Bhargavaea cecembensis gen. nov., sp. nov., isolated from the Chagos–Laccadive ridge system in the Indian Ocean

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A novel Gram-positive, rod-shaped, non-motile, non-spore-forming bacterium, strain DSE10T, was isolated from a deep-sea sediment sample collected at a depth of 5904 m from the Chagos–Laccadive ridge system in the Indian Ocean. Cells of strain DSE10T were positive for catalase, oxidase, urease and lipase activities and contained iso-C15 : 0, iso-C16 : 0 and anteiso-C15 : 0 as the major fatty acids. The major respiratory quinones were MK-6 and MK-8 and the major lipids were phosphatidylglycerol and diphosphatidylglycerol. The cell-wall peptidoglycan contained diaminopimelic acid as the diagnostic diamino acid. A Blast sequence similarity search based on 16S rRNA gene sequences indicated that the genera Planococcus, Planomicrobiurn, Bacillus and Geobacillus were the nearest phylogenetic neighbours to the novel isolate with gene sequence similarities ranging from 94.9 to 95.2 %. Phylogenetic analyses using neighbour-joining, minimum-evolution and maximum-parsimony methods indicated that strain DSE10T formed a deeply rooted lineage distinct from the clades represented by the genera Planococcus, Planomicrobiurn, Bacillus and Geobacillus. Further, strain DSE10T could be distinguished from the above-mentioned genera based on the presence of signature nucleotides G, A, C, T, C, A, G, C and T at positions 182, 444, 480, 492, 563, 931, 1253, 1300 and 1391, respectively, in the 16S rRNA gene sequence. Based on the phenotypic and phylogenetic characteristics determined in this study, strain DSE10T was assigned as the type species of a new genus, Bhargavaea gen. nov., as Bhargavaea cecembensis sp. nov. The type strain of Bhargavaea cecembensis gen. nov., sp. nov. is DSE10T (=LMG 24411T=JCM 14375T). The genomic DNA G+C content of strain DSE10T is 59.5 ± 2.5 mol%.

Many novel deep-sea bacterial species (close to 100 species) representing 28 new genera and belonging to the Proteobacteria (12 genera), Aquificae (3 genera), Deinococcus–Thermus group (3 genera), Cytophaga–Flavobacterium–Bacteroidetes (CFB) group (2 genera), Deferribacter (1 genus), Thermotogae (1 genus) and Firmicutes (6 genera) have been identified. The majority of these taxa have been isolated from hydrothermal vents (Nakagawa et al., 2004; Prokofeva et al., 2005; Miroshnichenko & Bonch-Osmolovskaya, 2006; Naganuma et al., 2007; Reysenbach et al., 2006), deep-sea-sediments (Arakawa et al., 2006; Kaneko et al., 2007; Kimura et al., 2007; Shivaji et al., 2007), deep-sea carbonate crusts (Heijts et al., 2006) and seawater (Lauro & Bartlett, 2008). In the present study, we describe another novel bacterium belonging to a new genus, Bhargavaea gen. nov., a member of the Firmicutes.

Strain DSE10T was isolated from a deep-sea sediment sample collected from the Chagos–Laccadive ridge system (111060 S, 721310 E), which is a part of the Chagos Archipelago in the Indian Ocean. The Chagos Archipelago is a group of seven atolls comprising more than 64 individual tropical islands about 300 miles south of the Maldives. The sediment sample was collected as described previously (Raghukumar et al., 2004). In brief, the sediment core (length 460 cm and diameter 10 cm) was collected at a depth of 5904 m using a gravity core made from polyvinyl carbonate (PVC). The core was then extruded into a plastic bag under sterile conditions and sectioned into 30 cm pieces from the top to the bottom. For isolation of bacteria, a 100 mg sediment sample from the centre of the 30 cm was recovered and suspended in 0.3 % saline solution (Shivaji et al., 2007) and vortexed for...
30 min. The suspension was allowed to settle and 100 µl supernatant was plated on ZoBell marine agar medium (ZoBell, 1941) and incubated at room temperature for 15 days. The viable bacterial cell count obtained was approximately 2.5–7.5 x 10^5 c.f.u. Based on colony morphology, a brown-coloured dominating morphotype was selected and characterized in the present study.

