Thalassomonas actiniarum sp. nov. and *Thalassomonas haliotis* sp. nov., isolated from marine animals

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Two marine heterotrophic bacteria, A5K-61^T and A5K-106^T, were isolated from marine animals. 16S rRNA gene sequence analysis data showed that the isolates were affiliated with the genus *Thalassomonas*; highest 16S rRNA gene sequence similarity values were found with *Thalassomonas viridans* DSM 13754^T (97.5 and 98.1 %, respectively). DNA–DNA hybridization values of strains A5K-61^T and A5K-106^T with *T. viridans* DSM 13754^T (22.2–49.1 %) were clearly below 70 %, the generally accepted limit for species delineation. The isolates produced a brown diffusible pigment. The major respiratory quinone was Q-8 and the predominant cellular fatty acids were $C_{16:1}\omega7c$ and $C_{16:0}$. Based on DNA–DNA hybridization data, some biochemical characteristics and 16S rRNA gene sequence comparison, the isolates represent two novel species of the genus *Thalassomonas*, for which the names *Thalassomonas actiniarum* sp. nov. (type strain A5K-106^T =MBIC08328^T =NCIMB 14418^T =NBRC 104231^T) and *Thalassomonas haliotis* sp. nov. (type strain A5K-61^T =MBIC08329^T =NCIMB 14417^T =NBRC 104232^T) are proposed.

The family *Colwelliaceae* (Ivanova *et al.*, 2004), of the order *Alteromonadales* (Bowman & McMeekin, 2005), class *Gammaproteobacteria*, comprises two genera, *Colwellia* and *Thalassomonas*. The genus *Thalassomonas* contains Gram-negative, rod-shaped, chemo-organotrophic, halophilic, mesophilic and strictly aerobic bacteria (Macián *et al.*, 2001), whereas the genus *Colwellia* contains psychrophilic and facultatively anaerobic bacteria (Deming & Junge, 2005). At the time of writing, the genus *Thalassomonas* accommodated four species: *Thalassomonas viridans*, isolated from an oyster homogenate (Macián *et al.*, 2001), *Thalassomonas ganghwensis*, from a tidal flat sediment (Yi *et al.*, 2004), *Thalassomonas loyana*, from a diseased coral (Thompson *et al.*, 2006) and *Thalassomonas agarivorans*, from shallow seawater (Jean *et al.*, 2006). In this study, two strains isolated from marine animals in Japan were analysed using a polyphasic taxonomic approach.

Two strains were isolated from Tateyama, Chiba prefecture, Japan, in August 2005: strain A5K-61^T was isolated from an abalone and strain A5K-106^T was from a sea anemone. They were isolated by using 1/10-strength marine agar [MA; 900 ml filtered seawater, 100 ml marine broth (MB; Difco) and 15 g agar], supplemented with 2 mg carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) l^{-1}. CCCP is proton-pump inhibitor. Each of these isolates was then cultured and maintained on 1/5-strength MA (800 ml filtered seawater, 200 ml MB and 15 g agar) at room temperature.

To identify the phylogenetic relationships of the isolates, 16S rRNA gene sequences, amplified by using universal primers (27F and 1492R; Weisburg *et al.*, 1991), were determined. Sequences that were similar to those of the isolates were obtained by using BLAST (Altschul *et al.*, 1990); the 16S rRNA gene sequences of the isolates were most similar to that of *T. viridans* DSM 13754^T. Multiple alignment was performed using the program CLUSTAL_X (version 1.83; Thompson *et al.*, 1997) [sequences used corresponded to positions 95–175, 193–442, 454–819 and 845–1393 of the sequence of *Aeromonas allosaccharophila* CECT 4199^T (GenBank accession no. S39232) according to the method of Ivanova *et al.* (2004)]. Phylogenetic trees were constructed according to three methods: the neighbour-joining method (NJ; Saitou & Nei, 1987) with the program CLUSTAL_X; the maximum-parsimony method (MP; Fitch, 1971) with the software MEGA4 (Tamura *et al.*, 2007); and the maximum-likelihood method (ML; Felsenstein, 1981) with PHYML (version 2.4.4; Guindon &

Abbreviations: ASW, artificial seawater; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains A5K-61^T and A5K-106^T are AB369381 and AB369380, respectively.

