# *Tepidibacillus decaturensis* sp. nov., a microaerophilic, moderately thermophilic iron-reducing bacterium isolated from 1.7 km depth groundwater

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A Gram-stain-negative, microaerophilic rod-shaped organism designated as strain Z9$^T$ was isolated from groundwater of 1.7 km depth from the Mt. Simon Sandstone of the Illinois Basin, Illinois, USA. Cells of strain Z9$^T$ were rod shaped with dimensions of 0.3×(1–10) μm and stained Gram-negative. Strain Z9$^T$ grew within the temperature range 20–60 °C (optimum at 30–40 °C), between pH 5 and 8 (optimum 5.2–5.8) and under salt concentrations of 1–5 % (w/v) NaCl (optimum 2.5 % NaCl). In addition to growth by fermentation and nitrate reduction, this strain was able to reduce Fe(III), Mn(IV), Co(III) and Cr(VI) when $H_2$ or organic carbon was available as the electron donor, but did not actively reduce oxidized sulfur compounds (e.g. sulfate, thiosulfate or $S^0$). The G+C content of the DNA from strain Z9$^T$ was 36.1 mol%. Phylogenetic analysis of the 16S rRNA gene from strain Z9$^T$ showed that it belongs to the class *Bacilli* and shares 97 % sequence similarity with the only currently characterized member of the genus *Tepidibacillus*, *T. fermentans*. Based on the physiological distinctness and phylogenetic information, strain Z9$^T$ represents a novel species within the genus *Tepidibacillus*, for which the name *Tepidibacillus decaturensis* sp. nov. is proposed. The type strain is Z9$^T$ (=ATCC BAA-2644$^T$=DSM 103037$^T$).

Microbial iron reduction has important biological and environmental implications due to the abundance of iron in the Earth's crust and the ubiquity of iron-reducing organisms across a broad range of natural environments (Melton *et al.*, 2014). The respiration of ferric iron minerals has been proposed as one of the earliest forms of microbial metabolism (Heimann *et al.*, 2010; Vargas *et al.*, 1998). Studies using culture-dependent and culture-independent methods have revealed iron-reducing organisms to be phylogenetically diverse and broadly distributed in aquatic, marine, terrestrial and engineered ecosystems (Lovley *et al.*, 2004; Nealson *et al.*, 2002; Roden *et al.*, 2012; Williams *et al.*, 2011). These organisms reduce iron either as an indirect consequence of fermentation or through direct microbial respiration that couples the oxidation of carbon or molecular hydrogen to the reduction of Fe(III) for energy conservation (termed dissimilatory iron reduction) (Lovley *et al.*, 2004). Given the ubiquity of Fe(III) oxides within soils and sedimentary systems, microbial Fe(III) reduction can have a profound impact on carbon cycling and degradation (Lalonde *et al.*, 2012; Nealson,

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA sequences of strain Z9$^T$ are KP898732 and KP898733.

Two supplementary figures are available with the online Supplementary Material.

1997). Microbial iron reduction was estimated to account for up to 50 % of carbon oxidation in non-sulfidogenic sediments (Canfield *et al.*, 1993; Thamdrup, 2000).

Of the diverse environments inhabited by iron-reducing organisms, deep subsurface groundwater ecosystems represent one of the more challenging environments for microorganisms to obtain enough usable energy to survive (Edwards *et al.*, 2012). Being physically and chemically isolated, water in the deep subsurface is typically saline and oligotrophic, so labile organic carbon is often lacking. Instead, more recalcitrant substrates such as buried kerogen, petroleum hydrocarbons, dissolved gases (e.g. $CH_4$) or metal oxides may enable deep subsurface microbes to conserve enough energy to survive (Fredrickson *et al.*, 1997; Liu *et al.*, 1997; Lovley & Chapelle, 1995). Despite the fact that some estimates place a significant proportion of the Earth's biomass in the so-called 'deep biosphere', very little is known about microbial life there, particularly in the terrestrial deep subsurface (Jorgensen, 2012). One such area is the Mt. Simon Formation, a thick sedimentary layer comprised primarily of highly porous and permeable quartz sandstones coated with ferric minerals and located within a deep intracratonic basin in North America known as the Illinois Basin (Bowen *et al.*, 2011). Groundwater from the Mt. Simon Formation has been shown to contain microbial life (Dong *et al.*, 2014a, b). The abundance of goethite and hematite in the sediment and the presence of ferrous iron in the groundwater (Dong *et al.*, 2014b) suggest dissimilatory metal-reducing bacteria (DMRB) might be active there. We have previously developed an active iron-reducing microbial enrichment culture from Mt Simon Formation water collected at a depth of 1.7 km (Dong *et al.*, 2014b). In this paper, we describe the isolation and characterization of a new strain of *Tepidibacillus* isolated from this iron-reducing enrichment culture, herein designated strain Z9$^T$. We show that this isolate represents a novel species within the phylum *Firmicutes* that is able to reduce the ferric iron minerals ferrihydrite and lepidocrocite, as well as other oxidized transition metals.

