Microbulbifer agarilyticus sp. nov. and *Microbulbifer thermotolerans* sp. nov., agar-degrading bacteria isolated from deep-sea sediment

Masayuki Miyazaki,[1] Yuichi Nogi,[1] Yukari Ohta,[1] Yuji Hatada,[1] Yoshihiro Fujiwara,[1] Susumu Ito[2] and Koki Horikoshi[1]

Correspondence
Yuichi Nogi
nogiy@jamstec.go.jp

[1]Extremobiosphere Research Center, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), 2-15 Natsushima-cho, Yokosuka 237-0061, Japan

[2]Creative Research Initiative Sousei (CRIS) in Hokkaido University, N21W10 Kita-ku, Sapporo 001-0021, Japan

Nine agar-degrading strains, designated JAMB A3^T, JAMB A7, JAMB A24, JAMB A33, JAMB A94^T, JAMM 0654, JAMM 0793, JAMM 1327 and JAMM 1340, were isolated from deep-sea sediment in Suruga Bay and Sagami Bay and off Kagoshima, Japan. On the basis of 16S rRNA gene sequence analysis, the strains were found to be closely affiliated with members of the genera *Microbulbifer* and *Thalassomonas*. The hybridization values for DNA–DNA relatedness between two of these strains and *Microbulbifer* reference strains were significantly lower than that accepted as the phylogenetic definition of a species. On the basis of their distinct taxonomic characteristics, six of the isolated strains represent two novel species of the genus *Microbulbifer*, for which the names *Microbulbifer agarilyticus* sp. nov. (type strain JAMB A3^T =JCM 14708^T =DSM 19200^T) and *Microbulbifer thermotolerans* sp. nov. (type strain JAMB A94^T =JCM 14709^T =DSM 19189^T) are proposed.

The deep sea is regarded as an extreme environment with high hydrostatic pressures and predominantly low temperatures. Micro-organisms living in the deep sea presumably have developed specific characteristics that allow them to thrive in such an environment and have great biotechnological potential for the production of hydrolytic enzymes (Ferrer *et al.*, 2005; Groudieva *et al.*, 2004; Hung *et al.*, 2005). Agar, which is present in the cell walls of some red algae, is composed of agarose and agaropectins. Agarases have potential applications in the food, cosmetic and medical industries for the production of oligosaccharides from agar (Kobayashi *et al.*, 1997; Yoshizawa *et al.*, 1995). Novel agar-degrading bacteria have been isolated from marine environments (Jean *et al.*, 2006; Kurahashi & Yokota, 2004; Nedashkovskaya *et al.*, 2004). However, the agarases reported to date have not found widespread industrial applications, because of their low levels of activity, stability and productivity. We have searched for novel agarase-producing micro-organisms in several deep-sea environments off Japan and have isolated novel strains of the genera *Microbulbifer* and *Thalassomonas* (Ohta *et al.*, 2004a, b, c, 2005). These genera comprise Gram-negative, facultatively anaerobic members of the *Gammaproteobacteria* (Gonzalez *et al.*, 1997; Macián *et al.*, 2001) and include agar-degrading species that are widely distributed in nature, especially in aquatic environments such as oceans and salt marshes (Yoon *et al.*, 2003a, b, 2004; Jean *et al.*, 2006). In this paper, we describe the results of taxonomic studies on nine of the agar-degrading strains isolated.

Strains JAMB A3^T, JAMB A7, JAMB A24, JAMB A33, JAMB A94^T, JAMM 0654, JAMM 0793, JAMM 1327 and JAMM 1340 were isolated using sterilized mud samplers from various deep sites in Japan, such as a rubbish dump in Suruga Bay, a bacterial mat in Sagami Bay and a location adjacent to whale carcasses in Sagami Bay and off Kagoshima (Table 1). *Microbulbifer elongatus* DSM 6810^T (Yoon *et al.*, 2003b), *Microbulbifer hydrolyticus* DSM 11525^T (Gonzalez *et al.*, 1997), *Microbulbifer salipaludis* JCM 11542^T (Yoon *et al.*, 2003a), *Microbulbifer maritimus* JCM 12187^T (Yoon *et al.*, 2004) and *Thalassomonas agarivorans* JCM 13379^T (Jean *et al.*, 2006) were used as reference strains. These bacteria were maintained on marine agar 2216 (MA; Difco) plates or in marine broth

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains JAMB A3^T and JAMB A94^T are AB158515 and AB124836, respectively.

