Pak. J. Bot., 45(SI): 41-50, January 2013.

A MODERATELY BORON-TOLERANT CANDIDATUS NOVEL SOIL BACTERIUM *LYSINIBACILLUS PAKISTANENSIS* SP. NOV. CAND., ISOLATED FROM SOYBEAN (*GLYCINE MAX* L.) RHIZOSPHERE

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Abstract

A Gram-positive, motile, rod-shaped, endospore-forming and moderately boron (B) tolerant novel candidatus strain, designated as NCCP-54^T, was isolated from rhizospheric soil of soybean (*Glycine max* L.) sampled from the experimental area of Research Farm, PMAS Arid Agriculture University, Rawalpindi, Pakistan. To delineate its taxonomic position, the strain was subject to polyphasic characterization. Cells of the strain NCCP-54^T can grow at 10-45°C (optimum at 28°C) at pH ranges of 6.5-9.0 (optimum at pH 7.0) and in 0-6% NaCl (w/v) in tryptic soya agar medium. It can also tolerate 150 mM boric acid in agar medium; however, optimum growth occurs in the absence of boric acid. Based on 16S rRNA gene sequence analysis, strain NCCP-54^T showed highest similarity to *Lysinibacillus xylanilyticus* KCTC13423^T (99.1%), *Lysinibacillus fusiformis* KCTC3454^T (98.5%), *Lysinibacillus boronitolerans* KCTC13709^T (98.4%), *Lysinibacillus parviboronicapiens* KCTC13154^T (97.8%), and *Lysinibacillus sphaericus* KCTC3346^T (97.5%) and less than 97% with other closely related taxa. The level of DNA–DNA relatedness between strain NCCP-54^T and the type strains of genus *Lysinibacillus* was <27 %. Strain was also studied chemotaxonomically. Whole-cell peptidoglycan of strain NCCP-54^T contained meso-diaminopimelic acid (meso-Dpm) as major diagnostic amino acids instead of lysine-aspartate (Lys-Asp) which is the characteristic of the genus *Lysinibacillus*. The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. MK-7 was the predominant menaquinones. The major cellular fatty acids were iso-$C_{15:0}$ (30.29%) followed by iso-$C_{16:0}$ (25.59%) and $C_{16:1}$w7calcohol (16.24%). The G + C contents of the strain is 37 mol%. The chemotaxonomic characteristics of the candidatus novel strain matched those described for the members of genus *Lysinibacillus* except diagnostic amino acids contained in peptidoglycans. Phenotypic and phylogenetic analyses thus indicate that strain NCCP-54^T may represent a novel candidatus status of species in the genus *Lysinibacillus*, for which the name *Lysinibacillus pakistanensis* is proposed. The type strain is NCCP-54^T (DSMZ 24784^T = KCTC= 13795^T). The DDBJ/EMBL/GenBank accession number of the 16S rRNA gene sequence of strain NCCP-54^T is AB558495.

Introduction

The boron, a non metal micronutrient is essential for the optimum growth of plants (Warington, 1923; Ahmed *et al*., 2011) and for some animals (Rowe & Eckhert, 1999) as well. It is also important in biological function because a quorum sensing signal molecule containing boron is produced by several bacterial species (Chen *et al*., 2002). However, Boron causes toxicity to the living cells if it crosses the critical limit and toxicity of B may occur in the soil or in groundwater naturally, or through mining, irrigation water and fertilizers etc (Nable *et al*., 1997; Ahmed *et al*., 2008). Soil bacterium belongs to *Bacillus boroniphilus* (Ahmed *et al*., 2007a), *Gracilibacillus boraciitolerans* (Ahmed *et al*., 2007b), *Chimaereicella boritolerans* (Ahmed *et al*., 2007c) and *Lysinibacillus boronitolerans* (Ahmed *et al*., 2007d) have been reported to tolerate 150 to 450 mM boric acid. Soya bean is an important cash crop and its agricultural importance has well been studied (Hamayun *et al*., 2010, 2010a &b).

In last few decades, a large numbers of bacteria including species of *Pseudomonas, Azospirillium, Azotobacter, Klebsiella, Enterobacter, Alcaligens, Arthrobacter, Bacillus* and *Serratia* are reported in the rhizosphere to perform different functions like plant growth (Okon & Labandera-Gonzalez, 1994; Glick, 1995, Khan *et al*., 2011). Bacillus a gram positive, aerobic or facultative endospore forming motile bacteria belongs to family *Bacillaceae* (Claus & Berkeley 1986) is one of the most common genus found in the variety of habitats (Heyrman *et al*., 2005; Glazunova *et al*., 2006). Bacillus, strains have wide genetic heterogeneity in term of DNA G + C composition which ranges from 32-69% of known *Bacillus* spp. Bacillus can resist and survive in a variety of environmental stresses and adverse conditions and considered as very important microbiota due to its diverse ecophysiology, direct and indirect functions. Up to 1986, a large number of phenotypically different bacterial species based upon the nutritional requirements, metabolic and physiological processes and DNA base composition were included in this genus. Priest *et al*., (1988) classified 368 *Bacillus* strains into 79 clusters depending upon phenotypic characters. The use of rRNA sequence analysis (Woese, 1987) as useful molecular chronometer further brought the changes in taxonomy and helped to establish the phylogenetic relationship among closely related species based upon their nucleotide sequence. Nearly 51 *Bacillus* species were separated into five distinct phylogenetic clusters by Ash *et al*., (1991). The genus *Bacillus* contained 250 species and 7 subspecies at the time of writing this manuscript (Euzéby: www.bacterio.cict.fr/b/bacillus.htm) with a variety of distinctive characteristics. Using 16S rDNA sequence analysis, *Bacillus* has been phylogenetically classified and reclassified into new genus like *Amphibacillus* (Niimura *et al*., 1990), *Alicyclobacillus* (Wisotzkey *et al*., 1992), *Paenibacillus* (Ash *et al*., 1993),

