Arch Microbiol (2017) 199:1303–1309
DOI 10.1007/s00203-017-1403-0

ORIGINAL PAPER

Bacillus alcaliphilum sp. nov., a bacterium isolated from a soda lake

Vishnuvardhan Reddy Sultanpuram[1] · Thirumala Mothe[1] · Sasikala Chintalapati[2] · Venkata Ramana Chintalapati[3]

Received: 18 April 2017 / Revised: 15 June 2017 / Accepted: 16 June 2017 / Published online: 26 June 2017

Abstract Two novel (14B^T and 7B) Gram-stain-positive, rod-shaped, motile and endospore-forming bacterial strains were isolated from Lonar soda lake, India. Based on 16S rRNA gene sequence analysis, the strains 14B^T and 7B were identified as belonging to the class *Firmibacteria* and were most closely related to *Bacillus halodurans* LMG 7121^T (99.7 and 99.8%, respectively), *Bacillus okuhidensis* LMG 22468^T (99.1 and 99.2%, respectively) and other members in the genus *Bacillus* (<97.0%). However, the DNA–DNA relatedness studies indicated that the strains 14B^T and 7B were distantly related to *B. halodurans* LMG 7121^T (49.1 ± 0.6 and 45.7 ± 0.6, respectively) and *B. okuhidensis* LMG 22468^T (40.9 ± 0.9 and 42.1 ± 0.5, respectively). The high 16S rRNA gene sequence similarity (99.9%) and DNA–DNA relatedness (88 ± 9) indicated that strains 14B^T and 7B were members of a single species. The strains grew optimally at a pH of 9.0–9.5 with 2–5% (w/v) NaCl and temperature of 37 °C. Strains 14B^T and 7B were catalase positive and oxydase negative. The cell wall of strain 14B^T contained *meso*-diaminopimelic acid as the diagnostic diamino acid. Polar lipids include diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), an unknown aminophospholipid (APL1) and three unknown lipids (L1–3). The predominant isoprenoid quinone is MK-7. *anteiso*-$C_{15:0}$ (30.8%) was the predominant fatty acid, and significant proportions of *iso*-$C_{15:0}$ (24.9%), *iso*-$C_{16:0}$ (17.9%) and *anteiso*-$C_{17:0}$ (12.3%) were also detected in strains 14B^T and 7B. The DNA G+C content of strains 14B^T and 7B was 41.6 and 41.3 mol%, respectively. The results of molecular, physiological and biochemical tests allowed a clear differentiation of strains 14B^T and 7B from all other members of the genus *Bacillus*, for which the name *Bacillus alcaliphilum* sp. nov. is proposed. The type strain is 14B^T (=KCTC 33777^T = CGMCC 1.15474^T).

Keywords *Bacillus alcaliphilum* sp. nov · Polyphasic taxonomy · Lonar soda lake

Introduction

The cells of the genus *Bacillus* are rod-shaped, straight or slightly curved, occurring singly and in pairs, some in chains and occasionally as long filaments. Endospores are formed in the cells of the members of the genus *Bacillus*; these spores are very resistant to many adverse conditions. Members of the genus are mostly Gram-stain-positive; however, they may be Gram-stain-positive only in early

Communicated by Erko Stackebrandt.

The EMBL accession numbers for the 16S rRNA gene sequences of strains 14B^T and 7B are LT617054 and LT837688, respectively. The DPD Taxonumber of the type strain 14B^T is TA00127.

Electronic supplementary material The online version of this article (doi:10.1007/s00203-017-1403-0) contains supplementary material, which is available to authorized users.

