Cecembia lonarensis gen. nov., sp. nov., a haloalkalitolerant bacterium of the family *Cyclobacteriaceae*, isolated from a haloalkaline lake and emended descriptions of the genera *Indibacter*, *Nitritalea* and *Belliella*

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A novel Gram-staining-negative, rod-shaped, non-motile bacterium, designated strain LW9^T, was isolated from a water sample collected from Lonar Lake of Buldhana district, Maharashtra, India. Colonies and broth cultures were reddish orange due to the presence of carotenoid pigments. Strain LW9^T was positive for catalase, ornithine decarboxylase and lysine decarboxylase activities and negative for gelatinase, oxidase, urease and lipase activities. The predominant fatty acids were iso-C_{15:0} (31.3 %), iso-C_{16:0} (9.3 %), anteiso-C_{15:0} (7.3 %), iso-C_{16:1} H (6.1 %), summed feature 3 (comprising C_{16:1}ω7c/C_{16:1}ω6c; 5.9 %), iso-C_{17:1}ω9c (5.4 %) and iso-C_{17:0} 3-OH (5.0 %). Strain LW9^T contained MK-7 as the major respiratory quinone. The polar lipids consisted of phosphatidylethanolamine, two unidentified aminolipids and seven unidentified lipids. The DNA G + C content of strain LW9^T was 40.5 mol%. 16S rRNA gene sequence analysis indicated that the type strains of *Indibacter alkaliphilus* and *Aquiflexum balticum*, two members of the family *Cyclobacteriaceae* (phylum 'Bacteroidetes') were the most closely related strains with sequence similarities of 93.0 and 94.0 %, respectively. Other members of the family *Cyclobacteriaceae* showed sequence similarities <93.0 %. Based on these phenotypic characteristics and on phylogenetic inference, strain LW9^T is proposed as the representative of novel species in a new genus, *Cecembia lonarensis* gen. nov., sp. nov. The type strain of the type species, *Cecembia lonarensis*, is LW9^T (=CCUG 58316^T=KCTC 22772^T). Emended descriptions of the genera *Indibacter*, *Nitritalea* and *Belliella* are also proposed.

The family *Cyclobacteriaceae* (a member of the class *Cytophagia*, phylum 'Bacteroidetes') was recently proposed (Ludwig *et al.*, 2008) to include the type genus *Cyclobacterium* (Raj & Maloy, 1990; Nedashkovskaya *et al.*, 2005; Ying *et al.*, 2006) and the recently described genera *Algoriphagus*, *Aquiflexum*, *Belliella*, *Chimaericella*, *Echinicola*, *Hongiella*, *Indibacter* and *Nitritalea*. Species belonging to the genera *Chimaericella* and *Hongiella* were transferred to the genus *Algoriphagus* on the basis of polyphasic taxonomy analyses (Nedashkovskaya *et al.*, 2004, 2007).

Abbreviation: PE, phosphatidylethanolamine.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain LW9^T is FN393747.

A supplementary figure is available with the online version of this paper.

Members of the family *Cyclobacteriaceae* have been recovered from a wide variety of habitats, such as marine surface water (Brettar *et al.*, 2004a, b), sediment from an oilfield (Ying *et al.*, 2006), a sea urchin (Nedashkovskaya *et al.*, 2006), alkaline ground water, coral, marine sediments and soil (Nedashkovskaya *et al.*, 2007; Yoon *et al.*, 2006), microbial mats from Antarctic lakes (Van Trappen *et al.*, 2004), a freshwater lake (Liu *et al.*, 2009), brown and green algae and tidal flat sediment (Nedashkovskaya *et al.*, 2004), a salt water lagoon (Copa-Patiño *et al.*, 2008), a marine solar saltern (Yoon *et al.*, 2005) and sea ice (Bowman *et al.*, 2003).