Aneurinibacillus and *Brevibacillus* (Shida *et al*., 1996a), *Virgibacillus* (Heyndrickx *et al*., 1998), *Gracilibacillus* (Wainø *et al*., 1999), *Salibacillus* (Wainø *et al*., 1999), *Filobacillus* (Schlesner *et al*., 2001), *Geobacillus* (Nazina *et al*., 2001), *Ureibacillus* (Fortina *et al*., 2001), *Jeotgalibacillus* (Yoon *et al*., 2001) and *Marinibacillus* (Yoon *et al*., 2001). In order to clarify the taxonomic situation within the genus *Bacillus* and *Paenibacillus*, Kämpfer *et al*., (2006) strongly recommended the inclusion of polar lipid pattern, menaquinone type and fatty acid profiles as important criteria for making taxonomic conclusions. On the basis of polyphasic taxonomic characteristics, especially cell-wall peptidoglycan structure the genus *Lysinibacillus* was proposed by Ahmed *et al*., (2007d) through the description of one novel species (*Lysinibacillus boronitolerans*) and the reclassification of two *Bacillus* species; *Lysinibacillus fusiformis* and *Lysinibacillus sphaericus*.

Pakistani soils are rich in microbial diversity and commendable efforts showing characterization of microbes for biofertilizers, bioremediation and in genetic manipulation etc. There is a need to explore novel taxa from this resource by adopting minimal standards (Logan *et al*., 2009) required for validation, characterization and preserve for future generations. Next to molecular markers, chemotaxonomic features are considered essential for correct identification at genus level. Chemotaxonomic assay dealt with low molecular weight components of bacterial cell involving cell wall composition, fatty acid, menaquinone and polar lipids etc. These traits may be variable within genus and family and will help for studying bacterial systematic, a neglected subject in Pakistan. This study focused to explore novel bacteria from Pakistani soil by examining exact taxonomic position of the type strain through phylogenetic analysis based upon the 16S rRNA gene sequencing and polyphasic taxonomic characterization.

Materials and Methods

Isolation, phenotypic and biochemical characterization: A bacterial strain (i.e. NCCP-54^T) was isolated from the rhizospheric soil of legume (soybean: *Glycine max*) sampled from the experimental area of Soil Science Department located within Research Farm of Pir Mehr Ali Shah (PMAS) Arid Agriculture University, Rawalpindi, northern Punjab, Pakistan. Samples were serially diluted in phosphate-buffered saline (PBS) solution, spread on to tryptic soya agar (TSA, Difco) medium and incubated at 28°C for 48 hrs. The strains were subcultured several times for purification and the purified cultures of the isolates were maintained on TSA medium and also stored in glycerol (35%, w/v) stocks at -80°C for further characterization. The reference strains used in this study were *L. xylanilyticus* KCTC13423^T, XDB9^T (Lee *et al*., 2010), *L. boronitolerans* KCTC13709^T, DSM 17140^T (Ahmed *et al*., 2007d), *L. parviboronicapiens* KCTC13154^T, NBRC103144^T (Miwa *et al*., 2009), *L. fusiformis* KCTC3454^T, DSM 2898^T (Priest *et al*., 1988; reclassified by Ahmed *et al*., 2007d) and *L. sphaericus* KCTC3346^T, IAM 13420^T (Claus & Berkeley, 1986; reclassified by Ahmed *et al*., 2007d). Cell morphology and motility were examined by light microscopy (Nikon E600). Colony morphology was observed on isolated colonies grown on nutrient agar (pH 7.0; Difco) for 2 days at 28°C. For pH optimization, various pH levels (6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0) were adjusted in in tryptic soy broth (TSB; Difco). For NaCl tolerance, the strain was inoculated in TSB with different NaCl concentrations from 0 to 10% (w/v) at optimum pH. During pH and NaCl optimization, the growth was assayed by measuring the absorbance on spectrophotometer at 600 nm after 12 and 24 hr. Temperature optimization was carried out by streaking bacterial strain on TSA plates adjusted with optimum pH and NaCl (%) and incubated at different temperatures from 4, 10, 16, 22, 28, 32, 37, 45 and 50°C. The strain was also cultured in TSB medium (pH 7.0) with different level of boron ranging from 0 (control) to 150 mM, while being shaken vigorously to measure OD_{600} using a spectrophotometer. Physiological and biochemical characterization were determined using API kits (API 20E, API zym; bioMérieux, France) and Biolog GP system to analyze different metabolic features of the strains. Gram staining was also performed using commercial kit (bioMérieux, France) and confirmed through KOH reaction. Oxidase and catalase activities were determined using Oxidase Kit (bioMérieux) and following the procedure described by Cowan & Steel (2004), respectively. All of these commercial kits were used according to the manufacturer's protocol. Boron tolerance of the type strain in TSB media (pH 7.0) was also measured using different level of boron ranging from 0 (control) to 150 mM (Ahmed *et al*., 2007a).

