements for chemolithoautotrophic processes. At numerous sites electron donors (e.g. $H_2$, $SO_3^{2-}$) and electron acceptors (e.g. $NO_3^-$, $O_2$) are available in appropriate combinations and in sufficient amounts together with plenty of inorganic carbon (Labrenz *et al.*, 2005; Madsen & Ghiorse, 1993). For organic carbon in groundwater ecosystems, two extremes are often met, either pristine systems that are depleted of dissolved organic carbon or polluted aquifers that are overloaded with complex mixtures of organic compounds that can be toxic and can only be degraded by individual micro-organisms (Alfreider *et al.*, 2003). Both situations support the argument that the role of chemolithoautotrophic members of the microbial community is significant. It may be the limited availability of dissolved organic carbon in the first case and the necessity to get rid of electrons in the latter case.

In the course of investigating the importance of autotrophic micro-organisms in groundwater ecosystems, we isolated a novel thiosulfate-oxidizing bacterium, designated strain D24TN$^T$, from sediments of a tar oil-contaminated aquifer under chemolithoautotrophic conditions. Strain D24TN$^T$ was phylogenetically most closely related to the genus *Thiobacillus* within the *Betaproteobacteria*.

Sediments from the quaternary homogeneous sandy aquifer were collected during a well-drilling campaign in June 2005. The aquifer was situated in the area of a former gasworks site in the Rhine valley in Duesseldorf-Flingern, Germany. Here, large amounts of tar oil phase were released into the subsurface during the first half of the 20th century, resulting in a contaminant plume with concentrations of BTEX (benzene, toluene, ethylbenzene and xylenes) of >100 mg $l^{-1}$ and polycyclic aromatic hydrocarbons of >10 mg $l^{-1}$. Since 1996, most of the oil phases in soil and groundwater have been removed by means of several remediation activities. Residual BTEX concentrations were still at about 20–60 mg $l^{-1}$ in the plume centre and the average concentrations of polycyclic aromatic hydrocarbons were about 10 mg $l^{-1}$. Further details concerning the field site are given by Anneser *et al.* (2008).

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *cbbL* and *cbbM* gene sequences of strain D24TN$^T$ are EU685841, EU746410 and EU746411, respectively.

Figures showing growth curves of cells of strain D24TN$^T$ under aerobic and anaerobic conditions and consensus phylogenetic trees based on *cbbL* and *cbbM* genes are available as supplementary material with the online version of this paper.

Strain D24TN$^T$ was isolated from anoxic sediment at 11.2 m below the soil surface and 4.9 m below the groundwater table. To protect the samples from contact with oxygen, sediment liners were removed from the borehole under argon and placed in a box flushed with argon gas for further processing. Sediment subsamples were collected using an autoclaved spoon, and transferred immediately to sterile Schott flasks filled with anoxic groundwater from the aquifer and stored at 4 °C in darkness. Enrichment cultures for chemolithoautotrophic growth were prepared using diluted Widdel freshwater medium (Widdel & Bak, 1992) (dilution 1 : 10; pH 7.3, anoxic), with sodium thiosulfate (10 mM) as electron donor and sodium nitrate (10 mM) as electron acceptor. Nitrate (Anneser *et al.*, 2008) as well as thiosulfate (F. Einsiedl, unpublished data) were detected in sufficient amounts in the aquifer. The redox indicator resazurin (1 mg $l^{-1}$) was used to confirm anoxic conditions during incubation. Ten grams of sediment (wet wt) was placed into 120 ml serum bottles containing 50 ml enrichment medium. The headspace was replaced by $N_2/CO_2$ gas (80 : 20, v/v) and the bottles were sealed with butyl stoppers (Ochs). Primary enrichment cultures were incubated at 16 °C (*in situ* aquifer temperature) in the dark for 3–4 months and transferred to fresh medium when visibly turbid. To isolate the most abundant cell type in the enrichment culture, two dilution series at appropriate dilutions (highest dilution used, $10^{-9}$; enrichment culture cell count was $1.4 \times 10^8$) were performed under the same culture conditions. To confirm purity, cells of strain D24TN$^T$ were examined using phase-contrast microscopy. Pure cultures were transferred every 2–3 weeks; cell growth was controlled by measuring optical density at 580 nm ($OD_{580}$; Varian) and occasionally by using microscopic total cell counts. Cell size and shape were determined using transmission electron microscopy. Cells of strain D24TN$^T$ were small rods (1.8–2.5 μm in length and 0.5–0.8 μm in diameter) and occurred singly; aggregate formation was not observed (Fig. 1).

