Extremophiles (2007) 11:33–39
DOI 10.1007/s00792-006-0006-5

ORIGINAL PAPER

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Tindallia texcoconensis sp. nov., a new haloalkaliphilic bacterium isolated from lake Texcoco, Mexico

Received: 8 December 2005 / Accepted: 25 May 2006 / Published online: 24 August 2006
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Abstract A new alkaliphilic and moderately halophilic, strictly anaerobic, fermentative bacterium (strain IMP-300^T) was isolated from a groundwater sample in the zone of the former soda lake Texcoco in Mexico. Strain IMP-300^T was Gram-positive, non-sporulated, motile and rod-shaped. It grew within a pH range from 7.5 to 10.5, and an optimum at 9.5. The organism was obligately dependent on the presence of sodium salts. Growth showed an optimum at 35°C with absence of growth above 45°C. It fermented peptone and a few amino acids, preferentially arginine and ornithine, with production of acetate, propionate, and ammonium. Its fatty acid pattern was mainly composed of straight chain saturated, unsaturated, and cyclopropane fatty acids. The G + C content of genomic DNA was 40.0 mol%. Analysis of the 16S rRNA gene sequence indicated that the new isolate belongs to the genus *Tindallia*, in the low G + C Gram-positive phylum. Phylogenetically, strain IMP-300^T has *Tindallia californiensis*, as closest relative with a 97.5% similarity level between their 16S rDNA gene sequences, but the DNA–DNA re-association value between the two DNAs was only 42.2%. On the basis of differences in genotypic, phenotypic, and phylogenetic characteristics, strain IMP-300^T is proposed as a new species of the genus *Tindallia*, *T. texcoconensis* sp. nov. (type strain IMP-300^T = DSM 18041^T = JCM 13990^T).

Keywords *Tindallia texcoconensis* · Fermentative bacterium · Haloalkaliphilic · Soda lake · Taxonomy

Communicated by G. Antranikian

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Introduction

Soda lakes are highly alkaline aquatic environments, characterized by the presence of high levels of sodium carbonate and their derivatives resulting from evaporative concentration in closed basins (Grant and Jones 2000). Their highly values of pH, ranging from 8 to 11, are associated with varying degrees of salinities and low concentration of both Mg^{2+} and Ca^{2+} ions.

In spite of their extreme nature, soda lakes are among the most productive environments, presumably because of the relative high ambient temperatures, high light intensities and availability of CO_2 in these carbonate-rich waters (Melak and Kilham 1974). Microbial communities of alkaline environments have received increasing attention during two last decades because of the biotechnological potential of their alkalistable enzymes (Horikoshi 1999; Gessesse and Gashe 1997; Martins et al. 2001). Alkaline lakes are usually dominated by dense blooms of cyanobacteria (notably *Spirulina* spp.) and other phototrophic bacteria, which constitute the main primary source of organic matter within these extreme environments. The rest of the microbial community inhabiting alkaline ecosystems comprised a diverse population of halophilic, alkaliphilic, and alkalitolerant representatives of all the major trophic groups of bacteria and archaea (Dubinin et al. 1995; Duckworth et al. 1996; Grant et al. 1999; Jones et al. 1998; Zavarzin et al. 1999).

Although anaerobic decomposition is dominating in these environments, the anaerobic alkaline saline microflora has been less studied than the aerobic one (Jones et al. 1998; Zavarzin et al. 1999). One of the reasons might be that obligate anaerobes were ‘secondary

organotrophs' utilizing products of primary hydrolysis provided by aerobes and facultative anaerobes (Jones and Grant 1999). Anaerobic alkaliphiles consist of fermentative, acetogenic, sulphate-reducing bacteria, and methanoarchaea.

