Cyclobacterium xiamenense sp. nov., isolated from aggregates of Chlorella autotrophica, and emended description of the genus Cyclobacterium

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A novel Gram-stain-negative, horseshoe-shaped, non-motile bacterium, designated strain KD51ᵀ, forming colonies coloured pink by carotenoid pigments, was isolated from aggregates of the alga Chlorella autotrophica collected from the coastal sea off the city of Xiamen, Fujian Province, China. 16S rRNA gene sequence comparison showed that strain KD51ᵀ was a member of the genus Cyclobacterium, forming a distinct lineage with Cyclobacterium lianum HY9ᵀ. The 16S rRNA gene sequence similarity between strain KD51ᵀ and the type strains of species of the genus Cyclobacterium ranged from 92.1 % to 95.2 %. Growth occurred at 4–40 °C (optimum, 28 °C), in the presence of 3–9 % NaCl (optimum, 3–5 %) and at pH 6–10 (optimum, pH 7.5). The dominant fatty acids (>20 %) of strain KD51ᵀ were iso-C₁₅ : 0 (32.2 %) and summed feature 3 (comprising C₁₆ : 1₀⁻⁷c and/or C₁₆ : 1₁⁻⁹c; 22.2 %). The DNA G + C content was 41.7 mol% and the only respiratory quinone was menaquinone-7. On the basis of phenotypic data and phylogenetic inference, strain KD51ᵀ represents a novel species of the genus Cyclobacterium, for which the name Cyclobacterium xiamenense sp. nov. is proposed. The type strain is KD51ᵀ (=CGMCC 1.12432ᵀ = KCTC 32253ᵀ). An emended description of the genus Cyclobacterium is also proposed.

The genus Cyclobacterium, type genus of the family Cyclobacteriaceae (phylum Bacteroidetes), was established by Raj & Maloy (1990) and its description has subsequently been emended by Ying et al., (2006). Members of the genus Cyclobacterium, displaying a unique ring-like or horseshoe-shaped cellular morphology, are common constituents of marine environments. At the time of writing, the genus Cyclobacterium contains five species with validly published names isolated from a variety of marine environments: Cyclobacterium marinum (Raj & Maloy, 1990), C. amurskyense (Nedashkovskaya et al., 2005), C. lianum (Ying et al., 2006), C. qasimii (Shivaji et al., 2012) and C. caemipelagi (Jung et al., 2013). In the present study, we characterized and determined the exact taxonomic position of a novel pink-pigmented bacterial strain with ring-like and horseshoe-shaped cells by using a polyphasic approach (Vandamme et al., 1996). Our data showed that the isolate represents a novel species of the genus Cyclobacterium.

In the course of a study on bacterial communities in marine algae off the coast of Xiamen city, Fujian Province, China (Zhou et al., 2013), we isolated strain KD51ᵀ in 2012 from aggregates of the alga Chlorella autotrophica. For isolation, 100 µl of a serially diluted sample was spread onto fresh marine agar 2216 (MA; Difco) and incubated at 28 °C for 7 days. A pink-pigmented colony, designated strain KD51ᵀ, was selected, subcultured on MA three times and stored at −80 °C in marine broth 2216 (MB; Difco) supplemented with 10 % (v/v) glycerol.

The genomic DNA of strain KD51ᵀ was extracted according to the method of Ausubel et al. (1995) and the 16S rRNA gene was amplified by PCR using the primer pair P27F and P1492R (DeLong, 1992). Purification of the PCR product was carried out using a TIANquick Midi purification kit (TIANGEN) and the purified PCR product was cloned into vector pMD19-T and sequenced. Sequences of related taxa were downloaded from the GenBank database and the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/);

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¹These authors contributed equally to this work.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain KD51ᵀ is KC206024.

Abbreviation: ECL, equivalent chain-length.
Kim et al., 2012). Phylogenetic analysis was performed using MEGA software version 4 (Tamura et al., 2007) after multiple alignment of data by DNAMAN (version 5.1). Evolutionary distances and clustering were performed by using the neighbour-joining method (Saitou & Nei, 1987). The resulting tree topology was evaluated by using bootstrap analysis, based on 1000 replicates.

