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Bhargavaea indica sp. nov., a Member of the Phylum *Firmicutes*, Isolated from Arabian Sea Sediment[§]

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A Gram-positive, aerobic, coccoid-rod shaped, non-motile, catalase- and oxidase-positive bacterium, designated strain KJW98^T, was isolated from the marine sediment of Karwar jetty, west coast of India. The strain was β-haemolytic, non-endospore-forming and grew with 0–8.5% (w/v) NaCl, at 15–48°C and at pH 6.5–9.0, with optimum growth with 0.5% (w/v) NaCl, at 42°C and at pH 7.0–8.0. Phylogenetic analyses based on 16S rRNA and *gyrB* gene sequences showed that strain KJW98^T forms a lineage within the genus *Bhargavaea*. The G+C content of the genomic DNA was 55 mol%. The DNA–DNA relatedness values of strain KJW98^T with *B. beijingensis* DSM 19037^T, *B. cecembensis* LMG 24411^T and *B. ginsengi* DSM 19038^T were 43.2, 39 and 26.5%, respectively. The major fatty acids were anteiso-$C_{15:0}$ (37.7%), iso-$C_{15:0}$ (19.7%), anteiso-$C_{17:0}$ (17.0%) and iso-$C_{16:0}$ (11.1%). The predominant menaquinone was MK-8 and the cell-wall peptidoglycan was of A4α type with L-lysine as the diagnostic diamino acid. The major polar lipids were diphosphatidylglycerol and phosphatidylglycerol. The phenotypic, genotypic and DNA–DNA relatedness data indicate that strain KJW98^T should be distinguished from the members of the genus *Bhargavaea*, for which the name *Bhargavaea indica* sp. nov. is proposed with the type strain KJW98^T (=KCTC 13583^T =LMG 25219^T).

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Introduction

The genus *Bhargavaea* is a member of the family *Planococcaceae* (class *Bacilli*). It was first reported by Manorama *et al.* (2009) with the species *Bhargavaea cecembensis* isolated from a deep-sea sediment sample collected from the Chagos-Laccadive ridge system, Indian Ocean. The genus *Bhargavaea* currently comprises three species, where two species *Bhargavaea beijingensis* and *Bhargavaea ginsengi* were reclassified from *Bacillus beijingensis* and *B. ginsengi* (Qiu *et al.*, 2009), respectively, based on their phylogenetic positions, morphological and chemotaxonomic characteristics by Verma *et al.* (2012).

The genus *Bhargavaea* is characterized by Gram-positive, non-endospore-forming, non-motile and rod-shaped bacteria. The main fatty acids are iso-$C_{15:0}$, anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{16:0}$, the main polar lipids are phosphatidylglycerol and diphosphatidylglycerol and the predominant menaquinone is MK-8. The cell-wall peptidoglycan is A4α type with L-lysine as the diagnostic diamino acid. During the course of our study on the microbial composition of coastal sediments, an aerobic, Gram-positive, non-motile, coccoid-rod shaped bacterial strain, designated KJW98^T, was isolated and subjected to systematic taxonomic investigation. On the basis of evidence from polyphasic taxonomic studies we propose that strain KJW98^T represents a novel species in the genus *Bhargavaea*.

Materials and Methods

Bacterial strains

Strain KJW98^T was isolated from sediments collected in the western coastal region of India (Karwar district, Karnataka). The samples were collected during January 2006 at 10.4 m water depth from Karwar jetty (14°47′N and 74°06′E). The pH, temperature and salinity of the environment were 6.6, 25°C and 30‰, respectively. Strain KJW98^T was initially isolated and purified from Luria-Bertani (LB) agar plates incubated at 28°C for 1 week, using a standard dilution-plating method. Three reference strains, *B. ginsengi* DSM 19038^T, *B. beijingensis* DSM 19037^T, and *B. cecembensis* LMG 24411^T were obtained from the respective culture collections. For comparison the same laboratory conditions were used and all the strains were cultured routinely on LB agar and maintained on it at 4°C and as a suspension in LB broth

Table 1. Characteristics that differentiate strain KJW98^T from the type strains of phylogenetically related species of the genus *Bhargavaea*
Strains: 1, KJW98^T; 2, *B. beijingensis* DSM 19037^T; 3, *B. ginsengi* DSM 19038^T; 4, *B. cecembensis* LMG 24411^T. Data for all species are from the present study except where indicated.
Symbols: +, positive; –, negative.