Strain D24TN$^T$ grew under oxic conditions. Therefore, the strain was characterized as a facultative anaerobe, exhibiting faster growth in aerobic medium ($\mu_{max}$=0.069 $h^{-1}$) than under anoxic conditions ($\mu_{max}$=0.051 $h^{-1}$). In addition, growth in a more concentrated Widdel medium was tested, i.e. a 1 : 2 dilution was used instead of a 1 : 10 dilution; cells of strain D24TN$^T$ grew faster in the more concentrated medium ($\mu_{max[Widdel\ medium\ 1:10]}$=0.054 $h^{-1}$; $\mu_{max[Widdel\ medium\ 1:2]}$=0.069 $h^{-1}$). Over time, precipitation of elemental sulfur could be observed (for details see below).

Catalase activity was determined by placing a solution of 3 % hydrogen peroxide on a cell pellet of a freshly grown culture. Oxidase activity was determined using the Fluka oxidase test (Fluka/Sigma Aldrich), according to the manufacturer's instructions. Strain D24TN$^T$ tested positive for both catalase and oxidase activities.

Unless otherwise described, routine cultivation and growth tests were performed in 120 ml serum bottles containing 50–60 ml Widdel freshwater medium (dilution 1 : 2, pH 7.3, oxic conditions) with sodium thiosulfate (10 mM) as electron donor and sodium nitrate (10 mM) as electron acceptor, in at least duplicate incubations. Strain D24TN$^T$ did not grow on agar under oxic conditions, either on Widdel freshwater medium containing agar or on nutrient agar plates (meat extract, peptone and agar) or on *Thiobacillus denitrificans* medium (M832; DSMZ). Growth on agar under anoxic conditions was only observed on medium M832. Strain D24TN$^T$ did not grow on agar plates of Widdel freshwater medium or on nutrient agar.

Cells of strain D24TN$^T$ were Gram-negative; Gram staining was performed using a kit from Sigma-Aldrich, according to the manufacturer's instructions, with *Bacillus subtilis* and *Pseudomonas putida* F1 as positive and negative controls, respectively. Spore formation was tested by using pasteurization. Cultures were heated for 10 min at 80 °C, transferred to fresh medium and incubated at 30 °C for several weeks. Under these conditions, strain D24TN$^T$ did not form spores.

The pH range and optimum pH for growth were determined by using 13 pH values ranging from 6.0 to 8.9. The initial pH was achieved using 1 M HCl or 0.5 M $Na_2CO_3$. As growth of strain D24TN$^T$ resulted in the pH of the medium being continuously lowered, the pH had to be readjusted every 2 days.

Growth occurred between pH 6.3 and 8.7, with an optimum at pH 7.5–8.3. The temperature range for growth was determined at temperatures ranging from −2 to 37 °C. Growth occurred at −2 to 30 °C (over 1–2 weeks; slower growth occurred at −2, 0 and 4 °C). The optimum temperature for growth was 25–30 °C. Growth rates related

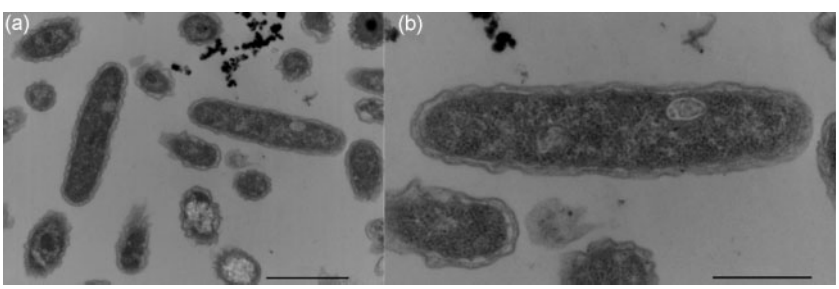


**Fig. 1.** Transmission electron micrographs of cells of strain D24TN$^T$. (a) Transverse and longitudinal thin sections of several single cells. Bar, 1 μm. (b) Magnified transverse thin section of a single cell. Bar, 500 nm.

to various pH values and temperatures were derived from optical density measurements at 580 nm.

