Gracilimonas tropica gen. nov., sp. nov., isolated from a *Synechococcus* culture

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An irregular, long, rod-shaped marine bacterium, designated CL-CB462^T, was isolated from a *Synechococcus* culture, which was established from surface water from the tropical Pacific Ocean. The physiological and biochemical features, fatty acid profile and phylogenetic position based on 16S rRNA gene sequences were investigated for the novel strain. Phylogenetic analysis of the 16S rRNA gene sequence showed that the closest relatives of strain CL-CB462^T were *Balneola vulgaris* and *Balneola alkaliphila*. Strain CL-CB462^T formed a robust clade with members of the genus *Balneola* in all phylogenetic trees constructed by three different methods. However, the 16S rRNA gene sequence similarity was very low (91.3–91.5 % similarity) and phenotypic and physiological features could clearly differentiate strain CL-CB462^T from the genus *Balneola*. Cells of the novel strain were non-motile and spore-forming. The strain was able to grow at 1–20 % (w/v) (optimum of 3–6 %) sea salt concentration, at temperatures of 20–40 °C and between pH 6 and 10. The fatty acids were dominated by 15:0 iso (41.2 %) and 17:1 ω 9c iso (21.4 %). The DNA G + C content was 42.7 mol%. Based on polyphasic evidence, strain CL-CB462^T was considered to represent a new genus. The name *Gracilimonas tropica* gen. nov., sp. nov. is proposed for the type strain of the type species (CL-CB462^T=KCCM 90063^T=DSM 19535^T).

According to Garrity *et al.* (2004), the family *Crenotrichaceae* within the *Bacteroidetes* consisted of five genera with validly published names. However, Stoecker *et al.* (2006) have presented molecular evidence that the genus *Crenothrix*, the type genus of the family, belongs to the order *Methylococcales* of the class *Gammaproteobacteria*. Consequently, other genera originally assigned to the family *Crenotrichaceae* within the order *Sphingobacteriales* are currently considered *Sphingobacteriales* genera *incertae sedis*, including the genera *Rhodothermus* (Alfredsson *et al.*, 1988) and *Salinibacter* (Antón *et al.*, 2002). Among these genera, two species have been isolated from extreme environments: *Rhodothermus marinus* was isolated from submarine hot springs in Iceland (Alfredsson *et al.*, 1988) and *Salinibacter ruber* was isolated from hypersaline crystallizer ponds in Spain (Antón *et al.*, 2002).

Abbreviation: ASW, artificial seawater.

The GenBank accession number for the 16S rRNA gene sequence of strain CL-CB462^T is EF988655.

Phylogenetic trees constructed with maximum-likelihood and maximum-parsimony methods and a comparison of fatty acid compositions for strain CL-CB462^T and related species are available with the online version of this paper.

Thus, they show thermophilic and extreme halophilic characteristics, respectively. Recently, the genus *Balneola*, was isolated from surface waters in the coastal Mediterranean Sea and it is not extremophilic (Urios *et al.*, 2006, 2008). In this study, strain CL-CB462^T, which is affiliated with *Balneola* species, was isolated from a *Synechococcus* culture that had been established from seawater from the tropical Pacific Ocean and a polyphasic taxonomic analysis of the strain was performed.

The *Synechococcus* culture was maintained by transferring small quantities of culture to f/2 medium (Guillard & Ryther, 1962) supplemented with ammonium chloride (final concentration 100 μ mol l⁻¹). The culture was incubated at 25 °C under a light–dark regime (14:10) at approximately 20 μ E m⁻² s⁻¹. To isolate other bacteria that had been maintained in the *Synechococcus* culture, 30 μ l culture at the exponential growth phase was spread on a marine agar 2216 (MA, Difco) plate and incubated at 25 °C for 1 week. Strain CL-CB462^T was isolated and subsequently purified with four rounds of subculture on MA at 30 °C. The strain was maintained both on MA at 4 °C and in marine broth 2216 (MB, Difco) supplemented with 30 % (v/v) glycerol at –80 °C.