Molecular identification using 16S rRNA gene sequencing: Nearly complete 16S rRNA gene sequences of the strain NCCP-54^T was amplified by the PCR as described by Katsivela *et al*., (1999) using universal forward and reverse primers: 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1510R (5'–GGCTACCTTGTTACGA-3'). PCR products were purified using PCR purification kit and the purified PCR product samples were sequenced using DNA sequencing service of MACROGEN, Korea (http://dna.macrogen.com/eng) using the BigDye™ Terminator Cycle Sequencing Kits and four universal 16S rRNA gene sequencing primers (9F, 515F (5'-GTGCCAGCAGCCGCGGT-3'), 926R (5'-CCGTCAATTCCTTTGAGTTT-3'), and 1510R), following the manufacturer's (Applied Biosystems) protocols using ABI PRISM® 3730 XL Genetic Analyzer. Software's used for sequence alignment and comparisons were CLUSTAL X (version 1.8msw; Thompson *et al*., 1997) and BioEdit (Hall, 1999). Sequences of closely related type strains used for constructing the phylogenetic tree were selected and retrieved from the NCBI (National Center for Biotechnology Information) and DDBJ (DNA Data Bank of Japan) databases by BLAST searches for bacteria. The alignment and editing was performed using CLUSTAL X (version 1.8msw) and BioEdit packages. Ambiguous positions and gaps were excluded during calculations. A phylogenetic tree was constructed using the neighbor-joining method (Saitou & Nei 1987) and plotted with NJ Plot software. The stability of the relationship was assessed by bootstrap analysis (Felsentein, 2005) by performing 1,000 re-sampling for the tree topology of the neighbour-joining data. DNA accession numbers of strain was obtained from DNA Data Bank of Japan (DDBJ). The type strain (NCCP-54^T) was also submitted to Korean Collection of Type Cultures (KCTC) and German Collection of Microorganisms and Cell Cultures i.e. The Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ).

Chemotaxonomic characterization: The strain NCCP-54^T was also characterized by chemotaxonomic traits i.e. fatty acid profile, menaquinones system, polar lipids, cell wall amino acids and DNA G + C contents. Cellular fatty acid composition was determined with Sherlock Microbial Identification system (MIDI). Fatty acid methyl esters were prepared from biomass grown at 28^{o}C on TSA (Difco) for 72 hrs. Extraction was performed according to Sasser (1990). Fatty acid methyl esters were analyzed by a Hewlett Packard 5890 series II gas chromatograph equipped with Ultra2 capillary column. Cellular fatty acid were identified by comparing the equivalent chain length (ECL) of each compound to a peak naming table that contains over 115 known standards. The quantity of each compound was determined as a percentage of fatty acid compounds present within bacterium. For isoprenoid quinones, dried cells (200 mg) were added in 20 ml chloroform-methanol (2:1 v/v) and stirred with stirrer bar for overnight at room temperature. The suspension was filtered and evaporated in vacuo. Quinones were extracted with 5 ml of acetone and evaporated in vacuo. The acetone fraction solved in 200 µl of ethyl alcohol. The extraction was developed with TLC plates (silica gel 60 F254, Merck, Darmstadt, Germany) in toluene. Quinone spots were detected under UV light at 275 nm. The spots were scrapped off and extracted with acetone. After the extraction was dried with a flow of N_2 gas, 100 µl of ethyl alcohol was added. The quinone solution was analyzed with HPLC, (model LC-10AD HPLC apparatus; Shimadzu, Japan) equipped with Cosmosil 5Ci8R column (Nacalin). Moreover, LC-MS (LCMS-QP-8000a, Shimadzu) was performed to detect saturation side chain. The peptidoglycan structure was determined as described previously (Schleifer, 1985; Schleifer & Kandler, 1972) with the modification that TLC on cellulose was applied instead of paper chromatography. The quantitative analysis of amino acids in the peptidoglycan was determined by gas chromatography according to Mackenzie (1987). We also confirmed the cell wall diagnostic amino acids of NCCP-54^T using DSMZ services. The whole cell of the strain was hydrolyzed (4N, HCl, 100^{o}C, 16 hr) and subjected the hydrolysate to thin-layer chromatography on cellulose plates using the solvent system of Rhuland *et al*., (1955). Polar lipids were extracted and separated from 100 mg freeze-dried cell material by the two-stage methods as described previously (Tindall, 1990). A culture of the strain was also grown at 28^{o}C on TSA (Difco) for 72 hours for the determination of DNA G + C contents. DNA was extracted using QIAGEN Genomic tips as described by manufacturer (Qiagen, Germany). DNA G+C content was analyzed according to the method described by Mesbah *et al*., (1989) using HPLC, (model LC-10Ad VP; Shimadzu, Japan) under the following condition: column, Cosmil 5C18R (Nacalai); mobile phase. 0.2 M ammonium phosphate: acetonitorile (40:1); column temperature, 40 ^{o}C. An additional DNA-DNA hybridization was also performed (Marmur, 1961; Sambrook *et al*., 1989; Ezaki *et al*., 1989) to precisely differentiate the closely related species.

