Loktanella agnita sp. nov. and *Loktanella rosea* sp. nov., from the north-west Pacific Ocean

Elena P. Ivanova,[1,2] Natalia V. Zhukova,[3] Anatoly M. Lysenko,[4] Nataliya M. Gorshkova,[2] Alexander F. Sergeev,[5] Valery V. Mikhailov[2] and John P. Bowman[6]

Correspondence
Elena P. Ivanova
eivanova@swin.edu.au

[1]Swinburne University of Technology, PO Box 218, Hawthorn, VIC 3122, Australia

[2]Pacific Institute of Bioorganic Chemistry of the Far-Eastern Branch of the Russian Academy of Sciences, Pr. 100 Let Vladivostoku 159, 690022 Vladivostok, Russian Federation

[3]Institute of Marine Biology of the Far-Eastern Branch of the Russian Academy of Sciences, 690041 Vladivostok, Russian Federation

[4]Institute of Microbiology of the Russian Academy of Sciences, 117811 Moscow, Russian Federation

[5]Pacific Oceanological Institute of the Far-Eastern Branch of the Russian Academy of Sciences, Baltiiskaya Str. 43, 690017 Vladivostok, Russian Federation

[6]School of Agricultural Science, University of Tasmania, Private Bag 54, Hobart, Tasmania 7001, Australia

One whitish and four pinkish strains of Gram-negative, non-motile, aerobic bacteria were isolated from sea-water and sediment samples collected in Chazhma Bay (Sea of Japan, Pacific Ocean). Analysis of 16S rRNA gene sequences revealed that these strains belonged to the '*Alphaproteobacteria*', having highest sequence similarity of about 94–97 % with species of the genus *Loktanella*. None of the strains degraded gelatin, casein, chitin, agar, DNA or starch and they had limited ability to utilize carbon sources. The four pinkish strains, Fg36^T, Fg1, Fg116 and Fg117, degraded Tween 80. Sea-water strain R10SW5^T grew at 3–6 % NaCl and a temperature range of 8–35 °C, whilst strains Fg36^T, Fg1, Fg116 and Fg117 grew at NaCl concentrations of 1–12 % and a temperature range of 4–35 °C. Phosphatidylglycerol (58/79 %), diphosphatidylglycerol (11/6 %) and phosphatidylcholine (28/22 %) were the major phospholipids. The predominant fatty acids were 16 : 0 (12·2/8·6 %) and 18 : 1ω7 (76·6/68·4 %). The DNA G+C content of strain R10SW5^T was 59·1 mol% and those of the four pinkish strains ranged from 60·5 to 61·8 mol%. Based on the results of phenotypic, genotypic, chemotaxonomic and phylogenetic investigation, two novel species, *Loktanella agnita* sp. nov. and *Loktanella rosea* sp. nov., are proposed. The type strains are R10SW5^T (=KMM 3788^T=CIP 107883^T) and Fg36^T (=KMM 6003^T=CIP 107851^T=LMG 22534^T), respectively.

The genus *Loktanella* was created in 2004 to accommodate three species, *Loktanella fryxellensis*, *Loktanella salsilacus* and *Loktanella vestfoldensis*, of heterotrophic '*Alphaproteobacteria*' isolated from microbial mat samples collected from different Antarctic lakes (Van Trappen *et al.*, 2004). One more species, *Loktanella hongkongensis*, was recently added to the genus (Lau *et al.*, 2004).

This study extends our previous investigations of the biodiversity of marine proteobacteria from the Sea of Japan, north-west Pacific Ocean, and other geographical locations (Ivanova *et al.*, 1996, 1998, 2000, 2004a, b). During isolation studies, bacteria of different taxonomic groups, including *Shewanella*, *Marinobacter*, *Halomonas* and *Pseudoalteromonas*, have been isolated (E. P. Ivanova, unpublished data; Ivanova *et al.*, 2001, 2004b). Here, we describe two *Roseobacter–Ruegeria–Sulfitobacter*-like phenotypes that appeared to represent novel members of the genus *Loktanella* (Van Trappen *et al.*, 2004). Strains examined in this study were isolated in November 2000 from water (salinity, 32 ‰; temperature, 13·6 °C) samples of two different horizons, from the first metre below the

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Loktanella agnita* R10SW5^T and *Loktanella rosea* Fg36^T are AY682198 and AY682199, respectively.

A supplementary table showing the polar lipid and cellular fatty acid compositions of *Loktanella agnita* and *Loktanella rosea* is available in IJSEM Online.

surface and from 1–2 m from the bottom (a varying depth of 9–13 m), in Chazhma Bay, Gulf of Peter the Great, Sea of Japan, Pacific Ocean, by using a standard hydrological plastic bathometer. Sample-handling and isolation procedures were described elsewhere (Ivanova *et al.*, 1996, 2004a, b).

