Tetrathiobacter kashmirensis gen. nov., sp. nov., a novel mesophilic, neutrophilic, tetrathionate-oxidizing, facultatively chemolithotrophic betaproteobacterium isolated from soil from a temperate orchard in Jammu and Kashmir, India

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Twelve chemolithotrophic strains were isolated from temperate orchard soil on reduced sulfur compounds as energy and electron sources and characterized on the basis of their physiological properties and ability to oxidize various reduced sulfur compounds. The new isolates could oxidize tetrathionate as well as thiosulfate, and oxidation of the latter involved conversion of thiosulfate to tetrathionate followed by its accumulation and eventual oxidation to sulfate, manifested in the production of acid. The mesophilic, neutrophilic, Gram-negative and coccoid bacteria had a respiratory metabolism. Physiologically and biochemically, all the strains were more or less similar, differing only in their growth rates and ability to utilize a few carbon compounds as single heterotrophic substrates. 16S rRNA gene sequence analysis was performed with five representative strains, which revealed a high degree of similarity ($\geqslant$99 %) among them and placed the cluster in the '*Betaproteobacteria*'. The strains showed low levels (93·5–95·3 %) of 16S rRNA gene sequence similarity to *Pigmentiphaga kullae*, *Achromobacter xylosoxidans*, *Pelistega europaea* and species belonging to the genera *Alcaligenes*, *Taylorella* and *Bordetella*. The taxonomic coherence of the new isolates was confirmed by DNA–DNA hybridization. On the basis of their uniformly low 16S rRNA gene sequence similarities to species of all the closest genera, unique fatty acid profile, distinct G+C content (54–55·2 mol%) and phenotypic characteristics that include efficient chemolithotrophic utilization of tetrathionate, the organisms were classified in a new genus, *Tetrathiobacter* gen. nov. In the absence of any significant discriminatory phenotypic or genotypic characteristics, all the new isolates are considered to constitute a single species, for which the name *Tetrathiobacter kashmirensis* sp. nov. (type strain WT001^T=LMG 22695^T=MTCC 7002^T) is proposed.

More than a century ago, Nathansohn (1902) described the first small, unicellular, Gram-negative bacterium capable of utilizing thiosulfate as a substrate for chemolithotrophic growth ['thiotroph', after Kelly (1989)]. For almost a century after this initial description, the metabolic ability to grow on reduced sulfur compounds as an energy and electron source was attributed to the so-called thiobacilli (Kelly, 1989), under which a huge number of species were accommodated. However, recent advances in molecular systematics have brought about a thorough reorganization of this heterogeneous and artificial genus *Thiobacillus* (Kelly, 1989), leading to a progressive widening of the phylogenetic and ecological spectra over which the thiotrophs are distributed (Fuchs *et al.*, 1996; Das *et al.*, 1996; Deb *et al.*, 2004). The systematic diversity (McDonald *et al.*, 1997; Moreira & Amils, 1997; Kelly & Wood, 2000) of sulfur chemolithotrophy is compounded by an apparent physiological multiplicity pertaining to the differential ability of organisms to utilize various reduced sulfur compounds as

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains WT001^T, 445a, 445c, WGT and WPT and are AJ864470–AJ864474, respectively.

An example AFLP PCR profile and a UPGMA phenogram are available as supplementary material in IJSEM Online.

chemolithotrophic substrates (Katayama *et al.*, 1995; Kelly *et al.*, 1997, 2000; Friedrich, 1998). Genetic studies with both chemo- and photolithotrophic sulfur-oxidizing alphaproteobacteria have led to the identification of a sulfur oxidation (*sox*) gene cluster that encodes a sulfur-oxidizing multienzyme complex (Friedrich *et al.*, 2001; Appia-Ayme *et al.*, 2001) governing the oxidation of thiosulfate, sulfite, sulfide and elemental sulfur. Interestingly, the proposed mechanism does not account for the dissimilatory oxidation of tetrathionate, which is utilized chemolithotrophically by many species belonging to the '*Alphaproteobacteria*', '*Betaproteobacteria*' and '*Gammaproteobacteria*' (Kelly *et al.*, 1997, 2000; Mukhopadhyaya *et al.*, 2000).

5S rRNA gene sequence analysis had earlier identified a phylogenetic cluster in which the mixotrophic sulfur-oxidizing betaproteobacterial species of *Thiomonas* were clustered with species belonging to *Pseudomonas*, *Burkholderia*, *Alcaligenes* and *Bordetella* (Lane *et al.*, 1985; Moreira & Amils, 1997), while 16S rRNA gene sequence-based phylogeny had indicated the obligately thermophilic chemolithotroph *Thermothrix azorensis* to be closely related to species of *Alcaligenes*, *Pseudomonas* and *Burkholderia* (Odintsova *et al.*, 1996). However, investigation with different hydrogen-oxidizing bacteria by Friedrich & Mitrenga (1981) had shown that *Alcaligenes eutrophus* and *Alcaligenes paradoxus* (weak reaction) do not oxidize thiosulfate.