Characteristics	1	2	3	4
Cell shape	coccoid rod single/pairs	coccoid rod single/pairs	coccoid rod single/pairs/short chains	rod single/pairs/short chains
Cell dimensions (in μm)	0.67–0.75 × 1.15–1.35	1.0 × 1.2	1.0 × 1.2–2.0	1.0 × 2.0–8.0
Optimum growth temperature (°C)	42	30	30	37
NaCl tolerance (%, w/v)	0–8.5	0–8.0	0–12	0–10
Acid production:				
Galactose	–	–	+	–
Salicin	–	+	+	–
Trehalose	–	+	+	+
Mannose	–	–	–	+
Rhamnose	–	–	–	+
Cellobiose	–	–	–	+
Maltose	–	–	+	–
Biochemical test:				
Urease	–	+	–	+
Sucrose fermentation in TSI	+	+	+	–
Lysozyme resistance	+	–	+	–
Utilization of substrates (Biolog):				
N-Acetyl-D-glucosamine	–	–	+	–
N-Acetyl-β-D-mannosamine	–	–	+	–
α-Hydroxybutyric acid	+	+	–	+
Dextrin	+	–	–	–
Glycogen	+	–	–	–
D-Malic acid	–	+	–	–
L-Malic acid	+	+	–	+
Mannan	+	–	–	–
Tween 80	+	–	–	–
Succinic acid	–	+	+	–
D-Alanine	+	+	–	+
L-Alanine	+	–	+	+
Glycyl-L-glutamic acid	+	+	–	–
L-Arabinose	+	–	–	–
D-Fructose	+	–	–	–
D-Galacturonic acid	+	–	–	–
Adenosine	+	+	+	–
2′-Deoxy adenosine	+	+	+	–
Inosine	+	+	+	–
D-Ribose	+	–	–	–
D-Xylose	+	–	–	–
Thymidine	+	+	+	–
Uridine	+	+	+	–
Adenosine-5′-monophosphate	–	–	+	–
Thymidine-5′-monophosphate	+	–	+	–
Uridine-5′-monophosphate	–	–	+	–
Growth on medium[a]:				
VY/2 agar	+	+	–	–
Alcaligenes xylosoxydans agar with benzoate	–	–	+	+
Arthrobacter agar	+	+	–	+
CGY agar[b]	+	+	–	–
Actinomyces agar (BBL)	+	+	–	+
DNA G+C content (mol %)	55	53.1[c]	50.2[c]	53.7[c]

[a] Media were prepared as the appended composition given in the DSMZ site (http://www.dsmz.de/catalogues/ catalogue-microorganisms/culture-technology/list-of-media-for-microorganisms.html).
[b] Pike *et al.* (1972)
[c] Data from Verma *et al.* (2012)

supplemented with 20% (v/v) glycerol at –80°C. Sub-cultivation, phenotypic, physiological, chemotaxonomic and molecular systematic studies were performed using strains grown on LB agar or in LB broth, else otherwise stated.

The proposed minimal standards to include species in the genus *Bacillus* (Kämpfer *et al*., 2006) and for genera accommodating species originally allocated to *Bacillus* (Logan *et al*., 2009) were followed, respectively.

Morphological, physiological, and biochemical characterization

Macroscopic properties were determined using the classical characterization of colony appearance. The cellular morphology was observed in the lag, exponential and stationary phases of growth and was determined by scanning electron microscopy (Zeiss DSM 982 Gemini). The motility of the cells was analyzed by the hanging drop method using phase-contrast microscopy (Eclipse E600; Nikon) and the semi-solid agar method. Gram staining was performed using a Gram-stain kit (HiMedia) according to manufacturer's instructions. Sporulation was observed using a Schaeffer & Fulton's spore staining kit (K006-1KT, HiMedia) according to the manufacturer's protocol with a culture grown on sporulation medium (M1018; HiMedia), tryptic soy agar (TSA) in combination with sporulation medium and TSA with $MnSO_4$ (50 mg/L).

All of the phenotypic and biochemical tests described below were performed on strain KJW98^T and its closest phylogenetic neighbors, *B. beijingensis* DSM 19037^T, *B. cecembensis* LMG 24411^T and *B. ginsengi* DSM 19038^T, under the same experimental conditions at 37°C in an aerobic environment, unless stated otherwise. Catalase and oxidase activities were determined using 3% (v/v) hydrogen peroxide and Kovacs' reagent (Kovacs, 1956), respectively. Haemolytic activities were determined by growing the cultures on LB agar with 5% (v/v) sheep blood and incubating for one week. Urease production, reduction of nitrate and nitrite, resistance to lysozyme, indole production from tryptophan, methyl red and Voges-Proskauer reaction and hydrolysis of casein and gelatin, utilization of citrate, phenylalanine deaminase activity, fermentative growth and H_2S production using triple sugar iron medium and acid production from various carbohydrates (Table 1 and Supplementary data Table S1) were determined as described previously (Verma *et al*., 2012). Growth at various temperatures (4, 10, 15, 20, 25, 30, 37, 40, 42, 45, 48, and 50°C) was measured on LB agar and in LB broth. The pH range for growth was determined in buffered LB broth adjusted to various pH values (pH 5.0–10.0, in increments of 0.5 pH units) and the pH was monitored at different stages of media preparation and during growth. Growth at various NaCl concentrations (0.0–13.0%, in increments of 0.5%, w/v) was investigated using LB broth prepared as per the formula except that no NaCl was used. The growth under anaerobic conditions was ascertained by incubating the inoculated plates under anaerobic conditions generated in an anaerobic chamber (HiMedia) with Anaerogas Pack (HiMedia) for 7 days. Before storage at 37°C, the anaerobic jar was kept overnight at 4°C to make sure that anoxic conditions had developed before allowing strain growth.