Fatty acid compositions of strains JAMB A3^T and JAMB A94^T and related strains are presented in a supplementary table available with the online version of this paper.

Table 1. Agar-degrading bacterial strains isolated from deep-sea sites

Strain	Sea area	Latitude	Longitude	Depth (m)	Source	Date	16S rRNA gene sequence accession no.	Reference
JAMB A94^T (=JCM 14709^T =DSM 19189^T)	Suruga Bay, Japan	34° 36.54′ N	138° 34.84′ E	2409	Sediment under rubbish dump	06/04/2001	AB124836	Ohta *et al.* (2004c)
JAMB A3^T (=JCM 14708^T =DSM 19200^T)	Sagami Bay, Japan	35° 00.173′ N	139° 13.141′ E	1174	Red bacterial mat	03/12/2001	AB158515	Ohta *et al.* (2004a)
JAMB A7	Sagami Bay, Japan	35° 00.173′ N	139° 13.141′ E	1174	Red bacterial mat	03/12/2001	AB107975	Ohta *et al.* (2004b)
JAMM 0654	Off Cape Nomamisaki, Japan	31° 20.998′ N	129° 59.156′ E	228	Sediment around bones of dead whale	28/07/2003	AB304799	
JAMB A33 (=DSM 17297)	Off Cape Nomamisaki, Japan	31° 20.998′ N	129° 59.158′ E	228	Blubber of dead whale	28/07/2003	AB162002	Ohta *et al.* (2005)
JAMB A24	Off Cape Nomamisaki, Japan	31° 20.720′ N	129° 59.285′ E	229	Sediment under bones of dead whale	28/07/2003	AB304803	
JAMM 0793	Off Cape Nomamisaki, Japan	31° 20.722′ N	129° 59.283′ E	225	Sediment beside bones of dead whale	27/07/2004	AB304800	
JAMM 1340	Sagami Bay, Japan	35° 04.992′ N	139° 13.013′ E	927	Sediment beside blubber of dead whale	20/01/2006	AB304802	
JAMM 1327	Sagami Bay, Japan	35° 04.989′ N	139° 13.015′ E	928	Sediment under bones of dead whale	20/01/2006	AB304801	

2216 (MB; Difco) were incubated aerobically for 2 or 3 days at the optimum temperature and stored at −80 °C in 15 % (v/v) glycerol. Unless otherwise indicated, physiological tests were performed by using a slight modification (with artificial seawater: 1 × artificial seawater consists of 2.75 % NaCl, 0.07 % KCl, 0.54 % $MgCl_2 . 6H_2O$, 0.68 % $MgSO_4 . 7H_2O$, 0.14 % $CaCl_2 . 2H_2O$, 0.02 % $NaHCO_3$) of the general procedures described by Barrow & Feltham (1993) and Baumann *et al.* (1972). Growth at various temperatures (0–60 °C) in MB was measured. The test strains retained their viability for about 7 days at optimal temperature. Growth at various NaCl concentrations was examined in medium containing 0.5 % peptone (Difco), 0.5 % yeast extract (Difco) and 0.32 % $MgSO_4 . 7H_2O$, with NaCl concentrations of 0–15 % (w/v). The optimal pH and pH range for growth were determined in 0.5 % peptone, 0.5 % yeast extract, 0.32 % $MgSO_4 . 7H_2O$ and 3.0 % NaCl (w/v) at pH 5.0–10.5. Acid production from sugars was assessed using modified OF medium (Hugh & Leifson, 1953) containing 0.5 × artificial seawater, 0.1 % $(NH_4)_2SO_4$, 0.1 % yeast extract (Difco), 0.1 % Tris, 1.4 % NaCl, 1 % test sugar and 0.006 % bromothymol blue (pH adjusted to 7.2 at 25 °C) with incubation at the optimum temperature. Oxidase activity was determined by spreading cell pellets on oxidase test paper (Nissui Pharmaceutical). Gelatinase, protease, amylase, lipase, chitinase and xylanase activities were detected on MA plates, using substrate concentrations of 1 % (w/v). DNase activity was assessed using DNase test agar (Difco). Hydrogen sulfide production from thiosulfate and the production of indole were assessed using sulfide indole motility agar (Nissui Pharmaceutical) stabs prepared with 0.5 × artificial seawater instead of water. Susceptibility to antimicrobial substances was examined on MA using the Sensi-Disc system (Becton Dickinson). Any sign of growth inhibition after 48 h incubation at the optimum temperature was recorded as indicating sensitivity to the respective antimicrobial agent. The following antibiotics (Becton Dickinson) were examined: ampicillin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), neomycin (30 μg), novobiocin (30 μg), penicillin (10 IU), streptomycin (10 μg) and tetracycline (30 μg).

