Antonie van Leeuwenhoek (2015) 107:133–139
DOI 10.1007/s10482-014-0310-5

ORIGINAL PAPER

Cohnella capsici sp. nov., a novel nitrogen-fixing species isolated from *Capsicum annuum* rhizosphere soil, and emended description of *Cohnella plantaginis*

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Received: 16 November 2013 / Accepted: 17 October 2014 / Published online: 4 November 2014

Abstract A novel bacterial strain designated YN-59^T was isolated from *Capsicum annuum* rhizosphere soil in China. The isolate was found to be aerobic, Gram-positive, rod-shaped and to form ellipsoidal or oval spores positioned centrally in swollen sporangia. On the basis of 16S rRNA gene sequence analysis, the isolated strain YN-59 was determined to be related to members of genus *Cohnella*. High levels of 16S rRNA gene sequence similarity were found between strain YN-59 and *Cohnella plantaginis* DSM 25424^T (98.5 %) and *Cohnella ginsengisoli* DSM18997^T (97.3 %); the 16S rRNA gene sequence similarities between strain YN-59 and the other strains recognized members of the genus *Cohnella* were below 97 %. The DNA–DNA hybridization values of strain YN-59 with *C. plantaginis* DSM 25424^T and *C. ginsengisoli* DSM18997^T were 44.2 ± 8.4 and 28.8 ± 5.8 %, respectively. The DNA G + C content of strain YN-59^T was determined to be 59.32 mol %. The major isoprenoid quinone was identified as MK-7 and the predominant fatty acids as anteiso-$C_{15:0}$ (45.32 %), iso-$C_{16:0}$ (19.19 %), iso-$C_{15:0}$ (9.65 %) and $C_{16:0}$ (8.91 %). The polar lipids of strain YN-59^T were found to consist of diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol; several unidentified phospholipids were also detected. The diagnostic diamino acid in the cell wall was identified as *meso*-diaminopimelic. On the basis of its phenotypic and genotypic characteristics and levels of DNA–DNA hybridization, strain YN-59^T is considered to represent a novel species of the genus *Cohnella*, for which the name *Cohnella capsici* sp. nov. (type strain YN-59^T - = CGMCC 1.12046^T = JCM 19168^T) is proposed.

Keywords *Cohnella capsici* sp. nov. · Nitrogen-fixing *Cohnella*

Introduction

The genus *Cohnella*, a member of the family *Paenibacillaceae* was created by Kämpfer et al.(2006). Members of the genus *Cohnella* differ from those of the genus *Paenibacillus* on the basis of 16S rRNA gene sequence analysis, polar lipid patterns and fatty acid composition. Members of *Cohnella* are spore-forming, aerobic and rod-shaped and contain MK-7. The major fatty acids are iso-$C_{16:0}$, anteiso-$C_{15:0}$ and $C_{16:0}$ (Kämpfer et al. 2006). At the time of writing the genus contains twenty-one species (http://www.bacterio.net/

Electronic supplementary material The online version of this article (doi:10.1007/s10482-014-0310-5) contains supplementary material, which is available to authorized users.

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cohnella.html) including *Cohnella arctica* (Jiang et al. 2012), *Cohnella boryungensis* (Yoon and Jung 2012), *Cohnella cellulosilytica* (Khianngam et al. 2012), *Cohnella damuensis* (Luo et al. 2010), *Cohnella ferri* (Mayilraj et al. 2013), *Cohnella fontinalis* (Shiratori et al. 2010), *Cohnella formosensis* (Hameed et al. 2013), *Cohnella ginsengisoli* (Kim et al. 2010), *Cohnella hongkongensis* (Kämpfer et al. 2006), *Cohnella laeviribosi* (Cho et al. 2007), *Cohnella luojiensis* (Cai et al. 2010), *Cohnella panacarvi* (Yoon et al. 2007), *Cohnella plantaginis* (Wang et al. 2012), *Cohnella phaseoli* (García-Fraile et al. 2008), *Cohnella soli* (Kim et al. 2011), *Cohnella suwonensis* (Kim et al. 2011), *Cohnella terrae* (Khianngam et al. 2010a), *Cohnella thailandensis* (Khianngam et al. 2010b), *Cohnella thermotolerans* (Kämpfer et al. 2006), *Cohnella yongneupensis* (Kim et al. 2010) and *Cohnella xylanilytica* (Khianngam et al. 2010a). *C. plantaginis* shows a capacity to fix atmospheric nitrogen in vitro (Wang et al. 2012). Here we show that a nitrogen-fixing bacterial strain YN-59^T, recently isolated from *Capsicum annuum* rhizosphere soil, represents a novel species of the genus *Cohnella* on the basis of phenotypic, chemotaxonomic characterization and 16Sr RNA gene sequence analysis.

