Thiomicrospira arctica sp. nov. and *Thiomicrospira psychrophila* sp. nov., psychrophilic, obligately chemolithoautotrophic, sulfur-oxidizing bacteria isolated from marine Arctic sediments

Katrin Knittel,[1] Jan Kuever,[1]† Anke Meyerdierks,[1] Ruth Meinke,[1] Rudolf Amann[1] and Thorsten Brinkhoff[1,2]

[1]Max-Planck-Institute for Marine Microbiology, Celsiusstraße 1, D-28359 Bremen, Germany

[2]Institute for Chemistry and Biology of the Marine Environment, University of Oldenburg, Carl von Ossietzky Straße 9–11, D-26129 Oldenburg, Germany

Correspondence
Thorsten Brinkhoff
t.brinkhoff@icbm.de

Two psychrophilic, chemolithoautotrophic, sulfur-oxidizing bacteria were isolated from marine Arctic sediments sampled off the coast of Svalbard with thiosulfate as the electron donor and CO_2 as carbon source. Comparative analysis of 16S rRNA gene sequences suggested that the novel strains, designated SVAL-D^T and SVAL-E^T, represent members of the genus *Thiomicrospira*. Further genotypic (DNA–DNA relatedness, DNA G+C content) and phenotypic characterization revealed that the strains represent members of two novel species. Both organisms are obligately autotrophic and strictly aerobic. Nitrate was not used as an electron acceptor. Chemolithoautotrophic growth was observed with thiosulfate, tetrathionate and sulfur. The temperature limits for growth of both strains were between −2 °C and 20·8 °C, with optima of 11·5–13·2 °C (SVAL-E^T) and 14·6–15·4 °C (SVAL-D^T), which is about 13–15 °C lower than the optima of all other recognized *Thiomicrospira* species. The maximum growth rate on thiosulfate at 14 °C was 0·14 h^{-1} for strain SVAL-E^T and 0·2 h^{-1} for strain SVAL-D^T. Major fatty acids of SVAL-D^T are $C_{16:1}$, $C_{18:0}$ and $C_{16:0}$, and those of SVAL-E^T are $C_{16:1}$, $C_{18:1}$, $C_{16:0}$ and $C_{14:1}$. Cells of SVAL-D^T and SVAL-E^T are rods, like those of their closest relatives. To our knowledge the novel strains are the first psychrophilic, chemolithoautotrophic, sulfur-oxidizing bacteria so far described. The names *Thiomicrospira arctica* sp. nov. and *Thiomicrospira psychrophila* sp. nov. are proposed for SVAL-E^T (=ATCC 700955^T=DSM 13458^T) and SVAL-D^T (=ATCC 700954^T=DSM 13453^T), respectively.

Most of the sea floor is at temperatures below 4 °C (Levitus & Boyer, 1994) and many bacteria living in this habitat are well adapted to the low temperature in their environment. Bacteria with very different metabolic properties have been isolated from cold marine sediments and their characterization has revealed that they were either psychrophilic or psychrotolerant (Knoblauch *et al.*, 1999; Rüger *et al.*, 2000; Humphry *et al.*, 2001). Psychrophilic sulfur-oxidizing bacteria (SOB), however, have been relatively scarcely studied and little is known regarding their occurrence and diversity. Within a 16S rRNA gene sequence clone library from organisms from permanently cold marine sediments, Ravenschlag *et al.* (1999) found that about 18 % of all clones were related to symbiotic or free-living SOB of the *γ-Proteobacteria*. Mats of *Beggiatoa* species were described for cold seeps (e.g. Barry *et al.*, 1996), but it is unknown whether these species are psychrophilic. Teske *et al.* (2000) reported that SOB from cold deep-sea sediments and from hydrothermal vent systems show habitat-related differences in growth temperature, but the temperature optima of these isolates were not determined.

We isolated two new SOB strains from Arctic sediments sampled off the coast of Svalbard. These strains are phylogenetically affiliated with members of the genus *Thiomicrospira* within the *γ-Proteobacteria*. *Thiomicrospira* species are obligately chemolithoautotrophic SOB, which have been

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†Present address: Bremen Institute for Materials Testing, Foundation Institute for Materials Science, Paul-Feller-Straße 1, D-28199 Bremen, Germany.

Abbreviation: SOB, sulfur-oxidizing bacteria.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *T. arctica* SVAL-E^T and *T. psychrophila* SVAL-D^T are respectively AJ404731 and AJ404732.

