Cocleimonas flava gen. nov., sp. nov., a gammaproteobacterium isolated from sand snail (*Umbonium costatum*)

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A Gram-stain-negative, aerobic, yellow-pigmented, heterotrophic, sulfur-oxidizing, non-motile, rod-shaped bacterium, designated KMM 3898^T, was isolated from an internal tissue of the sand snail *Umbonium costatum*, collected from the shallow sediments of the Sea of Japan. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain KMM 3898^T formed a distinct phylogenetic lineage within the class *Gammaproteobacteria* and was most closely related to *Leucothrix mucor* DSM 2157^T (89.2% 16S rRNA gene sequence similarity) and members of the genus *Thiothrix* (86.7–88.5%). Chemotaxonomically, strain KMM 3898^T contained the isoprenoid quinone Q-8, the polar lipids phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and an unknown phospholipid and the fatty acids C_{18:1ω7c}, C_{16:1ω7c} and C_{16:0} as predominant components (>10%). The DNA G+C content of strain KMM 3898^T was 43.4 mol%. On the basis of phenotypic features and phylogenetic analysis, strain KMM 3898^T represents a novel genus and species, for which the name *Cocleimonas flava* gen. nov., sp. nov. is proposed. The type strain is KMM 3898^T (=NRIC 0757^T =JCM 16494^T).

Marine coastal environments serve as a harbour for a wide array of hydrobionts, including molluscs, most of which live by burrowing into the shallow sand or mud. Marine molluscs can be considered to be natural accumulators of specific microbial communities due to their filter-feeding habit and provide an untapped potential source of novel micro-organisms. During a survey of bacteria associated with marine invertebrates inhabiting shallow sediments of the Sea of Japan, we have isolated and characterized several interesting bacterial groups. Of these, two unique pink-red-pigmented bacteria isolated from the sand snail *Umbonium costatum* were recently proposed to represent a novel genus and species, *Umboniibacter marinipuniceus*, in the class *Gammaproteobacteria* (Romanenko *et al.*, 2010). Strain KMM 3898^T was simultaneously isolated from the same snail specimen.

Strain KMM 3898^T was isolated from an internal tissue of a marine mollusc, the sand snail *Umbonium costatum* (Gastropoda, Trochidae), collected from the shallow sediments of the Sea of Japan, Russia as described previously (Romanenko *et al.*, 2005, 2008). Strain KMM 3898^T was maintained under aerobic conditions on marine 2216 agar (MA) or in marine broth (MB) at 25–30 °C and stored at –80 °C in MB supplemented with 30% (v/v) glycerol.

Growth under anaerobic conditions was determined following incubation for 1 week in MB after removing air by flushing with N₂/CO₂ (4:1, v/v). Motility was observed by the hanging-drop method as described by Gerhardt *et al.* (1994). Cell morphology and sulfur inclusions were observed by phase-contrast microscopy (AX70; Olympus). Gram-staining, oxidase and catalase activities, H₂S formation and hydrolysis of gelatin, casein, chitin, CM-cellulose, DNA, starch and Tweens 20, 40 and 80 were tested according to the standard methods described by Smibert & Krieg (1994). The oxidation/fermentation medium of Leifson (1963) was used to determine acid production from

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Two supplementary figures are available with the online version of this paper.

carbohydrates with 1 % (w/v) of each compound. The ability to grow in the presence of organic substrates as sole carbon and energy source was tested for 3 weeks on artificial seawater medium (containing l⁻¹: 30 g NaCl, 4.9 g MgCl₂, 3.9 g Na₂SO₄, 1.1 g CaCl₂, 0.66 g KCl, 0.2 g NaHCO₃, 0.096 g KBr, 0.026 g H₃BO₃, 0.024 g SrCl₂ and 0.003 g NaF) supplemented with (l⁻¹) 2 g NH₄Cl, 0.5 g yeast extract and 0.4 % carbon source. The ability to grow without additional organic growth factors was tested on the basal medium of Baumann & Baumann (1981) supplemented with 0.1 % glycerol, 0.1 % potassium acetate and 0.1 % potassium succinate. Growth at 4–40 °C, at pH 4.5–14.0, with 0–20 % NaCl and with antibiotics was studied as described previously (Romanenko *et al.*, 2005, 2008). Additional biochemical tests were carried out using the API 20 NE, API 32 GN and API ZYM systems (bioMérieux) according to the manufacturer's instructions, except that the culture was suspended in artificial seawater.

