Roseovarius aestuarii sp. nov., isolated from a tidal flat of the Yellow Sea in Korea

Jung-Hoon Yoon, So-Jung Kang and Tae-Kwang Oh

Correspondence

Jung-Hoon Yoon
jhyoon@kribb.re.kr

Korea Research Institute of Bioscience and Biotechnology (KRIBB), PO Box 115, Yusong, Taejeon, Republic of Korea

A Gram-negative, motile, ovoid to rod-shaped bacterial strain, designated strain SMK-122^T, was isolated from a Yellow Sea tidal flat located on the coast of Korea. Strain SMK-122^T grew optimally at pH 7.0–8.0 and 30 °C. It contained Q-10 as the predominant ubiquinone and possessed C_{18:1ω7c} and C_{16:0} as the major fatty acids. The DNA G+C content was 58.6 mol%. A phylogenetic analysis based on 16S rRNA gene sequences showed that strain SMK-122^T fell within the genus *Roseovarius*, being closest to *Roseovarius nubinhibens* ISM^T; the sequence similarities with respect to *Roseovarius* species ranged from 94.9 to 97.3%. The mean value for DNA–DNA relatedness between strain SMK-122^T and *Rva. nubinhibens* DSM 15170^T was 13%. Differential phenotypic properties of SMK-122^T, together with its phylogenetic and genetic distinctiveness, revealed that this strain is distinct from recognized *Roseovarius* species. On this basis, strain SMK-122^T represents a novel species of the genus *Roseovarius*, for which the name *Roseovarius aestuarii* sp. nov. is proposed. The type strain is SMK-122^T (=KCTC 22174^T =CCUG 55325^T).

The genus *Roseovarius*, a member of the *Alphaproteobacteria*, was proposed by Labrenz *et al.* (1999) and currently includes *Roseovarius tolerans* (Labrenz *et al.*, 1999), *Roseovarius nubinhibens* (González *et al.*, 2003), *Roseovarius crassostreae* (Boettcher *et al.*, 2005) and *Roseovarius mucosus* (Biebl *et al.*, 2005). In this study, we report the taxonomic characterization of a *Roseovarius*-like bacterial strain, SMK-122^T, isolated from a tidal flat sediment of the Yellow Sea, Korea.

Strain SMK-122^T was isolated from a Yellow Sea tidal flat, collected near Saemankum, Korea. A standard dilution technique was used for the isolation: the sample was suspended and diluted in a 0.85% (w/v) NaCl solution, plated on marine agar 2216 (MA; Difco) and incubated at 25 °C. The type strains of the four *Roseovarius* species, *Rva. tolerans* DSM 11457^T, *Rva. nubinhibens* DSM 15170^T, *Rva. crassostreae* DSM 16950^T and *Rva. mucosus* DSM 17069^T, which were used as reference strains for phenotypic characterization and DNA–DNA hybridization, were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. The morphological, physiological and biochemical characteristics of strain SMK-122^T were investigated using routine cultivation on MA at 30 °C. The cell morphology was examined by using light microscopy (E600; Nikon) and transmission electron microscopy. Flagellation was determined using a Philips CM-20 transmission electron microscope with cells from exponentially growing cultures: for this purpose, the cells

were negatively stained with 1% (w/v) phosphotungstic acid and the grids were examined after being air-dried. Growth under anaerobic conditions was determined after incubation in a Forma anaerobic chamber on MA and MA supplemented with potassium nitrate (0.1%, w/v), both of which had been prepared anaerobically using nitrogen. Growth in the absence of NaCl was investigated using trypticase soy broth prepared according to the formula of the Difco medium except that NaCl was excluded. Growth at various NaCl concentrations (0.5% and 1.0–10.0%, w/v, in increments of 1.0%) was investigated in marine broth 2216 (MB; Difco) or trypticase soy broth (Difco). Growth at various temperatures (4, 10, 15, 20, 22, 25, 28 and 30–40 °C, in increments of 1 °C) was measured on MA. For *in vivo* pigment-absorption spectrum analysis, strain SMK-122^T was cultivated aerobically in the dark at 30 °C in MB. The culture was washed twice by centrifugation using a MOPS buffer (MOPS/NaOH, 0.01 M; KCl, 0.1 M; MgCl₂, 0.001 M; pH 7.5) and disrupted by sonication with a Branson Sonifier S-450. After removal of cell debris by centrifugation, the absorption spectrum of the supernatant was examined on a Beckman Coulter DU800 spectrophotometer. Catalase and oxidase activities and hydrolysis of casein, starch and Tweens 20, 40, 60 and 80 were determined as described by Cowan & Steel (1965). Hydrolysis of hypoxanthine, tyrosine and xanthine was tested on MA using the substrate concentrations described by Cowan & Steel (1965). Hydrolysis of aesculin, gelatin and urea and reduction of nitrate were investigated as described previously (Lányi, 1987) with the modification that artificial seawater was used

