Salinibacterium amurskyense gen. nov., sp. nov., a novel genus of the family *Microbacteriaceae* from the marine environment

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Three bacterial strains were isolated from sea-water samples taken from Amursky Bay of the Gulf of Peter the Great, East Sea. The isolates, designated KMM 3670, KMM 3673^T and KMM 3928, were Gram-positive, non-motile, aerobic, non-spore-forming, irregular, rod-shaped actinobacteria. They grew well at mesophilic temperatures and neutral pH and tolerated up to 10 % NaCl, although salt was not essential for growth. The three strains shared all morphological properties and most physiological characteristics tested. Based on subsequent chemotaxonomic and phylogenetic analyses, it was evident that the strains represented a distinctive taxon in the family *Microbacteriaceae*. The strains contained a major amount of menaquinone with 11 isoprene units; lysine and ornithine were the major cell-wall diamino acids. In 16S rDNA analysis, the three strains formed an independent phylogenetic lineage within the tree encompassed by members of the family *Microbacteriaceae*. It is notable that the tested strains form the first described taxon of *Microbacteriaceae* that inhabits a marine aquatic environment. The three strains evidently merit recognition as a single species of a novel genus in the family *Microbacteriaceae*, for which the name *Salinibacterium amurskyense* gen. nov., sp. nov. (type strain, KMM 3673^T = KCTC 9931^T) is proposed.

The family *Microbacteriaceae* Park *et al.* (1993) emend. Rainey *et al.* 1997 (Stackebrandt *et al.* 1997) consists of actinobacteria that are irregularly shaped, aerobic, non-motile, non-spore-forming and Gram-positive and have G+C-rich genomic DNA. Fifteen genera with validly published names currently comprise the family *Microbacteriaceae* (Table 1), which includes various saprophytic members as well as some pathogenic members of agricultural importance (Yamada & Komagata, 1972; Davis *et al.*, 1984; Zgurskaya *et al.*, 1992, 1993; Groth *et al.*, 1996; Takeuchi *et al.*, 1996; Suzuki *et al.*, 1997; Takeuchi & Hatano, 1998; Kämpfer *et al.*, 2000; Männistö *et al.*, 2000; Evtushenko *et al.*, 2000, 2001, 2002; Tsukamoto *et al.*, 2001; Behrendt *et al.*, 2002).

Cell walls of microbacteria are characterized by the presence of diamino acids such as lysine, ornithine and diaminobutyric acid (DAB), the distribution of which is one of the key chemotaxonomic markers for generic differentiation (Schleifer & Kandler, 1972). Menaquinones with 9–12 isoprene units (MK-9–12) are the predominant isoprenoid quinones and straight or branched, saturated fatty acids constitute the major membrane lipid components (Jones & Collins, 1989; Collins & Bradbury, 1992). The cell-wall peptidoglycan is type B *sensu* Schleifer & Kandler (1972); no mycolic acid is found.

Members of the family *Microbacteriaceae* are widespread throughout natural and artificial habitats, such as plants, soil, dairy products, sewage, mushrooms, insects and groundwater. Some members, e.g. species of the genera *Microbacterium* and *Subtercola*, have been isolated from freshwater samples (Dias & Bhat, 1962; Dias *et al.*, 1962; Männistö *et al.*, 2000). However, there has not yet been any report of the isolation of microbacteria from the marine environment.

During the study of bacterial diversity in a marine bay area, actinobacteria were isolated from sea-water samples by

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Abbreviations: DAB, diaminobutyric acid; KCTC, Korean Collection for Type Cultures, Daejeon, Republic of Korea; KMM, Collection of Marine Microorganisms, Vladivostok, Russia.

The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences of strains KMM 3673^T, KMM 3670 and KMM 3928 are AF539697, AF539698 and AF539699, respectively.

Table 1. Differential characteristics for genera in the family *Microbacteriaceae*

Genera: 1, *Salinibacterium*; 2, *Agreia*; 3, *Agrococcus*; 4, *Agromyces*; 5, *Clavibacter*; 6, *Cryobacterium*; 7, *Curvibacterium*; 8, *Frigoribacterium**; 9, *Leifsonia*; 10, *Leucobacter*; 11, *Microbacterium*; 12, *Mycetocola*; 13, *Okibacterium*; 14, *Plantibacter*; 15, *Rathayibacter*; 16, *Subtercola*. Data from Suzuki *et al.* (1997), Evtushenko *et al.* (2000, 2001, 2002), Kämpfer *et al.* (2000), Männistö *et al.* (2000), Tsukamoto *et al.* (2001) and Behrendt *et al.* (2002).