Other biochemical tests, enzymatic activities and oxidation of different carbon substrates were determined using VITEK-2 GP compact system v. 04.01 (bioMérieux), API ZYM (bioMérieux) and GP2 MicroPlates (Biolog) following the manufacturer's instructions. The growth patterns on various standard agar media (Table 1 and Supplementary data Table S1) under aerobic condition at 37°C were observed for one week to ascertain the growth differences.

Phylogenetic analysis

Bacterial DNA isolation, PCR amplification and sequencing of the 16S rRNA gene were carried out as described (Hauben *et al*., 1997). As protein encoding genes evolve much faster than rDNAs and provide higher resolution than the use of the 16S rRNA gene (Yamamoto and Harayama, 1998), the partial sequence of DNA gyrase subunit B (*gyrB*) was determined according to Yamamoto and Harayama (1995). The 16S rRNA gene sequence of strain KJW98^T (1528 nt) was analyzed by the NCBI-BLAST (Altschul *et al*., 1997) and megaBLAST (Zhang *et al*., 2000) programs for the identification of closely related type strains with validly published names (Chun *et al*., 2007).

The *gyrB* gene sequence (1069 nt) was compared with respective reference gene sequences retrieved from the GenBank and EMBL databases. Translated NCBI-BLAST (blastx) was also used for finding proteins similar to those recovered by the *gyrB* nucleotide query. The 16S rRNA gene sequences with the highest scores were selected for the calculation of pairwise sequence similarity using a global alignment algorithm, which was implemented at the EzTaxon server (http://www.eztaxon.org/; Chun *et al*., 2007). The 16S rRNA gene and *gyrB* amino acid sequences of the closest relatives were downloaded and aligned with the CLUSTAL_X program (Thompson *et al*., 1997). The multiple sequence alignment was edited and corrected manually using DAMBE (Xia and Xie, 2001) and a degapped alignment of 1327 nt each for the 16S rRNA gene and 314 amino acid sequences for each of the *gyrB* gene were used to obtain an unambiguous sequence alignment. Evolutionary genetic distances were computed according to the algorithm of Kimura two-parameter (Kimura, 1980) and Poisson correction (Zuckerkandl and Pauling, 1965) for the 16S rRNA gene and *gyrB* amino acid sequences, respectively.

Tree topologies were inferred with the neighbor-joining (Saitou and Nei, 1987), minimum-evolution (Rzhetsky and Nei, 1992) and maximum-parsimony (Fitch, 1971) methods, using the MEGA program (version 5.05; Tamura *et al*., 2011) with 1,000 bootstrap replicates to obtain a strict consensus tree.

Nucleotide sequence accession numbers

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *gyrB* gene sequences of strain KJW98^T are FJ716700 and JF261160, respectively.

DNA-DNA hybridization

The genomic relationships of strain KJW98^T with *B. beijingensis* DSM 19037^T, *B. cecembensis* LMG 24411^T, and *B. ginsengi* DSM 19038^T were further examined using DNA-

DNA hybridization. The DNA-DNA hybridization was carried out spectrophotometrically and initial renaturation rates were recorded as described by De Ley *et al.* (1970) under consideration of the modifications described by Huss *et al.* (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6×6 multicell changer and a temperature controller with an *in situ* temperature probe (Varian).

Chemotaxonomy

The cell-wall peptidoglycan structure, quinones and polar lipids were determined using the identification service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany) by Dr. Peter Schumann and Dr. B. J. Tindall. The determination of peptidoglycan was carried out as described by Schleifer (1985) and Schleifer and Kandler (1972) with the modification that TLC on cellulose was applied instead of paper chromatography. Quantitative analysis of amino acids was performed by gas chromatography, after derivatization, according to MacKenzie (1987).

Quinones were extracted as described by Collins *et al.* (1977) and were examined by HPLC according to the method of Groth *et al.* (1996). Polar lipids were extracted from 100 mg freeze-dried cell material using the two-stage method described by Tindall (1990a, 1990b). Extraction was carried out by adjusting the methanol/0.3% (w/v) aqueous NaCl phase (containing the cell debris) to give a chloroform/methanol/0.3% (w/v) aqueous NaCl mixture (1: 2: 0.8, v/v). The extraction solvent was stirred overnight and the cell debris was then pelleted by centrifugation. Polar lipids were recovered into the chloroform phase by adjusting the chloroform/methanol/0.3% (w/v) aqueous NaCl mixture to a ratio of 1: 1: 0.9 (v/v).