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for the preparation of media. The artificial seawater contained (l^{-1} distilled water) 23.6 g NaCl, 0.64 g KCl, 4.53 g $MgCl_2 \cdot 6H_2O$, 5.94 g $MgSO_4 \cdot 7H_2O$ and 1.3 g $CaCl_2 \cdot 2H_2O$ (Bruns *et al.*, 2001). H_2S production was tested as described previously (Bruns *et al.*, 2001). Susceptibility to antibiotics was investigated on MA plates by using antibiotic discs with the following amounts:

polymyxin B (100 U), streptomycin (50 μ g), penicillin G (20 U), chloramphenicol (100 μ g), ampicillin (10 μ g), cephalothin (30 μ g), gentamicin (30 μ g), novobiocin (5 μ g) and tetracycline (30 μ g). Acid production from carbohydrates was tested as described by Leifson (1963). The utilization of various substrates for growth was determined as described by Baumann & Baumann (1981), using

Table 1. Differential phenotypic characteristics of strain SMK-122^T and type strains of *Roseovarius* species

Strains: 1, SMK-122^T; 2, *Rva. tolerans* DSM 11457^T; 3, *Rva. nubinihibens* DSM 15170^T; 4, *Rva. crassostreae* DSM 16950^T; 5, *Rva. mucosus* DSM 17069^T. Data were obtained in this study unless indicated. All of the strains are positive for the following: catalase and oxidase activities, growth at 37 °C (data for reference type strains taken from the papers indicated in the footnote), utilization of acetate, esterase (C4) and esterase lipase (C8) and susceptibility to ampicillin, carbenicillin, cephalothin, chloramphenicol, gentamicin, kanamycin, neomycin, penicillin G and streptomycin. All of the strains are negative for the following: Gram stain (data for reference type strains taken from the papers indicated in the footnote), hydrolysis of aesculin, gelatin, starch, Tween 80 and urea, utilization of D-glucose, D-fructose, D-galactose, cellobiose, D-mannose, trehalose, D-xylose, L-arabinose, sucrose, maltose, benzoate and salicin, acid production from L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, melezitose, melibiose, raffinose, L-rhamnose, sucrose, trehalose, D-xylose, *myo*-inositol and D-sorbitol, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase and susceptibility to lincomycin, polymyxin B and tetracycline. +, Positive; –, negative; w, weakly positive; ND, no data available.

Characteristic	1	2	3	4	5
Cell shape	Ovoid to rod-shaped	Rods ^{a*}	Rods ^b	Ovoid to rod-shaped ^c	Rods ^d
Motility	+	+/- ^a	+ ^b	- ^c	- ^d
Colony colour on MA	Cream	Cream	Cream	Cream	Pale yellow
Bacteriochlorophyll <i>a</i> production	–	+ ^a	ND	- ^c	Trace ^d
Growth at:					
4 °C	–	+ ^a	- ^b	- ^c	ND
10 °C	+	+ ^a	+ ^b	ND	ND
40 °C	–	+ ^a	+ ^b	ND	+ ^d
Reduction of nitrate to nitrite	+	–	–	+	–
Hydrolysis of:					
Casein	+	–	–	–	–
Hypoxanthine	+	–	–	+	–
Xanthine	–	–	–	w	w
Tyrosine	–	–	–	+	–
Tween 20	–	–	w	–	+
Tween 40	–	–	–	w	+
Tween 60	–	–	–	w	+
Utilization of:					
Citrate	–	–	–	+	–
Formate	–	–	–	–	+
L-Malate	+	+	–	+	+
Pyruvate	+	+	–	+	+
Succinate	+	–	–	+	+
L-Glutamate	–	–	–	+	+
Acid production from:					
D-Ribose	–	–	–	–	+
D-Mannitol	–	+	–	–	–
Enzyme activities (API ZYM)					
Alkaline phosphatase	w	+	+	+	–
Leucine arylamidase	–	+	+	+	+
Susceptibility to antibiotics					
Novobiocin	–	–	–	+	–
Oleandomycin	–	–	–	+	–

