Characterization of *Thiobacillus caldus* sp. nov., a moderately thermophilic acidophile

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Two isolates of a novel, moderately thermophilic *Thiobacillus* species have been studied. The isolates, KU and BC13, are Gram-negative, motile bacteria having a pH optimum for growth of 2–2.5 and an optimum growth temperature of 45 °C. Both isolates are capable of chemolithotrophic growth on reduced sulfur substrates. They can also use molecular hydrogen as an electron donor. These two isolates can grow mixotrophically with sulfur or tetrathionate and yeast extract or glucose. The G + C content is 63.1–63.9 mol% and the isolates exhibit no significant DNA homology to any other *Thiobacillus* species. Strains KU and BC13 both contain ubiquinone Q-8. 16S rRNA analysis indicates that these strains belong to a group of bacteria which includes other chemolithotrophic sulfur oxidizers such as *T. ferrooxidans* and *T. thiooxidans*. These characteristics distinguish KU and BC13 from any other species described previously and they thus represent the first acidophilic, thermophilic *Thiobacillus* species, named *T. caldus* sp. nov., to be described. The type strain, referred to as strain KU in this paper, has been deposited in the Deutsche Sammlung von Mikroorganismen, Braunschweig, FRG, with the accession number DSM 8584.

Keywords: *Thiobacillus caldus* sp. nov., sulfur-oxidizer, moderate thermophile, acidophile, chemolithotroph

INTRODUCTION

Bacteria belonging to the genus *Thiobacillus* are able to oxidize reduced sulfur compounds. Other substrates for these bacteria include ferrous iron (Ingledew, 1982), molecular hydrogen (Drobner *et al.*, 1990) and formate (Pronk *et al.*, 1991), in addition to various organic compounds and sulfide minerals. The ability to oxidize sulfidic ores, and subsequently solubilize metals, makes them useful in the industrial application of leaching metals from the ores or for the enhanced recovery of precious metals.

One of the practical applications of mineral biotechnology is the enhanced recovery of gold from pyrite- and arsenopyrite-containing ores (Lindström *et al.*, 1992). The

use of bioleaching in gold recovery is generally limited to stirred tank reactors where reaction conditions can be controlled (Lawrence, 1990). Leaching reactions are exothermic and thus bioleaching at elevated temperatures offers several advantages. For example, the leaching rates are faster and the need for cooling is reduced.

Studies with the extremely thermophilic archaeon *Sulfolobus* have shown that high temperature leaching (up to 70 °C) is possible yielding high oxidation rates (Lindström *et al.*, 1993). Concern for the sensitivity of *Sulfolobus* to high pulp densities of mineral has been raised (Norris & Barr, 1988). Other, moderately thermophilic, Gram-positive and Gram-negative iron- and sulfur-oxidizers have been isolated (Ghauri & Johnson, 1991; Marsh & Norris, 1983) which have potential use in bioleaching. In addition to these isolates, there has been a report of the isolation of sulfur-oxidizing bacteria, claimed to be *Thiobacillus thiooxidans*, from acidic soil around hot springs with growth at up to 55 °C (Fliermans & Brock, 1972).

In this paper, we characterize the properties of two moderately thermophilic, sulfur-oxidizing acidophiles

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Abbreviation: RDP, Ribosomal Database Project.

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isolated from coal spoils. We propose that these bacteria should be recognized as *Thiobacillus caldus* sp. nov., the first acidophilic species of thermophilic thiobacilli to be described.

METHODS

Bacteria and growth conditions. The two strains characterized in this study were *Thiobacillus* BC13 and KU. Strain BC13 was from Dr Paul Norris, University of Warwick, Coventry, UK, and a stock culture was made by streaking out the culture on solid medium. A single colony was used to inoculate liquid medium. Strain KU was isolated from a tetrathionate-maintained culture derived from a Kingsbury coal spoil enrichment culture (Marsh & Norris, 1983) by streaking a single colony three consecutive times on solid medium. The final single colony was inoculated into liquid medium. Both liquid cultures in mid-exponential phase were concentrated 50-fold in mineral salts medium, made to 7% (v/v) dimethyl sulfoxide and maintained as stock cultures at -80°C . These stock cultures have retained viability for over 3 years. Strain KU has been deposited in the Deutsche Sammlung von Mikroorganismen, Braunschweig, FRG, with the accession number DSM 8584.

