Nitratireductor aquimarinus sp. nov., isolated from a culture of the diatom *Skeletonema costatum*, and emended description of the genus *Nitratireductor*

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Two Gram-negative-staining, aerobic bacterial strains, designated CL-SC21^T and CL-SC22, were isolated from a culture of the diatom *Skeletonema costatum* (Korean Marine Microalgae Culture Center, KMMCC strain B-396) established from the East Sea, Korea. The two novel strains shared 99.9% 16S rRNA gene sequence similarity. Analysis of the 16S rRNA gene sequences showed an affiliation with the genus *Nitratireductor*, with the strains sharing 96.5–97.5% similarity with the type strains of recognized species of the genus *Nitratireductor* and being most closely related to *Nitratireductor aquibiodomus* NL21^T. Phylogenetic analyses of the 16S rRNA gene sequences showed that strain CL-SC21^T together with strain CL-SC22 belonged to the genus *Nitratireductor* and formed a robust clade among closely related *Nitratireductor* species. The polar lipids comprised phosphatidylcholine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylmonomethylethanolamine, two unidentified aminophospholipids, an unidentified aminolipid, an unidentified phospholipid and five unidentified lipids. Ubiquinone 10 was the major quinone. The major cellular fatty acids of strains CL-SC21^T and CL-SC22 were C_{18:1}ω7c (70.6–72.3%) and C_{19:0}ω8c cyclo (10.9–11.8%). The genomic DNA G+C contents of strains CL-SC21^T and CL-SC22 were 56.7 and 57.1 mol%, respectively. The level of DNA–DNA relatedness between strains CL-SC21^T and CL-SC22 was 86% (reciprocal 91%), indicating that the two isolates represented a single species. However, levels of DNA–DNA relatedness between *N. aquibiodomus* NL21^T and strains CL-SC21^T and CL-SC22 were 28% (reciprocal 45%) and 25% (reciprocal 50%), respectively. Phylogenetic analysis and the results of biochemical tests showed that strains CL-SC21^T and CL-SC22 were different from all recognized species of the genus *Nitratireductor*. Thus, strains CL-SC21^T and CL-SC22 represent a novel species of the genus *Nitratireductor*, for which the name *Nitratireductor aquimarinus* sp. nov. is proposed. The type strain is CL-SC21^T (=KCCM 90090^T=JCM 17288^T).

The genus *Nitratireductor*, a member of the family *Phyllobacteriaceae*, was formally described by Labbé *et al.* (2004) and, at the time of writing, comprised five recognized species, *Nitratireductor aquibiodomus*, *N. basaltis*, *N. kimnyeongensis*, *N. indicus* and *N. pacificus*. *N. aquibiodomus* NL21^T, a nitrate-reducing bacterium, was isolated from a marine denitrification system of the Montreal Biodome (Labbé *et al.*, 2004). *N. basaltis* was isolated from black beach sand of Jeju Island, Korea (Kim *et al.*, 2009). *N. kimnyeongensis* (Kang *et al.*, 2009) was isolated from a dried seaweed sample collected from Kimnyeong Beach in Jeju, Korea. *N. indicus* (Lai *et al.*, 2011b)

and *N. pacificus* (Lai *et al.*, 2011a) were isolated from deep-sea water of the Indian Ocean and deep-sea sediment of the Pacific Ocean, respectively. The aim of the present study was to determine the taxonomic positions of two *Nitratireductor*-like strains, CL-SC21^T and CL-SC22, isolated from a diatom culture.

A culture of the diatom *Skeletonema costatum* (KMMCC strain B-396) has been established from the east coast of Korea and maintained by transferring small quantities of culture to f/2 medium (Guillard & Ryther, 1962). For isolating bacteria from the diatom culture, an aliquot (10 µl) of the culture in the exponential phase was taken and spread on marine agar 2216 (MA; Difco) plates and incubated aerobically at 30 °C for 1 week. Two novel strains, CL-SC21^T and CL-SC22, were isolated and subsequently streaked onto fresh MA at 30 °C. The purification procedure

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains CL-SC21^T and CL-SC22 are HQ176467 and HQ176466, respectively.

One supplementary figure and a supplementary table are available with the online version of this paper.

was repeated four times. The two novel strains were able to grow well on trypticase soy agar (TSA; Difco) at 35 °C. The two strains were maintained on TSA at 35 °C and preserved in trypticase soy broth (TSB; Difco) supplemented with 30 % (v/v) glycerol at –80 °C.

