Pak. J. Agri. Sci., Vol. 51(3), 595-608; 2014
ISSN (Print) 0552-9034, ISSN (Online) 2076-0906
http://www.pakjas.com.pk

HEAVY METAL-TOLERANT AND PSYCHROTOLERANT BACTERIUM *Acinetobacter pakistanensis* SP. NOV. ISOLATED FROM A TEXTILE DYEING WASTEWATER TREATMENT POND

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A Gram-stain negative, cocci to short rod, non-motile, strictly aerobic, heavy metal-tolerant and psychrotolerant bacterium, designated NCCP-644^T, was isolated from a textile dyeing wastewater treatment pond. The optimum (and ranges of) temperature and pH for growth of strain NCCP-644^T was 20-25°C (3-37°C) and 7–8 (6-10), respectively. The strain can tolerate 0-3% NaCl (w/v) and heavy metals (Cr 2700 ppm, As 3000 ppm, Pb 2100 ppm and Cu 2700 ppm) in tryptic soya agar medium. The sequences of the 16S rRNA gene and three housekeeping genes, *gyrB, rpoB* and *atpD*, of strain NCCP-644^T showed the highest similarity (98.3, 91.9, 95.3 and 96.6 %, respectively) with the strain "*Acinetobacter kyonggiensis*" KSL5401-037^T. Phylogenetic analyses from the sequence of the16S rRNA, *gyrB* and *rpoB* genes also revealed the affiliation of NCCP-644^T with members of the genus *Acinetobacter.* The chemotaxonomic data [major quinones as Q-9; major cellular fatty acids as summed feature 3 (iso-$C_{15:0}$ 2OH/$C_{16:1}$ω7c) followed by $C_{18:1}$ ω9*c*, $C_{16:0}$, and $C_{12:0}$ 3-OH; major polar lipids as diphosphatidyl glycerol, phosphatidyl serine, phosphatidyl monomethyl ethanol, phosphatidyl inositol, phosphatidyl ethanolamine, phosphatidyl glycerol and one unknown phospholipid] also support the affiliation of strain NCCP-644^T with the genus *Acinetobacter.* The level of DNA–DNA relatedness between strain NCCP-644^T and the closely related type strains of the genus *Acinetobacter* was < 54.7 %. Based on the phylogenetic analyses, DNA–DNA hybridisation and biochemical characteristics, strain NCCP-644^T differed from the validly named *Acinetobacter* species and thus, it represents a new species. The name, *Acinetobacter pakistanensis* sp. nov.is proposed for the type strain NCCP-644^T (= LMG 28467^T = KCTC 42081^T = JCM 18977^T).

Keywords: Heavy metal tolerant, psychrotolerant, textile dyeing wastewater, *Acinetobacter pakistanensis*

INTRODUCTION

Brisou and Prévot (1954) proposed the genus *Acinetobacter* with the type species *A. calcoaceticus* in the family *Moraxellaceae*, and it comprises thirty three validly named species (Euzeby, 2014). Furthermore, there are seven other species, "*A. marinus*", "*A. seohaensis*" (Yoon *et al*., 2007), "*A. septicus*" (Kilic *et al*., 2008), "*A. antiviralis*" (Lee *et al*., 2009), "*A. kyonggiensis*" (Lee and Lee, 2010), "*A. oleivorans*" (Kang *et al*., 2011) and "*A. oryzae*" (Chaudhary *et al*., 2012), which have been published but yet not validated. Recently, three new species, *A. guangdongensis, A. bohemicus* and *A. apis* have been published (Feng *et al*., 2014; Kim *et al*., 2014; Krizova *et al*., 2014) but these are also still not validated names. Although several members of the genus *Acinetobacter* were isolated from clinical specimens and received; therefore, much higher interest, a majority of the members isolated from diverse environmental habitats, such as activated sludge, wetlands, forest soil, seawater, dump sites, wastewater, cotton, cankar bark, floral nectar and wild rice (Nishimura *et al*., 1988; Carr *et al*., 2003; Yoon *et al*., 2007; Lee and Lee, 2010; Vaz-Moreira *et al*., 2011; Malhotra *et al*., 2012; Álvarez-Pérez *et al*., 2013; Choi *et al*., 2013; Li *et al*., 2013; Li *et al*., 2014a; Li *et al*., 2014b). This genus consists of aerobic, Gram-negative coccobacillary rods, non-motile and non-fermentative bacteria. Many of these species can survive in a wide range of environmental conditions for a long period. Several of these organisms can grow over a wide temperature range on typical culture medium. Recently, *Acinetobacter harbinensis* has been reported to grow at temperatures as low as 2ºC (Li *et al*., 2014a). Several other *Acinetobacter* strains also identified from frozen food, therefore, these species attract particular interest.

Several microorganisms tolerate toxic concentrations of heavy metals (Affan *et al*., 2009; Zahoor and Rehman, 2009;

Tripathi *et al*., 2010; Tripathi *et al*., 2011). These heavy metal-tolerant bacteria offer an opportunity to exploit in the bioremediation of contaminated soil/water systems. During our studies of microbial diversity for heavy metal-tolerant bacteria, strain NCCP-644^T was isolated from a textile dyeing wastewater sample collected from a wastewater treatment pond. The objective of this study is to delineate the taxonomic position of the strain by phenotypic, chemotaxonomic and phylogenetic characterisation. Based on the results, it is concluded that strain NCCP-644^T represents a new species of the genus *Acinetobacter*.

MATERIALS AND METHODS

***Isolation and growth of strains*:** Strain NCCP-644^T was isolated from a wastewater sample collected from the treatment pond of textile dyeing wastewater plant of Kohinoor mills, Islamabad, Pakistan. The strain was recovered on tryptic soy agar (TSA, Difco) supplemented with different concentrations of heavy metals, including Cr^{+2}, As^{+2}, Pb^{+2} and Cu^{+2} using the dilution plate method. The purified strain was maintained on agar medium and stored in glycerol (35%, w/v) at –80ºC and used for the phenotypic and phylogenetic characterisation experiments. Based on the sequence similarity of the 16S rRNA gene, the type strains of the closely related taxa "*Acinetobacter kyonggiensis*" JCM 17071^T, *A. harbinensis* KCTC 32411^T, *A. bouvetii* JCM 18991^T, *A. beijerinckii* JCM 18990^T, and *A. johnsonii* JCM 20194^T and the type species of the genus *A. calcoaceticus* JCM 6842^T were used as reference strains for these characterisation experiments under the same laboratory conditions. The characterisation experiments were performed at 25ºC unless otherwise mentioned.

***Heavy metal tolerance*:** To determine the tolerance of the novel strain to toxic concentrations of heavy metals, the isolated strain NCCP-644^T and the reference strains were grown on TSA supplemented separately with different concentrations of the heavy metals Cr^{+2}, As^{+2}, Pb^{+2} and Cu^{+2} for five to seven days. The concentration of the heavy metals Cr^{+2}, As^{+2}, Pb^{+2} and Cu^{+2} in the agar media ranged from 300-3000 ppm and were prepared using the salts $K_2Cr_2O_7$, Na_2HAsO_4, $Pb(NO_3)_2$ and $CuSO_4.5H_2O$, respectively.

***Morphology and phenotypic characterisation*:** Colony morphology of strain NCCP-644^T was observed on well-isolated colonies, grown on TSA for 2 days. A phase-contrast microscope (Nikon Optiphot-2, Japan) was used to examine the cells of strain NCCP-644^T grown on TSA for 24-48 h. Gram staining was performed using a commercial kit (bio-Me´rieux, France) according to manufacturer's instructions. The motility of strain NCCP-644^T was determined with M medium (bioMérieux, France) and microscopy. The oxygen dependence was determined on TSA by incubating the cells in an anaerobic chamber (Mitsubishi Gas Chemicals Co., Inc.) for 10 days.