16S rRNA gene sequence-based trees constructed using the maximum-parsimony and maximum-likelihood methods and photographs of colonies of strains A5K-61^T and A5K-106^T are available as supplementary material with the online version of this paper.

Gascuel, 2003). The ML tree was calculated by using GTR (gamma distribution and invariable sites) as the substitution model. A bootstrap analysis was conducted from 1000 bootstrapped trials. Other reference sequences were obtained from GenBank. 16S rRNA gene sequence similarity values were calculated according to the method of Ivanova *et al.* (2004).

The MP and ML trees (see Supplementary Fig. S1, available in IJSEM Online) showed essentially the same topography as the NJ tree (Fig. 1). As shown in Fig. 1, the genus *Thalassomonas* was split into two groups with high bootstrap values. The two isolates belonged to the *T. viridans* group. Strains A5K-61^T and A5K-106^T showed 16S rRNA gene sequence similarity values of 98.4 % to each other and similarity values of 97.5 and 98.1 %, respectively, to *T. viridans* DSM 13754^T. The other group included *T. ganghwensis* KCTC 12041^T, *T. loyana* CBMAI 722^T and *T. agarivorans* TMA1^T; 16S rRNA gene sequence similarities between the two groups were 94–95 %. According to the NJ, MP and ML trees, the genus *Thalassomonas* could be split into two genera. However, the 16S rRNA gene sequence similarity values indicate that the isolates represent members of the genus *Thalassomonas* for the time being (Ivanova *et al.*, 2004; Stackebrandt & Goebel, 1994).

To determine genetic relatedness, a DNA–DNA hybridization experiment was performed between the isolates and *T. viridans* DSM 13754^T by using genomic DNA prepared by the method of Saito & Miura (1963). The experiment was carried out at 44.8 °C (calculated with correction for 50 % formamide) and measured fluorometrically by using the method of Ezaki *et al.* (1989). Results indicated that strains A5K-61^T and A5K-106^T shared a low level of DNA–DNA relatedness (41.5 %; 49.1 % reciprocally). DNA–DNA relatedness values of *T. viridans* DSM 13754^T with strains A5K-61^T and A5K-106^T were 24.3 % (40.6 % reciprocally) and 22.2 % (34.9 % reciprocally), respectively. These values are significantly lower than the value accepted as the phylogenetic definition of a species (Wayne *et al.*, 1987); strains A5K-61^T and A5K-106^T are therefore likely to represent two novel species of the genus *Thalassomonas*.

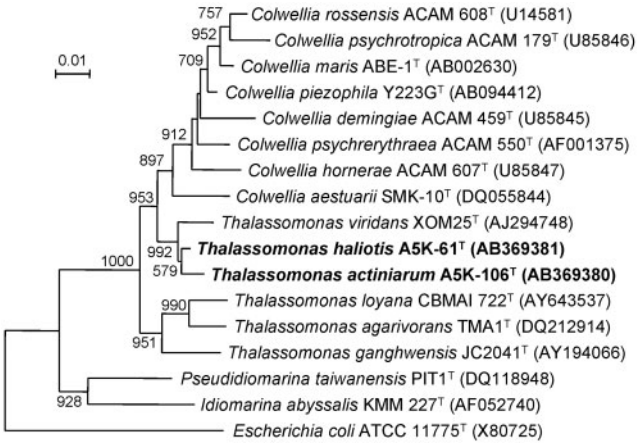

Fig. 1. Neighbour-joining phylogenetic tree of strains A5K-61^T and A5K-106^T and related members of the class *Gammaproteobacteria* based on 16S rRNA gene sequence analysis. Bootstrap values greater than 500 are shown. Bar, 0.01 substitutions per site.