From soil samples from a temperate orchard, we have isolated and characterized 12 mesophilic, neutrophilic and facultatively sulfur-chemolithoautotrophic strains that could grow on thiosulfate and tetrathionate. Polyphasic taxonomic studies were performed upon five representative strains to delineate the taxonomic identity of the isolates. It was found that these isolates, affiliated phylogenetically to the '*Betaproteobacteria*', were physiologically and biochemically similar to members of the *Alcaligenaceae* and belonged to a novel genus and species, for which we propose the name *Tetrathiobacter kashmirensis* gen. nov., sp. nov.

Three bulk-soil samples from a temperate apple orchard at Panthchawk in Srinagar in the state of Jammu and Kashmir, India, were collected and supplemented with $Na_2S_2O_3.5H_2O$ (5 %) and elemental sulfur powder (5 %) and incubated at 30 °C for 2 weeks with intermittent sprinkling of sterile water. Enriched soil samples were added (1 % w/v) to MSTY broth, a modified basal and mineral salts (MS) solution (Mukhopadhyaya *et al.*, 2000) supplemented with sodium thiosulfate (20 mM $Na_2S_2O_3.5H_2O$) and 5 g yeast extract l^{-1}. The soil-MSTY broth mixture was incubated at 30 °C on a rotary shaker until the colour of phenol red indicator added to the medium changed yellow. Serial dilutions from this mixture were plated on MSTY agar and incubated at 30 °C. Twelve neutrophilic, mesophilic and facultatively sulfur-chemolithotrophic bacterial strains distinguished in terms of colony morphology and rate and extent of acid production in chemolithotrophic media MST (MS-thiosulfate; 20 mM $Na_2S_2O_3.5H_2O$) and MSTr (MS-tetrathionate; 20 mM $K_2S_4O_6$) and mixotrophic media MSTY (details above) and MSTrY (MS-tetrathionate-yeast extract) were isolated as pure cultures and subjected to further studies. The new isolates were maintained in Luria–Bertani (LB) broth.

Chemolithotrophic and chemo-organotrophic growth experiments were performed at 30 °C in MS solutions supplemented with sulfur compounds or single carbon sources, respectively. To test the chemo-organotrophic utilization of various carbon sources, basal MS solution plus one organic carbon source at a concentration of 5 g l^{-1} was used. All the isolates were tested for their ability to use thiosulfate (20 mM), tetrathionate (10 mM), sulfide (2 mM), sulfite (3 mM), thiocyanate (2 and 5 mM), elemental sulfur (0·5 and 1·0 % w/v) or arsenite (5 mM) as substrates for chemolithotrophic growth. Phenol red was added (final concentration of 20 mg l^{-1}) as a pH indicator in media containing reduced sulfur compounds. To test mixotrophic utilization of sulfur compounds, 5 g yeast extract l^{-1} was added to the above formulations. The level of thiosulfate or tetrathionate in the medium was estimated by the cyanolytic method described by Kelly & Wood (1994). All other phenotypic tests were performed using standard techniques described elsewhere (Gerhardt *et al.*, 1994). A numerical analysis of all the available comparative physiological, biochemical and chemotaxonomic data was performed using the simple matching coefficient (S_{SM}) and the Jaccard coefficient (S_J) (Sneath & Sokal, 1973) followed by generation of phenograms using the UPGMA algorithm. Similar phenograms were generated with the two coefficients.

Cells of all 12 new isolates were Gram-negative, non-flagellated and oval to coccoid in shape and occurred singly or in pairs, chains, branched chains or clusters. Transmission electron micrographs revealed capsular coverings around the cells (Fig. 1). Colonies grown on LB agar plates

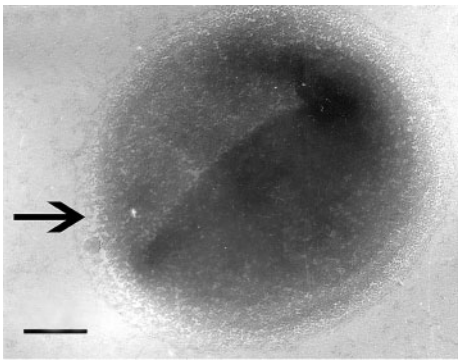

Fig. 1. Transmission electron micrograph of a cell of *Tetrathiobacter kashmirensis* WT001^T at ×30 000 magnification. The arrow points to the capsular covering around the cell. Bar, 0·4 μm.

were circular, entire, smooth, opaque and creamy white, reaching 4–6 mm in diameter after incubation for 2–3 days. On MSTY agar, colonies were smooth, glossy, concavely elevated and developed a central dark mound that was rusty red. The cells developed visible colonies on MST or MSTr agar plates after 4–6 days of incubation and the phenol red indicator turned completely yellow. Growth of all the isolates was completely inhibited by 200 μg ampicillin, 150 μg streptomycin, 100 μg nalidixic acid or rifampicin, 50 μg chloramphenicol, 40 μg neomycin and 20 μg tetracycline ml^{-1}.

