Tepidimicrobium ferriphilum gen. nov., sp. nov., a novel moderately thermophilic, Fe(III)-reducing bacterium of the order *Clostridiales*

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A moderately thermophilic, anaerobic bacterium (strain SB91^T) was isolated from a freshwater hot spring at Barguzin Valley, Buryatiya, Russia. Cells of strain SB91^T were straight to slightly curved rods, 0·5–0·6 μm in diameter and 3·0–7·0 μm in length. Formation of endospores was not observed. The temperature range for growth was 26–62 °C, with an optimum at 50 °C. The pH range for growth was 5·5–9·5, with an optimum at pH 7·5–8·0. The substrates utilized by strain SB91^T in the presence of 9,10-anthraquinone 2,6-disulfonate included peptone, tryptone, Casamino acids, yeast extract, beef extract, casein hydrolysate, alanine plus glycine, alanine plus proline, L-valine and n-propanol. Carbohydrates were not utilized. Strain SB91^T reduced amorphous Fe(III) oxide, Fe(III) citrate, Fe(III) EDTA or Fe(III) nitrilotriacetate with peptone, L-valine or n-propanol as an electron donor. Strain SB91^T reduced 9,10-anthraquinone 2,6-disulfonate, thiosulfate, elemental sulfur, fumarate and selenite. Strain SB91^T survived after exposure to gamma-radiation at a dose of 5·4 kGy. The G+C content of the DNA of strain SB91^T was 33 mol%. Analysis of the 16S rRNA gene sequence revealed that the isolated organism belonged to cluster XII of the clostridia. On the basis of its physiological properties and the results of phylogenetic analyses, it is proposed that strain SB91^T represents the sole species of a novel genus, *Tepidimicrobium*; the name *Tepidimicrobium ferriphilum* gen. nov., sp. nov. is proposed, with strain SB91^T (=DSM 16624^T=VKM B-2348^T) as the type strain.

Microbial processes play an essential role in controlling the solubility and mobility of radionuclides in environments contaminated by nuclear waste. The activities of dissimilatory Fe(III)-reducing micro-organisms are of particular importance because they can alter the solubility of radionuclides via direct enzymic reduction or by indirect mechanisms catalysed via a range of electron-shuttling compounds (Lloyd *et al.*, 2002; Anderson *et al.*, 2003). There is a need for bioremediating micro-organisms that are resistant to both radiation and high temperatures because of the existence of thermally insulated contaminated environments where temperatures are elevated by the decay of long-lived radionuclides (Brim *et al.*, 2003). The use of irradiation as a treatment method for the isolation of micro-organisms from thermal environments led to the isolation of hyperthermophilic archaea of the genus *Thermococcus* (Jolivet *et al.*, 2003) and aerobic bacteria of the genera *Rubrobacter* and *Deinococcus* (Yoshinaka *et al.*, 1973; Suzuki *et al.*, 1988; Ferreira *et al.*, 1997). In this article, we describe an anaerobic, moderately thermophilic, Fe(III)-reducing micro-organism, strain SB91^T, isolated from a continental hot spring after gamma-irradiation of the samples and enrichments, that belongs to a novel genus within the order *Clostridiales*.

Strain SB91^T was isolated from a sample of sediment collected under a cyanobacterial mat developing in a freshwater hot spring in the Alla River (Barguzin Valley, Buryatiya, Russia). The temperature at the sampling site was 50 °C and the pH was close to 8·0. After transportation to the laboratory, an aliquot of the sample was irradiated at 1·5 kGy with a gamma-ray source (^{60}Co) at a rate of 120 Gy min^{-1} (Institute of Physical Chemistry RAS,

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Abbreviation: AQDS, 9,10-anthraquinone 2,6-disulfonate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SB91^T is AY656718.