Materials and methods

Isolation of YN-59

The sample used for isolation of the strain YN-59^T was collected from the rhizosphere of *C. annuum* in Kunming, Yunnan province of China (25°12′ N, 102° 40′ E). One gram of soil was diluted in 9 ml sterile water and heated at 80 °C for 15 min, then the heated dilution (100 µl) was placed on nitrogen-free medium containing (per liter) 20 g sucrose, 0.1 g K_2HPO_4, 0.4 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.1 g NaCl, 0.01 g $FeCl_3$ and 0.002 g Na_2MoO_4. The strain was selected and purified after 3 days incubation at 30 °C. The reference strains *C. ginsengisoli* DSM 18997^T and *C. plantaginis* DSM 25424^T were maintained on LD medium agar (10 g tryptone, 5 g yeast extract, 2.5 g NaCl, pH 7.0). *C. ginsengisoli* DSM 18997^T was kindly provided by Dr Soon-Wo Kwon; *C. plantaginis* DSM 25424^T was isolated from plantain rhizosphere soil in our laboratory (Wang et al. 2012).

Nitrogenase activity assay

To confirm the nitrogen-fixing capacity, an assay for nitrogenase activity was carried out. Nitrogenase activity of YN-59^T was determined in comparison with reference strains according to the acetylene reduction method (Berge et al. 2002). The reference strains were *Paenibacillus brasilensis* DSM 13188^T, *Paenibacillus polymyxa* DSM 36^T, *Paenibacillus kribbensis* JCM 11465^T and *Paenibacillus zanthoxyli* DSM18202^T (obtained from our lab). The strains were grown in nitrogen-deficient medium containing (per liter) 26.3 g $Na_2HPO_4{\cdot}12H_2O$, 3.4 g KH_2PO_4, 26 mg $CaCl_2{\cdot}2H_2O$, 30 mg $MgSO_4$, 0.3 mg $MnSO_4$, 36 mg Ferric citrate, 7.6 mg $Na_2MoO_4{\cdot}2H_2O$, 10 µg p-aminobenzoic acid, 10 µg biotin, 0.4 %sucrose, and 0.1 %glutamate. After 48 h incubation at 30 °C, strains were incubated under acetylene for 3 days and then analyzed for ethylene production by gas chromatography.

Phenotypic characterization

To determine cell morphology, strain YN-59^T was grown on an endospore-forming medium agar plate [yeast extract 0.07 %, trypone 0.1 %, glucose 0.1 %, $(NH_4)_2SO_4$ 0.02 %, $MgSO_4{\cdot}7H_2O$ 0.02 %, K_2HPO_4 0.1 % (w/v), pH 7.2] for 72 h and then the morphology of cells was examined under scanning electrical microscopy (SEM). The flagellation type was determined by transmission electron microscopy (TEM) after 48 h incubation of strain YN-59^T on LD agar plate at 30 °C. Strain YN-59^T was tested for a range of phenotypic, physiological and biochemical characteristics together with the type strain of *C. ginsengisoli* DSM 18997^T and *C. plantaginis* DSM 25424^T. Catalase activity was analyzed by bubble formation in a 3 % (v/v) H_2O_2 solution.Nitrate reduction, production of dextrin, the Voges-Proskauerreaction and lysozyme test were performed according to (Gordon et al. 1973), (Priest et al. 1981) and Rhodes-Roberts (1981), respectively. Optimal temperature and temperature range for growth were determined after incubation at 4, 10, 20, 25, 30, 37, 40, 45 and 50 °C on LD agar. The pH range for growth was determined in LD broth adjusted to pH 4.0–10.0 (using increments of 1.0 pH unit) by using HCl and NaOH buffers. Growth in the absence of NaCl and in the presence of 1.0, 2.0 and 3.0 % (w/v) NaCl was

investigated by using LD broth. Hydrolysis of casein and starch was tested on LD agar, using the substrate concentrations described by Cowan and Steel (1965). Acid production was tested by using medium containing 1 g $(NH)_2HPO_4$, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.2 g KCl, 0.2 g yeast extract, 10 g sugars or alcohols dissolved in 1L water (Shirling and Gottlieb 1966). Utilization of substrates as sole carbon and energy sources was tested as described by Baumann and Baumann (1981). *Cohnella* reference strains were assayed under the same growth conditions as strain YN-59^T.

