Aquimarina macrocephali sp. nov., isolated from sediment adjacent to sperm whale carcasses

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A Gram-negative, rod-shaped, non-spore-forming, strictly aerobic strain with gliding motility, designated JAMB N27^T, was isolated from sediment adjacent to sperm whale carcasses off Kagoshima, Japan, at a depth of 219 m. Strain JAMB N27^T contained MK-6 as the major isoprenoid quinone and iso-C_{15:0}, iso-C_{15:1}, C_{16:1} and iso-C_{17:1} as the predominant fatty acids. Casein, chitin, gelatin and starch were degraded. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain JAMB N27^T represented a separate lineage within the genus *Aquimarina*. The DNA G+C content of strain JAMB N27^T was 33.1 mol%. DNA–DNA relatedness values between strain JAMB N27^T and type strains of species of the genus *Aquimarina* were significantly lower than the cut-off value accepted for the definition of a novel species. Therefore, strain JAMB N27^T represents a novel species, for which the name *Aquimarina macrocephali* sp. nov. is proposed. The type strain is JAMB N27^T (=JCM 15542^T=NCIMB 14508^T).

The genus *Aquimarina* was proposed by Nedashkovskaya *et al.* (2005) for a Gram-negative, heterotrophic and strictly aerobic bacterium with gliding motility, *Aquimarina muelleri*, belonging to the family *Flavobacteriaceae*. The species *Stanierella latercula* (Lewin, 1969; Reichenbach, 1989; Nedashkovskaya *et al.*, 2005) and *Gaetbulimicrobium brevivittae* (Yoon *et al.*, 2006) were reported to be neighbours of the genus *Aquimarina* and were reclassified into this genus, on the basis of 16S rRNA gene sequence analysis and cellular fatty acid composition, along with a novel species, *Aquimarina intermedia* (Nedashkovskaya *et al.*, 2006). Thus, four species of the genus *Aquimarina* have been recognized to date, all from marine environments: *Aquimarina muelleri* from seawater, *A. latercula* from the outflow of a marine aquarium, *A. intermedia* from a sea urchin and *A. brevivittae* from tidal flat sediment.

We have investigated an ecosystem of sperm whale carcasses off Kagoshima, Japan, for 3 years (Fujiwara *et al.*, 2007). About 800 aerobic bacteria have been isolated from the sediment adjacent to the carcasses and some novel species from this ecosystem have been described (Miyazaki *et al.*, 2008a, b, c). In this paper, we present a taxonomic study on another strain from this sediment.

Strain JAMB N27^T was collected from the marine sediment adjacent to sperm whale carcasses by an unmanned

remotely operated underwater vehicle, ROV *Hyper-Dolphin*, off Kagoshima, Japan (cruise NT0308, dive 198; 31° 23.865' N 129° 58.766' E) at a depth of 219 m. Sediment samples were collected by the vehicle's manipulator and placed in the sample holder of the sterilized sampler. Samples were stored with 15 % (v/v) glycerol in liquid nitrogen. A portion of each sample was cultivated on Daigo's artificial seawater SP agar (Nihon Pharmaceutical) at 8 °C for approximately 1 month. Strain JAMB N27^T was isolated and maintained on marine agar 2216 (MA; Difco) or in marine broth 2216 (MB; Difco) at 20 °C and stored with 15 % (v/v) glycerol at –80 °C.

Unless otherwise indicated, physiological tests were performed using the procedures described by Barrow & Feltham (1993) and Miyazaki *et al.* (2008c, d) with the modification of using artificial seawater (2.75 % NaCl, 0.07 % KCl, 0.54 % MgCl₂·6H₂O, 0.68 % MgSO₄·7H₂O, 0.14 % CaCl₂·2H₂O, 0.02 % NaHCO₃). Growth at 4–40 °C was measured in MB for 1 week. Growth with 0–6 % (w/v) NaCl was measured in medium containing 0.5 % peptone (Difco), 0.5 % yeast extract (Difco), 0.68 % MgSO₄·7H₂O, 0.54 % MgCl₂·6H₂O, 0.07 % KCl, 0.02 % NaHCO₃ and 0.14 % CaCl₂·2H₂O with the pH adjusted to pH 7.2 at 20 °C. Strain JAMB N27^T retained viability for about 1 week at 20 °C. In addition, tests for the hydrolysis of cellulose, chitin and xylan were performed using MA containing 1 % (w/v) substrate and susceptibility tests for rifampicin (5 µg) and vancomycin (30 µg) were also performed. The cultural, physiological and biochemical

Abbreviations: ML, maximum-likelihood; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JAMB N27^T is AB517144.

characteristics of strain JAMB N27^T are given in Table 1 and the species description.

