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Thermithiobacillus plumbiphilus sp. nov., a sulfur-oxidizing bacterium isolated from lead sulfide

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A novel sulfur oxidizer, strain wk12^T, was isolated from an industrially synthesized lead (II) sulfide. The G+C content of the genomic DNA was around 58.5 mol%. The major components in the cellular fatty acid profile were summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$), $C_{16:0}$ and summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$). The strain oxidized lead sulfide, thiosulfate and tetrathionate as electron donors to support autotrophic growth. Cells of strain wk12^T were motile, rod-shaped (0.5–1.0 × 0.7–2.2 μm), and Gram-stain-negative. For growth, the temperature range was 5–37 °C, and optimum growth was observed at 28–32 °C. The pH range for growth was 5.8–8.7, with optimum growth at pH 6.4–7.1. Optimum growth of the isolate was observed in medium without NaCl, and no growth was observed in the medium containing 0.5 M or more NaCl. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the isolate belongs to the class *Acidithiobacillia*. The closest relative with a validly published name was *Thermithiobacillus tepidarius* DSM 3134^T, with a 16S rRNA gene sequence similarity of 96 %. On the basis of phylogenetic and phenotypic properties, strain wk12^T represents a novel species of the genus *Thermithiobacillus*, for which the name *Thermithiobacillus plumbiphilus* sp. nov. is proposed. The type strain is wk12^T (=NBRC 111508^T=DSM 101799^T).

The class *Acidithiobacillia* was recently established within the phylum *Proteobacteria* to exclude the order *Acidithiobacillales* from the class *Gammaproteobacteria* (Williams & Kelly, 2013; Garrity *et al.*, 2005a). *Acidithiobacillales* is the type order of the class *Acidithiobacillia* and currently comprises two families, *Acidithiobacillaceae* and *Thermithiobacillaceae*. In the family *Thermithiobacillaceae*, only a single genus, *Thermithiobacillus*, is recognized which was established following the reclassification of *Thiobacillus tepidarius* as *Thermithiobacillus tepidarius* (Kelly & Wood, 2000; Garrity *et al.*, 2005b), which remains the sole recognized species of the genus at the time of writing. The type strain of this species was isolated from a water sample collected from the inflow of the Great Bath in Bath, UK having a temperature of 43 °C (Wood & Kelly, 1985), and was characterized as a moderately thermophilic and obligately chemolithoautotrophic sulfur oxidizer (Wood & Kelly, 1985; 1986). In this study, a novel sulfur oxidizer belonging to the family *Thermithiobacillaceae* was obtained and characterized.

Strain wk12^T was isolated from an industrially synthesized lead (II) sulfide, which was transferred into a medium having a slightly different composition from the original ATCC 290 S6 medium. The composition of the original ATCC 290 S6 medium was as follows (l^{-1}): 3 g $Na_2HPO_4 . 12H_2O$, 1.8 g KH_2PO_4, 0.1 g $MgSO_4 . 7H_2O$, 0.1 g $(NH_4)_2SO_4$, 0.04 g $CaCl_2 . 2H_2O$, 0.03 g $FeCl_3 . 6H_2O$, 0.03 g $MnSO_4 . 5H_2O$ and 10 g $Na_2S_2O_3$. For the first enrichment, thiosulfate concentration was decreased to 0.4 mM. The headspace of the culture bottle was filled with air, and 3 vol% CO_2 was added as a carbon source. The culture bottle was incubated in the dark at 22 °C. Approximately 1 month later, strain wk12^T was enriched, and the resulting culture was inoculated into the ATCC 290 S6 medium. For isolation, a colony was picked from agar-solidified ATCC 290 S6 medium, and maintained in the liquid medium. Purity of the culture was checked by phase-contrast light microscopy (Axioplan 2; Zeiss), by inoculation into media containing various heterotrophic substrates, and by sequencing of the 16S rRNA gene fragments amplified using various primer sets.

For characterization of strain wk12^T, the isolate was cultured in bicarbonate-buffered low-salt defined medium unless otherwise specified (Kojima *et al.*, 2015). The medium was composed as follows (l^{-1}): 0.2 g $MgCl_2 . 6H_2O$, 0.1 g $CaCl_2 . 2H_2O$, 0.1 g KH_4Cl, 0.1 g KH_2PO_4, 0.1 g KCl, 1 ml trace element solution, 1 ml selenite-tungstate solution, 1 ml vitamin solution DSM 141,

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain wk12^T is LC088006.