Cellular fatty acids and isoprenoid quinones were analysed. The isolated strains and reference strains were cultured in MB at optimal temperatures. Cells were washed twice with 0.7 % NaCl at 4 °C; this was followed by centrifugation at 8000 ***g*** and freeze-drying. Cellular fatty acids were analysed using a gas–liquid chromatograph/mass spectrometer and isoprenoid quinones were analysed using reversed-phase HPLC according to methods described previously (Miyazaki *et al.*, 2006).

The DNA G+C content was determined using reversed-phase HPLC (Tamaoka & Komagata, 1984). For analysis of relatedness, DNA–DNA hybridization was carried out at 47 °C for 4 h and measured fluorometrically using the method of Ezaki *et al.* (1989).

The 16S rRNA gene was amplified using the PCR method, with primers 27F and 1492R (Lane, 1991). The PCR product was sequenced with the dideoxynucleotide chain-termination method, using a DYEnamic ET terminator (MegaBACE) reagent premix (GE Healthcare UK) and a MegaBACE 1000 DNA sequencer (GE Healthcare UK). Primers 27F, 350F, 520R, 780F, 907R, 1100F and 1492R were used in the gene-sequencing reaction. The 16S rRNA gene sequence was compared with those in the GenBank nucleotide database using online BLAST searches. Nucleotide substitution rates (K_{nuc}; Kimura, 1980) were determined and a distance matrix tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) with the program CLUSTAL_X (Thompson *et al.*, 1997). Alignment gaps and unidentified base positions were not taken into consideration in the calculations. The topology of the phylogenetic tree was evaluated by performing a bootstrap analysis based on 1000 datasets.