Moscow, Russia). An enrichment culture was initiated by inoculating 10 % (w/v) of the irradiated sample into anaerobically prepared, bicarbonate-buffered, sterile (135 °C, 1 h) liquid medium with peptone (10 g l^{-1}) as an electron donor and amorphous Fe(III) oxide (90 mM) as an electron acceptor. Medium composition and preparation techniques were as described previously (Slobodkin *et al.*, 1999). The pH of the autoclaved medium was adjusted to 7·5–8·0 (at 25 °C) with 10 % (w/v) NaOH. After three subsequent 5 % (v/v) transfers of the initial enrichment, it was irradiated for the second time at 5·4 kGy under the same conditions. A pure culture of strain SB91^T was obtained from this irradiated outgrown enrichment by serial dilution in a medium in which amorphous Fe(III) oxide was replaced by 20 mM 9,10-anthraquinone 2,6-disulfonate (AQDS); this was followed by the selection of well-isolated colonies that had developed in agar shake tubes (1·5 % agar in growth medium). Physiological studies on substrate utilization and on temperature, pH and salinity ranges for growth were carried out in the medium containing AQDS unless noted otherwise. In electron-acceptor utilization experiments, AQDS was omitted. Light and electron microscopy, analytical techniques, DNA extraction and determination of the G + C content were performed as described previously (Slobodkin *et al.*, 1999). 16S rRNA gene amplification, sequencing and sequence analysis were done as described previously (Zavarzina *et al.*, 2002).

In agar-shake cultures, brown spherical colonies (1·0–1·5 mm in diameter) of strain SB91^T appeared after incubation at 50 °C for 18–24 h. Cells of strain SB91^T were straight to slightly curved rods, 0·5–0·6 μm in diameter and 3·0–7·0 μm in length (Fig. 1a). The cells occurred singly or in short chains, were peritrichously flagellated and exhibited slight tumbling motility. Formation of endospores was not observed. Ultrathin sectioning of strain SB91^T revealed a distinct thick peptidoglycan layer in its cell wall (Fig. 1b). The outermost layer consisted of small subunits lying outside the peptidoglycan layer.

The temperature range for growth of strain SB91^T was 26–62 °C, with an optimum at 50 °C. No growth was detected at 64 °C or at temperatures up to 25 °C after incubation for 3 weeks. The pH range for growth was 5·5–9·5, with an optimum between pH 7·5 and 8·0. No growth was detected at pH 5·0 or 10·0. Growth of strain SB91^T was observed at NaCl concentrations ranging from 0 to 3·5 % (w/v), but no growth was evident at 4·0 % (w/v). The substrates utilized by strain SB91^T included peptone, tryptone, Casamino acids, yeast extract, beef extract, casein hydrolysate (each at 10 g l^{-1}), L-valine (20 mM), DL-alanine plus glycine (both at 20 mM), DL-alanine (20 mM) plus L-proline (10 mM), and n-propanol (20 mM). Glucose, mannose, lactose, galactose, sucrose, fructose, maltose, L-arabinose, rhamnose, xylose, cellobiose (each at 25 mM), pyruvate, glycerol, formate, acetate, propionate, butyrate, lactate, malate, fumarate, methanol, ethanol, isopropanol, n-butanol, glycine, DL-alanine, L-arginine (each at 20 mM), L-proline

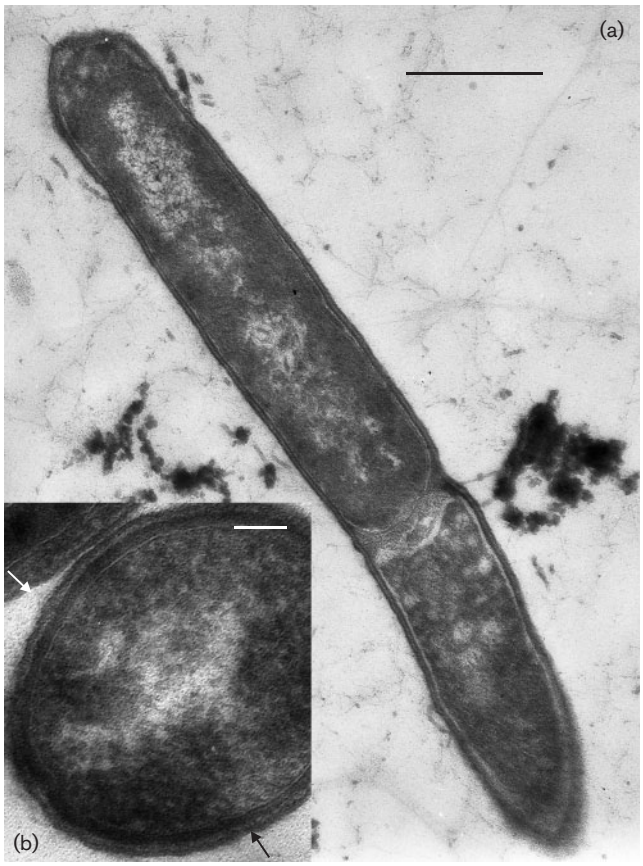

Fig. 1. Cell morphology of strain SB91^T grown in medium with peptone-Fe(III) EDTA. (a) Electron micrograph showing a dividing cell (ultrathin section). (b) Ultrathin sections showing cell wall layers. The outermost wall layer consisted of small subunits (see arrows). Bars, 0·5 μm (a) and 0·1 μm (b).