10 ml 1 M $Na_2S_2O_3$ solution and 30 ml 1 M $NaHCO_3$ solution. All stock solutions except for the vitamin solution were prepared as described previously (Widdel & Bak, 1992). Unless otherwise provided, the pH of the medium was adjusted to 7.1 with HCl solution. The headspace of the culture bottle was filled with air, and incubations were performed in the dark at 22 °C. Motility and morphology were observed by phase-contrast microscopy. The Gram-stain test was conducted with a kit (Fluka). Catalase activity was assessed by pouring 3 % H_2O_2 solution onto a pellet obtained by centrifugation of culture. Oxidase activity was tested using oxidase reagent (bioMérieux). The effect of the temperature on growth was tested by culturing the isolate at various temperatures (2, 5, 8, 13, 15, 18, 22, 25, 28, 30, 32, 34, 37 and 42 °C). To assess the effect of NaCl concentration on growth, the isolate was inoculated into media supplemented with varying concentrations of NaCl (0, 3.33, 6.67, 13.3, 26.7, 66.7, 107, 133, 160, 170, 180, 187, 206, 227, 249, 274, 301, 340, 374, 411, 453, 498, 548 and 602 mM). The effect of pH on growth was tested in the same manner as described previously (Kojima *et al.*, 2015). The buffering reagents (20 mM) and tested pH were as follows: sodium acetate buffer for pH 4.6, 4.7, 4.9, 5.6 and 5.9; MES for pH 5.8, 5.9, 6.1 and 6.3; PIPES for pH 6.3, 6.5 and 6.7; MOPS for pH 6.5 and 6.8; Tricine for pH 6.9, 7.1 and 7.2; and CHES for pH 6.5, 6.8, 7.1, 7.7, 8.6, 8.7, 9.2, 9.5, 9.7, and 9.8. To adjust pH, sodium acetate solution was used for the media buffered with sodium acetate, and NaOH solution was used for the media buffered with other reagents. Anaerobic growth was tested in medium supplemented with 20 mM nitrate under anoxic conditions (the headspace of the bottles was filled with N_2/CO_2). Utilization of electron donors was tested in modified basal medium (Watanabe *et al.*, 2016) each containing one of the substrates listed later. The medium was buffered with 20 mM MOPS-NaOH in addition to bicarbonate, and had a decreased concentration of thiosulfate (0.4 mM) and no vitamin solution. Thiosulfate could not be excluded from the medium because the isolate grew only when thiosulfate was present in the medium as a sulfur source. Growth under different conditions was assessed after incubation for 3−4 weeks. All tests were performed at least twice with appropriate negative and/or positive controls.

To determine the phylogenetic position of strain wk12^T, 16S rRNA gene fragments were amplified with the primer set 27F/1492R (Lane, 1991) and then sequenced. The G+C content of the genomic DNA was determined by HPLC analysis according to the previous study (Katayama-Fujimura *et al.*, 1984). The cellular fatty acid profile was identified using the Sherlock Microbial Identification System (version 6.0; database, TSBA6; MIDI) at Techno Suruga (Shizuoka, Japan).

Cells of strain wk12^T were motile, Gram-stain-negative rods (0.7−2.2 μm long and 0.5−1.0 μm wide) (Fig. 1). Spore formation was not observed. Catalase and oxidase tests were both negative. The G+C content of the genomic DNA of strain wk12^T was around 58.5 mol%. The cellular fatty acid profile was characterized by a high concentration of summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; 43.0 %), $C_{16:0}$ (27.0 %) and summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$; 17.2 %) (Table 1).