✉ Vishnuvardhan Reddy Sultanpuram
svr.micro@gmail.com

[1] Microbial Ecology Laboratory, Department of Applied Biosciences, Mahatma Gandhi University, Anneparthy, Yellareddygudem (PO), Nalgonda 508254, India

[2] Bacterial Discovery Laboratory, Centre for Environment, Institute of Science and Technology, J. N. T. University, Kukatpally, Hyderabad 500085, India

[3] Department of Plant Sciences, School of Life Sciences, University of Hyderabad, P.O. Central University, Hyderabad 500046, India

stages of growth and later exhibit Gram-stain-negative characteristic. A *meso*-diaminopimelic acid (*m*-DAP) direct murein cross-linkage type is most common, but L-Lys-D-Glu (Rheims et al. 1999; Nakamura et al. 2002), Orn-D-Glu (Abd El-Rahman et al. 2002) and L-Orn-D-Asp (Suresh et al. 2004) have also been occasionally reported. Most members of the genus are motile by means of peritrichous or degenerately peritrichous flagella, but sometimes they are even non-motile. The cells are mostly aerobes or facultative anaerobes, but a few species are described, which are strictly anaerobic. The terminal electron acceptor is mostly oxygen, but at times is replaced by alternatives in some species. The genus members have a wide diversity of physiological abilities, ranging from psychrophilic (e.g. *Bacillus insolitus*, now reclassified as *Psychrobacillus insolitus; Bacillus psychrosaccharolyticus*, etc) to thermophilic (e.g. *Bacillus schlegelii* and *Bacillus infermus*) and acidophilic (e.g. *Bacillus tusciae,* now reclassified as *Kyrpidia tusciae*; *Bacillus acidocaldarius*, now reclassified as *Alicyclobacillus acidocaldarius*; *Bacillus acidicola*, etc) to alkaliphilic (e.g. *Bacillus alcalophilus*; *Bacillus algicola* and *Bacillus clarkii*); some strains are salt tolerant (e.g. *Bacillus salitolerans* and *Bacillus solisalsi*), and some are halophilic (e.g. *Bacillus taeanensis* and *Bacillus chungangensis*). Catalase is produced by most species of the genus, but oxydase may be positive or negative. Members of the genus are predominantly isolated from soil or from environments that may have been contaminated directly or indirectly by soil, but also found in water, food and clinical specimens (Logan and De Vos 2009).

During the study of cultivable bacterial diversity from the alkalophilic Lonar soda lake, India, strains 14B^T and 7B were isolated. The present study focuses on the taxonomic position of the strains 14B^T and 7B based upon the polyphasic approach.

Materials and methods

Isolation and maintenance of cultures

Strains 14B^T and 7B were isolated from the alkaline Lonar Lake, located at Buldhana, Maharashtra, India (latitude 19°58′, longitude 76°36′), which is a unique basaltic rock meteorite impact crater, situated in the volcanic Deccan trap geological region. One gram of air-dried sediment (at the time of sample collection, the sample had a pH of 10.0, salinity of 5.0% and temperature of 31 °C) was serially diluted up to 10^{-8} in sterile saline solution (0.4%, NaCl solution), and 100 µl of the same was spread on a alkaline nutrient agar medium (pH 10.0) consisting of (g/L) peptone (5), NaCl (5), beef extract (1.5), yeast extract (1.5), agar (15) in 1 l $NaHCO_3$/Na_2CO_3 buffer (100 mM in deionized water). Pure cultures of strains 14B^T and 7B were obtained by repeated streaking of the isolates on alkaline nutrient agar plates. Pure cultures were then preserved in 15% glycerol stock solution at 4 °C for further use.