The fatty acid analysis of strain KJW98^T and the reference strains was carried out at the Belgian Co-ordinated Collections of Micro-organisms (BCCM/LMG) as described previously (Verma *et al.*, 2012) where standardization of the physiological age of cultures was taken into account.

The DNA G+C content was determined from reversed-phase HPLC analysis (Mesbah *et al.*, 1989). The solvent used was 0.3 M $(NH_4)H_2PO_4$/acetonitrile, 40:1 (v/v) at pH 4.4, the set conditions were a flow rate of 1.3 ml/min at a temperature of 45°C (adapted from Tamaoka and Komagata, 1984). Non-methylated bacteriophage lambda DNA (Sigma) was the calibration reference.

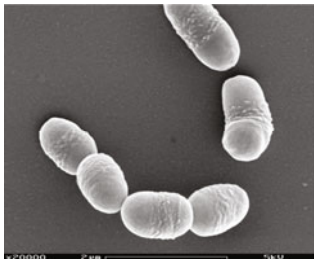

Fig. 1. Scanning electron micrograph of cells of strain KJW98^T grown on LB agar for 48 h at 37°C. Bar, 2 µm.

Results and Discussion

Morphological, physiological, and biochemical characteristics

Cells of strain KJW98^T were aerobic, Gram-positive, non-motile, non-endospore-forming, coccoid rod-shaped (Fig. 1), present as single cells or in pairs and β-haemolytic. These features are similar to those present in known species of genus *Bhargavaea*. Colonies grown on LB agar plates for 48 h at 37°C were circular, low-convex with entire margins, non-pigmented, light pale brown in colour, with butterous consistency and approximately 2.0–3.0 mm in diameter. Detailed results of physiological and biochemical analyses are given in Table 1, Supplementary data Table S1, and the species description. The API ZYM fingerprint is the same for strain KJW98^T and the reference strains. However the strain KJW98^T can be differentiated from the reference strains as negative for acid production from trehalose, positive for oxidation of substrates in Biolog like dextrin, glycogen, mannan, Tween 80, L-arabinose, D-fructose, D-galacturonic acid, D-ribose, D-xylose, and the negative result for phenylalanine arylamidase in the VITEK-2 GP compact system. Strain KJW98^T and the reference strains showed differential growth patterns on VY/2 agar, Alcaligenes xylosoxydans agar with benzoate, Arthrobacter agar, CGY agar and actinomyces agar (BBL) (Table 1). Thus, it is evident from the physiological and biochemical analyses that there are sev-

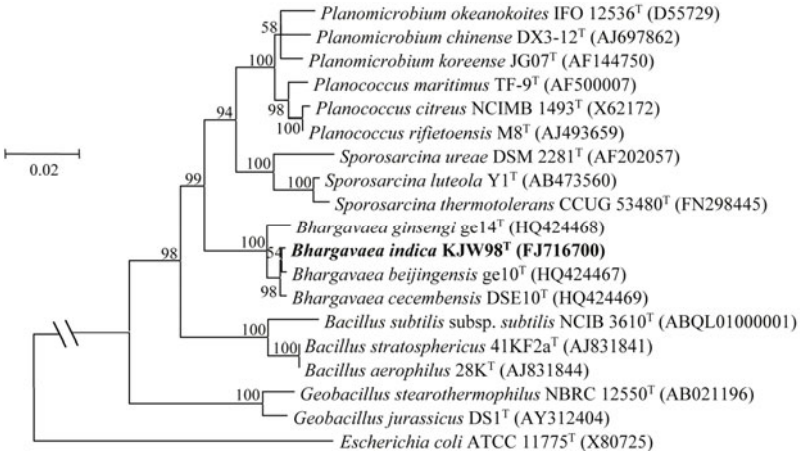

Fig. 2. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain KJW98^T, members of the genus *Bhargavaea* and some related representatives of the class *Bacilli*. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are shown at branch points. The sequence of *E. coli* ATCC 11775^T (X80725) was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

