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Solveig K. Petursdottir · Jakob K. Kristjansson

Silicibacter lacuscaerulensis gen. nov., sp. nov., a mesophilic moderately halophilic bacterium characteristic of the Blue Lagoon geothermal lake in Iceland

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Abstract Mesophilic, moderately halophilic bacteria were isolated from a silica-rich geothermal lake, the Blue Lagoon in Iceland. The isolates are strictly aerobic, but reduce nitrate to nitrite, and are oxidase- and catalase-positive. The nonsporeforming and nonmotile Gram negative rods are 0.6–0.8 μm in diameter and variable in length (9–18 μm), and contain gas vacuoles. The GC content in their DNA is 66.15%. The minimum, optimum, and maximum temperatures for growth are 22°C, 45°C, and 50°C, respectively. The isolates do not grow without added salt in the medium and can grow at up to 7% NaCl (w/v). The optimal salinity for growth is 3.5%–4% NaCl. The pH range for growth is 6.5–8.5, with the optimal pH at 7.0. At optimal conditions the bacterium has a doubling time of 80 min. The main cytochrome is a membrane-bound cytochrome *c* with an α-peak at 549 nm. Sequencing of 16S rRNA from the type strain ITI-1157 revealed it to be a proteobacterium of the α-subclass with the closest relatives being *Roseobacter litoralis* and *Paracoccus kocurii*. The new isolates do not contain bacteriochlorophyll *a* and are considered to represent a new genus and a new species, S*ilicibacter lacuscaerulensis*.

Key words Silica precipitation · Geothermal lagoon · Moderate halophile · *Silicibacter lacuscaerulensis* · *Erythrobacter longus* · *Roseobacter litoralis* · *Paracoccus kocurii*

Introduction

The Blue Lagoon is a shallow geothermal lake with an average temperature of 37°C, a pH of 7.5, and about 2.5% salinity. It was formed in 1976 from the effluent of the Svartsengi geothermal powerplant. It is saturated with silica which constantly precipitates in the lake. The lake has been colonized by a few types of microorganisms which proliferate in this unusual ecosystem (Petursdottir and Kristjansson 1996). In a previous study we concluded that the low species diversity in this ecosystem could be explained by the high rate of silica precipitation, which was estimated to be about 10 $mg.l^{-1}.h^{-1}$ (Petursdottir and Kristjansson, 1996). The silica particles may also have reflective effects similar to those of salt crystals in brines, thereby increasing the exposure of bacteria to both UV and visible light and thus reducing species diversity (Atlas and Bartha 1993).

A semicontinous enumeration of bacteria over a 7-month period showed that the average viable count was of the order of 10^5–10^6 ml^{-1}.

A numerical taxonomy analysis based on 54 phenotypic tests carried out on 99 randomly picked isolates from the Blue Lagoon revealed a dominant group at the 80% similarity level containing 85% of the strains (Petursdottir and Kristjansson 1996). Four representative strains from the dominant group were characterized further and compared to those bacteria that seemed to be phenotypically or phylogenetically closest to our isolates.

Based on sequencing of the 16S rRNA gene, the closest relatives seemed to be *Roseobacter litoralis* and *Paracoccus kocurii*. The new isolates were, however, very different from any previously described bacteria and are therefore considered to represent a new genus and a new species, for which we propose the name *Silicibacter lacuscaerulensis*.

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S.K. Petursdottir · J.K. Kristjansson
Department of Biotechnology, Technological Institute of Iceland, Reykjavík, Iceland

J.K. Kristjansson (✉)
Institute of Biology, University of Iceland, Keldnaholt, Is-112 Reykjavík, Iceland
Tel. +354-587-7000; Fax +354-587-7409
e-mail: Jakob.K.Kristjansson@iti.is

Materials and methods

Bacterial strains and media

The isolation of the apparently dominant bacterial type in the Blue Lagoon has been described elsewhere (Petursdottir and Kristjansson 1996). The representative type strain which was investigated in this study was ITI-1157. For

reference we used *Erythrobacter longus* (ATCC33941) and *Roseobacter litoralis* (ATCC49566) (Shiba 1991), obtained from the American Type Culture Collection, as well as data for *Paracoccus kocurii* (Ohara et al. 1990). Strain ITI-1157 was normally grown in medium PCA BL50% (Petursdottir and Kristjansson 1996) but the two reference strains were grown in Difco Marine Broth Medium (MBR; Difco, Detroit, MI, USA).