Morphological and biochemical characterization

The phenotypic characters of strains 14B^T and 7B were characterized following the minimum standards for describing new taxa of aerobic, endospore-forming bacteria recommended by Logan et al. (2009). Morphological properties, such as cell shape, cell size and motility (hanging drop method), were observed by phase-contrast light microscopy (Magnus MLX). Flagellum staining was performed as described by Kodaka et al. (1982). The pH (range 6–12, with an interval of 0.5 were tested with K_2HPO_4–KH_2PO_4 buffer for pH 6.0–8, $NaHCO_3$–NaOH buffer for pH 8.5–11 and $NaCO_3$–NaOH buffer for pH 11.5–12). The pH tests were conducted in triplicate, and the results reported were an average value of the two highest values found during the tests. The temperature (0, 4, 10, 16, 20, 28, 35, 37, 40, 45, 50, 55 and 60 °C) and salt concentration (0–25% w/v, with an interval of 0.5% w/v) ranges for growth were examined in LB broth medium, and the results were recorded after 48 h of incubation. The tests for temperature and salt concentration were performed in the medium emended with 10% Na_2CO_3 so as to get pH 9.0. Growth under anaerobic conditions was determined on modified NA supplemented with 0.5% (w/v) glucose and with or without 0.1% (w/v) nitrate using the anaerobic systems (Himedia). Various biochemical tests, such as hydrolysis of starch, tyrosine, xanthine, hypoxanthine, casein and gelatin, as well as urease, nitrate reduction, Voges–Proskauer test, methyl red test, H_2S production, indole production, oxydase and catalase activities, were carried out as mentioned by Smibert and Krieg (1981) and Oren et al. (1997) in the alkaline nutrient medium or the specified medium for these tests. In both media, the buffer was autoclaved separately and added after sterilization. Utilization of various substrates as sole carbon and energy sources or carbon, nitrogen and energy sources was determined using a basal medium with the following composition (g/L): yeast extract, 0.01; KNO_3, 1.0; KH_2PO_4, 1.0; $MgSO_4$ $7H_2O$, 0.2; $(NH_4)_2HPO_4$, 1.0; NaCl, 80; Na_2CO_3, 20. To this liquid medium, a 0.1% (w/v) filter-sterilized substrate was added. Carbohydrates were used at a final concentration of 0.2% (w/v). Antibiotic sensitivity tests were performed by growing the strain as a lawn on the alkaline nutrient agar plate and inserting discs containing ciprofloxacin (5 µg), amikacin (30 µg), gentamicin (120 µg), vancomycin (30 µg), tetracycline (30 µg), chloramphenicol (20 µg), streptomycin (10 µg), penicillin (10 µg), ampicillin (30 µg), erythromycin (15 µg), kanamycin (30 µg) and nalidixic acid (30 µg). The zone of

inhibition was measured to identify the antibiotic effect on the strain.

16S rRNA gene sequencing, phylogenetic analysis, DNA–DNA relatedness and G+C composition determination

Genomic DNA was extracted and purified according to the method of Marmur (1961). The 16S rRNA gene sequences of strains 14B^T and 7B were obtained by PCR as described earlier (Vishnuvardhan Reddy et al. 2013). Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the EzBioCloud server (http://www.ezbiocloud.net/; Yoon et al. 2016). The CLUSTAL_W algorithm of MEGA 5 (Tamura et al. 2011) was used for sequence alignments and the phylogenetic analysis of the near-complete (~1450 bp) sequence of the 16S rRNA gene. Distances were calculated by using the Kimura correction in a pairwise deletion manner (Kimura 1980). Neighbour-joining (NJ), minimum evolution (ME), maximum likelihood (ML) and maximum parsimony (MP) methods in the MEGA 5 software (Tamura et al. 2011) were used to construct phylogenetic trees. Percentage support values were obtained using a bootstrap procedure. The taxonomic relationship between strains 14B^T, 7B, *B. halodurans* LMG 7121^T and *B. okuhidensis* LMG 22468^T was examined using DNA–DNA hybridization, which was determined using a membrane filter technique (Tourova and Antonov 1987), using Nick translation kit (code no. LCK-1) supplied by BRIT, Jonaki, CCMB campus, Hyderabad. Hybridization was performed with three replications for each sample (control: reversal of strains was used for binding and labelling). α- P^{32} dCTP was used for labelling the probe. The DNA immobilized on the blots (nylon membranes) was probed with labelled DNA and then exposed to phosphor-imaging screen (Amersham Biosciences). The phosphor-imaging screen was scanned and quantified using a Typhoon (3480) variable mode imager. The per cent hybridization is calculated according to the formula: %Hybridization = (Counts obtained from heterologous *hybridization*/counts obtained from homologous *hybridization*) × 100. The mol% G+C of the DNA of strains 14B^T and 7B was determined by HPLC (Mesbah et al. 1989).