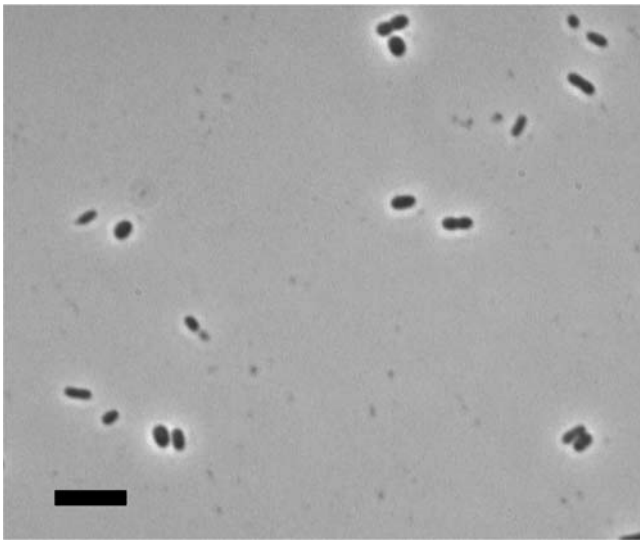

Fig. 1. Phase-contrast micrograph of cells of strain wk12^T grown on thiosulfate at 22 °C for 12 days. Bar, 5 μm.

Under anoxic conditions, nitrate did not support growth of the isolate. Autotrophic growth of strain wk12^T was observed in the presence of lead (II) sulfide (0.5 g l^{-1}),

Table 1. Cellular fatty acid contents (% of total fatty acids) of strain wk12^T

Fatty acid	wk12^T
$C_{12:0}$	4.3
$C_{14:1}\omega5c$	0.5
$C_{14:0}$	0.8
iso-$C_{15:0}$	0.2
$C_{15:1}\omega6c$	0.3
$C_{16:0}$	27.0
iso-$C_{17:0}$	0.2
$C_{17:1}\omega8c$	0.1
$C_{17:0}$	0.3
$C_{18:0}$	2.0
Summed features*	
2	4.1
3	43.0
8	17.2

*Summed features represent groups of two or three fatty acids that could not be separated using the MIDI system. Summed feature 2 comprises $C_{16:1}$ iso I and/or $C_{14:0}$ 3-OH; summed feature 3 comprises $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; summed feature 8 comprises $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$.

tetrathionate (10 mM) and thiosulfate (10 mM). Growth on sulfide (2 mM), elemental sulfur (1.5 g l^{-1}), sulfite (5 mM) and hydrogen (100 kPa in the headspace) was also tested, but no growth was observed. Heterotrophic growth of the isolate was not observed by the following organic substrates: yeast extract (5 mg l^{-1}), glucose, lactose, formate, fructose, sucrose and acetate (each 5 mM).

Strain wk12^T grew over a temperature range of 5−37 °C, a NaCl concentration range of 0−452.5 mM and a pH range of 5.8−8.7. Optimum growth was observed at 28−32 °C, pH 6.4−7.1 and without NaCl.

The 16S rRNA gene sequence analysis showed that strain wk12^T belongs to the class *Acidithiobacillia* (Fig. 2). The closest cultured relative with a validly published name was *Thermithiobacillus tepidarius* DSM 3134^T with 16S rRNA gene sequence similarity of 96 %. In the phylogenetic tree, strain wk12^T formed a bootstrap-supported cluster with an uncultured bacterium (clone 12L 191; GenBank accession no. KP183092).

While the isolate showed several physiological similarities to its closest cultured relative *Thermithiobacillus tepidarius* DSM 3134^T, there were apparent differences between these two organisms. *Thermithiobacillus tepidarius* DSM 3134^T could utilize elemental sulfur as an electron donor, but strain wk12^T could not sustain growth with elemental sulfur. The genomic DNA G+C content of the novel isolate (58.5 mol%) was lower than that of *Thermithiobacillus tepidarius* DSM 3134^T (66.6 mol%). One of the outstanding characteristics of *Thermithiobacillus tepidarius* is an adaptation to thermophilic conditions (up to 52 °C), as represented by the genus name. However, the higher limit of temperature for strain wk12^T growth was 37 °C, and strain wk12^T could grow at 5 °C. On the basis of phylogenetic and phenotypic properties, we conclude that strain wk12^T represents a novel species of the genus *Thermithiobacillus*, for which the name *Thermithiobacillus plumbiphilus* sp. nov. is proposed.

Description of *Thermithiobacillus plumbiphilus* sp. nov.

Thermithiobacillus plumbiphilus (plum.bi′phi.lus. L. neut. n. *plumbum* lead; N.L. adj. *philus -a -um* (from Gr. adj. *philos -ê -on*), friend, loving; N.L. masc. adj. *plumbiphilus* loving lead).

