# *Kistimonas scapharcae* sp. nov., isolated from a dead ark clam (*Scapharca broughtonii*), and emended description of the genus *Kistimonas*

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A Gram-negative, motile, facultatively anaerobic rod, designated A36$^T$, was isolated from a dead ark clam found on the south coast of Korea. The isolate was catalase- and oxidase-negative. 16S rRNA gene sequence analysis indicated that strain A36$^T$ was most closely related to *Kistimonas asteriae* KMD 001$^T$, with which it shared 98.2 % 16S rRNA gene sequence similarity. Strain A36$^T$ grew optimally at 30–37 °C, with 1 % (w/v) NaCl and at pH 8.0. The major respiratory quinone was ubiquinone-9 (Q-9). The major polar lipids were phosphatidylserine, phosphoethanolamine, phosphatidylglycerol and diphosphatidylglycerol. The major fatty acids were summed feature 3 (comprising $C_{16:1}\omega7c$ and/or iso-$C_{15}$ 2-OH) and $C_{16:0}$. The genomic DNA G+C content was 47.3 mol%. DNA–DNA relatedness between the isolate and *K. asteriae* JCM 15607$^T$ was $<25\pm3$ %. Strain A36$^T$ represents a novel species of the genus *Kistimonas*, for which the name *Kistimonas scapharcae* sp. nov. is proposed. The type strain is A36$^T$ (=KACC 16204$^T$ =JCM 17805$^T$). An emended description of the genus *Kistimonas* is also provided.

The genus *Kistimonas*, belonging to the class *Gammaproteobacteria*, was first described by Choi *et al.* (2010) to accommodate a bacterium isolated from the skin of a starfish (*Asteria samurensis*) found on the coast of the Korean East Sea. The genus *Kistimonas* is characterized as having ubiquinone-9 as the major quinone and iso-$C_{15:0}$, anteiso-$C_{15:0}$ and iso-$C_{14:0}$ as the major fatty acids. To date, only one species of the genus has been described: *Kistimonas asteriae*.

During an attempt to isolate and characterize tentative pathogenic bacteria following a mass mortality event within cage-cultured ark clams (*Scapharca broughtonii*), a dead ark clam was collected at Gang-jin Bay on the south coast of Korea. Homogenized tissue was suspended in PBS, serially diluted and then cultured on marine agar (MA; Difco) at 25 °C for 3 days. A pure culture, strain A36$^T$, was obtained after at least three subcultivations on MA.

Amplification of the 16S rRNA gene was performed by colony PCR using PCR Pre-Mix (Wizbio) with two universal bacteria-specific primers: forward primer 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1492R (5′-GGYTACCTTGTTACGACTT-3′). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis was conducted as described by Roh *et al.* (2008). Strain A36$^T$ was closely related to *K. asteriae* KMD 001$^T$ (98.2 % 16S rRNA gene sequence similarity). The 16S rRNA gene sequences of strain A36$^T$ and members of closely related species were aligned using the multiple sequence alignment program CLUSTAL W (Thompson *et al.*, 1994) and the phylogenetic relationships were determined using MEGA5 (Tamura *et al.*, 2011). A phylogenetic consensus tree was constructed using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Kluge & Farris, 1969) and maximum-likelihood (Felsenstein, 1981) methods, with 1000 random bootstrap replicates. According to the results of the phylogenetic analysis, strain A36$^T$ clustered with *K. asteriae* KMD 001$^T$ (Fig. 1).

To obtain a more comprehensive comparison of characteristics, *K. asteriae* JCM 15607$^T$ (=KMD 001$^T$) was obtained from the Japan Collection of Microorganisms and used as the reference strain. All experiments were conducted in triplicate. Growth at 4, 10, 15, 20, 25, 30, 37, 45 and 60 °C was tested in marine broth (MB; Difco). Growth at pH 4.0–11.0 (at intervals of one pH unit) was tested in MB with the pH adjusted using the following

Abbreviations: DPG, Diphosphatidylglycerol; PE, phosphoethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain A36$^T$ is JF811908.

A supplementary figure is available with the online version of this paper.

