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Isolation and characterisation of *Thiobacillus halophilus* sp. nov., a sulphur-oxidising autotrophic eubacterium from a Western Australian hypersaline lake

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Abstract. The isolation of a novel obligately chemolithotrophic, halophilic and extremely halotolerant *Thiobacillus* from a hypersaline lake is described. Attempts to demonstrate sulphur- and ferrous iron-oxidizing chemolithotrophs in neighbouring hypersaline lakes were unsuccessful. The organism isolated differs from any other *Thiobacillus* species previously described and is formally named as *Thiobacillus halophilus*. It possesses ribulose bisphosphate carboxylase and grows chemolithoautotrophically on thiosulphate, tetrathionate and sulphur, oxidising them to sulphate. Kinetic constants for oxidation of sulphide, thiosulphate, trithionate and tetrathionate are presented. The organism is obligately halophilic, growing best with 0.8–1.0 M NaCl, and tolerating up to 4 M NaCl. Optimum growth was obtained at about 30°C and pH 7.0–7.3. It contains ubiquinone Q-8 and its DNA contains 45 mol % G + C. Organisms of this type might contribute significantly to the autotrophic fixation of carbon dioxide in some hypersaline extreme environments of the kind described.

Key words: *Thiobacillus halophilus* – Obligate chemolithoautotroph – Obligately halophilic – Ubiquinone Q8 – Chemostat growth yields

The hypersaline lakes of the Western Australian wheatbelt are of mixed origin. Latterly the deforestation of the "bush" for agricultural purposes, has raised the water table and produced a proliferation of these lakes (Saunders and Hobbs 1989). Others are remnants of late Cretaceous or early Tertiary drainage systems, which have been preserved because of the tectonic stability of the area. Flow in these drainage systems has declined since the mid-Miocene, and evaporative concentration has produced the hypersaline lakes (van de Graaff et al. 1977). All the lakes sampled in our study belong to the latter category. Many of the lakes are only temporary features, appearing during the wet winters and drying up to produce salt pans at the end of the spring. As a consequence, organisms living in them must be able to tolerate high salt concentrations, and their life cycle needs to include a survival stage or annual reinoculation. These extreme environments are not only very salty, but may be acidic (pH 3–4), neutral, or alkaline (pH 9). Some lakes sustain a variety of short-lived animal and plant species, and at least one (Red Lake) supports only a monoculture of *Parartemia contracta* Linder 1941 (the Australian brine shrimp; A. Savage, personal communication).

It was of interest to determine the presence and contribution of autotrophic bacteria in these extreme conditions. Because of the apparent absence of cyanobacteria and other phototrophic bacteria from these lakes, we restricted our search for autotrophs to sulphur-oxidising bacteria of the *Thiobacillus*-type. Smith and Finazzo (1981) isolated a halophilic strain of *Thiobacillus intermedius* from salt marsh sediment. This required 1–5% (w/v) NaCl for maximum efficiencies of thiosulphate oxidation and carbon dioxide fixation, and grew optimally with 3% NaCl. There have been no reports of halophilic thiobacilli other than marine isolates (Saslawsky 1927; Adair and Gunderson 1968; Tilton 1968; Tuttle and Jannasch 1972; Wood and Kelly 1989).

We report here the isolation into pure culture of an obligately halophilic, obligately autotrophic *Thiobacillus* which differs from all previously described thiobacilli, and extends the range of environmental conditions known to be suitable for these organisms.

Materials and methods

Isolation of the organism

Anaerobic and aerobic enrichments were carried out on sediment samples obtained from the lakes described in Table 1, using sulphide (5 mM), dimethyl sulphide (5 mM), thiosulphate (20 mM) or tetrathionate (10 mM) as growth substrates. The medium described

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Table 1. Acid and neutral lakes of the Western Australian wheatbelt

Name	Location	Time of sampling	pH	Salinity (‰)
Red Lake	118°5′E 32°12′S	Winter (September)	4.4	93
		Late Spring (November)	2.8	Saturated
Lake O'Grady South	117°24′42″E 30°25′30″S	Winter (September)	5.5	110
		Late Spring (November)	3.6	300
Lake O'Grady North	117°24′45″E 30°25′30″S	Winter (September)	7.8	137
Lake Truslove	121°46′15″E 33°21′12″S	Winter (September)	6.7	152

Lake O'Grady North is a large lake, separated from Lake O'Grady South by a strip of land about 100 m wide. The coordinates of Lake O'Grady North represent a junction of lines on the southern shoreline, adjacent to the South O'Grady site. Lake O'Grady South coordinates are those of mid-lake

below was used for samples from the neutral pH lakes. The acid pH lakes were sampled into a low pH medium (Bounds and Colmer 1972). Ferrous sulphate (50 mM) was also tested as a sole energy substrate in the low pH medium. Media were adjusted to the appropriate salinity and all cultures were incubated at 20° C and 30° C. Incubations with volatile substrates were in sealed flasks or universal bottles.