Bacteria were grown in a medium consisting of the basal salts (g l^{-1}) $(\text{NH}_4)_2\text{SO}_4$ (3.0), $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ (3.2), KCl (0.1), K_2HPO_4 (0.05), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5) and $\text{Ca}(\text{NO}_3)_2$ (0.01), and the following trace elements (mg l^{-1}): $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (11.0), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.5), HBO_3 (2.0), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (2.0), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.8), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.6) and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.9). The basal salts were adjusted to pH 2.5 with H_2SO_4 and autoclaved before the filter-sterilized trace elements were added. The growth temperature was generally $45 \pm 1^{\circ}\text{C}$ and the growth medium was sparged with CO_2 -enriched air (2%, v/v). Media were solidified with 1.5% (w/v) Phytigel (Sigma) essentially as previously described (Lindström & Sehlén, 1989), with the exception that the pH of the double strength mineral salts solution was adjusted to 1.75 to ensure a final pH of 2.5 in the solid media.

Growth rates were determined in 1.5 l reactors with constant pH maintenance using NaOH. The effect of temperature on growth rate was determined at the indicated temperatures at pH 2.0. The determination of optimal pH for growth was performed at 45°C . The growth rates were calculated from the exponential increase in optical density measured at 440 nm.

Substrate utilization. The main growth substrate used in this study was tetrathionate. In addition, the following sulfur-containing substrates were tested: 0.5% flowers of sulfur; thiosulfate, added in pulses of 0.25 mM h^{-1} to minimize abiotic degradation in the acidic medium; and sulfide, as a gradient in 0.025% Phytigel over a 1% (w/v) sulfide-containing Phytigel base (Drobner *et al.*, 1992). Pyrite (FeS_2) was also tested as a growth substrate as a 1% (w/v) suspension in mineral salts medium with an initial pH of 1.8.

Growth with 50 mM FeSO_4 was tested in mineral salts medium at pH 1.7, with and without 0.05% yeast extract or 2.5 mM glucose. Heterotrophic growth was tested in basal salts amended only with 0.05% yeast extract, 0.05% Casamino acids, or 2.5 mM glucose. Oxidation of molecular hydrogen and formic acid was tested as previously described for *T. ferrooxidans* (Drobner *et al.*, 1990; Pronk *et al.*, 1991).

Transmission electron microscopy. Bacteria were fixed in 2.5% (w/v) glutaraldehyde and 1% (w/v) osmium tetroxide for 1 h

each before dehydration in a graded series of ethanol. After embedding, thin sections were post-stained with uranyl acetate and lead citrate. Flagella were observed by drying bacteria onto carbon-coated grids, washing with water and staining with uranyl acetate.

Chemotaxonomy. Ubiquinones were extracted from 500 mg wet weight of bacteria as described by DiSpirito *et al.* (1983) and were identified by UV spectroscopy following separation by HPLC. The standards used were Q-7, Q-9 and Q-10 (Sigma) with ubiquinones isolated from *Salmonella typhimurium* as the Q-8 standard. LPS was determined by polyacrylamide gel electrophoresis of untreated or proteinase K-treated whole cell lysates followed by LPS-specific silver staining (Hitchcock & Brown, 1983).

DNA analysis. Chromosomal DNA was extracted from strains KU and BC13 using a proteinase K-SDS procedure (Wilson, 1987) and subsequently purified on CsCl gradients. The G+C content was determined by melting point analysis in $0.1 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M NaCl , $0.015 \text{ M trisodium citrate}$, pH 7.0) (Marmur & Doty, 1962) with calf thymus DNA (42 mol% G+C) as standard. DNA-DNA homology was determined by filter hybridization (König, 1984). The indicated DNA was labelled with [α - ^{32}P]dCTP by nick translation. DNA from other organisms used in the homology experiments was kindly provided by Dr Harald Huber, University of Regensburg, FRG.