To investigate the taxonomic positions of the nine agar-degrading strains isolated, 16S rRNA gene sequence analysis and genomic DNA–DNA hybridization studies were performed. The generally recommended and accepted criteria for delineating bacterial species state that strains with a DNA–DNA relatedness of less than 70 % (as measured by hybridization) or with a 16S rRNA gene sequence dissimilarity greater than 3 % are considered to belong to separate species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002). However, bacterial strains with a difference in 16S rRNA gene sequence of less than 3 % cannot be allocated to the same species without support from DNA–DNA relatedness studies. The 16S rRNA gene sequence clustering demonstrated four groups that showed high levels of internal sequence similarity (99.7–100 %): (i) strains JAMB A24 and JAMB A33, (ii) strains JAMB A94^T, JAMM 1327 and JAMM 1340, (iii) strains JAMB A3^T, JAMM 0654 and JAMM 0793 and (iv) strain JAMB A7. In the neighbour-joining tree, the sequences form a distinct lineage, with species from the genera *Microbulbifer* and *Thalassomonas* as the closest relatives (Fig. 1). Strains JAMB A24 and JAMB A33 fall within the genus *Thalassomonas*. The DNA–DNA hybridization values between *T. agarivorans* JCM 13379^T and JAMB A24 and JAMB A33 were greater than 76 % (Table 2) and therefore these two strains were identified as belonging to the species *T. agarivorans*. Strains JAMB A3^T, JAMM 0654 and JAMM 0793, strains JAMB A94^T, JAMM 1327 and JAMM 1340 and strain JAMB A7 fall within the genus *Microbulbifer*. The results of DNA–DNA hybridization analysis indicated that the novel isolates could be divided into three groups (Table 2) matching those revealed by phylogenetic analyses, with more than 80 % DNA relatedness among the strains in each group. In the 16S rRNA gene sequence analysis, strains JAMB A3^T, JAMM 0654 and JAMM 0793 were closely related to the type strains of *M. salipaludis* (98.0 %) and *M. elongatus* (97.2 %). The respective DNA–DNA reassociation values between strain JAMB A3^T and these type strains were less than 35 % (Table 2) and therefore strains JAMB A3^T, JAMM 0654 and JAMM 0793 were identified as representing a novel species. Strains JAMB A94^T, JAMM 1327 and JAMM 1340 were closely related to the type strain of *M. maritimus* (97.1 % 16S rRNA gene sequence similarity). The DNA–DNA hybridization value between *M. maritimus* JCM 12187^T and strain JAMB A94^T was less than 50 % (Table 2) and therefore strains JAMB A94^T, JAMM 1327 and JAMM 1340 were identified as representing a second novel species. In the 16S rRNA gene sequence analysis, strain JAMB A7 was closely related to the type strains of *M. elongatus* (98.7 %) and *M. salipaludis* (98.3 %) and strain JAMB A3^T (98.2 %). The DNA–DNA hybridization value of 80 % (Table 2) indicated that *M. elongatus* DSM 6810^T and strain JAMB A7 represent the same species.

Physiological and biochemical characteristics of the six strains that represent novel *Microbulbifer* species and the type strains of existing *Microbulbifer* species are shown in Table 3. A comparison of the physiological and biochemical characteristics of the two groups showed some differences with regard to each other and to the

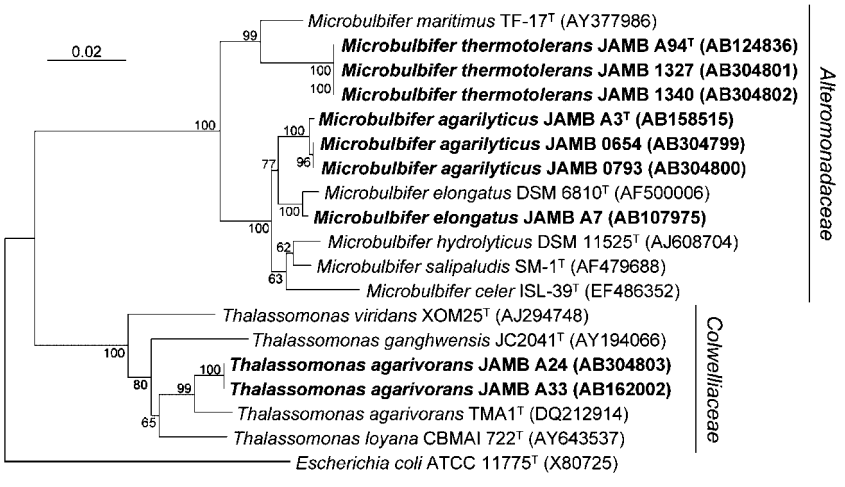

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the positions of the nine novel strains (shown in bold) with respect to members of the genera *Microbulbifer* and *Thalassomonas*. Bar, 0.02 substitutions per nucleotide position. Bootstrap percentages (based on 1000 datasets) are shown at branch points.