Morphological and physiological analyses were performed as follows. Unless otherwise specified, all characteristics of strains CL-SC21^T and CL-SC22 were based on cultures grown on TSA at 35 °C. Gram-staining was performed as described by Smibert & Krieg (1994). Motility of the cells was observed by the hanging-drop method (Suzuki *et al.*, 2001). Cell morphology and presence of flagella were examined by transmission electron microscopy (EX2; JEOL). The temperature range for growth was determined on the basis of colony formation on TSA incubated at 5–45 °C (increments of 5 °C) and 4 °C. Growth at pH 5.1, 5.6, 6.0, 6.5, 6.9, 7.3, 7.7, 8.2, 8.6, 9.0, 9.3 and 9.6 was determined by assessing changes in OD₆₀₀ over the incubation period (up to 10 days) in TSB at 35 °C; prior to autoclaving, pH (5–10, at increments of 0.5 pH units) was adjusted by using 1 M NaOH and 1 M HCl solutions. After autoclaving and cooling, the pH values were measured for the subsamples of each medium, before inoculation of cells. The tolerance of strains CL-SC21^T and CL-SC22 to NaCl was determined on the basis of colony formation on synthetic ZoBell agar (per litre distilled water: 5 g Bacto peptone, 1 g yeast extract, 0.1 g ferric citrate, 15 g Bacto agar) supplemented with 0–10 % (increments of 1 %) and 15 % (w/v) NaCl. Anaerobic growth was tested on TSA medium by using the GasPak anaerobic system (BBL) for 15 days. Resistance to antibiotics was determined by the disc diffusion plate method (Bauer *et al.*, 1966) by using custom-made antibiotic-impregnated discs.

For 16S rRNA gene amplification by PCR, DNA was extracted from a single colony by the boiling method (Englen & Kelley, 2000). The crude extracts served as the DNA template for PCR, which included *Taq* DNA polymerase (Bioneer) and primers 27F and 1492R (Lane, 1991). The PCR product was purified by using the AccuPrep PCR purification kit (Bioneer). Sequencing of the purified 16S rRNA gene was performed by using sequencing primers (27F, 518F, 800R, 1492R; Lane, 1991; Anzai *et al.*, 1997) with an Applied Biosystems automated sequencer (ABI3730XL) at Macrogen, Seoul, Korea. The almost-complete 16S rRNA gene sequences of strains CL-SC21^T (1417 bp) and CL-SC22 (1416 bp) were obtained and compared with available 16S rRNA gene sequences in the GenBank database by using BLASTN searches (Altschul *et al.*, 1990). The sequences of strains CL-SC21^T and CL-SC22 were manually aligned via the jPHYDIT program (Jeon *et al.*, 2005) with all available 16S rRNA gene sequences from the type strains of recognized species of the genus *Nitratireductor* and with those of the type species of other genera in the family *Phyllobacteriaceae*, obtained from the GenBank and Ribosomal Database Project II databases (Cole *et al.*, 2007). Accurate multiple alignment was made manually according to the 16S rRNA gene secondary-structure information implemented in the

jPHYDIT program. Phylogenetic trees were constructed with the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods. An evolutionary distance matrix for the neighbour-joining method was generated according to the model of Jukes & Cantor (1969). The robustness of tree topologies was assessed by bootstrap analyses based on 1000 replications for the neighbour-joining and maximum-parsimony methods. Phylogenetic analyses were carried out by using MEGA 4 (Tamura *et al.*, 2007) and PAUP 4.0 (Swofford, 1998). Likelihood parameters were estimated by using hierarchical ratio tests in MODELTEST, version 3.04 (Posada & Crandall, 1998). Levels of genomic DNA–DNA relatedness were determined by dot-blot hybridization (Kim *et al.*, 2007). Pre-hybridization, hybridization (45 °C) and detection were performed by using a DIG labelling and detection kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. The experiment was repeated on different days.