For polar lipid and fatty acid analyses, strain KMM 3898^T was cultivated on MA at 28 °C for 3 days. Lipids were extracted using chloroform/methanol (Bligh & Dyer, 1959) and analysed as described by Vaskovsky & Terekhova (1979). Fatty acid methyl esters were obtained by alkaline methanolysis (15 % NaOH/methanol), extracted by hexane and analysed using GLC-MS (model 6890; Hewlett Packard) equipped with a HP 5 MS 5 % phenyl-methylsiloxane capillary column (30 m × 250 µm × 0.25 µm) and connected to a mass spectrometer (model 5973; Hewlett Packard). Cells for respiratory lipoquinone analysis were obtained after culture in MB at 28 °C. Isoprenoid quinones were extracted using chloroform/methanol (2:1, v/v), purified by preparative TLC on silica gel 60 ADAMANT plates (Fluka) and analysed by HPLC (series 1100; Agilent) using a reversed-phase column (4.6 × 250 mm Hypersil ODS, 5 µm; Agilent) with methanol/2-propanol (65:35) as the mobile phase. Quinones were detected by monitoring absorbance at 270 nm. Pigments were extracted using hexane and then chloroform/methanol (2:1, v/v) and analysed with a CECIL 7250 spectrophotometer. The base composition of DNA isolated by the method of Marmur (1961) was determined as described by Marmur & Doty (1962) and Owen *et al.* (1969).

The 16S rRNA gene sequence (1512 nt) was determined for strain KMM 3898^T as described by Shida *et al.* (1997) and compared with sequences retrieved from public databases using FASTA (Pearson & Lipman, 1988). After multiple sequence alignment using CLUSTAL X version 1.83 (Thompson *et al.*, 1997), phylogenetic analysis was performed using MEGA4 (Tamura *et al.*, 2007). Phylogenetic trees were reconstructed by the neighbour-joining and maximum-parsimony methods and distances were calculated according to the Kimura two-parameter model. The robustness of the trees was estimated by bootstrap analysis using 1000 replicates.

Strain KMM 3898^T was a Gram-stain-negative, aerobic, yellow pigmented, heterotrophic, sulfur-oxidizing, non-motile

bacterium. The NaCl concentration and temperature ranges that supported optimal growth were narrow. Cells of strain KMM 3893^T were rods when on MA and in MB (Fig. 1a). In addition, oval-shaped and swollen cells with black precipitations and sulfur inclusions were observed during static cultivation in MB supplemented with 20 mM thiosulfate or 1 % (w/v) elemental sulfur (Fig. 1b, c). Chemotaxonomic analysis revealed Q-8 as the major isoprenoid quinone and phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and an unknown phospholipid as the polar lipids (Supplementary Fig. S1, available in IJSEM Online). The predominant fatty acids were C_{18:1}ω7c (25.8 %), C_{16:1}ω7c (25.5 %) and C_{16:0} (16 %) and the minor fatty acids were C_{18:1}ω9c (8.4 %), C_{14:0} (5.2 %), C_{18:0} (5.1 %), iso-C_{17:1} (2.4 %), iso-C_{15:1} (1.8 %) and C_{16:1}ω9c (1.7 %). The DNA G + C content was 43.4 mol%. Physiological, biochemical and metabolic characteristics of strain KMM 3898^T are listed in the genus and species descriptions and in Table 1.

Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain KMM 3898^T formed a deeply branching lineage in a cluster containing members of the genus *Leucothrix* in the family *Leucothrichaceae* (Brock, 1974) of the class *Gammaproteobacteria* (Fig. 2 and Supplementary Fig. S2). Strain KMM 3898^T shared 89.2 % 16S rRNA gene sequence similarity with *Leucothrix mucor* DSM 2157^T and 96.9 % 16S rRNA gene sequence similarity with an uncultured clone from crab (*Shinkaia crosnieri*) collected from hydrothermal vents of Okinawa (X. Ding & W. J. Yang, unpublished data). Members of the genus *Thiothrix* shared 86.7–88.5 % 16S rRNA gene sequence similarity with strain KMM 3898^T.