*Data obtained from: *a*, Labrenz *et al.* (1999); *b*, González *et al.* (2003); *c*, Boettcher *et al.* (2005); *d*, Biebl *et al.* (2005).

supplementation with 2 % (v/v) Hutner's mineral salts solution (Cohen-Bazire *et al.*, 1957) and 1 % (v/v) vitamin solution (Staley, 1968). Enzyme activities were determined by using the API ZYM system (bioMérieux).

Cell biomass for DNA extraction and for isoprenoid quinone analysis was obtained from cultures grown in MB at 30 °C. Chromosomal DNA was isolated and purified according to the method described by Yoon *et al.* (1996), except that RNase T1 was used in combination with RNase A to minimize the contamination of RNA. The 16S rRNA gene was amplified by using a PCR with two universal primers, 5'-GAGTTTGATCCTGGCTCAG-3' and 5'-AGA-AAGGAGGTGATCCAGCC-3', as described previously (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed as described by Yoon *et al.* (2003). Isoprenoid quinones were analysed as described by Komagata & Suzuki (1987) using reversed-phase HPLC. For cellular fatty acid analysis, cell mass of strain SMK-122^T and the type strains of the four *Roseovarius* species was harvested from MA plates after cultivation for 3 days at 30 °C. The fatty acids were extracted and fatty acid methyl esters were prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The DNA G+C content was determined by using the method of Tamaoka & Komagata (1984) with the modification that DNA was hydrolysed using nuclease P1 (Sigma) and the resultant nucleotides were analysed by reversed-phase HPLC. DNA-DNA hybridization was performed fluorometrically according to the method of Ezaki *et al.* (1989), using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained in each sample were excluded and the means of the remaining three values were quoted as DNA-DNA relatedness values.

Morphological, cultural, physiological and biochemical characteristics of strain SMK-122^T are given in the species description (see below) or are shown in Table 1. The almost-complete 16S rRNA gene sequence of strain SMK-122^T determined in this study comprised 1421 nt, representing approximately 96 % of the *Escherichia coli* 16S rRNA gene sequence. In the phylogenetic tree obtained using the neighbour-joining algorithm, strain SMK-122^T fell within the clade comprising *Roseovarius* species, clustering in particular with the type strain of *Rva. nubinihibens* at a bootstrap resampling value of 74.7 % (Fig. 1). Strain SMK-122^T exhibited 97.3 % 16S rRNA gene sequence similarity with respect to *Rva. nubinihibens* ISM^T and 94.9–96.0 % similarity with respect to the type strains of the other *Roseovarius* species.

The predominant ubiquinone detected in strain SMK-122^T was Q-10 (at a peak area ratio of approximately 97 %). The cellular fatty acid profile of strain SMK-122^T is shown in Table 2 together with those of *Roseovarius* species analysed in this study. Strain SMK-122^T contained large amounts of

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the positions of strain SMK-122^T, *Roseovarius* strains and some other related taxa. Bootstrap percentages (based on 1000 replications) >50 % are shown at branch points. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. The sequence of *Stappia stellulata* IAM 12621^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

unsaturated and straight-chain fatty acids, the major components (>10 % of total fatty acids) being C_{18:1}ω7c and C_{16:0}. This fatty acid profile of strain SMK-122^T was similar to those of the type strains of the four recognized *Roseovarius* species, although there were differences in the proportions of some fatty acids (Table 2). The DNA G+C content of strain SMK-122^T was 58.6 mol%. The data obtained from these chemotaxonomic analyses supported the results from the phylogenetic analysis, i.e. that strain SMK-122^T appeared to belong to the genus *Roseovarius*.