Unless otherwise specified, the cultures in this study were prepared using anaerobic culture techniques in 25 ml serum tubes or 100 ml serum bottles. The culture medium (medium MBM) was modified from the basal medium used by Roh *et al.* (2002), in which trace metal solution (Dworkin *et al.*, 2006) and vitamin supplement (ATCC MD-VS; ATCC) were used in place of those in the original protocol. The medium was buffered with 2.5 g $NaHCO_3$ $l^{-1}$ and equilibrated by bubbling with $N_2/CO_2$ (80 : 20, v/v), resulting in a final pH of 7.0–7.2. The serum tubes/bottles were sealed with butyl rubber stoppers and aluminium crimp seals. All the amendments were added from sterilized and anoxic stock solutions using sterile $N_2$-flushed syringes. All the treatments were prepared in triplicate. Abiotic controls were prepared under the same conditions but were not inoculated. The cultures were manually shaken once per day but otherwise incubated statically at 42 °C.

Strain Z9$^T$ was isolated from an iron-reducing enrichment culture initially amended with 10 mM Fe(III)-citrate as the electron acceptor and a mixture of fatty acids (5 mM each of acetate, lactate, pyruvate and formate) plus $H_2$ (5 ml per 25 ml serum tube) as electron donors (Dong *et al.*, 2014b). Before isolation efforts were begun, the enrichment culture had been grown for at least 6 months and was transferred to fresh media every 4–6 weeks.

Isolation was accomplished using anaerobic agar plates containing MBM medium, groundwater (10 % by volume) and 15 g agar $l^{-1}$. Since the parent enrichment demonstrated the ability to reduce nitrate (Dong *et al.*, 2014b), 5 mM nitrate and 5 mM each of formate and acetate were amended to the agar medium as the electron acceptor and electron donors, respectively. Prior to inoculation, the agar plates were kept in an anaerobic chamber (Coy Laboratory Products) filled with an atmosphere of mixed $N_2/H_2$ gas (95 : 5, v/v) for at least 2 days to remove residual $O_2$. Approximately 0.1 ml of the active enrichment culture was streaked on each plate. These inoculated plates were transferred to a Gaspak BBL anaerobic jar (BD Diagnostics), removed from the anaerobic chamber and flushed with a mixture of $N_2$ and $CO_2$ (80 : 20, v/v) prior to being resealed. The sealed anaerobic jar containing the plates was then transferred back to the anaerobic chamber and stored in a 37 °C incubator. Round-shaped beige colonies with smooth edges (0.5–1 mm in diameter) formed on the plates after 1 week of incubation. Three well-separated colonies were picked at random and inoculated into 10 ml of anaerobic liquid MBM medium. The cultures were amended with Fe (III)-citrate (10 mM) as well as a mixture of formate and acetate (5 mM each) as the electron acceptor and electron donors, respectively. When iron reduction was identified by increased Fe(II) concentration determined by ferrozine assay (Gibbs, 1976) and visible colour change (i.e. transition from reddish brown to greenish yellow), the isolation procedure was repeated inside the anaerobic chamber and one colony was picked and grown. The purity of the isolate was confirmed by microscopic examination and by cloning and sequencing the 16S rRNA genes.