Cell morphology was studied using light and transmission electron microscopy (TEM). For TEM, cells were negatively stained with 0.5 % (w/v) uranyl acetate and mounted on Formvar-coated copper grids. The grids were examined in TEM mode on a JEOL JEM-1010 electron microscope operated at 60 kV (Reddy et al., 2006). Motility was assessed on 0.4 % TSA agar plates and also by light microscopy. Spore staining was performed using a Schaeffer & Fulton’s spore staining kit (K006-1KT, HiMedia) according to the manufacturer’s protocols. Growth at different temperatures, tolerance to salt, biochemical characteristics, carbon assimilation, H2S production and the sensitivity of the cultures to different antibiotics were determined by previously described methods (Lanyi, 1987; Smibert & Krieg, 1994; Reddy et al., 2006). Biochemical characteristics were also double-checked with a Hi25 Enterobacteriaceae identification kit (KB003, HiMedia) and HiCarbohydrate kit parts A, B and C (KB009, HiMedia) according to the manufacturer’s protocol. Growth at different pH values was checked using TSA medium buffered either with citric acid–NaOH (for pH 5 and 6), phosphate (for pH 7 and 8), glycine–NaOH (for pH 9 and 10) or Tris (for pH 11 and 12) buffer. To check the tolerance of strain DSE10-T to pressure, cells were subjected to 2500 p.s.i. under sterile conditions in a French press for 30 min and then plated on TSA medium.

Fatty acid methyl esters were prepared and analysed by Sherlock Microbial Identification System (MIDI) according to the protocol described by Agilent Technologies. For this purpose, strain DSE10-T was grown on TSA medium (pH 7.5) at 25 °C for two days. Polar lipids were extracted and analysed according to the method described by Komagata & Suzuki (1987). Menaquinones were extracted by following a modified method. In this modified method, the cell pellet obtained from 500 ml freshly grown broth culture was suspended in a chloroform/methanol (2:1) mixture and homogenized for 5 min on ice. The lower layer containing quinones was collected by spinning the suspension at 6000 g for 10 min and dried with nitrogen gas. This process was repeated and 20 ml acetone was added to the remaining pellet, which was then homogenized. The upper acetone layer was collected and dried with nitrogen gas. The quinones obtained were initially separated by TLC (Collins et al., 1977) and further separated on HPLC using the isocratic solvent system of methanol/isopropyl ether (3:1) (Tamaoka et al., 1983).

Isolation of DNA and the determination of the DNA G + C content were conducted as described previously (Marmur, 1961; Marmur & Doty, 1962). The mean value from two independent experiments is given. The variation between the experiments was less than 2 %. For 16S rRNA gene sequencing, DNA was prepared using the MoBio microbial DNA isolation kit (MoBio Laboratories Inc.) and sequenced as described previously (Reddy et al., 2000). The resulting almost complete 16S rRNA gene sequence contained 1510 nucleotides [26–1536, according to the Escherichia coli 16S rRNA gene (GenBank accession no. J01695) numbering]. The 16S rRNA gene sequence was aligned with closely related sequences belonging to organisms of the genera Bacillus, Planococcus, Planomicrobium and Geobacillus using CLUSTAL W (Thompson et al., 1994). Pairwise evolutionary distances were computed using the DNADIST program with the Kimura two-parameter model as developed by Kimura (1980).

Phylogenetic trees were constructed using three tree-making algorithms, the minimum-evolution (ME), neighbour-joining (NJ) and maximum-parsimony (DNAPARS) methods, using the MEGA3 software package (Kumar et al., 2001) and the stability among the clades was assessed by employing 1000 replicate datasets.

Strain DSE10-T grew well on ZoBell marine broth 2216 (ZoBell, 1941) and on TSA medium. The major cellular fatty acids were (%): anteiso-C15:0 (31.2), iso-C15:0 (12.8), iso-C16:0 (10.7), iso-C14:0 (8.5), anteiso-C17:0 (6.2), C16:0 (4.7), C15:09c (4.7) and C16:1ω7c alcohol (4.5). The menaquinones were MK-6 and MK-8. Strain DSE10-T contained phosphatidylglycerol and diphosphatidylglycerol as the major lipids. The cell-wall peptidoglycan contained the amino acids alanine, glutamic acid and diaminopimelic acid in a ratio of 2:1:1. Cells of strain DSE10-T could tolerate a pressure of 2500 p.s.i. Additional characteristics of the novel strain are given in the species description.