Strain LW9^T was isolated from a water sample collected at a depth of 4.5 m from the haloalkaline Lonar Lake, located near Lonar town, Buldhana district, Maharashtra, India

(19° 58' N 76° 30' E) on 25th August 2008. The sample that yielded strain LW9^T had a pH of 10. For isolation of bacteria, a 100 µl water sample was plated on ZoBell marine agar (ZoBell, 1941) adjusted to pH 10.0 with a sodium carbonate solution (20 %, w/v) and incubated at room temperature for 15 days. The total viable bacterial count (per ml water) obtained was approximately 4.0 to 6.0 × 10⁵. Out of the two reddish orange coloured colonies that shared 100 % 16S rRNA sequence similarity, one was selected and characterized. For 16S rRNA gene sequencing, DNA was prepared using a microbial DNA isolation kit (Mo Bio Laboratories) and sequenced as described previously (Lane, 1991). The resultant almost complete sequence of the 16S rRNA gene (1513 nt) was subjected to a BLAST sequence similarity search (Altschul *et al.*, 1990) and EzTaxon (Chun *et al.*, 2007) was used to identify the nearest taxa. All 16S rRNA gene sequences of members of the family *Cyclobacteriaceae* were downloaded from the database (<http://www.ncbi.nlm.nih.gov>) and aligned using the program CLUSTAL_X (Thompson *et al.*, 1997); the alignment was then corrected manually. Phylogenetic trees were constructed using the maximum-likelihood and neighbour-joining methods (Saitou & Nei, 1987) using the program PhyML (Guindon *et al.*, 2005) and the PHYLIP package, version 3.5 (Felsenstein, 1993), respectively. The resultant tree topologies were evaluated by bootstrap analysis based on 1000 resamplings using the SEQBOOT and CONSENSE programs in the PHYLIP package. Pairwise evolutionary distances were computed using the DNADIST program with Kimura's two-parameter model as developed by Kimura (1980).

Strain LW9^T shared the highest 16S rRNA gene sequence similarity with *Indibacter alkaliphilus* LW1^T (94 %) and *Aquiflexum balticum* BA160^T (93 %). Phylogenetic analysis further indicated that strain LW9^T clustered with *I. alkaliphilus* forming a distinct branch in the family *Cyclobacteriaceae* on the neighbour-joining tree (Fig. 1). The topology of the maximum-likelihood tree was similar (data not shown). Both phylogenetic trees showed that the type strains of *A. balticum*, *Nitritalea halalkaliphila* and *Belliella baltica* were the next closest neighbours of strain LW9^T. Hence, the type strains of *I. alkaliphilus*, *A. balticum*, *N. halalkaliphila* and *B. baltica* were grown under the same conditions as strain LW9^T and used as reference strains in most phenotypic tests.

Cell morphology and motility were studied using a light microscope. Motility was assessed on trypticase soy agar (TSA) medium containing (l⁻¹) pancreatic digest of casein (17 g), papaic digest of soyabean meat (3 g), NaCl (5 g), dipotassium hydrogen phosphate (2.5 g), glucose (2.5 g) and agar (4.0 g). For assessment of colony morphology, the culture was grown on half-strength marine agar. Growth at 4, 10, 18, 30, 37 and 40 °C was ascertained using ZoBell marine agar medium and salt tolerance [at 0 (without NaCl), 1, 2, 3, 4, 5, 6, 8 and 10 % (w/v) NaCl] was ascertained using nutrient agar (NA) medium containing (l⁻¹) peptone (5 g), beef extract (3 g) and agar (20 g).

Biochemical characteristics such as oxidase, lysine decarboxylase and ornithine decarboxylase activities, nitrate reduction, hydrolysis of aesculin, gelatin, ONPG, starch, Tween 60 and Tween 80, carbon source assimilation, H₂S production and sensitivity to 32 different antibiotics using the disc diffusion method with commercially available discs (HiMedia) were determined by previously described methods (Lányi, 1987; Smibert & Krieg, 1994). Anaerobic growth was assessed in screw-capped tubes containing ZoBell marine broth with 0.05 % sodium ascorbate filled up to the top of the tube without an air gap. Biochemical characteristics were also assessed using the Hi25 *Enterobacteriaceae* identification kit (cat. no. KB003) and the HiCarbohydrate kit parts A, B and C (cat. no. KB009) (HiMedia) according to the manufacturer's protocol. Growth at pH 5.0, 6.0, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 11.0 and 12.0 was assessed on TSA buffered with citric acid/NaOH (for pH 5.0 and 6.0), NaHPO₄/Na₂HPO₄ (for pH 7.0–8.0), glycine/NaOH (for pH 9.0–10.0) or Tris (for pH 11.0 and 12.0). The phenotypic characteristics of strain LW9^T are listed in the genus and species descriptions and in Table 1.

