Roseomonas frigidaquae sp. nov., isolated from a water-cooling system

Mi Sun Kim,¹ Keun Sik Baik,¹ Seong Chan Park,¹ Moon Soo Rhee,² Hee-Mock Oh² and Chi Nam Seong¹

Correspondence
Chi Nam Seong
scnu@scnu.ac.kr

¹Department of Biology, College of Life Science and Natural Resources, Sunchon National University, Suncheon 540-742, Republic of Korea

²Korean Collection for Type Cultures, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-600, Republic of Korea

A non-motile, coccobacilli-shaped, pale-pink-pigmented bacterium, designated strain CW67^T, was isolated from a water-cooling system in Gwangyang, Republic of Korea. Cells were found to be Gram-negative, catalase-positive and oxidase-positive, the major fatty acids were C_{18:1ω7c} (43.6 %) and C_{16:0} (15.8 %), the predominant respiratory lipoquinone was Q-10 and the DNA G + C content was 69.5 mol%. A phylogenetic tree based on 16S rRNA gene sequence comparisons showed that strain CW67^T forms an evolutionary lineage within the radiation of the genus *Roseomonas* and that its closest relative is *Roseomonas gilardii* subsp. *rosea* MDA5605^T (94.7 % sequence similarity). Evidence from this polyphasic study showed that strain CW67^T could not be assigned to any recognized species. It therefore represents a novel species, for which the name *Roseomonas frigidaquae* sp. nov. is proposed, with CW67^T (=KCTC 22211^T =JCM 15073^T) as the type strain.

The genus *Roseomonas* was established by Rihs *et al.* (1993) for pink, coccoid isolates that cause bacteraemia and other human infections. A phylogenetic analysis based on 16S rRNA gene sequences showed that the genus *Roseomonas* falls within the *Alphaproteobacteria* (Gallego *et al.*, 2006). *Roseomonas* species have been isolated from blood, wounds, exudates, abscesses and genitourinary specimens (Han *et al.*, 2003; Sandoe *et al.*, 1997; Bibashi *et al.*, 2000; Subudhi *et al.*, 2001). *Roseomonas* species have also been isolated from environmental samples such as drinking water distribution systems (September *et al.*, 2004; Gallego *et al.*, 2006), freshwater sediment (Jiang *et al.*, 2006), a biofilm from a cooling tower (Furuhata *et al.*, 2007), deep-water marine invertebrates (Sfanos *et al.*, 2005) and a soil sample (Yoon *et al.*, 2007). 16S rRNA gene sequences related to the genus *Roseomonas* have also been detected frequently in the freshwater bacterial communities associated with cyanobacterial blooms in lakes (Eiler & Bertilsson, 2004). All of this suggests that members of the genus *Roseomonas* could be found widely in nature. At the time of writing, the genus comprises ten species and two subspecies with validly published names: *Roseomonas*

gilardii subsp. *gilardii* (Rihs *et al.*, 1993; Han *et al.*, 2003), *R. gilardii* subsp. *rosea* (Han *et al.*, 2003), *R. cervicalis* (Rihs *et al.*, 1993), *R. fauriae* [Rihs *et al.*, 1993; reported by Helsel *et al.* (2006) to be a synonym of *Azospirillum brasilense*], *R. mucosa* (Han *et al.*, 2003), *R. lacus* (Jiang *et al.*, 2006), *R. aquatica* (Gallego *et al.*, 2006), *R. terrae* (Yoon *et al.*, 2007), *R. aerilata* (Yoo *et al.*, 2008) and *R. vinacea* (Zhang *et al.*, 2008).

In the course of our study on the microbial diversity of a water-cooling system, a pink-pigmented bacterium, designated CW67^T, was isolated and was the subject of a taxonomic investigation.

Strain CW67^T was isolated, using the standard dilution-plating technique, from a cooled water sample from an oxygen-producing plant in Gwangyang, Republic of Korea. Isolation was achieved using plate-count agar (Difco) incubated at 25 °C for 7 days. The isolate was routinely cultured on tryptic soy agar (TSA; Difco) and maintained as a glycerol suspension (20 %, w/v) at –80 °C.