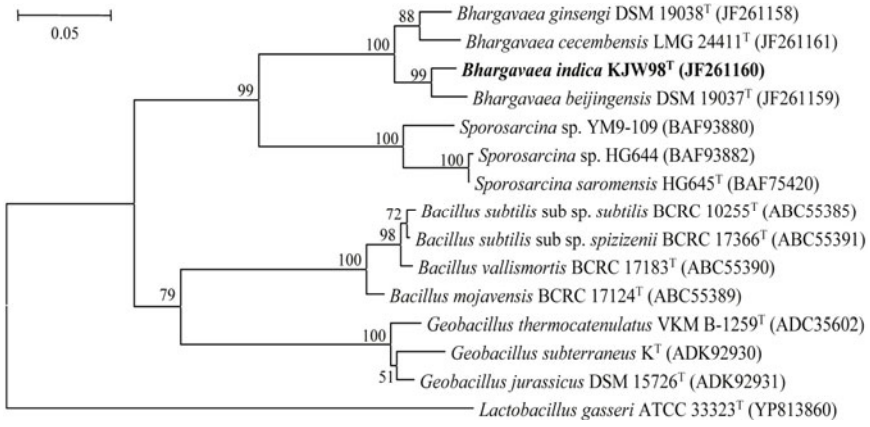

Fig. 3. Neighbor-joining phylogenetic tree based on *gyrB* amino acid sequences showing the relationships between strain KJW98^T, members of the genus *Bhargavaea* and some representatives of the class *Bacilli*. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are shown at branch points. The amino acid sequence of *Lactobacillus gasseri* ATCC 33323^T (YP813860) was used as an outgroup. Bar, 0.05 substitutions per amino acid position.

eral phenotypic characteristics that readily separate strain KJW98^T from phylogenetically related species (Table 1 and Supplementary data Table S1).

Phylogenetic analysis

Preliminary sequence comparison with 16S rRNA and *gyrB* gene sequences present in databases indicated that strain KJW98^T was closely associated with strains of the genus *Bhargavaea*. Strain KJW98^T showed the highest 16S rRNA gene sequence similarity to *B. beijingensis* DSM 19037^T (99.6%), *B. cecembensis* LMG 24411^T (99.5%) and *B. ginsengi* DSM 19038^T (98.7%). The 16S rRNA gene sequence similarity of strain KJW98^T with other representatives of the class *Bacilli* was ≤ 95.4%. The *gyrB* gene sequence similarities of strain KJW98^T with respect to its closest phylogenetic affiliates *B. beijingensis* DSM 19037^T, *B. cecembensis* LMG 24411^T and *B. ginsengi* DSM 19038^T were 85.9, 83, and 82.5%, respectively. Notwithstanding the high 16S rRNA gene sequence similarity values, the analysis of additional phylogenetic marker *gyrB* showed that strain KJW98^T may represent a distinct species of the genus *Bhargavaea*. The *gyrB* amino acid-based sequence similarity of strain KJW98^T with respect to other members of class *Bacilli* present in GenBank and EMBL database, was ≤79%.

Phylogenetic analysis based on 16S rRNA gene nucleotide and *gyrB* amino acid sequences among members of the class *Bacilli* showed that strain KJW98^T formed an evolutionarily distinct lineage within the genus *Bhargavaea* (Figs. 2 and 3), supported with high bootstrap-resampling values. The topologies of the phylogenetic trees built using the minimum-evolution (data not shown) and maximum-parsimony (see Supplementary data Figs. S1 and S2) algorithm also supported the notion that strain KJW98^T represents a species phylogenetically distinct from the closely related *B. beijingensis* DSM 19037^T, *B. cecembensis* LMG 24411^T, and *B. ginsengi* DSM 19038^T.

Table 2. Cellular fatty acid compositions (%) of strain KJW98^T and type strains of phylogenetically related *Bhargavaea* species
Strains: 1, KJW98^T; 2, *B. beijingensis* DSM 19037^T; 3, *B. ginsengi* DSM 19038^T; 4, *B. cecembensis* LMG 24411^T. Data for all species are from the present study. Symbols: Tr, Trace amount (<1%). Cellular fatty acids were determined after cultivation of strains on Luria Bertani agar at 37°C for 24 h.

Fatty acids	1	2	3	4
Straight chain saturated:				
$C_{14:0}$	Tr	Tr	1.1	1.0
$C_{16:0}$	2.6	2.1	3.7	3.9
Branched saturated:				
iso-$C_{14:0}$	5.3	4.2	4.9	8.9
iso-$C_{15:0}$	19.7	37.4	29.0	18.5
iso-$C_{16:0}$	11.1	5.9	8.6	14.0
iso-$C_{17:0}$	2.8	3.6	3.0	1.9
anteiso-$C_{15:0}$	37.7	27.0	32.6	33.4
anteiso-$C_{17:0}$	17.0	11.6	9.9	9.5
Monounsaturated:				
$C_{16:1}$ *ω11c*	Tr	1.6	1.4	1.5
$C_{18:1}$ *ω9c*	Tr	1.0	1.0	1.1
$C_{16:1}$ *ω7c* alcohol	1.7	2.2	2.5	4.8
Iso-$C_{17:1}$ *ω10c*	Tr	1.2	1.0	Tr
Summed feature 4[a]	1.0	2.2	1.5	1.5

[a] Summed feature represent group of two fatty acids that could not be separated by GLC with the MIDI system. Summed feature 4 contains iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B.