Phenotypic properties used for characterization of the new isolates were investigated by using standard procedures (Smibert & Krieg, 1994) and as described elsewhere (Ivanova *et al.*, 1996, 1998). To study the physiological properties, bacteria were grown under optimal conditions at 22–24 °C. Motility was studied in hanging-drop preparations. The following physiological and biochemical properties were examined: oxidation/fermentation of glucose (Hugh & Leifson, 1953), Gram stain, reduction of nitrate and nitrite, catalase [with 5 % (v/v) H_2O_2] and oxidase (Kóvacs, 1956) activities, gelatin liquefaction, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, poly-β-hydroxybutyrate and acetoin production (Voges–Proskauer test), sodium requirement [0, 1, 3, 6, 8, 10, 12 and 15 % (w/v) NaCl], indole and H_2S production and the ability to hydrolyse starch, Tween 80, casein, DNA and agar. The temperature range for growth was examined on marine agar incubated at 2, 4, 10, 30, 35, 37 and 42 °C. The haemolytic activity of the strains studied was detected on blood agar comprising 40 g trypticase–soy agar l^{-1}, 50 ml sheep blood and 950 ml water. Oxidative utilization of 95 carbon sources was tested by using Biolog GN Microplates (Rüger & Krambeck, 1994) as described elsewhere (Ivanova *et al.*, 1998).

For analysis of phospholipids and fatty acids, the strains were grown at 28 °C on marine agar 2216. After 48 h growth, cells were harvested. The lipids were extracted by a modification of the method of Bligh & Dyer (1959). Polar lipids were separated by two-dimensional microthin-layer chromatography in solvent systems described by Vaskovsky & Terekhova (1979). The detection and identification of lipids and fatty acids were performed as described elsewhere (Ivanova *et al.*, 2005). Phosphatidylglycerol, diphosphatidylglycerol and phosphatidylcholine were the major phospholipid constituents. In strain R10SW5^T and the four pinkish strains, they respectively accounted for: 58 and 69–79 %; 11 and 0–6 %; and 28 and 22–26 % of the total phospholipids, respectively. In addition, phosphatidylethanolamine was detected in strain R10SW5^T (3 %) and in trace amounts in strains Fg36^T, Fg1, Fg116 and Fg117. The major cellular fatty acids for R10SW5^T and the four pinkish strains, respectively, comprised the following: 16 : 0, 8·7 and 9–12 %; 18 : 1ω9, 2·6 and 5–7 %; 18 : 1ω7, 79 and 68–72 % (interstrain variations in cellular lipid composition are shown in the Supplementary Table, available in IJSEM Online).

DNA was isolated from the strains by following the method of Marmur (1961). The G+C content of the DNA was determined by using the thermal-denaturation method (Marmur & Doty, 1962). The DNA G+C content for strain R10SW5^T was 59·1 mol% and those for the four pinkish strains ranged from 60·5 to 61·8 mol%. DNA–DNA hybridization was performed spectrophotometrically and initial renaturation rates were recorded as described elsewhere (Marmur & Doty, 1962; De Ley *et al.*, 1970). DNA–DNA hybridization data revealed high interspecies levels of DNA relatedness among the four pinkish strains (96–98 %), indicating that they represent a single genospecies (Wayne *et al.*, 1987). DNA from the type strain of *L. vestfoldensis*, LMG 22003^T, showed intrageneric relatedness with R10SW5^T (35 %). These data indicated clearly that R10SW5^T constituted a distinct *Loktanella* species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994).

The small-subunit rRNA genes were sequenced as described elsewhere (Ivanova *et al.*, 2004b). 16S rRNA gene sequences of novel *Loktanella* species were aligned and analysed in the program BioEdit by using PHYLIP version 3.57c (Felsenstein, 1993). DNADIST was used to determine sequence similarities by using the maximum-likelihood algorithm option. Phylogenetic trees were constructed with maximum-likelihood distances and joined by neighbour joining using the program NEIGHBOR. The outgroups on the *Loktanella* trees were *Albidovulum inexpectatum* and *Rhodobacter veldkampii*. According to phylogenetic analysis (Fig. 1), strains R10SW5^T and Fg36^T, Fg1, Fg116 and Fg117 (the latter four sequences were almost identical, with sequence dissimilarity of $<0{\cdot}3$ %, to that of the type strain, therefore only Fg36^{T}and Fg1 were included in the resulting tree) formed separate clusters within species of the genus *Loktanella*. The most similar sequence was that