Nearly complete 16S rRNA gene sequences of strain Z9$^T$ were determined by Sanger sequencing and were deposited in the NCBI public database (accession nos KP898732 and KP898733). PCR-mediated amplification of the 16S rRNA genes was performed using primers 27F and 1492R as described (Dong *et al.*, 2014b). A clone library of 16S rRNA genes was constructed using pGEM-T Easy Vector Systems (Promega Corporation) following the manufacturer's instructions. Interestingly, the sequenced 16S rRNA reads showed two distinct genes that differed at the 5′ terminus in 92 bp nucleotide positions but are conserved in the remaining fraction (Fig. 1b). To verify that these two different sequences were from a single bacterial genome, a third round of sequential dilution and growth on agar-plate medium was performed. An additional 16S rRNA gene clone library was constructed from a single colony picked from the agar plate. Sequencing reads from 16 randomly

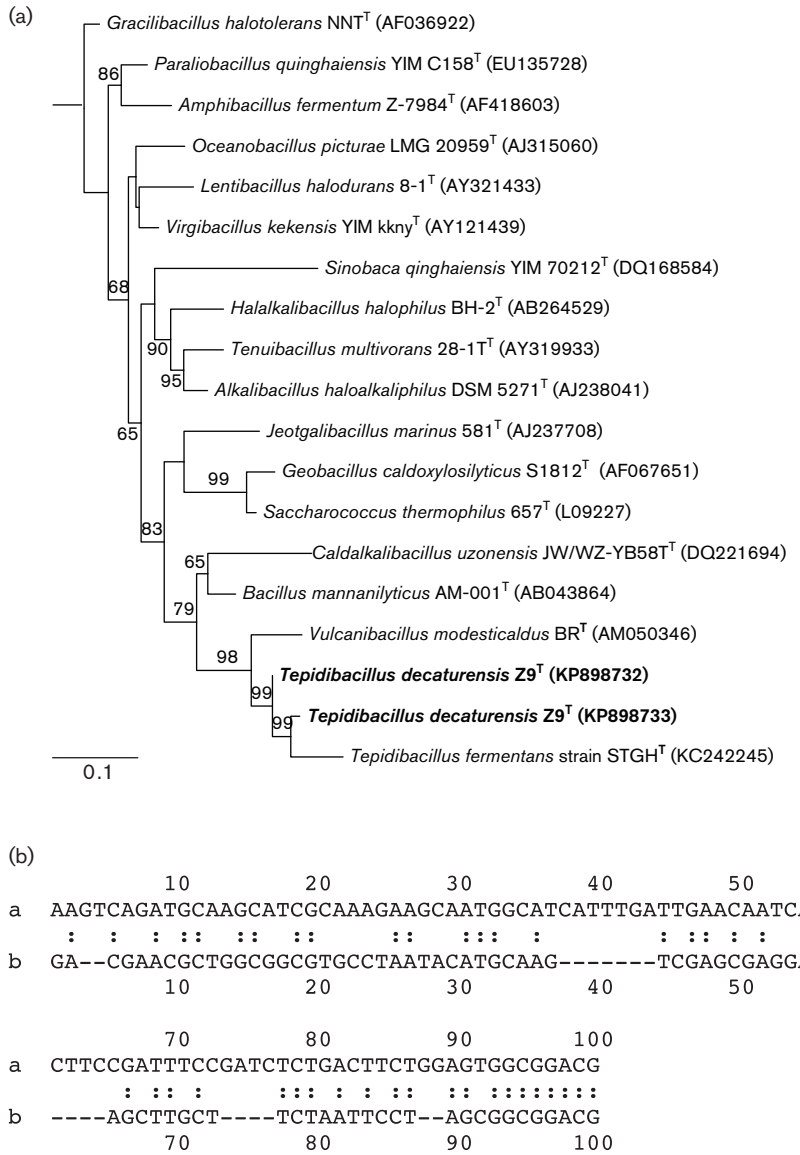


**Fig. 1.** Maximum-likelihood phylogenetic tree (a) based on 16S rRNA gene sequences (~1.5 kb) showing the phylogenetic relationship of the two 16S rRNA genes from strain Z9$^T$ to related species affiliated with the family *Bacillaceae*. Sequence analysis is shown of the intragenomic heterogeneity (b) in the two 16S rRNA genes found in strain Z9$^T$. The NCBI accession numbers of the type strains are listed in parentheses. Statistical confidence for the evolutionary tree was assessed by bootstrap analysis (1000 replicates) and shown as bootstrap values (%). Bar (a), 0.1 changes per nucleotide position. In (b), the same nucleotides at the same positions of aligned sequences are labelled with colons. The values above and below the sequences label every 10 nucleotides for the two sequences, respectively.

