Mariniradius saccharolyticus gen. nov., sp. nov., a member of the family Cyclobacteriaceae isolated from marine aquaculture pond water, and emended descriptions of the genus Aquiflexum and Aquiflexum balticum

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A novel marine, Gram-stain-negative, oxidase- and catalase- positive, rod-shaped bacterium, designated strain AK6T, was isolated from marine aquaculture pond water collected in Andhra Pradesh, India. The fatty acids were dominated by iso-C15:0, iso-C17:1ω9c, iso-C15:1 G, iso-C17:0 3-OH and anteiso-C15:0. Strain AK6T contained MK-7 as the sole respiratory quinone and phosphatidylethanolamine, one unidentified aminophospholipid, one unidentified phospholipid and seven unidentified lipids as polar lipids. The DNA G+C content of strain AK6T was 45.6 mol%. Phylogenetic analysis showed that strain AK6T formed a distinct branch within the family Cyclobacteriaceae and clustered with Aquiflexum balticum DSM 16537T and other members of the family Cyclobacteriaceae. 16S rRNA gene sequence analysis confirmed that Aquiflexum balticum DSM 16537T was the nearest neighbour, with pairwise sequence similarity of 90.1 %, while sequence similarity with the other members of the family was < 88.5 %. Based on differentiating phenotypic characteristics and phylogenetic inference, strain AK6T is proposed as a representative of a new genus and species of the family Cyclobacteriaceae, as Mariniradius saccharolyticus gen. nov., sp. nov. The type strain of Mariniradius saccharolyticus is AK6T (= MTCC 11279T = JCM 17389T). Emended descriptions of the genus Aquiflexum and Aquiflexum balticum are also proposed.

Members of the family Cyclobacteriaceae (order Cytophagaales, within the phylum Bacteroidetes) are Gram-stain-negative, aerobic, chemo-organoheterotrophic, straight, ring/circle or horse-shoe-shaped rods, usually non-motile (members of the genus Echinicola are motile by gliding; Srinivas et al., 2012), producing pink, red or orange pigments and containing predominantly branched and saturated fatty acids (e.g. iso-C15:0), menaquinone 7 (MK-7) and phosphatidylethanolamine (Nedashkovskaya & Ludwig, 2011). Members of the family are widely distributed in diverse habitats such as a hot spring (Kämpfer et al., 2010), soda lakes (Anil Kumar et al., 2010a, b, 2012; Yang et al., 2012), soil (Yoon et al., 2006), a freshwater lake (Liu et al., 2009), and various marine habitats such as marine surface water, marine organisms, salty water lagoon, sediment of an oilfield and solar saltern (Brettar et al., 2004a, b; Nedashkovskaya et al., 2004, 2006; Copa-Patiño et al., 2008; Ying et al., 2006; Yoon et al., 2005), and polar habitats (Bowman et al., 2003; Van Trappen et al., 2004). At the time of writing, the family Cyclobacteriaceae comprises 10 validly named genera: Algoriphagus (Bowlow et al., 2003), Aquiflexum (Brettar et al., 2004b), Belliella (Brettar et al., 2004a), Cecembia (Anil Kumar et al., 2012), Cyclobacterium (Raj & Maloy, 1990), Echinicola (Nedashkovskaya et al., 2006), Fontibacter (Kämpfer et al., 2010), Indibacter (Anil Kumar et al., 2010a), Mongoliitalea (Yang et al., 2012) and Nitritalea (Anil Kumar et al., 2010b). However, the genera Litoribacter (Tian et al., 2010) and Rhodonellum, members of the family Cytophagaceae, cluster with members of the family Cyclobacteriaceae in phylogenetic trees (All-Species Living Tree; Release LTPs106; Schmidt et al., 2006). In the present study, we focused on the characterization and classification of strain AK6T, by using a polyphasic approach (Vandamme et al., 1996). From the results of phylogenetic and phenotypic analyses, this strain...
was assigned to a novel species of a new genus in the family *Cyclobacteriaceae*.

Strain AK6\(^T\) was isolated from seawater of a fish culture pond at Bheemli (17° 53’ N 83° 26’ E), Andhra Pradesh, India. The sample (1 ml) was serially diluted (10-fold dilutions) in 2 % (w/v) NaCl solution and 100 µl of each dilution was plated on ZoBell marine agar (MA; HiMedia) and incubated at 30 °C. A reddish-orange-coloured colony observed after 5 days of incubation was selected and characterized in the present study. Subcultivation of the isolate was carried out on MA at 30 °C. Stock cultures were preserved at −80 °C in marine broth (MB; HiMedia) with 10 % glycerol.