Bacterial DNA preparation, PCR amplification and sequencing of the 16S rRNA gene were carried out as described previously (Chun & Goodfellow, 1995). The resultant sequence of strain CW67^T (1466 nt) was aligned manually against sequences obtained from the GenBank database. Phylogenetic trees were inferred from the regions available for all sequences (positions 22–1450; *Escherichia coli* numbering system) using the neighbour-joining

The GenBank/EMBL/DDJB accession number for the 16S rRNA gene sequence of strain CW67^T is EU290160.

A scanning electron micrograph, a Fitch–Margoliash phylogenetic tree based on 16S rRNA gene sequences and cellular fatty acid profiles of strain CW67^T and related strains are available as supplementary material with the online version of this paper.

(Saitou & Nei, 1987) and Fitch–Margoliash (Fitch & Margoliash, 1967) methods. Evolutionary-distance matrices were generated as described by Jukes & Cantor (1969). The resultant neighbour-joining tree topology was evaluated by means of bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. Alignment and phylogenetic analyses were carried out using the jPHYDIT program (available at <http://plaza.snu.ac.kr/~jchun/jphydit/>) and PAUP 4.0 (Swofford, 1998) as described previously (Chun *et al.*, 2000).

Preliminary sequence comparisons with 16S rRNA gene sequences held in GenBank indicated that our isolate was related to the genus *Roseomonas*. The newly determined sequence was then aligned manually against representatives of the genus *Roseomonas*. Strain CW67^T showed the highest 16S rRNA gene sequence similarity with respect to *R. gilardii* subsp. *rosea* MDA5605^T (94.7%), *R. aquatica* TR53^T (94.6%), *R. gilardii* subsp. *gilardii* ATCC 49956^T (94.5%), *R. aerilata* 5420S-30^T (94.4%) and *R. mucosa* ATCC 49957^T (94.3%); the sequence similarities with respect to all other species of the genus *Roseomonas* were below 94.3%. To elucidate the phylogenetic relationship between the novel isolate and other species of the genus *Roseomonas*, phylogenetic trees were constructed by using two different tree-making algorithms. The neighbour-joining tree (Fig. 1) showed that strain CW67^T was closely related to the type strains of the genus *Roseomonas*, occupying a distinct phylogenetic position. The tree based on the Fitch–Margoliash method showed an essentially similar topology (not shown).

Growth on various standard bacteriological media was tested by using nutrient agar, TSA, R2A agar, MacConkey agar and marine agar according to the instructions of the manufacturer (Difco). Cells of strain CW67^T grown on TSA at 25 °C for 3–7 days were used for physiological and biochemical tests. Motility was examined using phase-

contrast microscopy (TMS-F; Nikon) on cells grown in wet mounts. Growth at various NaCl concentrations (0–10%, w/v, in increments of 1.0%) was investigated in tryptic soy broth (TSB; Difco) prepared according to the formula of Difco medium except that no NaCl was used. The pH range for growth was determined in TSB adjusted to pH 3–12 (in increments of 1 pH unit) by the addition of HCl and NaOH. Growth temperature (4–42 °C) and growth in an anaerobic chamber (CO₂/H₂/N₂, 10:10:80; Sheldon Manufacturing) were checked using TSA, with incubation times of up to 1 week. Catalase and oxidase activities were determined using 3% (v/v) hydrogen peroxide and Kovács reagent (Kovács, 1956), respectively. Acid production from sugars was examined by using phenol red broth base (Difco) containing each sugar at 0.5% (Cowan & Steel, 1965). Nitrate reduction was tested in nitrate broth containing 0.2% KNO₃ (Skerman, 1967). Citrate utilization was tested on Simmons' citrate agar (Sigma) as described by Gallego *et al.* (2006). Indole production was determined with Kovács reagent in 1% tryptone broth (Gallego *et al.*, 2006). H₂S production was determined on Kligler's iron agar (Difco) according to Smibert & Krieg (1994). L-Phenylalanine deamination was examined using the method of Richard & Kiredjian (1995). Urease activity was tested on Christensen's medium (Christensen, 1946). Hydrolysis of casein (2% skimmed milk, w/v), starch (0.2% soluble starch, w/v), L-tyrosine (0.5%, w/v), Tween 20 (1%, w/v), Tween 80 (1%, w/v) and xylan (1%, w/v) was tested, using TSA as the basal medium (Barrow & Feltham, 1993). Methyl red and Voges–Proskauer tests were analysed as described by Smibert & Krieg (1994). DNase activity was determined with DNase test agar (Difco) as described by Satomi *et al.* (2007). Other physiological and biochemical tests were performed using API 20E, API 20NE and API 50 CH strips (bioMérieux). Enzyme activities were tested using an API ZYM kit (bioMérieux) according to the manufacturer's instructions.

