Thioclava pacifica gen. nov., sp. nov., a novel facultatively autotrophic, marine, sulfur-oxidizing bacterium from a near-shore sulfidic hydrothermal area

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Strain TL 2T was isolated on mineral medium with thiosulfate from a near-shore sulfidic hydrothermal area in Matupi Harbour on the island of New Britain, Papua New Guinea. The cells varied from long filaments with swollen ends, often aggregated, to short rods, depending on the growth conditions. The bacterium was obligately aerobic and grew autotrophically with thiosulfate as energy source or heterotrophically with organic acids and sugars. In thiosulfate-limited continuous culture, \( \mu_{\text{max}} \) and \( Y_{\text{max}} \) for autotrophic growth were 0·1 h\(^{-1} \) and 3 g protein mol\(^{-1} \), respectively. From the various reduced sulfur compounds tested, only thiosulfate and sulfide supported active respiration. Inorganic carbon was assimilated via the Calvin cycle. Presence of the 'green'-type of form I RubisCO gene was detected. Growth was possible from 15 to 47 °C with an optimum at 35 °C, pH 6·5–8·5 with an optimum at pH 8·0, and between 10 and 90 g NaCl l\(^{-1} \) with an optimum at 35 g l\(^{-1} \). Phylogenetic analysis based on 16S rRNA and \( \text{cbbL} \) gene sequences demonstrated that strain TL 2T forms a separate lineage within the alpha-3 subdivision of the Proteobacteria, distantly related to the genera Rhodovulum and Rhodobacter. On the basis of these results, a novel genus and species, Thioclava pacifica gen. nov., sp. nov., is proposed to accommodate strain TL 2T (\( = \) DSM 10166\(^T \) = UNIQUEST 229\(^T \)).

INTRODUCTION

The ability to grow chemolithoautotrophically with sulfur compounds has been demonstrated in many facultative species that differ from classical thiobacilli (Kelly, 1989). Facultative species are especially interesting in their metabolic flexibility, allowing them to dominate in environments with low fluxes of both reduced inorganic and organic carbon compounds, insufficient to maintain substantial populations of highly specialized obligate autotrophs or obligate heterotrophs (Beudecker et al., 1982; Kuenen, 1989).

A near-shore sulfidic hydrothermal area in Matupi Harbour on New Britain (Papua New Guinea) is an example of an environment that can be beneficial for proliferation of facultatively autotrophic sulfur bacteria. There, a hot sulfide-containing volcanic seep mixes with cold sea water containing oxygen and organic compounds, originated from decaying shore vegetation (Ferguson & Lambert, 1972). However, the concentration of these compounds fluctuates significantly because of the active water dynamics, which do not favour domination of obligate species. Enumeration of different types of sulfur-oxidizing bacteria demonstrated the dominance of heterotrophic forms in most of the samples oxidizing thiosulfate either to sulfate or to tetrathionate (Sorokin, 1991). Among the tetrathionate-producing heterotrophs, a peculiar coccoid
morphotype was dominant and was described as *Cateno-coccus thiocyclus* (Sorokin, 1992; Sorokin et al., 1996). Among the ten sulfate-forming heterotrophic strains, one isolate, strain TL 2, was able to grow autotrophically. The physiological properties and taxonomic position of this novel bacterium are described in this paper.

**METHODS**

The sampling point was situated above a hot sulfidic seep at 3 m depth, where the water temperature was 40 °C. The maximum sulfide concentration during low tide was up to 0.8 mM and the salinity was 30 %. The sulfur-oxidizing population from this sample was first enriched and then serially diluted on mineral medium with thiosulfate and sea water. The culture grown from the greatest dilution was streaked onto solid medium of the same composition and single colonies were isolated. When inoculated in a liquid mineral medium, these colonies always resulted in the growth of two types of bacteria. One type was represented by a *Thiomicrospira*-like, obligately autotrophic, sulfur-oxidizer unable to grow heterotrophically. Another type, represented by a filamentous heterotroph, was a minor component in mineral medium but dominated under purely organotrophic or mixotrophic conditions. Despite differences in metabolism, separation of the two organisms was difficult, because they grew in a stable association and even formed fused colonies. Finally, after eight to ten sequential passages on purely organic medium with acetate, the heterotrophic member was isolated in pure culture and designated strain TL 2T.