Results

Phenotypic, biochemical and morphological description: The type strain NCCP-54^T was motile rod, aerobic and produced endospores (ellipsoidal or spherical) terminally lie in a swollen sporangium and positive for catalase activity but variable for oxidase. The colonies were rough having dull surface and transparent. Texture was butyrous (butter like) and elevation was flat. Diameter of colony was 1-4 mm and margins were lubate in the older colonies. The color of colony was off white initially and turns to light yellow in older colonies. The form of colony was punctiform and 2nd to 3rd day spreads on the whole surface of the plate. Morphologically type strain NCCP-54^T was different species from reference taxa used in this study. Phase contrast microscopy confirmed short chain motile rods, and central ellipsoidal spores (Fig. 1). The isolated strain NCCP-54^T grew at pH 6.5-9.0 with optimum growth at pH 7.0 and no growth at pH 6.0 (Table 1). Cells of the strain NCCP-54 could grow at 10-45°C with optimum growth at 28°C and there was no growth at $\geq$ 50°C and only slight growth after several days at 10°C. These findings distinguished the strain from the closely related species. Type strain NCCP-54 grew upto 6% NaCl concentrations (w/v) on TSA (pH 7.0) at 28°C. Since mainly negative reactions were obtained with API 50CH and API 20E for utilization of various carbon sources, we analyzed an extended array of metabolic features of the strains using Biolog GP system (Table 1). The type strain NCCP-54^T showed mainly negative reactions especially for pyruvic acid, alanine, glutamic acid, adenosine, thymidine and nitrate reduction and positive for acetic acid, dextrin, lipase, and acid phosphatase, alkaline phosphatase (weak), chymotrypsin (weak), arginine (weak) and tryptophane deaminase (weak). The results of gram staining and KOH reaction are shown in Table 2.

Phylogenetic analysis: A nearly complete 16S rRNA gene sequence (NCCP-54^T 1481 bp) was compared with sequences of closely related type strain retrieved by BLAST search against the GenBank database. Based on 16SrRNA gene sequence data, the similarity (Table 3) of the novel strain NCCP-54^T was 99.1% to *Lysinibacillus xylanilyticus*, 98.5% to *Lysinibacillus fusiformis*, 98.4% to *Lysinibacillus boronitolerans*, 97.8% to *Lysinibacillus parviboronicapiens*, and 97.5% to *Lysinibacillus sphaericus*. Type strain (NCCP-54) and its closest *Lysinibacillus* relatives exhibited more than 97% sequence similarity. Recently, literature data compiled from hundreds of species descriptions has suggested that strains sharing less than 98.8% sequence similarity belong to different genospecies (Stackebrandt & Ebers, 2006; Jung *et al*., 2011). Therefore, additional DNA-DNA hybridization was performed to differentiate these closely related species more accurately. DNA-DNA hybridization values between strain NCCP-54^T and reference strains were <30% (Table 3). These values were less than the 70% threshold indicating that these strains represent a separate species of the genus *Lysinibacillus*. Phylogenetic interrelationship of NCCP-54^T with closely related species of *Lysinibacillus* and other related genera are shown in Fig. 2. The tree was generated and based on comparison of 1,302 nts and plotted using NJ-Plot software. The tree is rooted using *Bacillus subtilis* IAM 12118^T (AB042061). Bootstrap values (>70%) expressed as percentage of 1000 replications are indicated on nodes. Accession number of each type strain is shown in parenthesis.

Table 1. Characteristics that differentiate the novel candidatus strains (NCCP-54^T) from closely related reference strains.