Fig. 1. Neighbour-joining phylogenetic tree, based on almost-complete 16S rRNA gene sequences, showing the relationships between strain CW67^T, members of the genus *Roseomonas* and related members of the family *Acetobacteraceae*. Bootstrap percentages (based on neighbour-joining analyses of 1000 resampled datasets) are shown at nodes (when >50%). Bar, 0.02 nucleotide substitutions per position.

Antibiotic resistance was determined by using a disc-diffusion method (Bauer *et al.*, 1966) with commercial antibiotic-impregnated discs (BBL Becton Dickinson). The results were interpreted according to the guidelines set forth by the CLSI (2003).

Strain CW67^T grew on TSA, plate-count agar, R2A agar and nutrient agar, but grew only weakly on marine agar. The cells were non-spore-forming coccobacilli (Supplementary Fig. S1, available in IJSEM Online). On TSA, strain CW67^T was able to grow at 15–37 °C (optimally at 30 °C) but not at 42 °C. The detailed results of the physiological and biochemical analyses are given in Table 1 and the species description. There were several phenotypic characteristics (e.g. oxidase and urease production, nitrate reduction and assimilation of carbon sources) that serve to distinguish strain CW67^T from phylogenetically related species.

Cellular fatty acids from strain CW67^T grown for 2 days at 30 °C and pH 7.0 on TSA were prepared and analysed by GLC according to the instructions of the Microbial Identification System (MIDI, 1999). Isoprenoid quinone analysis of strain CW67^T grown on TSA for 2 days at 30 °C and pH 7.0 was performed using reversed-phase TLC as described by Collins (1985). The G + C content of the DNA was determined according to the thermal denaturation method of Marmur & Doty (1962).

The fatty acid profile of strain CW67^T (i.e. those fatty acids constituting >1.0% of the total) included unsaturated

fatty acids C_{18:1}ω7c (43.6%), C_{18:1}ω5c (2.0%) and C_{16:1}ω5c (1.0%), hydroxyl fatty acids C_{18:1} 2-OH (6.2%) and C_{18:0} 3-OH (1.1%), straight-chain fatty acids C_{16:0} (15.8%), C_{18:0} (6.5%) and C_{14:0} (1.1%), cyclo fatty acids C_{17:0} cyclo (8.6%) and C_{19:0}ω8c cyclo (2.7%) and summed feature 3 (comprising iso-C_{15:0} 2-OH and/or C_{16:1}ω7c) (9.6%) (Supplementary Table S1). This fatty acid profile was similar to those of *Roseomonas* species. However, there were differences between strain CW67^T and its nearest neighbour, *R. gilardii* subsp. *rosea* MDA5605^T, in terms of the contents of the predominant fatty acids, i.e. C_{18:1}ω7c, summed feature 3, C_{17:0} cyclo and C_{18:0}. Analysis of the quinone content of strain CW67^T revealed a spot that corresponded to Q-10. The DNA G + C content of strain CW67^T was 69.5 mol%, within the range reported for species of the genus *Roseomonas* (67.3–72.1 mol%).

Therefore, on the basis of the data presented, strain CW67^T represents a novel species within the genus *Roseomonas*, for which the name *Roseomonas frigidaquae* sp. nov. is proposed.

Description of *Roseomonas frigidaquae* sp. nov.

Roseomonas frigidaquae (fri.gi.da'quae. L. adj. *frigidus* cold; L. fem. n. *aqua* water; N.L. gen. n. *frigidaquae* from/of cold water, referring to the isolation of the type strain from a water-cooling system).

