Thiomonas islandica sp. nov., a moderately thermophilic, hydrogen- and sulfur-oxidizing betaproteobacterium isolated from a hot spring

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A novel, hydrogen- and sulfur-oxidizing bacterium, designated strain 6C^T, was isolated from a hot spring in Graendalur, south-western Iceland. Cells of this organism were Gram-reaction-negative, rod-shaped and motile. The strain grew aerobically and was capable of chemolithotrophic growth on thiosulfate and hydrogen, heterotrophic growth on pyruvate, oxalate, acetate and on glutamate in the presence of yeast extract and mixotrophic growth on several organic compounds, thiosulfate and/or hydrogen. During growth on thiosulfate, the final product was sulfate, resulting in a drop in pH from 6.8 to 2.7. Heterotrophic growth on pyruvate was observed at pH 4–7 (optimum pH 4) and 35–50 °C (optimum 45 °C). The DNA G + C content was 65.2 mol%. As determined by 16S rRNA gene sequence analysis, strain 6C^T represents a distinct species belonging to the class *Betaproteobacteria* and is most closely related to *Thiomonas intermedia* DSM 18155^T and *Thiomonas perometabolis* DSM 18570^T. DNA–DNA hybridization between strain 6C^T and *Thiomonas intermedia* DSM 18155^T and *Thiomonas perometabolis* DSM 18570^T gave relatedness values below 32%. These results, together with physiological characteristics, showed that strain 6C^T represents a novel species within the genus *Thiomonas*, for which the name *Thiomonas islandica* sp. nov. is proposed. The type strain is 6C^T (=DSM 21436^T =JCM 16107^T).

The genus *Thiomonas* was proposed by Moreira & Amils (1997) to accommodate four former species of the genus *Thiobacillus*: *Thiobacillus cuprinus*, *Thiobacillus intermedius*, *Thiobacillus perometabolis* and *Thiobacillus thermosulfata*. This classification was based on their ability to grow chemolithotrophically on reduced sulfur compounds, showing optimal growth under mixotrophic conditions with reduced sulfur compounds (or elemental sulfur) and various organic substrates (Moreira & Amils, 1997). Recently, two novel species have been assigned to the genus *Thiomonas*, *Thiomonas delicata* (Katayama-Fujimura *et al.*, 2006) and *Thiomonas bhubaneswarensis* (Panda *et al.*, 2009), as well as one species, ‘*Thiomonas arsenivorans*’, the name of which has not been validly published (Battaglia-Brunet *et al.*, 2006). A suggestion was made by Kelly *et al.* (2007) that the phylogenetic relationship between *Thiomonas cuprina* (formerly *Thiobacillus cuprinus*) and other *Thiomonas* species should be reassessed. Using full 16S rRNA gene sequence analysis, they revealed that *Thiomonas cuprina*,

Thiomonas delicata and ‘*Thiomonas arsenivorans*’ differ in only three nucleotides and are, for that reason, indistinguishable, but are still considered three separate species based on their physiological characteristics.

The first species of the genus *Thiomonas* were isolated from soils (London, 1963; London & Rittenberg, 1967). More recently, isolations have been made from mining sites where metals are often present in high concentrations (Dennison *et al.*, 2001; Coupland *et al.*, 2004). *Thiomonas cuprina* was originally isolated from a uranium mine and is capable of utilizing various sulfide ores (Huber & Stetter, 1990), whereas other species of the genus *Thiomonas* are able to oxidize iron (Coupland *et al.*, 2004; Johnson & Hallberg, 2005) and arsenite (Battaglia-Brunet *et al.*, 2002; Bruneel *et al.*, 2003). *Thiomonas thermosulfata* (Shooner *et al.*, 1996) and *Thiomonas bhubaneswarensis* (Panda *et al.*, 2009) are moderate thermophiles and were isolated from sewage sludge and hot-spring sediment, respectively.

In this study, we describe a novel sulfur- and hydrogen-oxidizing bacterium that was isolated from a hot-spring in Iceland and propose that it represents a novel species of the genus *Thiomonas*.

Samples of clayish liquid collected from an off-flow of a hot spring (45 °C, pH 6.6) were inoculated (10%, v/v) into

Abbreviations: HOX, hydrogen-oxidizing; SOX, sulfur-oxidizing.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 6C^T is EU625663.

Two supplementary figures are available with the online version of this paper.