Polar lipids were extracted according to the procedures described by Minnikin *et al.* (1984) and were identified by two-dimensional TLC followed by spraying with appropriate detection reagents (Komagata & Suzuki, 1987). The quinone system was determined according to Minnikin *et al.* (1984) and components were analysed by HPLC as described by Collins (1985) by using *Cohaesibacter gelatinilyticus* CL-GR15^T (Hwang & Cho, 2008) as a reference strain. Cellular fatty acids of strains CL-SC21^T and CL-SC22 were extracted and derivatized to their fatty acid methyl esters according to the protocol of Miller (1982). The samples were then prepared according to the instructions of the Sherlock Microbial Identification System (MIDI; version 3.10) and were analysed by GC at the Korean Culture Center of Microorganisms (Seoul, Korea). The strains were grown on TSA (Difco) at 35 °C for 3 days and on MA at 28 °C for 2 days. The DNA G + C content was analysed by HPLC (HP 100; Hewlett Packard) of deoxyribonucleosides as described by Mesbah *et al.* (1989), after DNA extraction according to Marmur (1961). *N. aquibiodomus* NL21^T was used as a standard.

In the following biochemical tests, *N. aquibiodomus* NL21^T was used as a reference strain. Catalase and oxidase were assayed according to the protocols described by Smibert & Krieg (1994). Strains were tested for the ability to reduce nitrate under anaerobic conditions at 35 °C in TSB supplemented with 10 mM NO₃[–] as KNO₃ and 10 µM phenol red (Vanparys *et al.*, 2005). The presence of nitrite and nitrate was tested after 2 and 5 days by using the procedure described by Smibert & Krieg (1994). The biochemical characteristics of strains CL-SC21^T, CL-SC22, *N. aquibiodomus* NL21^T, *N. basaltis* J3^T and *N. kimnyeongensis* KY 101^T were determined by using the API ZYM and API 20NE kits (bioMérieux) according to the manufacturer's instructions, except that the cell suspension was prepared with artificial seawater (per litre distilled water: 24 g NaCl, 10.9 g MgCl₂·6H₂O, 4 g Na₂SO₄, 1.5 g CaCl₂·2H₂O, 0.7 g KCl, 0.2 g NaHCO₃, 0.1 g KBr, 0.027 g H₃BO₃, 0.03 g SrCl₂·6H₂O, 0.003 g NaF; Lyman &

Fleming, 1940). For tests with the API ZYM and API 20NE kits, all strains were cultivated on MA at 35 °C for 2 days.

The level of 16S rRNA gene sequence similarity between strains CL-SC21^T and CL-SC22 was 99.9 %. Analysis of 16S rRNA gene sequences showed an affiliation with the genus *Nitrateductor*, the two novel strains sharing 96.5–97.5 % similarity with the type strains of recognized species of the genus *Nitrateductor* and being most closely related to *N. aquibiodomus* NL21^T. Phylogenetic analyses of the 16S rRNA gene sequences showed that strain CL-SC21^T formed a robust clade with strain CL-SC22, and that this clade clustered tightly with the nearest clade containing *N.*

aquibiodomus NL21^T and *N. kimnyeongensis* KY 101^T (Fig. 1). Thus, the distinct phylogenetic position of the two strains indicated that they represented a novel species of the genus *Nitrateductor*. The mean level of DNA–DNA relatedness between strains CL-SC21^T and CL-SC22 was 86 ± 1 % (reciprocal 91 ± 1 %), indicating that these two strains belonged to a single species. Mean levels of DNA–DNA relatedness between *N. aquibiodomus* NL21^T and strains CL-SC21^T and CL-SC22 were 28 ± 16 % (reciprocal 45 ± 18 %) and 25 ± 8 % (reciprocal 50 ± 1 %), respectively. These values are below the currently accepted cut-off (70 %) for the phylogenetic definition of a species (Stackebrandt & Goebel, 1994) and therefore the phylogenetic and genomic

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic positions of strains CL-SC21^T and CL-SC22, and the type strains of recognized species of the genus *Nitrateductor* and members of the family *Phyllobacteriaceae*; *Hyphomicrobium denitrificans* DSM 1869^T was used as the outgroup. Bootstrap values (percentages of 1000 resamplings) are shown at branch points; only values >60 % are shown. Solid circles indicate that the corresponding nodes were also obtained in the maximum-likelihood and maximum-parsimony trees. Bar, 0.01 nt substitutions per site.

evidence indicates that strains CL-SC21^T and CL-SC22 represent a single novel species of the genus *Nitrateductor*.