Preliminary comparison of the 16S rRNA gene sequence of strain KD51\textsuperscript{T} (1444 bp) with other sequences indicated that the novel isolate was closely related to the type strains of *Cyclobacterium lianum*, *C. marinum*, *C. qasimii*, *C. amurskyense* and *C. caenipelagi*, with pairwise sequence similarities of 95.2\%, 94.3\%, 94.0\%, 93.6\% and 92.1\%, respectively. Sequence similarities between strain KD51\textsuperscript{T} and other members of the family *Cyclobacteriaceae* were significantly lower. As shown in Fig. 1, strain KD51\textsuperscript{T} clustered together with *C. lianum* HY9\textsuperscript{T}. Consequently, *Cyclobacterium qasimii* KCTC 23011\textsuperscript{T}, *C. amurskyense* KCTC 12363\textsuperscript{T}, *C. lianum* HY9\textsuperscript{T} and *C. marinum* KCTC 2917\textsuperscript{T} were obtained from culture collections, grown under the same conditions as strain KD51\textsuperscript{T} and used as reference strains for a number of phenotypic tests.

Cell morphology and motility were observed by using transmission electron microscopy (JEM-2100HC; JEOL) and phase-contrast light microscopy (50i; Nikon), with cells from the early exponential phase grown on MA at 28 °C. Colony morphology was examined from cultures grown on MA for 7 days. Gliding motility was investigated as described by Bowman (2000). The Gram reaction was determined by using the bioMérieux Gram stain kit according to the manufacturer's instructions. Anaerobic growth was assessed on MA that was autoclaved and cooled to room temperature under a nitrogen atmosphere (99.999\% purity). Triplicate cultures were grown in 50 ml anaerobic serum bottles sealed with thick butyl rubber stoppers and aluminium caps, and incubated statically in the dark at 28 °C for 21 days. Growth in MB was tested at 4, 16, 20, 28, 30, 37 and 40 °C and at pH 3.0–10.0 (at 1 pH unit intervals). The pH of MB was adjusted prior to sterilization using the following buffers: citric acid/sodium citrate (for pH 3.0–6.0), Na\textsubscript{2}HPO\textsubscript{4}/citric acid (for pH 7.0–8.0) and lysine/NaOH (for pH 9.0–10.0). Verification of the pH values after autoclaving revealed only minor changes (Su et al., 2013). The NaCl concentration range and optimum for growth were determined in NaCl-free MB (containing 1\textsuperscript{1} distilled water: 5.0 g tryptone and 1.0 g yeast extract, pH 7.6–7.8), supplemented with 0–7\% (at 1\% intervals) and 9.0–13.0\% (at 2\% intervals) NaCl (w/v). Catalase and oxidase activities were assessed by the addition of 3\% hydrogen peroxide and 1\% potassium dichromate, respectively.

![Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship of *Cyclobacterium xiamenense* sp.nov. KD51\textsuperscript{T} with species of the genus *Cyclobacterium* and representative members of the family *Cyclobacteriaceae*. Bootstrap values >70\% (expressed as percentages of 1000 replications) are given at nodes. *Wandonia haliotis* Haldis-1\textsuperscript{T} was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.](image-url)
hydrogen peroxide to exponential-phase colonies and by using oxidase reagent (bioMérieux), respectively. Hydrolysis of starch, Tweens 20, 40, 60 and 80, casein, aesculin, gelatin, tyrosine, urea, chitin, and agar was tested using MA supplemented with 0.5 % (w/v) starch and 1 % (w/v) other substrates. DNA hydrolysis was assessed on toluidine Blue DNase agar (Dong & Cai, 2001). Cellulose hydrolysis was tested both by using cellulose overlay plates (1 % CM-cellulose) and by examining strips of filter paper in liquid bacterial cultures for dissolution (Smibert & Krieg, 1994).