It is conceivable that strain KMM 3898^T was intimately associated with its host as it displayed intrinsic morphological, physiological, cultural and metabolic properties and a phylogenetic position distinctly different from its closest neighbours. The species *Leucothrix mucor* represents an obligatory aerobic, heterotrophic, non-pigmented micro-organism that is widespread in marine environments and fresh water as a seaweed epiphyte. Morphologically, *L. mucor* is characterized by the presence of filaments, gonidia with gliding ability, rosettes, knots

Fig. 1. Micrographs of strain KMM 3898^T showing morphology of rod-shaped cells grown in MB (a) and oval-shaped (b) and swollen cells (c) grown in MB supplemented with 1 % (w/v) elemental sulfur. Arrow indicates sulfur inclusion. Bars, 5 µm.

Table 1. Differential characteristics of strain KMM 3898^T and closely related members of the class *Gammaproteobacteria*

Taxa: 1, *Cocleimonas flava* sp. nov. KMM 3898^T (data from this study); 2, *Leucothrix mucor* (33 strains; Kelly & Brock, 1969; Bland & Brock, 2005); 3, *Thiothrix* (7 species; Howarth *et al.*, 1999; Aruga *et al.*, 2002). +, Positive; v, variable; –, negative.

Characteristic	1	2	3
Isolation source	Marine sand snail	Fresh and marine water seaweeds	Natural sulfide-containing water, wastewater treatment systems
Nutrition	Heterotrophic	Heterotrophic	Facultatively autotrophic, mixotrophic, heterotrophic
Oxygen tolerance	Aerobic	Obligatory aerobic	Aerobic or microaerophilic
Pigmentation	Yellow	None	None
Gliding motility	–	+	+
Cell dimensions (µm)	0.3–0.4 × 1.6–1.8	1.8–2.8 (diameter)	0.7–4.0 × 0.7–5.5
Multicellular morphology	–	+	+
Formation of:			
Filaments	–	+	+
Sheaths	–	–	v
Gonidia	–	+	+
Holdfasts	–	+	+
Rosettes	–	+	v
Sulfur inclusion granules	+	–	+
Deletion in helix 18 of 16S rRNA	–	–	+
DNA G + C content (mol%)	43.4	48–51	44–55

and holdfasts and it can be directly observed under the microscope because the filaments form attachments to the surface of algal fronds (Brock, 1966; Kelly & Brock, 1969;

Bland & Brock, 2005). The genus *Thiothrix* is physiologically distinguishable from the genus *Leucothrix* and comprises colourless, filamentous, sulfur-oxidizing bacteria

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strain KMM 3898^T with related members of the class *Gammaproteobacteria*. Accession numbers in parentheses. Bootstrap values (>70%) based on 1000 replicates are shown at branch nodes. Bar, 0.02 substitutions per nucleotide position.

capable of depositing sulfur intracellularly. Their habitats include sulfide-containing waters and activated-sludge wastewater treatment plants (Larkin, 1989; Howarth *et al.*, 1999; Aruga *et al.*, 2002). Members of the genus *Thiothrix* share a characteristic deletion in helix 18 of the 16S rRNA gene (Howarth *et al.*, 1999), which was not found in strain KMM 3898^T (Table 1).

The phenotypic characteristics of strain KMM 3898^T and the low 16S rRNA gene sequence similarity values (86.7–89.2 %) suggest that strain KMM 3898^T should be classified in a novel genus and species, for which the name *Cocleimonas flava* gen. nov., sp. nov. is proposed.

Description of *Cocleimonas* gen. nov.

Cocleimonas (coc.le.i.mo'nas. L. n. *coclea* a snail; L. fem. n. *monas* a unit, monad; N.L. fem. n. *Cocleimonas* cell from a snail).

Gram-stain-negative, aerobic, oxidase- and catalase-positive, sulfur-oxidizing bacteria. Chemoorganoheterotrophic. Sodium ions are essential for growth. The predominant isoprenoid quinone is Q-8. The major polar lipids are phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and an unknown phospholipid. The major fatty acids are C_{18:1}, C_{16:1} and C_{16:0}. Isolated from marine invertebrates. On the basis of 16S rRNA gene sequence analysis, the genus represents a branch within the class *Gammaproteobacteria* that is related to the genera *Leucothrix* and *Thiothrix*. The type species of the genus is *Cocleimonas flava*.

Description of *Cocleimonas flava* gen. nov., sp. nov.

Cocleimonas flava (fla'va. L. fem. adj. *flava* yellow).