The mineral medium for primary enrichment culture was prepared on the basis of filter-sterilized sea water supplemented with 10 mM thiosulfate and the pH-indicator bromothymol blue (from water-saturated solution, 1 ml 1⁻¹). For further batch cultivation, an artificial medium was used containing (g 1⁻¹): NaCl 25, K2HPO4 0-5, MgCl2 0-5, MgSO4·7H2O 0-4, CaCl2·2H2O 0·1 and trace metal solution (Pfenning & Lippert, 1966) 1 ml 1⁻¹. The last three components were added after sterilization. TL 2T required biotin and thiamine for growth; pyridoxine had a growth-stimulating effect. Therefore, normally, a filter-sterilized mixture of these vitamins was supplied after medium sterilization to a final concentration of 50 μg 1⁻¹ each. Thiosulfate was added after sterilization from 2 ml stock solution to a final concentration of 10-20 mM. The initial pH was set at 7.5. Upon acidification, the pH of the medium was adjusted to 7·5 with a sterile 1 M NaHCO3 solution. For heterotrophic and mixotrophic growth, the mineral base was supplemented with an organic carbon source (10-20 mM) and yeast extract (0-2 g l⁻¹) instead of the vitamin mixture. Autotrophic growth with hydrogen was tested on a basic mineral medium plus the vitamin mixture in a closed jar under a 1:1 mixture of air/H2. Growth under denitrifying conditions was tested using acetate or thiosulfate as electron donor and nitrate, respectively. Thiosulfate and thionitrite were used. Respiratory activity of washed cells was measured in a 4 ml thermostatted cell with a Clark oxygen electrode (Yellow Spring Inc.) at 30 °C in 0-05 M potassium phosphate buffer, pH 7·5, supplemented with 25 g NaCl 1⁻¹ and 0·2 g MgCl2·6H2O 1⁻¹.

Cell-free extract was prepared by ultrasonic disruption of washed cell suspension (10-15 mg protein ml⁻¹) either in 0·05 M phosphate or Tris/HCl buffers at pH 7·0 and 8·0, respectively. Rubisco activity in cell-free extracts was measured according to Beudecker et al. (1980). Activities of sulfite-cytochrome c oxidoreductase and AMP-dependent sulfite dehydrogenase were determined in the cell-free extract as described by Kelly & Wood (1994). Qualitative tests for the presence of cytochrome oxidase and catalase were performed with single colonies from an acetate-grown culture using 1 % TMPD (N,N,N′,N′-tetramethyl-1,4-phenylenediamine dihydrochloride) and 3 % H2O2, respectively. Thiosulfate and tetrahydrothionate were analysed by cyanolation (Kelly et al., 1969), sulfite and sulfate colorimetrically according to Triipr & Schlegel (1964) and sulfate by HPLC (column ET 250/8/4 Nucleosil 10 Anion; 30 °C, carrier 0·025 M sodium salicylate, pH 4·0, flow rate 1·5 ml min⁻¹). Biomass protein was estimated by the Lowry method, after washing the pellet with a 2·5 % NaCl solution twice to remove interfering sulfur compounds. Pigments were extracted with a methanol/acetone mixture (3:7, v/v) at 4 °C for 12 h and the extract was analysed spectrophotometrically. For electron microscopy, cells were pre-fixed with glutaraldehyde (2·5 % v/v) and then post-fixed with 1 % OsO4 + 2 % NaCl (w/v), dehydrated and embedded into resin. Thin sections were stained with uranyl acetate and lead citrate.

Total DNA extraction, amplification and sequencing of 16S rRNA and of *cbbL* genes were performed as described previously (Sorokin et al., 2003; Spiridonova et al., 2004). Phylogenetic analyses based on nucleotide sequences of 16S rRNA genes and putative amino acid sequences deduced from *cbbL* genes were performed using different treeing algorithms realized in the TREECON (Van de Peer & De Wachter, 1994) and PHYLIP (Felsenstein, 1989) software packages.

**RESULTS**

**Morphology**

Cell morphology of strain TL 2T was highly variable, depending on the growth conditions. In diluted organic media, it grew as long non-motile filaments with swollen ends, often aggregated into tufts or rosettes (Fig. 1a-c). On rich organic media and in autotrophic chemostat culture, small uniform rods dominated, some cells being motile by means of a polar flagellum. The cell wall had a structure typical of Gram-negative bacteria. Autotrophically grown cells differed from cells grown under heterotrophic conditions by possessing an extended periplasm (Fig. 1d). Carboxysome-like structures were not observed in the cells grown autotrophically. Colonies of TL 2T on organic media were up to 10 mm in diameter, flat, convex and pinkish; old colonies turned yellowish. On thiosulfate mineral agar, colonies were much smaller (1-2 mm in diameter), colourless and without sulfur deposition.