Table 2. DNA–DNA reassociation between the isolated strains and closely related *Microbulbifer* and *Thalassomonas* strains

Strain	DNA–DNA reassociation (%) with labelled DNA from:				
	1	2	3	4	5
M. agarilyticus sp. nov.					
1. JAMB A3^T	100	–	–		
JAMM 0793	81	–	–		
JAMM 0654	92	–	–		
M. thermotolerans sp. nov.					
2. JAMB A94^T	22	100	–		
JAMM 1340	–	97	–		
JAMM 1327	–	99	–		
M. elongatus					
3. JAMB A7	17	20	100		
DSM 6810^T	16	22	80		
M. hydrolyticus DSM 11525^T	15	18	25		
M. salipaludis JCM 11542^T	35	22	34		
M. maritimus JCM 12187^T	28	50	41		
T. agarivorans					
4. JAMB A33				100	–
5. JAMB A24				76	100
JCM 13379^T				78	84
T. ganghwensis DSM 13754^T				15	11
T. viridans DSM 15355^T				7	8

Microbulbifer type strains. *M. elongatus* DSM 6810^T and the group of strains represented by JAMB A3^T grew at similar temperatures (10–38 °C) but showed differing results for motility, the ONPG test, hydrolysis of aesculin, reduction of nitrate and DNA G+C content. The group of strains represented by JAMB A94^T grew at higher temperatures (both optimum and maximum), was motile by means of peritrichous flagella, reduced nitrate and utilized the carbohydrate substrates D-fructose, D-mannitol and trehalose.

The whole-cell fatty acid compositions of strains JAMB A3^T and JAMB A94^T and reference strains are shown in Supplementary Table S1 (available in IJSEM Online). The major fatty acids in strain JAMB A3^T were iso-$C_{15:0}$ (isopentadecanoic acid), iso-$C_{17:1}$ (isoheptadecenoic acid) and $C_{18:1}$ (octadecenoic acid), while those for strain JAMB A-94^T were $C_{16:0}$ (hexadecanoic acid), iso-$C_{15:0}$, iso-$C_{17:1}$, $C_{16:1}$ (hexadecenoic acid) and $C_{18:1}$. For each of the isolated strains, the fatty acid profile showed low levels of similarity to those of the reference strains. For example, strain JAMB A3^T contained large amounts of $C_{18:1}$ and strain JAMB A94^T also contained relatively large amounts of $C_{16:1}$ and $C_{18:1}$, slightly less iso-$C_{15:0}$ (isopentadecanoic acid), contained $C_{10:0}$ 3-OH and did not contain $C_{17:1}$ (heptadecenoic acid).

On the basis of the phenotypic, genotypic and phylogenetic data, it is logical to conclude that six of the deep-sea, agar-degrading isolates we studied are members of two novel species within the genus *Microbulbifer*, for which the names *Microbulbifer agarilyticus* sp. nov. (type strain JAMB A3^T) and *Microbulbifer thermotolerans* sp. nov. (type strain JAMB A94^T) are proposed.

Description of *Microbulbifer agarilyticus* sp. nov.

Microbulbifer agarilyticus (a.gar.i.ly′ti.cus. N.L. n. *agarum* agar; N.L. adj. *lyticus* from Gr. adj. *lutikos* dissolving; N.L. part. adj. *agarilyticus* agar-dissolving).

Cells are Gram-negative, non-motile rods, 0.4–0.7 μm wide and 3.5–6.5 μm long. Colonies on MA are slightly irregular, smooth, cream-coloured and 2–4 mm in diameter after 3 days incubation at 37 °C. The optimal temperature for growth is 31–35 °C. No growth occurs at temperatures higher than 40 °C. Cells are able to grow at 7 % NaCl; optimal growth occurs at concentrations of approximately 2–3 %. No growth occurs in the absence of NaCl. Growth occurs at pH 6.5–9.5; the optimal pH is 7.5–8.0. Facultatively anaerobic chemo-organotroph with both respiratory and fermentative types of metabolism. Catalase, cytochrome oxidase, lipase, DNase and ONPG tests are positive. Agar, xylan, chitin, casein, gelatin, starch and Tweens 40 and 80 are hydrolysed. Nitrate is reduced to nitrite. Does not hydrolyse aesculin and does not produce H_2S or indole. Acid is formed oxidatively from cellobiose, D-glucose, maltose and xylose. No acid is produced from L-arabinose, D-fructose, glycerol, *myo*-inositol, D-lactose, D-mannitol, D-mannose, raffinose, L-rhamnose, D-sorbitol, sucrose or trehalose. Discs containing the following antibiotics cause zones of inhibition: ampicillin (10 μg),