The optimum and range of pH for growth was determined in tryptic soy broth (TSB; Difco) by adjusting the pH to a range of 4.0 to 10.0 at an increment of 1 pH unit, and the OD_{600} was monitored using a spectrophotometer (Beckman Coulter Model DU730, USA). The pH values were adjusted with HCl or Na_2CO_3 and buffers (Sorokin, 2005) and were verified after autoclaving. The temperature range for growth was determined on TSA (pH 7.0) by incubating the cells at different temperatures (3, 5, 10, 15, 20, 25, 30, 33, 37, 40, 45 and 50ºC) for 6 days. Growth in various NaCl concentrations was investigated in mTGE agar medium (Difco), which contains (per litre) beef extract (6 g), tryptone (10 g), dextrose (2 g), and agar (15 g), supplemented with various concentrations of NaCl (0–10%; w/v), with the pH adjusted to pH 7.0 and incubation for 6 days.

The physiological and biochemical characteristics were determined using the API 20E, API 20NE and API 50CH galleries (bioMérieux, France). Because negative reactions were primarily obtained with the API 50CH and API 20E for the utilisation of various carbon sources, we also analysed an extended array of biochemical features of the strains using the API Rapid 32 ID system (bioMérieux, France). The catalase and oxidase activities were determined using the API Color Catalase and API Oxidase Reagent (bioMérieux, France). Antibiotic resistance was assessed with an ATB-VET strip (bioMérieux, France), and the enzyme activities were determined with an API ZYM strip (bioMérieux, France). The API suspension medium was used to inoculate the strips. All commercial kits were used according to the manufacturers' protocols, except for the API Rapid 32 ID, for which the strains were grown aerobically on TSA at 25ºC rather than anaerobic growth on blood agar media.

***Amplification, sequencing and phylogenetic analysis of 16S rRNA and housekeeping genes, gyrase subunit B (gyrB), RNA polymerase beta subunit (rpoB) and ATP synthase beta subunit (atpD)*:** A nearly complete 16S rRNA gene was amplified as previously described (Ahmed *et al*., 2007). The housekeeping genes *gyrB* and *atpD* were amplified using the primers and PCR conditions described previously by Brady *et al*. (2008), whereas the *rpoB* gene was amplified and sequenced according to the method of La Scola *et al*. (2006). The purified PCR product was sequenced at Macrogen, Korea (http://dna.macrogen.com/eng) using universal primers for the 16S rRNA gene and internal primers for the housekeeping genes as described above (La Scola *et al*., 2006; Brady *et al*., 2008). The sequences were assembled using the BioEdit software to obtain a consensus sequence of the genes, which were then submitted to the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/). The strain was identified using the sequence of the 16S rRNA gene on the Ez-Taxon Server

(http://eztaxon-e.ezbiocloud.net) and a BLAST search of the housekeeping genes on the DDBJ/NCBI servers. The 16S rRNA gene sequences of closely related validly published type strains were retrieved from the EzTaxon Server database and phylogenetic trees were constructed as described previously (Ahmed *et al*., 2014) using three algorithms, the maximum parsimony (MP), neighbour joining (NJ) and maximum likelihood (ML) methods. The sequence similarities of the housekeeping genes *gyrB, rpoB* and *atpD* were estimated with the closely related available sequences using the Kimura 2-parameter model. The phylogenetic trees were constructed using nucleotide sequences of the *gyrB* and *rpoB* genes with the sequences of related species of the genus *Acinetobacter*. The stability of the relationship was assessed using bootstrap analysis with 1,000 re-samplings for the tree topology.

***DNA base composition, DNA–DNA hybridisation*:** For the DNA G+C content analysis and DNA–DNA hybridisation, the genomic DNA of strain NCCP-644^T and the reference strains were isolated using a Qiagen Genomic-tip 500/G following the manufacturer's protocol, with a minor modification in which the RNase T1 was used in addition to the RNase A. The DNA–DNA hybridisation of strain NCCP-644^T and the reference strains "*A. kyonggiensis*" JCM 17071^T, *A. harbinensis* KCTC 32411^T, *A. bouvetii* JCM 18991^T, *A. beijerinckii* JCM 18990^T, and *A. johnsonii* JCM 20194^T was performed at 45 ºC with photobiotin-labelled DNA and microplates as described by Ezaki *et al*. (1989). A Fluoroskan Ascent Plate Reader (Thermo Lab Systems, USA) was used for the fluorescence measurements.

To determine the DNA G+C contents, the genomic DNA was digested with P1 nuclease and alkaline phosphatase. The DNA G+C contents were analysed on an HPLC (model UFLC, Shimadzu, Japan) at 270 nm using solvent $NH_4H_2PO_4$ (0.02 M) -CH_3CN (v/v 20:1) with a Cosmosil 5C18 column (4.6 by 150 mm; Nacalai Tesque; reversed phase silica gel; C18).

***Chemotaxonomic analyses*:** For whole-cell fatty acid analysis, strain NCCP-644^T and the reference strains were grown on TSA for 24 h. The cellular fatty acid methyl esters were prepared (Sasser, 1990) and analysed on a GC (6890; Hewlett Packard) according to the standard protocol of the Sherlock Microbial Identification System (MIDI Sherlock version 4.5, MIDI database TSBA40 4.10). The respiratory quinone and polar lipids of strain NCCP-644^T and the closely related reference strain"*A. kyonggiensis*" JCM 17071^T were analysed from 150–200 mg lyophilised cells grown in TSB for 24 h as described by Minnikin *et al*. (1984). Isoprenoid quinones were purified by TLC and then analysed by HPLC. The polar lipids were examined by two-dimensional TLC, using HPTCL plates (10X10 cm) Silica gel 60 (Merck), as described by Kudo (2001).

RESULTS AND DISCUSSION

Strain NCCP-644^T formed off-white colonies, which are moist and raised with entire margins. The colonies are 1–2 mm in size after 1–2 days when grown on TSA (Difco) medium at 25°C. Old colonies (10 days) may become bigger (3-4 mm). Cells of the strain are Gram-stain negative, non-motile, strictly aerobic and cocci to short rod (coccobacillus), which occur primarily in pairs and sometimes in triplet form. The optimum growth of cells was at pH 7.0-8.0 (range of 6.0–10.0). Strain NCCP-644^T is sensitive to NaCl and can tolerate only up to 0–3% NaCl (w/v) (optimum without NaCl) in TSB (Difco) medium. The strain can grow at 3–37ºC (optimum 20–25ºC). No growth was observed at 40ºC and there was a slight growth at 37 ºC after many days. Strain NCCP-644^T exhibited many phenotypic features that were similar to that of closely related taxa "*A. kyonggiensis*" JCM 17071^T, *A. harbinensis* KCTC 32411^T, *A. bouvetii* JCM 18991^T, *A. beijerinckii* JCM 18990^T, *A. johnsonii* JCM 20194^T and *A. calcoaceticus* JCM 6842^T; but many biochemical and physiological characteristics also differentiated these species (Table 1). Of these, the most notable differentiating characteristics compared to the most closely related species, "*A. kyonggiensis*" JCM 17071^T, are growth of NCCP-644^T at 3°C, positive reactions for Voges-Proskauer and assimilation of capric acid. Strain NCCP-644^T also differed from the reference strains for tolerance against toxic concentrations of heavy metals (Table 1). Strain NCCP-644^T showed growth at 3°C, which is unusual for other species of the genus *Acinetobacter*, except *A. harbinensis.*

The isolated novel strain NCCP-644^T showed tolerance to toxic concentrations of heavy metals, including Cr^{+2}, As^{+2}, Pb^{+2} and Cu^{+2}. It grew well in TSA medium containing Cr 2700 ppm, As 3000 ppm, Pb 2100 ppm and Cu 2700 ppm. By comparison, the closely related reference strains were also tested for tolerance to heavy metals under similar conditions. It was found that the toxic effects of Cr^{+2}, Pb^{+2} and Cu^{+2} significantly differentiated the NCCP-644^T from the reference species (Table 1). However, the majority of the strains in this study tolerated high concentrations of As^{+2}, except "*A. kyonggiensis*" JCM 17071^T and *A. calcoaceticus* JCM 6842^T. By comparison with the previously reported heavy metal-tolerant bacteria (Affan *et al*., 2009; Zahoor and Rehman, 2009; Tripathi *et al*., 2010; Tripathi *et al*., 2011), strain NCCP-644^T is considered highly tolerant to toxic concentrations of heavy metals.