16S rRNA gene sequencing. 16S rDNA was amplified by PCR using a forward primer complementary to positions 8–27 of *Escherichia coli* 16S rRNA and a reverse primer complementing positions 1510–1492 (Lane, 1991). The amplification reaction (50 μl final volume) consisted of the following: 0.25 μg purified chromosomal DNA, 5 μl of $10 \times$ reaction buffer (200 mM Tris/HCl, pH 8.3, 25 mM MgCl_2 , 500 mM KCl, 0.5% Tween 20 and 1 mg gelatin ml^{-1}), 200 μM of each dNTP, 50 ng of each primer and 2.5 U of *Taq* polymerase. The reaction mixture was incubated in a thermal cycler at 95°C for 3 min before 25 cycles as follows: 95°C for 30 s, 50°C for 30 s and 72°C for 1 min (5 min on the last cycle). Following amplification, the product was cloned into the pT7Blue T-vector (Novagen) and sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) with internal primers of the 16S rRNA molecule (Lane, 1991).

The sequence obtained was compared to other sequences in the Ribosomal Database Project (RDP) using the similarity rank program (Larsen *et al.*, 1993). 16S rRNA sequences of various bacteria were obtained from the RDP and the sequence from strain KU was aligned with them. This alignment was used to construct a distance matrix (Jukes & Cantor, 1969) and the distance matrix was used to construct a phylogenetic tree by the neighbour joining method (Saitou & Nei, 1987). Both algorithms were provided in PHYLIP version 3.5c obtained from Dr Joseph Felsenstein, University of Washington, USA.

RESULTS

Morphology

Phase contrast microscopy revealed that the two isolates are short, rod-shaped organisms, frequently observed as pairs. Strain BC13 measured approximately $1.2 \mu\text{m} \times 0.7 \mu\text{m}$ and strain KU approximately $1.8 \mu\text{m} \times 0.8 \mu\text{m}$. Both strains showed a negative Gram reaction and both had a typical Gram-negative cell wall, as shown by transmission electron microscopy for KU (Fig. 1a).

Fig. 1. Electron micrographs of *Thiobacillus* strain KU showing (a) a thin section and (b) negatively stained cells with a single, polar flagellum. Bars, 1 µm.

Fig. 2. Growth rate of *Thiobacillus* strain KU as a function of (a) temperature at pH 2.0 and (b) pH at 45 °C.

Strains BC13 and KU are motile bacteria with one polar flagellum (Fig. 1b).

Growth conditions

The range of temperature for growth with tetrathionate as growth substrate at pH 2 was determined for each of the two strains. Strain KU grew at 32 ± 1 °C, the lowest temperature tested (Fig. 2a). The highest temperature for growth was 52 ± 1 °C with 45 ± 1 °C being optimal (Fig. 2a). A similar temperature profile was obtained for strain BC13. No growth was observed at 55 °C for either strain on tetrathionate, while growth of strain BC13 on sulfur at 55 °C has been previously reported (Norris *et al.*, 1986).

Strains BC13 and KU exhibited a broad pH range for growth in tetrathionate media at 45 °C. Growth at pH 4.0 was slow, with a generation time of 45 h. The growth rate of strain KU was reduced to 2.3 h at pH 2.0 and 2.5 (Fig. 2b). At pH 1.0, strain KU had a growth rate of 6.7 h (Fig. 2b), while at pH 0.5 no growth was observed. At pH 1.0 the cells grew in long filaments. As with temperature dependence, strain BC13 had nearly identical growth rates as strain KU at the various pH values.