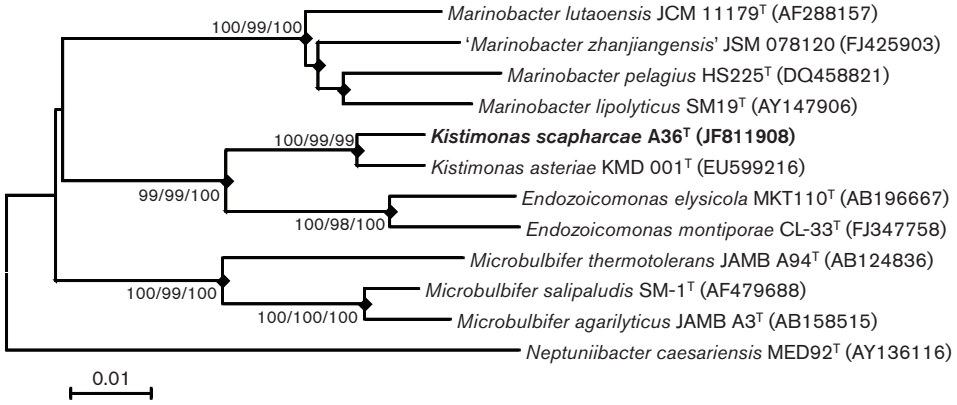


**Fig. 1.** Consensus 16S rRNA gene sequence phylogenetic tree constructed using the neighbour-joining, maximum-parsimony and maximum-likelihood algorithms showing the relationships of strain A36$^T$ with its closest relatives. Bootstrap values (>70 %) based on 1000 replicates for the neighbour-joining, maximum-parsimony and maximum-likelihood algorithms, respectively, are shown at branch nodes. Filled diamonds indicate that the corresponding nodes were recovered with all three algorithms. *Neptuniibacter caesariensis* MED92$^T$ was used as an outgroup. Bar, 0.01 substitutions per nucleotide.

buffers: 10 mM MES (pH 4–6), 10 mM TAPS (pH 7–9) and 10 mM $Na_2HPO_4$ (pH 10–11). Growth with 0–11 % (w/v) NaCl (at intervals of 1 %) was tested in MB that comprised all of the constituents except NaCl and was supplemented with appropriate concentrations of NaCl. Growth of strain A36$^T$ was observed at 10–45 °C, at pH 6.0–10.0 and with 0–5 % (w/v) NaCl, with optimum growth at 30–37 °C, at pH 8.0 and with 1 % (w/v) NaCl. Unless stated otherwise, all tests were performed with the isolate cultured under optimal growth conditions. Anaerobic growth was assessed in an anaerobic chamber ($N_2/H_2/CO_2$, 90 : 5 : 5) at 37 °C for 7 days. Strain A36$^T$ and *K. asteriae* JCM 15607$^T$ were cultured for 72 h at 30 °C in MB or on MA to analyse their physiological and biochemical characteristics. Motility was tested on semi-solid agar medium, as described by Tittsler & Sandholzer (1936). Gram-staining was performed using a Gram-staining kit (bioMérieux), according to the manufacturer's instructions. Cell morphology of strain A36$^T$, including cell shape and size, and Gram-staining were observed by light microscopy (Eclipse 50*i*; Nikon). The catalase and oxidase activities of strain A36$^T$ and the reference strain were tested using 3 % (v/v) hydrogen peroxide solution and 1 % (w/v) tetramethyl-*p*-phenylenediamine (bioMérieux), respectively. The ability to assimilate sole carbon sources was tested using the GN2 MicroPlate system with GN/GP inoculating fluid (Biolog) containing 1 % (w/v) NaCl, according to the manufacturer's instructions. Enzyme activities were characterized using the API ZYM system (bioMérieux), according to the manufacturer's instructions. Acid production from carbohydrates as the sole carbon source was tested using the API 50 CH system with 50 CHB/E medium (bioMérieux), according to the manufacturer's instructions. Strain A36$^T$ was facultatively anaerobic, motile, Gram-negative and catalase- and oxidase-negative. Strain A36$^T$ could be distinguished from *K. asteriae* JCM 15607$^T$ by a comparison of biochemical characteristics (Table 1).

For chemotaxonomic analysis, strain A36$^T$ and *K. asteriae* JCM 15607$^T$ were cultivated on MA or in MB at 30 °C for 72 h and *Undibacterium pigrum* KACC 13403$^T$ was cultured on R2A agar (Difco) at 25 °C for 5 days. Isoprenoid quinones were extracted using chloroform/methanol (2 : 1, v/v) (Collins & Jones, 1981b) and analysed by one-dimensional TLC on a silica gel 60 $F_{254}$ plate (Merck) using reversed-phase HPLC (Collins & Jones, 1981a) with a Thermo ODS Hypersil column (250 × 4.6 mm). Polar lipids were extracted from strain A36$^T$, *K. asteriae* JCM 15607$^T$ and *U. pigrum* KACC 13403$^T$ using the procedure of Xin *et al.* (2000) and separated using two-dimensional TLC on silica gel plates (Merck). Each component was detected and identified using appropriate spray reagents (Tindall, 1990). To identify the phospholipids, four standard phospholipid compounds, phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphoethanolamine (PE) and diphosphatidylglycerol (DPG), were obtained (Sigma) and phosphatidylserine (PS) was identified by comparison of PS in *U. pigrum* KACC 13403$^T$ (Kämpfer *et al.*, 2007). The standard compounds and phospholipids of strain A36$^T$, *K. asteriae* JCM 15607$^T$ and *U. pigrum* KACC 13403$^T$ were separated by one-dimensional TLC on a silica gel plate using chloroform/acetic acid/methanol/water (50 : 6 : 6 : 1, by vol.) as the solvent and detected by spraying with ninhydrin reagent and Zinzadze reagent. For the analysis of cellular fatty acids, strain A36$^T$ and *K. asteriae* JCM 15607$^T$ were cultured at 30 °C on MA for 3 days. The physiological ages of strain A36$^T$ and *K. asteriae* JCM 15607$^T$ were standardized to the exponential phase. The cellular fatty acids were extracted according to the protocol of the Sherlock Microbial Identification System (MIDI,