Strain SMK-122^T exhibited a mean DNA-DNA relatedness value of 13 % with respect to *Rva. nubinihibens* DSM 15170^T. Strain SMK-122^T is distinguishable from recognized *Roseovarius* species through differences in several phenotypic characteristics, as listed in Table 1. The phylogenetic and genetic distinctiveness and differential phenotypic properties of strain SMK-122^T are sufficient to categorize it as a member of a novel species of the genus *Roseovarius* (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). On the basis of the data presented, therefore, strain SMK-122^T represents a novel species of the genus *Roseovarius*, for which the name *Roseovarius aestuarii* sp. nov. is proposed.

Table 2. Cellular fatty acid compositions (%) of strain SMK-122^T and *Roseovarius* type strains

Strains: 1, SMK-122^T; 2, *Rva. tolerans* DSM 11457^T; 3, *Rva. nubinihibens* DSM 15170^T; 4, *Rva. crassostreae* DSM 16950^T; 5, *Rva. mucosus* DSM 17069^T. Data are from this study. Fatty acids that represented <0.5 % in all strains were omitted. –, Not detected; ECL, equivalent chain length.

Fatty acid	1	2	3	4	5
Straight-chain fatty acids					
C _{12:0}	5.1	–	5.1	–	6.6
C _{15:0}	0.2	1.9	0.6	–	–
C _{16:0}	16.2	10.3	13.8	11.8	8.1
C _{17:0}	0.3	5.8	0.6	0.3	0.3
C _{18:0}	1.0	1.2	0.9	1.5	1.0
Unsaturated fatty acids					
C _{17:1} ω8c	0.2	1.0	–	–	–
C _{18:1} ω7c	68.0	67.4	58.5	77.9	65.1
Hydroxy fatty acids					
C _{10:0} 3-OH	0.2	–	–	2.0	1.0
C _{12:0} 2-OH	–	0.6	–	–	1.5
C _{12:0} 3-OH	5.3	–	2.3	0.1	0.3
C _{12:1} 3-OH	–	2.6	–	–	2.8
C _{16:0} 2-OH	–	0.9	–	–	4.6
C _{16:0} 3-OH	–	–	–	0.6	–
C _{18:1} ω7c 11-methyl	0.8	6.1	–	1.5	0.9
C _{19:0} ω8c cyclo	1.5	0.2	14.2	–	4.3
Summed features*					
3	0.6	0.6	1.5	2.2	1.0
7	–	0.3	–	–	1.3
Unknown ECL 11.799	0.2	–	0.6	–	0.9

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contained C_{16:1}ω7c and/or iso-C_{15:0} 2-OH. Summed feature 7 contained C_{19:1}ω6c, C_{19:0}ω10c cyclo and/or unknown fatty acid ECL 18.846.

Description of *Roseovarius aestuarii* sp. nov.

Roseovarius aestuarii (aes.tu'a.ri.i. L. gen. n. *aestuarii* of the tidal flat, the place from which the type strain was isolated).

Cells are Gram-negative and ovoid to rod-shaped (0.3–0.6 × 0.8–3.5 µm). Motile by means of single polar flagella. Colonies on MA are circular, raised to slightly convex, glistening, smooth, cream-coloured and 1.0–1.5 mm in diameter after 7 days incubation at 30 °C. Growth occurs at 10 and 37 °C, but not at 4 or 38 °C. The optimal pH for growth is between 7.0 and 8.0; growth occurs at pH 6.0, but not at pH 5.5. Growth occurs in the presence of 7 % (w/v) NaCl, but not in the absence of NaCl or in the presence of more than 8 % (w/v) NaCl. Anaerobic growth does not occur on MA or on MA supplemented with nitrate. Bacteriochlorophyll *a* is not produced. H₂S is not produced. The predominant ubiquinone is Q-10. The major fatty acids (>10 % of total fatty acids) are C_{18:1}ω7c and C_{16:0}. The DNA G+C content of the type strain is 58.6 mol% (determined by HPLC). Other phenotypic characteristics are given in Table 1.

The type strain, SMK-122^T (=KCTC 22174^T =CCUG 55325^T), was isolated from a tidal flat sediment of the Yellow Sea at Saemankum, Korea.

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