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Vadicella arenosi gen. nov., sp. nov., a Novel Member of the Class *Alphaproteobacteria* Isolated from Sandy Sediments from the Sea of Japan Seashore

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Abstract A taxonomic study of three aerobic, Gram-negative, non-pigmented, non-motile rod-shaped bacterial strains, designated KMM 9008, KMM 9017, and KMM 9024^T, which were isolated from a sandy sediment sample collected from the Sea of Japan seashore, was undertaken. The DNA–DNA hybridization values of 88–96% obtained between novel strains confirm their assignment to the same species. An analysis of the nearly complete 16S rRNA gene sequences showed that the novel isolates were closely related to each other (99.6–100% sequence similarity) and shared highest sequence similarities to the described genera *Celeribacter* (96.2–95.9%), *Pseudoruegeria* (95.6–94.3%), and *Thalassobacter* (95.2–93.1%) within the class *Alphaproteobacteria*. The major isoprenoid quinone was Q-10, polar lipids were phosphatidylcholine, phosphatidylglycerol, phosphatidic acid, an unknown aminolipid and an unknown lipid as prevalent, and phosphatidylethanolamine was a minor component, and major fatty acids were $C_{18:1}\omega7c$, followed by 11-Methyl $C_{18:1}\omega7c$, $C_{12:1}$ and $C_{10:0}$ 3-OH in all strains. The DNA G+C content of strains KMM 9008, KMM 9017, and KMM 9024^T was in the range of 56.7–60 mol%. Based on distinctive phenotypic characteristics and phylogenetic distance, strain KMM 9024^T (=NRIC 0787^T = JCM 17190^T) represents the type strain of a novel species in a novel genus, for which the name *Vadicella arenosi* gen. nov., sp. nov. is proposed.

The DDBJ/GenBank/EMBL accession numbers of the 16S rRNA gene sequences of strains KMM 9008, KMM 9017, and KMM 9024^T are AB564597, AB564596, and AB564595, respectively.

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Introduction

Bacteria belonging to the *Roseobacter* clade (order *Rhodobacterales*, class *Alphaproteobacteria*) [8] are likely one of the most abundant groups of microbial communities associated with marine and saline environments, being isolated from seawater, sediments, polar sea ice, microbial mats, seaweeds, and animals [3]. In recent years, the *Roseobacter* clade has been considerably expanded with an inclusion of the genera *Thalassobius* [1], *Thalassobacter* [16, 22], *Shimia* [4], *Phaeobacter* and *Marinovum* [18], *Donghicola* [33], *Pseudoruegeria* [12, 34], and *Marivita* [10].

During the investigation of microorganisms inhabiting the shallow sediments of the Sea of Japan *Alphaproteobacteria*-like bacteria were found abundantly in many samples. We have recently proposed two novel genera within class *Alphaproteobacteria* to accommodate some of them [25, 26]. Here we describe the phenotypic and phylogenetic characterization of three strains, designated KMM 9008, KMM 9017, and KMM 9024^T, which were isolated from the shallow sediments of the Sea of Japan. On the basis of the phenotypic and molecular data obtained, a

novel genus and a novel species, *V. arenosi* gen. nov., sp. nov., is described.

Materials and Methods

Strains, Isolation, Cultivation, and Physiological Tests

Strains KMM 9008, KMM 9017, and KMM 9024^T were isolated from a sandy sediment sample, collected from the Sea of Japan seashore, Russia, as described previously [23, 24]. Bacteria were grown aerobically on marine 2216 agar (MA) or marine broth (MB), and stored at −80°C in the MB supplemented with 30% (v/v) glycerol. Motility was determined by the hanging drop method as described [9]. The Gram staining, oxidase and catalase, and hydrolysis of gelatin, casein, chitin, DNA, Tween 80, 40, 20, and H_2S production from thiosulfate were tested according to the standard methods [29]. Acid production from carbohydrates was examined using the oxidation/fermentation medium of Leifson [15]. Requirement for and tolerance of sodium chloride was tested on the artificial sea water (ASW)-based medium using various concentrations of NaCl ranging 0–20%, supplemented with 10.0 g l^{-1} Bacto peptone, 2.0 g l^{-1} yeast extract, 0.028 g l^{-1} $FeSO_4$, and 15.0 g l^{-1} agar. ASW was prepared according to the composition as described [2]. In addition, bacterial growth was tested on above medium, containing NaCl alone without any of the other sea salts components, $MgCl_2$, Mg_2SO_4, KCl, or $CaCl_2$. The ability of the strains to grow in the presence of organic substrates as a sole carbon and energy source was tested for 3 weeks on the ASW-based medium supplemented with 1 g NH_4Cl l^{-1}, 0.5 g yeast extract l^{-1}, and 0.4% carbon source. Growth was considered as negative if it was equal, or lesser, compared to that of that of the source-free control.