DNA isolation, 16S rRNA gene amplification and sequencing were done as described previously (Srinivas et al., 2011; Surendra et al., 2012). The 16S rRNA gene was sequenced using primers 530f and 907r (Johnson, 1994) in addition to the PCR primers, and the deoxy chain-termination method was used (Pandey et al., 2002). The 16S rRNA gene sequence of the isolate was subjected to BLAST sequence similarity search (Altschul et al., 1990) to identify the nearest taxa. The 16S rRNA gene sequences of closely related validly named taxa belonging to the family *Cyclobacteriaceae* were downloaded from the NCBI database (http://www.ncbi.nlm.nih.gov) and aligned using the CLUSTAL X program (Thompson et al., 1997) and the alignment was then corrected manually by deleting gaps and missing data. There were a total of 1258 positions in the final dataset. Phylogenetic trees were constructed using the maximum-likelihood method using the PhyML program (Guindon et al., 2005) and the neighbour-joining method (Saitou & Nei, 1987) using MEGA5 (Tamura et al., 2011) and the tree topologies were evaluated by bootstrap analysis based on 100 and 1000 replicates, respectively (Felsenstein, 1989). Evolutionary distances (expressed in number of base substitutions per site) were computed using the Kimura two-parameter method (Kimura, 1980).

The closest phylogenetic neighbour of strain AK6\(^T\) was *Aquiflexum balticum* DSM 16537\(^T\) with pair-wise 16S rRNA gene sequence similarity of 90.1 %, *Cecembia lonarensis* LW9\(^T\) (88.4 %), *Indibacter alkaliphilus* LW1\(^T\) (88.1 %) and several *Algoriphagus* species (87.8–87.6 %) were the next closest relatives of strain AK6\(^T\). The neighbour-joining tree (Fig. 1) confirmed the clear affiliation of strain AK6\(^T\) to the family *Cyclobacteriaceae* the new isolate clustered with *Aquiflexum balticum* DSM 16537\(^T\) and these two organisms clustered with the type strains of *Cecembia lonarensis*, *Fontibacter flavus* and *Indibacter alkaliphilus*. The topology of the maximum-likelihood tree was essentially the same (data not shown).

Colony morphology was studied after 48 h on MA at 30 °C. Cell morphology was investigated by phase-contrast microscopy (Olympus BX51; ×1000). The Gram reaction was determined by using the HiMedia Gram Staining kit according to the manufacturer’s protocol. Endospore formation was determined after malachite-green staining of the isolate grown on R2A (HiMedia) plates for 1 week. Flagellar motility was assessed on Motility-Indole-Lysine HiVeg medium (HiMedia) with 2 g agar l\(^{-1}\), by phase-contrast microscopy and gliding motility was determined according to Bernardet et al. (2002). Pigment characteristics were determined by previously described methods (Anil Kumar et al., 2012) except that the pigments were extracted into acetone.

Growth of strain AK6\(^T\) was assessed: (i) on MA, nutrient agar (NA; HiMedia) and tryptone soy agar (TSA; HiMedia); (ii) at 4, 10, 18, 25, 30, 35, 37, 42 and 45 °C in MB; (iii) in the presence of 0, 1, 2, 3, 4, 5, 6, 8, 10 % (w/v) NaCl in nutrient broth initially devoid of NaCl; and (iv) at pH 4, 5, 6, 7, 7.5, 8, 8.5, 9, 10, 11 and 12 in MB buffered with acetate buffer (pH 4–6), 100 mM NaH₂PO₄/Na₂HPO₄ buffer (pH 7–8), 100 mM NaHCO₃/Na₂CO₃ buffer (pH 9–10) or 100 mM Na₂CO₃/NaOH buffer (pH 11–12). Anaerobic growth was assessed by incubating agar slants under nitrogen atmosphere and by inoculating broth cultures in tightly closed tubes in the presence of ascorbic acid. Activity of catalase, oxidase, lysine decarboxylase, and ornithine decarboxylase; nitrate reduction; carbon source assimilation; acid production from carbohydrates; H₂S production; and sensitivity to 18 different antibiotics [using the disc diffusion method with commercially available discs (HiMedia)] were determined according to Lányi (1987) and Smibert & Krieg (1994) except that all media used contained 2.0 % (w/v) NaCl. Strain AK6\(^T\) was also tested in the Vitek 2 GN system (bioMérieux), according to the manufacturer’s protocol, except that a 2.0 % (w/v) NaCl sterile solution was used to prepare the inoculum.