(10 mM), benzoate (5 mM), betaine (5 mM), casein, olive oil, starch, xylan, carboxymethyl cellulose, filter paper, chitin (each at 10 g l^{-1}) and H_2/CO_2 (80 : 20, v/v) were not utilized. Strain SB91^T reduced AQDS (20 mM) to 9,10-anthrahydroquinone 2,6-disulfonate with all electron donors utilized. Strain SB91^T reduced amorphous Fe(III) oxide (90 mM), Fe(III) citrate (20 mM), Fe(III) EDTA (10 mM) and Fe(III) nitrilotriacetate (10 mM) with peptone (10 g l^{-1}), L-valine (20 mM) or n-propanol (20 mM) as an electron donor. No growth was evident on L-valine or n-propanol in the absence of Fe(III). L-Valine was incompletely oxidized to isobutyrate and n-propanol was oxidized to propionate with Fe(III) as an electron acceptor. Strain SB91^T reduced thiosulfate (20 mM) and elemental sulfur (150 mM) to hydrogen sulfide, fumarate (20 mM) to succinate, and selenite (20 mM) to elemental selenium with peptone (10 g l^{-1}) as an electron donor. Reduction of Fe(III), AQDS, thiosulfate, S^0 and fumarate enhanced final cell yields and growth rates of strain SB91^T two- to threefold in comparison with growth in pre-reduced medium (0·5 g $Na_2S.9H_2O$ l^{-1}) without an electron acceptor; reduction of

selenite did not stimulate growth. Strain SB91^T did not use nitrate (20 mM), sulfate (20 mM), selenate (20 mM) or oxygen (0·5, 4·0 or 20 %, v/v, in the gas phase) as electron acceptors with peptone (10 g l^{-1}) as the electron donor.

Cultures of strain SB91^T remained viable after gamma-irradiation at a dose of 5·4 kGy, but no growth was obtained from cultures irradiated at 10·0 kGy. While direct comparison of radiation-resistance data obtained for various micro-organisms is not always possible, because of differences in the irradiation conditions used, the level of radiation resistance of strain SB91^T could be considered as moderate. Thermophilic members of the genera *Deinococcus* and *Rubrobacter* withstand doses of gamma-radiation above 12 and 18 kGy, respectively (Ferreira *et al.*, 1997; Chen *et al.*, 2004), while the lethal dose for *Thermococcus* species exceeds 30 kGy (Jolivet *et al.*, 2003, 2004). The mechanism of radiation resistance of strain SB91^T is not connected with the formation of endospores. To date, ionizing-radiation resistance of vegetative cells of members of the *Clostridiales* has not been reported.

The G+C content of the genomic DNA of strain SB91^T was 33 mol% (T_m). Analysis using BLAST indicated that the highest levels of 16S rRNA gene sequence similarity were found with species of the genus *Clostridium* within the low-G+C-content Gram-positive subgroup of the *Bacteria*. A comparison of 1493 nt of the 16S rRNA gene sequence of strain SB91^T with the sequences of neighbouring reference bacterial strains and some representatives of thermophilic anaerobic bacteria showed that strain SB91^T belonged to cluster XII of the clostridia (nomenclature of Collins *et al.*, 1994) (Fig. 2); it formed a single phylogenetic cluster with *Clostridium ultunense*, the closest relative (92 % similarity). The levels of 16S rRNA gene sequence similarity with other members of phylogenetic cluster XII ranged between 88 and 91 %. Trees constructed by maximum likelihood and by maximum parsimony had the same topology (data not shown). Transversion analysis (Woese *et al.*, 1991) did not affect the phylogenetic position of strain SB91^T.

