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NOTE

Grimontia marina sp. nov., a Marine Bacterium Isolated from the Yellow Sea[§]

Ahyoung Choi[1], Kyung-Mi Kim[1], Ilnam Kang[1], Seok-Hyun Youn[2], Young-Sang Suh[2], Yoon Lee[2], and Jang-Cheon Cho[1]*

[1] *Division of Biology and Ocean Sciences, Inha University, Incheon 402-751, Republic of Korea*
[2] *National Fisheries Research and Development Institute (NFRDI), Busan 610-705, Republic of Korea*

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A novel species belonging to the genus *Grimontia* is described in this study. A Gram-negative, chemoheterotrophic, obligately aerobic, catalase- and oxidase-positive, motile by a single polar flagellum, and rod-shaped bacterium, designated IMCC5001^T, was isolated from surface seawater of the Yellow Sea. Strain IMCC5001^T grew optimally at 30°C in the presence of 3.5% NaCl. Phylogenetic analysis based on 16S rRNA gene sequences showed that the isolate was related most closely to *Grimontia hollisae* with a sequence similarity of 95.8%, and formed a robust phyletic lineage with *Grimontia hollisae*. Differential physiological characteristics between the new strain and *Grimontia hollisae* KCCM 41680^T and chemotaxonomic characterization including determination of DNA G+C content, fatty acid methyl esters, quinone composition, and polar lipid profiles justified the assignment of strain IMCC5001^T to the genus *Grimontia* as a novel species. In conclusion, strain IMCC5001^T represents a new species, for which the name *Grimontia marina* sp. nov. is proposed, with the type strain IMCC5001^T (=KCTC 22666^T =NBRC 105794^T).

Keywords: *Grimontia marina* sp. nov., taxonomy, 16S rRNA gene, new species, marine bacteria

Introduction

The genus *Grimontia* in the family *Vibrionaceae* was first proposed by Thompson *et al.* (2003) according to the reclassification of *Vibrio hollisae* (Hickman *et al.*, 1982) on the basis of distinct phenotypic and genotypic characteristics compared to other members of the genus *Vibrio*. The genus *Grimontia* comprises only one recognized species, *G. hollisae*, the type strain of which was isolated from stool samples of patients with diarrhea. The type strain of *G. hollisae* is characterized as being a Gram-negative, moderately halophilic, rod-shaped bacterium that is motile by a single polar flagellum. In this study, a *Grimontia*-like bacterium, designated strain IMCC5001^T, was isolated from a surface seawater sample of the Yellow Sea and subjected to a taxonomic consideration. The combined results of the present polyphasic taxonomic analysis shows that strain IMCC5001^T represents a novel species in the genus *Grimontia*.

Strain IMCC5001^T was isolated from a seawater sample collected from the surface (1 m) of the Yellow Sea (35°19′26″ N, 126°04′34″ E), Korea. The serially diluted seawater samples were spread on marine agar 2216 (MA; BD Difco, USA) and the agar plates were incubated aerobically at 25°C for 7 d. Strain IMCC5001^T formed small, circular, cream-colored colonies on MA plates. The strain was preserved at -80°C in marine broth 2216 (MB; BD Difco) supplemented with 20% glycerol (v/v). For phenotypic and chemotaxonomic comparison between strain IMCC5001^T and *G. hollisae,* the type strain *G. hollisae* KCCM 41680^T (= LMG 17719^T) was obtained from the KCCM (Korean Culture Center of Microorganisms) and used as a reference strain in the present study.