Chemotaxonomic characterization

Fatty acids, quinones and polar lipids of strain 14B^T and reference strains were analysed from cells grown in nutrient broth medium at 37 °C with 9.5 pH and 5% (w/v) NaCl. Cells were harvested by centrifugation (10,000*g* for 15 min at 4 °C) on reaching a cell density of 70% of the maximum optical density (100% = 0.8 OD540), and the lyophilized pellet was used for analysis. Cellular fatty acids, polar lipids, quinones and peptidoglycan of strain 14B^T and reference strains were extracted and analysed as described previously (Reddy et al. 2015).

Results and discussion

Morphological and biochemical characterization

Strains 14B^T and 7B form colourless colonies with entire margin in contrast to *B. halodurans* LMG 7121^T and *B. okuhidensis* LMG 22468^T. Further, the spore shape, position and the sporangium type (Table 1) were not similar to that of the reference strains (Nielsen et al. 1995 and Li et al. 2002, respectively). The substrates that supported the growth of the strains and the other biochemical characteristics of the strains are mentioned in the species description. Strains 14B^T and 7B were sensitive to ciprofloxacin (5 µg), gentamycin (120 µg), vancomycin (30 µg), tetracycline (30 µg), chloramphenicol (20 µg), streptomycin (10 µg), penicillin (10 µg), ampicillin (30 µg) and resistant to erythromycin (15 µg), kanamycin (30 µg) and nalidixic acid (30 µg). However, the two strains varied in their reaction to amikacin (30 µg); strain 14B^T was resistant to it, whereas strain 7B showed sensitivity to this antibiotic. The differentiating phenotypic properties of strain 14B^T from the related species of the genus *Bacillus* are summarized in Table 1.

16S rRNA gene sequencing and phylogenetic analysis

The almost complete 16S rRNA gene sequences (1478 and 1443 bp) of strains 14B^T and 7B were obtained. The results of phylogenetic analysis of the 16S rRNA gene sequences show that the strains 14B^T and 7B form a cluster with their nearest *Bacillus* neighbours *B. halodurans* LMG 7121^T and *B. okuhidensis* LMG 22468^T (a composite tree is shown in Fig. 1). EzBioCloud server search analysis revealed that strains 14B^T and 7B were most closely related to *B. halodurans* LMG 7121^T (99.7 and 99.8%, respectively), *B. okuhidensis* LMG 22468^T (99.1 and 99.2%, respectively) and other members in the genus *Bacillus* (<97.0%). Further, the DNA–DNA hybridization results suggest that the strains 14B^T and 7B are distantly related to *B. halodurans* LMG 7121^T (49.1 ± 0.6 and 45.7 ± 0.6, respectively) and *B. okuhidensis* LMG 22468^T (40.9 ± 0.9 and 42.1 ± 0.5, respectively), and the hybridization values are within the recommended standards to delineate a bacterial species (Stackebrandt and Goebel 1994; Stackebrandt and Ebers 2006; Meier-Kolthoff et al. 2013). However, high 16S rRNA gene

Table 1 Characteristics used to distinguish strain 14B^T from the type strains of phylogenetically related *Bacillus* species

Characteristic	1	2	3
Spore shape	Oval	Ellipsoidal	Ellipsoidal
Spore location	T	ST	T
Sporangium	Slightly swollen	Swollen	Unswollen
NaCl range (%, w/v)	2–24	2–15	2–12
NaCl optimal (%, w/v)	2–5	5–10	5–10
pH range	7.0–10.5	7.0–10.5	6.0–10.5
pH optimal	9.0–9.5	8.0–10.0	9.5
Temp range (°C)	15–50	15–55	30–60
Temp optimal (°C)	37	37	37–45
Oxydase	–	+	+
Catalase	+	+	+
Nitrate reduction	+	–	+
Hydrolysis of			
Hippurate	–	+	–
Cellulose	+	+	–
Tween 20	–	+	–
Gelatin	+	+	+
Urea	+	–	–
Arginine dihydrolase activity	+	–	+
H_2S production	+	–	+
Indole production	+	–	+
Voges–Proskauer test	+	+	–
Utilization of			
Cellobiose	+	±	+
Sorbitol	–	–	+
L-arabinose	+	–	+
Inositol	–	+	+
Lactose	+	–	+
D-mannose	+	+	–
Raffinose	+	–	+
Major fatty acids	*anteiso*-$C_{15:0}$, *iso*-$C_{15:0}$, *iso*-$C_{16:0}$, and *anteiso*-$C_{17:0}$.	*anteiso*-$C_{15:0}$, *iso*-$C_{15:0}$, *iso*-$C_{16:0}$, *iso*-$C_{17:0}$ and *anteiso*-$C_{17:0}$	$C_{16:0}$, *anteiso*-$C_{15:0}$, *iso*-$C_{15:0}$, *iso*-$C_{13:0}$-OH and summed feature 4
Major polar lipids	DPG, PG, PE, APL1, L1-3	DPG, PG, PE, APL2, L3-4	DPG, PG, PE, APL3, L5-7
G + C content (mol%)	41.6	42.8[a]	41.0[a]
Source	Soda lake sediment	Soil[a]	Hot water from spa[a]