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Diamino acid†	Lys, Orn	DAB, Orn	DAB	DAB	DAB	DAB	Orn	Lys	DAB	DAB	Orn, Lys	Lys	Lys	DAB	DAB	DAB
Acyl type	Acetyl	Acetyl	Acetyl	Acetyl	Acetyl	ND	Acetyl	Acetyl	ND	Acetyl	Glycolyl	Acetyl	Glycolyl	Acetyl	ND	ND
Major quinones (MK)	11	10	11, 12	11, 12	9, 10	10	9	9	11	11	11, 12, 13, 14	10	10, 11	10, 11	10	9, 10
Major fatty acids‡	S, A, I	S, A, I	S, A, I	S, A, I	S, A, I	S, A, I (12H)	S, A, I (H)	S, A, I	S, A, I	S, A, I	S, A, I	A	S, A, I	S, A, I	S, A, I	A, I
G + C content (mol%)	61	67.0	74	71–76	67–78	65	68–75	71.7	66–73	66.2	65–72	64–65	67	68–70	63–72	64–68
Motility	–	+	–	–	–	–	+, –	+	+	–	+, –	–	–	–	–	–
Optimal temperature (°C)	25–28	28	28	28	28	25–30	28	4–10	24–28	28	28	25	24–27	25	28	15–17

**Frigoribacterium* strains contain unsaturated anteiso fatty acids when grown at low temperatures (<10 °C).

†Lys, Lysine; Orn, D-ornithine.

‡A, Anteiso-methyl-branched; H, cyclohexyl fatty acid; 12H, 12-methyl tetradecenoic acid; I, iso-methyl-branched; S, straight-chain saturated.

using a conventional selective isolation technique and were subjected to further taxonomic investigation. The bacterial isolates shared a number of morphological and physiological properties and exhibited characteristic features that were consistent with their classification in the family *Microbacteriaceae*. From the results of a polyphasic taxonomic study, it is evident that the bacterial isolates merit recognition as a novel taxon within the family *Microbacteriaceae*, for which the name *Salinibacterium amurskyense* gen. nov., sp. nov. is proposed.

Sea-water samples were collected in June 2000 from Amursky Bay of the Gulf of Peter the Great, East Sea, at a depth of 5 m (salinity, 33 ‰; temperature, 15 °C) and strains KMM 3670 (= KCTC 9930), KMM 3673^T (= KCTC 9931^T) and KMM 3928 (= KCTC 9932) were isolated on marine agar 2216 (Difco). After primary isolation and purification, the strains were cultivated at 28 °C on marine agar 2216 (Difco) and stored at –80 °C in marine broth (Difco) supplemented with 20 % (v/v) glycerol.

To determine the oxidative–fermentative metabolism of glucose, bacterial strains were inoculated into modified oxidation–fermentation medium (Hugh & Leifson, 1953; Smibert & Kreig, 1994). Oxidase activity was determined by the tetramethyl *p*-phenylenediamine method (Tarrand & Gröschel, 1982). Catalase activity was tested by addition of 3 % (v/v) H₂O₂ solution to bacterial colonies to check for the appearance of bubbles. Hydrolysis of casein, cellulose, DNA, gelatin and urea, production of acetoin and indole (Voges–Proskauer reaction), nitrate reduction and hydrogen sulfide production were tested according to the methods described by Smibert & Kreig (1994). Degradation of polysaccharides and growth at different NaCl concentrations were tested by using medium that consisted of 5 g peptone, 2 g yeast extract, 1 g glucose, 0.02 g K₂HPO₄ and 15 g agar in 1 l water (1:1 natural sea water and distilled water) at pH 7.5–7.8. For the determination of optimal pH for growth, medium that consisted of 5 g peptone, 2 g yeast extract, 1 g glucose, 0.02 g KH₂PO₄ and 0.05 g MgSO₄·7H₂O in 1 l water (1:1 natural sea water and distilled water) was used and the pH of the medium was adjusted with 1 M solutions of K₃PO₄ and HCl. Hydrolysis of chitin (1 %, w/v) and alginate (sodium salt, 0.1 %) was determined by examining clear zones that developed around colonies. Arginine dihydrolase activity was determined by using the method of Thornley (1960), modified by Lelliott *et al.* (1966). Production of acid from carbohydrates and alcohols was tested on oxidation–fermentation medium for marine bacteria (Leifson, 1963). Susceptibility to antibiotics was examined by the conventional diffusion plate method. Discs were impregnated with the following antibiotics (amounts per disc): ampicillin (10 µg), benzylpenicillin (10 µg), carbenicillin (100 µg), gentamicin (10 µg), kanamycin (30 µg), lincomycin (15 µg), neomycin (30 µg), oleandomycin (15 µg), polymyxin B (300 U), streptomycin (10 µg) and tetracycline (30 µg). All tests were carried out at 28 °C. The G + C content of genomic DNA was determined