Phenotypic characterization

The following characterization tests have been described elsewhere (Petursdottir and Kristjansson 1996): growth on 34 single carbon sources; resistance against antibiotics; staining for Gram reaction, spores, and lipid-granules; motility; effects of NaCl and temperature on growth; presence of oxidase and catalase; and ability to grow anaerobically.

The effect of 4%, 6%, 7%, and 8% NaCl on the growth of the reference strains was tested in MBR medium with added NaCl. Tests for pH tolerance were done in PCA BL50% liquid medium with the following buffers: 0.1 M citric acid / 0.2 M Na_2HPO_4 (for pH 5.5 and 6.5); 0.2 M NaH_2PO_4 / 0.2 M Na_2HPO_4 for pH 7.5; and 0.2 M HCl / 0.2 M Tris for pH 8.5 and 9.5. After a 24-h incubation the absorbance at 600 nm was measured and the pH tolerance determined.

The susceptibility of ITI-1157 to five antibiotics was tested on PCA BL50% medium, and that of the reference strains on MBR medium. Oxoid 6-mm disks with chloramphenicol (30 μg); penicillin-G (10 units); streptomycin (10 μg); tetracycline (30 μg) and polymyxin B (300 units) were used and susceptability estimated by comparing the size of the haloes with reference tables in the Oxoid Manual (1982) (Oxoid, Basingstoke, Hampshire, UK).

DNA base composition

The GC mol content of ITI-1157 was determined by Dr. Fred Rainey at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM).

Spectroscopy and pigments

Bacteriochlorophyll *a* was determined by resuspending a pellet from 150-ml cultures in 10 ml methanol for pigment extraction. The methanol cell suspensions were then centrifuged at 12000 rpm for 20 min and the absorption spectrum determined between 250 and 900 nm.

The cytochrome composition was determined by resuspending the pellet from a 200-ml culture in TE buffer (10 mM Tris·Cl, 1 mM EDTA), pH 7.8 (5 $ml.g^{-1}$ of cells); the cells were then ruptured in a French press. DNAse (0.01 $mg.ml^{-1}$) was added and the extract incubated at 37°C for 30 min followed by centrifugation at 17000 rpm for 30 min. The pellet was dissolved in 2% Triton X-100 to release the membrane-bound proteins and then centrifuged again at 17000 rpm for 30 min. The oxidized versus reduced (with sodium dithionite) absorption spectrum was determined in the supernatant between 250 and 900 nm.

Samples of the crude extract, the membrane fraction, and the soluble fraction were also run on gradient (8%–25%) sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels to determine the size of the cytochromes. Duplicate samples were prepared and one was boiled for 2 min before running. The LMW electrophoresis calibration kit from Pharmacia Biotech (Uppsala, Sweden) was used as a standard. The gels were stained for heme proteins by immersing them for 30 min with constant shaking in 1.25 mM 3,3′,5,5′-tetramethylbenzidine (TMBZ) in 30:70 (v/v) methanol / 0.25 M sodium acetate at pH 5. Then, H_2O_2 was added to give 26 mM, and the gel was shaken further for 15 min. Then, the staining solution was replaced with fresh 30:70 (v/v) propanol / 0.25 M sodium acetate at pH 5 (Goodhew et al. 1986). The size of the cytochromes was determined by staining the same gels with 0.1% Coomassie blue R in 4:1:5 methanol/glacial acetic acid / H_2O mixture. The gels were destained in the same solution without Coomassie blue.

Electron microscopy

The cells of isolate ITI-1157 were processed for electron microscopy as previously described (Kristjansson et al. 1994). The electron micrographs were taken by Johann Arnfinnsson, Department of Anatomy, University of Iceland.

Utilization of tetramethylammonium chloride

Different concentrations (w/v) of tetramethylammonium chloride were made (0%, 0.1%, 0.2%, 0.4%, 0.8%, 1.0%) in liquid minimal medium, inoculated with fresh plate cultures of strain ITI-1157, and incubated for 48 and 96 h at 45°C, after which the absorbance was measured at 600 nm.