The bacteria could grow chemolithoautotrophically on reduced sulfur compounds or chemo-organoheterotrophically on several single carbon sources and required no yeast extract or vitamins under either conditions. All the isolates were facultative chemolithotrophs and showed similar phenotypes when grown with thiosulfate or tetrathionate as energy and electron source. Thiocyanate, soluble sulfides, elemental sulfur, sulfite and arsenite were not utilized for growth nor were they oxidized under the experimental conditions used. All the strains exhibited similar kinetics of sulfur compound oxidation (data not shown).

When batch cultures of the strains were grown in thiosulfate-containing mixotrophic (MSTY) or chemolithotrophic (MST) media (both containing 20 mM sodium thiosulfate, equivalent to 40 μg atoms sulfur ml^{-1}) with an initial pH of 7–7·5, there was an initial increase in pH up to 8·5 in the first 2 days of incubation. Subsequently, the pH of the two media came down to 5·5–5·7 over the next 2–3 days. Growth in MSTY or MST media involved consumption of 70–80 % of the thiosulfate in 4–5 days. In MSTr medium (10 mM potassium tetrathionate, equivalent to 40 μg atoms sulfur ml^{-1}), with an initial pH of 7·5, consumption of tetrathionate and a concomitant decrease in pH of the medium was recorded for all 12 strains. After 4 days of growth at 30 °C, all strains exhibited utilization of approximately 80 % of the tetrathionate and the pH of the spent medium was between 5 and 5·5.

Details of chemolithotrophic growth coupled with oxidation of thiosulfate or tetrathionate were studied in the representative strain WT001^T using an initial concentration of 20 mM sodium thiosulfate and 10 mM tetrathionate with subsequent periodic addition of the same amounts of filter-sterilized sodium thiosulfate or potassium tetrathionate solutions to the respective cultures over a total incubation period of 144 h (Fig. 2a, b). Utilization of 200 μg atoms sulfur ml^{-1} supported a final OD_{600} of 0·47 and 0·43 in the two media types, MST and MSTr, respectively, and a cellular yield of 500–600 mg cell protein (g atoms sulfur oxidized)$^{-1}$ was observed on both thiosulfate and tetrathionate. During chemolithotrophic growth on thiosulfate, the thiosulfate was converted to tetrathionate almost in equivalents with respect to sulfur atoms (Fig. 2b), which was then converted entirely to sulfate, as evident from the concomitant lowering of pH of the spent medium.

