Cyclobacterium amurskyense sp. nov., a novel marine bacterium isolated from sea water

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The taxonomic position of a novel marine, heterotrophic, aerobic, pigmented, non-motile bacterium isolated from sea water was determined. 16S rRNA gene sequence analysis revealed that strain KMM 6143^T is a member of the genus *Cyclobacterium*. The results of DNA–DNA hybridization experiments supported by phenotypic and chemotaxonomic data showed that the isolate represents a novel species of the genus *Cyclobacterium*, for which the name *Cyclobacterium amurskyense* sp. nov. is proposed. The type strain is KMM 6143^T (=KCTC 12363^T =LMG 23026^T).

The genus *Cyclobacterium*, comprising the single species *Cyclobacterium marinum* (Raj & Maloy, 1990), accommodates Gram-negative, strictly aerobic, heterotrophic, pink-pigmented and non-motile marine bacteria belonging to the phylum 'Bacteroidetes'. Strains of the genus can form ring-like cells. The genus *Cyclobacterium* forms a phylogenetic cluster with the recently described genera *Belliella* and *Aquiflexum* (Brettar *et al.*, 2004a, b).

During June 2000 we isolated an unknown bacterium, designated KMM 6143^T, from a sea-water sample collected in Amursky Bay, Gulf of Peter the Great. A polyphasic taxonomic study of the phenotypic, chemotaxonomic and genotypic characteristics and phylogenetic position of strain KMM 6143^T, cultured on marine agar 2216 (Difco), indicated that the isolate represents a novel species of the genus *Cyclobacterium*.

Genomic DNA extraction, PCR and sequencing of the 16S rRNA gene followed previously published procedures (Kim *et al.*, 1998). To establish the precise taxonomic position of strain KMM 6143^T, 1380 nucleotides of its 16S rRNA gene sequence was determined and 1365 bp of this sequence was used for comparative phylogenetic analysis. The sequence obtained was aligned with sequences of representative members of the family 'Flexibacteraceae' using PHYDIT version 3.2 (<http://plaza.snu.ac.kr/~jchun/phydit/>). 16S rRNA gene sequence analysis revealed that strain KMM 6143^T was a member of the family 'Flexibacteraceae' and formed a distinct subline within the genus *Cyclobacterium* (Fig. 1). The level of 16S rRNA gene sequence similarity between strains KMM 6143^T and *Cyclobacterium marinum* LMG 13164^T was 96.6% (47 nucleotide differences). Phylogenetic trees were inferred using suitable programs of the PHYLIP package (Felsenstein, 1993). Phylogenetic distances were calculated from the model of Jukes & Cantor (1969) and trees were constructed on the basis of the neighbour-joining (Saitou & Nei, 1987), least-squares (Fitch & Margoliash, 1967) and maximum-likelihood (Felsenstein, 1993) algorithms. Bootstrap analysis was performed with 1000 resampled datasets, using SEQBOOT and CONSENSE programs of the PHYLIP package.

Published online ahead of print on 1 July 2005 as DOI 10.1099/ij.s.0.63781-0.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Cyclobacterium amurskyense* KMM 6143^T is AY960985.

A micrograph of cells of *Cyclobacterium amurskyense* KMM 6143^T is available as supplementary material in IJSEM Online.

Fig. 1. Neighbour-joining evolutionary distance phylogenetic tree based on the 16S rRNA gene sequences of KMM 6143^T and representative members of related genera of the phylum 'Bacteroidetes'. The topology of the tree was not changed in the least-squares or maximum-likelihood trees (not shown). Numbers at nodes indicate bootstrap percentages; asterisks indicate branches. Bar, 0.1 substitutions per nucleotide position.

Genomic DNA was isolated by the method of Marmur (1961) and the G + C content of the DNA was determined by the thermal denaturation method (Marmur & Doty, 1962). The DNA G + C content of KMM 6143^T was 41.3 mol%.

To detect whole-cell fatty acids, strain KMM 6143^T and *C. marinum* LMG 13164^T (= KCTC 2917^T) were grown at 25 °C for 48 h on marine agar 2216 (Difco). The analysis of fatty acid methyl esters was carried out according to the standard protocol of the Microbial Identification System (Microbial ID Inc.). The predominant cellular fatty acids of KMM 6143^T and *C. marinum* LMG 13164^T were straight-chain unsaturated, branched-chain unsaturated and saturated, namely 15:0 iso (22.2 and 23 %, respectively), 15:0 anteiso (9.2 and 6.4 %), 15:1 iso (8.4 and 9.7 %), 17:1 iso ω 9c (4.3 and 6.3 %), 17:0 iso 3-OH (10.7 and 12.7 %) and summed feature 3 (24.3 and 23.4 %), comprising 16:1 ω 7c and/or 15:0 iso 2-OH.