For 16S rRNA gene amplification by PCR, DNA was extracted from a single colony by a boiling method (Englen & Kelley, 2000). The crude extracts served as the DNA template for PCR reactions with *Taq* DNA polymerase (Bioneer) and primers 27F and 1492R (Lane, 1991). PCR products were purified using the AccuPrep PCR Purification kit (Bioneer). Sequencing of the amplified 16S rRNA gene fragment was performed with an Applied Biosystems automatic sequencer (ABI 3730XL) at Solgent Co., Daejeon, Korea. The almost-complete 16S rRNA gene sequence of strain CL-CB462^T (1392 bp) was obtained and compared with 16S rRNA gene sequences available in GenBank using BLASTN (Altschul *et al.*, 1990). The sequence was aligned with those of type strains of genera that are phylogenetically related to strain CL-CB462^T using the ARB program (Ludwig *et al.*, 2004, available at www.arb-home.de) and the Silva database release 94 (Pruesse *et al.*, 2007, available at www.arb-silva.de). The alignment was checked manually using the ARB_EDIT sequence editor. Phylogenetic trees were obtained by neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods using the MEGA version 4 program (Tamura *et al.*, 2007). An evolutionary distance matrix for the neighbour-joining method was generated according to the model of Jukes & Cantor (1969). The robustness of the tree topologies was assessed by bootstrap analyses based on 1000 replications for neighbour-joining and maximum-parsimony methods. In addition, a maximum-likelihood tree was constructed using PAUP* version 4.0 (Swofford, 1998). Likelihood parameters were estimated by using the hierarchical ratio tests in MODELTEST version 3.04 (Posada & Crandall, 1998) and bootstrap analysis based on 100 replications was conducted.

Morphological and physiological analyses were also performed. Gram-staining was conducted as described by Smibert & Krieg (1994). Cell morphology and motility were examined by phase-contrast microscopy and transmission electron microscopy (EX2; JEOL) with cells grown for 3 days at 35 °C in MB and on MA. Anaerobic growth was checked on MA using the GasPak anaerobic system (BBL). Acid production from D-glucose was also tested on ZOF medium (Lemos *et al.*, 1985) supplemented with 1 % D-glucose. Endospores were detected by malachite green staining (Smibert & Krieg, 1994). The presence of flexirubin-type pigments was investigated by using the bathochromatic shift test with a 20 % (w/v) KOH solution (Bernardet *et al.*, 2002). Poly- β -hydroxybutyrate granules were examined by epifluorescence microscopy (BX60; Olympus) after Nile blue A staining (Ostle & Holt, 1982).

The temperature range for growth was determined on the basis of colony formation on MA plates that were incubated at 5–45 °C. The pH range for growth in MB was determined by changes in OD₆₀₀ with time for the pH range 5 to 11 by adjusting the final pH with NaOH and HCl solutions. Tolerance of strain CL-CB462^T to NaCl and sea salts was determined using synthetic ZoBell broth (1⁻¹ distilled water: 5 g Bacto peptone, 1 g yeast extract, 0.1 g

ferric citrate) with various concentrations [0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 18, 20, 23, 25 % (w/v)] of NaCl and sea salts (Sigma), respectively. Catalase and oxidase activities were determined according to the protocols described by Smibert & Krieg (1994), and gelatinase, amylase and degradation of Tweens 40 and 80 were examined as described by Hansen & Sørheim (1991). In addition, nitrate reduction, indole production, arginine dihydrolase, urease, gelatinase and β -galactosidase activities, acid production from glucose and hydrolysis of aesculin were tested using an API 20NE kit (bioMérieux) according to the manufacturer's instructions except that cell suspensions were prepared using artificial seawater (ASW) (1⁻¹ distilled water: 24 g NaCl, 5.1 g MgCl₂, 4 g Na₂SO₄, 1.1 g CaCl₂, 0.7 g KCl, 0.2 g NaHCO₃, 0.1 g KBr, 0.027 g H₃BO₃, 0.024 g SrCl₂, 0.003 g NaF; Lyman & Fleming, 1940) as a suspension medium. Other enzyme activities were also assayed by using the API ZYM kit (bioMérieux) with culture suspension in ASW. Carbon utilization was tested on a basal medium supplemented with yeast extract (1⁻¹ distilled water: 23.6 g NaCl, 0.64 g KCl, 4.53 g MgCl₂·6H₂O, 5.94 g MgSO₄·7H₂O, 1.3 g CaCl₂·2H₂O, 0.2 g NaNO₃, 0.2 g NH₄Cl, 0.05 g yeast extract; Bruns *et al.*, 2001) containing 0.4 % of the carbon source. Incubation was prolonged for 2 weeks.

The fatty acid methyl esters in whole cells were analysed by GC according to the instructions of the Microbial Identification System (MIDI) at the Korean Culture Center of Microorganisms in Seoul, Korea. The DNA G+C content was determined by HPLC analysis of deoxyribonucleosides as described by Mesbah *et al.* (1989) after DNA purification by the method of Marmur (1961).