Chemotaxonomy characterization

For determination of cellular fatty acid composition, strain YN-59^T and the type strains of *C. ginsengisoli* DSM 18997^T and *C. plantaginis* DSM 25424^T were incubated on LD agar at 30 °C for 48 h. The cellular fatty acid pattern was analyzed using the Sherlock Identification System (MIDI) (Sasser et al. 2005). Respiratory quinones were extracted, purified and analyzed on HPLC according to the method described by Collins (1985). Polar lipids extracted by the method of Minnikin et al. (1979) were identified by two-dimensional TLC and spraying with specific reagents as described by Collins et al. (1980). The isomer type of diamino acid of the cell wall peptidoglycan was determined as described by Schleifer and Kandler (1972).

Molecular characterization

A full-length sequence of the 16S rRNA gene was obtained from a PCR product amplified from strain YN-59^T by using the forward primer 27F (AGAGTTTGATCCTGGCTCAGAACGAACGCT) and the reverse primer 1492R (TACGGCTACCTTGTTACGACTTCACCCC). The obtained 16S rRNA gene was sequenced by the Invitrogen company. A preliminary phylogenetic analysis was performed using the EzTaxon-e database (Kim et al. 2012). The phylogenetic tree was constructed using the neighbour-joining method (Saitou and Nei, 1987). Evolutionary distances were calculated according to Kimura's two-parameter model (Kimura, 1980), Bootstrap analysis was on the basis of 1000 replications. The software package MEGA version 4.0 (Tamura et al. 2007) was used for the above analysis.

DNA was extracted and purified according to the method (Yoon et al. 1996) and the DNA G + C content of the strain YN-59^T was determined according to the method of De Ley et al. (1970); DNA–DNA hybridization experiments were performed according to the method of Ziemke et al. (1998).

Results and discussion

Phenotypic characteristics

Strain YN-59^T was found to be Gram-positive, aerobic, motile and rod-shaped (0.4–0.8 × 2.1–2.8 μm). It formed ellipsoidal spores, located in the centre of the swollen sporangia (Supplementary Fig. S1). The strain YN-59^T was found to be mobile by means of peritrichous flagella (Supplementary Fig. S1). Colonies on LD medium agar plates were observed to be circular, convex, opaque and glossy with entire margins after 72 h incubation at 30 °C. The strain was found to grow in 3 % NaCl (w/v) but not in 0.001 % (w/v) lysozyme. The temperature range for growth was determined to be 4–45 °C and optimal at 37 °C. Strain YN-59^T grows at pH 5.0–8.0 and optimally at pH 7.2. The catalase test was found to be positive, whereas the oxidase was negative. Compared to nitrogenase activity of four reference nitrogen-fixing strains (*P. zanthoxyli* DSM18202^T, 4917.6 ± 101.8 nmol C_2H_4 [mg protein h]$^{-1}$, *P. brasilensis* DSM 13188^T, 545.7 ± 9.1, *P. polymyxa* DSM 36^T, 455.1 ± 12.8 and *P. kribbensis* JCM 11465^T, 315.8 ± 7.8), strain YN-59^T showed relatively high nitrogenase activity (755.8 ± 12.8 nmol C_2H_4 [mg protein h]$^{-1}$). Compared to closely related *Cohnella* strains, isolate YN-59^T exhibited almost identical phenotypic characteristics, except for temperature range (4–45 °C) and pH range (5.0–8.0) for growth. The utilization of sucrose, inositol and fructose as sole carbon source and acid production from *N*-acetyl glucosamine differentiate strain YN-59 from its closest phylogenetic neighbours. The phenotypic characteristics that differentiate the novel strain YN-59 from the type strains of related phylogenetic species are shown in Table 1. The strain is sensitive to the antibiotics ampicillin (100 μg/ml), spectinomycin (100 μg/ml), streptomycin (40 μg/ml) and neomycin (7 μg/ml), but resistant to tetracycline (15 μg/ml), kanamycin (50 μg/ml).