To compare the phenotypic characteristics, strains IMCC 5001^T and *G. hollisae* KCCM 41680^T were routinely grown on MA at 30°C. Gram reaction, cell size and morphology, colony characteristics, presence of flagella and intracellular granules, motility, catalase, and oxidase activities were determined according to Smibert and Krieg (1994) and a previous report (Yang *et al.*, 2008) except that the bacterial cultures were grown on MA or in MB at 30°C for 2 d. The temperature range and optimum for growth were determined at 4–42°C (4, 10, 15, 20, 25, 30, 37, and 42°C) in artificial seawater medium (ASW; basic formula, per L: 5.9 g $MgCl_2{\cdot}6H_2O$, 3.24 g $MgSO_4{\cdot}7H_2O$, 1.8 g $CaCl_2{\cdot}2H_2O$, 0.55 g KCl, 0.16g $NaHCO_3$, 0.08 g KBr, 0.034 g $SrCl_2{\cdot}6H_2O$, 0.022 g H_3BO_3, 0.008 g $Na_2H_2PO_4$, 0.004 g Na_2SiO_3, 0.0024 g NaF, 0.0016 g KN_4NO_3) (Choo *et al.*, 2007), amended with peptone (5.0 g/L) and yeast extract (1.0 g/L). The pH range and optimum for growth were determined from pH 5.0 to 10.0 (at 0.5 pH unit intervals) in ASW supplemented with peptone (5.0 g/L) and yeast extract (1.0 g/L). The salinity range and optimum for growth were monitored in nutrient broth (BD Difco) with various NaCl concentrations (at 0–15%,

*For correspondence. E-mail: chojc@inha.ac.kr; Tel.: +82-32-860-7711; Fax; +82-32-232-0541
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w/v). Growth of strains IMCC5001^T and *G. hollisae* KCCM 41680^T on thiosulphate/citrate/bile salts/sucrose (TCBS) agar (BD Difco) was monitored for up to 7 d. Rugosity of colonies were examined by colony morphology after growing on tryptic soy agar (TSA) at 30°C and 37°C for 2 d. The MGC anaerobic system (Mitsubishi Gas Chemical) was employed to determine anaerobic growth and the agar plates were monitored up to 2 weeks. Triple Sugar Iron agar (BD Difco) supplemented with 3.0% NaCl was used to determine H_2S production. Hydrolysis of casein (10% skimmed milk, w/v), starch (0.2%, w/v), chitin (0.5%, w/v), CM-cellulose (0.2%, w/v), and Tween 80 (1.0%, v/v) was determined based on the formation of clear zones around colonies after adding appropriate solutions (Smibert and Krieg, 1994). Degradation of DNA was tested using DNase test agar (Difco) amended with 1.5% NaCl. API 20NE, API ZYM, API 50CH strips (bioMérieux) and Biolog GN2 microplates (Biolog) were used for biochemical characterization and substrate oxidation according to the manufacturer's suggestions except that the cells were suspended in ASW medium and inoculated on the strips. The following antibiotics were tested using the diffusion plate method: ampicillin (10 μg), chloramphenicol (25 μg), erythromycin (15 μg), gentamycin (10 μg), kanamycin (30 μg), penicillin G (10 μg), rifampicin (50 μg), streptomycin (10 μg), tetracycline (30 μg), and vancomycin (30 μg).

The DNA G+C content, quinone composition, fatty acid methyl esters (FAME), and polar lipids were determined for strains IMCC5001^T and *G. hollisae* KCCM 41680^T that were grown under the same culture conditions. The DNA G+C content of the strains was determined using the HPLC method according to Mesbah *et al.* (1989). Cellular FAME profiles of the strains were obtained from the cellular extracts of bacterial cultures grown on MA at 30°C for 2 d by using the Sherlock Microbial Identification System (MIDI, version 6.1, TSBA 6 database). Isoprenoid quinones were extracted by a thin-layer chromatography (TLC) according to Minnikin *et al.* (1984) and analyzed by HPLC (Collins, 1985). Polar lipids were extracted from lyophilized bacterial biomass and examined using two-dimensional TLC (Minnikin *et al.*, 1984).

The 16S rRNA gene amplification and sequencing were performed as described previously (Cho and Giovannoni, 2003). The 16S rRNA gene sequence (1,503 bp) of strain IMCC5001^T was aligned against closely related sequences held in the Silva rRNA database (Pruesse *et al.*, 2007) in the ARB software package (Ludwig *et al.*, 2004). The sequence similarities between strain IMCC5001^T and its phylogenic neighbors were determined with the ARB software on the basis of the multiple alignments considering the secondary structure of rRNA. Only unambiguously aligned nucleotide positions (1,375 bp), generated using the Maximum Frequency mask implemented in the ARB, were employed for phylogenetic inference in PAUP* 4.0 beta. Phylogenetic trees were generated using neighbor-joining (Saitou and Nei, 1987), maximum-parsimony (Fitch, 1971), and maximum likelihood (Felsenstein, 1981) with 1,000 bootstrap replicates.