Growth at different temperatures (4–40°C) and pH values (4.0–12.0), and antibiotic resistance were studied as described previously [23, 24]. In addition, biochemical tests were carried out using API ZYM, API 32GN, and API 20NE test kits (bioMérieux) according to the manufacturer's instructions, except that the cultures were suspended in ASW.

Lipid Analyses

For the analyses of polar lipids, cellular fatty acids and respiratory lipoquinone, the strains were grown on MA and MB at 28°C for 3 days. Lipids were extracted as described [7]. Two-dimensional thin layer chromatography of polar lipids was carried out on Silica gel 60 F_{254} (10 × 10 cm, Merck, Germany) using chloroform–methanol–water (65:25:4, v/v) for the first direction, and chloroform–methanol–acetic acid–water (80:12:15:4, v/v) for the second one [5].

Fatty acid methyl esters (FAMEs) were prepared according to a procedure of the Microbial Identification System (MIDI) [27]. The analysis of FAMEs was performed using the GC-17A chromatograph (Shimadzu, Kyoto, Japan) equipped with a capillary column (30 m × 0.25 mm I.D.) coated with Supecowax-10 and SPB-5 phases (Supelco, USA). Identification of FAMEs was accomplished by equivalent chain length values and comparing the retention times of the samples to those of standards. In addition, FAMEs were analyzed using a GLC-MS Shimadzu GC-MS model QP5050 (column MDM-5S, the temperature program from 140 to 250°C, at a rate of 2°C/min). Isoprenoid quinones were extracted and analyzed by HPLC as described [5, 19].

Production of bacteriochlorophyll α (Bchl α) was spectrophotometrically tested in methanolic extracts of cells grown on MA and MB in the dark as described [14].

Isolation of DNA and DNA–DNA Hybridization

The DNA base composition was determined as described [17, 20]. DNA–DNA hybridization between strains KMM 9008, KMM 9017, and KMM 9024^T was carried out by the photobiotin-labelled DNA probe microplate method [6].

DNA Sequence Analysis

The 16S rRNA gene sequences of 1440, 1445, and 1439 nucleotides were determined for strains KMM 9008, KMM 9017, and KMM 9024^T, respectively, as described [28]. The sequences obtained were compared with 16S rRNA gene sequences retrieved from the DDBJ/GenBank/EMBL databases by using the FASTA program [21].

Phylogenetic analysis of 16S rRNA gene sequences was performed using the software package MEGA 4 [30] after multiple alignments of data by CLUSTALX (version 1.83) [31]. Phylogenetic trees were constructed by the neighbor-joining and maximum-parsimony methods and the distances were calculated according to the Kimura two-parameter model. The robustness of phylogenetic trees was estimated by the bootstrap analysis of 1,000 replicates.

Results and Discussion

Comparative 16S rRNA gene sequence analysis showed that the novel isolates were closely related to each other (99.6–100% sequence similarity) and formed a separate branch within the class *Alphaproteobacteria* (Fig. 1). In the neighbor-joining and maximum-parsimony phylogenetic trees based on 16S rRNA gene sequences strains KMM