The 16S rRNA gene sequence of strain CL-CB462^T showed 91.5 % and 91.3 % sequence similarities to *Balneola vulgaris* and *Balneola alkaliphila*, respectively, and formed a robust clade with the *Balneola* species in three phylogenetic trees that were constructed with different methods (Fig. 1 and Supplementary Figs S1 and S2, available in IJSEM Online). However, strain CL-CB462^T did not form a clade with *R. marinus* and *S. ruber* and the sequence similarities with these species were much lower, ranging from 79.8 to 81.4 %. The low 16S rRNA gene sequence similarity between strain CL-CB462^T and *B. vulgaris* suggests that they could be recognized as representing different genera. Interestingly, the two extremophiles, *R. marinus* and *S. ruber*, and the three non-extremophiles, including strain CL-CB462^T, formed two distinct clades, and were not clustered together in the phylogenetic trees (Fig. 1). The habitats and their physiology are also greatly different between the two groups (Table 1).

Cells of strain CL-CB462^T in exponential growth phase were long irregular rods of 0.2–0.3 μ m in width and 6.5–17.7 μ m in length (Fig. 2). Spore-like dark and bright coccoid cells were also observed in the culture (Fig. 2) and densely packed endospores were detected inside the cells by malachite green staining (data not shown). Cells were non-

Fig. 1. Neighbour-joining tree showing the relationships between strain CL-CB462^T and other related species belonging to the phylum *Bacteroidetes*. Bootstrap values above 60 %, based on 1000 resamplings, are shown at the branching points. Solid circles indicate that the corresponding nodes are also recovered in maximum-parsimony and maximum-likelihood trees. *Methylococcus capsulatus* ACM 1292^T (X72770) was used as the outgroup (not shown). Bar, 0.02 nucleotide substitutions per site.

motile and did not possess poly- β -hydroxybutyrate granules inside the cells. Colonies on MA were circular, entire, convex and orange. In anaerobic conditions, strain CL-CB462^T grew very slowly and acid was produced from D-glucose. Flexirubin pigments were not detected.

Growth of strain CL-CB462^T was observed at temperatures of between 20 and 40 °C, with optimum growth at 35 °C. Growth occurred over the range pH 6–10 (Table 1). Strain

CL-CB462^T could grow with sea salt concentrations of 1–20 %, with optimum growth at 3–6 % (Table 1). The novel strain could not grow in media with NaCl as the sole salt. The strain could degrade starch, gelatin and Tweens 40 and 80. The results of other biochemical and physiological tests are given in Table 1 and the species description. The dominant fatty acids for strain CL-CB462^T were 15:0 iso (41.2 %), 17:1 ω 9c iso (21.4 %), 15:0 anteiso (8.4 %) and 15:0 iso 2-OH/16:1 ω 7c (6.8 %) (see Supplementary Table S1 in IJSEM Online).

Strain CL-CB462^T could be clearly differentiated from members of the genera *Salinibacter* and *Rhodothermus* by the differences in temperature and salinity ranges and the DNA G+C contents (Table 1). Further, the fatty acid profiles were conspicuously different (Supplementary Table S1). The phenotypic and physiological features of strain CL-CB462^T were also very different from those of the genus *Balneola*, which is the phylogenetically closest genus (Table 1). The novel strain forms long, irregular rods but the two *Balneola* species form relatively short rods. Other striking contrasts were also observed, namely spore formation, facultative anaerobic growth, sea salt requirement and growth at high salinity (up to 20 %). The fatty acid profile of strain CL-CB462^T was different from that of *B. vulgaris*, mainly in the proportions of 17:1 ω 9c iso, 15:1 ω 6c, 15:0 anteiso and 17:1 ω 9c anteiso (Supplementary Table S1). Enzyme activities, such as esterase, lipase, trypsin, naphthol-AS-BI-phosphohydrolase and N-acetyl- β -glucosaminidase, and carbon utilization patterns were also different between strain CL-CB462^T and species of the genus *Balneola* (Table 1). Thus,

Fig. 2. Transmission electron micrograph of negatively stained cells of strain CL-CB462^T. Bar, 5 μ m.

Table 1. Selected characteristics that differentiate strain CL-CB462^T from phylogenetically related strains

Strains: 1, CL-CB462^T (data from this study); 2, *B. vulgaris* 13IX/A01/164^T (Urios *et al.*, 2006); 3, *B. alkaliphila* CM41_14b^T (Urios *et al.*, 2008); 4, *S. ruber* M31^T (Antón *et al.*, 2002); 5, *R. marinus* (Alfredsson *et al.*, 1988; Sako *et al.*, 1996). +, Positive; –, negative; w, weakly positive; ND, no data available; v, variable.

