Aquimarina mytili sp. nov., isolated from the gut microflora of a mussel, *Mytilus coruscus*, and emended description of *Aquimarina macrocephali*

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An orange, rod-shaped, gliding bacterium, designated strain PSC33^T, was isolated from the gut microflora of a mussel collected from Gwangyang Bay, South Sea (Republic of Korea). Cells were Gram-reaction-negative, strictly aerobic, and catalase- and oxidase-positive. The major fatty acids were iso-C_{15:0}, iso-C_{17:0} 3-OH, iso-C_{15:1} G, C_{15:0} 3-OH and iso-C_{17:1} ω9c. The only isoprenoid quinone was menaquinone-6 (MK-6). The DNA G + C content of strain PSC33^T was 37.9 mol%. A phylogenetic tree based on 16S rRNA gene sequences showed that strain PSC33^T formed an evolutionary lineage within the radiation encompassing members of the genus *Aquimarina* with *Aquimarina macrocephali* JAMB N27^T as its nearest neighbour (96.3 % sequence similarity). A number of phenotypic characteristics distinguished strain PSC33^T from recognized members of the genus *Aquimarina*. On the basis of the data presented in this study, strain PSC33^T is considered to represent a novel species of the genus *Aquimarina*, for which the name *Aquimarina mytili* sp. nov. is proposed. The type strain is PSC33^T (=KCTC 23302^T=JCM 17454^T). An emended description of *A. macrocephali* is also provided.

The genus *Aquimarina* belongs to the family *Flavobacteriaceae* (phylum ‘*Bacteroidetes*’) and, at the time of writing, comprised seven recognized species: *Aquimarina muelleri* (type species), *A. latercula*, *A. brevivittae*, *A. intermedia* (Nedashkovskaya *et al.*, 2005, 2006), *A. macrocephali* (Miyazaki *et al.*, 2010), *A. spongiae* (Yoon *et al.*, 2011) and *A. addita* (Yi & Chun, 2011), all isolated from marine environments such as a marine aquarium, marine invertebrates, marine sediment and seawater. *Aquimarina* species are Gram-reaction-negative, strictly aerobic, chemo-organotrophic and pigmented bacteria. Here, we report the description of an *Aquimarina*-like strain, and show that this represents a novel species of the genus.

Strain PSC33^T was isolated from a sample of gut content obtained from a mussel (*Mytilus coruscus*) collected from Gwangyang Bay in the South Sea (34° 53′ N 127° 46′ E), Republic of Korea, in February 2009. Isolation was achieved by using the standard dilution plating technique on marine agar (MA; Becton Dickinson) (Yang *et al.*, 2006) incubated at 25 °C for 2 weeks. The isolate was routinely cultured on MA and preserved at –80 °C as a suspension in marine broth (MB; Becton Dickinson) containing glycerol (20 %, w/v). The following were used as reference

strains for all phenotypic tests: *A. brevivittae* KCTC 12390^T, *A. intermedia* JCM 13506^T, *A. latercula* JCM 8515^T, *A. macrocephali* JCM 15542^T, *A. muelleri* KCTC 12285^T and *A. spongiae* A6^T.

Bacterial DNA preparation, and PCR amplification and sequencing of the 16S rRNA gene were carried out as described by Chun & Goodfellow (1995). Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarities were achieved by using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Related sequences and the novel sequence were aligned by using CLUSTAL X (Thompson *et al.*, 1997), and the alignment was refined by using PHYDIT version 3.1 (<http://plaza.snu.ac.kr/~jchun/phydit/>). Phylogenetic analysis was performed by using the computer packages PHYLIP (Felsenstein, 1993) and PAUP* 4.0 (Swofford, 1998). Phylogenetic trees were inferred with the neighbour-joining (Saitou & Nei, 1987), Fitch–Margoliash (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1993) and maximum-parsimony (Fitch, 1971) algorithms. A distance matrix of the neighbour-joining dataset was generated according to the model of Jukes & Cantor (1969). The robustness of the topology in the neighbour-joining phylogenetic tree was evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings.

Preliminary comparisons with 16S rRNA gene sequences held in GenBank indicated that the novel isolate was closely related to members of the genus *Aquimarina*. The newly

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain PSC33^T is HM998910.