***Genotypic characterization*:** Comparative sequence analyses of the 16S rRNA, *gyrB, rpoB* and *atpD* genes confirmed the inter species relatedness of strain NCCP-644^T with the closely related type strain "*A. kyonggiensis*" JCM 17071^T and the other described *Acinetobacter* species. The sequence (1413 nucleotides) of 16S rRNA gene of strain NCCP-644^T

Table 1. Differentiating phenotypic and biochemical characteristics of strain NCCP-644^T and the type strains of closely related *Acinetobacter* species

	NCCP-644^T	"*A. kyonggiensis*" JCM17071^T	*A. harbinensis* KCTC32411^T	*A. bouvetii* JCM 18991^T	*A. beijerinckii* JCM18990^T	*A. johnsonii* JCM 20194^T	*A. calcoaceticus* JCM6842^T
Growth at:							
Temperature (ºC) range, (optimum)	3-37 (20-25)	5-30 (20-25)	2-35 (8-20)†	5-37 (20-30)	5-40 (25-33)	10-33 (20-30)	5-37 (20-30)
pH range (optimum)	6-10 (7-8)	6-9 (7-8)	6-8.5 (7.2)†	6-9 (6-8)	6-10 (7-8)	nd	nd
NaCl range (%), (optimum)	0-3 (0-1)	0-3 (0)	0-4 (0)†	0-3 (0-2)	0-4 (0-2)	0-4 (1-2)	0-2 (0-1)
Tolerance to heavy metals:							
Chromium (ppm)	2700	1200	nd	600	1800	300	300
Arsenic (ppm)	3000	2400	3000	3000	3000	900	1200
Lead (ppm)	2100	900	900	1500	1500	300	900
Copper (ppm)	2700	1200	900	1200	2100	300	300
Utilisation of citrate	–	–	–	–	+	–	–
Nitrate reduction to N_2	+	+	–	+	+	+	+
Voges-Proskauer reaction	+	–	+	+	+	+	+
***Acid from** N*-acetyl glucosamine	–	+	–	–	–	–	–
Assimilation of:							
Capric acid	+	–	+	+	+	+	–
Adipic acid	–	–	–	–	–	–	+
Malate	+	+	–	+	+	+	+
Trisodium citrate	–	–	–	w+	+	+	–
Phenyl acetic acid	–	–	–	–	–	–	w+
Enzyme activity							
(API-Zym and Rapid 32 ID):							
Alkaline phosphatase	+++	++	–	++	w+	w+	+++
Lipase (C 14)	+	+	w+	++	+++	++	w+
Cysteine arylamidase	+	+	w+	+	+	+	w+
Acid phosphatase	+	w+	+	++	++	+	+++
Napthol-As-BI-phosphohydrolase	+++	–	w+	+	++	+	++
Proline arylamidase	+	+	+	–	+	+	+
Glutamyl glutamic acid arylamidase	+	–	–	–	+	–	+
Resistance to **(µg mL^{-1})(API-ATB Vet)**							
Amoxycilin (4)	S	R	S	wR	R	R	R
Amox-clav. acid ((4/2)	S	wR	S	wR	wR	R	R
Oxacillin (2)	R	R	R	R	R	R	S
Chloramphenicol (8)	S	S	S	R	R	S	R
Tetracycline (4)	S	S	S	wR	R	S	S
Erythromycin (1)	wR	S	R	R	R	wR	S
Sulphamethizole (100)	S	S	wR	S	S	R	S
Nitrofurantoin (25)	R	R	R	R	R	R	S
Fusidic acid (2)	R	R	R	R	R	R	S
Rifamcin (4)	S	S	wR	S	R	S	S
G+C content, mol %	40.6	41.2–42.1†	45.5†	43.8†	42.0†	44.0–45.0†	39.0-42.0†

+++, Very strongly positive; ++, strongly positive; +, Positive; w+, weakly positive; –, negative; nd, no found; R, resistant to the antibiotic; wR, weakly resistant; S, sensitive; All data are from this study unless otherwise mentioned; † Data from previous studies (Bouvet and Grimont, 1986; Nishimura *et al*., 1987; Lee and Lee, 2010; Li *et al*., 2014a).

All strains were negative for the production of acid from glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, Methyl-*β*D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-αD-mannopyranoside, methyl-αD-glucopyranoside, amygdalin, arbutin, esculin, salicin, D-celiobiose, D-maltose, D-lactose, D-melibiose, D-saccharose (sucrose), D-trehalose, inulin, D-melezitose, D-raffinose, amidon (starch), glycogen, xylitol, gentiobiose, D-furanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate, and potassium 5-ketogluconate.

All strains were positive for catalase but negative for oxidase, *β*-galactosidase (2-nitrophenyl-*β*D galactopyranoside), arginine dihydrolase, lysine- and ornithine-decarboxylases, urease, tryptophan deaminase, indole production, H_2S production, and hydrolysis of esculin and gelatin. No oxidation/fermentation of D-glucose, D-mannitol, inositol, D-sorbitol, L-rhamnose, D-sucrose, D-melibiose, amygdalin, and L-arabinose was observed. All strains were positive for the assimilation of malate but negative for glucose, arabinose, mannose, mannitol, maltose and potassium gluconate.

High enzyme activity was observed in all strains for leucine arylamidase, esterase lipase (C 8), valine arylamidase, esterase (C 4), arginine arylamidase, leucyl glycine arylamidase, phenylalanine arylamidase, leucine arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase and serine arylamidase, whereas no enzyme activity was observed for trypsin, α-chymotrypsin, α- galactosidase, *β*-galactosidase, *β*-glucoronidase, α-glucosidase, *β*-glucosidase, *N*-acetyl-*β*-glucosaminidase, α-mannosidase, α-fucosidase, α-galactosidase, ß-galactosidase, ß-galactosidase, α -glucosidase, *ß*-glucosidase, α -arabinosidase, ß-glucuronidase, glutamic acid decarboxylase, α-fucosidase and pyroglutamic acid arylamidase.

All strains were resistant to the antibiotics (µg mL^{-1}) penicillin (0.25), cephalothin (8), cefoperazon (4), lincomycin (2), pristinamycin (2), tylosin (2) and metronidazol (4) but sensitive to the antibiotics streptomycin (8), spectinomycin (64), kanamicin (8), gentamicin (4), apramycin (16), doxycyclin (4), colistin (4), cotrimoxazol (2/38), flumequin (4), oxolinic acid (2) and enrofloxacin (0.5).

was compared with the sequences of closely related type strains on the Ez-Taxon Server database (http://www.ezbiocloud.net/eztaxon). The highest sequence similarity (98.3%) of16S rRNA gene of strain NCCP-644^T was observed with "*A. kyonggiensis*" KSL 5401-034^T (Gen Bank accession no. FJ527818), followed by 97.7% with *A. harbinensis* KCTC 32411^T (KC843488) and 97.6% with *A. bouvetii* DSM 14964^T (APQD01000004), whereas the similarity values were 94.3% (*A. towneri* DSM 14962^T) to 97.5% (*A. johnsonii* CIP 64.6^T) with the other related members in the genus *Acinetobacter*. The phylogenetic analyses (Fig. 1) showed that strain NCCP-644^T clustered with "*A. kyonggiensis*" KSL 5401-034^T (FJ527818) at high (98%) bootstrap support. The node of this cluster also appeared with the same species at high bootstrap values, 91 and 88%, when the phylogenetic trees were reconstructed using the MP and ML algorithms, respectively. This suggested a strong coherence of strain NCCP-644^T with "*A. kyonggiensis*" KSL 5401-034^T (Fig. 5 & 6). The sequence similarity values of the housekeeping genes *gyrB, rpoB* and *atpD* of strain NCCP-644^T were also highest with "*A. kyonggiensis*" JCM 17071^T (92.3, 95.3 and 96.6%, respectively); however, significantly low similarity values for the *gyrB* gene analysis (82.7% for *A. harbinensis* KCTC 32411^{T}to 71.1% for *A. Bouvetii* CCUG 50766^T) and *rpoB* gene analysis (85.3% for *A. gerneri* NIPH 2282^T to 76.7% for *A. soli* CCUG 59023^T) were observed with all other species of the genus. The *atpD* gene of strain NCCP-644^T also showed low sequence similarities (90.3% with *A. bouvetii* JCM 18991^T and 89.5% with *A. johnsonii* JCM 20194^T). The NJ phylogenetic tree based on the *gyrB* and *rpoB* genes sequence analyses with the type strains of the *Acinetobacter* species also showed strong coherence of strain NCCP-644^T with "*A. kyonggiensis*" JCM 17071^T with a high bootstrap value (100%, Fig. 2 & 3). This was also confirmed when a phylogenetic analyses was performed on the deduced amino acid sequences of the *gyrB* and *rpoB* genes. Based on some characteristics, the strain NCCP-644^T is closely related to "*A. kyonggiensis*" JCM 17071^T, However, many other data presented here also show some differences with these type strains of the genus *Acinetobacter* (Table 1).

