Shimia marina gen. nov., sp. nov., a novel bacterium of the *Roseobacter* clade isolated from biofilm in a coastal fish farm

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A rod-shaped marine bacterium, CL-TA03^T, isolated from a biofilm in a coastal fish farm in Tongyeong, Korea, was characterized for physiological and biochemical features, fatty acid profile and phylogenetic position based on 16S rRNA gene sequences. Analysis of the 16S rRNA gene sequence revealed a clear affiliation with the family *Rhodobacteraceae*. Phylogenetic analysis of the 16S rRNA gene sequence showed that the closest relatives of CL-TA03^T were *Thalassobius gelatinovorus* and *Thalassobius mediterraneus* (95·6 % similarity). The sequence similarities between CL-TA03^T and other type species of the *Roseobacter* lineage ranged from 92·4 to 95·4 %. Strain CL-TA03^T is motile and grows on marine agar as colourless or beige colonies. The strain is able to grow optimally in the range of 3–5 % sea salts. It grows within a temperature range of 15–35 °C and at pH 6–10. The fatty acids are dominated by 18 : 1ω7*c* (64·1 %) and 11-methyl 18 : 1ω7*c* (10·6 %). The DNA G+C content is 57·2 mol%. According to physiological data, fatty acid composition and phylogenetic analysis of the 16S rRNA gene sequence, CL-TA03^T is considered to represent a new genus in the family *Rhodobacteraceae* and the name *Shimia marina* gen. nov., sp. nov. is proposed. The type strain of *Shimia marina* is CL-TA03^T (=KCCM 42117^T=JCM 13038^T).

Strains in the *Roseobacter* clade, classified within the family *Rhodobacteraceae*, have been isolated mainly from various marine environments (seawater, sediment, hypersaline microbial mats and coastal biofilms) and from marine algae, invertebrates and vertebrates, and the *Roseobacter* clade is known to be one of the most abundant groups in marine environments (Giovannoni & Rappé, 2000; Selje *et al.*, 2004; Buchan *et al.*, 2005). The strains show diverse physiological and morphological features (e.g. phototrophy, aerobic sulfite oxidation, organic sulfur compound degradation, methylotrophy, gas vacuoles, poly-β-hydroxybutyrate granules, rosette formation) (Arahal *et al.*, 2005; Buchan *et al.*, 2005). Furthermore, phylogenetic analyses of marine *Roseobacter* sequences have shown that for two-thirds (68 %) of the *Roseobacter* diversity identified so far, it is not yet possible to access relevant physiological information through studies of cultured organisms (Buchan *et al.*, 2005). In early stages of biofilm establishment, clones affiliated with the *Roseobacter* clade accounted for 85 % of the total sequenced clones, suggesting that organisms belonging to the *Roseobacter* clade are ubiquitous and rapid colonizers of surfaces in coastal environments (Dang & Lovell, 2000).

In this study, a strain affiliated with the *Rhodobacteraceae*, CL-TA03^T, was isolated in October 2002 from a biofilm formed on an acrylic slide submerged for 1 month in surface water on a coastal fish farm in Tongyeong, Korea. The scraped biofilm was suspended in seawater that had been passed through a 0·2 μm filter and autoclaved. The suspension was spread on a marine agar 2216 (MA; Difco) plate and incubated at 25 °C for 1 week. Strain CL-TA03^T was isolated and subsequently purified four times 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.

The 16S rRNA gene was amplified from a single colony by PCR with *Taq* DNA polymerase (Bioneer) using primers 27F and 1492R (Lane, 1991). The PCR product was purified using the AccuPrep PCR Purification kit (Bioneer) and cloned using the pCR2.1 TOPO TA Cloning kit (Invitrogen). Sequencing of the 16S rRNA gene was performed with an Applied Biosystems automatic sequencer (ABI 3730xl) at Macrogen Corp., Seoul, Korea. An almost complete 16S rRNA gene sequence of strain CL-TA03^T (1382 bp) was obtained. The sequence of strain CL-TA03^T was compared with 16S rRNA gene sequences available in GenBank using

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CL-TA03^T is AY962292.