Table 3. Differential characteristics of *Microbulbifer* strains

Strains: 1, JAMB A3^T, JAMM 0654 and JAMM 0793 (*M. agarilyticus* sp. nov.); 2, strains JAMB A94^T, JAMM 1327 and JAMM 1340 (*M. thermotolerans* sp. nov.); 3, *M. elongatus* DSM 6810^T; 4, *M. hydrolyticus* DSM 11525^T; 5, *M. salipaludis* JCM 11542^T; 6, *M. maritimus* JCM 12187^T. All strains show the following properties: they are Gram-negative rods that do not form spores, they test positive for catalase, oxidase and hydrolysis of casein, DNA, gelatin, starch, Tween 40 and Tween 80, the major isoprenoid quinone type is Q-8, acid is produced from cellobiose, D-glucose and maltose, hydrogen sulfide and indole are not produced, nitrite is not reduced to nitrogen and acid is not produced from glycerol, D-lactose, *myo*-inositol, D-mannose, D-raffinose, L-rhamnose, D-sorbitol or sucrose.

Characteristic	1	2	3	4	5	6
Cell morphology	Rods	Cocci or rods	Cocci or rods	Rods	Rods	Rods
Colony colour	Cream	Brown	Yellowish brown	Cream	Greyish yellow	Yellowish brown
Motility	−	+	+	−	−	−
Temperature for growth (°C)						
Optimum	31–35	43–49	34	37	37	37
Maximum	35–38	52–54	38	41	45	48
Growth at 10 % NaCl	−	−	−	−	+	+
Agarase	+	+	+	−	+	−
ONPG test	+	−	−	−	+	−
Lipase	+	+	+	−	+	−
Xylanase	+	+	+	+	+	−
Chitinase	+	+	+	+	−	−
Hydrolysis of:						
Aesculin	−	+	+	+	−	−
Tween 20	+ (1/3)	+	−	+	−	+
Nitrate reduction	+	+	−	−	+	+
Acid from:						
L-Arabinose	−	−	−	+	−	−
D-Fructose	−	+	−	−	−	−
D-Galactose	+ (2/3)	−	−	−	−	−
D-Mannitol	−	+	−	−	−	−
Trehalose	−	+	−	−	−	+
Xylose	+	+	+	+	−	−
DNA G+C content (mol%)	55.2–55.3	56.0–56.2	57.1	57.5	58.1	60.2

chloramphenicol (30 μg) and nalidixic acid (30 μg). Discs containing gentamicin (10 μg), streptomycin (10 μg) or tetracycline (30 μg) do not cause any growth inhibition. The DNA G+C content of the type strain is 55.2 mol%. The major isoprenoid quinone is Q-8. The predominant cellular fatty acids are iso-$C_{15:0}$, iso-$C_{17:1}$ and $C_{18:1}$.

The type strain, JAMB A3^T (=JCM 14708^T =DSM 19200^T), was isolated from a deep-sea bacterial mat in Sagami Bay, Japan.

Description of *Microbulbifer thermotolerans* sp. nov.

Microbulbifer thermotolerans (ther.mo.to'le.rans. Gr. n. *thermê* heat; L. pres. part. *tolerans* tolerating; N.L. part. adj. *thermotolerans* heat-tolerating).