Micrographs of cells of strains SVAL-D^T and SVAL-E^T are available as supplementary material in IJSEM Online.

detected in different habitats worldwide. They have been found in several marine sediments, in intertidal mudflats and a continental shelf sediment, in hydrothermal vent systems, and also in hypersaline ponds, a saline spring and a freshwater pond (e.g. Kuenen & Veldkamp, 1972; Ruby & Jannasch, 1982; Jannasch *et al.*, 1985; Wood & Kelly, 1993; Brinkhoff & Muyzer, 1997). As indicated by molecular biological and microbiological studies, members of this genus appear to be ecologically significant at hydrothermal vent sites (Muyzer *et al.*, 1995; Brinkhoff *et al.*, 1999c), whereas in intertidal mudflat habitats *Thiomicrospira* strains have been found in much lower abundances than other SOB (Brinkhoff *et al.*, 1998).

Marine arctic sediments were sampled off the coast of Spitsbergen (Svalbard) in July 1998. Strain SVAL-D^T originated from Isfjorden sediment (78° 10·907′ N 14° 34·124′ E; water depth 246 m) and strain SVAL-E^T from Jonsfjorden sediment (78° 32·616′ N 12° 18·075′ E; water depth 168 m). The *in situ* temperature was around 0 °C at both sampling sites. Strains SVAL-D^T and SVAL-E^T were obtained from enrichment cultures inoculated with mud samples of the upper sediment layer (0–0·5 cm depth). The medium (TP) used and the isolation procedure were the same as described by Brinkhoff *et al.* (1999a), with the exception that the cultures were incubated at 4 °C.

Routine cultivation of the isolates, utilization of different substrates and determination of salinity optimum and range were performed in 15 ml tubes containing 5 ml TP medium or in 50 ml tubes containing 20 ml TP medium at 4 °C. Large-scale cultivation of strains SVAL-D^T and SVAL-E^T was performed in a chemostat at 4 °C and cultivation of *Thiomicrospira chilensis* DSM 12352^T was performed at 22 °C for subsequent analysis of fatty acids, determination of the G+C content, DNA–DNA hybridization experiments and protein analysis. In this regard, 3 and 20 l glass carboys were supplied with medium containing 40 mM thiosulfate; the pH was monitored by a sterilized pH electrode (Ingold) and readjusted by titration with Na_2CO_3 (1 M) to pH 7·5 through a personal computer program controlling a peristaltic pump. The program was developed by Volker Meyer at the Max-Planck-Institute for Marine Microbiology in Bremen. The chemostat was aerated with sterile pressurized air through sparging devices. The maximum growth rates for strains SVAL-D^T and SVAL-E^T in TP medium were determined in well-aerated cultures at 14 °C, by total 4′,6-diamidino-2-phenylindole (DAPI) cell counts (Porter & Feig, 1980).

An estimate of the optimal pH value and the lowest and highest values tolerated by the isolates was obtained by using TP medium adjusted to different initial pH values (in steps of 0·5) and supplied with pH indicators covering different pH ranges (bromocresol green, 3·8–5·4; bromocresol purple, 5·2–6·8; bromothymol blue, 6·0–7·5; phenol red, 6·8–8·4; phenolphthalein, 8·2–9·8). The pH range for growth was determined by screening for acidification on the basis of colour change of the pH indicator. For strain SVAL-E^T the optimum pH at 4 °C was additionally determined in a chemostat by measuring the oxygen turnover rates at different pH values between pH 6 and 9 in steps of 0·5. The experiment was started after the chemostat reached equilibrium. After the substrate supply was stopped, the medium was saturated with oxygen. Aeration of the chemostat was then stopped and the first pH value was adjusted. Substrate supply was switched on and the decrease of oxygen was measured. After oxygen concentration reached 0 %, the substrate supply was switched off, the chemostat was aerated and the next pH value was adjusted. The decrease of oxygen at different pH values was plotted against time and the gradient of the straight line in the range between 20 and 80 % oxygen saturation was determined by linear regression. This gradient was plotted against the pH values and the second-order polynomial was regressed. The pH optimum was determined from the curve obtained. Optimal growth temperature was determined in a thermally insulated aluminium block, which was heated electrically to 32 °C at one end and cooled to −3 °C with a refrigerated circulation thermostat at the other end. The block contained 30 rows of four holes, so that samples could be incubated simultaneously at temperature intervals of 0·5 °C with a maximum of four replicates. The temperature limits of growth were established by screening for acidification for 30 days. The optimal growth temperature was determined within 36–48 h following inoculation. The Na^+ requirement and the utilization of inorganic and organic electron donors, including growth on hydrogen, and tests for anaerobic growth were carried out as described by Brinkhoff *et al.* (1999a).