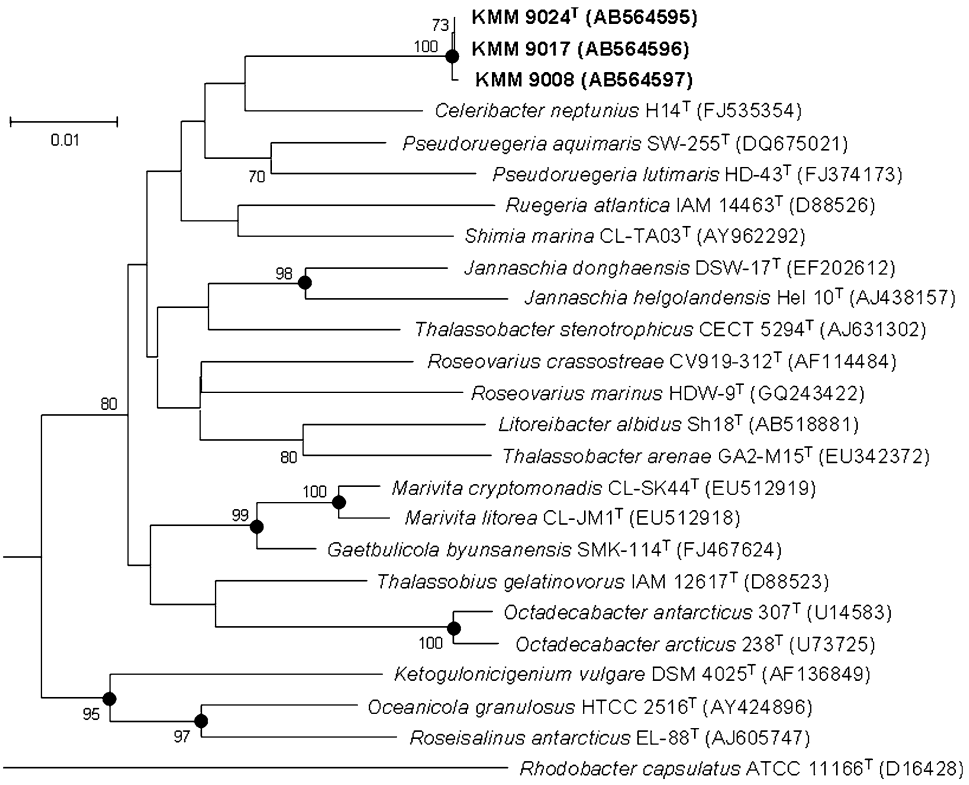

Fig. 1 Neighbor-joining phylogenetic tree based on the 16S rRNA gene sequences available from the GenBank/EMBL/DDBJ databases (accession numbers are given in *parentheses*) showing relationship of strains KMM 9008, KMM 9017, and KMM 9024^T and related genera of the class *Alphaproteobacteria*. Bootstrap values based on 1,000 replications are given as percentages at the branching points. *Numbers* indicate percentages greater than 70%. *Filled circles* indicate that the corresponding nodes were recovered in the tree generated with maximum-parsimony algorithm. *Bar*, 0.01 substitutions per nucleotide position

9008, KMM 9017, and KMM 9024^T were positioned as a distinct phylogenetic line adjacent to *Celeribacter neptunius*. The three strains shared highest sequence similarities to the members of genera, *Celeribacter* (96.2–95.9%), *Pseudoruegeria* (95.6–94.3%), and *Thalassobacter* (95.2–93.1%). The low sequence similarities found relatively to validly described species of the *Roseobacter* clade demonstrate that novel strains can be considered to represent a novel genus. Strains KMM 9008, KMM 9017, and KMM 9024^T showed a high level of DNA relatedness (88–96%), confirming their affiliation to the same species in accordance with the discriminative value of 70% recognized for the delineation of bacterial species [32].

Cultural, physiological, and metabolic properties of strains KMM 9008, KMM 9017, and KMM 9024^T are listed in Table 1 and in the genus and species descriptions. Novel strains were similar in their phenotypic characteristics, except for differences in carbohydrate utilization patterns and sensitivity to antibiotics (Table 1), and in their chemotaxonomic traits (Table 2). They gave negative results for the assimilation of carbon sources in API 20NE tests, but could utilize a number of substrates, preferring carbohydrates and organic acids, but not amino acids, when they were cultivated on ASW-based media containing substrates as a sole carbon and energy source.