Strain TL 2T is a mesophilic and neutrophilic bacterium, growing at temperatures from 15 to 47 °C (optimum 35 °C)
and at pH 6.5–8.5 (optimum pH 7.5–7.8). The bacterium was NaCl-dependent with the NaCl range suitable for growth between 10 and 80 (optimum 30–40) g l\(^{-1}\). It was oxidase- and catalase-positive. No specific light-absorbing pigments were detected in the methanol/acetone extract of TL 2\(^{T}\) cells between 300 and 1000 nm.

**Metabolism**

TL 2\(^{T}\) grew chemoautotrophically with thiosulfate as an electron donor and heterotrophically with acetate, succinate, malate, lactate, pyruvate, propionate, butyrate, glycolate, glyoxylate, malonate, ethanol, glycerol, mannitol, D-glucose, D-maltose, sucrose, D-cellobiose, proline, lysine, alanine, glutamate, glutamine, aspartate and asparagine. Growth was not observed with methanol, formate, methylamine, benzoate, D-ribose, D-fructose, D-galactose, D-melibiose, starch, casein, Tween 80, aromatic amino acids, leucine, arginine, threonine or serine. It was unable to ferment glucose or to grow anaerobically under denitrifying conditions either autotrophically or heterotrophically. Ammonium and nitrate, but not nitrite, urea or dinitrogen served as nitrogen sources.

In batch cultures with acetate, thiosulfate was oxidized mostly to sulfate during the exponential phase of growth (Fig. 2). Significant amounts of sulfite were detected in the medium as an intermediate of thiosulfate oxidation. To obtain a stable autotrophic culture of TL 2\(^{T}\) on mineral medium with thiosulfate as energy source, two or three adaptive transfers from the mixotrophic and five or six transfers from the heterotrophic media were necessary. Up to 5 mM sulfite was transiently produced in the course of thiosulfate consumption. The \(\mu_{\text{max}}\) and \(Y_{\text{max}}\) values in the activated autotrophic culture were 0.06 h\(^{-1}\) and 1.6–1.7 g protein (mol thiosulfate\(^{-1}\))\(^{-1}\), respectively. In continuous mixotrophic (acetate + thiosulfate) culture with 10 mM of each substrate, sulfite was not accumulated in the effluent at a dilution rate \((D)\) up to 0.05 h\(^{-1}\) but, when the influent thiosulfate concentration was increased up to 20 mM, sulfite started to accumulate at \(D > 0.04 \text{ h}^{-1}\). The autotrophic continuous culture with 40 mM thiosulfate started from the mixotrophic culture after 2 weeks adaptation period at low \(D \approx 0.03 \text{ h}^{-1}\) reached \(\mu = 0.1 \text{ h}^{-1}\), after which wash-out of the culture was observed. In contrast to batch cultures, no sulfite production was found in the autotrophic continuous culture and the molar growth yield was about

![Cell morphology of strain TL 2.](image)

**Fig. 1.** Cell morphology of strain TL 2. (a–c) Phase-contrast microphotographs of TL 2\(^{T}\) growing in filamentous aggregates on medium with 5 mM acetate and 0.1 g l\(^{-1}\) of yeast extract. (d) Electron micrograph of thin section of TL 2\(^{T}\) cells with extended periplasm (P) and polar polyphosphate granules (Pp) from autotrophic thiosulfate-limited chemostat culture; N, nucleoid. Bars, 10 μm (a–c) and 0.5 μm (d).

![Growth and oxidation of thiosulfate in batch mixotrophic culture.](image)

**Fig. 2.** Growth and oxidation of thiosulfate in batch mixotrophic culture (acetate + thiosulfate, 20 mM each) of strain TL 2\(^{T}\). •, Biomass (mg protein l\(^{-1}\)); ○, thiosulfate (mM); △, sulfate (mM); Δ, sulfite (mM).
2-fold higher (Table 1). One of the possible reasons for such differences might be more stable pH conditions in the continuous culture.

RubisCO activity was undetectable in TL 2T cells grown heterotrophically. It was very low, but detectable, in mixotrophically grown cells and increased significantly during autotrophic growth (Table 1). Oxygen-consumption experiments with washed cells demonstrated that TL 2T can oxidize thiosulfate and sulfide at almost equal rates, and activity was present in heterotrophically grown cells at a very low level and increased by two orders of magnitude in the autotrophic continuous culture (Table 1). The affinity constant for these substrates was very high (4–7 mM). Elemental sulfur and tetrathionate did not stimulate oxygen consumption. The ability of TL 2T to oxidize sulfite was of special interest because of its transient accumulation in batch cultures. Our experiments demonstrated that sulfite started to accumulate in those cultures where the thiosulfate loading exceeded the oxidizing capacity of the bacterium and when the culture was grown under inappropriate conditions (e.g. in the absence of vitamins or at an unsuitable pH). Hence, it might be speculated that sulfite was not a natural intermediate of thiosulfate oxidation in TL 2T. This was supported by the fact that washed cells were not capable of active sulfite oxidation (3–5 nmol mg protein⁻¹ min⁻¹) and did show sulfite dehydrogenase activity at a detectable level.