	NCCP-54^T	*Lysinibacillus xylanilyticus*	*Lysinibacillus boronitolerans*	*Lysinibacillus fusiformis*	*Lysinibacillus parviboronicapiens*	*Lysinibacillus sphaericus*
Characteristics						
Growth temperature range (°C) (optimum)	10-45 (28)	10-40 (30)	16-45 (37)	17-40	10-37 (30)	10-40
pH range (optimum)	6.0-9.0 (7.0)	5.0-9.0	5.5-9.5 (7.5)	6.0-9.5	6.0-8.0 (7.0)	6.0-9.5
Growth in 5% (w/v) NaCl	+	+	+	+	+	+
Growth in 6% (w/v) NaCl	+	-	-	+	+	ND
Growth in 7% (w/v) NaCl	-	-	-	+	-	-
Boron tolerance (mM)	0-150	0	150	60	50	75
ZYM					-	
Alkaline phosphatase	w	+	w	ND	ND	ND
Esterase (C4)	-	-	+	ND	-	ND
Esterase Lipase (C8)	+	-	w	ND	+	ND
Lipase (C14)	-	-	ND	ND	-	ND
Leucine arylamidase	-	-	w	ND	-	ND
Valine arylamidase	-	-	w	ND	-	ND
Cystine arylamidase	-	-		ND	-	ND
Trypsin	-	-	ND	-	-	ND
α-chymotrypsin	w	-	+	+	+	ND
Acid phosphatase	+	+	w	-	-	ND
Naphthol-AS-BI-phosphohydrolase	-	+	w	w	-	ND
α-galactosidase	-	-	ND	ND	-	ND
β-galactosidase	-	-	ND	-	-	+
α-glucosidase	-	-	ND	ND	-	ND
β-glucosidase	-	-	ND	ND	-	ND
N-acetyl-β-glucosaminidase	-	-	ND	ND	-	ND
α-mannosidase	-	-	ND	ND	-	ND
α-fucosidase	-	-	ND	ND	-	ND
20E	-					
2-nitrophphenyl-BD-galactopyranoside		ND	ND	ND	-	ND
L-arginine	w	-	+	-	-	-
L-lysine	-	-	-	ND	-	-
L-ornithine	-	-	-	ND	-	-
Trisodium cirate	-	+	+	ND	-	ND
Urea	-	-	+	+	+	+
L-tryptophane (Tryptophane DeAminase)	w	ND	+	-	w	-
L-tryptophane (Indole prouction)	-	-	-	-	-	-

Table 1. (Cont'd.).

	NCCP-54^T	*Lysinibacillus xylanilyticus*	*Lysinibacillus boronitolerans*	*Lysinibacillus fusiformis*	*Lysinibacillus parviboronicapiens*	*Lysinibacillus sphaericus*
Sodium pyruvate (Voges-Proskauer tset)	-	-	+	-	+	-
Gelatin	-	+	-	+	-	+
D-glucose	-	+		ND	ND	ND
D-mannitol	-	-	ND	ND	ND	ND
Inositol	-	-	ND	-	ND	-
D-sorbitol	-	+	ND	ND	ND	-
L-rhamnose	-	-	ND	-	ND	-
D-sucrose	-	+	-	v	-	-
D-melibiose	-	+	ND	ND	ND	-
Amygdalin	-	-	ND	ND	ND	-
L-arabinose	-	+	ND	-	ND	-
Oxidase	-	+	+	+	-	+
Nitrate reduction to NO_2	-	-	-	-	-	-
Biolog results						
Acetic acid	+	-	-	-	-	-
α-Ketovaleric acid	-	+	+	-	+	-
Pyruvatic acid methyl ester	-	-	-	+	+	+
Pyruvic acid	-	-	+	+	-	+
L-alaninamide	-	-	-	-	-	-
L-alanine	-	-	+	+	-	+
L-glutamic acid	-	-	+	+	-	+
Adenosine	-	-	-	+	-	+
Thymidine	-	-	-	-	-	-
Thymidine-5'-monophosphate	-	+	-	-	-	-
Dextrin	+	+	-	-	+	-
B-hydroxybutyric acid	-	-	-	-	-	-
D-lactic acid methyl ester	-	-	-	-	-	-
Succinic acid mono-methyl ester	-	-	-	-	-	-
Chemotaxonomic analysis						
Cell-wall type	meso-Dpm	Lys-Asp	Lys-Asp	Lys-Asp	Lys-Asp	Lys-Asp
Menaquinone system	MK-7	MK-7	MK-7	MK-7	MK-7	MK-7
Major fatty acid	iso-$C_{15:0}$	iso-$C_{15:0}$	iso-$C_{15:0}$	iso-$C_{15:0}$	iso-$C_{15:0}$	iso-$C_{15:0}$
G+C content (mol%)	37	37.2	36.5	35-36	38.7	37.3-38.3

ND, data not available; +, positive; w, weakly positive; -, negative; v, variable results