The analysis of respiratory lipoquinone revealed ubiquinone Q-10, and polar lipids included PC, PG, PA, an unknown aminolipid and an unknown lipid as the major components and PE in a minor amount in all strains (Supplementary material). Chemotaxonomic properties obtained for the novel isolates (ubiquinone Q-10, the predominance of $C_{18:1\omega7c}$, 11-Methyl $C_{18:1\omega7c}$, 3-OH $C_{10:0}$, and the presence of PC, PG, and PE) are in line with characteristics reported for the members of the *Roseobacter* clade. Together with the common characteristics, novel strains revealed some differences in their polar lipid and fatty acid profiles as compared with those of phylogenetically related bacteria. As seen from Table 2, unlike *Thalassobacter stenotrophicus* [16], *Thalassobacter arenae* [13], *Pseudoruegeria aquimaris* [34], and *Pseudoruegeria lutimaris* [12], the novel strains did not contain DPG, nor did they contain PL and GL which were found in *P. aquimaris* and *P. lutimaris*. Novel strains differed from *T. stenotrophicus* and *P. aquimaris* in the absence of $C_{19:1}$ or/and cyclo $C_{19:0}$; from *P. lutimaris* in the presence of 3-OH $C_{10:0}$ and in the absence of 3-OH $C_{12:}0$; and from *T. arenae* in the absence of $C_{20:1}$ and $C_{18:1}$. In contrast to their closest relative, *C. neptunius* [11], the novel strains contained 11-Methyl $C_{18:1\omega7c}$, a high amount of 3-OH $C_{10:0}$, $C_{12:1}$, a small amount of $C_{20:1}$, PA and L, but did not contain LPE (Table 2). The DNA G+C contents of 56.7, 57, and 60 mol% in strains KMM 9008, KMM 9017, and KMM 9024^T, respectively, were close to the G+C values reported for *C. neptunius* [11], *T. stenotrophicus* [16], and *T. arenae* [13], but clearly distinguished from those of *P. aquimaris* [34] and *P. lutimaris* [12] (Table 1).

Table 1 Differential characteristics of *V. arenosi* gen. nov., sp. nov. and related members of the *Alphaproteobacteria*

Characteristic	1	2	3	4	5	6	7	8
Pigmentation	None	None	None	None	Greyish yellow	Greyish yellow	Salmon pink	Beige-pinkish[a]
Bchl α	−	−	−	−	−	ND	+	ND
Oxidase	+	+	+	−	+	+	+	+
Motility	−	−	−	+	−	−	+	+
Growth at								
4°C	+	(+)	(+)	−	−	−	−	+[a]
37°C	(+)	+	(+)	−	+	+	+	−
Nitrate reduction	+	+	(+)	+	−	−	−	−
Utilization of								
D-galactose	(+)	−	−	ND	+	(+)	−	+
Sucrose	−	−	−	+	+	+	−	−
D-fructose	−	−	(+)	+	+	+	−	+
Cellobiose	+	+	+	+	+	+	−	−
D-xylose	−	−	−	+	+	(+)	−	+
D-mannose	−	−	−	ND	+	+	−	+
Glycerol	(+)	−	(+)	ND	ND	ND	−	+
D-mannitol	−	−	−	ND	ND	ND	+	+
β-galactosidase	+	+	+	−	+	+	−	+
Hydrolysis of								
Aesculin	+	+	+	+	+	−	+	+
Urea	−	−	−	+	−	−	ND	−
Tween 80	−	−	−	(+)	−	−	−	−[a]
DNA	+	(+)	(+)	−	ND	ND	−	−
L-tyrosine	−	−	−	ND	−	−	−	+[a]
DNA GC content (mol%)	60	57	56.7	59	67.0	73.5	59	56

Strain/species: *1* KMM 9024^T; *2* KMM 9017; *3* KMM 9008 (data from present study); *4 C. neptunius* data taken from [11]; *5 P. aquimaris* [34]; *6 P. lutimaris* [12]; *7 T. stenotrophicus* [13, 16]; *8 T. arenae* [13]. +, Positive reaction; −, negative reaction; (+), weak reaction; *ND* not determined. All bacteria are positive for D-glucose utilization and negative for gelatin and starch hydrolysis

[a] Data obtained from present study

The phylogenetic distinctiveness found for strains KMM 9008, KMM 9017, and KMM 9024^T was supported by a combination of phenotypic characteristics differentiating the novel strains from related *Alphaproteobacteria* (Table 1). Main phenotypic differential characteristics between novel strains and their closest relative *C. neptunius* [11] were the absence of flagella and non-motility, growth at 4 and 37°C, the presence of oxidase and β-galactosidase, positive (or weakly positive) reaction for hydrolysis of DNA, and negative reactions for hydrolysis of Tween-80 and urea, and utilization of sucrose and D-xylose (Table 1). Based on distinctive phenotypic and chemotaxonomic characteristics and phylogenetic evidence, strains KMM 9008, KMM 9017, and KMM 9024^T are considered to represent a novel genus and species, for which the name *V. arenosi* gen. nov., sp. nov. is proposed.