In summary, our strain NCCP-644^T is closely related to "*A. kyonggiensis*" and *A. Harbinensis* based on the analyses of the 16S rRNA, *rpoB* and *gyrB* gene sequences; however, it is distinct from all other recognised species of the genus *Acinetobacter*. Although the 16S rRNA gene sequence of our strain NCCP-644^T exhibited greater than 97% similarity with several species, such as "*A. kyonggiensis*", *A. harbinensis, A. bouvetii, A. johnsonii, A. beijerinckii, A. haemolyticus, A. tjernbergiae* and *A. tandoi* of the genus *Acinetobacter*, the findings of the three housekeeping genes sequences for *gyrB, rpoB* and *atpD* indicated that our strain NCCP-644^T is distinct from recognised species of the genus *Acinetobacter*. Previously, it was suggested that any strain with less than 95% sequence similarity of *rpoB* gene with the validly named species might belong to a new species in the genus *Acinetobacter* (La Scola *et al*., 2006; Gundi *et al*., 2009; Narciso-da-Rocha *et al*., 2013). Additionally, it was also proposed that a genetic distance of 0.041 for the *gyrB* gene sequences corresponds to a 70% DNA relatedness value, which is the upper limit to delineate any novel species in bacteria (Yamamoto *et al*., 1999). In this study, our strain NCCP-644^T showed sequence similarity of the *gyrB* and *rpoB* genes with "*A. kyonggiensis*" JCM 17071^T (95.3 and 92.3%, respectively), with lower values observed for the other type strains of the recognised species of the genus *Acinetobacter*. Therefore, the multilocus sequence analysis based on the three housekeeping genes supports the hypothesis that our strain NCCP-644^{T}is a novel species. To confirm this hypothesis, DNA– DNA hybridisation analysis was also performed. It was found that the DNA–DNA reassociation between strain NCCP-644^T and the reference strains "*A. kyonggiensis*" JCM 17071^T, *A. harbinensis* KCTC 32411^T, *A. johnsonii* JCM 20194^T, *A. beijerinckii* JCM 18990^T and *A. bouvetii* JCM 18991^T was 54.7, 19.6, 20.6, 12.3 and 10.3%, respectively, which are less than the 70% threshold that is required to delineate any strain to a new species (Wayne *et al*., 1987).

The G+C content of genomic DNA of strain NCCP-644^T was 40.6 mol% as determined by HPLC using a procedure previously described (Hayat *et al*., 2013). These results are in consistent with results previously reported in the members of the genus *Acinetobacter* (Bouvet and Grimont, 1986). However, the DNA G+C content of *A. johnsonii* JCM 20194^T was higher at 44.0-45.0 mol% (Bouvet and Grimont, 1986). This discrepancy may be a result of the different methodologies. In this study, the DNA G+C content was detected by HPLC, as opposed to detection with the spectrophotometrically denaturation method, followed by Bouvet and Grimont (1986).

Chemotaxonomic analysis: The cellular fatty acid profile of strain NCCP-644^T comprised predominantly of summed feature 3 (iso-$C_{15:0}$ 2OH / $C_{16:1}\omega7c$; 37.2 ± 3.4 %), followed by $C_{18:1}$ ω9*c* (16.8 ± 2.0 %), $C_{16:0}$ (12.5 ± 0.9 %), $C_{12:0}$ 3-OH (9.6 ± 1.8 %), $C_{12:0}$ (7.6 ± 0.7 %), $C_{10:0}$ (6.6 ± 0.8 %) and other minor components (Table 2). The major components of this profile are similar to those present in other members of the genus, although significant variation in the values of these components clearly differentiates our strain from the closely related reference strains. However, a significant amount of $C_{12:0}$ 2-OH (3.3 ± 2.4 %) is present in *A. calcoaceticus* JCM 6842^T, but only traces of this component are detected in NCCP-644^T and/or a small amount is present in the other reference strains (Table 2). Strain NCCP-644^T contained ubiquinone Q-9 as a major component (78%), whereas Q-8 was a minor component (12%), and traces of