Cells are motile, non-spore-forming, Gram-stain-negative rods, 0.7−2.2 μm in length and 0.5−1.0 μm in width. Catalase- and oxidase-negative. The major fatty acids are summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$), $C_{16:0}$ and summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$). Autotrophic growth occurs by the oxidation of lead (II) sulfide,

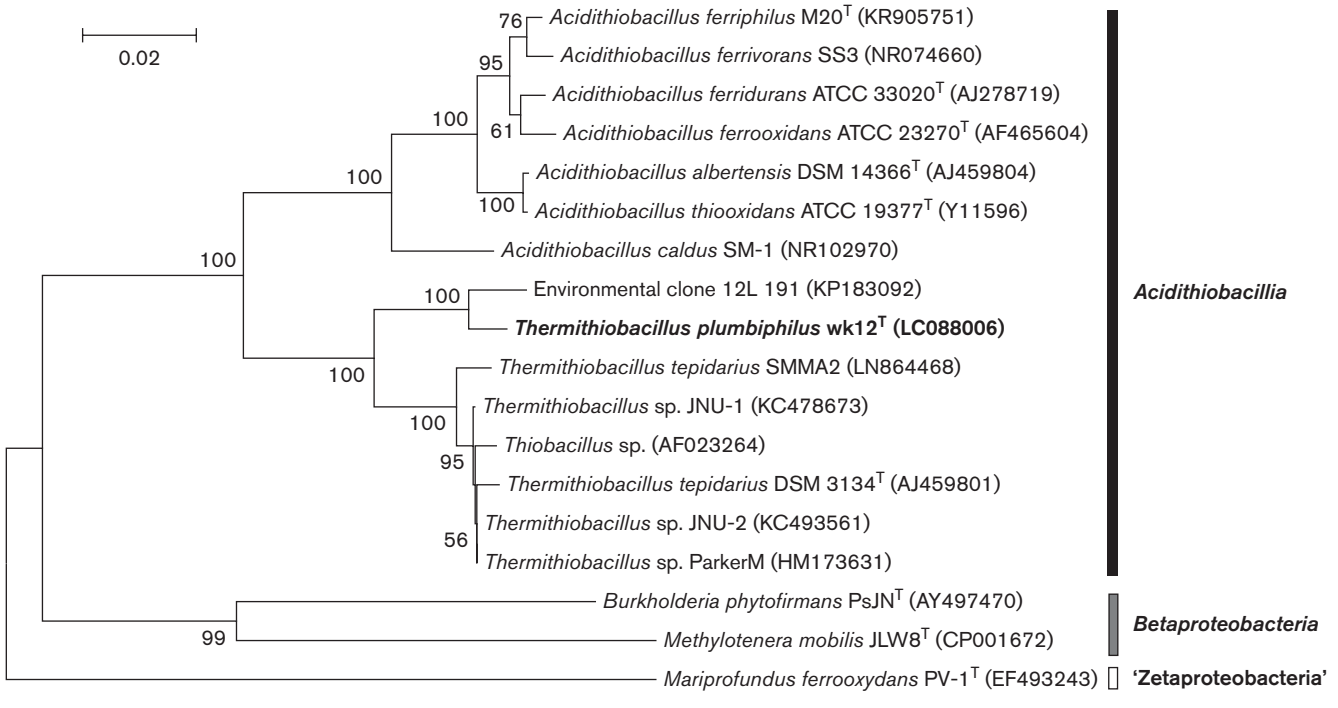

Fig. 2. Phylogenetic position of strain wk12^T within the class *Acidithiobacillia* based on 16S rRNA gene sequences aligned by CLUSTAL W (1334 comparable sites). *Mariprofundus ferrooxydans* PV-1^T was used as an outgroup. Tree was reconstructed by the neighbour-joining method with 1000 bootstrap resamplings; bootstrap values ≥50 % are shown at the nodes. Bar, 0.02 substitutions per nucleotide position.

tetrathionate and thiosulfate. Grows at a temperature range of 5−37 °C, a NaCl concentration range of 0−452.5 mM and a pH range of 5.8−8.7. Optimum growth is observed at 28−32 °C, pH 6.4−7.1 and in the absence of NaCl.

The type strain, wk12^{T} (=NBRC 111508^{T}=DSM 101799^{T}), was isolated from an industrially synthesized lead (II) sulfide. The G+C content of the genomic DNA of the type strain is around 58.5 mol%.

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