Strain D24TN$^T$ was also tested for its ability to grow with various salt concentrations (0.5, 1, 2, 3, 4, 5 and 8 % NaCl, w/v). Growth was observed after 2 days incubation with 0.5, 1 and 2 % NaCl; no growth occurred at concentrations of 3 % NaCl or above.

The ability of strain D24TN$^T$ to utilize various electron donors was tested by applying $H_2$ (30 ml $H_2$ in headspace of serum bottle), $NH_4^+$ (5 mM), $S^{2-}$ (5 mM), FeS (3 mM), $S4O_6^{2-}$ (10 mM) and $S^0$ (0.5 g $l^{-1}$) using oxygen and/or nitrate (5 mM) as electron acceptor. Growth occurred with thiosulfate, together with the production of sulfate and small amounts of elemental sulfur, and with tetrathionate as electron donors. Utilization of electron acceptors other than oxygen or nitrate was investigated by testing $SO_4^{2-}$ (5 mM) and Fe(III) (ferrihydrite 40 mM). Growth of strain D24TN$^T$ occurred only in medium containing $O_2$ or $NO_3$. Cells grown aerobically completely converted thiosulfate to sulfate (101–103 %) with only trace amounts of elemental sulfur being precipitated whereas, in cells grown aerobically, only 60–75 % of the thiosulfate was converted into sulfate (see Supplementary Fig. S1, available in IJSEM Online) with a significant amount of $S^0$ precipitation formed (data not shown). At the same time, part of the nitrate that disappeared during the aerobic incubations appeared as nitrite (32–41 %). In cultures grown in bottles under anaerobic conditions, 69–72 % of the nitrate consumed was converted to nitrite (Supplementary Fig. S1), suggesting that nitrite is an intermediate in total nitrate reduction.

Chemo-organoheterotrophic growth was tested using complex medium (nutrient broth) and Widdel freshwater medium (without thiosulfate as electron source) supplemented with either glucose (10 mM), fructose (10 mM), sodium acetate (10 mM) or formate (10 mM) under oxic conditions.

A similar test series was used to test the capacity of chemolithoheterotrophic growth under oxic conditions with thiosulfate (10 mM) as electron source. Growth was monitored via optical density measurements. In the case of sodium acetate, possible consumption was determined by means of ion chromatography. However, no growth was observed in the absence or presence of thiosulfate; and organic substrates could not be used as an electron or carbon source.

Extraction of genomic DNA from liquid cultures (50 ml) was performed using a modified protocol of Lueders *et al.* (2004) and Gabor *et al.* (2003). PCR components (Fermentas) consisted of 5 µl 10 × buffer, 3 µl 25 mM $MgCl_2$, 0.5 µl each deoxynucleoside triphosphate at 10 mM, 0.5 µl bovine serum albumin (0.25 mg $ml^{-1}$), 0.5 µl each primer at 50 µM (MWG), 1 µl template DNA, 0.2 µl (0.2 units) *Taq* polymerase and 38.8 µl Gibco water (Invitrogen). PCR was performed using an Eppendorf Mastercycler in a total volume of 50 µl per reaction.

For amplification of the 16S rRNA gene we used the universal 16S primers 27-F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492-R (5′-CGGYTACCTTGTTACGACTT-3′) (Weisburg *et al.*, 1991). Cycling parameters were as follows: an initial denaturation for 90 s at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 52 °C and 90 s at 70 °C, and extension for 5 min at 70 °C. Amplification products were analysed on a 2 % agarose gel to ensure the correct size (16S rRNA 1.4 kb).

