Arcobacter marinus sp. nov.

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A slightly curved, rod-shaped marine bacterium, designated strain CL-S1^T, was isolated from near Dokdo, an island in the East Sea, Korea. Cells were Gram-negative and grew well under either aerobic or microaerobic conditions. Analyses of the 16S rRNA and *gyrA* gene sequences of strain CL-S1^T revealed an affiliation with the genus *Arcobacter* within the class *Epsilonproteobacteria*. Phylogenetic analyses based on 16S rRNA and *gyrA* gene sequences showed that strain CL-S1^T formed a robust clade with *Arcobacter halophilus* LA31B^T, with sequence similarities of 96.1 and 88.2%, respectively. DNA–DNA relatedness between strain CL-S1^T and *A. halophilus* DSM 18005^T was 44%, indicating that they represent genomically distinct species. Strain CL-S1^T grew optimally at 30–37 °C, at pH 7 and in the presence of 3–5% NaCl. The dominant cellular fatty acids were iso-C_{15:0} 2-OH and/or C_{16:1}ω7c (28.4%), C_{16:0} (26.2%) and C_{18:1}ω7c (22.3%). The DNA G+C content of strain CL-S1^T was 28 mol%. Strain CL-S1^T differed phenotypically from *A. halophilus* LA31B^T based on its ability to grow aerobically at 10 °C and inability to grow under anaerobic conditions. Based on the data presented, strain CL-S1^T is considered to represent a novel species of the genus *Arcobacter*, for which the name *Arcobacter marinus* sp. nov. is proposed. The type strain is CL-S1^T (=KCCM 90072^T =JCM 15502^T).

The genus *Arcobacter* belongs to the family *Campylobacteraceae*, which also contains the genera *Campylobacter* and *Sulfurospirillum* (Vandamme *et al.*, 2005a). The genus *Arcobacter* was proposed by Vandamme *et al.* (1991); two *Campylobacter* species, [*Campylobacter*] *nitrofigilis* (McClung *et al.*, 1983) and [*Campylobacter*] *cryaerophilus* (Neill *et al.*, 1985), were reclassified as *Arcobacter nitrofigilis* and *Arcobacter cryaerophilus*, respectively (Vandamme *et al.*, 1991). [*Campylobacter*] *butzleri* (Kiehlbauch *et al.*, 1991) was later reclassified as *Arcobacter butzleri* (Vandamme *et al.*, 1992). Three other species of the genus (*Arcobacter skirrowii*, *Arcobacter cibarius* and *Arcobacter halophilus*) have been newly described (Vandamme *et al.*, 1992; Houf *et al.*, 2005; Donachie *et al.*, 2005). At the time of writing, the genus thus comprises six recognized species (Donachie *et al.*, 2005; Houf *et al.*, 2005; Vandamme *et al.*, 2005b). