Kistimonas asteriae gen. nov., sp. nov., a gammaproteobacterium isolated from *Asterias amurensis*

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A novel marine bacterium, strain KMD 001^T, was isolated from the starfish *Asterias amurensis*, which inhabits the East Sea of Korea. Strain KMD 001^T was aerobic, light-yellow pigmented and Gram-stain-negative. Analyses of the 16S rRNA gene sequence revealed that strain KMD 001^T represents a novel lineage within the class *Gammaproteobacteria*. Strain KMD 001^T is closely related to the genera *Endozoicomonas* and *Zooshikella*, which belong to the family *Hahellaceae* and to the order *Oceanospirillales*. The 16S rRNA gene sequence of strain KMD 001^T shows similarities of approximately 91.8–94.6 % with the above-mentioned genera. The DNA G + C content of KMD 001^T is 47.6 mol%. It contains Q-9 as the major isoprenoid quinone. The predominant fatty acids were determined to be anteiso-C_{15:0}, iso-C_{15:0}, iso-C_{14:0} and iso-C_{16:0}. Strain KMD 001^T should be assigned to a novel bacterial genus within the class *Gammaproteobacteria* based on its phylogenetic, chemotaxonomic and phenotypic characteristics. The name *Kistimonas asteriae* gen. nov., sp. nov. is proposed. The type strain is KMD 001^T (=KCCM 90076^T =JCM 15607^T).

Micro-organisms are prolific resources for drug discovery. Since the discovery of actinomycin, more than 13 000 bioactive metabolites have been described (Bérday, 2005). Unfortunately, the number of novel compounds isolated from cultured micro-organisms has been decreasing for more than a decade, while the demand for novel secondary metabolites that might have pharmaceutical benefits has increased in light of the dramatic resistance of tumour cells and pathogenic microbes. One promising method for the discovery of novel compounds is the utilization of bacteria that have not been cultured yet. Many new bacterial taxa have been discovered in marine environments. Some of them could serve as promising genetic resources for the development of new chemical structures. These taxa include the chemically and genetically prolific genera *Salinispora* and ‘*Marinispora*’, from which new structural classes of secondary metabolites have been discovered (Fenical & Jensen, 2006).

Our first attempt in the course of searching for new microbial taxa was to investigate the marine bacterial diversity associated with the starfish *Asterias amurensis*. This study resulted in the isolation of 18 culturable marine bacterial strains. NCBI nucleotide BLAST search results

based on partial 16S rRNA gene sequences of the 18 strains revealed that 16 strains had an affiliation with members of the class *Gammaproteobacteria*. The other two strains were related to the genera *Bacillus* and *Aquimarina*. Further analyses of the 16 gammaproteobacteria revealed that the 16S rRNA gene sequence of seven strains shared 99–100 % identity with species of the genus *Vibrio*, five strains clustered with species of the genus *Pseudoalteromonas* with sequence similarities of 99–100 %, and three strains were related to the genera *Halomonas*, *Shewanella* and *Colwellia*. Phylogenetic analysis of the remaining strain, KMD 001^T, revealed close relationships to the genera *Endozoicomonas*, *Zooshikella* and *Hahella*. The 16S rRNA gene sequences of KMD 001^T showed 89.7–94.7 % similarity with the aforementioned genera. The low sequence similarity suggested that this bacterium could be distinct from known genera within the class *Gammaproteobacteria*. In addition, the chemotaxonomic and phenotypic features supported the phylogenetic data, indicating that strain KMD 001^T belongs to a novel genus and species. In this paper, we propose the description of a new bacterial genus and species, *Kistimonas asteriae* gen. nov., sp. nov.