Strains 1, 14B^T; 2, *B. halodurans* LMG 7121^T; 3, *B. okuhidensis* LMG 22468^T. "+" positive; "−" negative

All data were obtained from this study unless indicated otherwise. All strains are endospore-forming, motile, Gram-stain-positive, facultative anaerobic rods. All strains are positive for hydrolysis of casein, gelatin and starch, but negative for nitrite reduction, methyl red test, hydrolysis of DNA, activities of phenylalanine deaminase and ornithine decarboxylase. All the strains were able to utilize D-fructose, melibiose, D-salicin, sucrose and D-xylose. All the strains contained *m*-Dpm as the diagnostic diamino acid and MK-7 as the major quinone

[a] Data from Nielsen et al. 1995 and Li et al. 2002, respectively

sequence similarity (99.9%) and DNA–DNA relatedness (88 ± 9) indicated that strains 14B^T and 7B were the members of a single species. The mol% G + C content of the DNA of strains 14B^T and 7B was 41.6 and 41.3%, respectively, which was similar to that of the nearest phylogenetic neighbours.

Chemotaxonomic characterization

Whole-cell fatty acid analysis of strain 14B^T revealed that *anteiso*-$C_{15:0}$ (30.8%) was the predominant fatty acid. However, significant proportions of *iso*-$C_{15:0}$ (24.9%), *iso*-$C_{16:0}$ (17.9%) and *anteiso*-$C_{17:0}$ (12.3%) were also