**Table 1.** Comparison of the characteristics of strain A36$^T$ and its closest phylogenetic relative

Strains: 1, *Kistimonas scapharcae* sp. nov. A36$^T$; 2, *K. asteriae* KMD 001$^T$ (Choi *et al.*, 2010). Both strains assimilate Tweens 40 and 80, succinic acid, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid and L-serine, produce acid from D-ribose, D-xylose, L-xylose, D-fructose, L-sorbose, aesculin, turanose, D-lyxose, D-tagatose and 5-ketogluconate and are positive for alkaline phosphatase, leucine arylamidase, trypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Both strains are negative for catalase and oxidase. +, Positive; W, weakly positive; −, negative.

| Characteristic | 1 | 2 |
|---|---|---|
| Growth at/with: | | |
| 10 °C | + | − |
| 45 °C | + | − |
| 0 % NaCl | + | − |
| 6 % NaCl | − | + |
| pH 5 | − | + |
| Optimum temperature (°C) | 30–37 | 25 |
| Utilization of:* | | |
| Pyruvic acid methyl ester | − | W |
| Bromosuccinic acid | W | − |
| Glycyl L-aspartic acid | − | + |
| L-Ornithine | + | − |
| D-Serine | − | + |
| D-Glucose 6-phosphate | − | W |
| Acid production from:* | | |
| D-Arabinose | − | W |
| Arbutin | W | − |
| Maltose | W | − |
| Xylitol | W | − |

*All data from this study.

1999) and analysed and identified using GC (Hewlett Packard 6890) and the Microbial Identification software Sherlock version 4.0 (Sasser, 1990) and the TSBA40 database. Strain A36$^T$ contained ubiquinone-9 (Q-9) as the major quinone, which is a characteristic feature of the genus *Kistimonas*. The polar lipids of strain A36$^T$ were PS, PE, PG and DPG, which are in accordance with the genus *Kistimonas* (Fig. S1, available in IJSEM Online). Although *K. asteriae* JCM 15607$^T$ also contained PS, PE, PG and DPG, it could be differentiated from strain A36$^T$ by an unidentified aminophospholipid and three unidentified lipids. Summed feature 3 (comprising $C_{16:1}\omega7c$ and/or iso-$C_{15}$ 2-OH) and $C_{16:0}$ were the major cellular fatty acids (>10 % of the total) in both strain A36$^T$ and *K. asteriae* JCM 15607$^T$. However, the isolate could be distinguished from the reference strain on the basis of differences in the proportions of minor fatty acids, such as $C_{18:1}\omega7c$ (Table 2).

Extraction of genomic DNA from strain A36$^T$ and *K. asteriae* JCM 15607$^T$ was performed as described by Rochelle *et al.* (1992). The DNA G+C content was

**Table 2.** Cellular fatty acid compositions of strain A36$^T$ and its closest phylogenetic relative

Strains: 1, *Kistimonas scapharcae* sp. nov. A36$^T$; 2, *K. asteriae* JCM 15607$^T$. All data were taken from this study. ECL, Equivalent chain-length; tr, trace (<0.5 %); −, not detected.

| Fatty acid (%) | 1 | 2 |
|---|---|---|
| $C_{10:0}$ | 1.66 | 0.50 |
| $C_{12:0}$ | 1.97 | 0.84 |
| $C_{13:0}$ | tr | − |
| $C_{14:0}$ | 5.22 | 5.80 |
| $C_{15:0}$ | tr | 0.76 |
| $C_{16:0}$ | 17.57 | 16.01 |
| $C_{17:0}$ | tr | 0.59 |
| $C_{18:0}$ | 3.41 | 3.93 |
| $C_{14:1}\omega5c$ | tr | 0.65 |
| $C_{15:1}\omega6c$ | − | tr |
| $C_{16:0}$ N alcohol | 1.47 | 0.53 |
| $C_{16:1}\omega5c$ | tr | tr |
| $C_{16:1}\omega7c$ alcohol | 1.26 | 1.28 |
| $C_{17:1}\omega8c$ | − | 0.54 |
| $C_{18:1}\omega7c$ | 5.74 | 10.98 |
| $C_{18:1}\omega9c$ | tr | tr |
| $C_{18:3}\omega6c$(6,9,12) | 0.64 | tr |
| $C_{10:0}$ 3-OH | 8.10 | 6.23 |
| $C_{11:0}$ 3-OH | − | tr |
| $C_{12:0}$ 3-OH | 2.72 | 1.56 |
| iso-$C_{14:1}$ E | tr | tr |
| 10-Methyl $C_{17:0}$ | 0.76 | 0.61 |
| 10-Methyl $C_{19:0}$ | − | tr |
| Summed features* | | |
| 1 | − | tr |
| 2 | 1.83 | 1.46 |
| 3 | 43.90 | 44.45 |
| 5 | tr | − |
| 7 | tr | − |
| Unknown ECL 11.799 | 1.80 | 1.69 |