Strain KMD 001^T was isolated from the skin of *A. amurensis* specimens, which were collected at a depth of 10 m near the port of Im-Won, located on the Korean East Sea coast (April, 2007). The skin of *A. amurensis* was

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain KMD 001^T is EU599216.

ground and diluted with sea water (1:10). The diluted suspension (100 µl) was spread onto A1+C agar plates. The A1+C medium contained 10.0 g starch, 4.0 g peptone, 2.0 g yeast extract, 1.0 g calcium carbonate and 18.0 g agar per litre filtered seawater. Plates were incubated for 2 weeks at 25 °C. Colonies were then isolated and purified on A1+C agar. The pure bacterial strains were subsequently cultured in liquid A1+C medium (25 ml) for 7 days at 25 °C. The stocks of all cultures were stored at -80 °C in culture medium containing 15 % glycerol until further use.

Cells that were cultured at 25 °C for 7 days on A1+C agar and marine agar plates were used for morphological studies. The ability of strain KMD 001^T to grow at different temperatures was examined using cultures on A1+C agar and marine agar plates at 4, 10, 15, 20, 25, 30, 37 and 42 °C. In addition, the ability to grow on different media was tested at 25 °C with the following media investigated: nutrient agar (Difco), marine agar (Difco), Tryptone Soy Broth Agar (TSA; Merck), starch agar (Difco) and xylan agar (Difco). Requirement for and tolerance of NaCl were examined in a medium that contained NaCl at different concentrations (0–15 %, w/v, with intervals of 1 %). Growth at different pH was tested on A1+C agar plates for which the pH was adjusted to 4–10 with intervals of 1 pH unit. Gram staining of KMD 001^T was performed according to the method described by Süßmuth *et al.* (1987). The ability of the strain to produce a fluorescent pigment was tested using King B medium (King *et al.*, 1954). Utilization of carbon sources was investigated by using the API 50CH tests (bioMérieux) according to the manufacturer's recommendations. In addition, API 20E, API 20NE and API ZYM kits (bioMérieux) using culture media containing 0.85 % NaCl were used to determine additional biochemical properties.

Fatty acid methyl esters were extracted and prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The DNA G+C content was determined by HPLC analysis as described by Martin *et al.* (1997). Isoprenoid quinones were extracted according to the method described by Bligh & Dyer (1959) and separated by HPLC, as described by Collins & Jones (1981). Cells that were cultured at 25 °C for 7 days in A1+C liquid medium were used for morphology observation using a scanning electron microscope (S-3500N; Hitachi).

The chromosomal DNA of strain KMD 001^T was isolated using the G-spin genomic DNA extraction kit (iNtRON Biotechnology). The 16S rRNA gene was amplified by PCR using the universal primers 27f and 1492r. These primers correspond to position 27 in the forward direction and 1492 in the reverse direction of the 16S rRNA gene of the *Escherichia coli* genome (Brosius *et al.*, 1978). Analysis of the DNA sequence was carried out by using the ABI prism BigDye terminator cycle sequencing Ready reaction kit

v.3.1 (Applied Biosystems). The PCR product was purified using the Montage dye removal kit (Millipore) according to the procedure described by the manufacturer. 16S rRNA gene sequences were determined by using a capillary DNA sequencer (Perkin-Elmer model ABI 3730XL; Applied Biosystems). Similarity of the 16S rRNA gene sequence (1506 bp) of strain KMD 001^T to previously known sequences was determined by performing BLAST searches in the GenBank/EMBL/DDBJ database (Altschul *et al.*, 1990). Multiple sequence alignment of representative sequences was performed using the CLUSTAL W program (version 1.8; Thompson *et al.*, 1994). Neighbour-joining analysis (Kimura, 1980) and a bootstrap analysis were performed using 1000 resampled datasets in order to determine the robustness of each topology. A phylogenetic tree was reconstructed by using the program MEGA4 (Tamura *et al.*, 2007).

Strain KMD 001^T is a Gram-stain-negative, aerobic, motile and short rod-shaped bacterium. It formed light-yellowish-pigmented colonies on A1+C medium, nutrient agar, marine agar and TSA medium at 25 °C. Growth was also observed on seawater base starch agar (17 g starch and 18 g agar per litre filtered seawater) and xylan agar medium (17 g xylan and 18 g agar per litre filtered seawater) at 25 °C. Growth of KMD 001^T was observed at pH 5–10 and at temperatures between 15 and 30 °C, with an optimal temperature of 25 °C. The strain did not grow at 4, 10, 37 or 42 °C. In addition, no growth was observed in A1+C medium containing 0 % or 11–15 % NaCl. Strain KMD 001^T did not produce a fluorescent pigment when cultured on King B medium. Detailed phenotypic data are shown in the species description and in Table 1.

The cellular fatty acid profiles of strain KMD 001^T, *Endozoicomonas elysicola* and *Zooshikella ganghwensis* are shown in Table 2. The predominant fatty acids of strain KMD 001^T are iso-C_{15:0}, anteiso-C_{15:0} and iso-C_{14:0}. The DNA G+C content of strain KMD 001^T is 47.6 mol%. Q-9 was detected as the predominant isoprenoid quinone. Scanning electron microscope observations of KMD 001^T revealed short rod-shaped bacterial cells, 1.0–1.6 µm in length and 0.3–0.5 µm in diameter (Fig. 1).

The 16S rRNA gene sequence (1506 bp) of strain KMD 001^T is most closely related to *E. elysicola* MKT110^T (94.6 %) and *Z. ganghwensis* JC2044^T (91.8 %) (Fig. 2). Strain KMD001^T also has 94.3–94.6 % sequence similarity to undescribed sponge isolates related to *E. elysicola*. A previously published study suggested that strain *E. elysicola* MKT110^T formed a sister clade with the genus *Zooshikella*, which belongs to the family *Hahellaceae* in the order *Oceanospirillales* (Kurahashi & Yokota, 2007). Strain KMD 001^T could also be considered another sister clade of *Zooshikella*; however, KMD 001^T is also closely related to several other genera, as shown in Fig. 2, such as *Oceanospirillum* (90.8–91.1 % similarity) (Satomi *et al.*, 1998), *Marinobacter* (89.5–91.1 % similarity) (Anzai *et al.*, 2000; Martín *et al.*, 2003; Yoon *et al.*, 2003b), *Balneatrix*

Table 1. Differential characteristics of strain KMD 001^T and its closest phylogenetic neighbours

Strains: 1, KMD 001^T; 2, *Endozoicomonas elysicola* MKT110^T (data from Kurahashi & Yokota, 2007); 3, *Zooshikella ganghwensis* JC2044^T (Yi *et al.*, 2003). +, Positive reaction or growth; –, negative reaction or no growth; NR, not recorded; NPG, *p*-nitrophenyl- β -D-galactopyranoside and *o*-nitrophenyl- β -D-galactopyranoside.

Characteristic	1	2	3
Colour of colonies	Light yellow	Beige	Yellowish-red or red
Cell size (μ m)	0.3–0.5 $\times$ 1.0–1.6	0.4–0.6 $\times$ 1.8–2.2	0.7–0.9 $\times$ 1.5–2.5
Growth at 37 °C	–	–	+
Nitrate reduction to nitrite	–	+	+
Hydrolysis of:			
Aesculin	–	+	–
Gelatin	+	–	+
Starch	+	NR	+
Casein	–	NR	+
NPG	+	–	–
Utilization of:			
Arginine	–	–	+
Cellobiose	+	NR	–
Ornithine	–	–	+
Fructose	+	–	+
Glycerol	+	NR	–
Inositol	+	NR	–
Lactose	+	–	–
Mannitol	+	–	–
Melibiose	+	–	NR
Ribose	+	–	+
Sucrose	+	–	–
DNA G + C content (mol%)	47.6	50.4	40–42
Major fatty acids	iso-C _{15:0} anteiso-C _{15:0} iso-C _{14:0}	C _{14:0} 3-OH C _{10:0} 3-OH C _{12:0} 3-OH	C _{16:0} C _{16:1} ω 7c C _{18:1} ω 7c