Analyses of cellular fatty acid composition and respiratory lipoquinones were performed at the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) using standard procedures (Tindall, 1990a, b). Determination of the G+C content and DNA–DNA hybridization experiments were also performed at the DSMZ, and as described by Brinkhoff *et al.* (1999a). For protein analysis cell pellets were suspended in SDS sample buffer [0·35 mM Tris/HCl, pH 6·8, 36 % (v/v) glycerol, 10 % (w/v) SDS, 9·3 % (w/v) DTT, 0·012 % (w/v) bromophenol blue], boiled and cooled on ice. Cell lysates were cleared by centrifugation at 14 000 r.p.m. for 1 min. The supernatant was separated on a 7·5 % denaturing SDS-polyacrylamide minigel according to the method of Laemmli (1970). Proteins were stained using Coomassie brilliant blue. PCR amplification of almost complete 16S rRNA genes, purification of PCR products and subsequent sequencing analysis were performed according to Brinkhoff & Muyzer (1997). Sequence data were analysed with the ARB software package (Ludwig *et al.*, 2004). A phylogenetic tree was calculated by maximum-likelihood analysis with different sets of filters. For tree calculation, only full-length sequences (>1300 bp) were considered.

Phylogenetic analysis of the 16S rRNA gene sequences of SVAL-D^T and SVAL-E^T demonstrated close affiliation with

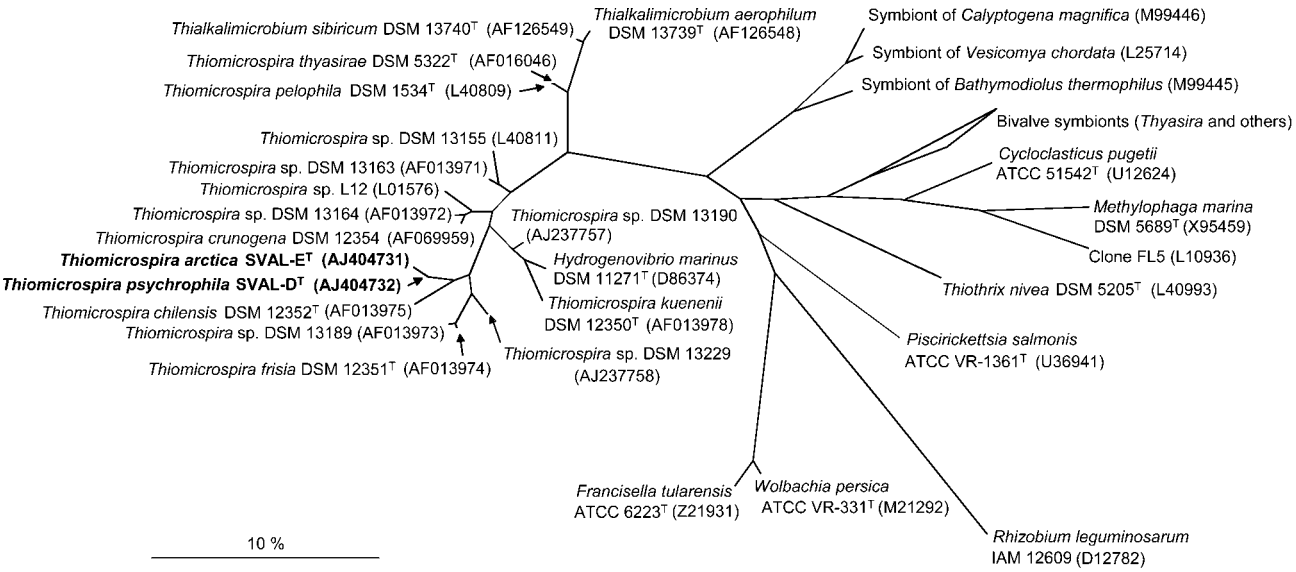

Fig. 1. Maximum-likelihood tree showing the affiliation of *Thiomicrospira psychrophila* sp. nov. SVAL-D^T and *Thiomicrospira arctica* sp. nov. SVAL-E^T to other *Thiomicrospira* species and selected reference sequences of the γ-*Proteobacteria*. *Rhizobium leguminosarum* was used as the outgroup. The tree is based on nearly complete 16S rRNA gene sequences. Bar, 10 % estimated sequence divergence.