Sequencing of 16S rRNA

Sequencing of the 16S rRNA of ITI-1157 was done by Dr. Fred Rainey at DSM. DNA extraction, PCR mediated amplification of the 16S rDNA, and purification of PCR products was carried out as described by Rainey et al.(1992) and Rainey and Stackebrandt (1993). Purified PCR products were sequenced using the Taq Dye-Deoxy TM Terminator Cycle sequencing kit (Applied Biosystems, Weiterstadt, Germany) as directed in the manufacturer's protocol. Sequence reactions were electrophoresed using the Applied Biosystems 373A sequencer. The 16S rDNA sequence was manually aligned against available representative sequences of members of the α-subclass of the proteobacteria. Pairwise evolutionary distances were computed using the correction of Jukes and Cantor (1969). The least squares distance method of DeSoete (1983) was used in the construction of the phylogenetic dendrogram from distance matrices.

Results and discussion

Phenotypic properties

The representative strain designated ITI-1157 is a Gram negative, nonmotile, nonsporeforming bacterium, which is obligately aerobic but reduces nitrate to nitrite. Colonies are tan colored, opaque, and 2–4 mm in diameter. It is oxidase positive and shows a weak positive reaction for catalase. It can utilize arginine, lysine, and pyruvate as sole carbon sources but not acetate, citrate, or glucose. It does not hydrolyze starch or casein.

The optimum temperature for growth is 45°C but it can grow at up to 50°C. The optimum salinity for ITI-1157 is 3.5% and it needs a minimum of 1.5% NaCl for growth. The maximum salinity for growth is 7% NaCl. It is therefore a moderate halophile. The optimum pH for growth is pH 7.0 but it grows at pH 6.5 and 8.5 (Petursdottir and Kristjansson 1996). ITI-1157 is susceptible to chloramphenicol, penicillin, tetracycline, and polymyxin B, but is resistant to streptomycin.

Growth on alkylated ammonium compounds is characteristic for *Paracoccus kocurii* (Ohara et al. 1990). Therefore, this was tested on the new isolate using tetramethyl ammonium chloride as the sole carbon source; this showed that ITI-1157 could tolerate the compound up to 0.2% but did not utilize it. This indicates that the Blue Lagoon isolate is different from that of *Paracoccus kocurii*.

Morphology and pigments

The bacterium is a long rod, 0.6–0.8 μm in diameter and 9–18 μm in length, containing vacuoles (Figs. 1, 2). Environ-

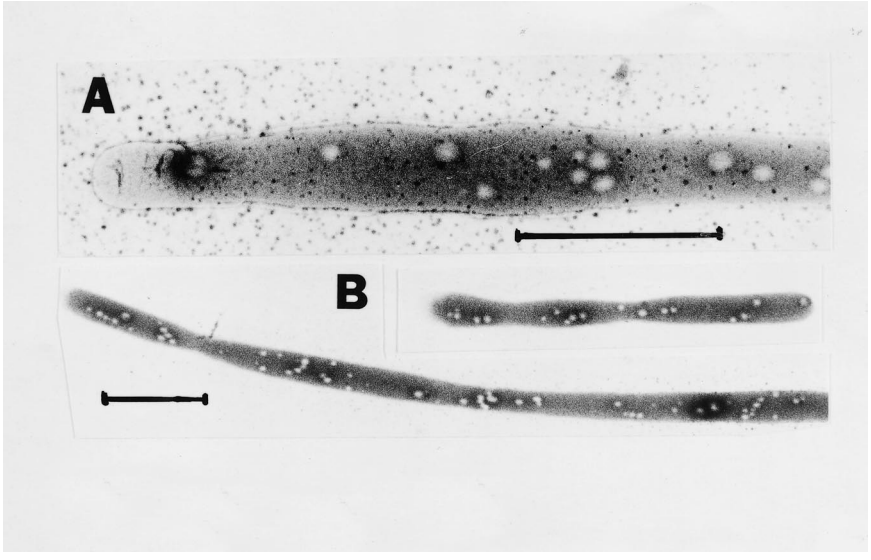

Fig. 1. Electron micrographs of *Silicibacter lacuscaerulensis*, strain ITI-1157. Negative staining of whole cells with vacuoles as *white dots* in cell. *Bar*, 2 μm. **a** Part of cell. Silica particles are visible as *small dark spots*. **b** Whole cells of different length