Two of the lakes (Table 1) were resampled at the end of spring (11 November 1988), when virtually waterless, by removing sediment samples from beneath the salt crust. This sediment was mixed with 1 M NaCl solution, dispensed (after settling) into flasks and the following substrates added (mM): tetrathionate (10), ferrous sulphate (50), peptone + yeast extract (0.2% w/v each), and tetrathionate (10) + yeast extract (0.2% w/v). All incubations were aerobic at 30° C.

Culture conditions

The standard medium used for isolation and maintenance contained (g/l distilled water): $Na_2HPO_4 \cdot 2\,H_2O$ (7.9), KH_2PO_4 (1.5), NH_4Cl (0.4), $MgSO_4 \cdot 7\,H_2O$ (0.1), NaCl (130), trace metal solution (Tuovinen and Kelly 1973, 10 ml). The phosphates were sterilised separately and mixed with the other components after cooling. In chemostat culture the NaCl was reduced to 50 g/l (0.86 M). For solid medium, Difco-Bacto agar (15 g/l) and saturated phenol red solution (1% v/v) were added. Anaerobic growth was tested using the same medium supplemented with 33 mM KNO_3 in completely filled 100 ml or 50 ml bottles. Aerobic cultures (50 or 100 ml) were shaken in 250 ml conical flasks in a Gallenkamp orbital incubator. All cultures were grown at 30° C. Growth was monitored by visual observation of turbidity or by measurement of absorbance at 440 nm. Organism dry weights, protein and total organic carbon were assayed as described previously for *Thiobacillus tepidarius* (Wood and Kelly 1986). Thiosulphate, tetrathionate, nitrate and nitrite assays have been previously described (Kelly et al. 1969; Kolthoff and Belcher 1957; Wood and Kelly 1983).

Chemostat cultures

These were grown essentially as described for *T. tepidarius* (Wood and Kelly 1986). The standard medium (with 0.86 M NaCl) was pre-mixed with sodium thiosulphate (20 mM) before being pumped into the culture vessel. The growth temperature was maintained at 30 ± 1° C and the pH maintained at 7.0 ± 0.2 by automatic titration with 2 M Na_2CO_3. The inoculum culture was grown on 20 mM thiosulphate with 0.43 M NaCl, initially at pH 7.3. Following inoculation (13% v/v), cultures (730 ml) were stirred at 250 rpm and aerated at 200 ml air/min. Growth was monitored and continuous flow of medium was commenced late in exponential growth.

Scanning electron microscopy

Cells were fixed overnight in 2.5% v/v glutaraldehyde, washed three times in water and resuspended in water for application to stubs. These were air-dried over silica gel for at least 2 days before examination in a JEOL JSM-T330A scanning electron microscope.

Oxygen electrode studies

Ability to oxidise a range of potential substrates was monitored by using an oxygen electrode cell (Rank Brothers, Cambridge, England), controlled at 30° C by a Churchill thermocirculator. Culture samples were removed directly from the chemostat, while in steady state at $D = 0.042\ h^{-1}$ or $0.048\ h^{-1}$, for use with the electrode.

Preparation of cell extracts

Collection, centrifugation and cell-free extract preparation were as described for *T. tepidarius* (Wood and Kelly 1985, 1986).

Ubiquinone analysis

The ubiquinone fraction of *T. halophilus* was isolated, purified and identified by the method described for *T. tepidarius* (Wood and Kelly 1985), using cells collected from the chemostat growing on thiosulphate (20 mM) at dilution rates of $0.056-0.065\ h^{-1}$.

DNA base composition

DNA was isolated using the procedure of Beji et al. (1987) and the mol % G + C determined by the method of Fredericq et al. (1961).

Assay of ribulose bisphosphate carboxylase

Ribulose bisphosphate carboxylase activity in permeabilized whole cells and in cell-free extracts was assayed at 30° C using a previously described method (Wood and Kelly 1989). Assays (1.7 ml reaction volumes) contained up to 7.5 mg cell protein, and were sampled over a period of 30 min. The presence of this enzyme was also determined by cross-reaction in Ouchterlony gels (Oakley 1971) with rabbit antiserum raised against ribulose bisphosphate carboxylase isolated and purified from *T. thyasiris* (T. Lanaras, C. M. Cook, A. P. Wood, G. A. Codd, D. P. Kelly, unpublished). Extracts from thiosulphate-grown *Thiobacillus neapolitanus, T. versutus* and *T. aquaesulis*, tetrathionate-grown *T. thyasiris, T. tepidarius* and *Sulfolobus brierleyi*, and sucrose- and formate-grown *T. versutus* were used as positive and negative controls.