Description of *Vadicella* gen. nov

Vadicella (Va.di. cel'la. L. n. *vadum*, a shallow place, a shallow; L. fem. n. *cella*, a chamber, a cell; N. L. fem. n., *Vadicella*, a cell from a shallow place)

Gram-negative, strictly aerobic, oxidase- and catalase-positive, rod-shaped bacteria. Chemoorganoheterotrophic. Sodium ions are essential for growth. The predominant isoprenoid quinone is Q-10. Bchl α is not produced. Polar lipids include phosphatidylcholine, phosphatidylglycerol, phosphatidic acid, an unknown aminolipid, and an unknown lipid as major and phosphatidylethanolamine as a minor component. The major fatty acids were $C_{18:1\omega7c}$, followed by 11-Methyl $C_{18:1\omega7c}$, $C_{12:1}$, and $C_{10:0}$ 3-OH. Isolated from the marine environments. On basis of the 16S rRNA gene sequence analysis the genus represents a separate branch within the *Alphaproteobacteria*, closely

Table 2 Fatty acid composition (%) and polar lipids of *V. arenosi* gen nov., sp., nov. and related members of the *Alphaproteobacteria*

Fatty acid	1	2	3	4	5	6	7	8
$C_{10:0}$ 3-OH	7.94	8.65	9.17	0.6	2.9	–	3.15	3.7
$C_{12:0}$ 3-OH	–	–	–	–	–	2.5	–	–
$C_{12:1}$	9.72	7.95	11.91	–	–	–	–	–
$C_{16:0}$	1.09	1.17	1.21	3.1	1.4	8.9	0.42	10.4
$C_{18:1\omega7c}$	52.84	58.45	52.84	82.4	72.9	88.6	78.09	74.3
$C_{18:1}$	8.80	7.36	8.05	5.1	0.8	–	1.23	–
11-Methyl $C_{18:1\omega7c}$	13.62	13.57	14.32	–	2.8	–	6.96	5.9
$C_{18:0}$	1.77	2.26	1.86	3.8	6.6	–	2.59	1.2
Cyclo $C_{19:0}$	–	–	–	–	5.9	–		–
$C_{19:1}$/cyclo$C_{19:0}$	–	–	–	–		–	1.47	–
ECL 11.799	–	–	–	–	2.8	–	3.55	3.0
$C_{20:1}$	1.02	0.60	0.66	–	0.9	–	1.04	–
PC[a]	+	+	+	+	–	+	+	+
PE	+	+	+	+	+	+	+	+
PG	+	+	+	+	+	+	+	+
DPG	–	–	–	–	+	+	+	+
AL	+	+	+	+	–	+	–	–
PL	–	–	–	–	+	+	+	–
L	+	+	+	–	–	+	–	–
PA	+	+	+	–	–	–	–	–
LPE	–	–	–	+	–	–	–	–
GL	–	–	–	–	+	+	–	–

Strain/species: *1* KMM 9024^T; *2* KMM 9017; *3* KMM 9008 (data from present study); *4 C. neptunius* data taken from [11]; *5 P. aquimaris* [34]; *6 P. lutimaris* [12]; *7 T. stenotrophicus* [13, 16]; *8 T. arenae* [13]

[a] *PC* phosphatidylcholine; *PE* phosphatidylethanolamine; *PG* phosphatidylglycerol; *DPG* diphosphatidylglycerol; *PA* phosphatidic acid; *LPE* lysophosphatidylethanolamine; *AL* an unknown aminolipid; *L* an unknown lipid; *PL* an unknown phospholipid; *GL* glycolipid

related to the genera *Celeribacter*, *Pseudoruegeria*, and *Thalassobacter*. The type species of the genus is *V. arenosi*.

Description of *V. arenosi* gen. nov., sp. nov.