Strain IMCC5001^T was related most closely to *G. hollisae* LMG 17719^T (95.8%), followed by *Enterovibrio coralii* LMG 22228^T (95.6%) and *Enterovibrio norvegicus* LMG 19839^T (95.3%) on the basis of alignment in the ARB database. In the sequence similarity analyses based on the Eztaxon server (Chun *et al.*, 2007), strain IMCC5001^T was most closely related to *Enterovibrio coralii* LMG 22228^T (96.0%), followed by *G. hollisae* LMG 17719^T (95.9%) and *Enterovibrio norvegicus* LMG 19839^T (95.6%). This discrepancy was due to the different alignment protocols between the Silva database and EzTaxon (e.g., with or without considering secondary structure of the rRNA molecule). All the phylogenetic trees generated in this study using three tree-generating algorithms (Fig. 1) showed that strain IMCC5001^T and *G. hollisae* LMG 17719^T formed a robust clade, suggesting the assignment of strain IMCC5001^T as a member of the genus *Grimontia*.

Morphological, cultural, physiological, and biochemical characteristics of strain IMCC5001^T are summarized in the species description and Table 1. Briefly, strain IMCC5001^T was a Gram-negative, chemoheterotrophic, obligately aerobic, catalase- and oxidase-positive, motile by a single polar flagellum, and rod-shaped bacterium (Supplementary data Fig. S1). Strains IMCC5001^T and *G. hollisae* KCCM 41680^T did not produce colonies on TCBS agar. Strain IMCC5001^T did not show any colony rugosity at 30°C and 37°C, whereas

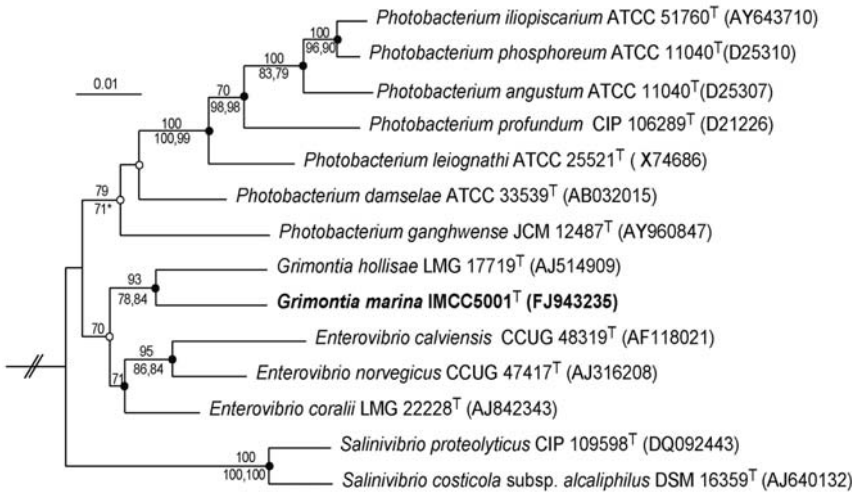

Fig. 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain IMCC5001^T among members of the family *Vibrionaceae*. Filled circles indicate the nodes also recovered by maximum parsimony and maximum likelihood trees and open circles indicate the nodes recovered by only two of three treeing methods. Bootstrap values shown above the nodes were obtained from neighbor-joining tree and those below the nodes from maximum parsimony and maximum likelihood trees in sequential order. *Ferrimonas balearica* PAT^T (X93021) was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Table 1. Differential characteristics between strains *G. marina* IMCC5001^T and *G. hollisae* KCCM 41680^T
Strains: 1, *G. marina* IMCC5001^T; 2, *G. hollisae* KCCM 41680^T.
All data are from this study. Both strains did not grow on TCBS agar. Both strains degraded starch, but not DNA, casein, CM-cellulose and chitin. Both strains did not produce H_2S.