BLASTN searches (Altschul *et al.*, 1990). The sequence of strain CL-TA03^T was aligned manually with those of type strains of species belonging to genera phylogenetically related to CL-TA03^T and with type species of other genera in the *Roseobacter* clade within the family *Rhodobacteraceae* obtained from GenBank and from the Ribosomal Database Project (Cole *et al.*, 2003) databases using known 16S rRNA secondary structure information. Phylogenetic trees were obtained by neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods. An evolutionary distance matrix for the neighbour-joining method was generated according to the model of Jukes & Cantor (1969). The robustness of tree topologies was assessed by bootstrap analyses based on 1000 replications for neighbour-joining and maximum-parsimony methods. Alignment analysis was carried out using the JPHYDIT program (Jeon *et al.*, 2005) and phylogenetic analyses were carried out using MEGA3 (Kumar *et al.*, 2004). The 16S rRNA gene sequence of CL-TA03^T showed 95·6 % sequence similarity to *Thalassobius gelatinovorus* IAM 12617^T and *Thalassobius mediterraneus* XSM19^T, 95·4 % to *Ruegeria atlantica* IAM 14463^T, 95·1 % to *Silicibacter lacuscaerulensis* ITI-1157^T and 92·4–94·8 % to other type species of the *Roseobacter* lineage. In the phylogenetic trees, however, strain CL-TA03^T did not form a robust clade with any species in the *Roseobacter* lineage (Fig. 1). The DNA G+C content was determined by HPLC analysis of deoxyribonucleosides as described by Mesbah *et al.* (1989) after DNA purification using the method of Marmur (1961) and was found to be 57·2 mol%.

Morphological and physiological analyses were performed. Gram staining was performed as described by Smibert & Krieg (1994). Cell morphology and motility were examined by phase-contrast microscopy and transmission electron microscopy (JEOL EX2) with cells grown for 1 day at 30 °C in MB and on MA. Anaerobic growth was checked on MA using the GasPak anaerobic system (BBL). Cells were rods of 0·3–0·6 μm wide and 0·8–3·6 μm long in exponential growth phase (Fig. 2). Cells occasionally formed small chains but not star-shaped aggregates. Cells were motile by several monopolar flagella (Fig. 2). Poly-β-hydroxybutyrate granules were not identified by transmission electron microscopy and Nile blue A staining (Ostle & Holt, 1982). Colonies on MA were circular, entire, convex, opaque and colourless or beige. After incubation for 1 week, colonies were approximately 2 mm in diameter. Bacteriochlorophyll *a* production was determined in 90 % acetone extracts from cells cultured in the dark and examined using a spectrophotometer (Ultraspec 2000; Pharmacia Biotech). Bacteriochlorophyll *a* was not detected in CL-TA03^T.

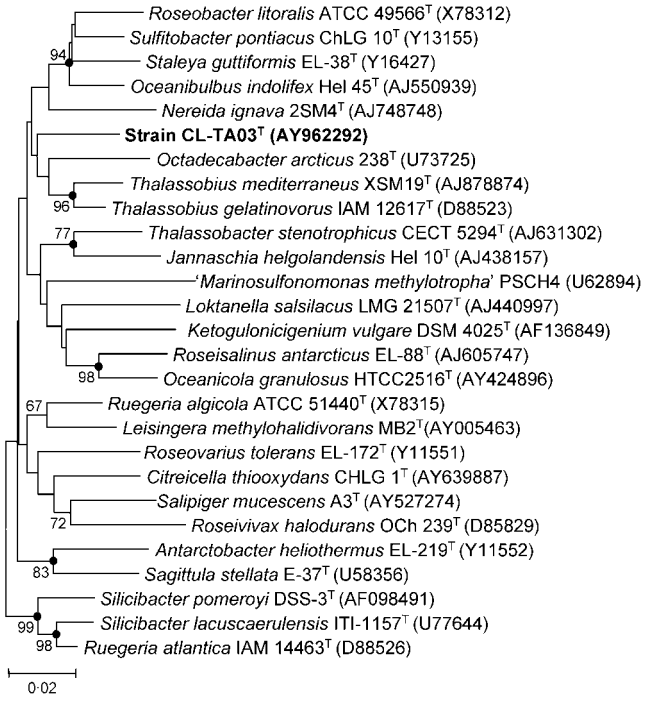

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the relationship between strain CL-TA03^T and other related species belonging to the *Roseobacter* clade of the family *Rhodobacteraceae*. Only bootstrap values above 60 % are shown (1000 resamplings) at the branching points. Solid circles indicate that the corresponding nodes were also recovered in the maximum-parsimony tree. *Idiomarina zobellii* KMM 231^T (GenBank accession no. AF052741) was used as an outgroup (not shown). Bar, 0·02 nucleotide substitutions per site.