The PCR products were purified using a MinElute PCR Purification kit (Qiagen) according to the manufacturer's instructions. For direct sequencing, a Big Dye Terminator labelling kit (Applera Norwalk) with an ABI PRISM 3730 DNA analyzer (Applied Biosystems) was used.

Sequence data were analysed with the ARB software package (Ludwig *et al.*, 2004). Phylogenetic trees were calculated by using maximum-likelihood (Olsen *et al.*, 1994) and neighbour-joining (Saitou & Nei, 1987) analyses. According to the phylogenetic analysis of the 16S rRNA gene sequence, strain D24TN$^T$ was closely affiliated with the genus *Thiobacillus* (Fig. 2), with the closest relative being *T. denitrificans* (97.6 % sequence similarity).

As the Calvin cycle is the most prominent $CO_2$ fixation pathway in aerobes and facultative anaerobes and, because of the close relatedness to *T. denitrificans*, strain D24TN$^T$ was tested for the corresponding marker genes, i.e. the *cbb* genes, encoding ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO), the key enzymes of the Calvin cycle.

The primer sets for Form I RuBisCO (*cbbL*G and *cbbL*R) *cbbL*G1-F (5′-GGCAACGTGTTCGGSTTCAA-3′) and *cbbL*G1-G (5′-TTGATCTCTTTCCACGTTTCC-3′), *cbbL*R1-F (5′-AAGGAYGACGAGAACATC-3′) and *cbbL*R1-R (5′-TCGGTCGGSGTGTAGTTGAA-3′) (Selesi *et al.*, 2005) and the primer set for Form II RuBisCO (*cbbM*) *cbbM*-f (5′-GGCACCATCATCAAGCCCAAG-3′) and *cbbM*-r (5′-TCTTGCCGTAGCCCATGGTGC-3′) (Alfreider *et al.*, 2003) were used to amplify the RuBisCO genes, with the following cycling parameters: an initial denaturation for 4 min at 94 °C followed by 32 cycles of 1 min at 94 °C, 1 min at 62 °C (*cbbL*G) or 57 °C (*cbbM* and *cbbL*R) and 1 min at 70 °C, ending with a 10 min extension at 70 °C.

Amplification products were analysed on a 2 % agarose gel to ensure correct size (*cbbL*G 1.1 kp, *cbbL*R 0.8 kb, *cbbM* 0.5 kb). PCR products were processed for sequencing as described above for the 16S PCR product. Sequence data were analysed with the ARB software package (Ludwig *et al.*, 2004), as described above. Phylogenetic trees were calculated by using maximum-likelihood (Olsen *et al.*, 1994) and neighbour-joining (Saitou & Nei, 1987) analyses. Strain D24TN$^T$ was positive for *cbbL* type green-like (GenBank no. EU746410) and *cbbM* (EU746411) genes, therefore possessing the potential to fix $CO_2$ via the Calvin cycle (see Supplementary Figs S2 and S3, available in IJSEM Online). The strain was negative for *cbbL* type red-like