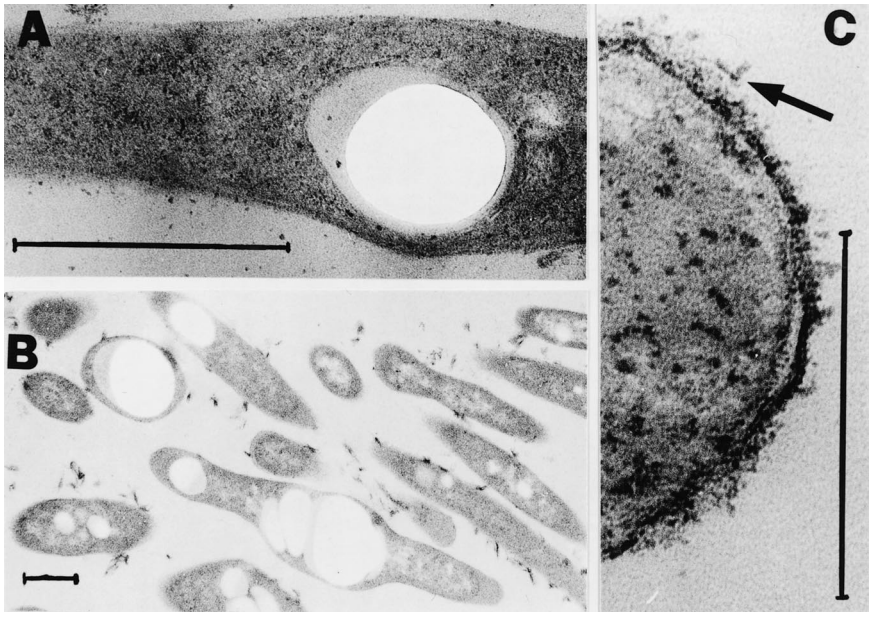

Fig. 2. Electron micrographs of *Silicibacter lacuscaerulensis*, strain ITI-1157. Thin sections. *Bar*, 0.5 μm. **a** Cells cultivated on plates. Swollen cells with large vacuoles. **b** Cells cultivated in liquid culture. **c** Cells cultivated in liquid culture. *Arrow* points to silica aggregates on the cell surface

Table 1. 16S rDNA similarity values betwen strain ITI-1157 and related taxa within the alpha subclass of the proteobacteria

Strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. Strain ITI-1157	–													
2. *Roseobacter denitrificans*	94.8	–												
3. *Roseobacter litoralis*	95.1	98.6	–											
4. SAR83	92.9	93.7	93.3	–										
5. *Rhodobacter veldkampii*	94.8	93.8	94.1	91.9	–									
6. *Paracoccus denitrificans*	93.2	91.9	92.1	90.4	94.0	–								
7. *Paracoccus kocurii*	95.1	93.2	93.3	90.6	94.4	95.7	–							
8. *Rhodobacter blastica*	92.5	91.9	91.9	90.5	94.6	94.1	93.6	–						
9. *Rhodobacter capsulatus*	93.1	92.6	92.9	90.9	95.3	94.6	95.2	95.5	–					
10. *Rhodobacter sphaeroides*	94.8	93.1	94.1	91.4	95.6	95.6	95.1	95.6	96.0	–				
11. *Rhodovulum adriaticum*	94.0	92.3	93.4	90.9	93.6	94.4	93.4	92.6	92.7	94.7	–			
12. *Rhodovulum sulfidophilum*	93.4	91.8	92.4	90.9	94.0	94.1	93.5	94.1	94.1	94.1	94.9	–		
13. *Rhodovulum euryhalinum*	93.9	90.8	91.7	88.9	93.5	92.9	93.7	93.7	93.0	94.0	93.4	95.5	–	
14. *Rhodobium marinum*	89.1	87.3	88.0	87.2	88.3	89.0	90.1	87.9	88.7	89.2	89.1	88.7	89.6	–