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 1 consisted of iso-$C_{15:1}$ H and/or $C_{13:0}$ 3-OH. Summed feature 2 consisted of $C_{14:0}$ 3-OH and/or iso-$C_{16:1}$ I. Summed feature 3 consisted of $C_{16:1}\omega7c$ and/or iso-$C_{15}$ 2-OH. Summed feature 5 consisted of $C_{18:2}\omega6,9c$ and/or anteiso-$C_{18:0}$. Summed feature 7 consisted of unknown ECL 18.846 and/or $C_{19:1}\omega6c$.

determined by a fluorimetric method using SYBR Gold I and a real-time PCR thermocycler (Gonzalez & Saiz-Jimenez, 2002). Genomic DNAs from *Escherichia coli* K-12, *Ruegeria pomeroyi* DSS-3$^T$ and *Ruminococcus obeum* ATCC 29174$^T$ were used as references for calibration. DNA–DNA hybridization was performed using genome-probing microarrays with reciprocal analysis to determine the genetic relatedness between strain A36$^T$ and *K. asteriae* JCM 15607$^T$, as described previously (Bae *et al.*, 2005; Chang *et al.*, 2008). The genomic DNA G+C content of strain A36$^T$ was 47.3 mol%. DNA–DNA relatedness

between strain A36$^T$ and *K. asteriae* JCM 15607$^T$ was 25±3 % (reciprocal 5±2 %). This value was below the threshold of 70 %, so strain A36$^T$ was considered as a distinct genospecies (Wayne *et al.*, 1987).

Based on clearly distinguished phylogenetic, phenotypic and genotypic characteristics, strain A36$^T$ represents a novel species of the genus *Kistimonas*, for which the name *Kistimonas scapharcae* sp. nov. is proposed. An emended description of the genus *Kistimonas* is also provided.

### Emended description of the genus *Kistimonas*

The genus description is based on that given by Choi *et al.* (2010) with the following amendments. Catalase- and oxidase-negative. The major polar lipids are PS, PG, PE and DPG. The predominant fatty acids are summed feature 3 (comprising $C_{16:1}\omega7c$ and/or iso-$C_{15}$ 2-OH) and $C_{16:0}$.

### Description of *Kistimonas scapharcae* sp. nov.

*Kistimonas scapharcae* [sca.phar′cae. N.L. gen. n. *scapharcae* of *Scapharca*, isolated from *Scapharca broughtonii* (ark clam)].

Cells are Gram-negative, motile, facultatively anaerobic rods (0.2–0.4 μm long and 0.6–3.9 μm wide). Colonies are beige, circular with entire margins and 0.5–1.5 mm in diameter after incubation for 3 days on MA at 30 °C. Growth occurs at 10–45 °C, with 0–5 % (w/v) NaCl and at pH 6.0–10.0. Optimal growth occurs at 30–37 °C, with 1 % (w/v) NaCl and at pH 8.0. Catalase- and oxidase-negative. With Biolog GN2, utilizes Tweens 40 and 80, succinic acid, bromosuccinic acid, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, L-ornithine and L-serine. With API ZYM, positive for alkaline phosphatase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and trypsin, but negative for esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. With API 50 CHB/E, produces acids from D-ribose, D-xylose, L-xylose, D-fructose, L-sorbose, arbutin, aesculin, maltose, xylitol, turanose, D-lyxose, D-tagatose and 5-ketogluconate. The predominant ubiquinone is Q-9. The polar lipid profile contains PS, PE, PG and DPG. The major cellular fatty acids are summed feature 3 (comprising $C_{16:1}\omega7c$ and/or iso-$C_{15}$ 2-OH) and $C_{16:0}$.

The type strain is A36$^T$ (=KACC 16204$^T$ =JCM 17805$^T$), which was isolated from a dead ark clam on the south coast of Korea. The DNA G+C content of the type strain is 47.3 mol%.

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