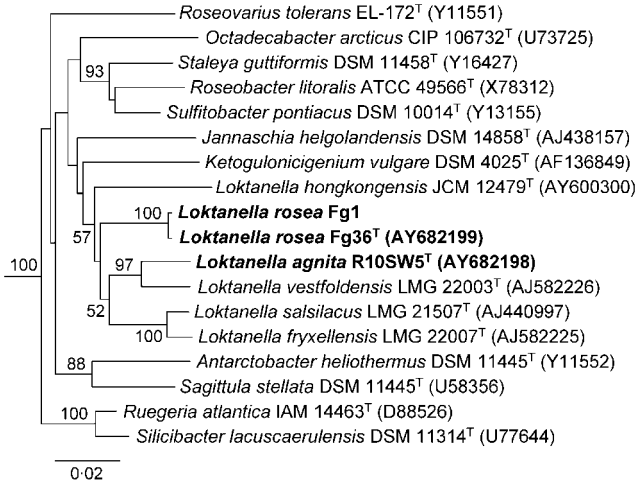

Fig. 1. Phylogenetic position of *Loktanella agnita* and *Loktanella rosea* according to 16S rRNA gene sequence analysis. The tree is based on maximum-likelihood distances and joined by neighbour joining; bootstraps are from 500 replications and only values >50 % are shown. Outgroups were *Albidovulum inexpectatum* and *Rhodobacter veldkampii*.

of *L. vestfoldensis*, sharing 97 and 95 % 16S rRNA gene sequence similarity with strains R10SW5^T and Fg36^T, respectively, followed by the remaining species with validly published names. Even though the 16S rRNA gene sequence of *L. vestfoldensis* showed 97 % similarity to that of R10SW5^T, DNA relatedness between the two strains was found to be low (35 %), which is in agreement with previous findings that bacteria that differ by $>2{\cdot}5$ % at the 16S rRNA gene sequence level are unlikely to exhibit more than 60–70 % DNA–DNA hybridization (Stackebrandt & Goebel, 1994; Keswani & Whitman, 2001; Rosselló-Mora & Amann, 2001). The new sequences of R10SW5^T and Fg36^T shared only 95 % 16S rRNA gene sequence similarity with each other. Notably, the two new phenotypes and the four existing species of the genus *Loktanella* all grouped together, with rather low bootstrap values (approx. 57 %). The group of four pinkish strains clearly represents an individual lineage and does not form a robust cluster with any other species of the genus (supported by the low bootstrap values). Based on these results, we could consider that they represent a species of a novel genus. However, we believe that, at the current stage, there are not enough differentiating pheno- and chemotypic characteristics to support such a proposal. Overall, our phylogenetic analysis indicated that the current taxonomic interpretation of the grouping of species of *Loktanella* and those of the genera *Roseobacter*, *Sulfitobacter*, *Oceanibulbus*, *Staleya*, *Silicibacter*, *Ruegeria* and some other related '*Alphaproteobacteria*' remains unsatisfactory (Rüger & Höfle, 1992; Uchino *et al.*, 1998, 1999; Söller *et al.*, 2000) and requires further phylogenetic analyses employing more housekeeping genes.

In addition to phylogenetic and genetic evidence, bacteria of the novel species can be distinguished from other *Loktanella* species by a number of phenotypic traits (Table 1). For example, in contrast to *L. vestfoldensis* and other species of the genus, except for *L. salsilacus* in which colony pigmentation is beige, strain R10SW5^T is non-pigmented, exhibits a weak oxidase reaction and requires NaCl at a limited range of 3–6 % for growth, whereas the four pinkish strains are essentially identical in their phenotypic characteristics, but differ from *L. hongkongensis* by lack of brown diffusible pigment and lack of ability to grow at 44 °C or in the presence of 14 % NaCl; they also differ from other species by their obligate requirement for NaCl and halophilicity (ability to grow in 12 % NaCl). Chemotaxonomically, all species of the genus possess the characteristic fatty acid 18 : 1ω7 at 68–87 % of total fatty acids. Nonetheless, a distinct species-specific pattern is observed for the new bacteria. Whilst the high proportion of 18 : 1ω7 is retained, strain R10SW5^T can be distinguished from other species of the genus by a greater proportion of the saturated fatty acids 14 : 0, 15 : 0 and 16 : 0, and of 14 : 1. The four strains of the other species, Fg36^T, Fg1, Fg116 and Fg117, can be distinguished by a lower proportion of 18 : 1ω7, 10 : 0 3-OH and 12 : 0 3-OH, and a greater proportion of 16 : 0. On the basis of these results, two novel species of the genus *Loktanella* are proposed: *Loktanella agnita* sp. nov. and *Loktanella rosea* sp. nov.

