INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
13–1196/2014/16–2–447–450
http://www.fspublishers.org

Short Communication

Description of *Lysinibacillus pakistanensis*

Iftikhar Ahmed[1*], Yeseul Sin[2], Jayoung Paek[2], Muhammad Ehsan[1], Rifat Hayat[1,3], Muhammad Iqbal[1] and Young Hyo Chang[2*]

[1]*National Institute for Genomics and Advanced Biotechnology (NIGAB), National Agricultural Research Center (NARC), Park Road, Islamabad-45500, Pakistan*

[2]*Korean Collection for Type Cultures, Biological Resources Centre, KRIBB, 52 Eoeun-dong, Daejeon, 305-806, Republic of Korea*

[3]*Department of Soil Science & SWC, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi-46300, Pakistan*

*For correspondence: iftikharnarc@hotmail.com; yhchang@kribb.re.kr

Abstract

The purpose of this addendum is to provide the additional information for validation of *Lysinibacillus pakistanensis* sp. nov. as a new name under the procedure described in the *Bacteriological Code* (1990 Revision). The strain NCCP-54^T recently published, however it does not meet the basic requirement as it lacks species description according to the rules of International Code of Nomenclature of Prokaryotes. Additionally, the data on DNA-DNA hybridization was required with all the validly recognized species having more than 97% similarity of 16S rRNA gene sequence. The results of this study showed that DNA-DNA relatedness of strain NCCP-54^T is below 70% with all the validly recognized species to date. The diagnostic amino acids in cell wall peptidoglycans were re-analyzed and contained Lys-Asp (type *A*4α). This addendum also provides the formal description of *Lysinibacillus pakistanensis* sp. nov. © 2014 Friends Science Publishers

Keywords: *Lysinibacillus pakistanensis*; Cell wall peptidoglycans type *A*4α; DNA-DNA hybridization

Introduction

Recently, strain NCCP-54^T has been characterized to describe a novel species, *Lysinibacillus pakistanensis* (Hayat *et al.*, 2013). To validate the name, *Lysinibacillus pakistanensis*, by International Committee of Systematics in Prokaryotes (ICSP), it must have to meet the basic requirements of the International Code of Nomenclature of Prokaryotes (*Bacteriological Code*, 1990 Revision). Unfortunately, the name published by the authors (Hayat *et al.*, 2013) did not meet the basic requirement as described in Rule 27 of the International Code of Nomenclature of Prokaryotes; and thus, it has been declined for validation as a new species by ICSP. Additionally, few other species, *Lysisnibacillus mangiferihumi* (Yang *et al.*, 2012), *L. macrolides* (Coorevits *et al.*, 2012) and *L. contaminans* (Kämpfer *et al.*, 2013) have been published and described in the genus *Lysinibacillus*, during or after the process of publication of *Lysinibacillus pakistanensis* (Hayat *et al.*, 2013). These species had 16S rRNA gene sequence similarity more than 97% with strain NCCP-54^T, which made it mandatory to provide further evidence to establish the novelty of strain NCCP-54^T by DNA-DNA hybridization (Stackebrandt and Goebel, 1994) and to describe strain NCCP-54^T as *Lysinibacillus pakistanensis* sp. nov. Previously, Hayat *et al.* (2013) have reported *meso*-diaminopimelic acid as a diagnostic amino acid in cell wall peptidoglycans, which is contradictory to the description of genus *Lysinibacillus.* Thus, the peptidoglycans analysis of strain NCCP-54^T is re-performed. This addendum is submitted to meet such requirements for the validation of *Lysinibacillus pakistanensis* sp. nov.

Materials and Methods

Phylogenetic Analyses

To understand the exact taxonomic position of strain NCCP-54^T, an updated phylogenetic analyses were performed with all the validly recognized species of genus *Lysinibacillus* as described previously (Roohi *et al.*, 2012). Sequences of closely related validly published type strains were retrieved from the database of EzTaxon Server (http://eztaxon-e.ezbiocloud.net). MEGA 5.10 was used to assess the molecular evolutionary distances following the Kimura 2-parameter model and phylogenetic trees were constructed using three algorithms: neighbor joining (NJ), maximum likelihood (ML) and maximum parsimony (MP). The bootstrap analysis was performed to assess the stability of relationship by performing 1,000 re-sampling for the tree topology.