Table 1 Differential phenotypic characteristics between strain YN-59^T and selected type strains of species in the genus *Cohnella*

Characteristic	1	2	3
Growth temperature (°C)	4–45	10–45	10–40
Growth pH	5.0–8.0	5.0–8.0	6.0–9.0
Growth in the presence of 3 % NaCl	+	+	−
Nitrate reduction	−	−	+
Dextrin	+	+	−
Assimilation of:			
Sucrose	+	+	−
Glycerin	−	+	+
Inositol	+	−	−
Fructose	+	+	−
Acid production from:			
D-xylose	+	−	−
Fucose	+	−	−
Lactose	+	+	−
N-acetyl glucosamine	+	+	−
Ribose	+	+	−
Sucrose	+	+	−
DNA G+C (%)	59.3	59.3	61.3

Species: *1* Strain YN-59^T (data from this study), *2 Cohnella plantaginis* DSM 25424^T(data from this study), *3 Cohnella ginsengisoli* DSM 18997^T(data from this study)

+ Positive reaction, − negative reaction, *ND* no data

Chemotaxonomic characteristics

The major fatty acid compositions of the strain YN-59^T, together with *C. ginsengisoli* DSM 18997^T and *C. plantaginis* DSM 25424^T are shown in Table 2. The major fatty acids were identified as anteiso-$C_{15:0}$ (45.3 %), iso-$C_{16:0}$ (19.2 %), iso-$C_{15:0}$ (9.7 %) and $C_{16:0}$ (8.9 %), which are also the predominant fatty acids of the members of the genus *Cohnella*. The major quinone componentMK-7 in the members of the genus *Cohnella*, wasalso the predominant isoprenoid quinone identified in the strain YN-59^T. The major polar lipids of strain YN-59^T were found to consist of diphosphatidylglycerol and phosphatidylglycerol; lesser amounts of phosphatidylethanolamine and several unidentified minor lipids were also detected (Supplementary Fig. 2). This profile is in agreement with the characteristics of the genus *Cohnella*. However, our reappraisal of the polar lipid data suggests that phosphatidylinositol and phosphatidylinositol mannosides are also unlikely to be present in *C. plantaginis* DSM 25424^T as originally reported (Wang et al. 2012). The isomer type of diamino acid of the cell wall peptidoglycan of strain YN-59^{T}was identified as *meso*-diaminopimelic acid.

Table 2 Fatty acid content (%) of strain YN-59^T and selected type strains of species in the genus *Cohnella*. All data from the present study

Fatty acid	1	2	3
Saturated fatty acids			
$C_{14:0}$	1.6	1.6	1.6
$C_{16:0}$	7.7	8.9	9.6
Branched fatty acids			
Iso-$C_{14:0}$	4.1	4.0	6.6
Iso-$C_{15:0}$	11.3	9.7	13.9
Iso-$C_{16:0}$	18.6	19.2	21.6
Iso-$C_{17:0}$	1.2	Tr	1.7
Anteiso-$C_{15:0}$	44.3	45.3	37.2
Anteiso-$C_{17:0}$	3.1	3.5	2.8
Unsaturated fatty acids			
$C_{18:1\ w9c}$	1.4	1.5	2.8

Species: *1* Strain YN-59^T, *2 Cohnella plantaginis* DSM 25424^T, *3 Cohnella ginsengisoli* DSM 18997^T

tr trace (<1 %)

Molecular characterization

Comparison of the 16S rRNA gene sequence of strain YN-59^T with sequences held in the EzTaxon-e database revealed that this bacterium clusters with species of the genus *Cohnella*. A phylogenetic tree based on 16S rRNA gene sequences was constructed using the software package MEGA version 4.0 (Tamura et al. 2007) using the neighbour-joining method. As show in Fig. 1, the phylogenetic analysis revealed that strain YN-59^T clusters together with *Cohnella* spp. and showed the position of the strain YN-59^T in relation to other *Cohnella* species. The highest 16S rRNA gene sequence similarity between YN-59 and *C. plantaginis* DSM 25424^T and *C. ginsengisoil* DSM18997^T was 98.5 and 97.3 %, while the 16S rRNA gene sequence similarity of YN-59^T with other closely related *Cohnella* spp. was lower than 97 %. Given the high 16S rRNA gene sequence similarity betweenYN-59 and it closest phylogenetic relatives, DNA–DNA hybridization analysis was conducted.