Pigments were extracted with 3 ml acetone/methanol (7:2, v/v) per g wet pellet and the absorption spectra were determined at 300–700 nm with a Hitachi U-2000 spectrophotometer. Cells of strain JAMB N27^T produced orange-coloured pigments with maximum absorption at 451.2 nm (Table 1).

Cells of strain JAMB N27^T were routinely observed with an Olympus BX51 microscope. Transmission electron microscopy of negatively stained cells was conducted as described by Nogi *et al.* (1998). Cells from mid-exponential growth phase on MA at 20 °C were used for electron microscope

observations (JEM-1210; JEOL). Cells of strain JAMB N27^T were rods, 0.4–0.6 µm in width and 3.0–25.5 µm in length (Fig. 1), and motile on solid substrata by gliding.

Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v) from 200 mg dried cells and purified by TLC. The purified isoprenoid quinones were analysed using reversed-phase HPLC (Komagata & Suzuki, 1987). The major isoprenoid quinone of strain JAMB N27^T was MK-6.

Cellular fatty acids were extracted and analysed as described by Komagata & Suzuki (1987). Briefly, strain JAMB N27^T was cultured in MB at 20 °C to mid-exponential growth phase and fatty acids were extracted

Table 1. Differential characteristics of strain JAMB N27^T and the type strains of species of the genus *Aquimarina*

Strains: 1, JAMB N27^T; 2, *A. muelleri* LMG 22569^T; 3, *A. latercula* LMG 1343^T; 4, *A. intermedia* KMM 6258^T; 5, *A. brevivitae* SMK-19^T. Data were taken from this study, Nedashkovskaya *et al.* (2005, 2006), Reichenbach (1989) and Yoon *et al.* (2006). +, Positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5
Colony colour	Orange	Yellow to brown	Orange–red	Reddish	Orange
Cell characteristics					
Width (µm)	0.4–0.6	0.3–0.5	0.3–0.4	0.4–0.5	0.2–0.3
Length (µm)	3.0–25.5	5–7	1–5	2.1–3.2	1.0–15.0
Gliding motility	+	+	–	+	+
Growth temperature (°C)					
Optimum	24	ND	ND	25–28	37
Range	8–30	4–34	4–34	4–36	10–41
Growth with NaCl (%)					
Optimum	2–3	ND	ND	2–5	2–3
Range	1–4	1–8	1–5	1–10	1–10
Production of H ₂ S	–	–	+	+	–
Enzymes					
Agarase	–	–	+	–	–
Amylase	+	+	–	+	+
Catalase	+	+	–	+	+
β-Galactosidase	+	–	+	+	–
Hydrolysis of:					
Aesculin	–	ND	ND	ND	+
Chitin	+	+	+	–	ND
Tweens 20, 40, 80	–	+	+	+	+
Acid from:					
Cellobiose	–	–	–	–	+
Maltose	+	–	–	–	ND
D-Mannose	+	–	ND	ND	–
Trehalose	+	ND	ND	ND	–
Susceptibility to:					
Ampicillin	–	+	+	+	+
Chloramphenicol	–	+	+	+	+
Streptomycin	–	–	+	–	+
Tetracycline	–	+	–	+	–
Maximum absorption of pigments (nm)	451.2	445.8	465.0	469.8	474, 505
DNA G + C content (mol%)	33.1	31.6–32.5	34	37.1	36.0

Fig. 1. Observations of colonies and cells of strain JAMB N27^T. (a) Colonies on MA. (b) Swarm colony on Daigo's artificial seawater SP agar at 1 month. (c) Transmission electron micrograph of a negatively stained cell. Bar, 2.0 μ m.

using the method of Miyazaki *et al.* (2006). The major fatty acids were iso-C_{15:0} (32.0%), iso-C_{15:1} (22.3%), C_{16:1} (13.6%), iso-C_{17:1} (10.5%), C_{13:0} (9.6%) and iso-C_{17:0} 3-OH (5.4%; Table 2).