The temperature range for growth was determined on the basis of colony formation on MA plates incubated at 5–45 °C. The pH range (pH 5–11) for growth was determined by changes in OD_{600} over time in MB. The final pH was adjusted using NaOH and HCl solutions. Tolerance of

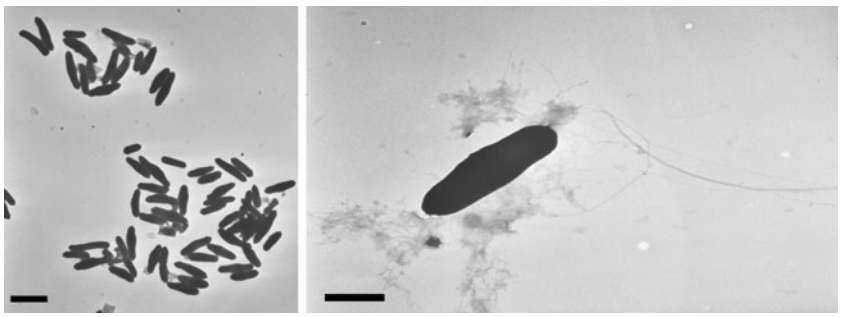

Fig. 2. Transmission electron micrographs of negatively stained cells of strain CL-TA03^T. Cells were grown at 30 °C on MA for 1 day. Bars, 2 μm (left) and 500 nm (right).

Table 1. Selected characteristics that differentiate strain CL-TA03^T from its closest phylogenetic relatives

Strains: 1, strain CL-TA03^T; 2, *T. gelatinovorus* IAM 12617^T; 3, *T. mediterraneus* CECT 5383^T. Data for reference strains were taken from Arahal *et al.* (2005) unless indicated otherwise. +, Positive; −, negative; W, weakly positive; ND, not detected or less than 1 %; ECL, equivalent chain length. All strains are Gram-negative, strictly aerobic and oxidase- and catalase-positive.

Characteristic	1	2	3
Motility	+	+*	−
Poly-β-hydroxybutyrate accumulation	−	+	+
Rosette formation	−	+†	−
Temperature range for growth (°C)	15–35	4–40	13–37
Growth on 1 % sea salts	−	+	−
DNA G+C content (mol%)	57·2	59	57
Enzyme activities			
Amylase	W	−†	−
Gelatinase	+	+†	−
Nitrate reductase	+	+†	−
API ZYM tests			
Alkaline phosphatase	+	+	−
Esterase lipase (C8)	W	+	+
Valine arylamidase	−	+	−
Acid phosphatase	W	+	−
β-Galactosidase	−	+	−
α-Glucosidase	−	+	−
Utilization of:			
Acetate	−	+	+
D-Glucose	−	+	+
D-Ribose	−	+	+
D-Fructose	−	+	+
L-Arginine	−	+	+
L-Aspartate	−	+	+
L-Glutamate	−	+	+
Sucrose	−	+	+
Glycine	+	+	−
Glycerol	−	+	+
D-Mannitol	−	+	+
myo-Inositol	−	+	+
Salicin	−	+	+
Sorbitol	−	+	+
Fatty acids (% of total)			
10 : 0	0·2	3·5	ND
12 : 0	1·7	3·1	ND
16 : 0	4·2	2·9	3·04
18 : 0	4·1	1·9	1·1
18 : 1ω7*c*	64·1	68·8	84·6
11-Methyl 18 : 1ω7*c*	10·6	7·5	ND
18 : 3ω6*c* (6,9,12)	3·3	ND	ND
2-OH 16 : 0	3·9	ND	ND
3-OH 10 : 0	2·1	1·5	1·9
3-OH 12 : 0	1·0	5·8	ND
3-OH 12 : 1	ND	ND	4·0
19 : 0 cyclo	ND	2·3	ND
ECL 11·799	1·2	ND	3·6‡

*Data from Uchino *et al.* (1998).
†Data from Rüger & Höfle (1992).
‡Reported as ECL 11·798.