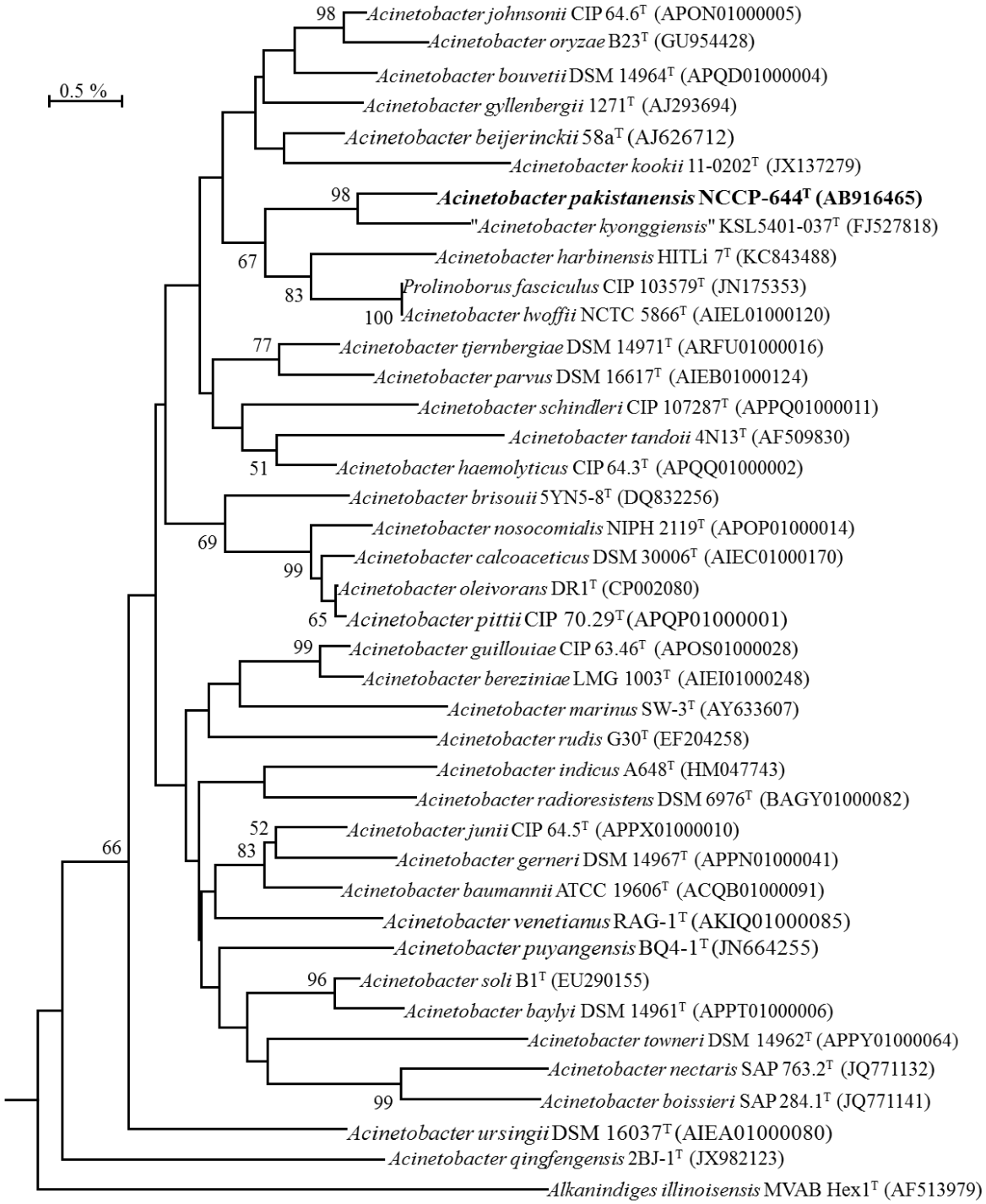

Figure 1. Neighbour-joining phylogenetic tree constructed from sequences of the 16S rRNA gene showing the inter-relationship of strain NCCP-644^T with the type strains of genus *Acinetobacter*, which is based on a comparison of 1232 nucleotides and is rooted using *Alkanindiges illinoisensis* MVAB Hex1^T (AF513979) as an out-group. The bootstrap values (only >50% are shown), expressed as a percentage of 1000 replications, are given at the branching points. The accession number of each type strain is shown in the parentheses.

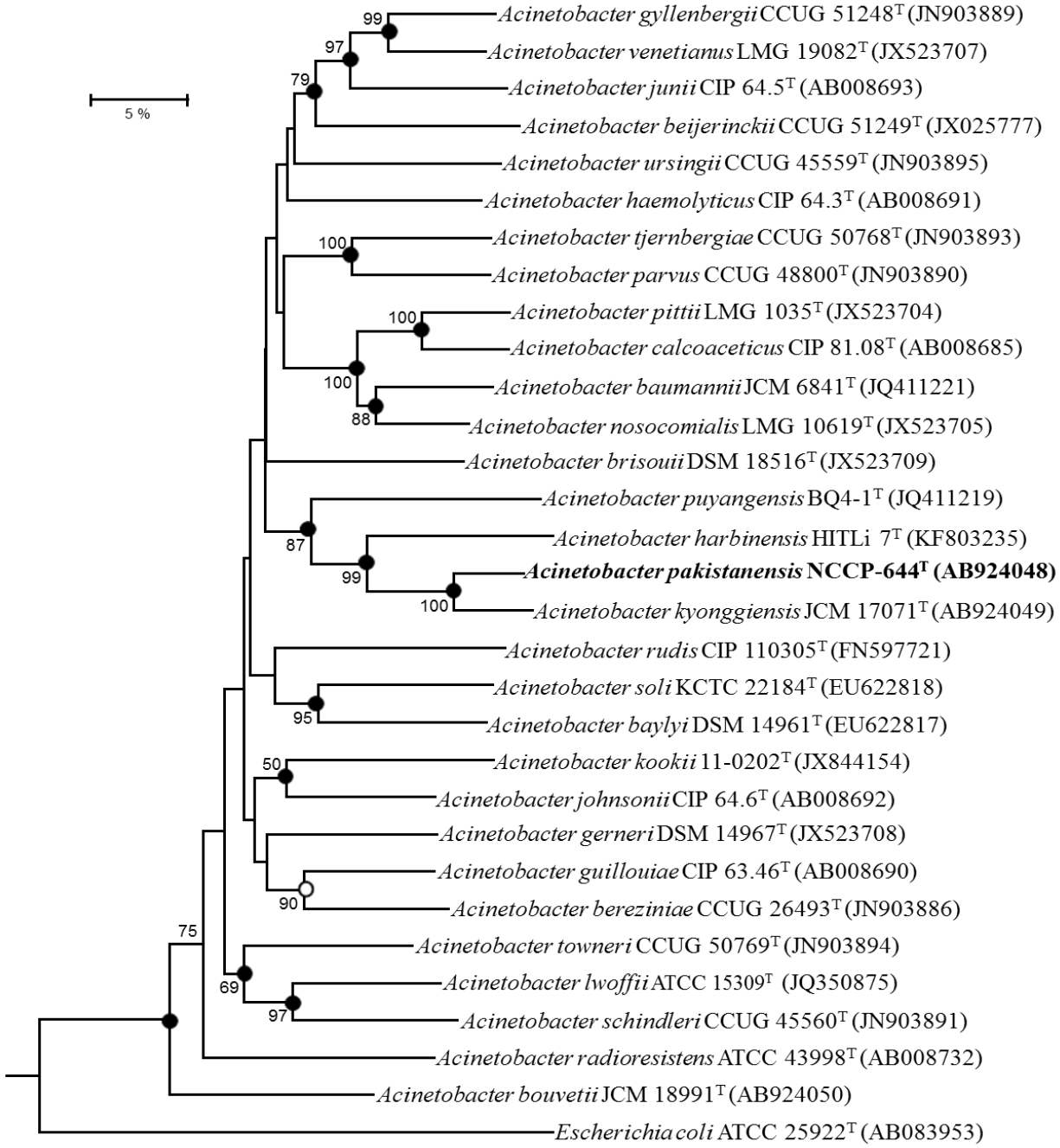

Figure 2. Neighbour-joining phylogenetic tree constructed from sequences of the *gyrB* gene showing the inter-relationship of strain NCCP-644^T with the closely related type strains of genus *Acinetobacter*, which is based on a comparison of 794 nucleotides and is rooted using *Escherichia coli* ATCC 25922^T (AB083953) as an out-group. The bootstrap values (only >50% are shown), expressed as a percentage of 1000 replications, are given at the branching points. The nodes indicated by empty circles were recovered by at least two algorithms, whereas the nodes with solid circles were recovered by three algorithms (NJ, MP and ML). The length of the bar is the 5 % sequence divergence. The accession number of each type strain is shown in the parentheses.