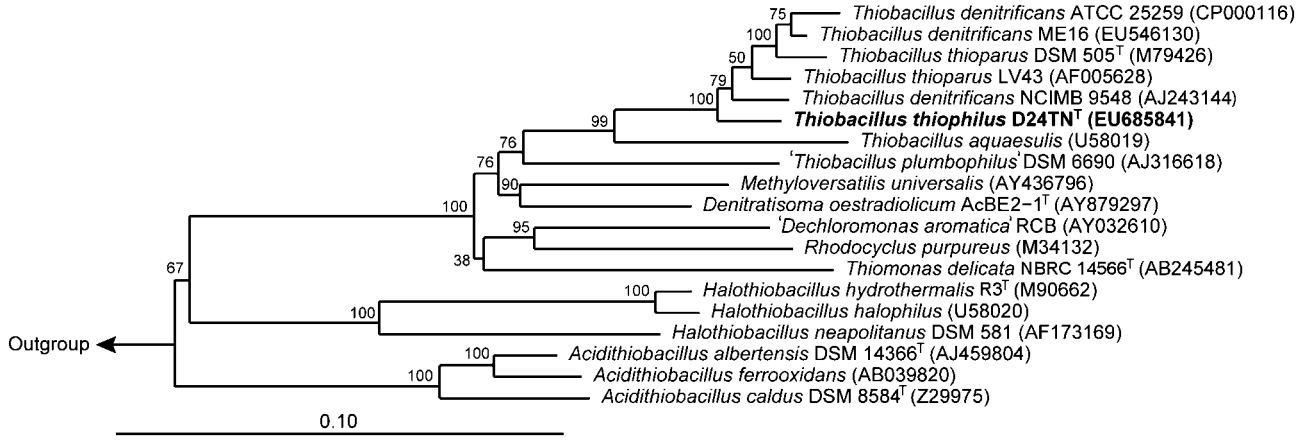


**Fig. 2.** Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strain D24TN$^T$ (*Thiobacillus thiophilus* sp. nov.) and some other related taxa. Bootstrap values (percentages of 1000 resamplings) are given at branch points. Bar, 10 % estimated sequence divergence.

genes. Sequence similarities to *cbbLG* sequences of *Thiobacillus thioparus* (DQ390449) and *T. denitrificans* (L42940) were 91.9 and 88.1 %, respectively, and similarities to *cbbM* sequences of *T. thioparus* (EU746412) and *T. denitrificans* (NC007404; L37437) were 88.4 % for each.

To confirm that strain D24TN$^T$ used the Calvin cycle for $CO_2$ fixation, RT-PCR was used to detect transcription of the *cbb* genes. For isolation of the total RNA, 50 ml dense cultures, grown aerobically and anaerobically, were centrifuged (4000 r.p.m., 15 min; Megafuge1.0R, Heraeus).

**Table 1.** Characteristics that differentiate strain D24TN$^T$ (*Thiobacillus thiophilus* sp. nov.) from its closest relatives *T. denitrificans* and *T. thioparus*

All taxa are oxidase positive and grow on or oxidize thiosulfate. All taxa are positive for tetrathionate and all are negative for chemo-organotrophic growth (data for *T. thioparus* and *T. denitrificans* are from Kelly & Wood, 2000). ND, No data.

| Characteristic | Strain D24TN$^T$ | *T. thioparus* | *T. denitrificans* |
|---|---|---|---|
| Morphology | Rods | Short rods | Rods |
| Cell length (μm) | 1.8–2.5 | 1.0–2.0 | 1.0–3.0* |
| Relation to $O_2$ | Facultative anaerobe | Aerobe* | Facultative anaerobe* |
| Sporulation | − | − | ND |
| Temperature optimum (°C) | 25–30 | 25–30† | 28–32* |
| Temperature range for growth (°C) | −2 to 30 | ND | ND |
| pH optimum | 7.5–8.3 | 6.0–8.0† | 6.8–7.4* |
| pH range for growth | 6.3–8.7 | 5.0–9.0‡ | ND |
| Catalase | + | ND | + |
| Growth on/oxidation of: | | | |
| Hydrogen | − | ND | ND |
| Sulfur | − | −‡ | +* |
| Ammonium | − | ND | ND |
| Sulfide | − | +‡ | +* |
| FeS | − | ND | +§ |
| Thiocyanate | − | +* | +* |
| DNA G+C content (mol%) | 61.5 | 61.0–66.0† | 63.0–68.0† |
| 16S rRNA sequence similarity with strain D24TN$^T$ (%) | (100) | 97.5 | 97.6 |

*Data from Kelly & Wood (2000).
†Data from Robertson & Kuenen (2006).
‡Data from Vlasceanu *et al.* (1997).
§Data from Beller *et al.* (2006).

Further preparation was performed as described by Schmitt *et al.* (1990).

To obtain pure RNA without DNA, 25 µl DNA/RNA extracts were digested with DNase I (20 U; Fermentas) at 37 °C for 45 min.