Metabolism

Several potential growth substrates were tested with both isolates. Chemolithoautotrophic growth of the two strains occurred with sulfur, tetrathionate, and thiosulfate. Sulfate was produced as the end product. Both strains grew as a turbid ring in soft Phytigel when provided a gradient of sulfide. Growth of these bacteria was enhanced by supplementing the air used for sparging with 2% (v/v) CO₂.

Heterotrophic growth did not occur with 0.05% yeast extract or Casamino acids, nor with 2.5 mM glucose as sole substrate. Growth occurred mixotrophically with tetrathionate and yeast extract or glucose and the final growth yields were higher than those of tetrathionate-grown cultures. The utilization of glucose by sulfur-oxidizing cultures of strain BC13 has been noted previously (Norris *et al.*, 1986). These authors also reported that strain BC13 could not grow on glucose alone after transfer from sulfur plus glucose, which is also the case for strains KU and BC13 grown on tetrathionate plus glucose.

In addition to the sulfur compounds, both isolates were able to oxidize molecular hydrogen.

Growth was not observed with formic acid, ferrous iron, or ferrous iron plus 0.05% yeast extract, nor with the mineral pyrite.

DNA base composition

Chromosomal DNA was isolated from strains KU and BC13 and was analysed for base composition. The G + C content for strain BC13 was 63.1 mol % and for strain KU it was 63.9 mol %. The G + C content of strain BC13 was previously determined to be 61.7 mol % (Norris *et al.*, 1986).

Fig. 3. PAGE analysis of whole cell lysates followed by staining with (a) silver for LPS or (b) Coomassie brilliant blue. Lanes: 1 and 3, untreated lysates of *Thiobacillus* strains KU and BC13, respectively; 2 and 4, lysates of strains KU and BC13 treated with proteinase K. Each gel was loaded with the same concentration of molecular mass standards (lane 5) to indicate the specificity of the LPS staining.

DNA hybridization studies

DNA–DNA hybridization studies were performed with DNA from strains KU and BC13 as well as from *Thiobacillus* type strains. Strains KU and BC13 exhibited 100% homology with each other (Table 1). No homology was observed with other *Thiobacillus* strains.

Chemotaxonomic markers

Both strains KU and BC13 contained ubiquinone Q-8. An unidentified menaquinone was also observed in the quinone extracts. These isolates can thus be considered to be members of *Thiobacillus* group III (Katayama-Fujimura

Table 1. Percentage DNA–DNA homology of *Thiobacillus* strains KU and BC13 with *Thiobacillus* type strains

Immobilized DNA	³² P-Labelled DNA (%)	
	Strain KU	Strain BC13
Strain KU	100	102
Strain BC13	108	100
<i>T. ferrooxidans</i> ATCC 23270*	6.4	2.2
<i>T. thiooxidans</i> ATCC 19377*	12	16
<i>T. cuprinus</i> DSM 5495*	5	6
<i>T. thioparus</i> DSM 505†	20	16

* Type strain.

† Type species.

Fig. 4. Phylogenetic relationship of *Thiobacillus* strains KU and BC13 to other selected proteobacteria based on 16S rRNA sequences contained in the RDP. Branch lengths are proportional to the calculated evolutionary distances. The tree was rooted using *Bacillus subtilis* as an outgroup.

et al., 1982). The LPS, a marker of Gram-negative bacteria, of both isolates appeared to be similar (Fig. 3).

16S rRNA analysis

A nearly complete sequence of the 16S rRNA gene (1469 bp) was obtained from strain KU. The structure of the helices between bp 180 and 220 (*E. coli* numbering) suggests that strain KU belongs to the β/γ subdivision of proteobacteria (Woese, 1987). Comparison of the sequence with sequences in the RDP indicated a close phylogenetic relationship to other chemolithotrophic members of the genus *Thiobacillus*. The sequence had an identity of 99.3% with the partial sequence of strain BC13

(Lane *et al.*, 1992). It must be pointed out here, as recently discussed by Goebel & Stackebrandt (1994), that the strain designated by Lane *et al.* (1992) as LM2 is in fact BC13 and the strain called BC is LM2 (Dr Paul Norris, personal communication). The correct nomenclature is used in this paper.