DNA-DNA Hybridization and Cell Wall Peptidoglycan Analysis

In addition to previously described reference strains (Hayat *et al.*, 2013), the additional strains used as reference species

in this study were *Lysinibacillus mangiferahumi* KCTC33154^T, *L. macroides* KCTC13806 ^{T} and *L. contaminans* KCTC33155^T. DNA of NCCP-54^T and the reference strains were extracted using the procedure described previously (Ahmed *et al.*, 2007a). DNA–DNA hybridization was performed with photobiotin-labelled DNA with five replications of each sample at 45ºC according to the procedure of Ezaki *et al.* (1989), using Fluoroskan Ascent Plate Reader (Thermo Labsystems, USA) for fluorescence measurements.

To determine the peptidoglycan structure of strain NCCP-54^T, cell walls were purified from 2 g wet cells of strain NCCP-54^T grown in tryptic soy broth (Difco) for 24 h at 30ºC according to the protocol described earlier (Kawamoto *et al.*, 1981). The cells were disrupted by sonication for more than 45 min in presence of phosphate buffer (0.05 M Na_2HPO_4/0.05 M KH_2PO_4; pH 7.2). The broken cells were reacted with 25% SDS solution at 100ºC for 40 min and after washing with phosphate buffer (pH 7.2), subsequently suspended in Pronase E solution, and incubated at 37˚C for 2 h. The broken cells were washed using phosphate buffer (pH 7.6) and treated with 5% trichloroacetic acid (TCA) solution at 100ºC for 20 min. The precipitated cell walls were washed with Milli-Q water, ethanol and diethyl ether and dried in vacuum. The purified cell walls were hydrolyzed with 6N HCl at 100ºC for 18 h. The amino acids in the cell wall peptidoglycans were analyzed quantitatively using automatic Amino Acid Analyzer (Hitachi, Japan).

Results

Based on comparison of 16S rRNA gene sequence (1484 bp) with closely related type strains, at least seven species showed the similarity values above 97% with strain NCCP-54^T. The results indicated that NCCP-54^T was most closely related to *L. macroides* (99.12%), *L. xylanilyticus* (99.11%), *L. fusiformis* (98.57%), *L. boronitolerans* (98.40%), *L. sphaericus* (98.38%). *L. contaminans* (97.63%) and *L. mangiferahumi* (97.52%). DNA-DNA hybridization was performed with the species having >97% similarity of 16S rRNA gene sequence (Table 1), which have not been included in comparison studies previously (Hayat *et al.*, 2013) to establish the novelty of NCCP-54^T. The results revealed that DNA-DNA hybridization values of strain NCCP-54^T with the reference species were less than 31% (Table 1). Strain NCCP-54^T contained alanine, glutamic acid, lysine and aspartic acid as diagnostic amino acids in cell wall peptidoglycans. An estimate of the molar ratio of these amino acids (alanine: glutamic acid: lysine: aspartic acid) in the peptidoglycan was obtained as 1.62: 1.00: 0.64: 0.62.

Discussion

An updated taxonomic position of strain NCCP-54^T was revealed by the phylogenetic analysis and the identification command on EzTaxon Server (http://eztaxon-e.ezbiocloud.net). Phylogenetic relationship of NCCP-54^T with closely related species of *Lysinibacillus* showed close association with *L. macroides*, *L. xylanilyticus*, and *L. boronitolerans* at high bootstrap values of >80% and formed a coherent cluster in neighbor-joining phylogenetic tree (Fig. 1). This association was also confirmed by MLH and MP algorithms.

Among the closely related taxa with more than 97% similarity of 16S rRNA gene sequence, three species, *Lysinibacillus macroides* (Coorevits *et al.*, 2012), *L. contaminans* (Kämpfer *et al.*, 2013) and *L. mangiferahumi* (Yang *et al.*, 2012), were described in the genus *Lysinibacillus*, during or after the process of publication of *L. pakistanensis* (Hayat *et al.*, 2013), whereas the others have been studied in comparison to strain NCCP-54^T previously (Hayat *et al.*, 2013). Thus, to establish the novelty of NCCP-54^T, the DNA-DNA hybridization was performed with the species having >97% similarity of 16S rRNA gene sequence, which have not been included in comparison studies previously (Hayat *et al.*, 2013). The results revealed that DNA-DNA hybridization values between strain NCCP-54^T and the reference strains were less than 31% (Table 1). These values were less than the 70% threshold indicating that NCCP-54^T still represents a separate species in the genus *Lysinibacillus* (Stackebrandt and Goebel, 1994).