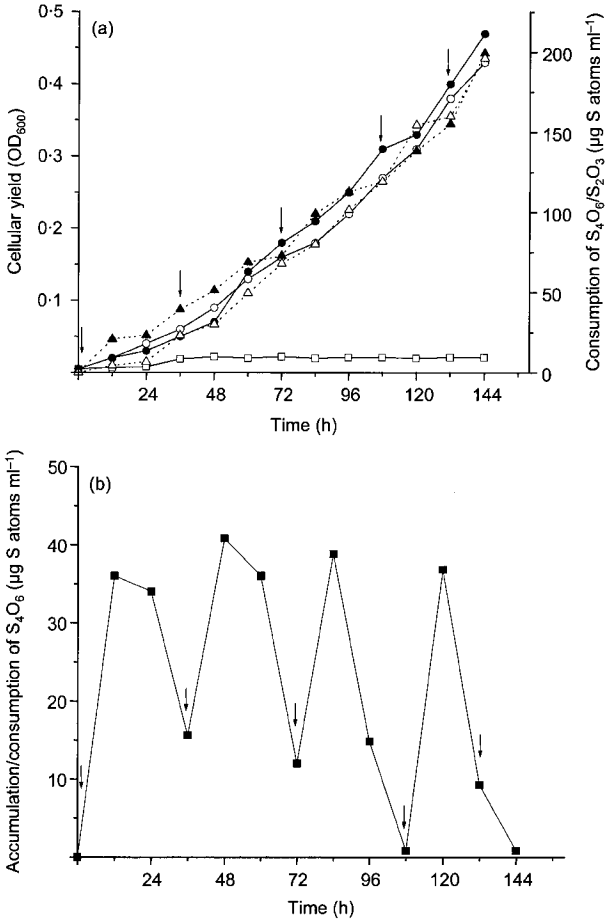

Fig. 2. Chemolithotrophic growth coupled with the oxidation of thiosulfate or tetrathionate by WT001^T using initial concentrations of 5 g thiosulfate or 3 g tetrathionate l^{-1} (both equivalent to 40 μg atoms sulfur ml^{-1}). Periodically, the same amounts of filter-sterilized sodium thiosulfate or potassium tetrathionate solutions were added four times to the respective cultures over a total incubation period of 144 h, which amounted to a total of 200 μg atoms sulfur ml^{-1} in both media. Cells were grown in MST or MSTr medium for 20 h and 1 % inocula were transferred to MS (control), MST or MSTr media. Cultures were incubated at 30 °C with shaking and sterile sodium carbonate solution (2 M) was added periodically to the flasks to adjust the pH of the medium to 7·4. (a) Solid and dotted lines respectively represent cellular yield and sulfur compound consumption for growth in control MS medium (□), MST medium (●,▲) and MSTr medium (○,△). Downward arrows indicate the times when filter-sterilized sodium thiosulfate (5 g l^{-1}) or potassium tetrathionate (3 g l^{-1}) solutions were added to the respective cultures. (b) Successive accumulation and consumption of tetrathionate over time by WT001^T during growth in MST medium. The tetrathionate concentration (in μg atoms sulfur ml^{-1}) in the spent medium is indicated. Downward arrows indicate times when filter-sterilized sodium thiosulfate (5 g l^{-1}) solution was added.

High-molecular-mass DNA was prepared as described by Ezaki *et al.* (1988) with minor modifications. No hybridization was detected (data not shown) when double-digested genomic DNA of all the isolates was transferred onto a nylon membrane and hybridized with DIG-labelled *soxT*, *soxYZ* or *soxBC* gene fragments, which were amplified by PCR from the thiosulfate- and tetrathionate-utilizing chemolithotrophic alphaproteobacterium *Pseudaminobacter salicylatoxidans* KCT001 (Mukhopadhyaya *et al.*, 2000; Deb *et al.*, 2004) using primers designed from the *sox* sequence of the bacterium (EMBL accession number AJ404005). This indicates the existence of a distinct *sox* genetic system in the new isolates that is unlikely to have any significant sequence similarity with the conserved *sox* gene cluster of alphaproteobacteria.

The new isolates were poorly distinguishable in terms of physiological and biochemical characteristics. Hence, comparison of AFLP patterns generated from *sox* PCR analysis was carried out, which also indicated that the isolates had a high degree of mutual genomic relatedness. Reproducible profiles were obtained for all isolates after gel-electrophoretic separation of the amplicons generated by PCR using different oligonucleotides bearing KCT001-specific sequences from discrete regions of its *sox* gene cluster as arbitrary primer pairs. The profile generated from a PCR with one such primer pair, $soxY_{\text{Forward}}$ and $soxA_{\text{Reverse}}$, is shown as Supplementary Fig. S1 in IJSEM Online. Though the pairwise similarity coefficients (S_{SM}) determined from the AFLP patterns for all 12 strains by manual as well as computer-aided qualitative and quantitative comparison were above 80 % (data not shown), the 12 strains could nonetheless be clustered into five genotypically related groups as follows: WT002 and WGT (represented by WGT); WPT, GT001, GT002, GT003 and GT004 (represented by WPT); 445b, 445c and 445d (represented by 445c); and WT001^T and 445a in single-strain groups. These five representative strains (Table 1) were selected for detailed polyphasic investigation.

16S rRNA genes were amplified by PCR with bacteria-specific primers f27 and r1492 (Gerhardt *et al.*, 1994) from DNA samples or boiled cell extracts of the isolates using a High fidelity PCR master kit (Roche Applied Science) according to the manufacturer's instructions. 16S rRNA gene sequences from PCR products were determined using universal primers (Gerhardt *et al.*, 1994), according to the manufacturer's specifications for *Taq* DNA polymerase-initiated cycle sequencing reactions using fluorescently labelled dideoxynucleotide terminators with an ABI PRISM 377 automated DNA sequencer. 16S rRNA gene sequences of the new isolates were compared against those in the EMBL, GenBank and DDBJ databases using FASTA (version 3.4t23, 18 March 2004; Pearson & Lipman, 1988). The 16S rRNA gene sequences of the five strains were very similar to each other, showing $\geqslant$99 % identity (Fig. 3). In contrast, the five strains had low levels (93–95 %) of 16S rRNA gene sequence similarity to species of the closest related genera, such as *Alcaligenes*, *Pigmentiphaga*, *Pelistega*, *Taylorella*, *Bordetella* and *Achromobacter*. Although the cluster of new sulfur-lithotrophic strains was phylogenetically closest to species of *Alcaligenes*, *Pigmentiphaga*, *Pelistega*, *Taylorella*, *Bordetella* and *Achromobacter*, the highest 16S rRNA gene sequence similarities found between the new isolates and any species of these genera were 95·3, 94·9, 94·9, 94·6, 94·6 and 94·3 %, respectively. Multiple alignment of sequences was executed using CLUSTAL W (Thompson *et al.*, 1994). Evolutionary distances (expressed in estimated numbers of changes per 100 nucleotides) were calculated by pairwise comparison of the aligned sequences (Jukes & Cantor, 1969) by the DNADIST program. A consensus neighbour-joining tree (Saitou & Nei, 1987) was

Table 1. Strains included in this study

LMG, Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium; MTCC, Microbial Type Culture Collection, IMTECH, Chandigarh, India.