The aforementioned tests were carried out on strain KD51T only, while the following tests were performed on strain KD51T and the four reference strains. Biochemical tests were carried out using API 20NE, API 20E and API ZYM strips (bioMérieux) according to the manufacturer’s instructions, except that the NaCl concentration in all tests was adjusted to 3.0 %. Susceptibility to antibiotics was tested on MA for 5 days by using filter-paper discs (OXOID) containing various antibiotics (La´nyi, 1987; Smibert & Krieg, 1994). All aforementioned tests were incubated at 28 °C. The novel isolate displayed the basic characteristics of members of the genus Cyclobacterium, e.g. pink-pigmented colonies and curved, ring-like or horseshoe-shaped cells (Fig. 2). Other phenotypic properties of strain KD51T are given in the species description and in Table 1.

Flexirubin-type pigments were absent from strain KD51T, as shown by the negative KOH test (Bernardet et al., 2002; Li et al., 2013). For pigment extraction, strain KD51T was inoculated into 1.5 ml MB, grown until exponential phase, and centrifuged at 6000 g at 4 °C for 10 min. The supernatant was discarded and the pellet was resuspended in 1.5 ml ethanol, mixed thoroughly on a vortex mixer and recentrifuged. The absorption spectrum of the supernatant was measured at 400–760 nm on a Lambda 35 spectrophotometer (Perkin Elmer) using ethanol as a blank (Shivaji et al., 2012). The broad peak with a maximum around 480 nm in the spectrum of the ethanol extract of strain KD51T was indicative of carotenoid pigments (Asker et al., 2007), as previously reported for C. qasimii M12-11B T (Shivaji et al., 2012).

For cellular fatty acid analysis, the fatty acids of strain KD51T and the four reference strains grown on MA at 28 °C for 5 days were saponified, methylated and extracted using the standard protocol of MIDI (Sherlock Microbial Identification System, version 6.0B). The fatty acids were analysed by GC (Agilent Technologies 6850) and identified by using the TSBA6 database of the Microbial Identification System (Sasser, 1990). The five strains had similar growth rates at 28 °C and the same physiological age at the time they were harvested. The dominant fatty acids (>20 %) of strain KD51T were iso-C15 : 0 (32.2 %) and summed feature 3 (comprising C16:1ω7c and/or C16:1ω6c; 22.2 %). Significant amounts (>7 %) of anteiso-C15 : 0 and summed feature 9 (comprising iso-C17:1ω9c and/or C16:0 10-methyl) were also present. The fatty acid compositions of the four reference strains were similar, with minor differences in the respective proportions of some fatty acids. Strain KD51T mainly differed from C. qasimii KCTC 23011T by the absence of iso-C11 : 0 and from C. amurskyense KCTC 12363T and C. lianum HY9T by the presence of C16:1ω5c and iso-C15 : 0 G, respectively (Table 2).

The G+C content of the DNA of strain KD51T was 41.7 mol%, as determined by thermal denaturation (Seidler & Mandel, 1971), a value within the range reported for members of the genus Cyclobacterium (Table 1). Respiratory quinones were also analysed at the CICC (China Center of Industry Culture Collection) on LDC Analytical HPLC (Thermo Separation Products) fitted with a reverse-phase column (Macherey-Nagel, 2 mm × 125 mm, 3 μm, RP18) using methanol/heptane (9:1, v/v) as the eluent. The only menaquione present in strain KD51T was menaquinone-7 (MK-7; 100 %), in line with other species of the genus Cyclobacterium (Jung et al., 2013; Shivaji et al., 2012).