Contradictory to the results of peptidoglycans reported by Hayat *et al.* (2013), strain NCCP-54^T contained Lys-Asp as a diagnostic amino acids in cell wall peptidoglycans, which corresponds to variation type *A*4α. The genus *Lysinibacillus* have also been reported with variation type *A*4α peptidoglycan (Ahmed *et al.*, 2007b) and thus, these results are in agreement with the genus description.

To meet the basic requirements for validation of a new name, *Lysinibacillus pakistanensis* sp. nov. according to the Rules of Bacteriological Code of Nomenclature of Prokaryotes, the description of species, *Lysinibacillus pakistanensis* sp. nov. has been compiled here mainly from the data reported by Hayat *et al.* (2013) and included as follows:

Description of *Lysinibacillus pakistanensis* sp. nov

Lysinibacillus pakistanensis (pa.kis.tan.en'sis. N.L. masc. adj. *pakistanensis* from Pakistan, where the organism was isolated).

Cells are rod-shaped, aerobic, motile and Gram-positive. Spherical or ellipsoidal endospores are produced terminally or sub-terminally in slightly bulging sporangia. The colonies are rough having dull surface and are transparent. Texture is butyrous (butter like) and elevation is flat. Diameter of colony is 1–4 mm and margins are lubate in the older colonies. The color of colony is off white initially and turns to light yellow in older colonies. The form of colony is punctiform and on 2nd to 3rd day spreads on the