Table 1. Characteristics that differentiate *Loktanella agnita* and *Loktanella rosea* from other species of the genus

Taxa: 1, *L. agnita*; 2, *L. rosea*; 3, *L. fryxellensis*; 4, *L. hongkongensis*; 5, *L. salsilacus*; 6, *L. vestfoldensis*. All species are catalase-positive. W, Weakly positive reaction; V, variable reaction depending on strain; ND, no data available.

Characteristic	1	2	3*	4†	5*	6*
DNA G+C content (mol%)	59	61	66	66	59–60	62–63
Colony pigmentation	Whitish	Pink	Pink–beige	Pink–white	Beige	Pink
Diffusible pigment	−	−	−	Brown	−	−
Oxidase	W	+	+	+	+	+
Growth on:						
Trypticase soy agar	−	−	−	−	−	W
Nutrient agar	−	−	−	−	−	W
Temperature range for growth (°C)	8–35	4–35	5–25	8–44	5–30	5–37
NaCl range for growth (%)	3–6	1–12	0–5	2–14	0–10	0–10
Reduction of nitrate to nitrite	W	−	−	−	−	−
Production of:						
Lipase (Tween 80)	−	W	+	−	+	+
Trypsin	−	−	−	ND	−	+
α-Galactosidase	+	−	−	ND	+	−
Hydrolysis of urea	−	−	−	−	−	+
Carbohydrate metabolism	−	−	−	V	−	−

*Data from Van Trappen *et al.* (2004).
†Data from Lau *et al.* (2004).

Description of *Loktanella agnita* sp. nov.

Loktanella agnita (ag.ni′ta. L. fem. part. adj. *agnita* recognized).

Rod-shaped cells, single, about 0·7–0·9 μm in diameter. Gram-negative. Non-motile. Chemo-organotroph with respiratory metabolism. Colonies are uniformly round, 1–3 mm in diameter, regular, convex, smooth and whitish after incubation for 48–74 h on marine agar. No diffusible pigment is released into the medium. Endospores are not formed. Na^+ or sea water is required for growth. Catalase reaction is positive, but oxidase reaction is only weakly positive. Growth occurs in media with 3–6 % NaCl. Temperature range for growth is 8–35 °C, with an optimum at 25 °C. No growth is detected at 37 °C. The pH for growth ranges from 6·0 to 10·0, with an optimum at 7·5–8·0. Does not decompose gelatin, agar, starch, casein, laminarin, chitin, Tween 80 or DNA. Negative for indole, H_2S, poly-β-hydroxybutyrate and acetoin production, arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase. Weakly reduces nitrate to nitrite. Non-haemolytic. Exhibits only a limited ability to utilize carbon sources, utilizing glycyl L-glutamic acid, alaninamide and glycyl L-aspartic acid (according to Biolog). The main cellular fatty acid is 18 : 1ω7 (approx. 77 %). The DNA G+C content is 59·1 mol%.

Isolated from sea water of Chazma Bay, Sea of Japan, Pacific Ocean. The type strain is R10SW5^T (=KMM 3788^T=CIP 107883^T).

Description of *Loktanella rosea* sp. nov.

Loktanella rosea (ro.se′a. L. fem. adj. *rosea* rose-coloured or rosy, referring to the pinkish colour of the colonies).

Rod-shaped cells, single, about 0·7–0·9 μm in diameter. Gram-negative. Non-motile. Chemo-organotroph with respiratory metabolism. Colonies are uniformly round, 1–3 mm in diameter, regular, convex, smooth, transparent and pinkish after incubation for 48–74 h on marine agar. No diffusible pigment is released into the medium. Endospores are not formed. Oxidase- and catalase-positive. Na^+ or sea water is required for growth. Growth occurs in media with 1–12 % NaCl. Mesophilic. Temperature range for growth is 4–35 °C, with an optimum at 25 °C. No growth is detected at 37 °C. The pH for growth ranges from 6·0 to 10·0, with an optimum at 7·5–8·0. Does not decompose gelatin, agar, starch, casein, laminarin, chitin or DNA. Tween 80 is utilized weakly. Negative for indole, H_2S, poly-β-hydroxybutyrate and acetoin production, arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase. Does not reduce nitrate to nitrite. Non-haemolytic. Exhibits only a limited ability to utilize carbon sources, utilizing glucuronamide, alaninamide and L-alanylglycine (according to Biolog). Phosphatidylglycerol, diphosphatidylglycerol and phosphatidylcholine are the major phospholipids. The main cellular fatty acid is 18 : 1ω7 (approx. 77 %). The G+C content of the DNA is 60·5–61·8 mol%.

Isolated from sediments of Chazma Bay, Sea of Japan, Pacific Ocean. The type strain is Fg36^T (=KMM 6003^T=CIP 107851^T=LMG 22534^T).

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