Characteristics	1	2
Growth range with NaCl (%)	1–8	1–12
Rugosity of colony morphology at 30°C	–	+
Anaerobic growth	–	+
Hydrolysis of Tween 80	+	–
Aesculin hydrolysis (API 20NE)	+	–
Indole production, urease (API 20NE)	–	+
Naphthol-AS-BI-phosphohydrolase activity (API ZYM)	–	+
Acid production from carbohydrates (API 50CH)		
D-Mannose	+	–
L-Arabinose, D-ribose, D-galactose, D-saccharose, D-trehalose, potassium gluconate	–	+
Carbon source oxidation (Biolog GN2)		
α-D-Lactose	+	–
L-Arabinose, D-fructose, D-galactose, maltose, D-mannose, sucrose, D-trehalose, turanose, pyruvic acid methyl ester, acetic acid, *cis*-aconitic acid, citric acid, D-gluconic acid, α-keto glutaric acid, D,L-lactic acid, propionic acid, succinic acid, bromosuccinic acid. succinamic acid, D-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, L-proline, urocanic acid, inosine, uridine, glycerol	–	+
Antibiotic susceptibility		
Ampicillin, chloramphenicol, erythromycin, gentamycin, kanamycin, streptomycin	–	+
Rifampicin	+	–
DNA G+C content (mol%)	52.6	51.0 †

+, Positive; –, negative.
† 49.8 mol% in the report of Hickman *et al.* (1982).

G. hollisae KCCM 41680^T produced rugous colonies at 30°C. Table 1 shows differential characteristics between strain IMCC5001^T and the type strain of *G. hollisae* on the basis of the phenotypic data obtained in this study. The two strains were differentiated from each other in a number of phenotypic characteristics, including rugose colony production, capability for anaerobic growth, enzyme activities, acid production from various carbohydrates, and carbon source oxidation pattern. The DNA G+C content of strain IMCC 5001^T and *G. hollisae* KCCM 41680^T was 52.6 mol% and 51.0 mol%, respectively. The major respiratory quinone detected in both strains was ubiquinone 8 (Q-8). The two strains shared diphosphatidylglycerol, phosphatidylglycerol, and phosphatidylethanolamine as major polar lipids, but they were differentiated by the presence of unidentified polar lipids (Fig. 2). The predominant cellular fatty acid constituents of strain IMCC5001^T were summed feature 3 ($C_{16:1}$ ω6c and/or $C_{16:1}$ ω7c, 40.5%), summed feature 8 ($C_{18:1}$ ω6c and/or $C_{18:1}$ ω7c, 22.8%) and $C_{16:0}$ (12.4%), which were similar to *G. hollisae* KCCM 41680^T (Table 2). The DNA G+C content, isoprenoid quinone composition, major polar lipids and fatty acid profile of strain IMCC5001^T were generally in good agreement with those of *G. hollisae*, suggesting that strain IMCC5001^T belonged to the genus *Grimontia*.

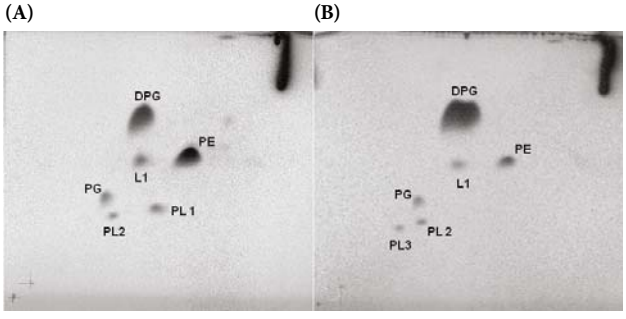

Fig. 2. Two-dimensional thin-layer chromatograms of total polar lipids extracted from strains IMCC5001^T (A) and *G. hollisae* KCCM 41680^T (B). DPG, Diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; L1, unidentified lipid; PL1-3, unidentified phospholipids.

Table 2. Cellular fatty acid composition (%) of strains *G. marina* IMCC 5001^T and *G. hollisae* KCCM 41680^T
Strains: 1, *G. marina* IMCC 5001^T; 2, *G. hollisae* KCCM 41680^T. Both strains were grown on marine agar 2216 (MA) at 30°C for 2 d. Only those fatty acids representing >1% of the total cellular fatty acids of either of the strains are shown.