For pigment extraction, 2 ml culture of exponential phase cells grown in ZoBell marine broth was centrifuged, the supernatant was discarded, 2 ml ethanol was added, and the mixture was mixed thoroughly on a vortex mixer. Following centrifugation, the supernatant was transferred to a new centrifuge tube. The absorption spectrum was measured at 300–800 nm on a Lambda 35 spectrometer (Perkin Elmer) using ethanol as a blank.

For cellular fatty acid analysis, strain LW9^T, *I. alkaliphilus* LW1^T, *A. balticum* DSM 16537^T, *B. baltica* DSM 15883^T and *N. halalkaliphila* LW7^T were grown on TSA at 30 °C for 48 h. Cellular fatty acid methyl esters were obtained from cells by saponification, methylation and extraction following the protocol of MIDI. Cellular fatty acid methyl esters were separated by GC (6850) and identified and qualified with MIDI System software (version 4.0, using the aerobe TSBA50 method and TSBA50 database). Polar lipids were extracted following the method of Bligh & Dyer (1959) and analysed by two-dimensional TLC followed by spraying with appropriate detection reagents (Komagata & Suzuki, 1987). Menaquinones were extracted as described by Collins *et al.* (1977) and analysed by HPLC (Groth *et al.*, 1997). Menaquinones and polar lipids were analysed on freeze-dried cells of strain LW9^T and the four reference strains. DNAs of strain LW9^T, *I. alkaliphilus* LW1^T, *A. balticum* DSM 16537^T, *N. halalkaliphila* LW7^T and *B. baltica* DSM 15883^T were isolated according to the procedure of Marmur (1961) and the G + C content was determined from melting point (*T*_m) curves (Sly *et al.*, 1986) obtained by using a Lambda 2 UV-Vis spectrophotometer (Perkin Elmer) equipped with the Templab 2.0 software package (Perkin Elmer). *Escherichia coli* strain DH5-α DNA was used as a standard in determining the DNA G + C content.

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship of *Cecembia lonarensis* LW9^T with members of the family Cyclobacteriaceae. Numbers at nodes are bootstrap values >70%. *Parapedobacter soli* DCY14^T was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

The absorption spectrum of the ethanol extract of strain LW9^T showed a broad peak with a maximum around 475 nm, which is typical for carotenoid pigments (Asker *et al.*, 2007). Alkalinization of the extract did not cause a bathochromic shift of the peak indicating the absence of flexirubin-type pigments, as often observed for marine members of the phylum 'Bacteroidetes' (Brettar *et al.*, 2004a, b; Yi & Chun, 2004). The cellular fatty acid composition of strain LW9^T showed a pronounced dominance of the branched saturated fatty acids iso-C_{15:0}, iso-C_{16:0}, anteiso-C_{15:0}, iso-C_{17:0} 3-OH and the unsaturated fatty acids iso-C_{16:1} H, summed feature 3 (comprising C_{16:1}ω7c/C_{16:1}ω6c) and iso-C_{17:1}ω9c (Table 2). Thus, the fatty acid composition of strain LW9^T differed from those of the four

reference strains (Table 2). Strain LW9^T had menaquinone MK-7 as respiratory quinone, which is similar to *I. alkaliphilus* LW1^T, *B. baltica* DSM 15883^T and *N. halalkaliphila* LW7^T. Polar lipids of strain LW9^T were phosphatidylethanolamine (PE), two unidentified amino-lipids (AL1, AL2) and seven unidentified lipids (L1–L7) (Fig. S1). The reference strains also possessed PE and some unknown lipids. In addition to PE, *I. alkaliphilus* LW1^T possessed one unidentified aminolipid (AL3) and five unidentified lipids (L1, L8–L11), *N. halalkaliphila* LW7^T possessed one unidentified aminolipid (AL3), two unidentified phospholipids (PL1, PL2) and six unidentified lipids (L1, L2, L5–L7, L12), and *B. baltica* DSM 15883^T possessed two unidentified phospholipids (PL1, PL3) and nine