picked clones showed the same two distinct 16S rRNA genes (Fig. 1b). This confirmed the intragenomic heterogeneity in the small subunit ribosomal gene for strain Z9$^T$, which has been observed previously in other bacteria (Boucher *et al.*, 2004; Sun *et al.*, 2013).

Phylogenetic analyses were carried out using mothur (Schloss *et al.*, 2009) and a phylogenetic tree of the isolate and related sequences was reconstructed (Fig. 1a) using the maximum-likelihood method RAxML (Mirarab *et al.*, 2015; Stamatakis, 2006). The phylogenetic tree indicates that strain Z9$^T$ robustly branches with *Tepidibacillus fermentans* and *Vulcanibacillus modesticaldus*. Based on the above analyses, strain Z9$^T$ is assigned to the genus *Tepidibacillus*, a member of the family *Bacillaceae* of the phylum *Firmicutes*. Similarity to 16S rRNA genes from *T. fermentans* (97 %) suggests that sufficient differences exist with strain Z9$^T$ to propose that it represents a novel species (Kim *et al.*, 2014; Stackebrandt, 2006; Stackebrandt & Goebel, 1994).

When viewed under a Zeiss Standard 25 microscope (Carl Zeiss AG), cells of strain Z9$^T$ were rod-shaped and motile. Morphological observations and cell-size measurements were made with strain Z9$^T$ fixed and stained using the method given by Roh *et al.* (2002). Further microscopic examination was performed using a Hitachi H600 transmission electron microscope (Hitachi High-Tech). The length of cells of strain Z9$^T$ was highly variable depending on the growth phase, exhibiting dimensions of 0.3×(1–10) μm with a singular polar flagellum. Endospores were observed in the stationary phase of the strain Z9$^T$ cultures under iron-reducing conditions (Table 1, Fig. S1, available in the online Supplementary Material). Based on Gram staining (Tindall *et al.*, 2007), strain Z9$^T$ stained Gram-negative. A further KOH test (Halebian *et al.*, 1981) indicated that Z9$^T$ was a Gram-positive strain. This organism showed negative activity for catalase assayed by mixing a pellet of fresh culture with a drop of $H_2O_2$ (10 %, v/v) and for oxidase when assayed using discs impregnated with dimethyl-*p*-phenylenediamine (Sigma-Aldrich).

The physiological properties of strain Z9$^T$ were evaluated using an approach similar to that previously used for a thermophilic metal-reducing culture (Roh *et al.*, 2002). The tolerance of this organism to temperature, pH, salinity and antibiotics was determined in MBM medium containing 10 mmol $l^{-1}$ of amorphous ferrihydrite as the electron acceptor and acetate (10 mM) as the electron donor, respectively. Un-inoculated control incubations were run in parallel. The bottles were incubated for 3 weeks and growth was assessed by direct cell counts and determination of Fe(II) concentration. Strain Z9$^T$ reduced ferrihydrite over a temperature range 20–60 °C, with the highest iron-reducing activity between 30 and 40 °C. Iron reduction by strain Z9$^T$ occurred in the presence of 1–5 % (w/v) NaCl with optimal growth at about 2.5 % (Table 1). Iron-reducing activity of strain Z9$^T$ was observed at pH 5–8 (optimum pH 5.2–5.8). Iron-reducing activity was assayed in the presence of individual antibiotics, including anisomycin, chloramphenicol, tetracycline, erythromycin and kanamycin (20 μg $ml^{-1}$ for chloramphenicol and 40 μg $ml^{-1}$ for the others) (Mouné *et al.*, 2000). The iron-reducing activity of strain Z9$^T$ was sensitive to chloramphenicol, tetracycline, kanamycin or erythromycin and partially inhibited by ampicillin. No significant decrease in iron-reducing activity compared to the cultures without antibiotic amendment was observed for anisomycin.