Phylogenetic analysis

The G+C content in the DNA of TL 2T was 63.1 ± 0.3 mol%. Phylogenetic analysis based on the almost complete 16S rRNA gene sequence of TL 2T placed the novel isolate into the α-3 group of the Proteobacteria within the order ‘Rhodobacteriales’. In the phylogenetic tree constructed with the neighbour-joining algorithm, strain TL 2T formed a separate branch located between the purple non-sulfur bacteria of the genera Rhodobacter and Rhodovulum (Fig. 3). However, a low value of bootstrap support (24%) showed an uncertain position for its branch point. The phylogenetic trees constructed either by maximum-parsimony or maximum-likelihood algorithms had the same topologies (data not shown). The nucleotide sequence similarity was almost the same with different species of the genera Rhodobacter and Rhodovulum, ranging from 92.4 to 95.0%. There was no close relatedness between the strain TL 2T and the recently described non-photosynthetic members of the Rhodobacter–Rhodovulum clade, Albidovulum inexpectatum (Albuquerque et al., 2002) and Pseudorhodobacter ferrugineus (Uchino et al., 2002), which had 92.8 and 92.5% sequence similarity to strain TL 2T, respectively.

Table 1. Parameters of growth and activity of strain TL 2T

<table>
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<tr>
<th>Growth conditions</th>
<th>Respiration rate [nmol O₂ (mg protein)⁻¹ min⁻¹]</th>
<th>RubisCO activity [nmol CO₂ (mg protein)⁻¹ min⁻¹]</th>
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<tr>
<td>Culture mode</td>
<td>S₀₂⁻ (mM)</td>
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ND, Not determined.

*Specific growth yield expressed as mg Corg (mmol S₀₂⁻)⁻¹ (Y₁) or mg protein (mmol S₀₂⁻)⁻¹ (Y₂); —, not possible to assign biomass to either substrate.
To obtain more information on the phylogenetic position of the novel bacterium among the Rhodobacter–Rhodovulum clade, an additional phylogenetic analysis of the translated sequence of the cbbL gene (encoding the large RubisCO subunit) was performed. Previous work indicated that most of the species of the genus Rhodobacter (Rhodobacter capsulatus, Rba. blasticus and Rba. veldkampii) contained the ‘green’-type of form I RubisCO, Rhodobacter azotoformans contained both ‘green’ and ‘red’ types and Rhodobacter sphaeroides contained only ‘red’-type (Paoli et al., 1998; Uchino & Yokota, 2003). Using our previously designed primer set for the two types of cbbL gene (Spiridonova et al., 2004), the ‘green’ type of form I RubisCO was detected in strain TL 2T and the type strains of three species of the genus Rhodovulum (Rhodovulum sulfidophilum, Rdv. adriaticum and Rdv. euryhalinum). Approximately 750-bp cbbL gene fragments were amplified and sequenced from the DNA of these strains. The identity values between the translated amino acid cbbL sequences of strain TL 2T, new cbbL sequences of Rhodovulum species and analogous sequences of Rhodobacter species available from GenBank ranged from 82·6 to 86·5 %; the identity values to analogous sequences of other autotrophic bacteria ranged from 75·0 to 85·3 %. These values are reflected in the branching pattern of the corresponding cbbL tree constructed with the maximum-likelihood-treeing algorithm (Fig. 4). Although the cbbL gene sequence of the TL 2T is more closely affiliated with the coherent cbbL sequence subcluster of the alphaproteobacterial Rhodobacter–Rhodovulum clade and the beta-proteobacterium Hydrogenophilus thermoluteolus within the ‘green’-type sequence cluster (bootstrap value of 76 % for the common branching point), it formed a novel lineage only distantly related to cbbL of this subcluster. The topology of the cbbL tree supported conclusions on the isolated position of the novel isolate among its photosynthetic neighbours within the Rhodobacter–Rhodovulum clade.