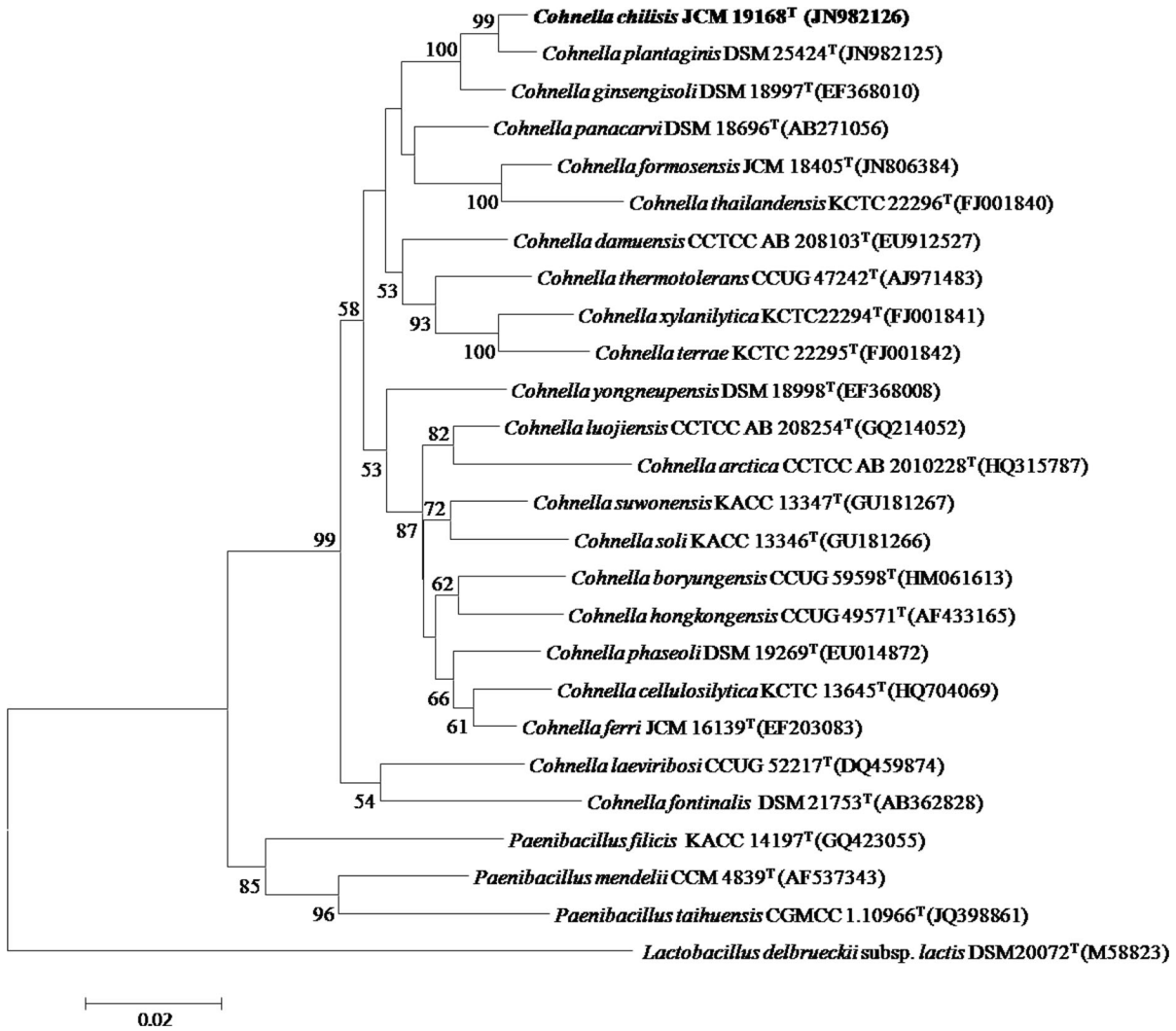

Fig. 1 Neighbour-joining phylogenetic tree based on 16Sr RNA gene sequences showing the position of strain YN-59^T among species of the genus *Cohnella*. *Lactobacillus delbrueckii* subsp. *lactis* DSM 20072^T (M58823) was used as an out group. Bootstrap analyses were performed with 1,000 cycles. Only bootstrap values >50 % are shown at the *branch points*. *Bar* 0.02 substitutions per nucleotide positions