An AccessQuick RT-PCR system (Promega) was used to monitor the transcription of RuBisCO Form I and II genes using the same primers as described above. Reactions were carried out in 50 µl volumes, according to the manufacturer's instructions. RT-PCR parameters were 30 min at 45 °C and 5 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 57 and 62 °C (depending on the specific primer pair), and 1 min at 68 °C, followed by 1 cycle of 5 min at 68 °C. Amplification products were separated on 2 % agarose gels in 1 × TAE buffer (0.04 M Tris/acetate; 0.001 M EDTA), stained with ethidium bromide and visualized under UV light. For each RT-PCR, a negative control PCR without avian myeloblastosis virus reverse transcriptase was performed to rule out DNA contamination. Cells grown aerobically and anaerobically were shown to transcribe *cbbM* and green-like *cbbL* genes.

In addition, enzyme activity tests were performed, to determine RuBisCO activity in cell extracts of aerobically grown cells of strain D24TN$^T$ as well as in extracts of anaerobically grown cells. The enzyme assays were performed as described by Hügler *et al.* (2003). RuBisCO activity was detected in aerobically as well as in anaerobically grown cells of D24TN$^T$ (data not shown).

Determination of the G+C content of the DNA was performed at the DSMZ. The content was calculated from the ratio of deoxyguanosine (dG) and thymidine (dT), according to the method described by Mesbah *et al.* (1989), by means of HPLC analysis (Shimadzu Corporation). The G+C content of the DNA of strain D24TN$^T$ was 61.5 %.

To determine the genomic relatedness between strain D24TN$^T$ and its closest relative *T. denitrificans*, DNA–DNA hybridization was performed. For each of the two species, 3 g cell material was centrifuged and resuspended 1 : 1 (v/v) in 2-propanol/MilliQ ultrapure water (Millipore). All other steps were carried out at the DSMZ as described by Huß *et al.* (1983) and De Ley *et al.* (1970), with the modifications of Cashion *et al.* (1977). Based on 16S rRNA gene sequence analysis, strain D24TN$^T$ belongs to the class *Betaproteobacteria*. According to Wayne *et al.* (1987), strains with DNA–DNA relatedness values greater than 70 % belong to the same species. DNA–DNA hybridization analysis between strain D24TN$^T$ and *T. denitrificans* resulted in a relatedness value of 24.8 %; therefore strain D24TN$^T$ is clearly distinct from *T. denitrificans* (Table 1). Therefore, strain D24TN$^T$ represents a novel species of the genus *Thiobacillus*, for which the name *Thiobacillus thiophilus* sp. nov. is proposed.

### Description of *Thiobacillus thiophilus* sp. nov.

*Thiobacillus thiophilus* (thi.o′phi.lus. Gr. n. *thion* sulfur; Gr. adj. *philos* loving; N.L. masc. adj. *thiophilus* sulfur-loving). Cells are Gram-negative, small rods (1.8–2.5 µm in length and 0.5–0.8 µm in diameter) that can grow aerobically and anaerobically. Oxidase- and catalase-positive. Does not grow aerobically on agar plates; anaerobically grown colonies are circular, smooth, shiny, convex and yellow in colour with a lighter-coloured fringe after 14 days incubation. Spores are not produced and aggregates are not formed. Growth occurs anaerobically as a chemolithoautotroph on thiosulfate, with nitrate as the final electron acceptor. Also grows as an aerobic chemolithoautotroph on thiosulfate. Heterotrophic growth is not observed. Temperature range for growth is −2 to 30 °C, with optimum growth at 25–30 °C. pH range for growth is 6.3–8.7, with optimum growth at pH 7.5–8.3. Growth occurs under saline conditions to an upper NaCl concentration of 2 % (w/v).

The type strain, D24TN$^T$ (=DSM 19892$^T$=JCM 15047$^T$), was isolated from sediment derived from a tar oil-contaminated aquifer. Strain D24TN$^T$ is obligately chemolithoautotrophic, oxidizes thiosulfate and has a DNA G+C content of 61.5 mol% (HPLC).

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