DISCUSSION

The novel bacterium strain TL 2T represents a relatively rare type of marine, facultatively autotrophic, sulfur-oxidizing bacterium. For unknown reasons, this type of organism is difficult to isolate from marine habitats, even using a chemostat technique (Gottschal & Kuenen, 1980) successfully applied for the enrichment of facultative species from freshwater samples. In the case of TL 2T, the presence of a small number of the cells with peculiar morphology in a uniform enrichment culture of obligately autotrophic, sulfur-oxidizing, Thiomicra-pha-like bacteria indicated the possible development of an interesting phenotype. Close association of the facultatively autotrophic TL 2T with an obligate autotroph resembles the well-known association between Acidithiobacillus ferrooxidans (formerly Thiobacillus ferrooxidans) and the facultatively autotrophic alphaproteobacterium Acidiphilium acidophilum (formerly Thiobacillus acidophilus) (Guay & Silver, 1975), which also grew in fused colonies where the latter consumed organic excretion products of the autotroph.

Phylogenetic analysis placed the novel bacterium in the x subclass of the Proteobacteria. This subclass is remarkable in the strong representation of lithoheterotrophic and facultatively autotrophic sulfur-oxidizing species. Moreover, some of the aerobic sulfur-oxidizing alphaproteobacteria still express non-functional bacteriochlorophyll a or contain its gene (Yurkov & Beatty, 1998). This might indicate direct descent from a common ancestral anaerobic, non-sulfur, purple bacterium. The facultatively
autotrophic strain TL $^{2}$T and the thiosulfate-oxidizing heterotroph *Albidovulum inexpectatum* (Albuquerque et al., 2002) are phylogenetically even more closely related to anaerobic phototrophs than to chemotrophic representatives of the α-Proteobacteria. But, apart of the ability to oxidize thiosulfate and similarity of their habitats (marine thermal waters), strain TL $^{2}$T and *Albidovulum inexpectatum* differed significantly in phenotypes, such as autotrophy, temperature and salt range. One of the interesting questions remaining to be answered is why this particular subclass, in contrast, for example, to the β- or γ-subclasses of the Proteobacteria, is almost completely lacking in obligate autotrophs.

Finally, regarding the unique phylogenetic position and distinct phenotype, this isolate is proposed to represent a novel genus and species with the name *Thioclava pacifica* gen. nov., sp. nov.

**Description of *Thioclava* gen. nov.**

*Thioclava* (Thi.o.clav’a. Gr. neut. n. thion sulfur; L. fem. n. clava stick, staff, cudgel, club; N.L. fem. n. *Thioclava* sulfur-oxidizing swollen rod).

Gram-negative rods of varying size from long filaments with swollen ends, clustered in aggregates, to single small rods, rarely motile by one polar flagellum. Obligately aerobic and facultatively autotrophic, sulfur-oxidizing bacteria. Grow chemoautotrophically with thiosulfate and heterotrophically with simple organic compounds. Unable to utilize hydrogen as electron donor and to grow methylotrophically. Inorganic carbon is assimilated via the Calvin cycle. Contain the ‘green form’ of type I RubisCO. UQ-10 is the only respiratory lipoquinone present. The type species of the genus is *Thioclava pacifica* sp. nov.

**Description of *Thioclava pacifica* sp. nov.**

*Thioclava pacifica* (pa.ci’fi.ca. L. fem. adj. pacifica pacific, peacemaking, and, by extension, pertaining to the Pacific Ocean, where the type strain was isolated). The description is identical to that of the genus, with the following additions. The following substrates are utilized for heterotrophic growth: acetate, succinate, malate, lactate, pyruvate, propionate, butyrate, glycolate, glyoxylate, malonate, ethanol, glycerol, mannitol, D-glucose, D-maltose, sucrose, D-cellobiose, proline, lysine, alanine, glutamate, glutamine, aspartate and asparagine. No growth on methanol, formate, methylamine, benzoate, D-ribose, D-fructose, D-galactose, D-melibiose, starch, casein, Tween 80, aromatic amino acids, leucine, arginine, threonine or serine. Unable to ferment glucose or to grow anaerobically under denitrifying conditions either autotrophically or heterotrophically. Ammonium salts and nitrate serve as nitrogen sources. Thiamine and biotin are required for growth. Oxidase- and catalase-positive. Grows within a pH range from 6.5 to 8.5 (optimum 7.5–7.8), temperature range from 15 to 47 °C (optimum 35 °C) and NaCl range from 0 to 80 g l$^{-1}$ (optimum 30–40 g l$^{-1}$). The G+C content of the DNA of the only strain known in the species is 63.1 ± 0.3 mol%.

The type strain, TL $^{2}$T (=DSM 10166$^{T}$ = UNIQEM 229$^{T}$), originated from sulfidic thermal sea water in Matupi Harbour, New Britain, Papua New Guinea.

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