CL-TA03^T to sea salts was determined using synthetic ZoBell broth (Bacto peptone, 5 g; yeast extract, 1 g; ferric citrate, 0·1 g; distilled water to 1 l) with various concentrations [0, 1, 3, 5, 7, 10, 15, 20 and 25 % (w/v)] of sea salts (Sigma). The ionic requirements of strain CL-TA03^T were determined after incubation for 21 days at 30 °C using synthetic ZoBell agar with the following combinations of salts: (i) 3 % (w/v) NaCl; (ii) 3 % (w/v) NaCl, 0·6 % (w/v) $MgCl_2.6H_2O$ and 0·3 % (w/v) $MgSO_4.7H_2O$; (iii) 3 % (w/v) NaCl, 0·6 % (w/v) $MgCl_2.6H_2O$, 0·3 % (w/v) $MgSO_4.7H_2O$ and 0·06 % (w/v) KCl; (iv) 3 % (w/v) NaCl, 0·6 % (w/v) $MgCl_2.6H_2O$, 0·3 % (w/v) $MgSO_4.7H_2O$, 0·06 % (w/v) KCl and 0·2 % (w/v) $CaCl_2.2H_2O$. Strain CL-TA03^T was unable to grow on ZoBell agar with Na^+, Mg^{2+} and K^+, and was able to grow only on medium additionally supplemented with Ca^{2+}. Catalase and oxidase activities were determined according to the protocols described by Smibert & Krieg (1994) and gelatinase, amylase, DNase, nitrate reductase activities and degradation of Tween 80 were examined as described by Hansen & Sørheim (1991). In addition, nitrate reduction, production of indole, arginine dihydrolase, urease, gelatinase, β-galactosidase, acid production from glucose and hydrolysis of aesculin were tested using the API 20NE kit (bioMérieux) according to the manufacturer's instructions, except that the cell suspension was prepared using artificial seawater (NaCl, 24 g; $MgCl_2$, 5·1 g; Na_2SO_4, 4 g; $CaCl_2$, 1·1 g; KCl, 0·7 g; $NaHCO_3$, 0·2 g; KBr, 0·1 g; H_3BO_3, 0·027 g; $SrCl_2$, 0·024 g; NaF, 0·003 g; distilled water to 1 l; Lyman & Fleming, 1940) as a suspension medium. Other enzyme activities were also assayed using the API ZYM kit (bioMérieux) and artificial seawater as a suspension medium. Carbon utilization was tested on basal agar medium supplemented with yeast extract (NaCl, 23·6 g; KCl, 0·64 g; $MgCl_2.6H_2O$, 4·53 g; $MgSO_4.7H_2O$, 5·94 g; $CaCl_2.2H_2O$, 1·3 g; $NaNO_3$, 0·2 g; NH_4Cl, 0·2 g; Bacto agar, 15 g; yeast extract, 0·05 g; distilled water to 1 l; Choi *et al.*, 2006) containing 0·2 % of the carbon source. Incubation was prolonged for 1 month and growth was scored as positive when visible colonies were observed. Growth of CL-TA03^T was observed at temperatures of 15–35 °C, with optimum growth between 30 and 35 °C. Growth occurred from pH 6 to 10. Strain CL-TA03^T grew at sea salt concentrations of 3–7 % and could degrade starch and gelatin and reduce nitrate to nitrite. CL-TA03^T was positive for cytochrome oxidase, catalase and alkaline phosphatase, but negative for valine arylamidase, β-galactosidase and α-glucosidase (Table 1). The results of the other biochemical and physiological tests are given in Table 1 and in the species description.

Isoprenoid quinones were isolated according to Minnikin *et al.* (1984) and analysed by HPLC as described by Collins (1985). The major isoprenoid quinone in CL-TA03^T is UQ-10. The fatty acid methyl esters in whole cells were analysed by gas chromatography according to the instructions of the Microbial Identification System (MIDI) at the Korean Culture Center of Microorganisms in Seoul, Korea. The dominant fatty acid for CL-TA03^T was 18 : 1ω7*c*

(64·1 %), which is a characteristic common to the *Roseobacter* clade, followed by 11-methyl 18 : 1ω7*c* (10·6 %), 16 : 0 (4·2 %) and 18 : 0 (4·1 %) (Table 1).