A BLAST sequence similarity search, based on the 16S rRNA gene sequence of strain DSE10-T resulted in the identification of various strains of cultivable bacterial strains without validly published names and environmental sequences of the genus Bacillus with similarity of more than 97 %. The phylogenetic neighbours of strain DSE10-T with validly published names included Planococcus rifietoensis (Romano et al., 2003), Planococcus maritimus (Yoon et al., 2003), Planococcus citreus (Farrow et al., 1992), Bacillus bataviensis (Heyman et al., 2004), Bacillus fumaroli (Logan et al., 2000), Bacillus fortis (Scheldeman et al., 2004), Bacillus foraminis (Tiago et al., 2006), Bacillus novalis (Heyman et al., 2004), Bacillus niacin (Nagel & Andreesen, 1991), Bacillus methanolicus (Arfman et al., 1992), Brevibacterium frigoritolerans (Delaporte & Sasson, 1967) and Geobacillus thermoglucosidasius (Nazina et al., 2001), which had gene sequence similarities ranging from 94.9 % to 95.2 %. The BLAST results indicated that the novel strain could be a member of the genera Planococcus, Planomicrobium, Bacillus or Geobacillus. However, phylogenetic analyses, using NJ, ME and DNAPARS, resulted in the positioning of strain DSE10-T as a deeply rooted lineage distinct from the clades represented by the genera Planococcus and Planomicrobium, with a bootstrap value of 100 (Fig. 1).
The ambiguous clustering and less than 95% 16S rRNA gene sequence similarity with the nearest phylogenetic neighbours (Ludwig et al., 1998) clearly supported the creation of a higher taxon to accommodate strain DSE10T. Further evidence in support of genus status for strain DSE10T comes from an analysis based on a comparison of the 16S rRNA gene sequences. This indicated that strain DSE10T exhibited a difference of 93, 102, 97 and 56 nucleotides with the genera Bacillus, Planococcus, Planomicrobium and Geobacillus, respectively, in the highly variable region of the 16S rRNA gene. It is also interesting to note that strain DSE10T possessed nine unique nucleotides in the highly conserved region of the 16S rRNA gene. The bases G, A, C, T, C, A, G, C and T at positions 182, 444, 480, 492, 563, 931, 1253, 1300 and 1391 (positions with respect to the E. coli 16S rRNA gene sequence with GenBank accession number X80725) were present only in strain DSE10T and not in members of the genera Planococcus, Planomicrobium, Bacillus or Geobacillus (see Supplementary Table S1 in IJSEM Online). The conserved bases in strain DSE10T may be considered as the signature nucleotides that separate it from the genera Planococcus, Planomicrobium, Bacillus and Geobacillus. In addition, strain DSE10T shared the highest 16S rRNA gene sequence similarity of 95% with species of the genera Bacillus, Planococcus, Planomicrobium and Geobacillus. It is worth mentioning that the genera Planococcus and Planomicrobium also share 16S rRNA gene sequence similarity of more than 95%, justifying the creation of a new genus to accommodate strain DSE10T.

In addition to the above genotypic and phylogenetic differences, strain DSE10T can be differentiated from members of the genera Planococcus and Planomicrobium in that strain DSE10T is non-motile, contains diaminopimelic acid as the diagnostic diamino acid of the cell-wall peptidoglycan and has MK-6 and MK-8 as the predominant menaquinones (Table 1). Further, strain DSE10T can be differentiated from the genera Bacillus and Geobacillus with respect to sporulation, fatty acid methyl esters and menaquinones (Table 1). Thus, the combined differences that strain DSE10T exhibits when compared with the above genera unambiguously support the creation of a new genus and novel species, for which the name Bhargavaea cecembensis gen. nov., sp. nov. is proposed.

Description of Bhargavaea gen. nov.

Bhargavaea (Bhar.ga.va’ e.a. N.L. fem. n. Bhargavaea named in honour of Pushpa Mittra Bhargava, the renowned Indian biologist).

Cells are Gram-positive, aerobic, non-spore-forming rods that give a positive result in tests for catalase, oxidase, urease and lipase activities. The major fatty acids are iso-
**Table 1.** Characteristics that differentiate the genus *Bhargavaea* gen. nov. from other closely related genera

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tr>
<td>Sporulation*</td>
<td>Non-spore-forming</td>
<td>Non-spore-forming</td>
<td>Non-spore-forming</td>
<td>Spore-forming</td>
<td>Spore-forming</td>
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<td>Cell shape</td>
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<td>Cocci</td>
<td>Cocci/short rods/rods</td>
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<td>-</td>
<td>V</td>
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<td>Motile</td>
<td>Motile/non-motile</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>V</td>
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<td>-</td>
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<td>D</td>
<td>-</td>
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<tr>
<td>Gelatinase</td>
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<td>-</td>
<td>-</td>
<td>D</td>
<td>-</td>
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<td>Major isoprenoid quinones</td>
<td>MK-6, MK-8</td>
<td>MK-7, MK-8</td>
<td>MK-6, MK-7, MK-8</td>
<td>MK-7</td>
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<td>Major cellular fatty acids</td>
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<td>iso-C_{14:0}, anteiso-C_{15:0}, iso-C_{16:0}, iso-C_{17:0}</td>
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<td>PE, PG, DPG</td>
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<td>m-DAP</td>
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<td>DNA G+C content (mol%)</td>
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<td>39–52</td>
<td>35–47</td>
<td>32–69</td>
<td>49–58</td>
</tr>
</tbody>
</table>

 ستُميز هذه الجزيئات في تصنيف ***Bhargavaea*** gen. nov. من بين الأصناف القريبة.