DSM81 medium, a mineral medium for chemolithotrophic growth, with the addition of 0.1 mg resazurin oxygen indicator l^{-1} . The composition of the medium was as follows ($g\ l^{-1}$): KH_2PO_4 (2.3), $Na_2HPO_4 \cdot 2H_2O$ (2.9), NH_4Cl (1.0), $MgSO_4 \cdot 7H_2O$ (0.5), $NaHCO_3$ (0.5), $CaCl_2 \cdot 2H_2O$ (0.01) and ferric ammonium citrate (0.05). A trace element solution was added ($5\ ml\ l^{-1}$) with the following composition ($g\ l^{-1}$): $ZnSO_4 \cdot 7H_2O$ (0.1), $MnCl_2 \cdot 4H_2O$ (0.03), H_3BO_3 (0.3), $CoCl_2 \cdot 6H_2O$ (0.2), $CuCl_2 \cdot 2H_2O$ (0.01), $NiCl_2 \cdot 6H_2O$ (0.02) and $Na_2MoO_4 \cdot 2H_2O$ (0.03). The cultivation bottles used contained atmospheric air but were pressurized with 101 kPa (1 atm) of a H_2 and CO_2 mixture (80:20, v/v) resulting in a final gas phase of 202 kPa (2 atm) consisting of $H_2/CO_2/N_2/O_2$ (40:10:40:10, v/v); the gas was filter-sterilized. The 120 ml bottles contained 20 ml medium. Strain 6C^T was isolated by using repeated enrichments (10% inoculation, v/v) and end-point serial dilution. A pure culture of strain 6C^T was obtained by plating medium from the last bottle in the dilution series on a solid medium. The solid medium was prepared by adding agar ($15\ g\ l^{-1}$) to liquid DSM81 medium. After sterilization, the medium was poured into plates, inoculated and incubated in an airtight jar containing the same gas composition as before. The resulting four greyish round colonies (~1 mm diameter) were reinoculated into liquid medium and 16S rRNA gene sequence analysis was performed. Phase-contrast microscopy and scanning electron microscopy (SEM) were used to examine purity of the cultures and cell morphology. For SEM, the cells were fixed with 2% glutaraldehyde and dried in ethanol prior to critical-point drying. Before examination, the sample was coated with gold. Gram-staining was assayed as described by Smibert & Krieg (1994) and motility was confirmed by growing the isolate in liquid DSM81 medium under heterotrophic conditions with pyruvate and microscopic examination in a hanging-drop preparation. Cells of strain 6C^T were Gram reaction-negative, singly occurring, motile rods, 1.7 μm long and 0.4 μm wide (see Supplementary Fig. S1, available in IJSEM Online).

Growth characteristics were determined from optical density. All experiments were done in duplicate. Experiments to determine the optimum pH and temperature for growth were performed using the same medium (DSM81) under heterotrophic conditions with 20 mM pyruvate. The experimental bottles were prepared as before and the pH was adjusted accordingly. For determination of the optimum temperature for growth, the isolate was grown at pH 6.8 and at 20–75 °C. For determination of the optimum pH for growth, the isolate was grown at 50 °C and at pH 3–10. The strain grew at 35–50 °C with optimum growth at 45 °C. No growth was observed at 30 °C or below or at 55 °C and higher. The strain was able to grow at pH 4–7 with an optimum of pH 4.

The ability of strain 6C^T to utilize various substrates heterotrophically was tested by growing the isolate under aerobic conditions at 50 °C in 5 ml liquid DSM81 medium supplemented with filter-sterilized substrates (final concentration 20 mM or 2 $g\ l^{-1}$ in the case of yeast extract,

beef extract, peptone and tryptone). The samples were grown in 23 ml bottles closed with butyl rubber stoppers and aluminium caps. Similar experiments were also done with *Thiomonas perometabolis* DSM 18570^T and *Thiomonas intermedia* DSM 18155^T. Substrate utilization was tested in the presence and absence of yeast extract for a cultivation period of 5 days. Growth was monitored by measuring OD₆₀₀. Growth was observed on pyruvate, oxalate, acetate

Table 1. Characteristics of strain 6C^T and type strains of other closely related species of the genus *Thiomonas*

Strain: 1, *Thiomonas perometabolis* DSM 18570^T; 2, *Thiomonas intermedia* DSM 18155^T. Data for heterotrophic and chemolithotrophic growth was determined in this study. Data on temperature- and pH-dependent growth characteristics as well as DNA G+C content for *Thiomonas perometabolis* DSM 18570^T and *Thiomonas intermedia* DSM 18155^T are from Katayama-Fujimura *et al.* (1983, 1984). All strains were positive for chemolithotrophic growth on sulfur and thiosulfate and negative for heterotrophic growth on formate, propionate, butyrate and crotonate. w, Weakly positive.