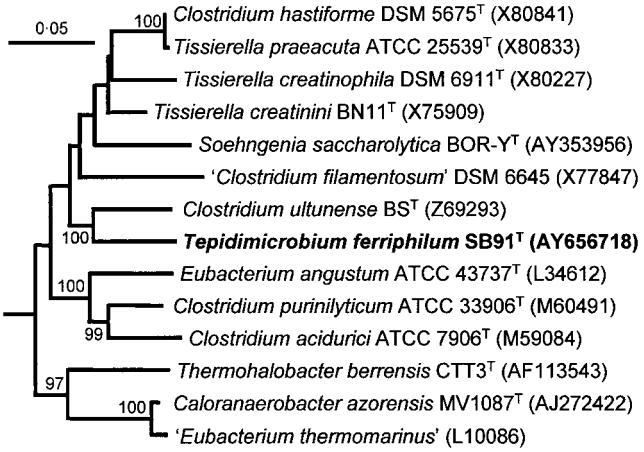

Fig. 2. Phylogenetic tree showing the position of strain SB91^T within cluster XII of the clostridia. Bootstrap values (from 100 replications) are shown at branch points; values greater than 80 were considered significant. Bar, 5 substitutions per 100 nt.

On the basis of 16S rRNA gene sequence analysis, the closest phylogenetic relative of strain SB91^T is *C. ultunense* (Schnurer *et al.*, 1996). In addition to the fact that there is a significant phylogenetic distance between *C. ultunense* and strain SB91^T, the former is a mesophilic micro-organism with an optimum growth temperature of about 37 °C. It also differs from strain SB91^T by its ability to ferment glucose and to form endospores. Cluster XII of the clostridia comprises mesophilic (*Tissierella* or *Soehngenia*) as well as thermophilic (*Thermohalobacter* and *Caloranaerobacter*) genera, but the low level of 16S rRNA sequence similarity (89 %) does not permit placement of strain SB91^T within any of these genera. All thermophilic species of cluster XII require marine (or even higher) levels of salinity for growth, whereas strain SB91^T grows optimally in freshwater medium. Dissimilatory Fe(III) reduction has not been reported previously for micro-organisms from this phylogenetic cluster. On the basis of the physiological properties and the results of phylogenetic analyses, we propose that strain SB91^T represents the sole species of a novel genus, *Tepidimicrobium ferriphilum* gen. nov., sp. nov.

Description of *Tepidimicrobium* gen. nov.

Tepidimicrobium (Te.pi.di.mi.cro′bi.um. L. adj. *tepidus* moderately warm; N.L. neut. n. *microbium* microbe; N.L. neut. n. *Tepidimicrobium* a microbe from a hot spring).

Rod-shaped bacteria. Member of cluster XII of the clostridia (nomenclature of Collins *et al.*, 1994). Anaerobic and moderately thermophilic. Neutrophilic. Gram-positive-type cell wall. Grow organotrophically on a number of proteinaceous substrates. Reduce Fe(III), AQDS, thiosulfate, elemental sulfur, fumarate and selenite. Resistant to gamma-radiation at a dose of 5–10 kGy. The type species is *Tepidimicrobium ferriphilum*.

Description of *Tepidimicrobium ferriphilum* sp. nov.

Tepidimicrobium ferriphilum (fer.ri.phi′lum. L. n. *ferrum* iron; Gr. adj. *philos* loving; N.L. neut. adj. *ferriphilum* iron-loving).

Shows the following properties in addition to those given in the genus description. Cells are straight to slightly curved rods, 0·5–0·6 μm in diameter and 3·0–7·0 μm in length. Cells occur singly or in short chains and exhibit tumbling motility due to peritrichous flagellation. The temperature range for growth is 26–62 °C, with an optimum at 50 °C. The pH range for growth is 5·5–9·5, with an optimum at pH 7·5–8·0. Growth occurs at NaCl concentrations in the range 0–3·5 % (w/v). Substrates utilized include peptone, tryptone, Casamino acids, yeast extract, beef extract, casein hydrolysate, valine, alanine plus glycine, alanine plus

proline, and n-propanol. Glucose, mannose, lactose, galactose, sucrose, fructose, maltose, arabinose, rhamnose, xylose, cellobiose, pyruvate, glycerol, formate, acetate, propionate, butyrate, lactate, malate, fumarate, benzoate, methanol, ethanol, isopropanol, n-butanol, glycine, alanine, arginine, proline, betaine, casein, olive oil, starch, xylan, carboxymethyl cellulose, filter paper, chitin and H_2/CO_2 are not utilized. Reduces amorphous Fe(III) oxide, Fe(III) citrate, Fe(III) EDTA, Fe(III) nitrilotriacetate, thiosulfate, elemental sulfur, fumarate and selenite. Does not use nitrate, sulfate, selenate or oxygen as electron acceptors. Survives exposure to gamma-radiation at a dose of 5·4 kGy. The G + C content of the genomic DNA is 33 mol% (T_m).

The type strain is SB91^T (= DSM 16624^T = VKM B-2348^T), isolated from a freshwater hot spring at Barguzin Valley, Buryatiya, Russia.