Table 1. Characteristics that differentiate *Cecembia lonarensis* gen. nov., sp. nov., from closely related members of the family *Cyclobacteriaceae*

Strains: 1, *Cecembia lonarensis* LW9^T; 2, *Indibacter alkaliphilus* LW1^T; 3, *Aquiflexum balticum* DSM 16537^T; 4, *Nitritalea halalkaliphila* LW7^T; 5, *Belliella baltica* DSM 15883^T. Data for all strains are from the present study except for DNA G+C content of *A. balticum* DSM 16537^T (Brettar *et al.*, 2004b) and *B. baltica* DSM 15883^T (Brettar *et al.*, 2004a). All strains are positive for catalase activity, and utilization of xylose, glucose, fructose, lactose and mannose. All strains are negative for phenylalanine deaminase activity, methyl red and Voges-Proskauer reactions, indole production, agar and urea hydrolysis and utilization of sodium gluconate, dulcitol, sorbitol, mannitol, methyl α -D-glucoside, ribose, xylitol, sorbose and glycerol. +, Positive; –, negative; w, weak positive reaction. All taxa contain PE as major phospholipid.

Characteristic	1	2	3	4	5
pH growth range	7.5–10.0	7.5–12.0	7–9	7.5–12.0	6–10
NaCl tolerance (% w/v)	0–4	0–8	0–6	1–22	0–6
Oxidase	–	+	+	+	+
Lysine decarboxylase	+	+	–	+	–
Ornithine decarboxylase	+	+	–	+	–
Nitrate reduction	–	+	+	–	+
Hydrolysis of:					
Aesculin	–	+	+	–	+
Gelatin	–	–	+	+	–
ONPG	–	–	+	+	+
Starch	–	+	+	–	–
Utilization of:					
Citrate	+	–	+	+	–
Malonate	+	–	+	+	–
D-Arabinose	+	+	–	–	+
L-Arabinose	+	+	–	–	+
Adonitol	+	+	–	–	–
Rhamnose	+	+	–	–	–
Cellobiose	+	+	–	+	+
Melibiose	+	+	–	+	+
Melezitose	–	–	–	–	+
Raffinose	+	+	–	+	+
Trehalose	–	+	–	+	+
Maltose	+	+	–	–	+
Galactose	–	+	–	–	+
Sucrose	+	+	–	+	+
Glucosamine	–	+	–	–	–
Methyl α -D-mannoside	–	–	–	–	+
Inositol	–	–	+	+	–
Inulin	–	–	–	–	+
Salicin	–	–	–	–	+
DNA G+C content (mol%)	40.5	43	38.4	49	35.4

Table 2. Fatty acid compositions of *Cecembia lonarensis* gen. nov., sp. nov., and related members of the family *Cyclobacteriaceae*

Strains: 1, *Cecembia lonarensis* LW9^T; 2, *Indibacter alkaliphilus* LW1^T; 3, *Aquiflexum balticum* DSM 16537^T; 4, *Nitritalea halalkaliphila* LW7^T; 5, *Belliella baltica* DSM 15883^T. Results are presented as a percentage of the total fatty acids. Fatty acids amounting to 5% or more of the total fatty acids are in bold. Data for all taxa are from the present study. The five strains were grown on TSA at 30°C for two days. Tr, Trace (<1%); ND, not detected.