Strain	Reference/source
Tetrathiobacter kashmirensis	
WT001^T (=LMG 22695^T=MTCC 7002^T)	Novel soil isolate from Jammu and Kashmir, India
WGT (=LMG 22696)	As above
445a	As above
445c	As above
WPT	As above
Alcaligenes defragrans LMG 18538^T	Foss *et al.* (1998)
Pigmentiphaga kullae LMG 21665^T	Blümel *et al.* (2001)
Pelistega europaea LMG 10982^T	Vandamme *et al.* (1998)
Taylorella equigenitalis LMG 6222^T	Sugimoto *et al.* (1983)
Achromobacter xylosoxidans MTCC 491^T	Yabuuchi *et al.* (1998)
Alcaligenes faecalis MTCC 126^T	Kersters & De Ley (1984)
Pseudaminobacter salicylatoxidans KCT001	Deb *et al.* (2004)

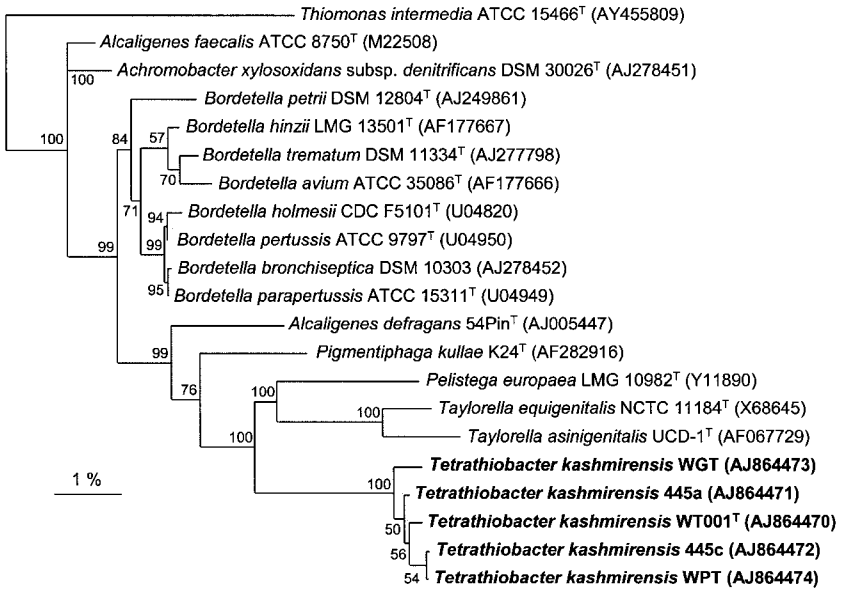

Fig. 3. Neighbour-joining tree showing the phylogenetic relationships between strains of *Tetrathiobacter kashmirensis* and selected strains from closely related taxa based on 16S rRNA gene sequences. The outgroup species *Thiomonas intermedia* belongs to another related betaproteobacterial cluster. Bar, 1 % nucleotide difference.

constructed following the majority rule and strict consensus out of 100 phylogenetic trees produced using the program NEIGHBOR in PHYLIP version 3.572c (Felsenstein, 1993). Bootstrap values (100 replicates) were calculated by the method of Felsenstein (1985) to validate the reproducibility of the branching pattern.

Phylogenetic analysis on the basis of 16S rRNA gene sequences (Fig. 3) clearly suggested that the five strains did not belong to and diverged from the classical branch of the family *Alcaligenaceae* (Yabuuchi *et al.*, 1998), comprising *Alcaligenes*, *Achromobacter* and *Bordetella* species. Instead, the new strains were phylogenetically closest to *Alcaligenes defragrans* (95·3 %), *Taylorella equigenitalis* (95·2 %), *Pelistega europaea* (94·9 %) and *Pigmentiphaga kullae* (94·8 %) (in each case, strain 445a had the highest similarity). In all the neighbour-joining trees constructed, the novel isolates formed a subtree with the two species of *Taylorella* and its closest relative, *Pelistega europaea*. *Alcaligenes defragrans* also clustered with the new strains, along with *Pigmentiphaga kullae*, and was found to be relatively distant from *Alcaligenes faecalis* ATCC 8750^T. From the high values of 16S rRNA gene sequence similarity ($\geqslant$99 %) observed between the new chemolithotrophic strains, together with their unequivocally low similarity to species of all the closest genera, it can be concluded that WT001^T, WGT, WPT, 445a and 445c form a taxonomically coherent assemblage and represent a unique phylogenetic lineage. The stability of the cluster is also reflected in its bootstrap value of 100 %, which signifies that the five isolates form a consolidated and homogeneous phylogenetic group.