Cells are coccobacilli (0.6–0.8 × 0.8–1.0 μm in size) that occur singly and in pairs. Gram-negative, non-motile, non-

Table 1. Characteristics that serve to differentiate strain CW67^T from members of the genus *Roseomonas*

Strains: 1, CW67^T; 2, *R. gilardii* subsp. *gilardii* ATCC 49956^T; 3, *R. gilardii* subsp. *rosea* (data for eight strains); 4, *R. aquatica* TR53^T; 5, *R. cervicalis* E7107^T; 6, *R. fauriae* C610^T; 7, *R. mucosa* MDA5527^T; 8, *R. lacus* TH-G33^T; 9, *R. terrae* DS-48^T; 10, *R. aerilata* 5420S-30^T; 11, *R. vinacea* CPCC 100056^T. Data are from Rihs *et al.* (1993), Han *et al.* (2003), Gallego *et al.* (2006), Jiang *et al.* (2006), Yoon *et al.* (2007), Yoo *et al.* (2008), Zhang *et al.* (2008) and this study. +, Positive; –, negative; v, variable; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Isolation source	Cooled water	Potable water	Blood	Drinking water	Cervix	Wound	Blood	Freshwater lake sediment	Soil	Air	Soil
Optimum growth temperature (°C)	30	35	35	28	35	35	35	30	25	30	30–32
Oxidase activity	+	–	v	–	+	+	v	+	+	+	+
Reduction of nitrates to nitrites	+	–	–	+	–	+	–	+	–	–	+
Urease activity	–	+	+	+	+	ND	+	+	+	–	–
Assimilation of (API 50 CH):											
L-Arabinose	–	+	+	–	v	+	+	+	–	–	+
Citrate	–	+	+	–	v	v	+	–	–	–	–
Glycerol	–	+	ND	–	–	+	ND	–	–	–	–
Fructose	–	+	ND	–	+	+	ND	–	–	–	–
Malate	–	+	+	+	ND	ND	+	+	–	–	–
Major (minor) quinone(s)	Q-10	ND	ND	Q-10	ND	ND	ND	Q-10	Q-10 (Q-7, Q-11)	Q-10	Q-10
DNA G + C content (mol%)	69.5	67.6	ND	68.6	70.4	68	ND	71.9	69.3	72.1	67.3

*Data from Yoon *et al.* (2007).

spore-forming, strictly aerobic and pale-pink-pigmented. Grows best on media such as TSA, nutrient agar and R2A agar. Grows on MacConkey agar. Colonies on TSA are convex, circular, smooth and opaque with entire margins, approx. 2 mm in diameter after 5 days at 30 °C (pH 7). Growth occurs in 0–3 % (w/v) NaCl (optimally at 0.5 %), at pH 6–10 (optimally at pH 7) and at 15–37 °C (optimally at 30 °C). Oxidase and catalase activities are present. H₂S and indole are not produced. Tween 20 is hydrolysed, but casein, DNA, aesculin, gelatin, starch, tyrosine, Tween 80, urea and xylan are not hydrolysed. The Voges–Proskauer test and the methyl red reaction are negative. In the API 20E strip, the ONPG reaction is negative. Nitrate is reduced to nitrite, but with no gas. Acid is not produced from the following (at 0.5 %, w/v): cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, rhamnose, starch, sucrose and xylose. Citrate is not utilized. None of the substrates in the API 20NE and API 50 CH strips is utilized as a sole carbon source. In the API ZYM gallery, acid phosphatase, alkaline phosphatase, esterase (C4) and α -glucosidase activities are positive and esterase lipase (C8) is weakly positive. Cells are sensitive to (μ g per disc, unless otherwise indicated) amikacin (30), ampicillin (10), chloramphenicol (30), erythromycin (15), gentamicin (10), kanamycin (30), nalidixic acid (30), penicillin G (10 IU), streptomycin (10) and tetracycline, but resistant to polymyxin B (300 IU) and vancomycin (30). Major fatty acids are C_{18:1} ω 7c and C_{16:0} (see Supplementary Table S1 for the complete fatty acid composition of the type strain). Q-10 is the predominant quinone. The DNA G + C content of the type strain is 69.5 mol%.

The type strain, CW67^T (=KCTC 22211^T =JCM 15073^T), was isolated from a water-cooling system at an oxygen-producing plant in Gwangyang, Republic of Korea.

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