Chemotaxonomy

L-Lysine was the diagnostic diamino acid in the cell-wall peptidoglycan of all four strains. The four strains showed peptidoglycan type A4α: KJW98^T yielded L-Lys–L-Ala–D-Asp (type A11.34 according to www.peptidoglycan-types.info), whereas *B. cecembensis* yielded L-Lys–D-Asp (type A11.31), *B. beijingensis* and *B. ginsengi* yielded L-Lys–D-Glu (type A11.33) (Verma *et al.*, 2012). The major cellular polar lipids detected in strain KJW98^T were diphosphatidylglycerol and posphatidylglycerol, with minor amounts of an unidentified aminolipid and two unidentified phospholipids (see Supplementary data Fig. S3). The quinone of strain KJW98^T was dominated by MK-8 (87%), with minor amounts of MK-7 (8%) and MK-9 (5%). The major polar lipids and quinone present in strain KJW98^T were essentially the same as those present in reference strains (Verma *et al.*, 2012), supporting that strain KJW98^T belongs to genus *Bhargavaea*. The fatty acid profile of strain KJW98^T contained large quantities of branched saturated fatty acids, especially anteiso-$C_{15:0}$, iso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{16:0}$. The fatty acid profile of strain KJW98^T was related to that of reference strains *B. beijingensis*, *B. ginsengi* and *B. cecembensis* qualitatively, but

differs quantitatively mainly for fatty acids, anteiso-$C_{17:0}$, anteiso-$C_{15:0}$ and iso-$C_{15:0}$ (Table 2).

The DNA G+C content of strain KJW98^T was 55 mol%, which does not differ much from the closely related reference species *B. beijingensis*, *B. ginsengi*, and *B. cecembensis* (Table 1), supporting the affiliation of strain KJW98^T to the genus *Bhargavaea*. The DNA-DNA relatedness values of strain KJW98^T with *B. beijingensis* DSM 19037^T, *B. cecembensis* LMG 24411^T, and *B. ginsengi* DSM 19038^T were 43.2, 39, and 26.5%, respectively. These similarity values were well below the value of 70% generally accepted as the threshold level for the delineation of a novel bacterial species (Wayne *et al.*, 1987), backing the proposal of strain KJW98^T as a separate species of the genus *Bhargavaea*.

Taxonomic conclusion

The results of DNA-DNA hybridization experiments in combination with physiological, biochemical, phylogenetic (16S rRNA and *gyrB* gene sequences), chemotaxonomic (fatty acid, peptidoglycan, quinone and polar lipid compositions) and DNA G+C content data demonstrated that the new strain KJW98^T represented a novel species of the genus *Bhargavaea*, for which the name *Bhargavaea indica* sp. nov. is proposed.

Description of *Bhargavaea indica* sp. nov.

Bhargavaea indica (in′di.ca. L. fem. adj. *indica* of India, Indian).