5S rRNA gene sequence analysis has previously demonstrated that the root of the '*Betaproteobacteria*' is apparently located near the phylogenetic branch consisting of the strictly chemolithoautotrophic species now classified under the '*Gammaproteobacteria*' (Lane *et al.*, 1985). In our present study, however, the type strains of *Alcaligenes defragrans*, *Alcaligenes faecalis*, *Achromobacter xylosoxidans*, *Pelistega europaea*, *Taylorella equigenitalis* and *Pigmentiphaga kullae* tested negative for both oxidation and utilization of thiosulfate and tetrathionate, while 16S rRNA gene sequence-based phylogenetic analysis indicated that the cluster formed by the new chemolithotrophic isolates is distantly related to the sulfur lithotrophs of both the β1 ($<$90 % similarity) and γ ($<$86 % similarity) groups of the *Proteobacteria*. Preliminary studies showed that thiosulfate oxidation in the new isolates involved conversion of thiosulfate to tetrathionate, followed by accumulation and eventual oxidation of the latter to sulfate (Fig. 2b). The biochemical mechanism of thiosulfate as well as tetrathionate oxidation in the new isolates is likely to be similar to that employed by the beta- and gammaproteobacteria, following the tetrathionate intermediate (S_4I) pathway (Kelly, 1989; Kelly *et al.*, 1997). Most sulfur-oxidizing beta- and gammaproteobacterial species are obligately chemolithoautotrophic and/or extremophilic and hence are not amenable to genetic studies. The novel soil isolates reported here, being fast-growing, nutritionally versatile, mesophilic, neutrophilic, tetrathionate-oxidizing facultatively chemolithotrophic betaproteobacteria, may well fit the bill and provide a system of choice for the investigation of the S_4I pathway of thiosulfate oxidation as well as dissimilatory tetrathionate oxidation at the molecular level.

To determine the genomic relatedness of the new isolates, dot-blot hybridization experiments were carried out with DIG-labelled DNA as described previously (Labrenz *et al.*, 2000) using the detection kit from Roche Applied Sciences following the manufacturer's instructions. Colorimetric quantification of dot intensities was done using the Molecular Analyst software (Bio-Rad) by determining mean pixel densities in equal-sized circles. Genomic DNA probes were

prepared from the new isolates and a 100- to 1000-fold excess of the probe was used in hybridizing against frames of three to five target DNAs taken in quantitative triplicates of 25, 50 and 100 ng at a time. The hybridization temperature was 60 °C and the membranes were washed under highly stringent conditions (twice with 2 × SSC/0·1 % SDS at room temperature for 10 min; once with 0·1 × SSC/0·1 % SDS at 68 °C for 15 min). A high level of genomic relatedness (DNA–DNA hybridization values greater than or equal to 85 %) was observed between the new isolates, while the representative strain WT001^T exhibited uniformly low levels of DNA–DNA binding (between 10 and 15 %) with all the phylogenetically closest species.

In view of the high physiological, biochemical, phylogenetic and genetic similarities among the new isolates, fatty acid compositions were determined for WT001^T and WGT, which exhibited identical fatty acid profiles (no qualitative difference and $<0{\cdot}5$ % quantitative variation). After an incubation period of 24 h at 30 °C on LB agar, a loopful of well-grown cells was harvested and the preparation, separation and identification of fatty acids were performed using the Sherlock Microbial Identification System (Microbial ID, Inc.) at the DSMZ (Braunschweig, Germany). The fatty acids detected in strain WT001^T are listed in Table 2. Members of the *Alcaligenaceae* characteristically have large amounts of 16 : 0 and 17 : 0 cyclo and 14 : 0 3-OH fatty

Table 2. Fatty acid profile of *Tetrathiobacter kashmirensis* gen. nov., sp. nov. in comparison with related taxa

Taxa: 1, *Tetrathiobacter kashmirensis* WT001^T (data from this study); 2, range of values for strains of *Alcaligenes faecalis* (data from Dees & Moss, 1975) and *Alcaligenes defragrans* (Foss *et al.*, 1998); 3, *Achromobacter xylosoxidans* [range adapted from Dees & Moss (1978) and Holmes *et al.* (1993)]; 4, *Taylorella equigenitalis* (Vandamme *et al.*, 1998); 5, *Pelistega europaea* (Vandamme *et al.*, 1998); 6, *Pigmentiphaga kullae* (Blümel *et al.*, 2001). tr, Trace amount (<1 %) (for all species except *Tetrathiobacter kashmirensis*); −, not detected.