DNA–DNA relatedness between YN-59^T and *C. plantaginis* DSM 25424^T and *C. ginsengisoil* DSM18997^T was 44.2 ± 8.4 % and 28.8 ± 5.8 % respectively. Values below 70 % are a key marker for the identification of a novel species (Wayne et al. 1987). These data show that strain YN-59^T can be considered a novel species of the genus *Cohnella*. The DNA G + C content of strain YN-59^T was determined to be 59.32 % (Table 1).

In summary, phylogenetic analysis based on the full-length 16S rRNA gene sequence, DNA G + C content and chemotaxonomic properties revealed that strain YN-59^T can be clearly affiliated with the genus *Cohnella*. The phenotypic characteristics and levels of DNA–DNA hybridization values below 70 % further demonstrate that the isolate strain YN-59^T should be classified a novel species of the genus *Cohnella*, for which the name *Cohnella capsici* sp. nov. is proposed.

Description of Cohnella capsici sp. nov.

Cohnella capsici (cap'si.ci. N.L. gen. n. *capsici* of the plant Capsicum, referring tothe plant *C. annuum*, the

source of the rhizosphere soil from which the strain was isolated).

Cells are mobile Gram-positive rods (approximately 0.4–0.8 × 2.1–2.8 μm) that can form ellipsoidal spores centrally in swollen sporangia. Colonies on LD agar are circular, convex, opaque and glossy with entire margins and usually measure 1.0–2.5 mm in diameter. Growth occurs at 4–45 °C (optimum, 30 °C), at pH 5.0–8.0 (optimum, 7.0) and in the presence of 0–2 % NaCl (w/v). Cannot grow in 0.001 %(w/v) lysozyme. Catalase test is positive, whereas oxidase is negative. Voges-Proskauer, methyl red reaction and nitrate reduction are negative. Starch is hydrolysed but gelatin and casein are not. β-Galactosidase is produced, but not urease arginine dihydrolase, phenylalanine deaminase and indole. Acid is produced from *N*-acetyl glucosamine, inulin, lactose, ribose, D-xylose, fucose, sucrose, mannitol and raffinose, but not from mannose, galactose, glucose, glycerin, maltose, fructose, sorbitol, sorbose, rhamnose, inositol, creatine, gluconic acid sodium salt, sodium succinate, sodium malate or sodium citrate. Cannot use the following substrates: glycerol, fructose, sorbose, creatine, sodium citrate. The major fatty acids are anteiso-$C_{15:0}$, iso-$C_{16:0}$, iso-$C_{15:0}$, and $C_{16:0}$. Contains menaquinone with seven isoprene units (MK-7) as the predominant quinone. Diphosphatidylglycerol and phosphatidylglycerol are the major polar lipids and phosphatidylethanolamine is present. The isomer type of diamino acid of the cell wall peptidoglycan is *meso*-diaminopimelic acid. The G+C content of the type strain is 59.32 mol %.

The type strain of the species is YN-59^T(=CGMCC 1.12046^T = JCM 19168^T), which was isolated from *C. annuum* rhizosphere soil collected in Kunming, Yunnan province of China. The GenBank (EMBL) accession number for the 16S rRNA gene sequence of strain YN-59^T is JN982126.

Emended description of Cohnella plantaginis Wang et al. 2012

The description is as given in Wang et al. (2012) except thatphosphatidylinositol and phosphatidylinositol mannosides cannot confirmed to be present and the polar lipid profile should be emended as follows: the polar lipids contain of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, lyso-phosphatidylglycerol and unidentified lipids.

Acknowledgments We thank Dr Soon-Wo Kwon for providingthe reference type strain. We are grateful to Ph.D.Takuji Kudo for depositing the strain in JCM. This work was supported by the 863 High Technology Program (Grant No. 2013AA102802-04).

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