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

As levels of 16S rRNA gene sequence similarity between strain PSC33^T and the type strains of recognized *Aquimarinu* species were below the level indicative of relatedness at the species level (97%; Tindall *et al.*, 2010), no DNA–DNA hybridization experiments were needed to define novel species status.

glucose and 15 g agar). Cell morphology was observed by phase-contrast microscopy (TMS-F; Nikon) and scanning electron microscopy (S-4800; Hitachi) of cells of exponentially growing cultures. Gliding motility was examined by observing cells grown in wet mounts by using phase-contrast microscopy (DS-Fi1; Nikon). Cells of strain PSC33^T and the six reference strains grown on MA at 25 °C for 3 days were used for physiological and biochemical tests. Growth was assessed in MB at 4, 10–50 (at 5 °C intervals), 37 and 42 °C. Growth was assessed in MB adjusted to pH 4.0–10.0 (at 1 pH unit intervals) by using 100 mM acetate buffer (pH 4–5), 100 mM NaH₂PO₄/Na₂HPO₄ buffer (pH 6–8) and 100 mM NaHCO₃/Na₂CO₃ buffer (pH 9–10) (Yumoto *et al.*, 2004). The requirement for and tolerance to NaCl were determined in NaCl-free artificial seawater medium (ASW, containing per litre distilled water: 5.9 g MgCl₂·6H₂O, 3.24 g MgSO₄·7H₂O, 1.8 g CaCl₂·2H₂O, 0.55 g KCl, 0.16 g NaHCO₃, 0.08 g KBr, 0.034 g SrCl₂·6H₂O, 0.022 g H₃BO₃, 0.008 g Na₂H₂PO₄, 0.004 g Na₂SiO₃, 0.0024 g NaF and 0.0016 g NH₄NO₃) supplemented with 5.0 g peptone, 1.0 g yeast extract and various concentrations of NaCl (Sigma) (final concentration 0–10 %, using increments of 1 %) (Yang & Cho, 2008). Anaerobic growth was tested on MA in a jar containing an AnaeroPak (Mitsubishi Gas

Chemical), which works as an oxygen absorber and CO₂ generator, for up to 14 days at 25 °C. Acid production from sugars was tested at 25 °C by using phenol red broth base (Becton Dickinson) with a modification that 2 % NaCl solution was used instead of distilled water. The presence of flexirubin-type pigments was determined by the bathochromic shift test by using 20 % (w/v) KOH solution (Bernardet *et al.*, 2002; McCammon & Bowman, 2000). The presence of catalase activity was revealed by the production of oxygen bubbles by the cells when mixed with 3 % (v/v) H₂O₂ on a glass slide. The presence of oxidase activity was determined by oxidation of a 1 % (w/v) *N,N,N',N'*-tetramethyl-*p*-phenylenediamine solution. Indole production was determined with Kovacs' indole reagent on SIM agar (Becton Dickinson) supplemented with 2 % NaCl. H₂S production was determined on Kligler iron agar (Becton Dickinson) supplemented with 2 % NaCl. Degradation of the following macromolecules was tested by using MA as the basal medium and incubation at 25 °C for 10 days (all w/v; all Sigma): carboxymethylcellulose (CM-cellulose) (1 %), casein (2 % skimmed milk), starch (0.2 %), Tween 20 (1 %) and Tween 80 (1 %). Degradation was revealed by formation of clear zones around the colonies either directly or after flooding with adequate staining solutions (Smibert & Krieg, 1994). Hydrolysis of L-tyrosine (0.5 %, w/v; Sigma) and xylan (1 %, w/v; Sigma) was tested by using MA as the basal medium (Barrow & Feltham, 1993). Hydrolysis of gelatin (1 %, w/v; Sigma) and urea (2 %, w/v; Sigma) was determined as described by Lányi (1987) with a modification that 2 % NaCl solution was used instead of distilled water. DNase activity was determined with DNase test agar (Becton Dickinson) supplemented with 2 % NaCl. Other biochemical tests and enzyme activity tests were performed by using the API 20NE and API ZYM kits (bioMérieux) prepared according to the manufacturer's instructions except that bacterial strains were suspended in distilled water supplemented with 2 % (w/v) NaCl. Antibiotic resistance was determined with the disc diffusion method by using commercial antibiotic-impregnated discs (BBL Becton Dickinson). After 7 days of incubation at 25 °C on MA, the results were interpreted according to the guidelines provided by CLSI (2009).