On the basis of morphological, physiological and chemotaxonomic characteristics, as well as phylogenetic inference, strain KD51T represents a novel species of the genus Cyclobacterium, for which the name Cyclobacterium xiamenense sp. nov. is proposed. An emended description of the genus

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**Fig. 2.** Transmission electron micrographs of cells of strain KD51 T grown on MA for 5 days at 28 °C. Bars, 1 μm.
Table 1. Differential characteristics between strain KD51¹ and members of the genus Cyclobacterium

Strains: 1, KD51¹; 2, *C. qasimii* KCTC 23011T; 3, *C. amurskyense* KCTC 12363T; 4, *C. lianum* HY9T; 5, *C. marinum* KCTC 2917T. Data from this study except where otherwise indicated. All strains were ring-like and horseshoe-shaped, non-motile rods. All strains were positive for production of carotenoid pigments; catalase, alkaline and acid phosphatase, leucine and valine arylamidase and \(\beta\)-galactosidase activities; aesculin hydrolysis; utilization of citrate and \(\alpha\)-maltose. All strains were susceptible to ampicillin, carbenicillin, cefazolin, cephradine, chloramphenicol, ciprofloxacin, erythromycin, minomycin, norfloxacin, novobiocin, ofloxacin, oxacillin, rifampicin, vancomycin, doxycycline and cephalaxin. All strains were weakly positive for lipase C14, cysteine arylamidase and trypsin activities. All strains were negative for production of flexirubin pigments; hydrolysis of gelatin, urea, chitin, starch, casein, filter paper and Tween 80; nitrate reduction; \(\alpha\)-chymotrypsin and \(\alpha\)-fucosidase and tryptophan deaminase activities; production of H₂S and indole; utilization of citrate, potassium gluconate, capric acid and phenylacetic acid. All strains were resistant to clindamycin, metronidazole, neomycin, polymyxin B and streptomycin. +, Positive; −, negative; w, weakly positive; ND, no data available.

<table>
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<td>0–10†</td>
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<td>ND†</td>
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<td>−†</td>
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<td>+</td>
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<td>−†</td>
<td>W‡</td>
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<td>+</td>
<td>−*</td>
<td>ND†</td>
<td>ND‡</td>
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Members of the genus *Cyclobacterium* shown. TR, Traces (<0.5%); C16:0 10-methyl.

Table 2. Cellular fatty acid content of strain KD51<sup>T</sup> and other members of the genus *Cyclobacterium*

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<td>0.7</td>
<td>1.8</td>
<td>–</td>
<td>–</td>
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<tr>
<td>iso-C&lt;sub&gt;10&lt;/sub&gt;:0</td>
<td>–</td>
<td>1.4</td>
<td>–</td>
<td>–</td>
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<td>0.9</td>
<td>–</td>
<td>2.0</td>
<td>–</td>
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<td>–</td>
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<td>1.9</td>
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<td>TR</td>
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<td>1.3</td>
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<tr>
<td>C&lt;sub&gt;18&lt;/sub&gt;:1 9&lt;sup&gt;9&lt;/sup&gt;c</td>
<td>TR</td>
<td>1.1</td>
<td>2.0</td>
<td>0.9</td>
<td>–</td>
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*Summed features*<sup>+</sup>

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<td>4.3</td>
<td>5.6</td>
<td>10.8</td>
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*As indicated by Montero-Calasanz et al. (2013), summed features are groups of two or three fatty acids that are treated together for the purpose of evaluation in the MIDI system and include both peaks with discrete equivalent chain-lengths (ECLs) as well as those where the ECLs are not reported separately. Summed feature 3 was listed as C<sub>16</sub>:1 07c and/or C<sub>16</sub>:1 06c; summed feature 4 was listed as iso-C<sub>17</sub>:1 1 and/or anteiso-C<sub>17</sub>:1 4; summed feature 8 was listed as C<sub>18</sub>:1 07c and/or C<sub>18</sub>:1 06c; summed feature 9 was listed as iso-C<sub>17</sub>:1 09c and/or C<sub>16</sub>:0 10-methyl.*

*Data from Shivaji et al. (2012).† Data from Nedashkovskaya et al., (2005a).‡ Data from Ying et al. (2006).§ Data from Raj & Maloy (1990).*

**Cyclobacterium xiamenense** is also proposed on the basis of new data obtained in this study.

**Description of Cyclobacterium xiamenense** sp. nov.

*Cyclobacterium xiamenense* (xia.men.en’se. N.L. neutr. adj. *xiamenense* of Xiamen, a city in Fujian Province, China, where the type strain was isolated).