the genus *Thiomicrospira* (Fig. 1). Sequence similarity between the closest described relative *T. chilensis* DSM 12352^T and strains SVAL-D^T and SVAL-E^T was 96·9 and 96·1 %, respectively. Sequence similarity between SVAL-D^T and SVAL-E^T was 99·2 %, indicating that the two isolates belong to one species (Stackebrandt & Goebel, 1994). However, DNA–DNA hybridization analysis with these organisms as well as with *T. chilensis* DSM 12352^T revealed values below 70 % (49·2 % DNA–DNA relatedness between SVAL-D^T and SVAL-E^T, 19·9 % between *T. chilensis* DSM 12352^T and SVAL-E^T and 19·2 % between *T. chilensis* DSM 12352^T and SVAL-D^T). According to Wayne *et al.* (1987), the phylogenetic definition of a species generally includes strains with greater than 70 % DNA–DNA relatedness. Thus, strains SVAL-D^T and SVAL-E^T are clearly distinguishable from recognized *Thiomicrospira* species and from each other. Cells of strains SVAL-D^T and SVAL-E^T appear as single rods (see supplementary figure in IJSEM Online), like those of *Thiomicrospira frisia* DSM 12351^T and *T. chilensis* DSM 12352^T, as well as those of the two strains *Thiomicrospira* sp. DSM 13189 and *Thiomicrospira* sp. DSM 13229. These organisms form a phylogenetic subcluster within the genus *Thiomicrospira* (Fig. 1), indicating a common ancestor for the rod-shaped morphology. Cells of strains SVAL-D^T and SVAL-E^T showed reduced levels of motility and were Gram-negative and spore formation was absent. Both strains were strictly aerobic and grew autotrophically on thiosulfate, tetrathionate and sulfur, but not on sulfite, thiocyanate or formate. Growth of strain SVAL-D^T on thiosulfate lowered the pH to 5·5, whereas strain SVAL-E^T lowered the pH to 5·1. Intermediate formation of elemental sulfur was observed with solid and liquid media. No growth occurred in TP medium supplemented with any of the organic substrates tested. Oxidation of thiosulfate was not inhibited by any of the organic substrates, except by acetate. Addition of vitamin B12 enhanced growth, but was not essential for growth. For strains SVAL-D^T and SVAL-E^T growth was observed between pH 6·5 and 9·0. SVAL-D^T and SVAL-E^T were able to grow at Na^+ concentrations between 40 and 1240 mM. For both isolates a Na^+ concentration of 250 mM resulted in optimal growth. The optimum pH for SVAL-D^T was 7·5–8·5. The optimum pH for SVAL-E^T was 7·5–8·0 as determined by indicator colour change, and 7·3–7·6 as determined in the chemostat experiment by measuring the oxygen turnover rates at different pH values. The G+C contents of strains SVAL-D^T and SVAL-E^T were 42·5 and 42·4 mol%, respectively. Ubiquinone 8 was the sole respiratory lipoquinone detected in both strains. This quinone is present in all *Thiomicrospira* species investigated so far (Brinkhoff *et al.*, 1999b).

The cellular fatty acid profiles of SVAL-D^T and SVAL-E^T were identical with respect to the presence of specific fatty acids, but differed in their relative amounts (Table 1). The profile of *T. chilensis* DSM 12352^T was also similar, but three fatty acids found for the novel strains could not be detected ($C_{12:0}$, $C_{14:0}$, $C_{14:1}$). The major fatty acid in all three strains was palmitoleic acid ($C_{16:1}$; 39·1–43·4 % of the total cellular fatty acids). Furthermore, high levels of palmitic acid ($C_{16:0}$) were found in all three species. Whereas high levels of stearic acid ($C_{18:0}$) and low levels of $C_{18:1}$ fatty acids were found in SVAL-D^T (32·0 and 3·2 %, respectively), the opposite was detected for SVAL-E^T (0·8

Table 1. Cellular fatty acid composition of *Thiomicrospira* species

Strains: 1, *T. chilensis* DSM 12352^T (growth at 22 °C); 2, *T. psychrophila* sp. nov. SVAL-D^T (growth at 4 °C); 3, *T. arctica* sp. nov. SVAL-E^T (growth at 4 °C). Proportions of fatty acids are given as percentages of whole-cell fatty acids. ND, Not detected.