Asaccharolytic acetogens that only ferment amino acids, also called acetogenic ammonifers, have been isolated from soda lakes. They include *Natronoincola histidinovorans* (Zhilina et al. 1998) and *Tindallia magadiensis* (Kevbrin et al. 1998; Validation List 1999) recovered from lake Magadi (Kenya), and *T. californiensis* (Pikuta et al. 2003) recovered from Mono lake (California). All these microorganisms were also related to members of cluster XI of the order *Clostridiales*. They fermented only a few amino acids, peptone and some organic acids and are involved in the anaerobic degradation of proteinaceous material derived from died phototrophic microorganisms (Tindall 1988).

Saline and soda lakes are found throughout Mexico. Lake Texcoco is the last remaining vestige of lakes existing in the pre-Hispanic times in the valley of Mexico. It now occupies only a small area surrounded by salt marshes. Lake Texcoco is a saline lake that has been exploited commercially for decades to produce soda (sodium carbonate and sodium chloride) and until recently *Spirulina* on an industrial scale.

In this paper, we describe a new haloalkaliphilic, anaerobic, proteolytic bacterium isolated from an underground water sample in the former lake Texcoco area in Mexico. Phylogenetic analysis based on 16S rDNA sequences indicates that strain IMP-300^T is proposed to be a novel species of the genus *Tindallia* with the name *Tindallia texcoconensis* sp. nov. (type strain IMP-300^T = DSM 18041^T = JCM 13990^T).

Materials and methods

Origin of samples

Lake Texcoco is located at the northeast (25 km) of Mexico city (geographic coordinates: lat. 19.7°, long. 99°). It has an extension of approximately 5,000 ha, between 2,270 and 2,750 m above the sea level. The water-bearing zone is near the surface (1.2–4 m). The underground water samples were collected from a 3 m deep borehole in the former lake Texcoco area. The water sample was saline (90 g l^{-1}) and alkaline (pH 9.1). It has an electrical conductivity of 0.72 S m^{-1}. The samples were collected under anaerobic conditions, transported at ambient temperature to our laboratory, and stored at 4°C until they were used.

Isolation and culture techniques

Standard anaerobic culture techniques were used throughout this study (Balch et al. 1979; Hungate 1969). Selective medium for isolation included (g l^{-1}): NH_4Cl, 1; K_2HPO_4, 0.3; KCl, 0.1; $CaCl_2$, 0.2; $MgCl_2 \bullet 6H_2O$, 2; NaCl, 100; $NaHCO_3$, 5; Na_2CO_3, 2.5; yeast extract, 1; peptone (Difco), 5; cysteine-HCl, 0.5; resazurin, 0.001; and 10 ml/l trace mineral element solution (Balch et al. 1979). The pH was adjusted to 9.0 with 10 M KOH and the medium was boiled under a stream of O_2-free N_2 gas and cooled to room temperature. $NaHCO_3$, and Na_2CO_3were added after the medium has cooled. Prior to inoculation, $Na_2S \bullet 9H_2O$ was injected from anaerobic sterile stock solutions to final concentrations of 0.04% (w/v). Five ml aliquots were dispensed into Hungate tubes and 20 ml in serum bottles, under a stream of N_2–CO_2 (80:20, v/v) gas and the sealed vessels were autoclaved for 45 min at 110°C. Serum bottles were inoculated with groundwater samples (5% v/v). The bottles were incubated seven days at 35°C. These cultures were subcultured into fresh medium (inoculum, 10%). Isolation was performed in the same medium supplemented with Noble agar (1.6%) (Difco laboratories, Detroit, MI, USA) using the roll tube method (Hungate 1969). The culture was serially diluted tenfold in roll tubes and single colonies that developed were picked according to Hungate (1969) in a N_2 atmosphere. The process of serial dilution was repeated twice. The purity of strains was checked by phase-contrast microscopy.