The major polar lipids of strain CL-SC21^T were phosphatidylcholine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylmonomethyl-ethanolamine, two unidentified aminophospholipids, an unidentified aminolipid and three unidentified lipids (see Supplementary Fig. S1 in IJSEM Online). An unidentified phospholipid and two unidentified lipids were detected in minor amounts (Supplementary Fig. S1). All the above polar lipids were commonly detected in strain CL-SC21^T and in *N. aquibiodomus* NL21^T (Supplementary Fig. S1). The major

quinone was ubiquinone 10 (Q-10) for strains CL-SC21^T, CL-SC22 and *N. aquibiodomus* NL21^T. The fatty acid profiles of strains CL-SC21^T and CL-SC22 were similar (Supplementary Table S1): the major components were C_{18:1}ω7c (70.6–72.3 %) and C_{19:0}ω8c cyclo (10.9–11.8 %). These fatty acids are also found as major components in related species of the genus *Nitrateductor* (Supplementary Table S1). The genomic DNA G+C contents of strains CL-SC21^T and CL-SC22 (56.7 and 57.1 mol%, respectively) were within the range reported for members of the genus *Nitrateductor*, but differed distinctly from that of the type strain of *N. pacificus* (63 mol%; Lai *et al.*, 2011a). Strains CL-SC21^T and CL-SC22 had traits differentiating them from

Table 1. Differential characteristics between strains CL-SC21^T and CL-SC22 and the type strains of recognized species of the genus *Nitrateductor*

Strains: 1, CL-SC21^T and CL-SC22; 2, *N. aquibiodomus* NL21^T (unless indicated otherwise, data from Labbé *et al.*, 2004); 3, *N. basaltis* J3^T (Kim *et al.*, 2009); 4, *N. kimmyenensis* KY 101^T (Kang *et al.*, 2009). +, Positive; –, negative; w, weakly positive. All strains are Gram-negative-staining and oxidase- and catalase-positive. In API ZYM and API 20NE tests, all strains are positive or weakly positive for alkaline phosphatase, acid phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase, but negative for lipase (C14), α-chymotrypsin, α-fucosidase, β-glucuronidase, β-glucosidase, α-mannosidase and gelatinase, indole production, glucose fermentation and assimilation of caprate and phenylacetate.

Characteristic	1	2	3	4
Temperature range for growth (optimum) (°C)	20–40 (30–35)	10–40 (30–35)	15–45 (35)*	10–45 (30)
pH range for growth (optimum)	6.5–9.0 (7.7–8.2)	6.5–9.0 (7.0–7.5)	5.5–10.0 (7.0–8.1)*	6.1–12.1 (7.0)*
NaCl range for growth (optimum) (%)	1–7 (3–4)	0–5 (1)	0–8 (3)*	0–7 (3)*
DNA G + C content (mol%)	56.7 (57.1)†	57	56.7	60.4
API ZYM results*				
Cystine arylamidase	w	w‡	–	–‡
α-Galactosidase	–	–	+	–
β-Galactosidase	–	–	w	–
α-Glucosidase	–	w‡	–	+
β-Glucosidase	–	–	–	–
N-Acetyl-β-glucosaminidase	+	+	–	–
API 20NE results*				
Nitrate reduction	+	+	–‡	–
Arginine dihydrolase	–	w	–	–
Urease	+	–	–	–
β-Glucosidase (aesculin hydrolysis)	–	–	+\$	–
β-Galactosidase (PNPG)	+	–	+	–
Assimilation (API 20NE) of:*				
Arabinose	+	+	–‡	–
Mannose	+	+	–	–
Mannitol	–	–	+	+
Maltose	–	–	–‡	–
Gluconate	–	–	+	–
Glucose	+	+	+	+
Adipate	–	+	–	–
Malate	–	+	–	–
Citrate	+	+	–	–

*Data from this study.

†Data in parentheses are for strain CL-SC22.

‡Positive in each original study.