Cells are 0.4–0.6 μm wide and 2.5–5.5 μm long; both rods and coccoid cells occur. Cells are Gram-negative and motile by means of peritrichous flagella. Colonies on MA are slightly irregular, smooth, brown-coloured and 2–4 mm in diameter after 3 days incubation at 40 °C. The optimal temperature for growth is 43–49 °C; no growth occurs at temperatures higher than 55 °C. Cells are able to grow with NaCl at 7 %; optimal growth occurs at concentrations of approximately 1–2 %. No growth occurs in the absence of NaCl. Growth occurs at pH 5.5–9.0; the optimal pH is 6.5–7.5. Facultatively anaerobic chemo-organotroph with both respiratory and fermentative types of metabolism. Catalase, cytochrome oxidase, lipase and DNase tests are positive. Agar, xylan, chitin, casein, gelatin, starch, aesculin and Tweens 20, 40 and 80 are hydrolysed. Nitrate is reduced to nitrite. Does not produce H_2S or indole. Acid is formed oxidatively from cellobiose, D-fructose, D-glucose, maltose, D-mannitol, trehalose and xylose. No acid is produced from L-arabinose, D-galactose, glycerol, *myo*-inositol, D-lactose, D-mannose, raffinose, L-rhamnose, D-sorbitol or sucrose. Discs containing the following antibiotics cause zones of inhibition: ampicillin (10 μg), chloramphenicol (30 μg), gentamicin (10 μg), kanamycin (30 μg), neomycin (30 μg), penicillin (10 IU) and streptomycin (10 μg). The DNA G+C content of the type strain is 56.2 mol%. The major isoprenoid quinone is Q-8. The predominant cellular fatty acids are iso-$C_{16:0}$, iso-$C_{15:0}$, iso-$C_{17:1}$, $C_{16:1}$ and $C_{18:1}$.

The type strain, JAMB A94^T (=JCM 14709^T =DSM 19189^T), was isolated from deep-sea sediment from Suruga Bay, Japan.

References

Barrow, G. I. & Feltham, R. K. A. (1993). *Cowan and Steel's Manual for the Identification of Medical Bacteria*, 3rd edn. New York: Cambridge University Press.

Baumann, L., Baumann, P., Mandel, M. & Allen, R. D. (1972). Taxonomy of aerobic marine eubacteria. *J Bacteriol* **110**, 402–429.

Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.

Ferrer, M., Golyshina, O. V., Chernikova, T. N., Khachane, A. N., Martins Dos Santos, V. A., Yakimov, M. M., Timmis, K. N. & Golyshin, P. N. (2005). Microbial enzymes mined from the Urania deep-sea hypersaline anoxic basin. *Chem Biol* **12**, 895–904.

Gonzalez, J. M., Mayer, F., Moran, M. A., Hodson, R. E. & Whitman, W. B. (1997). *Microbulbifer hydrolyticus* gen. nov., sp. nov., and *Marinobacterium georgiense* gen. nov., sp. nov., two marine bacteria from a lignin-rich pulp mill waste enrichment community. *Int J Syst Bacteriol* **47**, 369–376.

Groudieva, T., Kambourova, M., Yusef, H., Royter, M., Grote, R., Trinks, H. & Antranikian, G. (2004). Diversity and cold-active hydrolytic enzymes of culturable bacteria associated with Arctic sea ice, Spitzbergen. *Extremophiles* **8**, 475–488.

Hugh, R. & Leifson, E. (1953). The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram negative bacteria. *J Bacteriol* **66**, 22–26.

Hung, V. S., Hatada, Y., Goda, S., Lu, J., Hidaka, Y., Li, Z., Akita, M., Ohta, Y., Watanabe, K. & other authors (2005). α-Glucosidase from a strain of deep-sea *Geobacillus*: a potential enzyme for the biosynthesis of complex carbohydrates. *Appl Microbiol Biotechnol* **68**, 757–765.

Jean, W. D., Shieh, W. Y. & Liu, T. Y. (2006). *Thalassomonas agarivorans* sp. nov., a marine agarolytic bacterium isolated from shallow coastal water of An-Ping Harbour, Taiwan, and emended description of the genus *Thalassomonas*. *Int J Syst Evol Microbiol* **56**, 1245–1250.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

Kobayashi, R., Takisada, M., Suzuki, T., Kirimura, K. & Usami, S. (1997). Neoagarobiose as a novel moisturizer with whitening effect. *Biosci Biotechnol Biochem* **61**, 162–163.

Kurahashi, M. & Yokota, A. (2004). *Agarivorans albus* gen. nov., sp. nov., a γ-proteobacterium isolated from marine animals. *Int J Syst Evol Microbiol* **54**, 693–697.

Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.

Macián, M. C., Ludwig, W., Schleifer, K. H., Garay, E. & Pujalte, M. J. (2001). *Thalassomonas viridans* gen. nov., sp. nov., a novel marine γ-proteobacterium. *Int J Syst Evol Microbiol* **51**, 1283–1289.

Miyazaki, M., Nogi, Y., Usami, R. & Horikoshi, K. (2006). *Shewanella surugensis* sp. nov., *Shewanella kaireitica* sp. nov. and *Shewanella abyssi* sp. nov., isolated from deep-sea sediments of Suruga Bay, Japan. *Int J Syst Evol Microbiol* **56**, 1607–1613.

Nedashkovskaya, O. I., Suzuki, M., Vancanneyt, M., Cleenwerck, I., Lysenko, A. M., Mikhailov, V. V. & Swings, J. (2004). *Zobellia amurskyensis* sp. nov., *Zobellia laminariae* sp. nov. and *Zobellia russellii* sp. nov., novel marine bacteria of the family *Flavobacteriaceae*. *Int J Syst Evol Microbiol* **54**, 1643–1648.

Ohta, Y., Hatada, Y., Nogi, Y., Li, Z., Zhang, H.-M., Ito, S. & Horikoshi, K. (2004a). Thermostable β-agarase from a deep-sea *Microbulbifer* isolate. *J Appl Glycosci* **51**, 203–210.

Ohta, Y., Hatada, Y., Nogi, Y., Miyazaki, M., Li, Z., Akita, M., Hidaka, Y., Goda, S., Ito, S. & Horikoshi, K. (2004b). Enzymatic properties and nucleotide and amino acid sequences of a thermostable β-agarase from a novel species of deep-sea *Microbulbifer*. *Appl Microbiol Biotechnol* **64**, 505–514.

Ohta, Y., Nogi, Y., Miyazaki, M., Li, Z., Hatada, Y., Ito, S. & Horikoshi, K. (2004c). Enzymatic properties and nucleotide and amino acid sequences of a thermostable β-agarase from the novel marine isolate, JAMB-A94. *Biosci Biotechnol Biochem* **68**, 1073–1081.

Ohta, Y., Hatada, Y., Miyazaki, M., Nogi, Y., Ito, S. & Horikoshi, K. (2005). Purification and characterization of a novel alpha-agarase from a *Thalassomonas* sp. *Curr Microbiol* **50**, 212–216.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

Stackebrandt, E., Frederiksen, W., Garrity, G. M., Grimont, P. A. D., Kämpfer, P., Maiden, M. C. J., Nesme, X., Rosselló-Mora, R., Swings, J. & other authors (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* **52**, 1043–1047.

Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Yoon, J. H., Kim, I. G., Shin, D. Y., Kang, K. H. & Park, Y. H. (2003a). *Microbulbifer salipaludis* sp. nov., a moderate halophile isolated from a Korean salt marsh. *Int J Syst Evol Microbiol* **53**, 53–57.

Yoon, J. H., Kim, H., Kang, K. H., Oh, T. K. & Park, Y. H. (2003b). Transfer of *Pseudomonas elongata* Humm 1946 to the genus *Microbulbifer* as *Microbulbifer elongatus* comb. nov. *Int J Syst Evol Microbiol* **53**, 1357–1361.

Yoon, J. H., Kim, I. G., Oh, T. K. & Park, Y. H. (2004). *Microbulbifer maritimus* sp. nov., isolated from an intertidal sediment from the Yellow Sea, Korea. *Int J Syst Evol Microbiol* **54**, 1111–1116.

Yoshizawa, Y., Ametani, A., Tsunehiro, J., Nomura, K., Itoh, M., Fukui, F. & Kaminogawa, S. (1995). Macrophage stimulation activity of the polysaccharide fraction from a marine alga (*Porphyra yezoensis*): structure-function relationships and improved solubility. *Biosci Biotechnol Biochem* **59**, 1933–1937.