Chemicals

Ribulose bisphosphate (tetrasodium salt), ubiquinone Q-10, proteinase K and ribonuclease were all obtained from the Sigma Chemical Company (Poole, Dorset, England).

Results and discussion

Enrichment cultures

As described above (Materials and methods; Table 1), the occurrence of sulphur- or iron-oxidizing chemolitho-

trophs in a number of hypersaline lakes was investigated, in order to see whether halophilic varieties of such organisms commonly exploit these habitats. Samples taken in winter from lakes Red, O'Grady South and Truslove produced no positive enrichments on thiosulphate, tetrathionate, sulphide, DMS or ferrous iron substrates. At that time these lakes represented a pH range of 4.4–6.7, and salinities of 93–152‰. Resampling the acid lakes (Red and O'Grady South) in late spring also produced no growth on ferrous iron or tetrathionate in a period of 13 days, although media containing organic substrates showed substantial growth of uncharacterised, halophilic, heterotrophic bacteria. We conclude that any chemolithotrophs of the types sought in these lakes were below the levels detectable by our enrichment procedures.

One successful enrichment was, however, achieved using these procedures on samples from Lake O'Grady North, which was of similar salinity to the other lakes, but was at pH 7.8 when sampled. The substrate supporting chemolithoautotrophic growth in this enrichment was thiosulphate. Tetrathionate, sulphide, DMS and ferrous iron did not give positive enrichments. This thiosulphate enrichment was taken through a series of successful subcultures in the same medium, and a pure culture obtained by isolation and subculture of single colonies from agar plates. This organism is described in this paper as *Thiobacillus halophilus*.

Morphological and physiological characteristics

Light microscopy showed *T. halophilus* to be a Gram negative rod-shaped eubacterium, which had no capsule and contained no storage inclusions or spores. Motility was not observed. Scanning electron microscopy showed the cells be about $0.3-0.5 \times 1.0-1.2$ μm in size and to occur singly, in pairs or in short chains.

This organism was isolated on thiosulphate-medium initially at pH 7.3, which appeared optimal for growth, although some growth could also be obtained on medium with a starting pH as high as pH 8.4. It grew autotrophically on thiosulphate, tetrathionate and sulphur. Growth on thiosulphate agar and in liquid batch culture produced a decrease in pH (to about pH 5.5–6.0), often with a sulphur precipitate.

Growth experiments with salt concentrations from 0 to 4 M showed *T. halophilus* to require NaCl for growth, with an optimum at about 1 M, and to tolerate up to 4 M NaCl. Routinely growth media contained 0.86 M NaCl. These properties make this organism the most halophilic and halotolerant of any *Thiobacillus* yet described, and indicate that such strains could be active during a substantial part of the year in the lake from which this isolate came, possibly contributing significantly to carbon input to the lake system. At the time of isolation the lake was at pH 7.8 and at a salinity of 137‰ (approx. 2.3 M NaCl), which would have allowed growth.

Using batch culture methods the temperature range for growth on thiosulphate was shown to be fairly narrow, with no growth being obtained at 20, 37 and 42°C. Growth at 26°C was very slow and fastest growth occurred at 30–32°C. Doubling times were difficult to determine because of deposition of sulphur during growth. This makes the measurement of absorbance unreliable, and interferes with microscopic direct counting or plate counting.

Anaerobic growth on thiosulphate was not observed although nitrate supplied was, in part, converted to nitrite. *T. halophilus* could not grow heterotrophically on a wide range of substrates (mM): acetate (20), arabinose (10), citrate (10), galactose (10), glycollate (10), lactose (10), maltose (10), raffinose (10), succinate (10), xylose (10), tryptone (0.1%), yeast extract (0.1%), or nutrient broth (0.13%). It did not grow on methylamine (20–25 mM) or on formate (50 mM).