*Sporeulation medium (M1018; HiMedia) and sporulation medium in combination with TSA were used for induction of sporulation.*

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C_{14:0}, iso-C_{15:0}, iso-C_{16:0} and anteiso-C_{15:0}. MK-6 and MK-8 are the predominant respiratory quinones. The cell-wall peptidoglycan contains diaminopimelic acid, lysine and glutamic acid in the ratio of 1:2:1. The type strain of the type species, DSE10T, contains the signature nucleotide G, A, C, T, C, A, G, C and T at positions 182, 444, 480, 492, 563, 931, 1253, 1300 and 1391, respectively, in the 16S rRNA gene sequence. The G+C content of the genomic DNA is 59.5 ± 2.5 mol%. The genus is affiliated to the family *Planococcaceae* of the order *Planococcales* of the class *Firmicutes*. The type species is *Bhargavaea cecembensis*.

### Description of *Bhargavaea cecembensis* sp. nov.

*Bhargavaea cecembensis* [ce.cem.ben’sis. N.L. fem. adj. *cecembensis* pertaining to CCMB, arbitrary adjective formed from the acronym of the Centre for Cellular and Molecular Biology (CCMB), where the taxonomic studies on this novel species were performed].

Has the following characteristics in addition to those given for the genus above. Cells are non-motile and rod-shaped (2.0 μm in length x 6.0 μm in width) and occur singly, in pairs or in groups (Fig. 2). Colonies on TSA medium are convex, 1–2 mm in diameter, smooth, regular and opaque. Cells grow from 15 to 55 °C with an optimum temperature of 37 °C and tolerate up to 6.0% (w/v) NaCl. Growth occurs over a pH range of 7–7.5. The doubling time is 5 h.

Tests for catalase, oxidase and urease activities are positive, but tests for phosphatase, β-galactosidase, arginine dihydrolase, ornithine decarboxylase, lysine decarboxylase and tryptophan deaminase are negative. Tyrosine is not degraded, indole is not produced. The methyl red and Voges–Proskauer reactions are negative. Tween 60 is hydrolysed but Tween 80, starch, aesculin and gelatin are not hydrolysed. Nitrate is reduced to nitrite and H2S gas is produced. Cells do not produce either acid or gas from adonitol, D-arabinose, dulcitol, erythritol, D-fructose, D-galactose, D-glucose, inositol, lactose, maltose, mannitol, D-mannose, raffinose, L-rhamnose, salicin, sucrose, D-sorbitol, trehalose or xylose even after incubation at an optimum temperature for 1 week. Assimilates adonitol, D-fructose, melezitose and maltose, but not N-acetylgalcosamine, D-arabinose, arbutin, cellubiose, citric acid, dulcitol, galactose, D-glucose, glycerol, glyoxylic acid, inositol, inulin, lactose, D-mannose, mannitol, melibiose, methyl α-D-glucoside, polyethylene glycol, acetate, raffinose, L-rhamnose, ribose, salicin, gluconate, sorbitol, L-sorbose, sucrose, trehalose, xylitol or D-xylose.

![Fig. 2. TEM images of negatively stained cells (a) and thin sectioned cells (b) of strain DSE10](http://ijs.sgmjournals.org)

Bars, 0.5 μm (a) and 200 nm (b).
Cells utilize L-arginine and L-proline but not the remaining eighteen amino acids, including L-ornithine and L-creatine. Cells are resistant to (μg disc−1) cefazolin (30), colistin (10), co-trimoxazole (25), lincomycin (2), nalidixic acid (30) and polymyxin-B (50 U) but susceptible to amikacin (30), ampicillin (10), bacitracin (10), carbenicillin (100), cefotaxime (30), chloramphenicol (30), ciprofloxacin (5), erythromycin (15), gentamicin-G (30), kanamycin (30), lomefloxacin (30), nitrofurantoin (300), norfloxacin (10), novobiocin (30), oleandomycin (15), penicillin-G (10), rifampicin (30), spectinomycin (100), tetracycline (30), tobramycin (10) and vancomycin (30). The peptidoglycan contains diaminopimelic acid, glutamic acid and alanine. The polar lipids comprise phosphatidylglycerol and diphosphatidylglycerol. The isoprenoid quinones are MK-6 (8%) and MK-8 (92%). The major cellular fatty acids are anteiso-C₁₅:₀, iso-C₁₅:₀, iso-C₁₆:₀ and iso-C₁₄:₀.

The type strain, DSE10T (=LMG 24411T=JCM 14375T), was isolated from a deep-sea sediment sample collected from the Chagos–Laccadive ridge system (111060 S, 721310 E), Indian Ocean.

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References


Bhargavaea cecembensis gen. nov., sp. nov.