Cells of strain PSC33^T were strictly aerobic, Gram-reaction-negative, motile by gliding and rod-shaped (see Fig. S1 available in IJSEM Online). The detailed results of physiological and biochemical analyses are given in Table 1 and the species description below. As shown in Table 1, several phenotypic characters readily distinguished strain PSC33^T from phylogenetically related species. In particular, *N*-acetyl- β -glucosaminidase activity was found in strain PSC33^T exclusively.

For cellular fatty acid analysis, strain PSC33^T and the six reference strains were grown on MA for 3 days at 25 °C and the methyl esters were analysed by GLC (HP 6890; Hewlett Packard) and the MIDI aerobe method (Sherlock version 4.0) according to the instructions of the Microbial Identification System (MIDI, 1999; TSBA40 database). For

G+C content analysis, the DNA of strain PSC33^T was prepared in duplicate and analysed by the thermal denaturation method of Marmur & Doty (1962). Polar lipids were analysed by using standard procedures (Minnikin *et al.*, 1984). Extracted lipids were separated by two-dimensional TLC and identified by spraying with the appropriate detection reagents (Minnikin *et al.*, 1984; Komagata & Suzuki, 1987). Isoprenoid quinones were extracted from 200 mg freeze-dried cells, purified according to the method of Minnikin *et al.* (1984) and analysed by HPLC as described by Collins (1994).

The cellular fatty acid profile of strain PSC33^T is described in Table 2. The predominant components were saturated fatty acids iso-C_{15:0} (34.0 %), iso-C_{17:0} 3-OH (22.3 %), iso-C_{15:1} G (12.6 %) and C_{15:0} 3-OH (7.5 %) and mono-unsaturated fatty acid iso-C_{17:1} ω 9c (7.6 %). The fatty acid profile of strain PSC33^T was essentially similar to those of the six reference strains except for the presence of C_{15:0} 3-OH and the absence of iso-C_{15:0} 3-OH (Table 2). The only isoprenoid quinone of strain PSC33^T was menaquinone-6 (MK-6; 100 %); MK-6 is the only or major respiratory quinone in all *Aquimarina* species and all members of the family *Flavobacteriaceae*. The polar lipid compositions of strain PSC33^T and *A. macrocephali* JCM 15542^T were very similar, with an unknown amino lipid (AL1) and two unknown polar lipids (L1–L2) as the major components (Fig. 2). Strain PSC33^T differed from *A. macrocephali* JCM 15542^T by a large amount of phosphatidylethanolamine and the presence of two additional unidentified aminolipids (Fig. S2). The DNA G+C content of strain PSC33^T was 37.9 $\pm$ 0.3 mol% (mean $\pm$ SD of 3 determinations), a value in the range of recognized *Aquimarina* species (Table 1).

Phylogenetic inference and phenotypic data clearly indicate that strain PSC33^T represents a novel species of the genus *Aquimarina*, for which the name *Aquimarina mytili* sp. nov. is proposed. On the basis of new data obtained in this study, an emended description of *A. macrocephali* is also presented.

Description of *Aquimarina mytili* sp. nov.

Aquimarina mytili (my'ti.li. L. n. *mytilus* -i a kind of mussel, sea mussel, an edible mussel, and also a scientific genus name; L. gen. n. *mytili* of a sea mussel, *Mytilus*, reflecting isolation of the type strain from *Mytilus coruscus*).

Cells are Gram-reaction-negative, strictly aerobic rods, motile by gliding, approximately 0.3–0.4 μ m in diameter and 1.2–3.0 μ m in length. Colonies on MA are circular with entire margins, convex, smooth, opaque, orange-pigmented and approximately 1.0–2.0 mm in diameter after 3 days at 25 °C (pH 7). Growth occurs on MA, but not on GYEA, NA, R2A agar or TSA. Growth occurs with 1–4 % (w/v) NaCl (optimum, 2 %), at pH 7–9 and at 15–37 °C (optimum, 25–30 °C). Oxidase- and catalase-positive. Flexirubin-type pigments are absent (KOH-test-negative). Nitrate is reduced. Arginine dihydrolase activity is absent.