Phenotypic features of the strain studied were tested as described previously (Nedashkovskaya *et al.*, 2003, 2004). Physiological and biochemical properties of KMM 6143^T and *C. marinum* LMG 13164^T were also determined using API 20E, API 20NE, API ZYM and API 50 CH (bioMérieux) and the Biolog GN2 Microplate system (Biolog Inc.) according to the manufacturers' instructions. Gliding motility was

determined as described by Bowman (2000). For determination of cell morphology, the samples were fixed in 2.5 % paraformaldehyde/glutaraldehyde mixture buffered with 0.1 M phosphate (pH 7.2) for 2 h and then fixed in 1 % osmium tetroxide in the same buffer for 1 h, dehydrated in graded ethanol and substituted with isoamyl acetate. Samples then underwent critical-point drying in CO₂. Finally the samples were sputtered with gold in a sputter coater (SC502; Polaron) and observed using a scanning electron microscope (SEM 515; Philips). Cells of KMM 6143^T were ring-like and horseshoe-shaped with an outer diameter ranging from 0.9 to 1.2 μ m and a width of 0.3–0.4 μ m (micrograph available as a supplementary figure in IJSEM Online).

The physiological, morphological and biochemical characteristics of the strains studied are given in the species description and Table 1. It should be noted that Raj & Maloy (1990) described *C. marinum* LMG 13164^T as a halophilic bacterium; however, the results of our study indicated that this strain can grow without Na⁺ ions or sea water. Also, the G + C content of the DNA for the type strain of *C. marinum* was 41.9 mol% (thermal denaturation method), according to data obtained in this work, in contrast to 33.7 mol% reported by Raj & Maloy (1990) for this bacterium. Moreover, we found that *C. marinum* LMG 13164^T demonstrated good growth at 42 °C. These results are different from those obtained by Raj & Maloy (1990). The similarities in the phenotypic characteristics, cellular fatty acid composition and G + C content of the DNA support the inclusion of strain KMM 6143^T in the genus *Cyclobacterium*. However, strain KMM 6143^T differed from *C. marinum* by an inability to grow at 42 °C, to hydrolyse Tween 40 and to utilize glucose 6-phosphate or mannitol. The presence of lipase (C14) and trypsin activities, acid production from L-arabinose, DL-xylose, starch and N-acetylglucosamine, and the ability to utilize D-gluconate also distinguish strain KMM 6143^T from *C. marinum*. Moreover, susceptibility to benzylpenicillin and kanamycin and resistance to streptomycin and tetracycline clearly separate strain KMM 6143^T from *C. marinum* (Table 1).

The above-mentioned phenotypic features (Table 1) in association with molecular differences allow the differentiation of strain KMM 6143^T from *Cyclobacterium marinum*. Thus, we propose that strain KMM 6143^T should be placed in the genus *Cyclobacterium*, as the type strain of *Cyclobacterium amurskyense* sp. nov.

Description of *Cyclobacterium amurskyense* sp. nov.

Cyclobacterium amurskyense (a.mur.sky.en'se. N.L. neut. adj. *amurskyense* pertaining to Amursky Bay, in which the type strain was isolated).

Cells are Gram-negative, strictly aerobic with respiratory metabolism, chemo-organotrophic, non-motile, asporogenic, ring-like and horseshoe-shaped with an outer diameter

Table 1. Phenotypic characteristics of *Cyclobacterium amurskyense* sp. nov. KMM 6143^T

Both strains were positive for following: oxidase, catalase, β -galactosidase, alkaline and acid phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, naphthol-AS-BI-phosphohydrolase, α - and β -galactosidase, α - and β -glucosidase, *N*-acetyl- β -glucosaminidase and α -mannosidase activities; growth at 0–10% NaCl and at 4–40 °C; hydrolysis of aesculin; acid formation from D-cellobiose, L-fucose, D-galactose, D-lactose, L-raffinose, D-melibiose, L-rhamnose, D-trehalose, D-maltose and D-sucrose; susceptibility to ampicillin, carbenicillin, lincomycin and oleandomycin; and utilization of glucose, D-mannose, *N*-acetyl-D-glucosamine, D-fructose, methyl α -D-mannoside, methyl α -D-glucoside, amygdalin, arbutin, salicin, inulin, melezitose, gentiobiose, D-turanose, lyxose, tagatose, D-fucose, L-fucose, ribose, sorbose, D-xylose and methyl β -D-xyloside. Both strains were negative for following: α -chymotrypsin, β -glucuronidase, α -fucosidase, arginine dihydrolase, lysine and ornithine decarboxylase activities; gliding motility; Na⁺ requirement for growth; requirement for organic growth factors; nitrate reduction; flexirubin pigments; H₂S, indole and acetoin production; degradation of agar, casein, gelatin, DNA, starch, cellulose (CM-cellulose, filter paper), chitin, urea, and Tweens 20 and 80; acid production from D-glucose, L-sorbose, adonitol, dulcitol, glycerol, *myo*-inositol, mannitol, malate, fumarate and citrate; utilization of glycerol, *i*-erythritol, adonitol, dulcitol, *myo*-inositol, D-sorbitol, glycogen, xylitol, D-arabitol, L-arabitol, gentiobiose, 2-ketogluconate, 5-ketogluconate, caprate, adipate, malate, citrate and phenylacetate; and susceptibility to gentamicin, neomycin and polymyxin B.