The capacity of strain Z9$^T$ to ferment sugars and other organic substrates was examined using the cultures grown on MBM medium amended with one of the test substrates indicated in Table 1. Growth was assessed by measuring $OD_{600}$. Within 48 h of incubation, fructose, galactose, glucosamine, glucose, glycerol, glycine, lactose, maltose, mannitol, mannose, peptone, starch, sucrose and trehalose supported growth of strain Z9$^T$, while no significant growth was observed when betaine, fumarate or glutamate was provided (Table 1). An additional set of cultures was grown in tightly sealed 160 ml serum bottles containing 80 ml of modified MBM medium, in which bicarbonate was removed and the medium was equilibrated with $N_2$ to avoid background $CO_2$/$HCO_3^-$. Glucose (10 mM) fermentation was monitored for ~10 days. The presence of fermentation products (organic acids, alcohols) in culture liquids was verified by using an HPLC system (Agilent Technologies 1200 series) equipped with a Rezex ROA-Organic Acid $H^+$ (8 %) column (Phenomenex). The analytes were eluted with 5 mM $H_2SO_4$ at 42 °C. The gaseous fermentation products (e.g. $CO_2$ and $H_2$) were measured using an SRI 8610C gas chromatograph coupled with a sequential thermal conductivity detector discharged into an SRI Model 810C reduction gas detector (SRI Instruments). End products of glucose fermentation by strain Z9$^T$ were acetate, formate, ethanol, lactate and $CO_2$. No growth of strain Z9$^T$ on glucose was observed under aerobic conditions during incubation with atmospheric concentration of oxygen. To check for microaerobic growth, various amounts of air were injected in the headspace of the serum culture tubes containing the MBM medium prepared anaerobically but without cysteine and $Na_2S$. In the presence of 5 mM glucose, strain Z9$^T$ grew microaerobially when the oxygen concentrations in the gaseous phase were no higher than 15 %.

The ability of strain Z9$^T$ to reduce different inorganic electron acceptors was assessed in MBM medium amended with acetate and one of the following compounds (concentrations are 5 mM unless specified): Co(III)-EDTA (1.5 mM), chromate (0.5 mM), $MnO_2$, nitrate, nitrite (2.5 mM), Fe(III) compounds [i.e. Fe(III)-citrate, lepidocrocite, hematite, goethite] and sulfur compounds (i.e. $S^0$, thiosulfate and sulfate) (Table 1). Growth of the isolate was determined by measuring $OD_{600}$, and the concentrations of the electron acceptors were quantified using HPLC or colorimetric methods (Caccavo *et al.*, 1994; Gibbs, 1976). Similar to its phylogenetic relatives (Slobodkina *et al.*, 2013), strain Z9$^T$ grew using nitrate as an electron acceptor and produced nitrite as the final product. No reduction or growth was observed with nitrite, $S^0$, thiosulfate or sulfate. For the ferric compounds, about 50 % of the Fe(III)-citrate and 12–18 % of the ferrihydrite and lepidocrocite (5 mmol $l^{-1}$) were reduced after about 20 days of incubation. In comparison, hematite and goethite were not reduced during the same incubation period. Strain Z9$^T$ appeared to also reduce transition metals such as Co(III) [Co(III)-EDTA], Cr(VI) (chromate) and Mn(IV) ($MnO_2$) based on the change in colour from yellow/orange to clear, purple to pink and brown to clear, respectively, and compared to abiotic controls. However, cellular growth with these metals was not established. Reduction of iron or other transition metals has not been previously reported for *Tepidibacillus* species, although *T. fermentans* STGH$^T$ has been shown to reduce both $S^0$ and thiosulfate (Slobodkina *et al.*, 2013).