(90.3 % similarity) (Dauga *et al.*, 1993), *Oleiphilus* (90.2 % similarity) (Golyshin *et al.*, 2002), *Alcanivorax* (90.1 % similarity), *Microbulbifer* (91.1–92.0 % similarity) (Yoon *et al.*, 2003, 2004) and *Pseudomonas* (89.5–91.5 % similarity) (Anzai *et al.*, 1997; Dabboussi *et al.*, 1999; Peix *et al.*, 2005; Ramette *et al.*, 2001; Sikorski *et al.*, 2001; Tvrtzová *et al.*, 2006; Verhille *et al.*, 1999). In addition, the 16S rRNA gene sequence of strain KMD 001^T showed similarities of 89.7 % and 89.4 % with *Hahella ganghwensis* and *Hahella chejuensis*, respectively (Baik *et al.*, 2005; Lee *et al.*, 2001). Strain KMD 001^T also showed similarity (87.0–90.3 %) with various species belonging to the order *Oceanospirillales*.

According to our results, strain KMD 001^T is clearly distinguishable from its phylogenetic neighbours, *E. elysicola* and *Z. ganghwensis*, by its fatty acid profile, the ability to reduce nitrate, β -galactosidase activity, and the utilization of lactose and mannitol (Tables 1 and 2) (Kurahashi & Yokota, 2007; Yi *et al.*, 2003). In the case of fatty acid composition, 3-hydroxy fatty acids and a

C_{16:1} ω 7c unsaturated fatty acid were detected in *E. elysicola* and *Z. ganghwensis*, but not in KMD 001^T. However, iso- and anteiso-fatty acids were the dominant forms in KMD 001^T. The differences between KMD 001^T and the species shown in Fig. 2 are also clearly supported by fatty acid composition, DNA G + C content and the major isoprenoid quinone, as well as the very low 16S rRNA gene sequence similarity. In particular, species of the genus *Oceanospirillum* contain Q-8 as the major isoprenoid quinone (Satomi *et al.*, 2002). Species of the genera *Marinobacter*, *Microbulbifer*, *Litoricola*, *Oleiphilus* and *Alcanivorax* have DNA G + C contents of over 52 mol% and contain C_{16:1} ω 7c or C_{18:1} ω 7c unsaturated fatty acid (Yoon *et al.*, 2003a,b, 2004; Kim *et al.*, 2007; Golyshin *et al.*, 2002; Liu & Shao, 2005). No iso- or anteiso-fatty acids were detected in species of the genera *Pseudomonas* and *Hahella* (Peix *et al.*, 2005; Baik *et al.*, 2005). On the basis of the phenotypic, chemotaxonomic and genotypic features, strain KMD 001^T should be assigned to a novel genus in the class

Table 2. Cellular fatty acid profiles of strain KMD 001^T and its closest phylogenetic neighbours

Strains: 1, KMD 001^T; 2, *Endozoicomonas elysicola* MKT110^T (data from Kurahashi & Yokota, 2007); 3, *Zooshikella ganghwensis* JC2044^T (data from Yi *et al.*, 2003).

Fatty acid (%)	1	2	3
iso-C _{14:0}	11.96	—	—
iso-C _{15:0}	21.31	—	—
iso-C _{16:0}	6.39	—	—
iso-C _{17:0}	1.45	—	—
iso-C _{18:0}	0.27	—	—
iso-C _{17:1} ω10c	3.85	—	—
anteiso-C _{15:0}	31.36	—	—
anteiso-C _{17:0}	3.5	—	—
C _{10:0} 3-OH	—	3.1	2.1
C _{12:0} 3-OH	—	2.8	5.1
C _{12:1} 3-OH	—	—	<0.5
C _{14:0} 3-OH	—	4.1	—
C _{10:0}	—	1	2.6
C _{12:0}	—	6.5	<0.5
C _{14:0}	0.72	9.3	6.0
C _{15:0}	—	0.7	—
C _{16:0}	1.06	18.9	31.9
C _{18:0}	—	0.8	0.7
C _{16:1} ω7c	—	54.5	37.1
C _{16:1} ω7c alcohol	7.01	—	—
C _{16:1} ω11c	4.49	—	—
C _{18:1} ω7c	—	5.5	14.5
C _{18:1} ω9c	0.36	—	—
Summed feature 4*	6.26	—	—