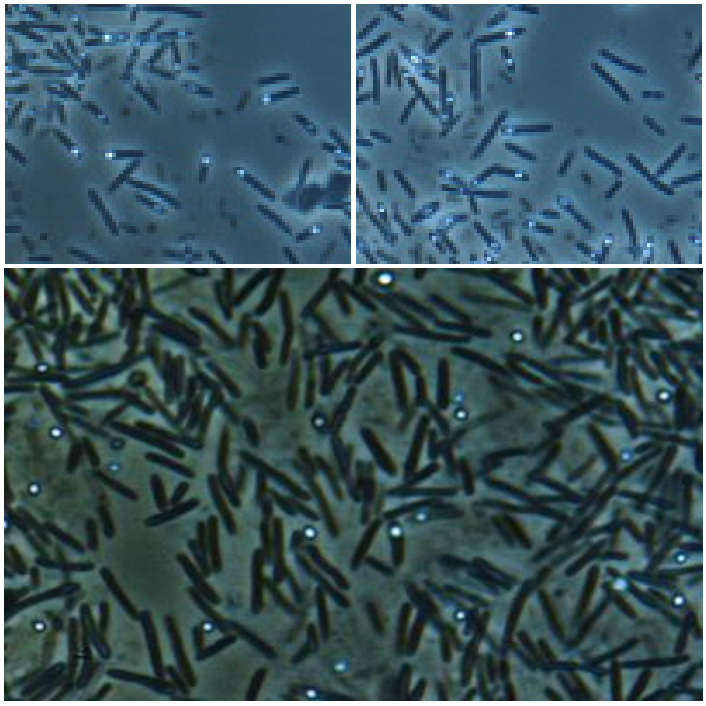

Fig 1. Photomicrograph of sporangia and vegetative cells of the type strain *Lysinibacillus Pakistanensis* NCCP-54^{T} (AB558495), viewed by phase-contrast microscopy (Nikon E600); spherical and slightly ellipsoidal spores lie terminally and subterminally in sporangia that are slightly swollen. Bar, 2 µm.

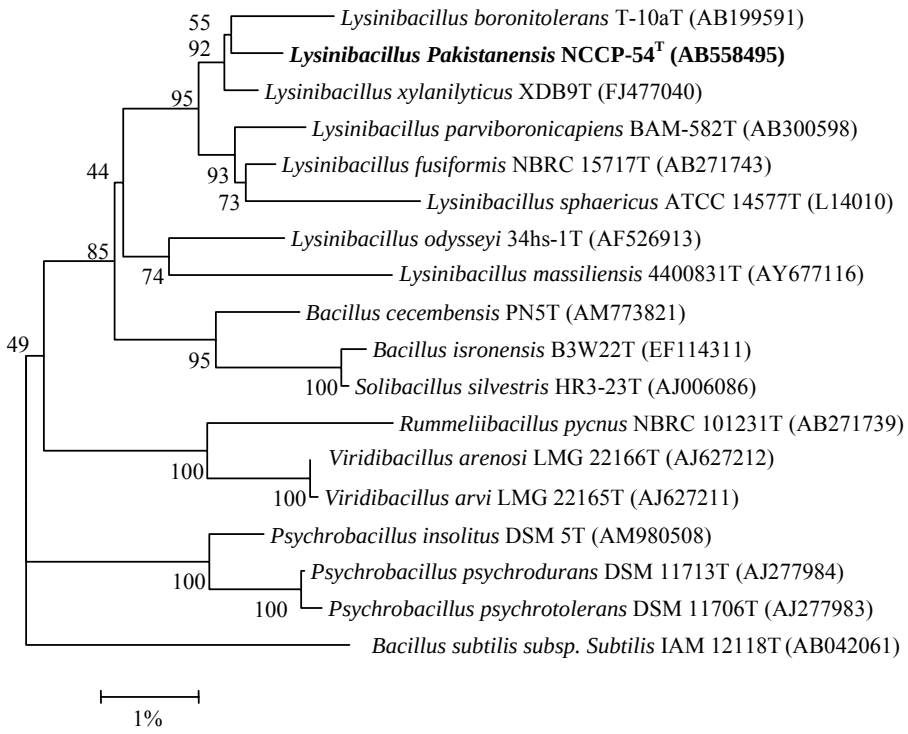

Fig. 2. Phylogenetic tree showing interrelationship of *Lysinibacillus Pakistanensis* NCCP-54^{T} (AB558495) with closely related species of *Lysinibacillus* and other related genera. The tree is generated using Neighbor-Joining method contained in PHYLIP software package (Felsentein, 2005) based on comparison of 1,302 nts and plotted using NJ-Plot software. The tree is rooted using *Bacillus subtilis* IAM 12118^{T} (AB042061). Bootstrap values (>70%) expressed as percentage of 1000 replications are indicated on nodes. Accession number of each type strain is shown in parenthesis.

Table 2. Results of gram-staining and KOH-reaction.

S. No.	Isolate(s)	Gram stain		KOH reaction		Reference
		72h incubation	24h incubation	72h incubation	24h incubation	
1.	*L. pakistanensis* (NCCP-54^T) KCTC13795	v	v	-	-	novel
2.	*L. xylanilyticus* KCTC13423	v	v	+	+	+
3.	*L. boronitolerans* KCTC13709	v	v	-	-	+
4.	*L. parviboronicapiens* KCTC13154	+	v	+	+	+
5.	*L. spaericus* KCTC 3346	-	v	-	-	+
6.	*L. fusiformis* KCTC 3454	-	v	+	+	+

Table 3. DNA-DNA relatedness among the strains (NCCP-54^T) and their most closely related species.