Table 1. Differential phenotypic characteristics of strain PSC33^T and the type strains of recognized *Aquamarina* species

Strains: 1, PSC33^T; 2, *A. brevivittae* KCTC 12390^T; 3, *A. intermedia* JCM 13506^T; 4, *A. latercula* JCM 8515^T; 5, *A. macrocephali* JCM 15542^T; 6, *A. muelleri* KCTC 12285^T; 7, *A. spongiae* A6^T. +, Positive/susceptible; –, negative/resistant. Data are from this study unless indicated otherwise. All strains were positive for hydrolysis of casein, gelatin and starch, activity of oxidase, acid phosphatase, alkaline phosphatase, esterase (C4), leucine arylamidase and naphthol-AS-BI-phosphohydrolase, and susceptibility to amikacin, gentamicin, kanamycin, polymyxin B and streptomycin, but negative for production of indole, acid production from D-fructose, D-galactose, lactose, D-mannitol, L-rhamnose, sucrose and D-xylose, hydrolysis of tyrosine and urea, and activity of cystine arylamidase, α -fucosidase, α -galactosidase, α -glucosidase, β -glucuronidase, lipase (C14) and α -mannosidase.

Characteristic	1	2	3	4	5	6	7
Catalase activity	+	+	+	–	+	+	+
Gliding motility	+	+	+	–	+	+	–
Flexirubin-type pigments	–	–	+	–	–	+	–
Nitrate reduction	+	+	–	+	+	–	+
Production of H ₂ S	–	–	+	+	–	–	–
Acid production from:							
D-Glucose	–	–*	–	–	+	–	–
Maltose	–	–	–	–	+	–	–
D-Mannose	–	–	–	–	+	–	–
Hydrolysis of:							
Agar	–	–	–	+	–	–	–
Aesculin	+	+	+	+	–	–	+
CM-cellulose	–	–	–	+	–	–	+
DNA	–	–	+	+	–	–*	–*
Tween 20	–	–*	+	+	–	+	–
Tween 80	+	+	+	+	–	+	+
Enzyme activities (API ZYM)							
N-Acetyl- β -glucosaminidase	+	–	–	–	–	–	–
α -Chymotrypsin	–	+	–	+	–	+	+
Esterase lipase (C8)	–	+	–	–	–	+	–*
β -Galactosidase	–	–	+	+	–	–	–
β -Glucosidase	–	+	–	–	–	–	–
Trypsin	–	+	–	–	–	–	–*
Valine arylamidase	+	+	+	+	–	+	+
Antibiotic susceptibility (μ g per disc)							
Ampicillin (10)	–	–	–	–	+	+	–
Chloramphenicol (30)	–	–	–	–	–	+	–
Erythromycin (15)	–	–	–	–	–	–	+
Nalidixic acid (30)	+	–	+	–	+	+	+
Penicillin (10)	–	–	–	–	+	+	–
Tetracycline (30)	+	–	–	–	–	+	+
Vancomycin (30)	–	–	–	–	–	+	–
DNA G + C content (mol%) [†]	37.9	36.0 ^a	37.1 ^b	34.0 ^c	33.1 ^d	31.9	36.0 ^e

*Opposite results were reported by Yoon *et al.* (2006), Miyazaki *et al.* (2010), Nedashkovskaya *et al.* (2005) and Yoon *et al.* (2011).

[†]Data from: a, Yoon *et al.* (2006); b, Nedashkovskaya *et al.* (2006); c, Lewin (1969); d, Miyazaki *et al.* (2010); e, Yoon *et al.* (2011).

H₂S and indole are not produced. Acid is not produced from D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannitol, D-mannose, L-rhamnose, sucrose or D-xylose. Hydrolyses aesculin, casein, gelatin, starch and Tween 80, but not agar, CM-cellulose, DNA, Tween 20, L-tyrosine, urea or xylan. In API ZYM strips, positive for N-acetyl- β -glucosaminidase, acid phosphatase, alkaline phosphatase, esterase (C4), leucine arylamidase, naphthol-AS-BI-phosphohydrolase and valine arylamidase activities, but negative for α -chymotrypsin, cystine arylamidase, esterase lipase