The medium for cultivation and maintenance was formulated after growth optimization. It contained (g l^{-1}): NH_4Cl, 1; K_2HPO_4, 0.3; KCl, 0.1; $CaCl_2$, 0.2; $MgCl_2 \bullet 6H_2O$, 0.5; NaCl, 75; $NaHCO_3$, 20; Na_2CO_3, 3; yeast extract, 0.5; peptone, 5; cysteine-HCl, 0.5; $Na_2S \bullet 9H_2O$, 0.4; resazurin, 0.001; and 10 ml^{-1} trace mineral element solution (Balch et al. 1979). The pH was adjusted to 9.0 with 10 M KOH.

Morphological studies

Light-microscope examination of morphology was performed using a Nikon Eclipse 800 phase-contrast microscope. Gram reaction was performed by the Hucker staining method (Murray et al. 1994). For electron microscopy, exponentially grown cells were negatively stained with 1% sodium phosphotungstic acid (pH 7.2). Whole cells were observed with a Hitachi model H600 electron microscope at an accelerating voltage of 75 kV. The presence of spores was analyzed by phase-contrast microscopic observations of young and old cultures and pasteurization tests performed at 80, 90, and 100°C for 10 and 20 min.

Determination of physiological characteristics

Growth experiments were performed in duplicate, using Hungate tubes containing the optimized culture medium. When substrate utilization was studied, peptone was omitted and substrates were tested at a final concentration of 10 mM for amino acids and 20 mM for others substrates. Turbidity (580 nm) was used to assess

growth. Amino acid utilization via the Stickland reaction was tested in a basal medium without cysteine but in the presence of 0.2% yeast extract; the end products formed were determined and compared to control without amino acids. Temperature, pH and NaCl ranges for growth were determined in the optimized growth medium. The temperature range for growth was tested from 20 to 55°C. The medium in the Hungate tubes was adjusted prior to sterilization to the desired pH values (between 6.5 and 11) by adding $NaHCO_3$ or Na_2CO_3 from 10 and 8% (w/v) sterile anaerobic solutions, respectively. After autoclaving, the pH of the medium was measured and re-adjusted with sterile KOH if necessary. The strain was subcultured at least once under the same experimental conditions prior to determination growth rates. For studies on the NaCl requirements, different amounts of NaCl were weighted directly into the Hungate tubes (to obtain final NaCl concentrations of 1–20%) prior to dispensing 5 ml growth medium and pH 9 was maintained with 20 mM Tris-base buffer. Dependence upon the sodium ions was tested by substituting potassium salts for the sodium salts in the culture medium. Dependence upon the carbonate ions was tested on a medium in which $NaHCO_3$ and Na_2CO_3 were replaced with equimolar amounts of NaCl and the pH was buffered with 20 mM Tris-base buffer.

Analytical methods

The end products from peptone and amino acid fermentation, and Stickland reaction were determined by HPLC using an Aminex HPX-87H (Biorad) column with 5 mM H_2SO_4 as the motile phase. Ammonium was determined with Nessler's reagent after isothermal microdistillation of free ammonia. Fe (II) was determined with the ferrozine assay after HCl extraction as described by Lovley and Phillips (1988).

Fatty acid analysis

The determination of fatty acids composition of strain IMP-300^T was done at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Fatty acid methyl esters (FAMEs) were extracted from fresh biomass and identified following the procedure recommended by microbial identification system (MIDI, Sherlock microbial identification system version 4.0, MIS operating manual March 2001, Newark, Del.). The MIS system was used to compare the fatty acid methyl esters of strain IMP-300^T with fatty acid patterns stored in MIDI fatty acid database.

DNA sequencing and 16S rRNA gene sequence analysis

The 16S rDNA gene of strain IMP-300^T was amplified using the universal primers Fd1 and Rd1 and its sequence was determined and analysed as described (Maidak et al. 2001; Ben Dhia Thabet et al. 2004; Weisburg et al. 1991).