The ability of strain Z9$^T$ to utilize various electron donors to support iron reduction was assessed by amending MBM medium with 10 mmol $l^{-1}$ ferrihydrite and one of a suite of different organic and inorganic substrates at a concentration of 5 mM unless otherwise indicated. The parental strain Z9$^T$ culture that had been grown on ferric citrate (5 mM) and acetate (5 mM) was inoculated into fresh MBM media at a

**Table 1.** Phenotypic characteristics of strain Z9$^T$ and its closest phylogenetic relative

Reference data were taken from L'Haridon *et al.* (2006) and Slobodkina *et al.* (2013).

| Characteristic | Strain Z9$^T$ | *Tepidibacillus fermentans* STGH$^T$ |
|---|---|---|
| Sample characteristics | | |
| Source | 1.7 km below surface, Illinois Basin, IL, USA | Underground gas storage reservoir |
| Morphology (width×length) (μm) | Rod [0.3×(1–10)] | Rod [0.3×(2–4)] |
| Spore formation | + | + |
| Gram stain | – | + |
| G+C content of DNA (mol%) | 36.1 | 34.8 |
| Oxidase | – | – |
| Catalase | – | – |
| NaCl range (optimal) (%) | 1–5 (2.5) | 1–4(1.0) |
| Temperaturerange (optimal) (°C) | 20–60 (30–40) | 36–65 (50–52) |
| pH range (optimal) | 5.2–8 (5.2–5.8) | 5.5–8 (7.0–7.5) |
| Major fatty acids | $C_{15:0}$, ai-$C_{15:0}$, $C_{16:0}$, $C_{18:0}$ | i-$C_{15:0}$, ai-$C_{15:0}$, $C_{16:0}$, i-$C_{16:0}$ |
| Microaerobic growth | + | + |
| Fermentation substrates* | | |
| Betaine | – | No data |
| Fructose | + | + |
| Galactose | + | – |
| Glucose | + | + |
| Glycerol | + | – |
| Lactose | + | – |
| Maltose | + | + |
| Starch | + | – |
| Sucrose | + | – |
| Electron acceptors† | | |
| Nitrate | + | + |
| Nitrite | – | – |
| Fe(III)-citrate | + | – |
| Ferrihydrite | + | – |
| $S^0$ | – | + |
| Thiosulfate | – | + |
| Sulfate | – | – |
| Glucose fermentation products | Formate, acetate, ethanol, lactate, $CO_2$ | Succinate, lactate, acetate |

*5 mM fermentable substrates were amended and growth of strain Z9$^T$ was determined based on $OD_{600}$.
†Substrates were added to achieve final concentrations of 5 mM except for ferric citrate (10 mM) and nitrite (2.5 mM) in the presence of 5 mM acetate as the electron donor; growth of cells was determined based on $OD_{600}$ for the cultures amended with nitrate, nitrite and sulfur compounds; the concentrations of nitrate, nitrite, sulfate and thiosulfate were quantified using HPLC; Fe(II) concentrations were determined using the ferrozine method (Gibbs, 1976).

dilution ratio of 1 : 20 (v/v). Inoculated medium without additional electron donors was used as a control. Strain Z9$^T$ utilized a broad diversity of organic and inorganic substrates coupled to iron reduction. With the exception of glycine, enhanced growth and iron reduction occurred with all the other organic and inorganic substrates tested, including $H_2$ (5 ml per 25 ml serum tube), acetate, benzoate, butyrate, citrate, ethanol, formate, glutamate, lactate, methanol, peptone, phenol (2.5 mM), propionate, pyruvate, succinate and trimethylamine (Fig. 2). In comparison, in the presence of nitrate as the electron acceptor, *T. fermentans* STGH$^T$showed growth with peptone, tryptone, yeast extract, glucose, fructose, maltose, fumarate, lactate, malate, pyruvate and succinate, but not acetate, formate, propionate, butyrate, methanol, ethanol, glycerol, citrate, sucrose, xylose, cellobiose, arabinose, lactose, galactose, starch or carboxymethyl-cellulose (Slobodkina *et al.*, 2013). In addition, strain Z9$^T$ reduced Fe(III) coupled to the fermentation of selected substrates (i.e. fructose, galactose, glucose, lactose, starch, tryptone and yeast extract).