Using an alignment of the sequences of the 16S rRNA from strain KU and other bacteria, a phylogenetic tree was constructed (Fig. 4). Within the cluster of bacteria related to strain KU are included both acidophiles and neutrophiles, including the moderately thermophilic, neutrophilic *T. tepidarius*. Strain BC13 exhibits a very close relationship to strain KU, as would be expected.

DISCUSSION

Moderately thermophilic bacteria have been isolated from various habitats. The bacteria are usually differentiated by their optimal growth temperature of about 45 °C–50 °C, apparent lack of sulfate assimilation and spore formation (Norris, 1990). One group of isolates is related to *Sulfobacillus thermosulfidooxidans* (Karavaiko *et al.*, 1990) and are usually Gram-positive or yield ambiguous Gram stain results. Three isolates, TH3, LM2 and ALV, have been shown by 16S rRNA sequencing to belong to the Gram-positive division of the eubacteria (Lane *et al.*, 1992).

The two moderately thermophilic bacteria described in this paper are rod-shaped, Gram-negative and can utilize reduced sulfur compounds as electron donors for growth. Based upon these characteristics, they belong to the genus *Thiobacillus*. These bacteria, unlike other described acidophilic members of the genus, have an optimal growth temperature of 45 °C and thus represent the first moderately thermophilic, acidophilic *Thiobacillus* species. Two thermophilic *Thiobacillus* species, *T. tepidarius* (Wood & Kelly, 1985) and '*T. aquaesulis*' (Wood & Kelly, 1988), have been previously described, but both are neutrophilic. Strains KU and BC13 did not show any DNA homology with other *Thiobacillus* type strains, including *T. ferrooxidans*, *T. thiooxidans* and *T. thioparus*. The two strains exhibit 100 % homology between each other and therefore can be considered to be two isolates of the same species.

It has been reported previously that strain BC13 cannot grow by oxidation of pyrite in pure culture while it can grow when in mixed culture with the iron-oxidizing '*Leptospirillum ferrooxidans*' (Norris, 1990). Using strain-specific antibodies raised against strain KU, it has recently been shown by slot immunobinding assay that these bacteria are significant constituents of a moderately thermophilic mixed culture used in pilot scale leaching studies (Amaro *et al.*, 1994). Also, a group of moderately thermophilic, sulfur-oxidizing bacteria represented by strain C-SH12, were isolated from laboratory-scale continuous bioreactors operating at 40 °C (Goebel & Stackebrandt, 1994). By 16S rDNA sequence analysis (Goebel & Stackebrandt, 1994), these isolates were shown to be highly related to strain BC13, referred to as *Thiobacillus* BC. These data indicate that, although these

bacteria cannot oxidize ores, they can be a significant part of bioleaching cultures in reactors which are operating at elevated temperatures.

Description of *Thiobacillus caldus*

The description of *T. caldus* (cal.dus L. M. adj., warm) given here is based upon the type strain DSM 8584, referred to in this paper as strain KU. Cells are short, motile, Gram-negative rods. *T. caldus* is capable of chemolithoautotrophic growth with thiosulfate, tetrathionate, sulfide, sulfur and molecular hydrogen. *T. caldus* can grow mixotrophically with tetrathionate and glucose or yeast extract. Growth does not occur with ferrous iron or sulfidic ores, with or without an organic amendment. Colonies formed on solid tetrathionate medium are small, circular, convex, smooth and transparent, with precipitated sulfur in the centre of the colony. Growth occurs from 32 °C to 52 °C, the optimum temperature being 45 °C, and at pH 1.0–3.5, 2.0–2.5 being optimal. The DNA contains 63.9 mol % G+C. No significant DNA–DNA hybridization has been detected with other *Thiobacillus* type strains. The respiratory chain contains ubiquinone Q-8 and a menaquinone.

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