Characteristic	6C ^T	1	2
Heterotrophic growth on:			
Acetate	+	w	–
Pyruvate	+	w	–
Oxalate	+	–	w
Lactate	–	–	+
Malate	–	+	+
Succinate	–	+	+
Aspartate	–	+	+
Alanine	–	+	+
Glycine	–	+	–
Serine	–	+	–
Threonine	–	+	–
Histidine	–	+	w
Glutamate	+*	+	+
Xylose	–	+	+
Glucose	–	+	+
Mannose	–	+	+
Fructose	–	w	+
Sucrose	–	w	+
Galactose	–	w	–
Yeast extract	–	+	+
Peptone	–	+	+
Bacto tryptone	–	+	+
Beef extract	–	+	+
Casamino acids	–	+	+
Chemolithotrophic growth on H_2	+	–	–
Growth temperature (°C)			
Range	35–50	15–42	15–37
Optimum	45	35–37	30–35
Growth pH			
Range	4.0–7.0	5.0–7.0	5.0–7.5
Optimum	4.0	5.5–6.0	5.5–6.0
DNA G+C content (mol%)	65.2	65–66	65–67

*Positive in the presence of yeast extract.

and on glutamate in the presence of yeast extract (Table 1). No growth was observed on many other compounds, as described in the species description.

Special growth experiments were done under hydrogen-oxidizing (HOX), sulfur-oxidizing (SOX) and mixotrophic conditions on pyruvate, hydrogen and thiosulfate. In all cases, growth experiments were done in 120 ml serum bottles with a gas volume of 100 ml and medium (DSM81) volume of 20 ml. Growth experiments under HOX conditions were done under conditions used during the isolation of the strain. Hydrogen levels were analysed according to Örylgsson & Baldursson (2007). After a lag phase of more than 20 h, hydrogen uptake was detected and growth was observed under HOX conditions (Fig. 1a). During the active-growth phase, the strain utilized hydrogen from the gas phase at a rate of 0.17 mmol H_2

$\text{l}^{-1} \text{ h}^{-1}$ and the concentration of hydrogen dropped from 22.3 to 8.0 mmol l^{-1} . The pH at the end of the experimental time was 6.5. Growth experiments on thiosulfate (SOX conditions) were performed at 50°C using DSM81 medium supplemented with thiosulfate (40 mM). The gas phase had a pressure of 202 kPa (2 atm) and consisted of $\text{N}_2/\text{CO}_2/\text{O}_2$ (80:10:10, v/v). Growth was measured by monitoring OD_{600} , the levels of sulfate formed and drops in pH in the medium. The amount of sulfate formed was determined according to Tabatabai (1974). A similar lag phase was observed, as under HOX conditions (Fig. 1b). Growth was observed under SOX conditions but the increase in biomass (OD_{600}) was much less pronounced than that of cultures grown under HOX conditions. The end product of thiosulfate oxidation was sulphate, which accumulated to 14.6 mM, with a drop in pH to 2.7 at the end of the cultivation time.

Growth experiments under mixotrophic conditions were performed using hydrogen, thiosulfate (40 mM) and pyruvate (20 mM) as energy sources and carbon dioxide as the carbon source. Similar growth characteristics were observed to those under SOX conditions and the hydrogen oxidation rate ($0.09 \text{ mmol H}_2 \text{ l}^{-1} \text{ h}^{-1}$ from the gas phase) was far slower compared with those observed under HOX conditions only. The final sulfate concentration was similar to that for SOX conditions only, being 13.4 mM at the end of the experimental time with a resulting pH of 2.6 (Fig. 1c). Additional experiments on the physiological characteristics of the strain under heterotrophic and mixotrophic conditions are presented as supplementary data (see Supplementary Fig. S2a–c).

It has been known for a long time that hydrogen oxidizers are present in various environments (Kaserer, 1906; Ruhland, 1924; Schatz & Bovell, 1952), but relatively few data have been reported on the hydrogen uptake rates of these bacteria. In the few experiments reported, the culture conditions used were different and are, therefore, not comparable to the data presented in this study. Previous studies in our laboratory have focused on the hydrogen uptake rates of strains *Cupriavidus necator* (previously *Wautersia eutropha*) DSM 428, *Hydrogenophilus hirschii* DSM 11420^T, *Hydrogenobacter hydrogenophilus* DSM 2913^T and *Sulfurihydrogenibium azoreense* DSM 15241^T (data not shown). Compared to these strains, strain 6C^T had slightly lower hydrogen oxidation rates. During the growth period of strain 6C^T, oxygen was depleted from the culture bottles, which could be observed visibly by the resazurin indicator. The depletion of oxygen and the drop in pH to 2.7 during both chemolithotrophic (Fig. 1b) and mixotrophic (Fig. 1c) conditions could explain the inability of the strain to utilize the hydrogen added to the bottles completely. This will be addressed in further physiological studies of the strain.