DNA G+C contents were determined by HPLC according to the method of Tamaoka & Komagata (1984). The DNA G+C contents of strains A5K-61^T and A5K-106^T were 50 and 47 mol%, respectively. Respiratory quinone and cellular fatty acid compositions were determined by previously described methods (Katsuta *et al.*, 2005) on cells that had been grown for 12 h at 30 °C in MB. The major respiratory quinone of the isolates and members of the genus *Thalassomonas* was Q-8; strain A5K-106^T also contained Q-7 (6 %) and Q-9 (13 %), which were present in trace amounts in strain A5K-61^T and other *Thalassomonas* species (Table 1). The major fatty acid of the isolates and of *T. viridans* DSM 13754^T was $C_{16:1}\omega7c$; however, the isolates also contained $C_{16:0}$, which was a minor component of *T. viridans* DSM 13754^T (Table 1). The fatty acid composition of strain A5K-106^T was $C_{16:1}\omega7c$ (44.6 %), $C_{16:0}$ (31.9 %), $C_{18:1}\omega7c$ (5.5 %), $C_{17:1}\omega8c$ (4.8 %), $C_{17:0}$ (1.9 %), $C_{12:0}$ (1.8 %), $C_{14:0}$ (1.8 %), $C_{12:0}$ 3-OH (1.7 %), $C_{15:0}$ (1.5 %) and $C_{18:0}$ (1.3 %). The fatty acid composition of strain A5K-61^T was $C_{16:1}\omega7c$ (39.2 %), $C_{16:0}$ (31.3 %), $C_{17:1}\omega8c$ (6.4 %), $C_{15:0}$ (4.0 %), $C_{17:0}$ (3.4 %), $C_{18:1}\omega7c$ (3.2 %), $C_{14:0}$ (2.7 %), $C_{15:1}\omega8c$ (2.3 %), $C_{12:0}$ (2.0 %) and $C_{12:0}$ 3-OH (1.7 %).

Cells were grown for 24 h at 30 °C on MA and observed by transmission electron microscopy after negative staining with uranyl acetate. Gram staining was performed using the Hucker staining method described by Smibert & Krieg (1994). Growth at different temperatures (8–42 °C) was examined on MA. The pH range for growth was tested on half-strength SP5 agar (1/2 SP5 agar; Hosoya *et al.*, 2006) using 10 mM MES to adjust to pH 5.5–7.0, 10 mM TAPS to adjust to pH 8.0–9.0 and a 100 mM $NaHCO_3/Na_2CO_3$ buffer to adjust to pH 10.0. Salt tolerance was tested on salt-free 1/2 SP5 agar (adjusted to pH 8.0) supplemented with 0.1–1.5 × artificial seawater (ASW; Hosoya *et al.*, 2006), giving NaCl concentrations of 0.3–4.5 % (w/v). Oxidase activity was tested by spreading cell pellets on oxidase test paper (Eiken) and catalase activity was tested by using a 3 % H_2O_2 solution. Growth under anaerobic conditions was tested on MA for 1 week with the AnaeroPack system (Mitsubishi Gas Chemical).

Cells of the isolates were Gram-negative, rod-shaped, aerobic and oxidase- and catalase-positive. On MA plates, the isolates grew at 15–30 °C, but not at 8 or 37 °C. Cell morphology and other data are given in the species description.

The following phenotypic characteristics were determined; results are given in the species description and shown in Table 1. Pigment production was examined on MA;

Table 1. Differential characteristics of strains A5K-106^T and A5K-61^T and members of the genus *Thalassomonas*

Species: 1, strain A5K-106^T; 2, strain A5K-61^T; 3, *T. viridans* DSM 13754^T; 4, *T. ganghwensis* DSM 15355^T; 5, *T. loyana* LMG 22536^T; 6, *T. agarivorans* JCM 13379^T. Fatty acid compositions were determined using cells that had been grown for 12 h at 30 °C on MB. +, Positive; −, negative or not detected.

Characteristic	1	2	3	4	5	6
Pigment	Brown	Brown	Green	Yellow	Cream	Off-white
β-Galactosidase (API ZYM)	−	−	+	+	+	+
Assimilation of (API 20NE/50CH):						
Glucose	+	+	+	−	−	−
n-Capric acid	−	−	+	−	−	−
Glycerol	−	+	−	−	−	−
Erythritol	+	−	−	−	−	−
Ribose	−	+	−	−	−	−
Amygdalin	−	+	−	−	−	−
Cellobiose	+	+	−	−	−	−
Glycogen	+	−	−	−	−	−
Hydrolysis of:						
Alginate	−	−	−	−	+	+
Lecithin	−	−	+	−	−	−
Acid production from (API 50CH):						
Glucose	−	−	+	−	−	+
Amygdalin	+	−	+	−	−	−
Arbutin	−	−	+	−	−	−
Salicin	+	−	+	−	−	−
Lactose	−	−	+	−	−	+
Melibiose	−	+	−	−	−	−
Xylitol	−	+	−	−	−	−
2-Ketogluconate	−	+	+	+	+	−
5-Ketogluconate	−	+	−	+	−	−
DNA G+C content (mol%)	47	50	49	42	43	43
Respiratory quinones	Q-8 (81 %), Q-9 (13 %), Q-7 (6 %)	Q-8 (98 %), Q-7 (1 %), Q-9 (1 %)	Q-8 (98 %), Q-7 (1 %), Q-9 (1 %)	Q-8 (96 %), Q-9 (4 %)	Q-8 (97 %), Q-7 (2 %), Q-9 (1 %)	Q-8 (97 %), Q-7 (2 %), Q-9 (1 %)
Major fatty acids (% of total):						
$C_{16:0}$	31.9	31.3	4.0	26.6	16.6	25.1
$C_{16:1}\omega7c$	44.6	39.2	51.9	40.8	33.7	9.7
$C_{18:1}\omega7c$	5.5	3.2	14.0	−	14.5	3.4