As glucose was consumed in the presence of ferrihydrite, Fe (II), formate, acetate, ethanol, lactate and $CO_2$ were produced, which is slightly different from those by *T. fermentans* (i.e. succinate, lactate and acetate) (Slobodkina *et al.*,

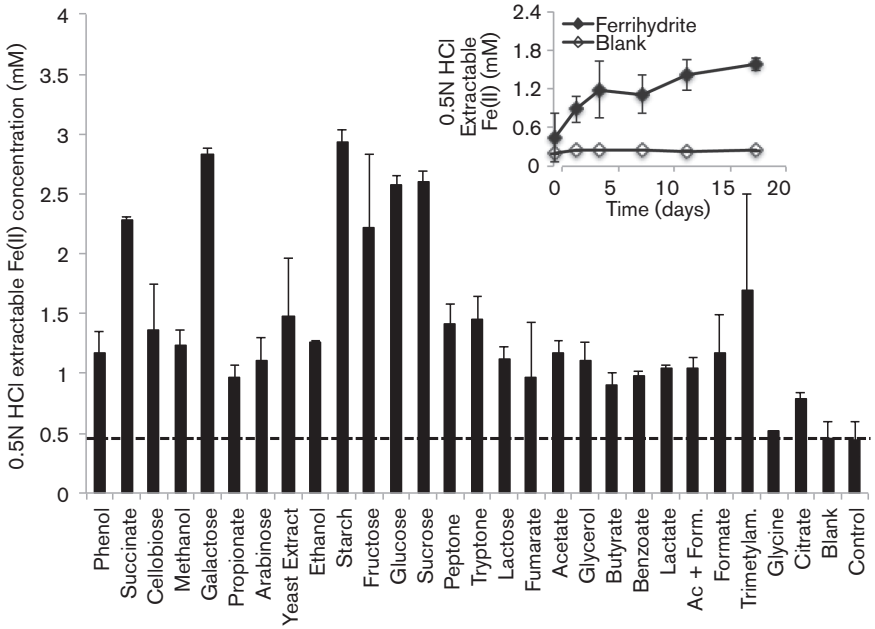


**Fig. 2.** Substrates that supported ferrihydrite reduction by strain Z9$^T$ as illustrated as the final Fe(II) concentrations after 21 days of incubation. 'Blank' indicates the cultures without cell inoculation, while 'Control' indicates the cultures with cell inoculation but without additional electron donors. For each culturing condition, a 'Blank' was prepared. Since similar and low final Fe(II) concentrations were observed in all the blank samples, the condition amended with acetate was selected as representative for illustration. The dashed line shows the final Fe(II) concentrations in the control samples. The inset illustrates production of Fe(II) during ferrihydrite reduction by this organism when acetate was used as the electron donor. The medium contained 0.5 g yeast extract $l^{-1}$; 'Ac+Form.' indicates mixture of acetate and formate (5 mM each).

2013). During concurrent iron reduction and fermentation, iron reduction may act as a minor side reaction for energy production, as indicated by the use of only a small fraction (<5 %) of available electron equivalents for reduction of ferric iron (Boone *et al.*, 1995; Lehours *et al.*, 2010). Thus, ferric iron acts as an electron sink that creates more favourable thermodynamic conditions for coexisting fermentation, which promotes substrate consumption and results in higher biomass production (Lehours *et al.*, 2010; Lovley, 1991; Lovley & Phillips, 1986; Pollock *et al.*, 2007). Quantitative PCR (qPCR) targeting the 16S rRNA genes (Ritalahti *et al.*, 2006) for strain Z9 grown with glucose versus glucose and ferrihydrite showed that the maximal cell concentrations were $(3.1\pm1.3)\times10^7$ and $(3.5\pm1.3)\times10^8$ cells $ml^{-1}$, respectively (Fig. S2). This suggests significantly enhanced growth of strain Z9$^T$ during fermentative iron reduction compared to fermentation alone.

Biomass of strain Z9$^T$ was obtained by cultivation under fermentative conditions with 10 mM glucose at 42 °C. The cellular fatty acid composition was identified using the method of Singh *et al.* (2011). The major fatty acids for strain Z9$^T$ were $C_{15:0}$, ai-$C_{15:0}$, $C_{16:0}$ and $C_{18:0}$ (Tables 1 and 2). $C_{15:0}$ and $C_{24:0}$ were detected for strain Z9$^T$ but not in its phylogenetic relative *T. fermentans* STGH$^T$. In contrast, $C_{14:0}$ and i-$C_{14:0}$ were only detected in *T. fermentans* STGH$^T$ (Tables 1 and 2). The G+C content of the chromosomal DNA of strain Z9$^T$ was determined based on its sequenced genome (Dong *et al.*, 2016). The G+C content of this isolate's DNA was 36.1 mol%, similar to that of *T. fermentans* (34.8 mol%) and *V. modesticaldus* (34.5 mol%) (L'Haridon *et al.*, 2006; Slobodkina *et al.*, 2013).