Phenotypic characteristics of strain AK6\(^T\) are given in Table 1 and in the genus and species descriptions.

For cellular fatty acid analysis, strains AK6\(^T\) and *Aquiflexum balticum* DSM 16537\(^T\) were grown on TSA with 2 % (w/v) NaCl at 30 °C. The physiological age of the two strains was standardized using the protocol (http://www.microbialid.com/PDF/TechNote_101.pdf) given by the Sherlock Microbial Identification System (MIDI). The two strains were collected after 2 days of incubation. Cellular fatty acid methyl esters were obtained by saponification, methylation and extraction following the standard protocol of the Sherlock Microbial Identification System. Cellular fatty acid methyl esters were separated by GC (Agilent 6890 series GC system), identified and quantified with the Sherlock Microbial Identification System Software (version.6.0, using the aerobe TSBA6 method and the TSBA6 database). Polar lipids and respiratory quinones were analysed from freeze-dried cells. The polar lipids of strains AK6\(^T\) and *Aquiflexum balticum* DSM 16537\(^T\) were extracted from cells grown on MA at 30 °C for 3 days under aerobic conditions (Bligh & Dyer, 1959) and analysed by two-dimensional TLC followed by spraying with appropriate detection reagents (Komaga & Suzuki, 1987). Isoprenoid quinones were extracted as described by Collins et al. (1977) and analysed by HPLC (Groth et al., 1997). The solvent was acetonitrile/2-propanol.
(65:35) and the flow rate was 1 ml min⁻¹. The DNA of strain AK6ᵀ was isolated according to the procedure of Marmur (1961) and the G+C content was determined from melting point (Tₘ) 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). The DNA of *Escherichia coli* was used as a standard.

The absorption spectrum of the acetone extract of strain AK6ᵀ showed a broad peak with a maximum at approximately 480 nm, which was typical for carotenoid pigments (Anil Kumar et al., 2012; Asker et al., 2007). The cellular fatty acid composition of strain AK6ᵀ was dominated by branched saturated and hydroxy fatty acids, especially iso-C₁₅:₀, iso-C₁₇:₁₀₉c, iso-C₁₅:₁ G, iso-C₁₇:₀ 3-OH and anteiso-C₁₅:₀. This profile was quite different from that of *Aquiflexum balticum* DSM 16537ᵀ (Table 2). The sole respiratory quinone of strain AK6ᵀ was menaquinone 7 (MK-7) in line with all other members of the family *Cyclobacteriaceae* (Nedashkovskaya & Ludwig, 2011). The polar lipids of strain AK6ᵀ consisted of phosphatidylethanolamine, one unidentified aminophospholipid, one unidentified phospholipid and seven unidentified lipids (Fig. 2). The polar lipids of *Aquiflexum balticum* DSM 16537ᵀ were phosphatidylethanolamine, the same unidentified aminophospholipid and phospholipid as strain AK6ᵀ and five unidentified lipids (four of which were shared with strain AK6ᵀ and one additional lipid). Strain AK6ᵀ had a DNA G+C content of 45.6 mol%, clearly distinct from that of *Aquiflexum balticum* DSM 16537ᵀ (38.4 mol%; Brettar et al., 2004b) (Table 1).

Hence, phenotypic distinctiveness and phylogenetic inference warrant the description of a new genus and species to accommodate strain AK6ᵀ, for which the name *Mariniradius saccharolyticus* gen. nov., sp. nov. is proposed. On the basis of new data obtained in this study, emended descriptions of the genus *Aquiflexum* and *Aquiflexum balticum* are also proposed.