Fatty acid	1	2	3	4	5		6
					Cluster I	Cluster II	
12 : 0	3·12	0–2	−	−	4·4	4·4	−
Unknown (ECL 13·957)	0·24	−	−	−	−	−	−
14 : 0	0·18	4–8	⩽4	tr	9·7	10·8	4
Summed feature 2*	10·13	−	−	10·7†	12·7†	12·4†	4·6†
14 : 0 3-OH	−	7–12	2–7	−	−	−	−
Summed feature 3	28·02	−	−	−	−	−	−
16 : 1ω7*c*	−	−	−	tr	21·7	41·4	−
16 : 0	21·69	22–40	21–35	36·8	15·7	25·2	39·9
17 : 0 cyclo	3·53	4–28	22–33	−	−	−	21·9
17 : 0	0·27	4–28	⩽6	−	−	−	−
16 : 0 3-OH	0·80	−	−	tr	1·2	1·2	−
18 : 1ω7*c*	28·31	11–32	63–69	−‡	−‡	−‡	−‡
Summed feature 7	−	−	−	41·6	27·8	1·5	9·8
18 : 0	1·86	4–6	2–10	6·8	tr	tr	−
19 : 0 cyclo ω8*c*	1·32	−	−	−	−	−	12·2
19 : 0 10-methyl	0·18	−	−	2·2	tr	−	−
20 : 1ω7*c*	0·34	−	−	−	−	−	−
10 : 0 3-OH	−	−	−	−	−	−	2·9
16 : 0 2-OH	−	−	⩽3	−	−	−	4·8
Summed feature 1	−	−	−	−	tr	2·7	−
15 : 0	−	<2	−	−	−	−	−
16 : 1	−	5–24	−	−	−	−	−
12 : 0 2-OH	−	0–5	<5	−	−	−	−
14 : 0 2-OH	−	⩽5	⩽7	−	−	−	−
18 : 1 2-OH	−	−	2–12	−	−	−	−
19 : 0	−	−	0–2	−	−	−	−
19 : 0 cyclo	−	−	<5	−	−	−	−
16 : 1ω5*c*	−	−	−	−	5·2	tr	−

*Summed features consist of the following fatty acids, which could not be separated. Summed feature 1, 14 : 1ω5*c* and/or ω5*t*; summed feature 2, 14 : 0 3-OH and/or 16 : 1 iso I; summed feature 3, 16 : 1ω7*c* and/or 15 : 0 iso 2-OH; summed feature 7, 18 : 1ω7*c*, ω9*t* and/or ω12*t*.

†Referred to as summed feature 3 in these studies.

‡See summed feature 7.

acids (Foss *et al.*, 1998; Blümel *et al.*, 2001), and the newly isolated strains synthesized significant amounts of these (Table 2). However, the new lithotrophs contained less than 4 % 17 : 0 cyclo, though 16 : 0 and 14 : 0 3-OH (as summed feature 2) were present in larger amounts (Table 2). The new strains are distinguished from their phylogenetic relatives by the presence of trace amounts of 20 : 1ω7*c* and high concentrations of summed features 2 and 3 (Table 2).

Genomic DNA was hydrolysed with P1 nuclease and the nucleotides were dephosphorylated with bovine alkaline phosphatase. From the resulting deoxyribonucleosides, G+C contents (mol%) were analysed using HPLC (Mesbah *et al.*, 1989). The new isolates had G+C contents ranging between 54·0 and 55·2 mol%, which are distinct from all members of *Alcaligenaceae* except *Alcaligenes faecalis*, which has a G+C content of 56–59 mol%. Phylogenetically, however, in a comparison with all other related genera, *Alcaligenes faecalis* was only distantly related to the new strains.

Comparison of all available phenotypic and chemotaxonomic characteristics of the five new isolates with their nearest phylogenetic relatives and numerical analysis of the data (UPGMA phenogram available as Supplementary Fig. S2 in IJSEM Online) corroborated the phylogenetic distinctiveness of the new cluster. Key physiological and biochemical characters that distinguish the new isolates from phylogenetically related species are shown in Table 3. It is evident from these findings that the new mesophilic and neutrophilic, facultatively chemolithotrophic, tetrathionate-oxidizing isolates constitute a single phylogenetic and genomic group that merits generic treatment. They are hence classified in a new genus comprising a single species, for which we propose the name *Tetrathiobacter kashmirensis* gen. nov., sp. nov.

Description of *Tetrathiobacter* gen. nov.

Tetrathiobacter (Tet.ra.thi.o.bac′ter. Gr. adj. *tetra* four; Gr. n. *thium* sulfur; N.L. masc. n. *bacter* from Gr. neut. n. *bakterion* a rod; N.L. masc. n. *Tetrathiobacter* a tetrathionate-oxidizing bacterium).