S. No.	Strains	*L. pakistanensis* (NCCP-54^T)	
		% Identity of 16S rRNA gene sequence	DNA-DNA hybridization
1.	*L. pakistanensis* (NCCP-54^T)KCTC13795	100	100
2.	*L. xylanilyticus* KCTC13423	99.1	23
3.	*L. boronitolerans* KCTC13709	98.4	24
4.	*L. parviboronicapiens* KCTC13154	97.8	19
5.	*L. spaericus* KCTC 3346	98.5	27
6.	*L. fusiformis* KCTC 3454	97.5	19

Table 4. Polar lipids composition of NCCP-54^T along with type strains.

	1	2	3	4	5	6
Polar lipids	DPG	DPG	DPG	DPG	DPG	DPG
	PG	PG	PG	PG	PG	PG
	PE	PE	GL1	PE	GL1	GL1
	PL3		PE	GL1		L1
	PL2			L1		

PL1-PL4 = phospholipids; PG = phosphatidylglycerol; PE = phosphatidylethanolamine;
DPG = Diphosphatidylglycerol; L1 = Unidentified lipid; PNI-PN2 = Aminophopholipids; GL1-GL2 = Glycolipids
Strains: 1, *L. pakistanensis* (NCCP-54^T); 2, *L. xylanilyticus* KCTC13423; 3, *L. boronitolerans* KCTC13709; 4, *L. parviboronicapiens* KCTC13154; 5, *L. fusiformis* KCTC3454; 6, *L. sphaericus* KCTC3346

Table 5. Cellular fatty acid composition of NCCP-54^T along with type strains.

Fatty acid	1	2	3	4	5	6
14:0 ISO	1.58	1.87	1.77	-	1.24	3.66
14:0	-	1.01	0.57	-	-	-
15:0 ISO	30.29	49.01	39.27	33.51	48.58	49.85
15:0 ANTEISO	1.82	9.25	16.22	11.81	9.27	3.34
15:0	-	-	-	-	-	-
16:1 w7c alcohol	16.24	7.93	9.25	5.11	8.29	14.46
16:0 ISO	25.59	5.51	12.7	4.66	5.15	12.12
16:1 w11c	4.25	2.2	2.35	4.38	3.54	1.72
16:0	2.7	-	1.68	3.04	1.02	-
ISO 17:1 w10c	4.33	5.87	1.33	7.33	5.92	3.23
17:0 ISO	10.59	7.22	5.79	12.08	4.7	8.07
17:0 ANTEISO	1.6	4.81	6.86	9.25	3.38	1.07
SUMMED Feature 4	1	3.21	2.22	6.26	3.61	1

Strains: 1, *L. pakistanensis* (NCCP-54^T); 2, *L. xylanilyticus* KCTC13423; 3, *L.boronitolerans*KCTC13709; 4, *L. parviboronicapiens* KCTC13154; 5, *L. fusiformis* KCTC3454; 6, *L. sphaericus* KCTC3346

Chemotaxonomic characterization: The DNA G + C contents of the novel strain NCCP-54^T were 37 mol% and MK-7 (88%) was determined as the predominant quinone system in the novel strains (Table 1). Whole-cell peptidoglycan of strain NCCP-54^T contained meso-diaminopimelic acid (meso-Dpm) as major diagnostic amino acid (Table 1) instead of lysine-aspartate (Lys-Asp) which is the characteristics of genus *Lysinibacills.* The type strain NCCP-54^T shared a similar polar lipids profile (Table 4) with L. *boronitolerans* KCTC 13709^T, L. *xylanilyticus* KCTC 13423^T and L. *parviboronicapiens*, which consisted predominantly diphosphatidylglycerol (DPG), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) (Table 4; Fig. 3). The pre dominant cellular fatty acids of the type strain (NCCP-54^T) are iso-$C_{15:0}$ (30.29%), iso-$C_{16:0}$ (25.59%) and $C_{16:1}$w7calcohol (16.24%) (Table 5).