The nearly complete sequence (1,494 bp) of the 16S rDNA of IMP-300^T was manually aligned with a representative set of 16S rDNA sequences obtained from the GenBank and EMBL databases using the alignment editor BioEdit v 5.0.9 (Hall 1999). Positions of sequence with alignment uncertainties were omitted, and in total 1,344 positions of alignment were used in the analysis. A phylogenetic tree was constructed using various algorithms implemented in the TREECON (van de Peer and De Watcher 1994) and PHYLIP software packages (Felsenstein 1993). The 16S rRNA sequence of strain IMP-300^T was submitted to GenBank under accession number DQ234901.

DNA base composition and DNA–DNA hybridization

The G + C content of the DNA was determined at the DSMZ. DNA was isolated and purified by chromatography on hydroxyapatite and its G + C content was determined by using HPLC as described by Mesbash et al. (1989). DNA–DNA hybridization was performed at DSMZ as described by De Ley et al. (1970) with modifications reported by Escara and Hutton (1980) and Huss et al. (1983), using a model 2600 spectrophotometer equipped with a model 2527-R thermoprogrammer and plotter (Gilford instrument laboratory inc, Oberlin, Ohio, USA). Renaturation rates were computed with the TRANSFER.BAS program (Jahnke 1992).

Results

Morphology

The colonies obtained in roll tubes were round, white in colour, and 2–4 mm in diameter after seven days incubation at 35°C. Several strains similar in morphology were isolated in pure cultures and maintained in liquid medium. One of them, strain IMP-300^T, which showed the best growth, was chosen for further characterization.

Strain IMP-300^T was a Gram positive, thin straight rod, 3–5 μm in length and about 0.5 μm in diameter, occurring singly or in pair (Fig. 1a). Cells were motile by means of peritrichous flagella (Fig. 1b). Ultrathin sections revealed a rather thin, Gram-positive type, homogenous cell wall (results not shown). Sporulation has never been observed.

Physiological characteristics

Strain IMP-300^T was a strictly anaerobic bacterium. It was mesophilic and grew at temperatures ranging from 25 to 45°C, with an optimum at 35°C. The strain

required NaCl, and it did not grow when NaCl was replaced by equimolar amount of Na_2CO_3 + $NaHCO_3$. Strain IMP-300^T did not grow when potassium salts replaced sodium-containing salts. The isolate was moderately halophilic and grew in the presence of NaCl concentrations ranging from 25 to 250 g l^{-1}, with an optimum between 50 and 100 g l^{-1}. It was alkaliphilic; the optimum pH for growth was 9.5 and growth occurred between pH 7.5 and 10.5.

The nutritional characteristics of strain IMP-300^T were found to be restricted to a limited number of amino acids and some organic acids (Table 1). Strain IMP-300^T possessed fermentative metabolism. It grew on peptone, casaminoacids, L-arginine, L-histidine, L-alanine, L-lysine, L-ornithine, L-threonine, pyruvate, and citrate as substrates. No growth was detected on glucose, fructose, mannose, galactose, mannitol, starch, acetate, formate, propionate, butyrate, malate, succinate, fumarate, lactate, ethanol, methanol, glycerol, choline, betaine, methylamine, trimethylamine, L-cystine, L-isoleucine, L-leucine, L-proline, L-serine, L-valine, L-tryptophan, glycine, L-aspartate, L-glutamine, D-histidine, or D-threonine. It reduced Fe^{3+} to Fe^{2+} but was incapable of dissimilatory reduction of nitrite, nitrate, or sulfur compounds (thiosulfate and sulfate). Growth of strain IMP-300^T in medium containing yeast extract (5 g l^{-1}) as the only organic substrate was extremely poor.

The main products of amino acids fermentation were acetate, propionate and hydrogen. With peptone as the substrate, isovalerate was detected, ethanol being a very minor fermentation product. The new isolates was capable of respiration by the Stickland reaction on the limited amino acid pairs: L-leucine + L-proline, L-leucine + L-tryptophan, L-isoleucine + glycine, L-isoleucine + L-proline, and L-isoleucine + L-tryptophan.