Basic phenotypic characteristics suggest that strain 6C^T belongs to the genus *Thiomonas*, being Gram-negative, rod-shaped and capable of chemolithotrophic (on sulfur

Fig. 1. Growth of strain 6C^T during: HOX conditions (a), SOX conditions (b) and mixotrophic conditions on hydrogen, thiosulfate and pyruvate (c). Plots represent hydrogen level (▲), growth (○) and sulfate level (□).

compounds) and mixotrophic growth. The isolate is, however, phenotypically different in several aspects from all other previously described species of the genus *Thiomonas*. The major difference is the ability of strain 6C^T to use hydrogen as an electron donor and energy source. Until now, no other species of the genus *Thiomonas* have been shown to possess this ability, but the species formerly known as *Thiobacillus ferrooxidans* (now *Acidithiobacillus ferrooxidans*) (Kelly & Wood, 2000) is able to oxidize hydrogen (Drobner *et al.*, 1990). The hydrogen oxidation rates of this species have not, to our knowledge, been determined. It is, however, well known that many sulfur-oxidizing bacteria are capable of hydrogen oxidation (Aragno, 1992). Phenotypic characteristics that differentiate strain 6C^T from its closest phylogenetic neighbours, *Thiomonas intermedia* DSM 18155^T and *Thiomonas perometabolis* DSM 18570^T are given in Table 1. Strain 6C^T has a higher optimum temperature (45 °C compared with 30–35 °C) (Katayama-Fujimura *et al.*, 1984) and a lower optimum pH (pH 4.0 compared with pH 5.5–6.0) for growth compared with *Thiomonas intermedia* DSM 18155^T and *Thiomonas perometabolis* DSM 18570^T (Katayama-Fujimura *et al.*, 1984). *Thiomonas intermedia* DSM 18155^T and *Thiomonas perometabolis* DSM 18570^T were able to utilize a significantly larger number of carbon substrates than strain 6C^T, which could only grow on acetate, pyruvate, oxalate or on glutamate with the addition of yeast extract (Table 1). There were, however, several discrepancies concerning earlier reports on the carbon degradation capabilities of *Thiomonas intermedia* DSM 18155^T and *Thiomonas perometabolis* DSM 18570^T. In the present investigation, *Thiomonas perometabolis* DSM 18570^T showed weak growth on acetate, pyruvate, fructose, sucrose and galactose, which was not observed by Katayama-Fujimura *et al.* (1983). *Thiomonas intermedia* DSM 18155^T has been reported to be negative for growth on sucrose and positive for growth on serine, contrary to our findings (Table 1). Strain 6C^T is also phenotypically different and phylogenetically more distant from other species of the genus *Thiomonas*. Most

clearly, the temperature optima are different for all other strains (Huber & Stetter, 1990; Moreira & Amils, 1997; Kelly & Wood, 2005; Kelly *et al.*, 2007; Panda *et al.*, 2009) compared with strain 6C^T. All other strains are mesophiles, except for *Thiomonas thermosulfata* ATCC 51520^T, and show much lower temperature optima (20–37 °C). *Thiomonas thermosulfata* ATCC 51520^T has a higher temperature optimum (50.0–52.5 °C) and can grow at up to 65 °C (Shooner *et al.*, 1996). *Thiomonas thermosulfata* ATCC 51520^T is also distinguished from strain 6C^T by a lower DNA G + C content (61 mol%) and by its inability to degrade acetate and pyruvate (Shooner *et al.*, 1996).

For 16S rRNA gene sequence analysis, 1366 unambiguous bases were used. PCR was performed using primers F9 and R1544 (Skirnisdottir *et al.*, 2000), specific for bacterial genes, to amplify 16S rRNA genes from DNA. The PCR products were sequenced with universal 16S rRNA primers F9, F515, F1392, R357, F1195 and R1544 by using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). Subsequently, the DNA was analysed using a 3730 DNA analyser (Applied Biosystems). The nucleotide sequence was displayed and analysed with Sequencher sequence assembly software (Gene Code Corporation) (Skirnisdottir *et al.*, 2000). Sequences from the 16S rRNA gene sequence analysis were compared to sequences in the NCBI database using the nucleotide-nucleotide BLAST (BLASTN) tool. The Ribosomal Database Project was also used to obtain sequences of related strains. The most similar sequences obtained from the databases were aligned with the sequencing results in the programs BioEdit (Hall, 1999) and CLUSTAL_X (Thompson *et al.*, 1997) where final alignments were performed to generate phylogenetic trees using the TREECON program (Van de Peer & De Wachter, 1997); this was also used to view the trees. A diverse group of proteobacteria were used as reference species and *Pseudomonas aeruginosa* strain X06684 was used as an outgroup. Examination of the 16S rRNA gene sequences revealed that strain 6C^T showed 97.5 and 97.4 % 16S rRNA gene sequence similarity to *Thiomonas intermedia* DSM 18155^T and *Thiomonas perometabolis* DSM 18570^T,