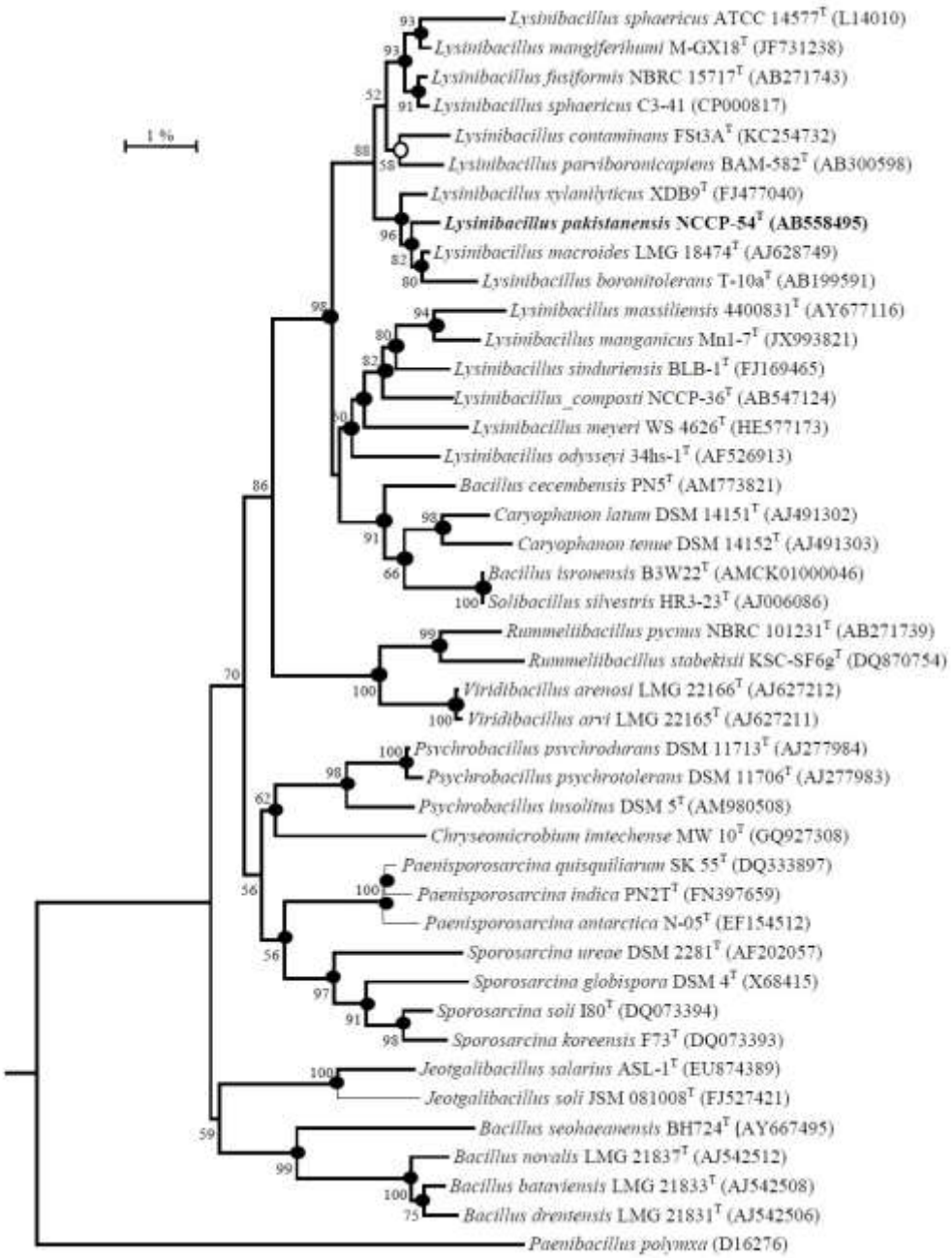

Fig. 1: Phylogenetic tree showing interrelationship of strain NCCP-54^T with closely related species of *Lysinibacillus* and other related genera inferred from 16S rRNA gene sequences. Tree was generated using the Neighbour-Joining method based on a comparison of approximately 1324 nucleotides and was rooted by using *Paenibacillus polymxa* (D16276) as an out group. Bootstrap values (more than 50%), expressed as percentage of 1000 replications, and are indicated at the nodes. Nodes indicated by empty circles were recovered by at least two algorithms, whereas nodes with solid circles were recovered by three algorithms (NJ, MLH and MP). Accession number of each type strain is shown in parentheses

whole surface of the plate. Cells can grow at 10–45°C (optimum at 28°C), at pH ranges of 6.0–9.0 (optimum at pH 7.0) and can survive in medium with 0–6% (w/v) NaCl. It can also be able to tolerate 0–150 mM boron (optimum growth without boron) in the media. Positive for catalase and can reduce nitrate to nitrogen but negative for oxidase. No acid production observed from sugar contained in API-50CH and API-20E galleries (bioMérieux, France). Strong enzyme activity for acid phosphatase and Esterase Lipase (C8), but weak activity for alkaline phosphatase and α-

Table 1: 16S rRNA gene sequence similarity and DNA-DNA relatedness between strain NCCP-54^{T} and the closely related type species of genus *Lysinibacillus* [additional to data presented by Hayat *et al.* (2013)]

Strains	*Lysinibacillus pakistanensis* NCCP-54^{T} (AB558495)	
	16S rRNA gene sequence similarity (%)	DNA–DNA Hybridization value (%)
Lysinibacillus pakistanensis NCCP-54^{T} (AB558495)	100.0	100.0
Lysinibacillus macroides LMG18474^{T} (AJ628749)	99.12	25.1
Lysinibacillus contaminans KCTC33155^{T} (KC254732)	97.63	21.6
Lysinibacillus mangiferahumi M-GX18^{T} (JF731238)	97.52	22.2
Lysinibacillus composti NCCP-36^{T} (AB547124)	96.90	27.0
Lysinibacillus sinduriensis KCTC13296^{T} (FJ169465)	96.38	31.0
Lysinibacillus odyssey KCTC 3961^{T} (AF526913)	95.81	22.0
Lysinibacillus massiliensis KCTC13178^{T} (AY677116)	95.77	19.0