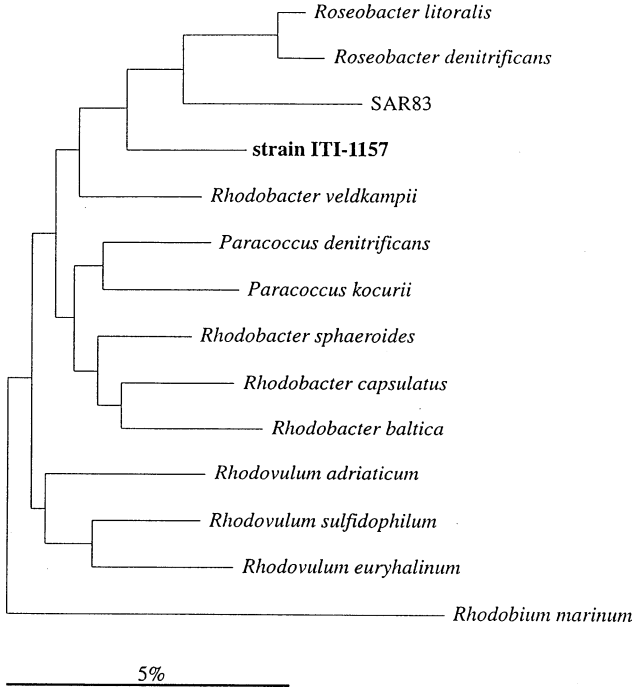

Fig. 3. Dendrogram of several α-proteobacteria based on 16S rRNA sequencing. Strain ITI-1157 forms a branch with *Roseobacter* sp.

mental conditions in the Blue Lagoon should be favorable for many organisms, but the constant precipitation of silica is an unusual factor which probably restricts the number of species capable of living there (Petursdottir and Kristjansson 1996). The presence of vacuoles would certainly enable the bacterium to float and thus counter the sieving effect of the constantly sinking silica particles, and may explain the dominance of this species in the Blue Lagoon.

The absorption spectrum of a methanol extract of cell membranes from ITI-1157 did not reveal any peaks in the area between 700 and 900 nm, as would be expected for bacteriochlorophyll *a* (Shiba 1991). This therefore rules out its placement in the genus *Roseobacter*.

The main cytochrome is a membrane-bound cytochrome *c* with α-peak at 549 nm. Cytochromes *a* and *b* could not be detected spectrophotometrically (Pettigrew and Moore 1987). The sizes of the cytochromes obtained by SDS-PAGE, heme staining, and Coomassie staining are 87, 59, 40, and 24 kDa (data not shown). No soluble cytochromes could be detected spectrophotometrically and only a faint band was revealed by SDS-PAGE analysis of the soluble fraction. Inspection of absorption spectra for *R. litoralis* show a much more complicated pattern: the presence of oxidase in the area of around 620 nm was indicated (data not shown) but cytochrome *c* could not be detected as a distinctive peak by the method used.

DNA base composition and sequencing

The GC mol content of ITI-1157 was determined as 66.15%. Sequencing of the 16S rRNA gene of ITI-1157 revealed it to be a eubacterium of the α-subclass of proteobacteria (GenBank U77644). Its closest relatives were found to be *Roseobacter litoralis* and *Paracoccus kocurii* as shown in the pairwise similarity matrix, with both species giving similarity values of 95.1% (Table 1). In a dendrogram with the 13 most similar α-proteobacteria, our isolate groups with *Roseobacter* sp., together with the uncultivated species SAR 83 from the Sargasso sea (Fig. 3).

Comparison with other bacteria

Our isolate ITI-1157 is not only phylogenetically distant from other bacteria but also phenotypically very different in many respects. The main characteristics of the new isolate are compared with its closest relatives *Roseobacter litoralis* (Shiba 1991), *Erythrobacter longus* (Shiba and Simidu 1982), and *Paracoccus kocurii* (Ohara et al. 1990) in Table 2.

The most striking differences are the bacterial size and presence of vacuoles, lack of motility and bacteriochlorophyll *a*, the high optimum and maximum growth

Table 2. Main characteristics differentiating the new genus and species, *Silicibacter lacuscaerulensis*, from related bacteria