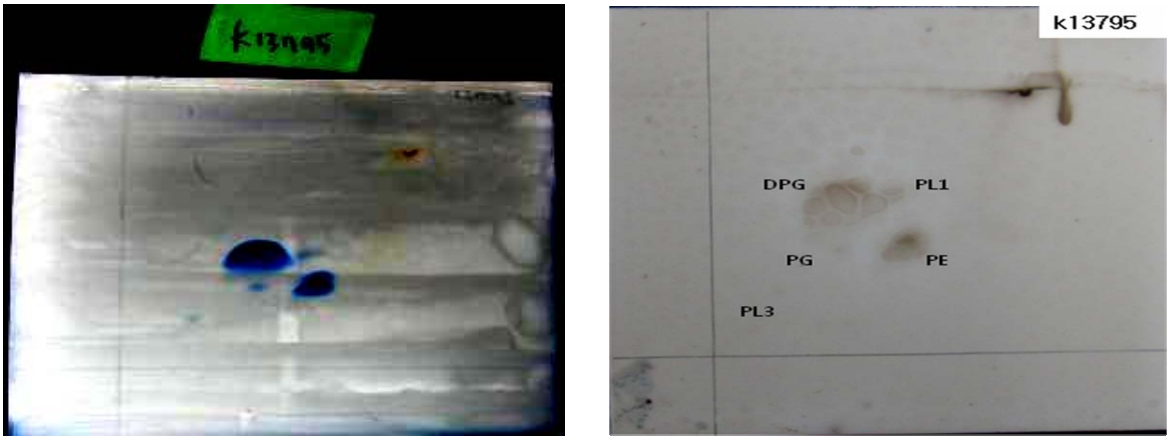

Fig. 3. Polar lipid profile of *Lysinibacillus Pakistanensis* NCCP-54^T (AB558495) after separation by two-dimensional thin layer chromatography and spraying with 5% molybdatophosphoric acid to show all lipids. DPG: diphosphatidylglycerol, PG: phosphatidylglycerol, PE: phosphatidylethanolamine, PL1-PL3 = phospholipids.

Discussion

The genus *Lysinibacillus* was proposed by Ahmed *et al*., (2007d) through the description of one novel species and the reclassification of two *Bacillus* species on the basis of polyphasic taxonomic characteristics, especially cell-wall peptidoglycan structure i.e. type A4a (Lys-Asp). At the time of writing, the genus *Lysinibacillus* was composed of 5 species with validly published names: *Lysinibacillus boronitolerans*, *Lysinibacillus fusiformis* and *Lysinibacillus sphaericus* (Ahmed *et al*., 2007d) *Lysinibacillus parviboronicapiens* (Miwa *et al*., 2009) and *Lysinibacillus* xylanilyticus (Lee *et al*., 2010). As for the taxonomic position of *Bacillus massiliensis* and *Bacillus odysseyi*, these species seemed to be distantly phylogenetically related to *R. pycnus* or *S. silvestris*. Although it has been discussed previously (Ahmed *et al*., 2007d; Krishnamurthi *et al*., 2009), there was a need to transfer these species into new or existing genera. Recently, Jung *et al*., (2011) re-evaluated the taxonomic position of *B. massiliensis* and *B. odysseyi*, and also described a new spore-forming species, *Lysinibacillus sindruensis* isolated from tidal flat sediment, based upon its phenotypic, genetic and chemotaxonomic properties and reclassified *B. massiliensis* and *B. odysseyi* as *Lysinibacillus Massiliensis* and *Lysinibacillus odyssey*.

The novel candidates strain NCCP-54^T tolerated upto 6% (w/v) NaCl indicating its halo tolerant nature. This differed from *Lysinibacillus* xylanilyticus which can only tolerate upto 5% (Lee *et al*., 2010) where as *Lysinibacillus fusiformis* can tolerate upto 7% (w/v) NaCl (Priest *et al*., 1988). The whole-cell peptidoglycan of strain NCCP-54^T contained meso-diaminopimelic acid (meso-Dpm) as major diagnostic amino acids (Table 1) instead of lysine-aspartate (Lys-Asp) which was major differentiating characteristic of the genus *Lysinibacillus* (Ahmed *et al*., 2007d). This characteristic was found different as compared to the type species of the genus *Lycinibacillus*. The occurrence of meso-diaminopimelic acid (meso-Dpm) has been reported up to now only for the peptidoglycan type A1γ (and A1γ' with glycine instead of L-alanine) and for three variations of peptidoglycan type A4γ (A31.1, A31.2 and A31.3). The variations of peptidoglycan type A4γ based on meso-Dpm have been found so far exclusively in members of the genera *Brachybacterium, Devriesea* and *Dermabacter* (family: *Dermabacteraceae*).

Description of *Lysinibacillus pakistanensis* sp. nov. cad. (NCCP-54^T): *Lysinibacillus pakistanensis*: origin Pakistan isolated from rhizospheric soil of legume (*Glycine max* L.) sampled from the experimental area of Soil Science Department located at Research Farm of PMAS Arid Agriculture University, Rawalpindi, northern Punjab, Pakistan. It was further concluded during this study that cell wall peptidoglycans predominant in the genus *Lysinibacillus* may be Lys-Asp or meso-Dpm or both. The DDBJ/EMBL/GenBank accession number of the 16S rRNA gene sequence of the novel candidatus strain NCCP-54^T (DSMZ 24784^T = KCTC= 13795^T) is AB558495.

Acknowledgments

Initial funds for this study were provided by Higher Education Commission of Pakistan under talent training program for young faculty members for training from Plant Biotechnology Program, National Institute for Genomics and Advanced Biotechnology, NARC, Pakistan and from Institute of Molecular and Cellular Biosciences, The University of Tokyo, Japan. This work was also supported by Pakistan Science Foundation through project # PSF/Res/P-PMAS.AAUR/Agr-374 and the KRIBB Research Initiative Program funded by the Ministry of Education, Science and Technology, Republic of Korea. The contribution of Yeseul Sin from KRIBB and Prof Akira Yokota from IMCB, Japan is highly acknowledged.

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(Received for publication 1 September 2012)