Cells are Gram-positive, aerobic, non-motile, non-endospore-forming, coccoid rod-shaped (0.67–0.75 × 1.15–1.35 μm), present as single cells or in pairs. After 48 h of growth on LB agar at 37 °C, colonies are circular, low-convex with entire margins, non-pigmented, light pale brown in colour, with butterous consistency and approximately 2.0–3.0 mm in diameter. Growth occurs with 0–8.5% (w/v) NaCl (optimum, 0.5%), at pH 6.5–9 (optimum, pH 7–8) and at 15–48°C (optimum, 42°C). Catalase- and oxidase-positive. Hydrolyze casein and gelatin. Reduce nitrate and nitrite. Show β-haemolysis. Negative for indole production, Simmon's citrate, methyl red and Voges–Proskauer reaction, phenylalanine deaminase, urease, glucose fermentation and H_2S production in TSI. Positive for sucrose fermentation in TSI and lysozyme resistance. Produce acid from fructose but not from adonitol, arabinose, galactose, salicin, trehalose, mannose, rhamnose, cellobiose, maltose, dextrose, dulcital, inositol, inulin, lactose, mannitol, melibiose, raffinose, sorbitol, sucrose, and xylose. In the VITEK-2 GP gallery, reaction is positive for Ala-phe-pro-arylamidase, ellman, L-pyrrolydonyl-arylamidase, L-aspartate-arylamidase, leucine arylamidase, and tyrosine arylamidase. The remaining reactions of the VITEK-2 GP gallery are negative (Supplementary data Table S1). Metabolic fingerprinting using API ZYM showed negative reaction for alkaline phosphatase, lipase (C14), N-acetyl-β-glucosaminidase, α-mannosidase, valine arylamidase, cystine arylamidase, trypsin, β-glucuronidase, α-glucosidase, α-chymotrypsin, acid phosphatase, α-galactosidase, β-galactosidase, β-glucosidase, and α-fucosidase, while positive for esterase (C4), leucine arylamidase, esterase lipase (C8), and napthol-AS-BI-phosphohydrolase. Based on Biolog tests, gives a positive result for oxidation of Tween 40, β-hydroxybutyric acid, α-ketoglutaric acid, α-ketovaleric acid, acetic acid, pyruvatic acid methyl ester, succinic acid mono-methyl ester, pyruvic acid, succinamic acid, L-alaninamide, L-alanyl-glycine, L-glutamic acid, β-cyclodextrin, L-serine, glycerol, α-hydroxybutyric acid, dextrin, glycogen, L-malic acid, mannan, Tween 80, D-alanine, L-alanine, glycyl-L-glutamic acid, L-arabinose, D-fructose, D-galacturonic acid, adenosine, 2′-deoxy adenosine, inosine, D-ribose, D-xylose, thymidine, uridine and thymidine-5′-monophosphate, but negative for α-cyclodextrin, inulin, amygdalin, D-arabitol, arbutin, D-cellobiose, L-fucose, D-galactose, gentiobiose, D-gluconic acid, α-D-glucose, m-inositol, L-asparagine, α-D-lactose, lactulose, maltose, maltotriose, D-mannitol, D-mannose, D-melezitose, D-melibiose, α-methyl-D-galactoside, β-methyl-D-galactoside, 3-methyl-glucose, N-acetyl-L-glutamic acid, α-methyl-D-glucoside, β-methyl-D-glucoside, α-methyl-D-mannoside, palatinose, D-psicose, D-raffinose, L-rhamnose, salicin, sedoheptulosan, D-sorbitol, stachyose, sucrose, D-tagatose, D-trehalose, turanose, xylitol, γ-hydroxybutyric acid, p-hydroxy-phenylacetic acid, lactamide, D-lactic acid methyl ester, L-lactic acid, propionic acid, L-pyroglutamic acid, putrescine, 2,3-butanediol, D-fructose-6-phosphate, α-D-glucose-1-phosphate, D-glucose-6-phosphate, D-L-α-glycerol phosphate, N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, D-malic acid, succinic acid, adenosine-5′-monophosphate and uridine-5′-monophosphate. Able to grow on trypticase soy broth agar, MacConkey agar, GYM streptomyces medium, caso agar, wolinella succinogenes medium, leucothrix medium, medium for campylobacter DSM 806, VY/2 agar, Arthrobacter agar, CGY agar and Actinomyces agar (BBL). Growth does not occur on SM1 agar medium, universal medium for yeasts, halobacterium medium, semisolid NFb medium, glycerol asparagine agar, MRS medium, soybean mannitol medium, YPM medium, pityrosporum medium and Alcaligenes xylosoxydans agar-with benzoate (Table 1 and Supplementary data Table S1). The major cellular fatty acids are anteiso-$C_{15:0}$, iso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{16:0}$. Polar lipids present are diphosphatidylglycerol, posphatidylglycerol, unidentified aminolipid and two unidentified phospholipids. The cell-wall peptidoglycan contains L-lysine as the diagnostic diamino acid and type is A4 α: L-Lys–L-Ala-D-Asp. MK-8 is present as the major quinone. The DNA G+C content of the type strain is 55 mol%.

The type strain, KJW98^T (=KCTC 13583^T =LMG 25219^T) was isolated from sediments collected in the tropical western coastal region of India (Karwar district, Karnataka).

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References

Altschul, S.F., Madden, T.L., Schaeffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*. **25**, 3389–3402.

Chun, J., Lee, J.-H., Jung, Y., Kim, M., Kim, S., Kim, B.K., and Lim, Y.W. 2007. EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int. J. Syst. Evol. Microbiol.* **57**, 2259–2261.

Collins, M.D., Pirouz, T., Goodfellow, M., and Minnikin, D.E. 1977. Distribution of menaquinones in *Actinomycetes* and *Corynebacteria*. *J. Gen. Microbiol.* **100**, 221–230.

De Ley, J., Cattoir, H., and Reynaerts, A. 1970. The quantitative measurement of DNA hybridization from renaturation rates. *Eur. J. Biochem.* **12**, 133–142.

Fitch, W.M. 1971. Toward defining the course of evolution: minimum change for a specific tree topology. *Syst. Zool.* **20**, 406–416.

Groth, I., Schumann, P., Weiss, N., Martin, K., and Rainey, F.A. 1996. *Agrococcus jenensis* gen. nov., sp. nov., a new genus of *Actinomycetes* with diaminobutyric acid in the cell wall. *Int. J. Syst. Bacteriol.* **46**, 234–239.

Hauben, L., Vauterin, L., Swings, J., and Moore, E.R.B. 1997. Comparison of 16S ribosomal DNA sequences of all *Xanthomonas* species. *Int. J. Syst. Bacteriol.* **47**, 328–335.

Huss, V.A.R., Festl, H., and Schleifer, K.H. 1983. Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst. Appl. Microbiol.* **4**, 184–192.

Kämpfer, P., Rossello-Mora, R., Falsen, E., Busse, H.J., and Tindall, B.J. 2006. *Cohnella thermotolerans* gen. nov., sp. nov., and classification of '*Paenibacillus hongkongensis*' as *Cohnella hongkongensis* sp. nov. *Int. J. Syst. Evol. Microbiol.* **56**, 781–786.