Fatty acid	1	2	3
Saturated fatty acids			
$C_{12:0}$	ND	1·6	2·4
$C_{14:0}$	ND	0·5	0·8
$C_{16:0}$	18·9	9·7	12·7
$C_{18:0}$	3·5	32·0	0·8
Unsaturated fatty acids			
$C_{12:1}$	3·4	4·5	3·2
$C_{14:1}$	ND	5·0	11·6
$C_{16:1}$	43·4	40·0	39·1
$C_{18:1}$	27·8	3·2	26·5
Hydroxy fatty acids			
$C_{10:0}$ 3-OH	1·7	0·7	0·4
$C_{14:1}$ 3-OH*	2·1	2·2	1·6

*Classification uncertain.

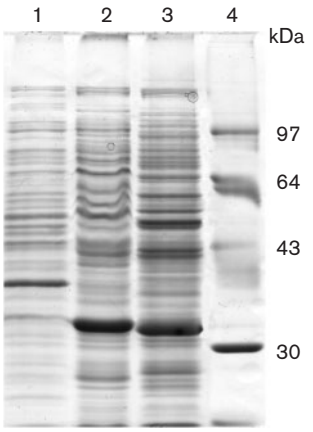

Fig. 2. SDS-PAGE of proteins of *Thiomicrospira* species. All strains were grown in a fermenter, *T. chilensis* DSM 12352^T (lane 1) at 22 °C and *T. arctica* sp. nov. SVAL-E^T (lane 2) and *T. psychrophila* sp. nov. SVAL-D^T (lane 3) at 4 °C. Lane 4 contains markers.

and 26·5 %). The fatty acids $C_{16:0}$, $C_{16:1}$ and $C_{18:1}$ are characteristic for most aerobic, Gram-negative bacteria, including *Thiomicrospira*-related genera (Nishihara *et al.*, 1991). Protein patterns of SVAL-D^T and SVAL-E^T, which were grown under similar conditions, showed some differences, and were clearly different from the pattern of *T. chilensis* DSM 12352^T (Fig. 2). Differences in fatty acid and protein patterns of SVAL-D^T and SVAL-E^T from those of *T. chilensis* DSM 12352^T might partially be explained by differences in their cultivation conditions (4 °C and 22 °C, respectively).

Temperature limits for growth of SVAL-D^T and SVAL-E^T were between −2·0 and 20·8 °C. The optimum growth temperature for strain SVAL-D^T was between 14·6 and 15·4 °C, and was even lower for strain SVAL-E^T (11·5–13·2 °C). A widely accepted definition for psychrophilic bacteria is that they have an optimal growth temperature below 15 °C, a maximum growth temperature of 20 °C and the ability to grow at 0 °C (Morita, 1975; Russell & Hamamoto, 1998). According to this definition our novel strains are psychrophilic. Cells of strain SVAL-E^T, which were pre-incubated for 2 months at 10, 14 and 20 °C, grew over a slightly broader temperature range (−2·0 to 24 °C) and also showed a higher optimum growth temperature (14·5–17·3, 15·3–17·6 and 15·7–17·3 °C). Phenotypic properties that distinguish strains SVAL-E^T and SVAL-D^T from recognized *Thiomicrospira* species are given in Table 2.

It is known that the ability of psychrophilic and psychrotolerant micro-organisms to grow at low but not moderate temperatures depends on adaptive changes in cellular proteins and lipids (Gounot & Russell, 1999). Changes in lipids can be genotypic or phenotypic and are important in regulating membrane fluidity and permeability. The upper growth temperature limit can result from the inactivation of a single enzyme type or system, including protein synthesis or energy generation (Russell, 1990). The present study demonstrates that the range of the worldwide-distributed genus *Thiomicrospira* is also extended to cold habitats. *Thiomicrospira* species appear to be adaptable to different environmental conditions and the main condition for their occurrence seems to be the presence of utilizable reduced sulfur compounds.

Description of *Thiomicrospira arctica* sp. nov.

Thiomicrospira arctica (arc′ti.ca. L. fem. adj. *arctica* from the Arctic, referring to the site where the type strain was isolated).