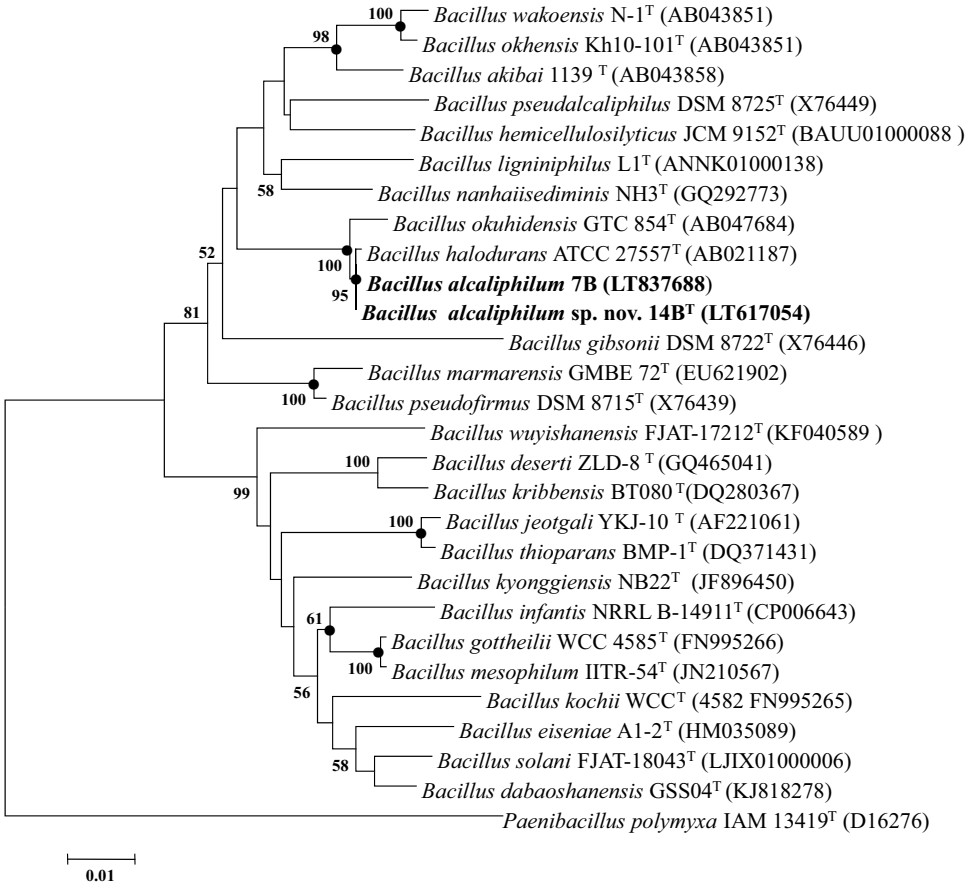

Fig. 1 Phylogenetic analysis of strains 14B^T and 7B with other closely related members based on 16S rRNA gene sequences available from the EMBL database (accession numbers are given in *parentheses*). Multiple alignments, distance calculations (distance options according to the Kimura two-parameter model) and clustering with the neighbour-joining method were performed by using the software package MEGA version 5 (Tamura et al. 2011). Bootstrap values based on 1000 replications are listed as *percentages* at the branching points. *Bar* 0.01 nucleotide substitutions per nucleotide position. *Black circle* indicates that these branches are clustering similarly in different algorithms tested

Table 2 Fatty acid compositions of strain 14B^T and phylogenetically closely related *Bacillus* species

Peak name	1	2	3	4
$C_{14:0}$	–	–	1.9	1.2
$C_{15:0}$	–	–	1.0	1.6
$C_{16:0}$	3.5	3.1	3.9	**6.6**
$C_{17:0}$	–	–	–	2.0
$C_{18:0}$	–	–	–	–
iso- $C_{13:0}$	–	–	–	–
iso- $C_{14:0}$	4.5	2.9	**13.1**	4.8
iso- $C_{15:0}$	**24.9**	**20.1**	**22.8**	**20.5**
iso- $C_{16:0}$	**17.9**	**19.1**	**14.5**	4.9
iso- $C_{17:0}$	3.9	2.1	**7.5**	4.9
anteiso- $C_{15:0}$	**30.8**	**34.6**	**22.6**	**10.9**
anteiso- $C_{17:0}$	**12.3**	**15.4**	**11.9**	**–**
iso- $C_{13:0}$-OH	**–**	1.1	**–**	**21.2**
$C_{18:1}$ ω9c	1.9	1.1	–	–
Summed feature 4*	–	–	–	**20.9**

Data above 5% are shown in bold

Strains 1, 14B^T; 2, 7B; 3, *B. halodurans* LMG 7121^T; 4, *B. okuhidensis* LMG 22468^T. Data are percentages of the total fatty acid content. – not detected. All data were from this study

* Summed features are groups of two or three fatty acids that cannot be separated by GLC using the MIDI system. Summed feature 4 comprised iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B

detected in strain 14B^T (Table 2). Polar lipids of strain 14B^T include diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), unknown aminophospholipid (APL1) and three unknown lipids (L1–3) (Supplementary Fig. S1). These profiles are somewhat similar to the polar lipid profile of *Bacillus subtilis* subsp. *subtilis* DSM 10^T (Kämpfer et al. 2006). Major quinone of strain 14B^T is MK-7 (97.5%) with traces of MK-6 (2.5%). The type species of the genus *Bacillus* (*Bacillus subtilis* subsp. *subtilis*) also contains a MK-7 predominant quinone system (Collins and Jones 1980). The peptidoglycan cell-wall amino acids of strain 14B^T contain *meso*-DAP as the diagnostic diamino acid with the peptidoglycan-type A1γ (Schleifer and Kandler 1972) or A31 (Schumann 2011).