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References

Anderson, R. T., Vrionis, H. A., Ortiz-Bernad, I. & 10 other authors (2003). Stimulating the *in situ* activity of *Geobacter* species to remove uranium from the groundwater of a uranium-contaminated aquifer. *Appl Environ Microbiol* **69**, 5884–5891.

Brim, H., Venkateswaran, A., Kostandarithes, H. M., Fredrickson, J. K. & Daly, M. J. (2003). Engineering *Deinococcus geothermalis* for bioremediation of high-temperature radioactive waste environments. *Appl Environ Microbiol* **69**, 4575–4582.

Chen, M.-Y., Wu, S.-H., Lin, G.-H., Lu, C.-P., Lin, Y.-T., Chang, W.-C. & Tsay, S.-S. (2004). *Rubrobacter taiwanensis* sp. nov., a novel thermophilic, radiation-resistant species isolated from hot springs. *Int J Syst Evol Microbiol* **54**, 1849–1855.

Collins, M. D., Lawson, P. A., Willems, A., Cordoba, J. J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H. & Farrow, J. A. E. (1994). The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *Int J Syst Bacteriol* **44**, 812–826.

Ferreira, A. C., Nobre, M. F., Rainey, F. A., Silva, M. T., Wait, R., Burghardt, J., Chung, A. P. & da Costa, M. S. (1997). *Deinococcus geothermalis* sp. nov. and *Deinococcus murrayi* sp. nov., two extremely radiation-resistant and slightly thermophilic species from hot springs. *Int J Syst Bacteriol* **47**, 939–947.

Jolivet, E., L'Haridon, S., Corre, E., Forterre, P. & Prieur, D. (2003). *Thermococcus gammatolerans* sp. nov., a hyperthermophilic archaeon from a deep-sea hydrothermal vent that resists ionizing radiation. *Int J Syst Evol Microbiol* **53**, 847–851.

Jolivet, E., Corre, E., L'Haridon, S., Forterre, P. & Prieur, D. (2004). *Thermococcus marinus* sp. nov. and *Thermococcus radiotolerans* sp. nov., two hyperthermophilic archaea from deep-sea hydrothermal vents that resist ionizing radiation. *Extremophiles* **8**, 219–227.

Lloyd, J. R., Chesnes, J., Glasauer, S., Bunker, D. J., Livens, F. R. & Lovley, D. R. (2002). Reduction of actinides and fission products by Fe(III)-reducing bacteria. *Geomicrobiol J* **19**, 103–120.

Schnurer, A., Schink, B. & Svensson, B. H. (1996). *Clostridium ultunense* sp. nov., a mesophilic bacterium oxidizing acetate in syntrophic association with a hydrogenotrophic methanogenic bacterium. *Int J Syst Bacteriol* **46**, 1145–1152.

Slobodkin, A. I., Tourova, T. P., Kuznetsov, B. B., Kostrikina, N. A., Chernyh, N. A. & Bonch-Osmolovskaya, E. A. (1999). *Thermoanaerobacter siderophilus* sp. nov., a novel dissimilatory Fe(III)-reducing, anaerobic, thermophilic bacterium. *Int J Syst Bacteriol* **49**, 1471–1478.

Suzuki, K., Collins, M. D., Iijima, E. & Komagata, K. (1988). Chemotaxonomic characterization of a radiotolerant bacterium *Arthrobacter radiotolerans*: description of *Rubrobacter radiotolerans* gen. nov., comb. nov. *FEMS Microbiol Lett* **52**, 33–40.

Woese, C. R., Achenbach, L., Rouviere, P. & Mandelco, L. (1991). Archaeal phylogeny: reexamination of the phylogenetic position of *Archaeoglobus fulgidus* in light of certain composition-induced artifacts. *Syst Appl Microbiol* **14**, 364–371.

Yoshinaka, T., Yano, K. & Yanaguchi, H. (1973). Isolation of a highly radioresistant bacterium, *Arthrobacter radiotolerans* nov. sp. *Agric Biol Chem* **37**, 2269–2275.

Zavarzina, D. G., Tourova, T. P., Kuznetsov, B. B., Bonch-Osmolovskaya, E. A. & Slobodkin, A. I. (2002). *Thermovenabulum ferriorganovorum* gen. nov., sp. nov., a novel thermophilic, anaerobic, endospore-forming bacterium. *Int J Syst Evol Microbiol* **52**, 1737–1743.