\$Negative in Kim *et al.* (2009).

||Weakly positive in Labbé *et al.* (2004).

recognized species of the genus *Nitratireductor*. For example, the minimum temperature for growth of strains CL-SC21^T and CL-SC22 (20 °C) was higher than those of *N. aquibiodomus*, *N. basaltis* and *N. kimnyenogensis* (10–15 °C, Table 1). Strains CL-SC21^T and CL-SC22 could be distinguished from *N. aquibiodomus*, *N. basaltis* and *N. kimnyenogensis* based on the presence of urease activity (Table 1). Strains CL-SC21^T and CL-SC22 differed from their closest phylogenetic neighbour, *N. aquibiodomus*, based on inability to grow without NaCl, the absence of arginine dihydrolase and α -glucosidase activities, and the presence of β -galactosidase (API 20NE) activity (Table 1). Strains CL-SC21^T and CL-SC22 could be distinguished from *N. basaltis* and *N. kimnyenogensis* by their inability to grow without NaCl and the presence of *N*-acetyl- β -glucosaminidase activity (Table 1). They could be distinguished from *N. pacificus* and *N. indicus* by their inability to grow without NaCl, and inability to grow at pH 5 and at 10–15 °C under the same conditions as described in Lai *et al.* (2011a, b) (data not shown). Thus, on the basis of the data from the present polyphasic study, strains CL-SC21^T and CL-SC22 are considered to represent a single novel species of the genus *Nitratireductor*, for which the name *Nitratireductor aquimarinus* sp. nov. is proposed.

Emended description of the genus *Nitratireductor* Labbé *et al.* 2004

With the properties given in the descriptions of species of the genus *Nitratireductor* in earlier studies (Kang *et al.*, 2009; Kim *et al.*, 2009; Lai *et al.*, 2011a, b) and the present study, the description of the genus given by Labbé *et al.* (2004) is emended as follows. Cells are rods, short rods or coccoid. Motility is variable. Colony colour is white, light yellow, smooth grey or cream. Optimum temperature for growth is 25–35 °C. Growth occurs at pH 5–12. Nitrate reduction is variable. Major polar lipids are phosphatidylcholine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylmonomethyl-ethanolamine, two unidentified aminophospholipids and three unidentified lipids. Major quinone is Q-10. DNA G+C content is 56.7–63 mol%.

Description of *Nitratireductor aquimarinus* sp. nov.

Nitratireductor aquimarinus (a.qui.ma.ri'nus. L. fem. n. *aqua* water; L. adj. *marinus*-, -a, -um marine, of the sea; N.L. masc. adj. *aquimarinus* pertaining to seawater).

Cells are rod-shaped, approximately 0.4–0.7 μ m wide and 1.3–2.0 μ m long. Cells are Gram-negative-staining, strictly aerobic and motile by means of a polar flagellum. After 3 days on TSA and MA plates at 35 °C, colonies are smooth, circular, convex and creamy in colour. Growth occurs at 20–40 °C (optimum 30–35 °C), at pH 6.5–9.0 (optimum pH 7.7–8.2) and in the presence of 1–7 % (w/v) NaCl (optimum 3–4 % NaCl). Positive for oxidase and catalase. Nitrate is reduced. According to API ZYM tests,

positive for acid phosphatase, alkaline phosphatase, cystine arylamidase (weak), esterase (C4), esterase lipase (C8; weak for strain CL-SC22), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, trypsin, valine arylamidase (weak) and *N*-acetyl- β -glucosaminidase activities, but negative for α -chymotrypsin, α -fucosidase, α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, β -glucuronidase, lipase (C14) and α -mannosidase activities. According to API 20NE tests, positive for nitrate reduction, β -galactosidase, urease, and assimilation of arabinose, *N*-acetylglucosamine, citrate, glucose and mannose, but negative for arginine dihydrolase, aesculin hydrolysis, gelatinase, glucose fermentation, indole production, and assimilation of adipate, caprate, gluconate, malate, maltose, mannitol and phenylacetate. Cells are sensitive to (μ g per disc) ampicillin (6), chloramphenicol (20), cephalixin (20), erythromycin (10), gentamicin (6), kanamycin (20), polymyxin B (25), streptomycin (6), tetracycline (20) and vancomycin (20), but resistant to nalidixic acid (20), mitomycin C (0.6) and penicillin G (6). The major cellular fatty acids are C_{18:1 ω 7c} and C_{19:0 ω 8c} cyclo. The DNA G+C content is 56.7–57.1 mol%.

The type strain, CL-SC21^T (=KCCM 90090^T=JCM 17288^T), was isolated from a culture of the diatom *Skeletonema costatum*. CL-SC22, isolated from the same source, is a second strain of the species.

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