**Description of Mariniradius gen. nov.**

*Mariniradius* (Ma.ri.ni.ra.dis. us. L. adj. marinus of the sea, marine; L. masc. n. radius a staff, rod; N.L. masc. n. *Mariniradius* a marine rod, isolated from marine aquaculture pond water).
**Table 1. Features that distinguish strain AK6<sup>T</sup> from *Aquiflexum balticum* DSM 16537<sup>T</sup>**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell size (μm) (MA, 30 °C, 48 h)</td>
<td>0.5–1 x 4–6</td>
<td>0.3–0.7 x 1–4</td>
</tr>
<tr>
<td>Colony pigmentation</td>
<td>Reddish-orange</td>
<td>Red</td>
</tr>
<tr>
<td>Temperature growth range (°C)</td>
<td>25–42</td>
<td>4–40</td>
</tr>
<tr>
<td>Optimum growth temperature (°C)</td>
<td>30–37</td>
<td>30</td>
</tr>
<tr>
<td>NaCl concentration range (% w/v)</td>
<td>0–4</td>
<td>0–6</td>
</tr>
<tr>
<td>pH growth range</td>
<td>7–8</td>
<td>7–9</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>H₂S production</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>γ-Glutamyl-transferase activity</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Glycine arylamidase activity</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Acid production from:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inositol, rhamnose, galactose</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Raffinose, mannose, arabinose, xylose</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Susceptibility to:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Novobiocin (30 μg)</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>Penicillin G (2 U)</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>45.6</td>
<td>38.4</td>
</tr>
</tbody>
</table>

Cells are Gram-stain-negative, non-spore-forming, strictly aerobic, straight rods devoid of flagellar and gliding motility. Oxidase- and catalase-positive. Cells contain carotenoids but no flexirubin-type pigments. The major fatty acids (≥13 %) are iso-C<sub>15:0</sub> and iso-C<sub>17:0</sub> 3-0c. MK-7 is the sole respiratory quinone. The predominant polar lipids are phosphatidylethanolamine, one unidentified aminophospholipid and one unidentified lipid. A member of the family *Cyclobacteriaceae* (class *Cytophagia*, phylum *Bacteroidetes*). The type species is *Mariniradius saccharolyticus*.

**Description of *Mariniradius saccharolyticus* sp. nov.**

*Mariniradius saccharolyticus* [sac.cha.ro.ly’ti.cus. Gr. n. sakchar sugar; N.L. masc. adj. lyticus (from Gr. masc. adj. lutikos), able to loosen, able to dissolve; N.L. masc. adj. saccharolyticus breaking up polysaccharides].