	Silicibacter lacuscaerulensis	Erythrobacter longus	Roseobacter litoralis	Paracoccus kocurii
Cell form	Long rod	Long rod	Ovoid or rod	Ovoid short rod
Cell size (diameter × length)	0.6–0.8 × 9–18 μm	0.3–0.4 × 2–5 μm	0.6–0.9 × 1.0–2 μm	0.5–0.8 × 0.7–1.1 μm
Vacuoles	+	−	−	−
Colony color	Tan	Orange	Pink	Yellowish white
Growth at				
4°C	−	n.d.	n.d.	−
22°C	+	+	+	+
30°C	+	+	+	+
40°C	+	−	−	−
45°C	+	−	−	−
50°C	+	−	−	−
55°C	−	−	−	−
Growth at				
0% NaCl	−	+	+	+
2% NaCl	+	+	+	+
4% NaCl	+	+	+	−
6% NaCl	+	−	−	−
8% NaCl	−	−	−	−
Growth at				
pH 5.5	−	−	−	−
pH 6.5	+	+	−	+
pH 7.5	+	+	+	+
pH 8.5	+	+	+	+
pH 9.5	−	+	+	−
GC mol% in DNA	66.2	57.4	59.6	71
Motility	−	+	+	−
Oxidase	+	+	+	+
Catalase	+	+	+	+
Susceptability to:				
chloramphenicol	+	+	+	n.d.
penicillin	+	−	−	n.d.
tetracycline	+	−	−	n.d.
streptomycin	−	−	−	n.d.
polymyxin B	+	−	−	n.d.
Utilization as a sole carbon source:				
Tetramethylammonium	−	n.d.	n.d.	+
acetate	−	−	+	n.d.
pyruvate	+	V	+	+
glucose	−	V	+	−
citrate	−	V	+	−
Bacteriochlorophyll *a*	−	+	+	−
Nitrate reduction	+	+	−	+

V, variable; n.d., not determined.

temperatures, the moderate halophilicity, and the different GC mol% in DNA. All of these properties are of such a fundamental nature that they clearly exclude the new isolate, ITI-1157, from any of other three species listed in Table 2. The pattern of antibiotic susceptibility, utilization of carbon sources, and colony color further strengthens this conclusion (Table 2).

The GC mol content of DNA for ITI-1157 is 66.15%, which is 8.8% and 6.6% higher than for *E. longus* and *R. litoralis*, respectively, but 4.8% lower than for *P. kocurii*. It is now generally accepted that bacteria differing by more than 5% in GC content should not be assigned to the same species and those differing by more than 10% GC should not be classified within the same genus (Goodfellow and O'Donnell 1993). The similiarity value obtained from sequence alignments of the 16S rRNA between ITI-1157 and *R. litoralis* is 95.1%; the same value is obtained when ITI-1157 is compared to *P. kocurii*. That is the highest similarity value obtained between ITI-1157 and any other 16S rRNA sequences in the α-proteobacteria group.

Strains that show 70% or more DNA–DNA reassociation are currently defined as belonging to the same species (Amann et al. 1995). As 50% DNA–DNA corresponds to about 99% 16S rRNA similarity, the 95% 16S rRNA

similarity found here would correspond to a much lower DNA–DNA reassociation and therefore provide solid evidence of a novel species (Amann et al. 1995).

Description of *Silicibacter* Petursdottir and Kristjansson gen. nov.

Silicibacter (Si.li.ci.bac′ter L. masc. n. *silex*, silica; M.L. masc. n. *bacter*, rod-shaped bacterium; M.L. masc. n. *Silicibacter*, rod-shaped bacterium living in silica (-rich) habitats). Cells are Gram negative, unicellular, nonmotile, nonsporeforming, long rods, about 9–18 μm in length and 0.6–0.8 μm wide. Colonies are tan colored. Obligately aerobic, mesophilic heterotrophs growing optimally at 45°C and at temperatures up to 50°C. Growth strictly salt-dependent. Oxidase and catalase positive. Reduction of nitrate to nitrite. Do not hydrolyze starch or casein. Growth on common amino acids such as arginine and lysine. No growth on glucose. Cells are susceptible to chloramphenicol, penicillin, tetracycline, and polymyxin B, but resistant to streptomycin. No bacteriochlorophyll *a*. No utilization of tetramethylammoniumchloride but tolerance up to 0.2%. The DNA composition is about 66% mol% GC.

Type species: *Silicibacter lacuscaerulensis* (DSM 11314).

Description of *Silicibacter lacuscaerulensis* Petursdottir and Kristjansson sp. nov.

Silicibacter lacuscaerulensis (*la.cus.cae.ru.len′ sis.* L. masc. n. *lacus*, lake; L. adj. *caeruleus*, blue; M.L. adj. *lacuscaerulensis*, pertaining to the blue lake). Description as for the genus. Membrane-bound cytochrome *c* with a distinctive peak at 549 nm. The GC content of type strain ITI-1157 is 66.15 mol%. Optimal growth at 45°C and pH 7.5 and about 3.5% (w/v) NaCl. Growth occurs at up to 7% (w/v) NaCl.

Type strain: *Silicibacter lacuscaerulensis* ITI-1157 (isolated from Blue Lagoon, near Svartsengi geothermal powerplant, Iceland).

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