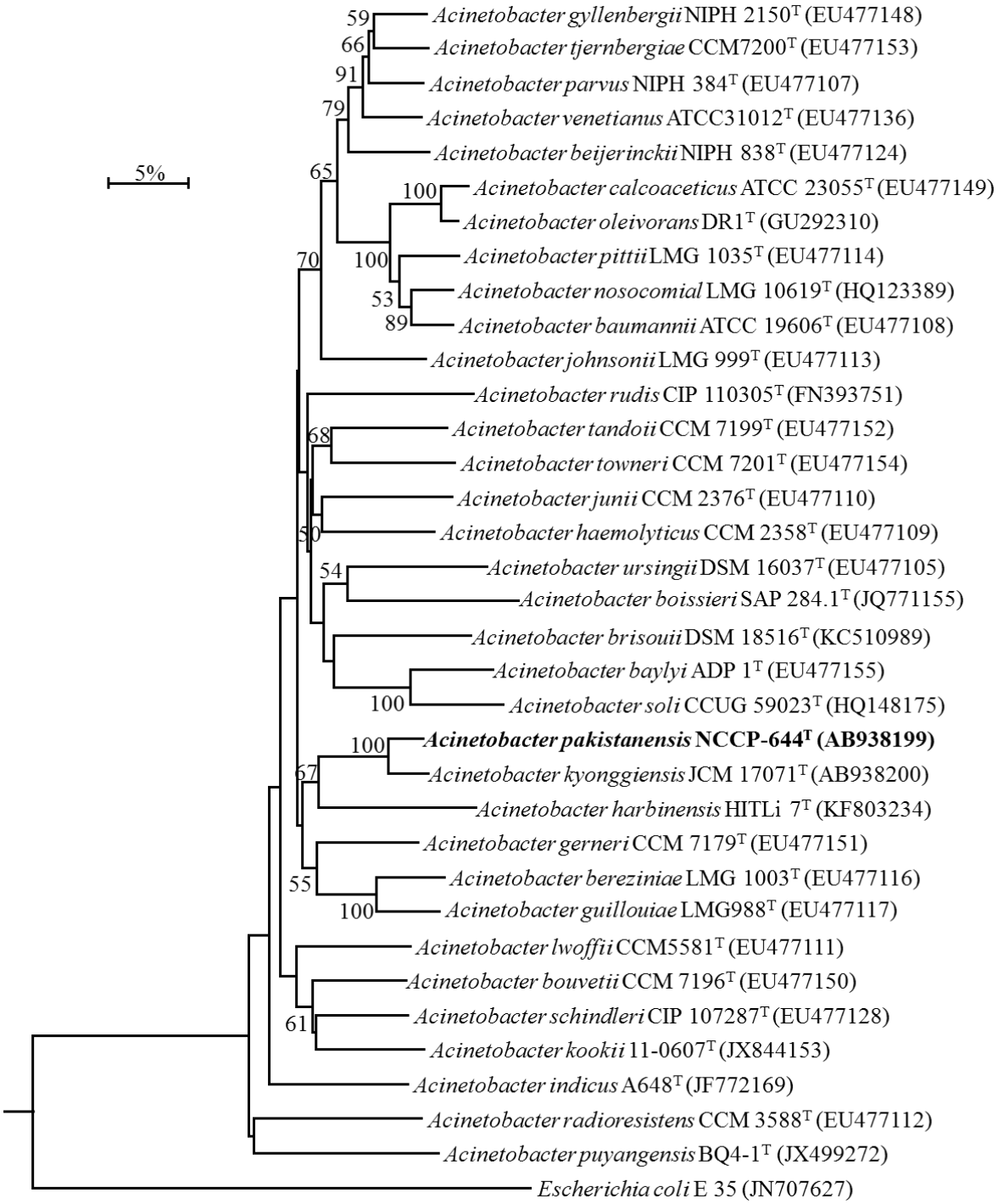

Figure 3. Neighbour-joining phylogenetic tree constructed from sequences of the *rpoB* gene showing inter-relationship of strain NCCP-644^T with the closely related type strains of genus *Acinetobacter*, which is based on a comparison of 842 nucleotides and is rooted using *Escherichia coli* E35 (JN707627) as an out-group. The bootstrap values (only >50% are shown), expressed as a percentage of 1000 replications, are given at the branching points. The length of the bar is the 5 % sequence divergence. The accession number of each type strain is shown in the parentheses.

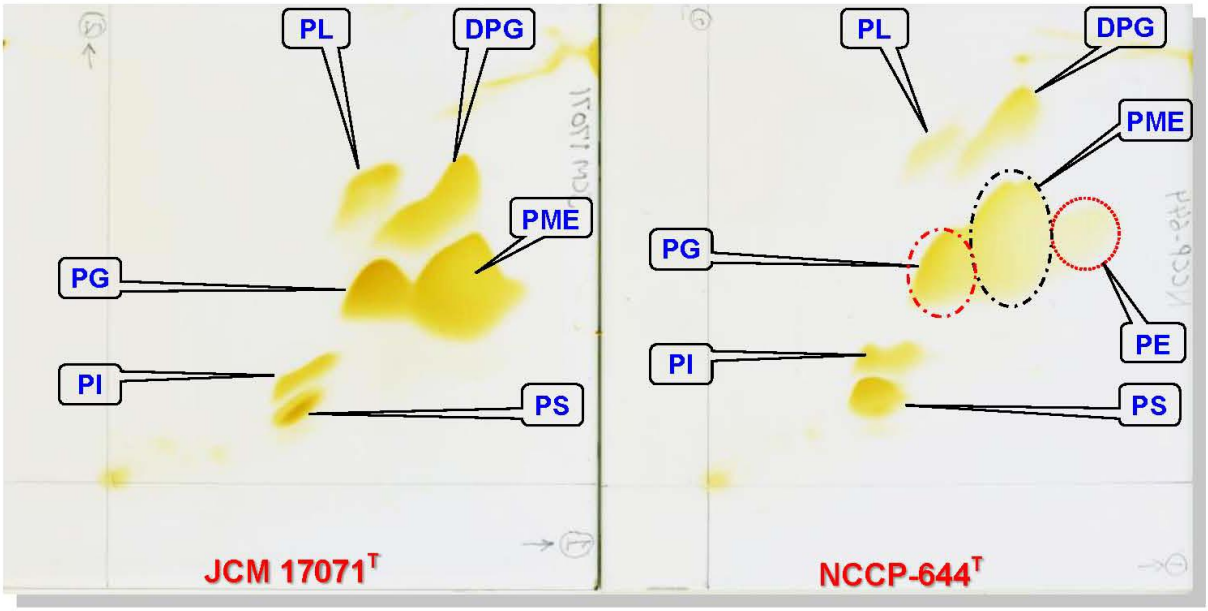

Figure 4. Polar lipid profiles of strain NCCP-644^T compared to the closely related strain "*Acinetobacter kyonggiensis*" JCM 17071^T (reference strain). DPG (diphosphatidyl glycerol), PG (phosphatidyl glycerol), PE (phosphatidyl ethanolamine), PME (phosphatidyl monomethyl ethanol), PI (phosphatidyl inositol), PS (phosphatidyl serine) and PL (one phospholipid of unknown structure).

Table 2. Cellular fatty acid profiles (%) of strain NCCP-644^T compared to the reference type strains of the genus *Acinetobacter*.

Characteristics	NCCP-644^T	"*A. kyonggiensis*" JCM17071^T	*A. harbinensis* KCTC32411^T	*A. bouvetii* JCM 18991^T	*A. beijerinckii* JCM18990^T	*A. johnsonii* JCM 20194^T	*A. calcoaceticus* JCM6842^T
$C_{10:0}$	6.60 ±0.82	4.31 ±0.25	1.91	2.61 ±0.11	1.28 ±0.55	0.28 ±0.01	0.23 ±0.08
$C_{12:0}$	7.57 ±0.66	3.32 ±0.18	5.1	8.91 ±0.20	6.96 ±2.32	10.00 ±3.38	10.16 ±1.77
$C_{14:0}$	0.56 ±0.02	1.14 ±0.02	0.5	1.05 ±0.11	0.78 ±0.32	0.93 ±0.21	0.88 ±0.02
$C_{16:0}$	12.53 ±0.93	16.38 ±3.37	11.93	17.40 ±2.44	14.52 ±0.10	14.12 ±0.08	11.60 ±4.18
$C_{18:0}$	0.38 ±0.01	1.73 ±0.67	1.98	0.54 ±0.13	0.68 ±0.07	0.56 ±0.16	1.29 ±0.09
$C_{10:0}$ 2-OH	nd	nd	nd	nd	2.09 ±0.59	nd	0.03 ± 0
$C_{12:0}$ 2-OH	0.27 ±0.08	0.18 ±0.02	0.18	0.21 ±0.04	2.90 ±1.12	1.67 ±0.75	3.27 ±2.40
$C_{12:0}$ 3-OH	9.56 ±1.79	5.39 ±0.57	4.63	6.84 ±0.44	9.12 ±1.37	6.79 ±1.37	6.09 ±2.40
$C_{16:0}$ N alcohol	0.71 ±0.60	2.69 ±2.67	nd	0.07 ±0.05	0.19 ±0.09	1.01 ±0.57	1.41 ±1.39
$C_{16:1}$ ω9*c*	0.68 ±0.34	1.12 ±0.64	0.81	nd	0.76 ±0.04	0.48 ±0.17	0.37 ± 0
$C_{17:1}$ ω8*c*	0.76 ±0.40	0.08 ±0.01	0.04	0.15 ±0.04	1.51 ±0.03	0.18 ±0.04	1.21 ±0.26
$C_{18:1}$ ω7*c*	2.43 ±0.59	3.00 ±0.52	2.48	2.53 ±1.19	0.49 ±0.10	3.66 ±1.54	1.72 ±1.12
$C_{18:1}$ ω9*c*	16.76 ±2.01	26.71 ±3.46	37.05	13.52 ±2.70	24.95 ±7.84	23.22 ±8.90	14.15 ±4.14
$C_{18:3}$ ω6*c* (6, 9, 12)	0.27 ±0.24	0.92 ±1.10	nd	nd	0.23 ±0.05	0.51 ±0.28	1.41 ±1.41
Summed features 2*	0.22 ±0.04	0.12 ±0.02	0.07	0.16 ±0.01	0.33 ±0.06	0.68 ±0.11	2.59 ±0.12
Summed features 3*	37.20 ±3.43	31.53 ±2.40	32.66	45.27 ±1.90	30.08 ±0.03	34.91 ±3.92	34.44 ±6.66

*Summed feature 2 comprises one or more of iso-$C_{16:1}$ I/ $C_{14:0}$ 3OH, and summed feature 3 comprises one or more of $C_{16:1}$ ω7c / iso-$C_{15:0}$ 2OH,which could not be separated by the MIDI system; All data were obtained in this study. The data are the mean of two values except for *A. harbinensis* KCTC32411^T. The values are the percentages of total fatty acid detected; nd, not detected; The cellular fatty acid component values were deleted if present at less than 1% in all species and/or absent in some species.