Conclusion

The phenotypic and genotypic distinctiveness of strains 14B^T and 7B supports the proposal of the isolates as a new member of the genus *Bacillus* for which the name *Bacillus alcaliphilum* sp. nov. is proposed.

Description of *Bacillus alcaliphilum* sp. nov

Bacillus alcaliphilum (al.ca.li.phi1'unm. M.L. *alcali* alkali [from Arabic *a1* end; *qaliy* soda ash]; Gr. adj. *philum* loving; M.L. adj. *alcaliphilum* liking alkaline media or conditions).

Cells are motile with peritrichous flagella, rod-shaped [0.3–0.4 μm (*w*) × 1–5 μm (*l*)], Gram-stain-positive and terminal oval endospore forming in the slightly swollen sporangium. Facultative anaerobe. Strain forms colourless colonies with entire margins on alkaline nutrient agar. Positive for nitrate reduction and catalase activities, whereas oxydase and lipase show negative activity. Growth occurs between pH 7.0 and 10.5. NaCl is essential for growth; optimum growth occurs at 2–5% and can tolerate up to 24%. Optimal growth occurs after 3 days of incubation on nutrient agar at 37 °C and pH 9.0–9.5. Casein, cellulose and starch are hydrolysed, whereas hippurate, esculin, DNA and Tween 20 are not hydrolysed. Gelatin is liquefied. The indole production from tryptophan, VP test and citrate utilization is positive. Produce H_2S and show positive result for arginine dihydrolase, but, show negative results for phenylalanine deaminase and ornithine decarboxylase activities. Show negative result for methyl red test. Acids are not produced from most of the sole carbon sources tested. But, growth of the strain is supported by lactose, D-maltose, D-mannitol, cellobiose, D-glucose, sucrose and D-fructose are readily utilized as the sole carbon source. Ammonium chloride and urea are the most suitable nitrogen sources, but growth is also observed with glutamate and aspartate. Major (>5%) fatty acids are *anteiso*-$C_{15:0}$, *iso*-$C_{15:0}$, *iso*-$C_{16:0}$, and *anteiso*-$C_{17:0}$. Polar lipids include diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), an unknown aminophospholipid (APL1) and three unknown lipids (L1–3). The predominant isoprenoid quinone is MK-7. The DNA G + C content of the type strain is 41.6 mol%. Type strain is $14B^T$ (=KCTC 33777^T = CGMCC = 1.15474^T).

The type strain is isolated from a sediment sample of Lonar Lake, India. An additional strain 7B with almost similar features and DNA G + C content of 41.3 mol% is also isolated from the same sediment sample. The additional strain 7B differed from the type strain $14B^T$ in amikacin sensitivity, positive lipase activity and inability in utilization of lactose as a sole carbon source.

Acknowledgements SVR and MT thank Department of Science & Technology, Government of India, for providing FTSYS project Grants (SB/FT/LS-115/2012 and SB/FT/LS-320/2012, respectively).

References

Collins M, Jones D (1980) Lipids in the classification and identification of coryneform bacteria containing peptidoglycans based on 2, 4-diaminobutyric acid. J Appl Microbiol 48:459–470

EL-Rahman HAA, Fritze D, Spröer C, Claus D (2002) Two novel psychrotolerant species, *Bacillus psychrotolerans* sp. nov. and *Bacillus psychrodurans* sp. nov., which contain ornithine in their cell walls. Int J Syst Evol Microbiol 52:2127–2133

Kämpfer P, Rosselló-Mora R, Falsen E, Busse HJ, Tindall BJ (2006) *Cohnella thermotolerans* gen. nov., sp. nov., and classification of '*Paenibacillus hongkongensis*' as *Cohnella hongkongensis* sp. nov. Int J Syst Evol Microbiol 56:781–786

Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Kodaka H, Armfield AY, Lombard GL, Dowell VR (1982) Practical procedure for demonstrating bacterial flagella. J Clin Microbiol 16:948–952