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequence comparisons showing the relationship between strain 6C^T and related type strains. The tree was reconstructed using the neighbour-joining method, following distance analysis calculated by using the Jukes and Cantor method (Jukes & Cantor, 1969). Bootstrap values greater than 50 % are shown at branching points. *Pseudomonas aeruginosa* ATCC 10145^T was used as an outgroup. Bar, 0.1 substitutions per nucleotide site.

respectively, with less than 97% similarity to strains of other species of the genus *Thiomonas*. The results of the complete 16S rRNA gene sequence analysis are presented as a phylogenetic tree (Fig. 2) showing the position of strain 6C^T within the genus *Thiomonas* and other closely related species. Together, strain 6C^T, *Thiomonas thermosulfata* ATCC 51520^T, *Thiomonas bhubaneswarensis* DSM 18181^T, *Thiomonas perometabolis* DSM 18570^T and *Thiomonas intermedia* DSM 18155^T form a subcluster within the genus that branches off above the other two type strains of species of *Thiomonas*, *Thiomonas cuprina* DSM 5495^T and *Thiomonas delicata* DSM 17897^T. Intracluster 16S rRNA gene sequence similarity values for strain 6C^T within the genus *Thiomonas* ranged from 94.4 to 97.5%. The DNA G+C content was analysed by DSMZ using HPLC according to Mesbah *et al.* (1989) and was 65.2 mol%, which is similar to that of *Thiomonas intermedia* DSM 18155^T (64.9 mol%) and *Thiomonas perometabolis* DSM 18570^T (65.0 mol%) (Katayama-Fujimura *et al.*, 1984).

Genomic DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out, as described by De Ley *et al.* (1970) and under consideration of the modifications described by Huß *et al.* (1983), using a Cary 100 Bio UV-Vis spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with an *in-situ* temperature probe (Varian). The results of DNA–DNA hybridization between strain 6C^T and *Thiomonas perometabolis* DSM 18570^T and *Thiomonas intermedia* DSM 18155^T were 30.6% (31.7%) and 26.8% (21.1%), respectively (values in parentheses are results of measurements in duplicate). Therefore, considering 70% DNA–DNA relatedness as a cut-off point for species delineation (Wayne *et al.*, 1987), strain 6C^T should be regarded as representative of a novel species of the genus *Thiomonas*.

The phylogenetic relationship between strain 6C^T and other species of the genus *Thiomonas* and the differences in their physiological properties suggest that strain 6C^T represents a novel species, for which the name *Thiomonas islandica* sp. nov. is proposed.

Description of *Thiomonas islandica* sp. nov.

Thiomonas islandica (is.lan'di.ca. N.L. fem. adj. *islandica* Icelandic, describing the place of first isolation).

Cells are straight rods, 1.7 × 0.4 µm, occurring singly, motile and Gram-reaction-negative. Growth occurs under aerobic conditions. Colonies grown on solid DSM81 medium are greyish, round and ~1 mm in diameter. Moderately thermophilic with an optimum temperature for growth of 45 °C. No growth occurs at 30 °C or below or at 55 °C and higher. Optimum pH for growth under heterotrophic conditions is pH 4. Shows heterotrophic, chemolithotrophic and mixotrophic growth properties. Shows heterotrophic growth on acetate, oxalate, pyruvate

and on glutamate in the presence of yeast extract. No growth occurs on propionate, butyrate, formate, crotonate, lactate, malate, succinate, aspartate, alanine, glycine, serine, threonine, histidine, xylose, glucose, mannose, fructose, galactose, sucrose, yeast extract, peptone, tryptone, beef extract or Casamino acids. The DNA G+C content of the type strain is 65.2 mol%. No growth factors are required.

The type strain, 6C^T (=DSM 21436^T =JCM 16107^T), was isolated from a clayish liquid sample of a hot spring in Graendalur, south-western Iceland.

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