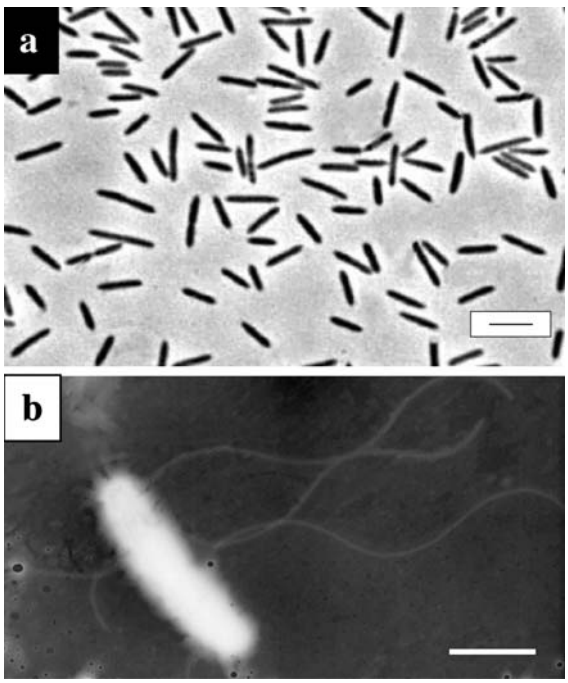

Fig. 1 Morphology of strain IMP-300^T: **(a)** vegetative cells of a culture under phase-contrast microscope, *bar* = 5 μm; **(b)** negatively stained cells with peritrichous flagella, *bar* = 1 μm

Fatty acid analysis

The cellular fatty acid composition of strain IMP-300^T is summarized in Table 2. For comparison, lipid profiles previously described of *Tindallia californiensis* and *T. magadiensis* are included. The fatty acid pattern of our isolate is mainly composed of straight chain saturated, unsaturated and cyclopropane fatty acids. High amounts of dimethylacetals (DMA) were found in addition. A small percentage of aldehydes were present too. This composition of fatty acids differs from the fatty acid patterns of the two previously described *Tindallia*. Furthermore, the MIS system could not assign strain IMP-300^T to any fatty acid patterns stored in MIDI fatty acids database.

16S rRNA gene sequence analysis

Phylogenetic analysis of the almost complete sequence (1,494 base pairs) of the 16S rDNA gene of strain IMP-300^T was compared with all sequences presently available in the GenBank and RDPII databases and showed its affiliation to cluster XI of the order Clostridiales. It was most closely related to the recently described *T. californiensis* (97.5% similarity) isolated from sediments of the Mono lake, USA (Pikuta et al. 2003) than to *T. magadiensis* (96.4% similarity) isolated from lake Magadi, Kenya (Kevbrin 1998). The phylogenetic dendrogram shows the relationships between strain IMP-300^T and the closely related described species (Fig. 2). The cluster consisting of *T. magadiensis*, *T. californiensis*, and strain IMP-300^T was strongly supported by maximum like-hood and by bootstrap analysis (distance matrix method).

G + C content and DNA–DNA relatedness

The G + C content of the DNA was 40.0 mol% as determined by HPLC method, comparable to values quoted for the other members of the genus *Tindallia*, *T. magadiensis* (37.6%) and *T. californiensis* (44.4%). DNA–DNA homology between strain IMP-300^T and *Tindallia californiensis* was 42.2%, indicating that IMP-300^T represents a distinct species within the genus *Tindallia*.