pigments were stable. Degradation of starch was tested on 1/2 SP5 agar (adjusted to pH 8.0) containing 0.2 % soluble starch by the method of Smibert & Krieg (1994). Hydrolysis of Tweens 20, 40, 60 and 80 was determined on a modified Tween 80 medium (Hosoya & Yokota, 2007). Degradation of L-tyrosine was tested on 1/2 SP5 agar (adjusted to pH 8.0) containing 0.5 % L-tyrosine by the method of Barrow & Feltham (1993). Hydrolysis of DNA was determined on DNA agar (Nissui) adjusted to pH 8.0 and supplemented with half-strength ASW. Degradation of casein was tested on 1/2 SP5 agar (adjusted to pH 8.0) supplemented with 4 % (w/v) litmus milk (Difco). Hydrolysis of alginate (1 % w/v sodium alginate) and 1 % (w/v) lecithin was tested on MA. Acid production from sugars was assessed by using API 50CH (bioMérieux); cells for inoculation of these API test strips were suspended in an agar-free Leifson modified O/F medium (Smibert & Krieg, 1994). Enzyme activities were assessed by using API ZYM (bioMérieux). Physiological characteristics were examined by using API 20NE (bioMérieux); cells for inoculation of these API test strips were suspended in half-strength ASW. Carbon source assimilation (API 20NE and API 50CH) was tested by using a suspension medium consisting of 0.05 % $NH_4H_2PO_4$, 0.005 % yeast extract, 0.05 % Tris, 0.15 % agar and half-strength ASW. The API 20NE and API 50CH tests were read after 48 h incubation at 30 °C; the API ZYM tests were read after 4 h incubation at 37 °C.

As shown in Table 1, phenotypic differences between the two isolates and the most closely related strain, *T. viridans* DSM 13754^T, were observed in pigment colour

(Supplementary Fig. S2), β-galactosidase activity, assimilation of n-capric acid and the ability to produce acid from nine carbon sources.

Results of phylogenetic analysis based on 16S rRNA gene sequences suggested that the isolates belonged to the genus *Thalassomonas*. Sequence similarity values and DNA–DNA hybridization data indicated that the isolates represent two novel species of the genus *Thalassomonas*. On the basis of results described above, it is proposed that the isolates belong to two novel species within the genus *Thalassomonas*, *Thalassomonas actiniarum* sp. nov. and *Thalassomonas haliotis* sp. nov.

Description of *Thalassomonas actiniarum* sp. nov.

Thalassomonas actiniarum (ac.ti.ni.a′rum. N.L. gen. pl. n. *actiniarum* of the Actiniaria, sea anemones and related animals).