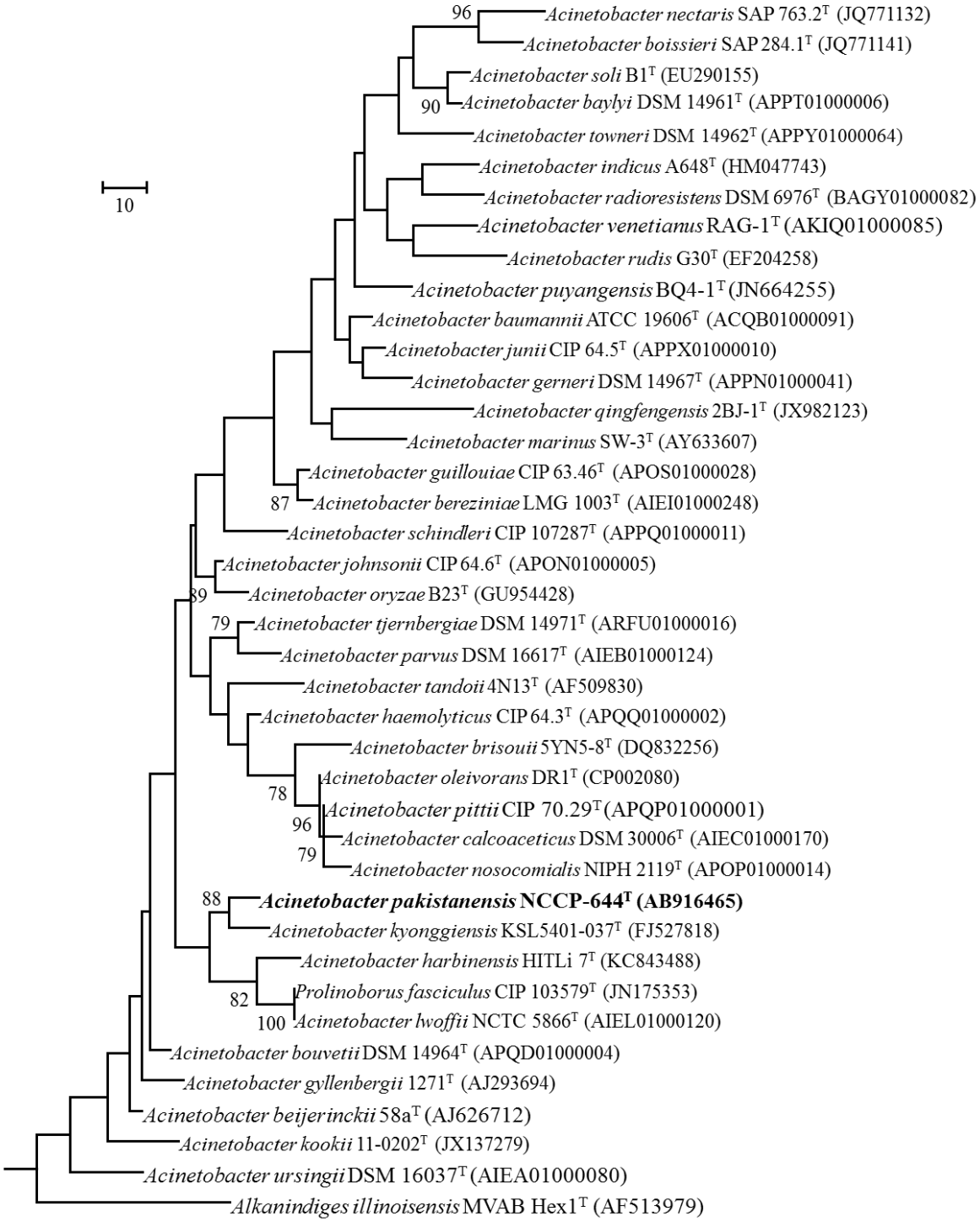

Figure 5. Maximum parsimony phylogenetic tree inferred from the sequences of the 16S rRNA gene showing the inter-relationship of strain NCCP-644^T with the type strains of genus *Acinetobacter*, which is based on a comparison of 1232 nucleotides and is rooted using *Alkanindiges illinoisensis* MVAB Hex1^T (AF513979) as an out-group. The bootstrap values (only >50% are shown), expressed as a percentage of 1000 replications, are given at the branching points. The accession number of each type strain is shown in the parentheses.

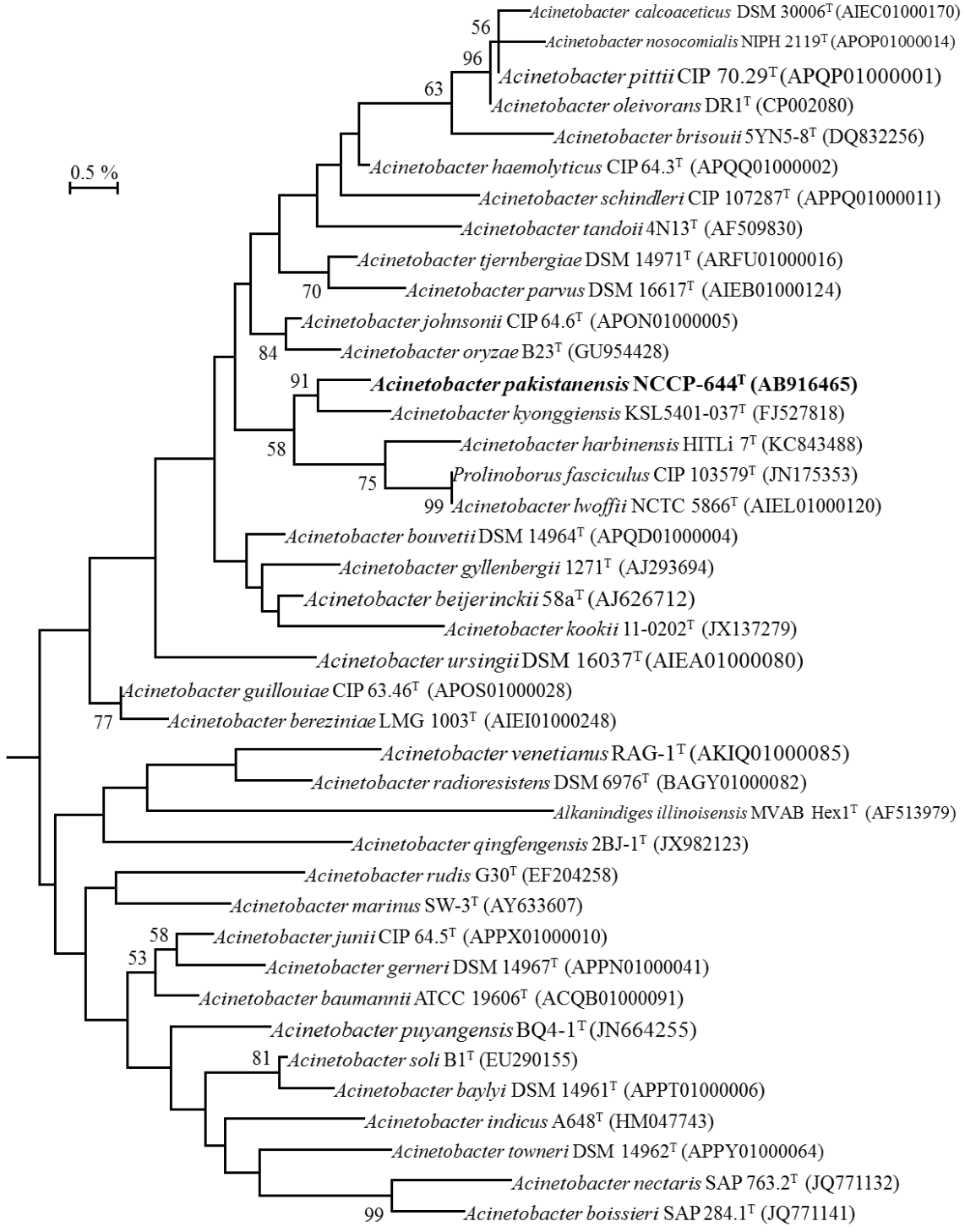

Figure 6. Maximum-likelihood phylogenetic tree inferred from the sequences of 16S rRNA gene showing the inter-relationship of strain NCCP-644^T with the type strains of genus *Acinetobacter*, which is based on a comparison of 1232 nucleotides. The bootstrap values (only >50% are shown), expressed as a percentage of 1000 replications, are given at the branching points. The accession number of each type strain is shown in the parentheses