V. arenosi (a.re.no'si. L. gen. n. *arenosi*, of a sandy place, dwelling in marine sand)

In addition to properties given in the genus description the species is characterized as follows: cells are rods 0.6–0.8 μm in diameter and 2.5–4.5 μm in length. Non-motile. Strains formed non-pigmented, translucent, smooth and shiny colonies with the regular edges of 2–3 mm in diameter on MA. Required NaCl for growth; growth occurred at concentrations of 1–7% NaCl (w/v) and is optimal in 3–4%, weak growth in 0.5–1% NaCl, and no growth in 8% NaCl. Grow in/on basal media, containing NaCl alone without any of sea salts components, $MgCl_2$, KCl, $CaCl_2$, $MgSO_4$ addition. The temperature range for growth was 4–37°C with optimum growth at 25–30°C; growth at 4 and 37°C is weak and strain-dependent, and no growth occurred above 37°C. The pH range is 5.5–9.5 with an optimum of 7.0–8.0. Positive or weakly positive for nitrate reduction. Negative for hydrolysis of casein, starch, gelatin, chitin, L-tyrosine, Tweens 20, 40, 80, and for H_2S production. On the L-tyrosine containing medium, strains did not produce any pigments or/and clearance zones. Hydrolysis of DNA is positive for the type strain and weakly positive for the rest strains. No acid production observed from D-glucose, D-maltose, D-galactose, sucrose, D-lactose, D-mannose, D-cellobiose, D-xylose, L-arabinose, L-rhamnose, D-melibiose, D-ribose, fructose, L-sorbose, raffinose, *N*-acetylglucosamine, glycerol, D-*myo*-inositol, and D-mannitol. According to the API 20NE, positive for PNPG test, hydrolysis of aesculin and nitrate reduction, and negative for indole production, D-glucose acidification under anaerobic conditions, arginine dihydrolase, urease, gelatin hydrolysis, and assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, maltose, D-gluconate, caprate, adipate, L-malate, citrate, and phenylacetate. According to the API 32 GN, assimilation of 3-hydroxybutyric acid and lactic acid is strain-dependent (the reaction of the type strain is positive for 3-hydroxybutyric acid and negative for assimilation of lactic acid); negative for assimilation of D-ribose, D-sucrose, D-maltose, itaconic acid, suberic acid, sodium malonate, sodium acetate, inositol, D-melibiose, L-proline, L-rhamnose, *N*-acetylglucosamine, L-alanine, potassium 5-ketogluconate, glycogen, 3-hydroxybenzoic acid, L-serine, D-mannitol, D-glucose, salicin, L-fucose, D-sorbitol, L-arabinose, propionic acid, capric acid, valeric acid, trisodium citrate, L-histidine, potassium 2-ketogluconate, and 4-hydroxybenzoic acid. According to the API ZYM tests, positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, β-galactosidase, α-glucosidase, and β-glucosidase, and negative for lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. Production of acid phosphatase and naphthol-AS-BI-phosphohydrolase is strain-dependent (the reaction of the type strain for acid phosphatase is positive, and negative for naphthol-AS-BI-phosphohydrolase).

Utilized D-glucose, maltose, D-cellobiose, citrate, acetate, fumarate, and malate as a carbon and energy source; some strains could weakly utilized glycerol, fructose, D-galactose; did not utilize L-rhamnose, L-arabinose, D-mannose, D-xylose, sucrose, raffinose, D-mannitol, inositol, aminoacetic acid, L-tyrosine, ornithine, DL-leucine, L-α-alanine, DL-β-phenylalanine, DL-lysine, L-arginine,

L-asparagine, and L-methionine. A detailed fatty acid composition and polar lipids are displayed in Table 2 and Supplementary Fig. 1. All strains were susceptible to antibiotics (content per disk): ampicillin (10 μg), benzylpenicillin (10 U), carbenicillin (100 μg), gentamicin (10 μg), rifampicin (5 μg), streptomycin (30 μg), vancomycin (30 μg), oxacillin (10 μg), neomycin (30 μg), kanamycin (30 μg), oleandomycin (15 μg), erythromycin (15 μg), cephazolin (30 μg), cephalexin (30 μg), tetracycline (30 μg), and chloramphenicol (30 μg). Sensitivity to nalidixic acid (30 μg), lincomycin (15 μg), ofloxacin (5 μg), and polymyxin (300 U) is strain-dependent; the type strain is susceptible to lincomycin (15 μg), ofloxacin (5 μg), and polymyxin (300 U) and resistant to nalidixic acid (30 μg). The DNA G+C content was 56.7–60 mol% (T_m). Isolated from a sandy sediment sample collected from the Sea of Japan seashore, Russia. The type strain of the species is strain KMM 9024^T (=NRIC 0787^T = JCM 17190^T).

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