chymotrypsin, whereas negative for all other enzymes of API-Zym (bioMérieux, France). Positive for L-alaninamide, α-ketovaleric acid, dextrin and acetic acid; whereas negative for pyruvic acid, alanine, pyruvatic acid methyl ester, thymidine, glutamic acid, *β*-hydroxybutyric acid, adenosine, thymidine-5'-Monophosphate, succinic acid mono-methyl ester and D-lactic acid methyl ester (Biolog GP2 MicroPlate system; BIOLOG, USA). Major polar lipids are phosphatidylglycerol diphosphatidylglycerol and phosphatidylethanolamine. The predominant cellular fatty acids are iso-$C_{15:0}$, iso-$C_{16:0}$, iso-$C_{16:1}\omega7c$ alcohol, iso-$C_{17:0}$, iso-$C_{17:1}$ ω10c, iso-$C_{16:1}$ ω11c, $C_{16:0}$, anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, and anteiso-$C_{14:0}$. Diagnostic amino acids of cell wall peptidoglycans were Lys-Asp (which corresponds to peptidoglycan variation type *A*4α). MK-7 is the major menaquinone. The DNA G+C contents of the type strain is 37 mol%.

Strain NCCP-54^{T} (=DSMZ 24784^{T} = KCTC13795^{T} = JCM 18776^{T}) is the type strain, isolated from rhizospheric soil of legume (*Glycine max* L.) sampled from the experimental area of Soil Science Department located at Research Farm of Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan.

The DDBJ/EMBL/GenBank accession number of the 16S rRNA gene sequence of the novel strain NCCP-54^{T} is AB558495.

Acknowledgments

We gratefully acknowledge the kind help of Dr. Takuji Kudo from JCM, Tsukuba, Japan in analysis of cell wall peptidoglycans.

References

Ahmed, I., A. Yokota and T. Fujiwara, 2007a**.** A novel highly boron tolerant bacterium, *Bacillus boroniphilus* sp. nov., isolated from soil, that requires boron for its growth. *Extremophiles*, 11: 217–224

Ahmed, I., A. Yokota, A. Yamazoe and T. Fujiwara, 2007b**.** Proposal of *Lysinibacillus boronitolerans* gen. nov sp nov., and transfer of *Bacillus fusiformis* to *Lysinibacillus fusiformis* comb. nov and *Bacillus sphaericus* to *Lysinibacillus sphaericus* comb. nov. *Int. J. Syst. Evol. Microbiol.*, 57: 1117–1125

Coorevits, A., A.E. Dinsdale, J. Heyrman, P. Schumann, A. Van Landschoot, N.A. Logan and P. De Vos, 2012**.** *Lysinibacillus macroides* sp. nov., nom. rev. *Int. J. Syst. Evol. Microbiol.*, 62: 1121–1127

Ezaki, T., Y. Hashimoto and E. Yabuuchi, 1989**.** Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int. J. Syst. Bacteriol.*, 39: 224–229

Hayat, R., I. Ahmed, J. Paek, M. Ehsan, M. Iqbal and Y.H. Chang, 2013**.** A moderately boron-tolerant candidatus novel soil bacterium *Lysinibacillus pakistanensis* sp. nov. cand., isolated from soybean (Glycine max L.) rhizosphere. *Pak. J. Bot.*, 45: 41–50

Kämpfer, P., K. Martin and S.P. Glaeser. 2013**.** *Lysinibacillus contaminans* sp. nov., isolated from a surface water. *Int. J. Syst. Evol. Microbiol.*, 63: 3148-3153

Kawamoto, I., T. Oka and T. Nara, 1981. Cell-Wall Composition of Micromonospora-Olivoasterospora, Micromonospora-Sagamiensis, and Related Organisms. *J. Bacteriol.*, 146: 527–534

Roohi, A., I. Ahmed, M. Iqbal and M. Jamil, 2012**.** Preliminary isolation and characterization of halotolerant and halophilic bacteria from salt mines of Karak, Pakistan. *Pak. J. Bot.*, 44: 365–370

Stackebrandt, E. and B.M. Goebel, 1994**.** Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Evol. Microbiol.*, 44: 846–849

Yang, L.L., Y. Huang, J. Liu, L. Ma, M.H. Mo, W.J. Li and F.X. Yang. 2012**.** *Lysinibacillus mangiferahumi* sp. nov., a new bacterium producing nematicidal volatiles. *Antonie van Leeuwenhoek*, 102: 53–59

(Received 30 September 2013; Accepted 24 October 2013)