Gram-negative, non-flagellated, oval to coccoid-shaped bacteria occurring singly or in pairs, chains, branched chains or clusters. Capsular coverings are present around cells of some strains. Cells are approximately 1·0–1·8 μm long and 0·6–1·5 μm broad. Colonies are circular, entire, smooth, opaque and creamy white and develop central dark mounds that sometimes turn rusty red. Growth is observed between 10 and 42 °C and between pH 4·5 and 8·5. Oxidase, catalase and urease activities are observed, but not gelatinase activity. Facultatively chemolithoautotrophic organisms that use thiosulfate or tetrathionate as electron and energy source. Thiocyanate, soluble sulfides, elemental sulfur, sulfite and arsenite are not utilized as chemolithotrophic substrates. The DNA G+C content, as determined by HPLC, is 54–55·2 mol%. The genus belongs to the '*Betaproteobacteria*' and is phylogenetically closest to *Alcaligenes defragrans*, *Taylorella equigenitalis*, *Pelistega europaea* and *Pigmentiphaga kullae*. The type species is *Tetrathiobacter kashmirensis*. Until further taxa are identified, the genus comprises a single species.

Table 3. Phenotypic characteristics useful in differentiating *Tetrathiobacter kashmirensis* from related taxa

Species: 1, *Tetrathiobacter kashmirensis*; 2, *Alcaligenes faecalis* (data from Kersters & De Ley, 1984); 3, *Alcaligenes defragrans* (Foss *et al.*, 1998); 4, *Achromobacter xylosoxidans* (Yabuuchi *et al.*, 1998); 5, *Taylorella equigenitalis* (Sugimoto *et al.*, 1983); 6, *Pelistega europaea* (Vandamme *et al.*, 1998); 7, *Pigmentiphaga kullae* (Blümel *et al.*, 2001). +, Positive; −, negative; d, strain-dependent; NA, data not available.

Characteristics	1	2	3	4	5	6	7
Assimilation of single carbon compounds:							
D-Glucose, D-xylose	+	−	−	+	−	−	−
D-Fructose	+	−	−	d	−	−	−
L-Arabinose, D-galactose, lactose	+	−	−	−	−	−	−
Aesculin	−	−	NA	+	−	−	NA
Adipate	−	−	−	+	−	−	+
Phenylacetate	−	d	NA	+	−	−	−
Urease activity	+	−	−	−	−	d	NA
Growth at 42 °C	+	+	−	+	−	+	+
Chemolithotrophic growth on S	+	−	−	−	−	−	−
Reduction of nitrate	+	−	+	+	−	−	NA
G+C content (mol%)	54–55·2	56–59	67	66–69·8	36–37	42–43	68·5
Diagnostic fatty acid(s)*	20 : 1ω7*c*, SF3	15 : 0, 16 : 1	15 : 0, 16 : 1	18 : 1 2-OH, 19 : 0 cyclo, 19 : 0	−	16 : 1ω5*c*, SF1	10 : 0 3-OH

*SF, Summed feature (for details see Table 2).

Description of *Tetrathiobacter kashmirensis* sp. nov.

Tetrathiobacter kashmirensis (kash.mir.en′sis. N.L. masc. adj. *kashmirensis* of Kashmir, after the name of the province from where the original strains of the species were isolated.)

Can reduce nitrate to nitrite, utilize ammonium salts and urea but not glutamate, aspartate or nitrate as the nitrogen source and requires no yeast extract or vitamins for growth. Can grow in 1 M NaCl. The following sole carbon compounds are assimilated chemo-organotrophically: succinate, citrate, malate, acetate, D-fructose, D-glucose, D-xylose, D-lactose, D-galactose, L-glutamate, L-lysine, L-arabinose and L-cysteine. None of following compounds are utilized: benzoate, sucrose, glycerol, L-histidine, L-leucine, L-isoleucine, *myo*-inositol, oxalate, mandelate, D-mannitol, D-raffinose, DL-lactate, L-tyrosine, L-threonine and L-serine. Utilization of D-mannose, D-maltose, L-arginine, L-tryptophan and L-aspartic acid as the sole carbon source is strain dependent. The following fatty acid components are present: 12 : 0, an unknown fatty acid having an equivalent chain-length of 13·957, 14 : 0, summed feature 2, summed feature 3, 16 : 0, 17 : 0 cyclo, 17 : 0, 16 : 0 3-OH, 18 : 1ω7*c*, 18 : 0, 19 : 0 cyclo ω8*c*, 19 : 0 10-methyl and 20 : 1ω7*c*.

The type strain is WT001^T (= LMG 22695^T = MTCC 7002^T) and its characteristics are as described above for the species. In addition, the type strain utilizes D-mannose, L-tryptophan and L-aspartic acid but not maltose or L-arginine as single heterotrophic substrates. The G+C content of the type strain is 55·1 mol% (by HPLC). All strains of the species identified to date have been isolated from bulk soils of a temperate orchard in Srinagar, Jammu and Kashmir, India.

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