Characteristic	<i>C. amurskyense</i> KMM 6143 ^T	<i>C. marinum</i> LMG 13164 ^T
Lipase (C14), trypsin activities	+	–
Degradation of Tween 40	–	+
Growth at 42 °C	–	+
Acid from L-arabinose, DL-xylose, starch, <i>N</i> -acetyl-D-glucosamine	+	–
Utilization of:		
Glucose 6-phosphate, mannitol	–	+
D-Gluconate	+	–
Susceptibility to:		
Benzylpenicillin, kanamycin	+	–
Streptomycin, tetracycline	–	+
DNA G + C content DNA (mol%)	41.3	41.9

of 0.9–1.2 μ m and cell width of 0.3–0.4 μ m. Oxidase-, catalase-, β -galactosidase- and alkaline phosphatase-positive. Colonies are circular, low-convex, shiny with entire edges, 1–3 mm in diameter on marine agar 2216. Produces non-diffusible pink pigments. Grows at 0–10% NaCl. Flexirubin pigments are absent. Growth occurs at 4–40 °C. Degrades aesculin. Does not hydrolyse agar, casein, gelatin, starch, cellulose (CM-cellulose and filter paper), chitin, DNA, urea or Tweens 20, 40 or 80. Forms acid from L-arabinose, starch, D-cellobiose, L-fucose, D-galactose, D-lactose, D-maltose, D-melibiose, D-sucrose, L-raffinose, L-rhamnose, D-trehalose, DL-xylose and *N*-acetylglucosamine, but not from D-glucose, L-sorbose, adonitol, dulcitol, glycerol, inositol, mannitol, malate, fumarate or citrate. According to the Biolog GN2 Microplate test system, utilizes dextrin, *N*-acetyl-D-glucosamine, cellobiose, D-fructose, D-galactose, gentiobiose, α -D-glucose, α -lactose, α -D-lactose, lactulose, maltose, D-mannose, D-melibiose, methyl β -D-glucoside, psicose, D-raffinose, L-rhamnose, sucrose, D-trehalose, turanose, methylpyruvate, D-galacturonic acid, D-glucuronic acid, α -DL-glycerol phosphate and glucose 1-phosphate; can oxidize glycogen, *N*-acetyl-D-galactosamine, L-arabinose, L-fucose, D-mannitol, monomethyl succinate,

acetic acid, α -ketobutyric acid, α -ketoglutaric acid, DL-lactic acid, glucuronamide, L-glutamic acid, L-serine, L-threonine and glycerol; does not utilize α -cyclodextrin, Tweens 40 and 80, adonitol, D-arabitol, *i*-erythritol, *myo*-inositol, D-sorbitol, xylitol, *cis*-aconitic acid, citric acid, formic acid, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, α -hydroxybutyric acid, β -hydroxybutyric acid, γ -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α -ketovaleric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, D-serine, DL-carnitine, γ -aminobutyric acid, uronic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol or glucose 6-phosphate. Nitrate is not reduced. H₂S, indole and acetoin (Voges–Proskauer reaction) are not produced. Susceptible to ampicillin, benzylpenicillin, carbenicillin, kanamycin, oleandomycin and lincomycin. Resistant to neomycin, streptomycin, gentamicin, polymyxin B and tetracycline. The predominant cellular fatty acids are

straight-chain unsaturated, branched-chain unsaturated and saturated fatty acids, namely 15:0 iso (22.2%), 15:0 anteiso (9.2%), 15:1 iso (8.4%), 17:1 iso ω 9c (4.3%), 17:0 iso 3-OH (10.7%) and summed feature 3 (24.3%), comprising 16:1 ω 7c and/or 15:0 iso 2-OH fatty acids. The G+C content of the DNA is 41.3 mol%.

The type strain is KMM 6143^T (=KCTC 12363^T=LMG 23026^T), isolated from sea water, collected in Amursky Bay, Gulf of Peter the Great, East Sea (also known as the Sea of Japan).

Acknowledgements

This research was supported by grants of the Federal Agency for Sciences and Innovations of the Ministry for Education and Sciences of the Russian Federation no. 2-2.16, the Russian Foundation for Basic Research no. 05-04-48211 and the Program of Fundamental Investigations of the Presidium of the Russian Academy of Sciences 'Molecular and Cell Biology'. This work was also supported by the Eco-Technopia-21 Project. K.H.L. and K.S.B. are grateful for the support from the KRIBB Research Initiative Program.

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