In terms of phenotypic features, strain CL-TA03^T could be differentiated from the closely related genus *Thalassobius* by the absence of granules inside cells, the presence of weak amylase activity and the absence of growth on acetate, D-glucose, D-ribose, D-fructose, D-mannitol, *myo*-inositol, sorbitol, salicin, glycerol, L-arginine, L-aspartate, L-glutamate and sucrose as the sole carbon source. Furthermore, fatty acid profiles could distinguish CL-TA03^T from the two *Thalassobius* species (Table 1). The fatty acid profile of strain CL-TA03^T was different from that of *T. mediterraneus* mainly by the proportions of 18 : 1ω7*c* and 11-methyl 18 : 1ω7*c*, and from that of *T. gelatinovorus* mainly by the proportions of minor fatty acids including 10 : 0, 19 : 0 cyclo, 18 : 3ω6*c* (6,9,12) and 2-OH 16 : 0. Moreover, phylogenetic analysis of the 16S rRNA gene sequence clearly showed that strain CL-TA03^T could not be classified as a member of any known genera in the *Roseobacter* clade. Therefore, phylogenetic analyses based on 16S rRNA gene sequences, fatty acid profile and phenotypic features indicated that strain CL-TA03^T should be classified as a novel genus and species, for which the name *Shimia marina* gen. nov., sp. nov. is proposed.

Description of *Shimia* gen. nov.

Shimia (Shi′mi.a. N.L. fem. n. *Shimia* named in honour of Dr Jae H. Shim, for his contributions to marine plankton ecology in Korea).

Cells are Gram-negative and rod-shaped. Growth is heterotrophic and strictly aerobic. Catalase- and oxidase-positive. Absence of granules inside cells. The predominant isoprenoid quinone is UQ-10. The dominant fatty acid is 18 : 1ω7*c*. Cells do not contain bacteriochlorophyll *a*. The genus is a member of the family *Rhodobacteraceae*. The type species is *Shimia marina*.

Description of *Shimia marina* sp. nov.

Shimia marina (ma.ri′na. L. fem. adj. *marina* of or belonging to the sea, marine).

Displays the following properties in addition to those given in the genus description. Cells are approximately 0·3–0·6 μm wide and 0·8–3·6 μm long. Cells are motile by several monopolar flagella. On MA medium, colonies are circular, entire, convex, opaque and colourless or beige. Grows at 15–35 °C (optimum 30–35 °C) and at pH 6–10. Growth occurs at sea-salt concentrations of 3–7 % (w/v). No growth without sea salts in the medium. Amylase, gelatinase, nitrate reductase, DNase and Tween 80 hydrolysis activities are present. According to API 20NE tests, nitrate reductase, indole production, acid production from glucose, arginine dihydrolase, aesculin hydrolysis, gelatinase and urease activities are not detected. According to API ZYM tests, alkaline phosphatase and leucine arylamidase activities are present and esterase (C4), esterase lipase (C8) and acid phosphatase activities are weakly present, whereas lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, naphthol-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are absent. Major fatty acids are 18 : 1ω7*c*, 11-methyl 18 : 1ω7*c*, 16 : 0, 18 : 0, 18 : 3ω6*c* (6,9,12) and 2-OH 16 : 0. Growth occurs on acetone, α-ketobutyric acid, citrate, ethanol, glycine, glycogen, L-leucine, L-lysine, L-ornithine, pyruvate, D-raffinose, succinate, tartrate and urea. No growth occurs on acetamide, L-ascorbate, benzoate, acetate, D-cellobiose, D-galactose, D-glucose, lactose, D-mannose, D-ribose, D-xylose, D-fructose, formic acid, glycerol, inulin, 2-propanol, L-arabinose, L-aspartate, L-arginine, L-glutamate, L-asparagine, L-proline, D-mannitol, maleic acid, *myo*-inositol, *N*-acetylglucosamine, L-rhamnose, salicylate, salicin, sorbitol, sucrose, thiamine or D-trehalose. The DNA G + C content of the type strain is 57·2 mol%.

The type strain is CL-TA03^T (=KCCM 42117^T=JCM 13038^T), isolated from a biofilm in a coastal fish farm in Korea.

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