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

Kovacs, N. 1956. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. *Nature* **178**, 703.

Logan, N.A., Berge, O., Bishop, A.H., Busse, H.-J., De Vos, P., Fritze, D., Heyndrickx, M., Kämpfer, P., Rabinovitch, L., Salkinoja-Salonen, M.S., and *et al.* 2009. Proposed minimal standards for describing new taxa of aerobic, endospore-forming bacteria. *Int. J. Syst. Evol. Microbiol.* **59**, 2114–2121.

MacKenzie, S.L. 1987. Gas chromatographic analysis of amino acids as the N-heptafluorobutyryl isobutyl esters. *J. Assoc. Off. Anal. Chem*. **70**, 151–160.

Manorama, R., Pindi, P.K., Reddy, G.S.N., and Shivaji, S. 2009. *Bhargavaea cecembensis* gen. nov., sp. nov., isolated from the Chagos–Laccadive ridge system in the Indian Ocean. *Int. J. Syst. Evol. Microbiol.* **59**, 2618–2623.

Mesbah, M., Premachandran, U., and Whitman, W.B. 1989. Precise measurement of the G+C content of deoxyribonucleic acid by high performance liquid chromatography. *Int. J. Syst. Bacteriol.* **39**, 159–167.

Pike, E.B., Carringtoen, E.G., and Ashburner, A.P. 1972. An evaluation of procedures for enumerating bacteria in activated sludge. *J. Appl. Bacteriol.* **35**, 309–321.

Qiu, F., Zhang, X., Liu, L., Sun, L., Schumann, P., and Song, W. 2009. *Bacillus beijingensis* sp. nov. and *Bacillus ginsengi* sp. nov., isolated from ginseng root. *Int. J. Syst. Evol. Microbiol.* **59**, 729–734.

Rzhetsky, A. and Nei, M. 1992. A simple method for estimating and testing minimum evolution trees. *Mol. Biol. Evol.* **9**, 945–967.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406–425.

Schleifer, K.H. 1985. Analysis of the chemical composition and primary structure of murein. *Methods Microbiol.* **18**, 123–156.

Schleifer, K.H. and Kandler, O. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol. Rev.* **36**, 407–477.

Tamaoka, J. and Komagata, K. 1984. Determination of DNA base composition by rever-sed-phase high-performance liquid chromatography. *FEMS Microbiol. Lett.* **25**, 125–128.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol. Biol. Evol.* **28**, 2731–2739.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. 1997. The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**, 4876–4882.

Tindall, B.J. 1990a. A comparative study of the lipid composition of *Halobacterium saccharovorum* from various sources. *Syst. Appl. Microbiol.* **13**, 128–130.

Tindall, B.J. 1990b. Lipid composition of *Halobacterium lacusprofundi*. *FEMS Microbiol. Lett.* **66**, 199–202.

Verma, P., Pandey, P.K., Gupta, A.K., Seong, C.N., Park, S.C., Choe, H.N., Baik, K.S., Patole, M.S., and Shouche, Y.S. 2012. Reclassification of *Bacillus beijingensis* and *Bacillus ginsengi* Qiu *et al.*, 2009 as *Bhargavaea beijingensis* comb. nov. and *Bhargavaea ginsengi* comb. nov. and emended description of the genus *Bhargavaea*. *Int. J. Syst. Evol. Microbiol.* **62**, 2495–2504.

Wayne, L.G., Brenner, D.J., Colwell, R.R., Grimont, P.A.D., Kandler, O., Krichevsky, M.I., Moore, L.H., Moore, W.E.C., Murray, R.G.E., Stackebrandt, E., and *et al.* 1987. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int. J. Syst. Bacteriol.* **37**, 463–464.

Xia, X. and Xie, Z. 2001. DAMBE: Data analysis in molecular biology and evolution. *J. Hered.* **92**, 371–373.

Yamamoto, S. and Harayama, S. 1995. PCR amplification and direct sequencing of *gyrB* genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. *Appl. Environ. Microbiol.* **61**, 1104–1109.

Yamamoto, S. and Harayama, S. 1998. Phylogenetic relationships of *Pseudomonas putida* strains deduced from the nucleotide sequences of *gyrB*, *rpoD* and 16S rRNA genes. *Int. J. Syst. Bacteriol.* **48**, 813–819.

Zhang, Z., Schwartz, S., Wagner, L., and Miller, W. 2000. A greedy algorithm for aligning DNA sequences. *J. Comput. Biol.* **7**, 203–214.

Zuckerkandl, E. and Pauling, L. 1965. Evolutionary divergence and convergence in proteins, pp. 97–166 in Evolving Genes and Proteins. *In* Bryson, V. and Vogel, H.J. (eds.). Academic Press, New York, N.Y., USA.