Fatty acid	1	2	3	4	5
C _{14:0}	ND	ND	2.9	Tr	ND
iso-C _{14:0}	1.1	ND	1.8	Tr	ND
iso-C _{15:0} G	2.4	ND	ND	ND	8.7
iso-C _{15:0}	31.3	47.0	15.6	59.6	21.1
anteiso-C _{15:0}	7.3	7.6	61.0	3.1	3.8
C _{15:0} 3-OH	2.8	3.7	ND	ND	2.1
iso-C _{15:0} 3-OH	ND	2.1	ND	2.1	2.9
C _{15:1} ω 6c	3.4	ND	ND	1.6	ND
C _{16:0}	ND	3.5	5.9	1.2	6.0
iso-C _{16:0}	9.3	2.3	2.1	1.8	2.9
C _{16:0} 3-OH	2.0	ND	ND	ND	1.6
iso-C _{16:0} 3-OH	2.4	ND	ND	Tr	ND
iso-C _{16:1} H	6.1	ND	ND	Tr	ND
C _{16:1} ω 5c	2.8	ND	ND	2.4	8.9
C _{17:0} 2-OH	1.1	ND	ND	ND	ND
iso-C _{17:0}	ND	3.5	ND	1.4	1.8
anteiso-C _{17:0}	ND	ND	3.1	Tr	ND
iso-C _{17:0} 3-OH	5.0	8.2	ND	8.9	6.5
C _{17:1} ω 6c	2.8	ND	ND	1.4	3.98
iso-C _{17:1} ω 9c	5.4	17.6	ND	ND	9.5
anteiso-C _{17:1} ω 9c	ND	ND	1.2	ND	ND
C _{18:0}	ND	ND	1.6	ND	ND
iso-C _{18:0}	ND	ND	ND	1.5	ND
Summed features*					
3	5.9	3.3	4.9	3.7	20.2
4	ND	ND	ND	1.4	ND
9	ND	ND	ND	2.7	ND

*Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 3 contains C_{16:1} ω 7c and/or C_{16:1} ω 6c; summed feature 4 contains iso-C_{17:1} I and/or anteiso-C_{17:1} B; summed feature 9 contains C_{16:0} 10-methyl and/or iso-C_{17:1} ω 9c.

unidentified lipids (L1, L2, L7–L9, L11, L13–L15) (Fig. S1, available in IJSEM Online). *A. balticum* DSM 16537^T possessed PE, one unidentified aminophospholipid and five unidentified lipids. The DNA G+C content of strain LW9^T was 40.5 mol% (Table 1).

Strain LW9^T could be differentiated from its closest phylogenetic neighbours by several traits such as oxidase activity, nitrate reduction, salt tolerance, hydrolysis of gelatin and starch, fatty acid and polar lipid composition and DNA G+C content (Tables 1 and 2 and Fig. S1).

Hence, the combination of phenotypic properties and phylogenetic inference unambiguously supports the proposal of a novel species in a new genus in the family *Cyclobacteriaceae* for which the name *Cecembia lonarensis* gen. nov., sp. nov. is proposed.

Description of *Cecembia* gen. nov.

Cecembia [Ce.cem'bi.a. N.L. fem. n. *Cecembia* arbitrary name derived from the abbreviation CCMB (Centre for Cellular and Molecular Biology)].

Cells are Gram-staining-negative, strictly aerobic rods, catalase-positive and oxidase-negative. Carotenoid pigments are produced. The major fatty acids are iso-C_{15:0}, iso-C_{16:0}, anteiso-C_{15:0}, iso-C_{16:1} H, summed feature 3 (comprising C_{16:1}ω7c/C_{16:1}ω6c), iso-C_{17:1}ω9c and iso-C_{17:0} 3-OH. MK-7 is the only respiratory quinone. The polar lipids consist of PE, two unidentified aminolipids and seven unidentified lipids. The genus is affiliated to the family *Cyclobacteriaceae*. The type species is *Cecembia lonarensis*.

Description of *Cecembia lonarensis* sp. nov.

Cecembia lonarensis (lo.na.ren'sis. N.L. fem. adj. *lonarensis* of or belonging to Lonar lake, referring to the isolation source of the type strain).