by the thermal denaturation method (Marmur & Doty, 1962), in which an *Escherichia coli* strain (G+C content, 50 mol%) served as a reference. DNA extraction, PCR and sequencing of 16S rDNA followed previously described procedures (Kim *et al.*, 1998). The obtained sequence data were aligned with those of representative members of selected genera that belong to the family *Microbacteriaceae* by using PHYDIT version 3.1 (Chun, 2001). Phylogenetic trees were inferred by using suitable programs of the PHYLIP package (Felsenstein, 1993). Phylogenetic distances were calculated from the model of Jukes & Cantor (1969) and trees were constructed following the Fitch–Margoliash (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1981) and neighbour-joining (Saitou & Nei, 1987) algorithms. Bootstrap analysis was performed with 1000 resampled datasets by using the SEQBOOT and CONSENSE programs of PHYLIP. Preparation of cell walls and TLC determination of cell-wall diamino acids followed procedures described by Bousfield *et al.* (1985); HPLC for the quantitative analysis of amino acids was performed by the service at the Korea Basic Science Institute (Daejeon, Republic of Korea). Peptidoglycan acyl type was determined according to the procedure of Uchida & Seino (1997). Menaquinones and phospholipids were analysed by using standard procedures (Minnikin *et al.*, 1984). To determine fatty acid methyl esters, the strains were cultivated on marine agar (Difco) at 25 °C for 24–48 h. Extraction and analysis of cellular fatty acids were performed according to the procedures for the SHERLOCK Microbial Identification system (MIDI).

Isolates KMM 3670, KMM 3673^T and KMM 3928 grew well on marine agar plates. All strains were Gram-positive, non-motile, aerobic, non-spore-forming, irregular, rod-shaped cells. Growth occurred between 4 and 37 °C (optimal temperature, 25–28 °C) and between pH 4.5 and 11.0 (optimal pH range, 7.2–7.5). The strains tolerated up to 10 % NaCl with an optimal concentration of approximately 1–3 %, although salt was not required for growth. All three strains were arginine dihydrolase-, oxidase- and urease-negative, catalase-positive and did not reduce nitrate or produce hydrogen sulfide. Indole and acetoin were not produced (Voges–Proskauer reaction). Growth did not occur on MacConkey agar.

Cell-wall hydrolysates of the strains contained major amounts of lysine and ornithine as diagnostic cell-wall diamino acids; the molar ratios of alanine:glycine:glutamic acid:lysine:ornithine were estimated to be 1.0:1.0:0.4:0.25:1.0. Cell extracts contained MK-11 as the major isoprenoid quinone and also a smaller amount of MK-10. Phosphatidylglycerol and diphosphatidylglycerol were the major characteristic phospholipids. The membrane fatty acids of strain KMM 3673^T consisted of 12-methyl tetradecanoic acid (anteiso-C_{15:0}; 40.4 %), 14-methyl pentadecanoic acid (iso-C_{16:0}; 34.7 %), 12-methyl tridecanoic acid (iso-C_{14:0}; 14.7 %), 13-methyl tetradecanoic acid (iso-C_{15:0}; 6.6 %) and 14-methyl hexadecanoic acid

(anteiso-C_{17:0}; 2.8 %). The DNA G+C content of KMM 3673^T was 61.0 mol%. Chemotaxonomic profiles of the tested strains, together with their morphological properties, were consistent with their classification in the family *Microbacteriaceae* (Jones & Collins, 1989; Collins & Bradbury, 1992).