(C8), α -fucosidase, α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, β -glucuronidase, lipase (C14), α -mannosidase and trypsin activities. Cells are sensitive to (μ g per disc, unless otherwise indicated) amikacin (30), gentamicin (10), kanamycin (30), nalidixic acid (30), polymyxin B (300 IU), streptomycin (10) and tetracycline (30), but resistant to ampicillin (10), chloramphenicol (30), erythromycin (15), penicillin (10 IU) and vancomycin (30). The major fatty acids (>10% of the total) are iso-C_{15:0}, iso-C_{17:0} 3-OH and iso-C_{15:1} G. The complete fatty acid

Table 2. Fatty acid compositions (%) of strain PSC33^T and the type strains of recognized *Aquimarina* species

Strains: 1, PSC33^T; 2, *A. brevivittae* KCTC 12390^T; 3, *A. intermedia* JCM 13506^T; 4, *A. latercula* JCM 8515^T; 5, *A. macrocephali* JCM 15542^T; 6, *A. muelleri* KCTC 12285^T; 7, *A. spongiae* A6^T. All data are from this study. All strains studied were grown on MA at 25 °C for 3 days. Tr, Trace (<1%); –, not detected. Fatty acids amounting to <1% of the total in all strains are not shown.

Fatty acid	1	2	3	4	5	6	7
C _{14:0}	Tr	Tr	Tr	Tr	2.8	1.4	–
C _{15:0}	2.0	3.8	1.9	4.8	1.7	–	2.4
C _{16:0}	3.3	1.0	4.4	5.5	3.9	10.2	1.9
C _{18:0}	1.7	Tr	3.3	3.9	–	6.7	1.0
C _{17:1ω6c}	–	–	Tr	1.2	–	–	–
iso-C _{13:0}	Tr	Tr	Tr	Tr	4.4	1.9	Tr
iso-C _{15:0}	34.0	21.5	27.1	19.2	16.8	21.1	38.1
iso-C _{16:0}	1.4	2.5	1.6	4.2	Tr	–	1.3
iso-C _{15:1} G	12.6	12.3	12.5	11.0	8.7	6.1	15.5
iso-C _{16:1} H	–	1.6	1.2	–	–	–	–
iso-C _{16:1} G	–	–	–	1.6	–	–	1.2
iso-C _{17:1ω9c}	7.6	5.59	9.2	5.6	6.0	9.4	8.2
anteiso-C _{15:0}	1.0	Tr	Tr	Tr	–	–	Tr
C _{15:0} 3-OH	7.5	Tr	–	–	–	–	Tr
C _{16:0} 3-OH	Tr	1.5	Tr	2.0	14.5	1.5	0.7
iso-C _{15:0} 3-OH	–	4.9	4.5	4.1	4.8	4.7	5.3
iso-C _{16:0} 3-OH	–	2.4	1.1	1.0	–	–	Tr
iso-C _{17:0} 3-OH	22.3	30.4	23.3	23.5	24.4	28.8	18.1
Summed feature 3*	3.9	4.8	3.5	4.2	8.9	8.2	3.4
Unknown ECL	Tr	1.8	Tr	Tr	–	–	–
16.582†							

*Summed feature 3 comprises C_{16:1ω7c} and/or iso-C_{15:0} 2-OH.

†ECL, Equivalent chain-length.

Fig. 2. Two-dimensional TLC pattern of the total polar lipids from strain PSC33^T (a) and *Aquimarina macrocephali* JCM 15542^T (b). PE, Phosphatidylethanolamine; L1–L4, unidentified polar lipids; AL1–3, unidentified aminolipids. The solvent systems used were chloroform/methanol/water (65:25:3.8, by vol.) for the first dimension (F) and chloroform/methanol/acetic acid/water (40:7.5:6:1.8, by vol.) for the second dimension (S). Phosphomolybdic acid (Sigma) was used to detect all lipids.

composition is given in Table 2. The only isoprenoid quinone is MK-6. The major polar lipids are phosphatidylethanolamine, an unknown aminolipid and two unknown polar lipids.

The type strain, PSC33^T (=KCTC 23302^T=JCM 17454^T), was isolated from a mussel, *Mytilus coruscus*, collected from the South Sea, Republic of Korea. The DNA G + C content of the type strain is 37.9 mol%.

Emended description of *Aquimarina macrocephali* Miyazaki *et al.* 2010

The description is as given by Miyazaki *et al.* (2010) with the following addition. Polar lipids are phosphatidylethanolamine, an unknown aminolipid and four unknown polar lipids.

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