The main characteristics are the same as those given for the genus. In addition, cells are non-motile rods, 0.5–0.6 µm wide and 1.5–3.0 µm long, occurring singly, and multiply by binary fission. Colonies on half-strength marine agar are circular, 2–3 mm in diameter, smooth, reddish orange, translucent and raised with entire margins. Grows at 10 to 40 °C with an optimum temperature of 30–37 °C. Tolerates 0–4 % (w/v) NaCl and exhibits optimum growth in the presence of 2 % (w/v) NaCl. Grows at pH 7.5–10.0, with optimum growth at pH 8.0. Ornithine decarboxylase and lysine decarboxylase activities are present but urease, β-galactosidase, arginine dihydrolase and tryptophan deaminase activities are absent. The methyl red and Voges–Proskauer reactions are negative. Starch, aesculin, Tween 60, Tween 80 and gelatin are not hydrolysed. Nitrate is not reduced and H₂S gas is not produced. Produces acid from D-xylose, maltose, D-fructose, L-arabinose, cellobiose, D-glucose, D-mannose, methyl α-D-glucoside, methyl α-D-mannoside, melibiose, glycogen, creatinine, ornithine, starch, sorbose, citric acid, sodium gluconate, pyruvic acid, cellulose, rhamnose, erythritol, adonitol, inositol, D-mannitol and raffinose after 1 week of incubation at optimum temperature and pH. Assimilates adonitol, lactose, xylose, maltose, fructose, glucose, raffinose, melibiose, sucrose, L-arabinose, mannose, rhamnose, cellobiose, D-arabinose, citrate and malonate but not galactose, trehalose, inulin, sodium gluconate, glycerol, salicin, glucosamine, dulcitol, inositol, sorbitol, mannitol, methyl α-D-glucoside, ribose, melezitose, methyl α-D-mannoside, xylitol, sorbose or amygdalin. Susceptible to (µg per disc unless indicated)

co-trimoxazole (25), lincomycin (2), nalidixic acid (30), ampicillin (10), bacitracin (10), carbenicillin (100), cefotaxime (30), chloramphenicol (30), ciprofloxacin (5), erythromycin (15), gentamicin (30), lomefloxacin (30), nitrofurantoin (300), norfloxacin (10), novobiocin (30), oleandomycin (15), penicillin G (10), rifampicin (30), spectinomycin (100), tetracycline (30), doxycycline (10), cefuroxime (30), cefoperazone (75), roxithromycin (30), streptomycin (10) and vancomycin (30) and resistant to amikacin (30), cefazolin (30), colistin (10), polymixin B (50 units/disc), kanamycin (30) and tobramycin (10). The polar lipids comprise PE, two unidentified aminolipids (AL1, AL2) and seven unidentified lipids (L1–L7). The complete fatty acid composition is given in Table 2.

The type strain is LW9^T (=CCUG 58316^T=KCTC 22772^T), isolated from a water sample collected at a depth of 4.5 m from haloalkaline Lonar Lake, Lonar, Buldhana district, Maharashtra, India. The DNA G+C content of the type strain is 40.5 mol%.

Emended description of the genus *Indibacter* Anil Kumar *et al.* 2010

The description is as given previously (Anil Kumar *et al.*, 2010a) with the following modifications. The major fatty acids are iso-C_{15:0}, iso-C_{17:1}ω9c, iso-C_{17:0} 3-OH and anteiso-C_{15:0}. The polar lipids consist of PE, one unidentified aminolipid and five unidentified lipids. The type species is *Indibacter alkaliphilus*.

Emended description of the genus *Nitritalea* Anil Kumar *et al.* 2010

The description is as given previously (Anil Kumar *et al.*, 2010b) with the addition that the polar lipids consist of PE, one unidentified aminolipid, two unidentified phospholipids and six unidentified lipids.

Emended description of the genus *Belliella* Brettar *et al.* 2004

The description is as given previously (Brettar *et al.*, 2004a) with the following modifications. The major fatty acids are iso-C_{15:0}, C_{16:1}ω7c/C_{16:1}ω6c, iso-C_{17:1}ω9c, C_{16:1}ω5c, iso-C_{15:1} G, iso-C_{17:0} 3-OH and C_{16:0}. MK-7 is the only respiratory quinone. The polar lipids consist of PE, two unidentified phospholipids and nine unidentified lipids.

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