Displays the following properties in addition to those given in the genus description. Cells are rods (0.3–0.4 µm wide and 1.6–1.8 µm long) after growth on MA or in MB at 28 °C for 3 days. Under static conditions in MB containing 1 % (w/v) elemental sulfur or 20 mM thiosulfate, cells become enlarged (0.8–1.0 µm wide and 2.6–3.0 µm long) and deposit sulfur. Non-motile. Produces yellow pigmented, opaque colonies with regular edges (2–3 mm in diameter) after 3 days on MA at 28 °C. Grows with 2–5 % (w/v) NaCl (optimum 3–4 % NaCl). Grows with peptone media containing NaCl alone without MgCl₂, KCl, CaCl₂, NaNO₃, K₂HPO₄, KCl, NaSO₄, NaHCO₃, NaF or FeSO₄. Psychrotolerant: grows at 4–33 °C (optimum 25–28 °C). Slightly alkaliphilic: grows at pH 5.5–11.0 (optimum pH 8.5–9.5). Positive for hydrolysis of Tweens 20, 40 and 80, but not casein, chitin, CM-cellulose, DNA, gelatin or starch. Does not produce H₂S. On L-tyrosine-containing medium, does not produce melanin-like pigments or a clearance zone. Does not produce acid from L-arabinose, cellobiose, D-galactose, D-glucose, lactose, maltose, D-mannose, L-rhamnose, sucrose, D-xylose, D-mannitol or D-sorbitol. No growth on basal medium

supplemented with 0.1 % glycerol, 0.1 % potassium acetate and 0.1 % potassium succinate unless D-glucose is added. With conventional methods, utilizes D-galactose, D-glucose, L-rhamnose, D-mannitol, citrate, L-alanine and L-asparagine as sole carbon and energy source, but not L-arabinose, cellobiose, lactose, maltose, D-mannose, melibiose, raffinose, D-ribose, sucrose, D-xylose, N-acetylglucosamine, glycerol, acetate, glutamic acid, L-arginine, L-lysine, DL-methionine, L-phenylalanine or L-valine. With API 20 NE, positive for gelatin hydrolysis (slowly in 3–5 days) and negative for aesculin hydrolysis, PNPG test, nitrate reduction, indole production, acidification of D-glucose under anaerobic conditions, arginine dihydrolase, urease and assimilation of L-arabinose, D-glucose, maltose, D-mannose, D-mannitol, N-acetylglucosamine, adipate, caprate, D-gluconate, citrate, L-malate and phenylacetate. With API ID 32 GN, negative for assimilation of L-arabinose, L-fucose, D-glucose, maltose, melibiose, L-rhamnose, D-ribose, sucrose, N-acetylglucosamine, glycogen, capric acid, itaconic acid, lactic acid, propionic acid, suberic acid, valeric acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 3-hydroxybutyric acid, sodium acetate, trisodium citrate, sodium malonate, potassium 2-ketogluconate, potassium 5-ketogluconate, L-alanine, L-histidine, L-proline, L-serine, inositol, D-mannitol, D-sorbitol and salicin. With API ZYM, positive for alkaline phosphatase, esterase (C4), leucine arylamidase and acid phosphatase, weakly positive for naphthol-AS-BI-phosphohydrolase and esterase lipase (C8) and negative for lipase (C14), cystine arylamidase, valine arylamidase, trypsin, α-chymotrypsin, α-fucosidase, N-acetyl-β-glucosaminidase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, β-glucuronidase and α-mannosidase. Susceptible to (µg per disc unless otherwise stated) kanamycin (30), neomycin (30), polymyxin M (300 U), rifampicin (5), cephazolin (30) and erythromycin (15) and resistant to ampicillin (10), benzylpenicillin (10 U), carbenicillin (100), gentamicin (10), streptomycin (30), vancomycin (30), nalidixic acid (30), ofloxacin (5), oxacillin (10), cephalixin (30), tetracycline (30), chloramphenicol (30), lincomycin (15) and oleandomycin (15). The predominant fatty acids (>10 %) are C_{18:1ω7c}, C_{16:1ω7c} and C_{16:0} and the minor fatty acids are C_{18:1ω9c}, C_{14:0}, C_{18:0}, iso-C_{17:1}, iso-C_{15:1} and C_{16:1ω9c}. One non-polar carotenoid pigment is present (absorption peaks at 453 and 480 nm and shoulder at 429 nm). The DNA G + C content of the type strain is 43.4 mol% (T_m).

The type strain is KMM 3898^T (=NRIC 0757^T =JCM 16494^T), isolated from an internal tissue of sand snail, *Umbonium costatum*, collected from the shallow sediments of the Sea of Japan, Russia.

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