In summary, strain Z9$^T$ shares many physiological characteristics with its closest phylogenetic relative, *T. fermentans*. However, its clear phylogenetic distance, nutrient profile, and capacity to reduce ferric iron and other inorganic compounds distinguished it from *T. fermentans*. Therefore, we suggest that strain Z9$^T$ represents a novel species of the genus *Tepidibacillus*, for which we propose the name *Tepidibacillus decaturensis* sp. nov.

## Description of *Tepidibacillus decaturensis* sp. nov.

*Tepidibacillus decaturensis* sp. nov. (de.ca.tur.en′sis. N.L. masc. adj. *decaturensis* pertaining to Decatur, Illinois, USA). Cells are motile, rod shaped, 1–10 μm in length by 0.3 μm in width and possess a polar flagellum. Endospores are observed at the stationary phase of iron reduction. Cells are microaerophilic and stain Gram-negative. Growth occurs at 20–60 °C (optimum 30–40 °C), at pH 5–8 (optimum 5.2–5.8) and with salt concentrations of 1–5 % (w/v)

**Table 2.** Cellular fatty acid composition of strain Z9$^T$ and its closest phylogenetic relative

Minor fatty acids comprising <0.1 % for both strains were not included and those comprising >10 % for strain Z9$^T$ are highlighted in bold. Strain Z9$^T$ was grown with 10 mM glucose at 42 °C. Cells of *T. fermentans* STGH$^T$ were grown with 1 g yeast extract $l^{-1}$, 5.6 mM glucose and with nitrate at 50 °C (Slobodkina *et al.*, 2013). –, Not present.

| Fatty acid | Strain Z9$^T$ | *Tepidibacillus fermentans* STGH$^T$ |
|---|---|---|
| $C_{14:0}$ | – | 2.3 |
| i-$C_{14:0}$ | – | 7.8 |
| **$C_{15:0}$** | **14.5** | – |
| i-$C_{15:0}$ | 0.1 | 10.6 |
| **ai-$C_{15:0}$** | **12.8** | 17.1 |
| **$C_{16:0}$** | **31.0** | 33.0 |
| i-$C_{16:0}$ | 0.1 | 19.6 |
| $C_{17:0}$ | 5.5 | 0.8 |
| i-$C_{17:0}$ | 1.8 | 1.5 |
| ai-$C_{17:0}$ | 0.1 | 2.9 |
| **$C_{18:0}$** | **30.7** | 4.5 |
| $C_{24:0}$ | 1.8 | – |

NaCl (optimum 2.5 %). Strain Z9$^T$ reduces soluble and insoluble ferric iron compounds [e.g. Fe(III)-citrate, ferrihydrite and lepidocrocite] and other oxidized metals [e.g. $MnO_2$, Co(III)-EDTA and $Cr_2O_7^{2-}$], while more crystalline goethite and hematite are not reduced. Strain Z9$^T$ is capable of reducing nitrate to nitrite, but cannot reduce nitrite, sulfate, thiosulfate or $S^0$. It supports iron reduction using a broad range of inorganic and organic compounds including $H_2$, acetate, butyrate, citrate, ethanol, formate, fructose, galactose, glucose, glutamate, lactate, lactose, methanol, peptone, phenol, propionate, pyruvate, starch, succinate, trimethylamine, tryptone and yeast extract. Fructose, galactose, glucosamine, glucose, glycerol, glycine, lactose, maltose, mannitol, mannose, peptone, starch, sucrose, trehalose and trimethylamine can also be fermented and support growth.

The type strain, Z9$^T$ (=ATCC BAA-2644$^T$=DSM 103037$^T$), was isolated from 1.7 km depth groundwater of the Illinois Basin, Decatur, IL, USA. The DNA G+C content of the type strain is 36.1 mol%.

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