Chromosomal DNA was purified using the phenol extraction method (Saito & Miura, 1963). The DNA G+C content was determined using reversed-phase HPLC (Tamaoka & Komagata, 1984). DNA–DNA hybridization was performed at 38 °C for 4 h and DNA–DNA relatedness was measured fluorometrically using the method of Ezaki *et al.* (1989). The 16S rRNA gene sequence of strain JAMB N27^T was obtained by direct sequencing of PCR-amplified DNA as described previously (Miyazaki *et al.*, 2008b). The sequence was aligned with 16S rRNA gene sequences obtained from GenBank by using the FastAligner utility from the ARB program (Ludwig *et al.*, 2004) and the phylogenetic relationships of each taxon were inferred by neighbour-joining (NJ) and maximum-likelihood (ML) methods, using PAUP* 4.0 beta 10 (Swofford, 1998). NJ trees (Saitou & Nei, 1987) were inferred from Jukes–Cantor distances. ML trees (Felsenstein, 1981) were inferred by using the default starting parameters (NJ using a Jukes–Cantor model of evolution). Bootstrap analysis of the tree topologies was performed using 1000 and 500 replications for the NJ and ML trees, respectively.

The results of the 16S rRNA gene sequence phylogenetic analysis are shown in Fig. 2. Strain JAMB N27^T showed high sequence similarities with the type strains of species of the genus *Aquimarina*: 97.3% with *A. muelleri* KMM 6020^T, 95.4% with *A. latercula* ATCC 23177^T, 95.9% with *A. intermedia* KMM 6258^T and 94.2% with *A. brevitate* SMK-19^T. DNA–DNA hybridization experiments showed that strain JAMB N27^T had less than 8.4% DNA–DNA relatedness with *A. muelleri* KMM 6020^T and less than 1.6% DNA–DNA relatedness with *A. latercula* ATCC 23177^T. The recommended and accepted criteria for delineating bacterial species state that more than 3% 16S rRNA gene sequence dissimilarity (Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002) and less than 70% DNA–

DNA relatedness (Wayne *et al.*, 1987) indicate that strains belong to separate species. Thus, strain JAMB N27^T was clearly separated from the type strains of species of the genus *Aquimarina* according to the recommended and accepted criteria and, therefore, the strain was identified as representing a novel species.

Strain JAMB N27^T exhibited phenotypic features that distinguished it from the type strains of species of the genus *Aquimarina* (Table 1). Specifically, strain JAMB N27^T and *A. muelleri* KMM 6020^T differed in the following characteristics: colony colour, cell dimensions, temperature and NaCl concentration for growth, β -galactosidase activity, hydrolysis of Tweens 20, 40 and 80, production of acid from maltose and D-mannose, susceptibility to ampicillin, chloramphenicol and tetracycline and the maximum absorption spectra of pigments (Table 1). Moreover, the fatty acid profile showed differences with those of the reference strains (Table 2). For example, strain JAMB N27^T contained higher amounts of fatty acid iso-C_{15:1} and lower amounts of iso-C_{15:0} 3-OH and iso-C_{17:0} 3-OH.

From the results of the phenotypic, genotypic and phylogenetic analyses, it is evident that strain JAMB N27^T represents a novel species within the genus *Aquimarina*. Therefore, we propose a novel species, *Aquimarina macrocephali* sp. nov.

Description of *Aquimarina macrocephali* sp. nov.

Aquimarina macrocephali (mac.ro.ceph'a.li. N.L. gen. n. *macrocephali* of macrocephalus, isolated from sediment adjacent to carcasses of *Physeter macrocephalus*, a sperm whale).