Comparison of chemotaxonomic properties, including cell-wall diamino acids, isoprenoid quinones, acyl type, fatty acid profiles and DNA G+C content, is a key criterion for the differentiation of genera in the family *Microbacteriaceae* (Table 1; Tsukamoto *et al.*, 2001; Evtushenko *et al.*, 2002). However, generic separation based on chemotaxonomic profiles is not always straightforward; for example, the genera *Leifsonia* and *Leucobacter* cannot be distinguished by using these chemical criteria (Takeuchi *et al.*, 1996; Evtushenko *et al.*, 2000). 16S rDNA sequence analysis gives a more clear-cut view of their relationship, as the two genera form distinctive clades in phylogenetic trees (Evtushenko *et al.*, 2000; Fig. 1). It is therefore clear that combined comparison of chemotaxonomic properties and 16S rDNA sequences enables the separation of each member of the family *Microbacteriaceae*.

Strains KMM 3670, KMM 3673^T and KMM 3928 shared identical 16S rDNA sequences and formed a distinctive phylogenetic lineage in 16S rDNA analysis of 1397 nucleotide positions, with *Leucobacter komagatae* as the nearest neighbour (Fig. 1). However, the branch that consisted of the tested strains and *L. komagatae* was neither supported strongly by bootstrap analysis nor recovered in trees constructed by using other algorithms (Fig. 1). In contrast, the tested strains were related to the clade that includes *Agreia* and *Subtercola* in Fitch–Margoliash and maximum-likelihood trees (data not shown). In the comparison of sequence similarity, the tested strains were related more closely to *Subtercola boreus* DSM 13056^T (95.3 %), *Subtercola pratensis* DSM 14246^T (96.6 %) and *Agreia bicolorata* VKM Ac-1804^T (96.4 %) than to *L. komagatae* NBRC 15245^T (94.8 %). *L. komagatae* NBRC 15245^T was also close to *Okibacterium fritillariae* VKM Ac-2059^T and *Plantibacter flavus* DSM 14012^T (94.8 % similarity with both species). In any of the trees, the tested strains were clearly shown to form an independent clade on their own.

It was notable from the phylogenetic tree that the three members of the genus *Subtercola* failed to form a single phylogenetic clade (Fig. 1). *A. bicolorata* VKM Ac-1804^T was consistently recovered as a clade with *S. pratensis* DSM 14246^T (99.7 % similarity) and *S. boreus* DSM 13056^T (97.0 %) in the phylogenetic trees. The taxonomic position of *Subtercola frigoramans* was in doubt, as it formed an independent lineage in Fitch–Margoliash and maximum-likelihood trees (data not shown). Further studies may be needed to resolve the ambiguity between the genera *Agreia* and *Subtercola*.

Separation of the tested strains from related genera, such as

Fig. 1. Phylogenetic tree showing the relationship between the novel KMM strains and other representative members of the family *Microbacteriaceae*. Asterisks indicate generic branches that were also recovered by using the Fitch–Margoliash and maximum-likelihood algorithms. Numbers at generic nodes and above indicate levels of bootstrap support (%) from 1000 resampled datasets. Bar, 0.01 substitutions per nucleotide position.

Agreia, *Leifsonia*, *Leucobacter* and *Subtercola*, is also supported clearly by comparison of their chemotaxonomic and phenotypic characteristics (Table 1). Members of these related genera contain a major amount of DAB in their cell walls, which is not present in those of the tested strains. The tested strains lack motility, which separates them from *Agreia* and *Leifsonia*.

There are only a few records of isolation of members of the family *Microbacteriaceae* from the aquatic environment, e.g. *Microbacterium barkeri* and *Microbacterium laevaniformans* from activated sludge (Dias & Bhat, 1962; Dias *et al.*, 1962) and *S. boreus* and *S. frigoramans* from groundwater (Männistö *et al.*, 2000). However, all reported isolations were from freshwater samples and there has been no report of isolation from marine samples.

It is still to be confirmed whether the tested strains are indeed indigenous to the particular environment where the

Table 2. Differential phenotypic properties of the strains tested

Strains: 1, KMM 3673^T; 2, KMM 3670; 3, KMM 3928.