Discussion

In soda lakes, the anoxygenic decomposition of the biomass produced by microbial photosynthetic blooms

Table 1 Main characteristics differentiating strain IMP-300^T from the two other species of the genus *Tindallia*

Characteristics	IMP-300Ta	*Tindallia californiensis*[b]	*Tindallia magadiensis*[c]
Cell size (μm)	0.4–0.6 × 3–5	0.55–0.7 × 1.7–3.0	0.5–0.6 × 1.2–2.5
Sporulation	−	+	−
Temperature (°C) range (optimum)	25–45 (35)	10–48 (37)	19–47 (37)
pH range (optimum)	7.5–10.5 (9.5)	8–10.5 (9.5)	7.5–10.5 (8.5)
NaCl (%) range (optimum)	2.5–25 (7.5)	1–20 (3)	1–10 (3–6)
Substrates			
Peptone	+	+	+
Casaminoacids	+	+	*ND*
L-Arginine	+	+	+
L-Ornithine	+	*ND*	+
L-Alanine	+	−	−
L-Histidine	+	+	+
L-Threonine	+	−	(+)
L-Serine	−	+	(+)
L-Lysine	+	+	*ND*
L-Glutamine	−	−	(+)
L-Asparate	−	−	(+)
Glycine	−	−	(+)
Pyruvate	+	+	+
Citrate	+	−	+
DNA G + C mol%	40.0	44.4	37.6

Tests scored as: +, positive; −, negative; (+), weak growth; *ND* not determined.
[a]This study
[b]Data from Pikuta et al.(2003)
[c]Data from Kevbrin et al.(1998)

Table 2 Fatty acid methyl ester profiles of strain IMP-300^T, Tindallia *californiensis*, and *Tindallia magadiensis*

Compounds	Total fatty acid (%)		
	IMP-300$^{T\ a}$	*T. californiensis*[b]	*T. magadiensis*[c]
C12: 0	0.75		
C13: 0	1.07		
C14: 0	6.47		1.8
C15: 0	2.98		2.3
C15: 1			1.5
C16: 0	12.09	13.5	4.8
C16: 1 ω7c	10.23	4.47	
C16: 1 ω9c	2.74	35.44	33.4
C15: 0 DMA	2.11		
C16: 0 DMA	22.93	15.32	
C17: 0 DMA	1.77		
C18: 0 DMA		10.05	0.3
C16: 1 ω9c DMA	1.44		
Aldehyde-C15			
Aldehyde-C16: 1 Δ 9			1.7
Aldehyde-C16: 1 Δ 11			0.5
Aldehyde-C16	4.92		0.7
C17: 1 Δ9			25.5
C17: 1 Δ11			2
C18: 0	1.03		3.7
C17 cyclopropane	12.68		
C18: 1 ω9c	1.95		

[a]This study
[b]Data from Pikuta et al. (2003)
[c]Data from Zhilina et al. (1998)

is performed by an anaerobic community adapted to high alkaline environments (Dubinin et al. 1995). Strain IMP-300^T was isolated from an alkaline groundwater in the zone of the former soda lake Texcoco. Phenotypically, it was a representative of the alkaliphilic moderately halophilic proteolytic microorganisms of the anaerobic community inhabiting soda lake environments. Growth characteristics of the isolate with regard to its pH and salinity optima for growth fit the physicochemical conditions existing in lake Texcoco, suggesting that this bacterium is indigenous to this aquatic environment. Its taxonomical assignment to the genus *Tin-*