Cells are rod-shaped, 0.4–0.6 μ m in width and 3.0–25.5 μ m in length. Cells are Gram-negative and motile by gliding. Colonies on MA are orange, circular, shiny with entire edges, smooth and convex, 0.5–1.0 mm in diameter after 2–3 days at 20 °C. Grows at 8 and 30 °C (optimum 24 °C), but not at 32 °C. Grows with 1–4% (w/v) NaCl

Table 2. Fatty acid compositions of strain JAMB N27^T and the type strains of species of the genus *Aquimarina*

Strains: 1, JAMB N27^T; 2, *A. muelleri* LMG 22569^T; 3, *A. latercula* LMG 1343^T; 4, *A. intermedia* KMM 6258^T; 5, *A. breviviae* SMK-19^T. Data from this study and Nedashkovskaya *et al.* (2006). tr, Trace (<1.0%); —, not detected; ND, no data available.

Fatty acid (%)	1	2	3	4	5
Straight-chain					
C _{13:0}	9.6	ND	ND	ND	ND
C _{14:0}	tr	ND	ND	ND	ND
C _{15:0}	1.1	1.0	5.3	1.4	4.5
C _{16:0}	2.0	—	—	—	1.2
Unsaturated					
C _{15:1}	—	—	1.6	tr	—
C _{16:1}	13.6	—	—	—	—
C _{17:1}	—	—	1.6	tr	1.4
C _{18:1}	—	—	—	1.0	—
Branched					
iso-C _{13:0}	—	1.5	tr	tr	—
iso-C _{14:0}	—	—	—	—	1.3
iso-C _{15:0}	32.0	22.2	18.4	25.9	20.2
iso-C _{15:1}	22.3	5.2	7.3	7.8	5.2
iso-C _{16:0}	tr	tr	2.7	tr	4.0
iso-C _{16:1}	—	—	1.4	tr	1.4
iso-C _{17:1}	10.5	9.9	4.7	12.7	3.7
Hydroxy					
C _{15:0} 2-OH	—	—	1.1	—	—
iso-C _{15:0} 3-OH	1.9	9.7	7.0	7.0	6.9
C _{16:0} 3-OH	—	1.5	2.0	tr	—
iso-C _{16:0} 3-OH	—	tr	2.5	tr	2.9
C _{17:0} 3-OH	—	—	1.1	—	1.0
iso-C _{17:0} 3-OH	5.4	37.6	30.5	32.6	31.9
Summed feature 3†	—	6.8	6.4	5.0	3.7

†Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 consisted of iso-C_{15:0} 2-OH and/or C_{16:1}ω7.

(optimum 2–3 % NaCl), but not without NaCl or with 5 % NaCl. Strictly aerobic. Catalase- and cytochrome oxidase-positive. H₂S and indole are not produced. Nitrate and

nitrite are not reduced. Casein, chitin, gelatin and starch are degraded. Aesculin, agar, cellulose, urea, xylose and Tweens 20, 40 and 80 are not hydrolysed. Lipase (tri-*n*-butyrin)

Fig. 2. 16S rRNA gene sequence phylogenetic tree constructed with the NJ and ML methods showing the relationships between strain JAMB N27^T and type strains of species in the family *Flavobacteriaceae*. Bootstrap values (>50 %) based on 1000/500 replicates for NJ/ML, respectively, are shown at branch nodes. Bar, 0.01 substitutions per nucleotide position.

and β -galactosidase are produced. Acid is produced from maltose, D-mannose and trehalose, but not from L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, glycerol, *myo*-inositol, lactose, D-mannitol, raffinose, L-rhamnose, D-sorbitol, sucrose or xylose. Susceptible to erythromycin, vancomycin and rifampicin and resistant to ampicillin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, neomycin, penicillin, streptomycin and tetracycline. Produces pigment with maximum absorption at 451.2 nm. The major isoprenoid quinone is MK-6. The dominant cellular fatty acids are iso-C_{15:0}, iso-C_{15:1}, C_{16:1}, iso-C_{17:1}, C_{13:0} and iso-C_{17:0} 3-OH.

The type strain, JAMB N27^T (=JCM 15542^T=NCIMB 14508^T), was isolated from the sediment adjacent to a sperm whale carcass off Kagoshima, Japan. The DNA G+C content of the type strain is 33.1 mol% (HPLC).

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