Characteristic	1	2	3
Hydrolysis of Tween 80	—	—	+
Acid from:			
Arabinose	—*	+	—*
L-Fucose	—*	+	+
Mannitol	—	+	+
Susceptibility to (µg per disc):			
Benzylpenicillin (10)	—	+	—
Lincomycin (15)	+	+	—
Oleandomycin (15)	+	+	—

*Alkaline reaction.

samples were taken from. However, considering that multiple strains were isolated, all strains grew well in the presence of salt and a few other Gram-positive strains that displayed phenotypic characteristics similar to these strains were also isolated (data not shown), their isolation is not likely to be incidental. From the above evidence, strains KMM 3670, KMM 3673^T and KMM 3928 obviously form a distinct centre of taxonomic variation at the generic level in the family *Microbacteriaceae*; accordingly, a novel genus is proposed to accommodate this group of bacteria from the marine environment, namely *Salinibacterium* gen. nov.

Although strains KMM 3670, KMM 3673^T and KMM 3928 shared an identical 16S rDNA sequence, they could be distinguished from one another by comparison of phenotypic characteristics (Table 2). The strains shared most biochemical properties, such as enzyme activities and degradation of organic compounds, but were different in acid-forming ability from selected carbohydrates. There were no apparent differences in morphological features, as the three strains shared key morphological properties (such as cell shape and staining properties), lacked motility and spore formation and also formed colonies that were phenotypically indistinguishable on agar plates. The above evidence is considered to be sufficient to assign the three strains to a single species, for which the name *Salinibacterium amurskyense* sp. nov. is proposed.

Description of *Salinibacterium* gen. nov.

Salinibacterium (Sa.li.ni.bac.te'ri.um. L. n. *salinum* salt-cellar; Gr. dim. n. *bakterion* a small rod; N.L. neut. n. *Salinibacterium* a saline bacterium).

Gram-positive, high-G + C, non-motile, aerobic, non-spore-forming, irregular rods. Mycolic acid is absent. Metabolism is respiratory, but acids can also be formed from some sugars. Arginine dihydrolase-, oxidase- and urease-negative, but catalase-positive. Nitrate is not reduced and hydrogen sulfide is not produced. Grows in the presence of NaCl. Grows at mesophilic temperatures and around

neutral pH. Cell-wall peptidoglycan is type B; lysine and ornithine are the major diamino acids. Muramic acid contains acetyl residues. Principal phospholipids are phosphatidylglycerol and diphosphatidylglycerol. Cellular fatty acids consist of branched, saturated species; major components are 12-methyl tetradecanoic acid (anteiso-C_{15:0}) and 14-methyl pentadecanoic acid (iso-C_{16:0}). Predominant menaquinone is MK-11; a smaller amount of MK-10 is also present.

The type and only species to date is *Salinibacterium amurskyense*. Member of the family *Microbacteriaceae*; characteristics that differentiate *Salinibacterium* gen. nov. from the other genera of the family are given in Table 1.

Description of *Salinibacterium amurskyense* sp. nov.

Salinibacterium amurskyense (a.mur.sky.en'se. N.L. neut. adj. *amurskyense* referring to Amursky Bay, the geographical location where the organism was first isolated).

General morphological and chemotaxonomic properties are as given in the generic description. Grows between 4 and 37 °C (with optimal growth at 25–28 °C), between pH 4.5 and 11.0 (with optimal growth at around pH 7.2–7.5) and in the presence of 0–10 % NaCl (with optimal growth at 1–3 %). Gelatin and DNA are degraded, but agar, casein, starch, cellulose, alginate, chitin and Tweens 20 and 40 are not. Acid is produced from rhamnose and glycerol. Acid is not produced from acetate, *N*-acetylglucosamine, adonitol, cellobiose, dulcitol, fumarate, galactose, glucose, inositol, lactose, malate, maltose, melibiose, raffinose, sorbitol, sorbose, sucrose or xylose. Susceptible to ampicillin (10 µg per disc), carbenicillin (100 µg) and streptomycin (10 µg), but resistant to gentamicin (10 µg), kanamycin (30 µg), neomycin (30 µg), polymyxin B (300 U) and tetracycline (30 µg). DNA G + C content of the type strain is 61.0 mol%.

The type strain is KMM 3673^T (=KCTC 9931^T). Isolated from sea-water samples taken from Amursky Bay of the Gulf the Peter the Great, East Sea.

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