*Summed features are groups of two or three fatty acids that cannot be separated by GLC using the MIDI system. Summed feature 4 comprises iso-C_{17:1} I and/or anteiso-C_{17:1} B.

Gammaproteobacteria. We propose that strain KMD 001^T be classified as the novel genus and species *Kistimonas asteriae* gen. nov., sp. nov.

Fig. 1. Scanning electron microscope image showing short rods of strain KMD 001^T. The organism was grown on A1 + C medium at 25 °C, 200 r.p.m. for 7 days. Bar, 5 μm.

Description of *Kistimonas* gen. nov.

Kistimonas [Kis.ti.mo'nas. N.L. n. *kistum* acronym of the Korea Institute of Science and Technology (KIST); L. fem. n. *monas* a unit, monad; N.L. fem. n. *Kistimonas* Kist monad].

Do not produce a fluorescent pigment on King B medium. Isoprenoid quinone Q-9 is detected. The predominant fatty acids are iso-C_{15:0}, anteiso-C_{15:0} and iso-C_{14:0}. The DNA G+C content of the type strain of the type species is 47.6 mol%. The genus *Kistimonas* is a member of the class *Gammaproteobacteria*. The type species is *Kistimonas asteriae*.

Description of *Kistimonas asteriae* sp. nov.

Kistimonas asteriae (as.te'ri.ae. N.L. gen. n. *asteriae* of *Asterias amurensis*, a starfish).

Cells are Gram-stain-negative, aerobic, motile and rod-shaped. Light-yellow-pigmented colonies are formed on A1 + C medium, nutrient agar, marine agar and TSA medium. Grows between 15 and 30 °C, with an optimal temperature of 25 °C. Does not grow at 4, 10, 37 or 42 °C. No growth observed in A1 + C medium prepared with deionized water without NaCl. No colonies detected when medium contains 11–15 % NaCl. Growth is reduced by NaCl at concentrations higher than 9 % (w/v). Grows at pH 5–10 but not at pH 4. Can utilize the following carbon sources in the API 50CH test: glycerol, ribose, galactose, D-glucose, D-fructose, inositol, mannitol, methyl-α-D-glucopyranoside, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, melezitose, raffinose, starch, glycogen, β-gentiobiose, turanose, D-tagatose, L-arabitol and potassium 5-ketogluconate. Can also use gelatin and xylan as a carbon source, but not casein or alginate. Shows β-galactosidase activity as determined by a nitrophenyl-β-D-galactopyranoside reaction in API 20E and API 20NE tests (Table 1). Does not produce H₂S, indole or acetoin. Positive for the oxidation of glucose, mannitol, sucrose and melibiose, and the assimilation of glucose, mannitol, N-acetylglucosamine, maltose, gluconate and malate in API 20E and API 20NE tests. Shows cytochrome oxidase activity. API ZYM tests using naphthyl-substrates indicate positive results for the activities of alkaline phosphatase, esterase (C4) and esterase lipase (C8). However, test results are negative for lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase.

The type strain KMD 001^T (KCCM 90076^T = JCM 15607^T) was isolated from the skin of *Asterias amurensis* collected at the Im-Won port in Samcheok, East Sea in Korea.

Fig. 2. Phylogenetic tree based on the 16S rRNA gene sequences of strain KMD 001^T and members of related genera within the class Gammaproteobacteria. Bar, 0.01 substitutions per nucleotide position. Bootstrap confidence values (%) obtained with 1000 resamplings are given at the branch-points.

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