Fatty acid	1	2
$C_{12:0}$	4.0	3.7
$C_{14:0}$	1.3	2.2
$C_{16:0}$	12.4	14.6
$C_{18:0}$	4.0	3.0
$C_{16:1\ \omega}$9c	-	5.0
$C_{17:1}$ ω8c	tr	1.1
$C_{18:1}$ ω9c	4.3	6.1
$C_{12:0}$ 3-OH	2.2	2.2
Summed features*		
2 ($C_{14:0}$ 3-OH and/or iso-$C_{16:1}$)	1.7	2.9
3 ($C_{16:1}$ ω6c and/or $C_{16:1}$ ω7c)	40.5	32.7
8 ($C_{18:1}$ ω6c and/or $C_{18:1}$ ω7c)	22.8	20.8

–, Not detected; tr, trace (less than 1.0%).
* Summed features represent groups of two fatty acids that could not be separated by GLC with the MIDI system (MIDI version 6.1, TSBA 6 database).

It is appropriate, based on a 16S rRNA gene sequence similarity less than 97% (95.8%), formation of a robust clade with *G. hollisae* in the phylogenetic analyses (Fig. 1) and differential phenotypic characteristics (Table 1) that strain IMCC5001^T be assigned as a member of a species that is distinct from the currently recognized *Grimontia* species, *G. hollisae* (Wayne *et al.*, 1987; Stackebrandt and Goebel, 1994). Therefore strain IMCC5001^T should be classified as a novel species in the genus *Grimontia*, for which the name *Grimontia marina* sp. nov. is proposed. Based on differential phenotypic characteristics of strain IMCC5001^T and additional characteristics of *G. hollisae* obtained in this study, an emended description of the genus *Grimontia* (Thompson *et al.*, 2003) is additionally presented.

Emended description of *Grimontia* Thompson *et al.* 2003

The description of the genus *Grimontia* is as given by Thompson *et al.* (2003) with the following amendments. Catalase-positive. Oxygen requirement for growth is species-dependent. Indole production is variable with species. The major respiratory quinone is Q-8. Major polar lipids include diphosphatidylglycerol, phosphatidylglycerol, and phosphatidylethanolamine.

Description of *Grimontia marina* sp. nov.

Grimontia marina (ma.ri'na. L. fem. adj. *marina* of the sea, marine)

Cells are Gram-negative, oxidase- and catalase-positive, chemoheterotophic, obligately aerobic, and rod-shaped, with motility conferred by a single polar flagellum. Cells are 1.7–2.2 μm in length and 0.5–0.7 μm in width. Colonies grown on MA at 30°C for 2 d are circular, cream-colored, convex, with entire margin, glistening and 1.0 mm in diameter. Does not grow on TCBS agar. Does not produce rugose colonies. Growth occurs at 15–37°C (optimum, 30°C), pH 6.0–9.5 (optimum, pH 8.0), and 1.0–8.0% NaCl (optimum, 3.5% NaCl). Starch and Tween 80 are hydrolyzed, but DNA, CM-cellulose, chitin, and casein are not. H_2S is not produced. Positive for nitrate reduction, acid production from glucose, arginine dihydrolase, aesculin hydrolysis, gelatin liquefaction, and β-galactosidase (PNPG) in API 20NE, but negative for indole production and urease activity. Positive for the following enzyme activities; alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, and cystine arylamidase in API ZYM. In API 50CH, acids are produced from D-glucose, D-fructose, D-mannose, N-acetylglucosamine, esculin ferric citrate, and potassium 2-ketogluconate. In Biolog GN2 microplate, the following carbon substrates are aerobically oxidized; Tween 40, Tween 80, N-acetyl-D-glucosamine, α-D-glucose, α-D-lactose, and L-alanine. Susceptible to rifampicin, but resistant to ampicillin, chloramphenicol, erythromycin, gentamycin, kanamycin, penicillin G, streptomycin, tetracycline, and vancomycin. The major cellular fatty acids are summed feature 3 (iso $C_{16:1}$ ω6c and/or $C_{16:1}$ ω7c), summed feature 8 ($C_{18:1}$ ω6c and/or $C_{18:1}$ ω7c) and $C_{16:0}$. The respiratory quinone detected is ubiquinone 8 (Q-8). The major polar lipids contain diphosphatidylglycerol, phosphatidylglycerol, and phosphatidylethanolamine. The DNA G+C content of the type strain is 52.6 mol%. The type strain is IMCC5001^T (=KCTC 22666^T =NBRC 105794^T), isolated from surface seawater of the Yellow Sea, Korea.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain IMCC5001^T is FJ943235.

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