Cells are Gram-negative, aerobic and rod-shaped, approximately 1.0–2.0 μm long by 0.4–0.6 μm wide. Cells are motile by a subpolar flagellum. Releases a stable, brown, diffusible pigment into the medium. Cytochrome oxidase- and catalase-positive. Optimal growth temperature is 25–30 °C and can grow at 15 °C, but no growth occurs at 8 or 37 °C. The pH range for growth is 6.5–8.5; optimum pH is 8.0. Growth occurs in 1/2 SP5 agar medium supplemented with 0.4–1.4× ASW [NaCl concentration range of 1.2–4.2 % (w/v)], but no growth occurs in NaCl alone. Positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Positive for degradation of casein, DNA, aesculin, gelatin, starch, Tweens 20, 40, 60 and 80 and tyrosine. Nitrate is reduced to nitrite (API 20NE). Acid is produced from *N*-acetylglucosamine, amygdalin, salicin, cellobiose, maltose, sucrose, trehalose, starch, glycogen and gentiobiose (API 50CH). Does not decompose alginate, lecithin or urea. Does not produce indole. Negative for arginine dihydrolase, lipase (C4), cystine arylamidase, valine arylamidase, trypsin, chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Does not produce acid from glycogen, erythritol, DL-arabinose, ribose, DL-xylose, adonitol, methyl β-D-xylopyranoside, galactose, glucose, fructose, mannose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, arbutin, lactose, melibiose, inulin, melezitose, raffinose, xylitol, turanose, D-lyxose, D-tagatose, DL-fucose, DL-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. In API 20NE tests, positive for assimilation of glucose as a carbon source, but negative for assimilation of L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, maltose, gluconate, n-capric acid, adipic acid, DL-malate, citrate and phenylacetate. Positive for utilization of erythritol, cellobiose and glycogen as carbon sources (API 50CH). The major respiratory quinones are Q-8, Q-9 and Q-7. The fatty acid profile comprises $C_{16:1}\omega7c$, $C_{16:0}$, $C_{18:1}\omega7c$, $C_{17:1}\omega8c$, $C_{17:0}$, $C_{12:0}$, $C_{14:0}$, $C_{12:0}$ 3-OH, $C_{15:0}$ and $C_{18:0}$.

The type strain is A5K-106^T (=MBIC08328^T =NCIMB 14418^T =NBRC 104231^T), isolated from a sea anemone in Futtsu, Chiba Prefecture in Japan. The DNA G+C content of the type strain is 47 mol%.

Description of *Thalassomonas haliotis* sp. nov.

Thalassomonas haliotis (ha.li.o′tis. N.L. gen. n. *haliotis* of *Haliotis*, systematic name of a genus of abalone).

Cells are Gram-negative, aerobic and rod-shaped, approximately 1.0–2.0 μm long by 0.5–0.7 μm wide. Cells are motile by a subpolar flagellum. Releases a stable, brown, diffusible pigment into the medium. Cytochrome oxidase- and catalase-positive. Optimal growth temperature is 25–30 °C and can grow at 15 °C, but no growth occurs at 8 or 37 °C. The pH range for growth is 6.5–8.5; optimum pH is 8.0. Growth occurs in 1/2 SP5 agar medium supplemented with 0.4–1.4× ASW [NaCl concentration range of 1.2–4.2 % (w/v)]; no growth occurs in NaCl alone. Positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Positive for degradation of casein, DNA, aesculin, gelatin, starch, Tweens (20, 40, 60 and 80) and tyrosine. Nitrate is reduced to nitrite (API 20NE). Acid is produced from *N*-acetylglucosamine, cellobiose, maltose, melibiose, sucrose, trehalose, starch, glycogen, xylitol, gentiobiose, 2-ketogluconate and 5-ketogluconate (API 50CH). Does not decompose alginate, lecithin or urea. Does not produce indole. Negative for arginine dihydrolase, lipase (C4), cystine arylamidase, trypsin, chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Does not produce acid from glycerol, erythritol, DL-arabinose, ribose, DL-xylose, adonitol, methyl β-D-xylopyranoside, galactose, glucose, fructose, mannose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, amygdalin, arbutin, salicin, lactose, inulin, melezitose, raffinose, turanose, D-lyxose, D-tagatose, DL-fucose, DL-arabitol or gluconate (API 50CH). In API 20NE tests, positive for assimilation of glucose as a carbon source, but negative for assimilation of L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, maltose, gluconate, n-capric acid, adipic acid, DL-malate, citrate and phenylacetate. Positive for utilization of glycerol, ribose, amygdalin and cellobiose as carbon sources (API 50CH). The major respiratory quinone is Q-8 (>98 %). The fatty acid profile comprises $C_{16:1}\omega7c$, $C_{16:0}$, $C_{17:1}\omega8c$, $C_{15:0}$, $C_{17:0}$, $C_{18:1}\omega7c$, $C_{14:0}$, $C_{15:1}\omega8c$, $C_{12:0}$ and $C_{12:0}$ 3-OH.

The type strain is A5K-61^T (=MBIC08329^T =NCIMB 14417^T =NBRC 104232^T), isolated from an abalone in Futtsu, Chiba Prefecture in Japan. The DNA G+C content of the type strain is 50 mol%.

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