Characteristic	1	2	3	4	5
Isolation source	Seawater	Seawater	Seawater	Crystallizer ponds of salterns	Submarine hot springs, hydrothermal vent
Colony colour	Orange	Orange	Pale yellow	Red	Reddish
Cell characteristics					
Shape	Long irregular rods	Rods	Rods	Straight or slightly curved rods	Rods
Dimensions (µm)	6.5–17.7 × 0.2–0.3	2.0–3.0 × 0.2	2.6 ± 0.1 × 0.7 ± 0.05	2.0–6.0 × 0.4	2–10 × 0.5–1.0
Motility	–	+	–	+	–
Spore-formation	+	–	–	–	–
Temperature (°C)					
Range	20–40	10–40	15–37	27–52	50–85
Optimum	35	30	25	37–47	65–80
Salinity (g l ^{–1})					
Range	10–200	0–50	10–80	150–300	10–60
Optimum	30–60	20	30	200	20–30
Growth on NaCl medium	–	+	+	ND	+
pH for growth:					
Range	6–10	5.0–10.0	6–10	6.0–8.5	5.5–9.0
Optimum	7–8	8	8–9	6.5–8.0	7
Anaerobic growth	w	–	–	–	–
Enzyme activities					
Cystine arylamidase	+	w	–	ND	ND
Esterase (C4)	+	–	–	ND	ND
Esterase lipase (C8)	+	w	–	ND	ND
Lipase (C14)	w	–	–	ND	ND
Oxidase	+	–	–	+	v
Trypsin	–	+	+	ND	ND
Naphthol-AS-BI-phosphohydrolase	+	–	–	ND	ND
N-Acetyl-β-glucosaminidase	+	–	–	ND	ND
Utilization of:					
Acetate	–	+	+	–	+
Aspartate	+	–	–	ND	+
Citrate	–	–	+	ND	–
Fructose	+	+	+	–	ND
Galactose	–	–	–	–	+
Glucose	+	+	w	–	+
Lactose	–	w	–	–	+
Mannitol	–	w	–	–	ND
Sucrose	–	–	–	–	+
DNA G + C content (mol%)	42.7	41.8 ± 1.1	39 ± 1	66.3–67.7	64.4–66.6

polyphasic evidence suggests that strain CL-CB462^T should be classified as a novel genus and species, for which the name *Gracilimonas tropica* gen. nov., sp. nov. is proposed.

Description of *Gracilimonas* gen. nov.

Gracilimonas [Gra.ci.li.mo'nas. L. adj. *gracilis* slim, slender, thin; L. fem. n. *monas* a unit, monad; N.L. fem. n.

Gracilimonas thin unit (bacterium), relating to its shape].

Cells are Gram-negative and irregular rods. Growth is heterotrophic. Aerobic and facultatively anaerobic. Positive for endospore staining. Catalase- and oxidase-positive. Absence of poly-β-hydroxybutyrate granules. Dominant fatty acids are 15:0 iso and 17:1ω9c iso. The type species is *Gracilimonas tropica*.

Description of *Gracilimonas tropica* sp. nov.

Gracilimonas tropica [tro'pi.ca. L. fem. adj. *tropica* tropical, of or pertaining to the tropic(s), relating to its isolation from a tropical ocean].

Exhibits the following properties in addition to those given in the genus description. Cells are long and thin rods, approximately 0.2–0.3 µm wide and 6.5–17.7 µm long. Cells are non-motile. On MA, colonies are circular, entire, convex, opaque and orange. Does not produce flexirubin pigments. Grows within the temperature range of 20–40 °C (optimum 35 °C), at pH 6–10 and at sea salt concentrations of 1–20 % (w/v). No growth without sea salts in the medium. Hydrolyses starch, gelatin and Tweens 40 and 80. According to API 20NE tests, positive for gelatinase activity but negative for nitrate reductase, arginine dihydrolase and urease activities, indole production, acid production from glucose and aesculin hydrolysis. Acid is produced from glucose in ZOF medium. According to API ZYM tests, alkaline phosphatase, esterase (C4), esterase lipase (C8), weak lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl-β-glucosaminidase activities are present, whereas α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase and α-fucosidase activities are absent. Growth occurs on fructose, glucose and aspartate but not on acetate, citrate, galactose, mannitol, inositol, lactose, succinate or sucrose.

The type strain, CL-CB462^T (=KCCM 90063^T =DSM 19535^T), was isolated from a *Synechococcus* culture established from surface water of the tropical Pacific Ocean. The DNA G + C content of the type strain is 42.7 mol%.

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