Growth in chemostat culture

T. halophilus was grown in chemostat culture with thiosulphate as growth-limiting substrate. Individual steady states at specified dilution rates were stable, but the organism had a tendency to produce a sulphur deposition following a large change in dilution rate. The sulphur was oxidised by the organism during recovery of steady state conditions. Growth yield on thiosulphate (19.5 $\pm$ 0.6 mM) did not vary greatly when measured at dilution rates of 0.019, 0.026, 0.035, 0.042, 0.048, 0.056 and 0.065 h^{-1}, being 6.4 ± 0.5 g dry weight mol^{-1}. This is comparable to thiobacilli such as *T. neapolitanus* (Kelly 1982, 1990), but greater than seen with the halotolerant marine isolate, *T. thyasiris* (Wood and Kelly 1989). Washout of the culture occurred at a dilution rate of 0.072 h^{-1}. Addition of carbon dioxide (1% v/v) to the air supply, during the steady state at $D = 0.048$ h^{-1} had no effect on growth yield, suggesting that CO_2 limitation was not responsible for the unchanged growth yields.

Oxygen electrode studies

Thiosulphate-grown organisms were capable of oxidising a range of sulphur compounds. K_m and V_{max} values were calculated by the Lineweaver-Burk and Eadie-Hofstee procedures. Values for thiosulphate were $K_m = 5.7$ and 5.4 μM; $V_{max} = 850$ and 810 nmol O_2 min^{-1} (mg dry weight)$^{-1}$, respectively, calculated from 12 data values with a Pearson correlation coefficient of 0.8–0.9 for each calculation procedure. Values for trithionate from 7 data values were $K_m = 14.9$ and 14.0 and $V_{max} = 900$ and 1090 respectively, with a Pearsons correlation coefficient of 0.95–0.98. Values for tetrathionate (from 17 data points) were $K_m = 2.1$ and 1.6 and $V_{max} = 730$ and 700 with Pearsons correlation coefficient of 0.7–0.9. Sulphide gave values of K_m 9.8 and 9.9 and V_{max} 890 with correlation coefficient > 0.99. Hexathionate oxidation gave non-computable results, possibly due to a combination of diffusion and chemical interferences.

Ubiquinone content

The presence of either ubiquinone Q-8 or Q-10 has been used as a taxonomic feature for the thiobacilli (Kelly and

Harrison 1989). All known obligately autotrophic species contain Q-8 and none contains Q-10. The ubiquinone isolated from *T. halophilus* was identified as ubiquinone Q-8 by its mobility in thin layer chromatography as compared with the markers of authentic Q-8, isolated from *T. tepidarius* and *T. thyasiris* and commercially available Q-10. It is thus like all other obligately autotrophic thiobacilli in this respect.

DNA base composition

The mol% G + C of the DNA was 45.0 ± 0.5% (15 determinations on two separate preparations). This is below the range of 50–68% reported for the thiobacilli (Kelly and Harrison 1989).

Ribulose bisphosphate carboxylase

Activity could not be detected in permeabilized whole cells or cell-free extracts. The reason for this is unclear, but it may be due to inappropriate assay conditions: a possible requirement for NaCl was not tested. Immunological assay using the Ouchterlony gel procedure with antiserum raised against purified enzyme gave positive results for autotrophically-grown *T. aquaesulis, T. neapolitanus, T. tepidarius, T. thyasiris* and *T. versutus*, while autotrophically grown *Sulfolobus* and heterotrophically-grown *T. versutus* were negative as expected. *T. halophilus* gave a positive reaction to the antiserum and was therefore indicated to possess ribulose bisphosphate carboxylase even though activity was not demonstrated.

Description of *Thiobacillus halophilus*

The description of *T. halophilus* (hal.o.phil.us. L.n. halophil...salt loving) given below is based on the type strain deposited with the Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany (DSM 6132). Cells are Gram negative rods. This organism has an obligate requirement for salt and will tolerate up to 4 M NaCl although its optimum is about 1 M. It is obligately chemolithotrophic and autotrophic, being able to grow aerobically on thiosulphate, tetrathionate and sulphur, reducing the pH of the growth medium to pH 5.5–6.0. It oxidises thiosulphate, tetrathionate, sulphide, hexathionate, trithionate and sulphur. Ribulose bisphosphate carboxylase is present in the organism. It cannot grow on formate or methylamine. On agar medium containing thiosulphate, colonies are circular, small (1 to 3 mm diameter), opaque, smooth, convex and become yellow/white with deposited sulphur. The temperature for optimum growth is 30–32° C. The best growth occurs at pH 7.0–7.3. Nitrate is reduced to nitrite, but anaerobic growth does not occur on thiosulphate. The DNA contains 45.0 mol% G + C. Ubiquinone Q-8 is present in the respiratory chain. The organism was isolated from a salt lake in the wheatbelt of Western Australia.

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