Q-10 were also detected. The closely related type strain of the reference species "*A. kyonggiensis*" JCM 17071^T also contained a similar quinone system (Q-9, 83%; Q-8, 10% and traces of Q-10). These results are consistent with previous reports for the members of *Acinetobacter* (Vaz-Moreira *et al*., 2011); however, our results for "*A. kyonggiensis*" JCM 17071^T differed from those reported previously by Lee and Lee (2010), who described the presence of only Q-8 as the major quinone system in "*A. kyonggiensis*" JCM 17071^T.Polar lipids profile of strain NCCP-644^T is similar (Fig. 4) to "*A. kyonggiensis*" JCM17071^T, which comprised of diphosphatidyl glycerol, phosphatidyl monomethyl ethanol, phosphatidyl glycerol, phosphatidyl serine, phosphatidyl inositol and one unknown phospholipid. However, the presence of phosphatidyl ethanolamine differentiates our strain NCCP-644^T from "*A. kyonggiensis*" JCM 17071^T, in which it is absent (Fig. 4).

Based on the physiological and biochemical characteristics, chemotaxonomic data and phylogenetic analyses of the 16S rRNA and *gyrB* genes along with genotypic (DNA-DNA relatedness) data presented in this paper, the isolated strain NCCP-644^T is concluded to be a new species in the genus *Acinetobacter* with the proposed name, *A. pakistanensis* sp. nov., the type strain NCCP-644^T and its description is provided below.

Description of Acinetobacter pakistanensis sp. nov.

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

The cells are Gram-stain negative, cocci to short rod (coccobacillus), occur primarily in pairs and sometimes triplet form, which are non-motile and strictly aerobic. The colonies are moist, raised, off-white in colour with entire margins and are 1–2 mm in size after 1–2 days when grown on TSA (Difco) medium at 25°C. The cells grow at 3–37°C (optimum at 20–25°C), at pH ranges of 6.0–10.0 (optimum at pH 7–8) and in 0–3% NaCl (w/v) (optimum without NaCl) in TSB (Difco) medium. Positive for Voges-Proskauer reaction, nitrate reduction to N_2 and catalase but negative for oxidase, arginine dihydrolase, *β*-galactosidase (2-nitrophenyl-*β*D-galactopyranoside), lysine- and ornithine-decarboxylases, tryptophan deaminase, H_2S production, citrate utilisation and indole production. In addition, esculin, urea and gelatine are not hydrolysed by this strain. There is no oxidation/fermentation of D-melibiose, L-arabinose, amygdalin, D-sucrose, D-mannitol, L-rhamnose, D-glucose, D-sorbitol and inositol. The strain can assimilate malate and capric acid but not glucose, mannose, mannitol, arabinose, maltose, adipic acid trisodium citrate or potassium gluconate, phenyl acetic acid. The strain is negative for acid production from D-mannitol, D-galactose, inulin, D-sorbitol, L-xylose, L-rhamnose, D-mannose, methyl-*β*D-xylopyranoside, *N*-acetyl glucosamine, D-trehalose, arbutin, D-glucose, D-fucose, inositol, erythritol, D-lyxose, D-adonitol, D-melibiose, L-fucose, methyl-α D-mannopyranoside, D-melezitose, D-xylose, D-maltose, dulcitol, amygdalin, D-tagatose, D-cellobiose, D-ribose, potassium gluconate, methyl-αD-glucopyranoside, D-raffinose, xylitol, L-arabinose, D-fructose, D-arabitol, glycerol, gentiobiose, esculin, D-lactose, salicin, D-saccharose (sucrose), L-sorbose, potassium 2-ketogluconate, amidon (starch), D-arabinose, glycogen, D-furanose, L-arabitol and potassium 5-ketogluconate (API-50CH, bioMérieux, France). The strain has high enzyme activity for leucine arylamidase, napthol-As-BI-phosphohydrolase, lipase (C 14), leucyl glycine arylamidase, arginine arylamidase, esterase lipase (C 8), proline arylamidase, alkaline phosphatase, cysteine arylamidase, glycine arylamidase, tyrosine arylamidase, alanine arylamidase, valine arylamidase, esterase (C 4), phenylalanine arylamidase, histidine arylamidase, serine arylamidase, esterase (C 4), glutamyl glutamic acid arylamidase, leucine arylamidase and acid phosphatase. However, the strain has no enzyme activity forα-arabinosidase, α-fucosidase, α-chymotrypsin, glutamic acid decarboxylase, α-glucosidase, α-mannosidase, α- galactosidase, α-fucosidase, *β*-glucosidase, *β*-glucuronidase, trypsin, *N*-acetyl-*β*-glucosaminidase, *β*-galactosidase, α- & ß-galactosidase, α- & ß-glucosidase, ß-glucuronidase and pyroglutamic acid arylamidase (API-Zym and API Rapid 32 ID, bioMérieux, France). Major polar lipids are phosphatidyl monomethyl ethanol (PME), phosphatidyl inositol (PI) diphosphatidyl glycerol (DPG), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS), phosphatidyl glycerol (PG) and one unknown phospholipid. Major cellular fatty acids are summed feature 3 (iso-$C_{15:0}$ 2OH / $C_{16:1}$ ω7c as defined by MIDI), followed by $C_{18:1}$ ω9*c*, $C_{16:0}$, $C_{12:0}$ 3-OH, $C_{12:0}$ and $C_{10:0}$. The major component of quinone system detected as Q-9 and minor component as Q-8. The genomic DNA G+C content of the type strain is 40.6 mol%.

Strain NCCP-644^T (= LMG 28467^T = KCTC 42081^T = JCM 18977^T) is the type strain isolated from a sample of textile dyeing wastewater collected from a treatment pond at Kohinoor mills, Islamabad, Pakistan.

The GenBank/EMBL/DDBJ accession numbers for strain NCCP-644^T (JCM 18977^T = KCTC 42081^T) are AB916465 (16S rRNA gene), AB924048 (*gyrB* gene), AB938199 (*rpoB* gene) and AB924051 (*atpD* gene); for strain JCM 17071^T, these are AB924049 (*gyrB* gene), AB938200 (*rpoB* gene) and AB924052 (*atpD* gene); for strain JCM18990^T, it is AB924053 (*atpD* gene); and for strain JCM 18991^T, the accession numbers are AB924050 (*gyrB* gene) and AB924054 (*atpD* gene).

Acknowledgments**:** The financial support from the Higher Education Commission of Pakistan to S.A. under the International Research Support Initiative Program (IRSIP) is gratefully acknowledged. This study was partially supported by financial assistance from the PSDP funded Project *Research for Agricultural Development Project* (RADP) under a sub-project (Grant No. CS-55/RADP/PARC) titled "Establishment of Microbial Bio-Resource Laboratories: National Culture Collection of Pakistan (NCCP)" from the Pakistan Agricultural Research Council (PARC), Islamabad, Pakistan, and partially from the Japan Society for Promotion of Science (JSPS) under a fellowship program to I.F.

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