Cells are Gram-stain-negative, non-motile, strictly aerobic and heterotrophic, curved, horseshoe-shaped or ring-like, 1.5–2.0 μm in length and 0.4–0.6 μm in diameter. Colonies grown for 5 days on MA are pink, circular with entire margins, 2–3 mm in diameter, smooth, translucent and raised. Carotenoid pigments are produced but flexirubin pigments are not. Growth occurs at 4–40 °C (optimum, 28 °C), with 3–9% (w/v) NaCl (optimum, 3–5%) and at pH 6–10 (optimum, pH 7.5). Tweenes 20, 40 and 60 and aesculin are hydrolysed; starch and DNA are weakly hydrolysed; Tween 80, tyrosine, casein, urea, chitin, gelatin, agar, filter paper and CM-cellulose are not hydrolysed. Catalase-positive and oxidase-negative. In the API ZYM strip, alkaline and acid phosphatases, esterase C8, leucine arylamidase, valine arylamidase, β-galactosidase, β-glucosidase, N-acetyl-β-glucosaminidase and α-mannosidase activities are present; weak esterase C4, lipase C14, cystine arylamidase, naphthol-AS-B1-phosphohydrolase, β-galactosidase, β-glucosidase activities are present; trypsins, α- and β-chymotrypsins, β-glucuronidase and α-fucosidase activities are absent. All tests in the API 20 NE strip are negative, except β-glucosidase activity (aesculin hydrolysis) and maltose assimilation. All tests in the API 20 E strip are negative except arginine dihydrolase and gelatinase activities and utilization of citrate. Susceptible to (μg per disc unless otherwise indicated): ampicillin (10), carbenicillin (100), cefazolin (30), cephradin (30), chloramphenicol (30), ciprofloxacin (5), erythromycin (15), kanamycin (30), minomycin (30), norfloxacin (10), novobiocin (5), ofloxacin (5), oxacillin (1), penicillin G (10), piperacillin (100), polymyxin B (30 IU), rifampicin (5), vancomycin (30), ceftazidime (30), doxycycline (30), cephalexin (30) and sulphamethoxazole (25)/trimethoprim (5). Resistant to clindamycin (2), gentamicin (10), metronidazole (5), neomycin (30), streptomycin (10), sulphafurazole (300) and tetracycline (30). The major fatty acids (>20%) are...
iso-C\textsubscript{15:0} and summed feature 3 (comprising C\textsubscript{16:1\textit{\alpha}7c} and/or C\textsubscript{16:1\textit{\omega}6c}). The complete fatty acid composition is given in Table 2. The only respiratory quinone is MK-7.

The type strain is KD51\textsuperscript{T} (=CGMCC 1.12432\textsuperscript{T}=KCTC 32253\textsuperscript{T}), isolated from aggregates of Chlorella autotrophica in Xiamen, China. The DNA G+C content of the type strain is 41.7 mol%.

**Emended description of the genus *Cyclobacterium***

This emended description of the genus *Cyclobacterium* is based on the description of *Cyclobacterium caeniipelagi* published by Jung et al. (2013) and on this study.

Cells are Gram-stain-negative, strictly aerobic, curved, ring-like or horseshoe-shaped, non-flagellated and non-motile. Colonies on MA are pink- or orangish-red-pigmented and shiny. Carotenoid pigments are produced but flexirubin pigments are not. Catalase-positive. The major or only respiratory quinone is MK-7. The major cellular fatty acids (>10\%) are iso-C\textsubscript{15:0} and summed feature 3 (comprising C\textsubscript{16:1\textit{\alpha}7c} and/or C\textsubscript{16:1\textit{\omega}6c}). The DNA G+C content is 40.5–45.2 mol%.

**Acknowledgements**

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**References**