Li Z, Kawamura Y, Shida O, Yamagata S, Deguchi T, Ezaki T (2002) *Bacillus okuhidensis* sp. nov., isolated from the Okuhida spa area of Japan. Int J Syst Evol Microbiol 52:1205–1209

Logan NA, De Vos P (2009) Genus *Bacillus*. *Bergey Manual of Systematic Bacteriology,* Second Edn, Vol 3, *The Firmicutes*, p 21, Bergey's Manual Trust and Springer

Logan NA, Berge O, Bishop AH, Busse H-J, De Vos P, Fritze D et al (2009) Proposed minimal standards for describing new taxa of aerobic, endospore forming bacteria. Int J Syst Evol Microbiol 59:2114–2121

Marmur J (1961) A procedure for the isolation of deoxyribonucleic acid from microorganisms. J Mol Biol 3:208–218

Meier-Kolthoff JP, Alexander FA, Klenk HP, Göker M (2013) Genome sequence-based species delimitation with confidence intervals and improved distance functions. BMC Bioinform 14:60

Mesbah M, Premachandran U, Whitman WB (1989) Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. Int J Syst Bacteriol 39:159–167

Nakamura LK, Shida O, Takagi H, Komagata K (2002) *Bacillus pycnus* sp. nov. and *Bacillus neidei* sp. nov., round-spored bacteria from soil. Int J Syst Evol Microbiol 52:501–505

Nielsen P, Fritze D, Priest FG (1995) Phenetic diversity of alkaliphilic *Bacillus* strains: proposal for nine new species. Microbiol 141:1745–1761

Oren A, Ventosa A, Grant WD (1997) Proposed minimal standards for description of new taxa in the Order Halobacteriales. Int J Syst Bacteriol 47:233–238

Reddy SV, Thirumala M, Farooq M, Sasikala Ch, Venkata Ramana Ch (2015) *Bacillus lonarensis* sp. nov., an alkalitolerant bacterium isolated from a soda lake. Arch Microbiol 197:27–34

Rheims H, Fruhling A, Schumann P, Rohde M, Stackebrandt E (1999) *Bacillus silvestris* sp. nov., a new member of the genus *Bacillus* that contains lysine in its cell wall. Int J Syst Bacteriol 49:795–802

Schleifer KH, Kandler O (1972) Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol Rev 36:407–477

Schumann P (2011) Peptidoglycan structure. In: Rainey F, Oren A (eds) Taxonomy of prokaryotes, methods in microbiology, vol 38. Academic Press, London, pp 101–129

Smibert RM, Krieg NR (1981) General characterization. In: Gerhardt P, Murray RGE, Costilow RN, Nester EW, Wood WA, Krieg NR, Phillips GB (eds) Manual of Methods for General Microbiology. American Society for Microbiology, Washington, DC, pp 409–443

Stackebrandt E, Ebers J (2006) Taxonomic parameters revisited: tarnished gold standards. Microbiol Today 33:152–155

Stackebrandt E, Goebel BM (1994) Taxonomic note: a place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol 44:846–849

Suresh K, Prabagaran SR, Sengupta S, Shivaji S (2004) *Bacillus indicus* sp. nov., an arsenic-resistant bacterium isolated from an aquifer in West Bengal, India. Int J Syst Evol Microbiol 54:1369–1375

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis (MEGA) using maximum likelihood, evolutionary distance and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Tourova TP, Antonov AS (1987) Identification of microorganisms by rapid DNA–DNA hybridization. Methods Microbiol 19:333–355

Vishnuvardhan Reddy S, Aspana S, Tushar DL, Sasikala CH, Ramana V (2013) *Spirochaeta sphaeroplastigenens* sp. nov., a halo-alkaliphilic, obligately anaerobic spirochaete isolated from soda lake Lonar. Int J Syst Evol Microbiol 63:2223–2228

Yoon SH, Ha SM, Kwon S, Lim J, Kim Y, Seo H, Chun J (2016) Introducing EzBioCloud: a taxonomically united database of 16S rRNA and whole genome assemblies. Int J Syst Evol Microbiol. doi:10.1099/ijsem.0.001755