Cells are Gram-negative, motile and rod-shaped (0·5–0·6 × 1·2–1·5 μm). Strictly aerobic and grows autotrophically on thiosulfate, tetrathionate and sulfur, but not on sulfite or thiocyanate. Does not grow heterotrophically. When thiosulfate is used as the primary energy source small amounts of sulfur are produced. During growth on reduced sulfur compounds the pH decreases from neutrality to around 5·1. Autotrophic growth on thiosulfate occurs between pH 6·5 and 9·0 and at temperatures of −2·0 to 20·8 °C; optimum growth occurs at pH 7·3–8·0 and at 11·5–13·2 °C. The optimal Na^+ concentration for growth is 250 mM; growth is possible between Na^+ concentrations of 40 and 1240 mM. Nitrate is not used as a terminal electron acceptor. On thiosulfate agar, cells produce yellow, smooth, entire colonies [mean diameter on 1 % (w/v) agar is 1 mm after 4–6 weeks], in which sulfur is deposited and acid is produced. Ubiquinone Q-8 is present in the respiratory chain. Major fatty acids are $C_{16:1}$,

Table 2. Phenotypic properties that differentiate *T. arctica* sp. nov. SVAL-E^T and *T. psychrophila* sp. nov. SVAL-D^T from phylogenetically related *Thiomicrospira* species

Strains: 1, *T. pelophila* DSM 1534^T; 2, *T. frisia* DSM 12351^T; 3, *T. chilensis* DSM 12352^T; 4, *T. arctica* SVAL-E^T; 5, *T. psychrophila* SVAL-D^T. Data are from Kuenen & Veldkamp (1972), Brinkhoff *et al.* (1999a, b) and this study.

Character	1	2	3	4	5
Motility	+	+	+	+*	+*
G+C content (mol%)	45·7	39·6	49·9	42·4	42·5
Maximum growth rate (h^{-1})	0·3	0·45	0·4	0·14	0·2
Optimal pH	7·0	6·5	7·0	7·3–8·0	7·5–8·5
pH range	5·6–9·0	4·2–8·5	5·3–8·5	6·5–9·0	6·5–9·0
Optimal temperature (°C)	28–30	32–35	32–37	11·5–13·2	14·6–15·4
Temperature range (°C)	3·5–42	3·5–39	3·5–42	−2·0 to 20·8	−2·0 to 20·8
Optimal Na^+ concentration (mM)	470	470	470	250	250

*Only few cells showed motility.

$C_{18:1}$, $C_{16:0}$ and $C_{14:1}$. As determined by 16S rRNA gene sequence analysis, *Thiomicrospira arctica* belongs to the *γ-Proteobacteria* and is closely related to previously described members of the genus *Thiomicrospira*.

The type strain is SVAL-E^T (=ATCC 700955^T=DSM 13458^T). The G+C content of the DNA is 42·4 mol%. Isolated from marine Arctic sediments taken off the coast of Svalbard.

Description of *Thiomicrospira psychrophila* sp. nov.

Thiomicrospira psychrophila (psy.chro′phi.la. Gr. adj. *psychros* cold; Gr. adj. *philos* loving; N.L. fem. adj. *psychrophila* cold-loving).

Cells are Gram-negative, motile and rod-shaped (0·5–0·6 × 1·3–1·7 μm). Strictly aerobic and grows autotrophically on thiosulfate, tetrathionate and sulfur, but not on sulfite or thiocyanate. Does not grow heterotrophically. When thiosulfate is used as the primary energy source small amounts of sulfur are produced. During growth on reduced sulfur compounds the pH decreases from neutrality to around 5·5. Autotrophic growth on thiosulfate occurs between pH 6·5 and 9·0 and at temperatures of −2·0 to 20·8 °C; optimum growth occurs at pH 7·5–8·5 and at 14·6–15·4 °C. The optimal Na^+ concentration for growth is 250 mM; growth is possible between Na^+ concentrations of 40 and 1240 mM. Nitrate is not used as a terminal electron acceptor. On thiosulfate agar, cells produce yellow, smooth, entire colonies [mean diameter on 1 % (w/v) agar is 1 mm after 4–6 weeks], in which sulfur is deposited and acid is produced. Ubiquinone Q-8 is present in the respiratory chain. Major fatty acids are $C_{16:1}$, $C_{18:0}$ and $C_{16:0}$. As determined by 16S rRNA gene sequence analysis, *Thiomicrospira psychrophila* belongs to the *γ-Proteobacteria* and is closely related to previously described members of the genus *Thiomicrospira*.

The type strain is SVAL-D^T (=ATCC 700954^T=DSM 13453^T). The G+C content of the DNA is 42·5 mol%. Isolated from marine Arctic sediments taken off the coast of Svalbard.

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