Exhibits the following properties in addition to those given in the genus description. Cells are 0.5–1 μm in diameter and 4–6 μm in length. Colonies on MA are circular, 2–3 mm in diameter, smooth, reddish-orange in colour, opaque and convex with entire margin. Grows at 25–42 °C (optimum, 30–37 °C) and at pH 7–8 (optimum, pH 7.5). Growth occurs in the presence of 0–4 % (w/v) NaCl (optimum, 2 %). H₂S is not produced. In the Vitek 2GN system phosphatase, Ala–Phe–Pro-arylamidase, β-galactosidase, N-acetyl-β-glucosaminidase, glutamyl arylamidase, β-glucosidase, β-xylodolyl-arylamidase, L-proline arylamidase, tyrosine arylamidase, γ-glutamyltransferase, α-glucosidase, α-galactosidase and Glu–Gly–Arg-arylamidase activities are present; lipase, β-lipolydolyl-arylamidase, β-alanine arylamidase, N-acetyl-β-galactosaminidase, β-glucuronidase, glycine arylamidase, lysine decarboxylase and ornithine decarboxylase activities are absent; negative for L-lactate and succinate alkalization, ELLMAN reaction, fermentation of glucose, resistance to the vibriostatic compound O/129, and utilization of adonitol, L-arabitol, cellobiose, D-glucose, maltose, D-mannitol, D-mannose, palatinose, D-sorbitol, sucrose, D-tagatose, trehalose, citrate, malonate, 5-keto-D-glucosamine, coumarate, L-malate, L-lactate and L-histidine. Acid is produced from sucrose, glucose, raffinose, fructose, mannose, arabinose, xylose and inulin, but not from inositol, dulcitol, rhamnose, galactose, maltose, melibiose, cellobiose, salicin, adonitol, and 4–6 mm in diameter, smooth, reddish-orange in colour, opaque and convex with entire margin. Grows at 25–42 °C (optimum, 30–37 °C) and at pH 7–8 (optimum, pH 7.5). Growth occurs in the presence of 0–4 % (w/v) NaCl (optimum, 2 %). H₂S is not produced. In the Vitek 2GN system phosphatase, Ala–Phe–Pro-arylamidase, β-galactosidase, N-acetyl-β-glucosaminidase, glutamyl arylamidase, β-glucosidase, β-xylodolyl-arylamidase, L-proline arylamidase, tyrosine arylamidase, γ-glutamyltransferase, α-glucosidase, α-galactosidase and Glu–Gly–Arg-arylamidase activities are present; lipase, β-lipolydolyl-arylamidase, β-alanine arylamidase, N-acetyl-β-galactosaminidase, β-glucuronidase, glycine arylamidase, lysine decarboxylase and ornithine decarboxylase activities are absent; negative for L-lactate and succinate alkalization, ELLMAN reaction, fermentation of glucose, resistance to the vibriostatic compound O/129, and utilization of adonitol, L-arabitol, cellobiose, D-glucose, maltose, D-mannitol, D-mannose, palatinose, D-sorbitol, sucrose, D-tagatose, trehalose, citrate, malonate, 5-keto-D-glucosamine, coumarate, L-malate, L-lactate and L-histidine. Acid is produced from sucrose, glucose, raffinose, fructose, mannose, arabinose, xylose and inulin, but not from inositol, dulcitol, rhamnose, galactose, maltose, melibiose, cellobiose, salicin, adonitol,
trehalose, lactose, mannitol or sorbitol after 3 weeks of incubation. Susceptible to (μg per disc unless indicated) polymyxin-B (300), tetracycline (30), bacitracin (8 U), spectinomycin (100), ciprofloxacin (10), vancomycin (30), chloramphenicol (30), cefadroxil (30), novobiocin (30), amoxicillin (30), chlorotetracycline (30), ampicillin (25) and lincomycin (2), but resistant to novobiocin (30), penicillin G (2 U), ceftazidime (30) and gentamicin (10). In addition to the major fatty acids listed above, intermediate amounts (≥6%) of iso-C₁₅:₀ G, iso-C₁₅:₀ 3-OH and anteiso-C₁₅:₀ and significant amounts (>2%) of summed feature 3 (comprising iso-C₁₅:₀ 2-OH and/or C₁₁:₀ Iranian, iso-C₁₅:₀ 3-OH, C₁₆:₀ 3-OH, iso-C₁₆:₁ G, C₁₆:₁ Iranian, iso-C₁₇:₀ 3-OH and C₁₆:₀ are also present. The complete cellular fatty acid composition is given in Table 2. In addition to the major polar lipids listed above, significant amounts of one unidentified phospholipid and six unidentified lipids are also present.

The type strain, AK₆ᵀ (=MTCC 11279ᵀ=JCM 17389ᵀ), was isolated from marine water collected from the sea shore at Visakhapatnam, Andhra Pradesh, India. The DNA G+C content of the type strain is 45.6 mol%.

### Emended description of the genus *Aquiflexum* Brettar et al. 2004

The description is as given previously (Brettar et al., 2004b) with the following modifications. MK-7 is the sole respiratory quinone. The predominant polar lipids are phosphatidylethanolamine and two unidentified lipids.

### Emended description of *Aquiflexum balticum* Brettar et al. 2004

The description is as given by Brettar et al. (2004b) with the following modification. In addition to the predominant polar lipids cited above, detectable amounts of one unidentified phospholipid, one unidentified aminophospholipid and three unidentified polar lipids are also present.

### Acknowledgements

We thank Dr J. Euzéby for his expert suggestion for the correct genus and species epithets and Latin etymology. We also thank the Council of Scientific and Industrial Research (CSIR) and the Department of Biotechnology, Government of India, for financial assistance. We thank Mr Deepak Bhatt for his excellent help in 16S rRNA gene sequencing. The laboratory facility was extended by MMRF of NIO, RC, Kochi, funded by the Ministry of Earth Sciences, New Delhi.

![Fig. 2. Two-dimensional thin-layer chromatogram of the total polar lipids of strain AK₆ᵀ (a) and *Aquiflexum balticum* DSM 16537ᵀ (b) revealed by 5% ethanolic molybdatephosphoric acid. PE, phosphatidylethanolamine; APL, unidentified aminophospholipid; L1–8, unidentified lipids; PL, unidentified phospholipid.](image-url)
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References


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