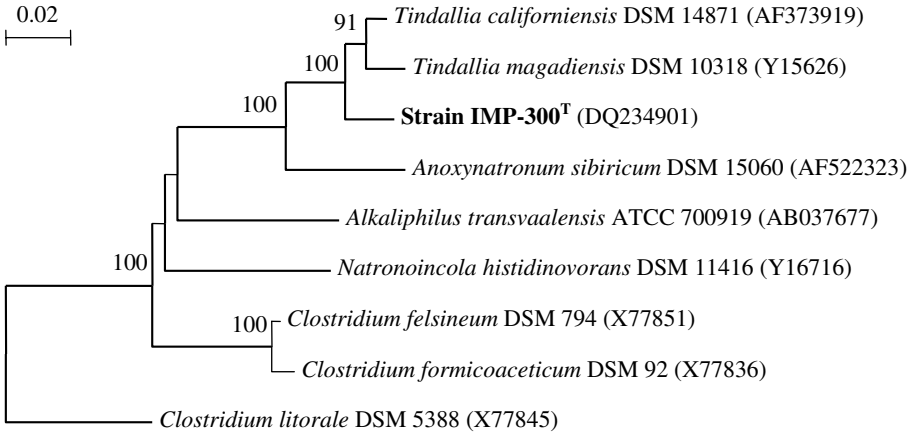

Fig. 2 Phylogenetic dendrogram based on 16S rDNA comparison indicating the position of strain IMP-300^T amongst the cluster XI (*Clostridium felsineum* group) of the order *Clostridiales* and related bacteria. *Clostridium litorale* was taken as outgroup. Bar corresponds to two nucleotide substitutions per 100 nucleotides. Bootstrap values, expressed as a percentage of 100 replications, are shown at branching points. Only values above 80% are shown. Only values above 90% were considered significant and therefore reported

dallia, within the cluster XI of the order *Clostridiales*, is ascertained by phylogenetical studies of the 16S rDNA gene sequence, *T. californiensis* being its most closely phylogenetic relative (similarity of 97.5%). However, phenotypic differences were observed between strain IMP-300^T and both *Tindallia* spp. In contrast to *T. californiensis*, strain IMP-300^T (a) uses L-alanine and L-histidine, but not L-serine, (b) does not produce spores, and (c) is motile (Table 1). Major differences between strain IMP-300^T and *T. magadiensis*, the second representative of the genus *Tindallia* are also reported in Table 1. In addition, strain IMP-300^T exhibited a different fatty acid pattern from the two *Tindallia* sp. described so far (Table 2). This isolate differs not only phenotypically but also genotypically from *T. californiensis*, its closest phylogenetic relative, as the latter microorganism has (a) a lower G + C content in the DNA, and (b) a low DNA–DNA hybridization relatedness with our isolate (< 70%) (Wayne et al. 1987). Therefore, based on the overall characteristics of strain IMP-300^T described above, we propose it to be assigned to a novel species of the genus *Tindallia*, *T. texcoconensis* sp. nov.

Description of *Tindallia texcoconensis* sp. nov.

Tindallia texcoconensis (tex.co.co.nen'sis N. L. adj. *texcoconensis* from Texcoco lake, Mexico from which this microorganism was isolated). Thin straight rods, with a mean of 3–5 μm in length and a width of about 0.4–0.6 μm. Usually occurring single or in pairs motile by peritrichous flagella; Gram-positive, strictly anaerobic, asporogenous, alkaliphilic; the optimum pH for growth is 9.5, with the range of pH 7.5–10.5. Obligately depends on sodium ions. The optimal concentration of NaCl is between 50 and 100 g l^{-1}. Mesophile; growth occurs at temperatures from 25 to 45°C with optimum at 35°C. Under optimal growth conditions, the generation time is 28 h. Fermentative; uses only a few organic compounds as substrates: arginine, ornithine, alanine, peptone, casamino-acids, pyruvate, and citrate. Reduces Fe (III) to Fe (II) with peptone as electron donor. The fatty acid pattern is mainly composed of straight chain saturated, unsaturated and cyclopropane fatty acids. The G + C content of genomic DNA is 40.0 mol% (by HPLC method). Belongs to the branch of low G + C clostridia.

Habitat: type strain has been isolated from underground water sample near the athalassic saline alkaline Texcoco lake in Mexico. The type strain is IMP-300^T, deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig as DSM 18041^T, and at the Japan collection of microorganisms as JCM 13990^T.

The GenBank accession number for the 16S rDNA sequence of strain IMP-300^T is DQ234901.

Acknowledgments This work was partially supported by project No 31371-B from Consejo Nacional de Ciencia y Tecnología (CONACyT), Mexico.

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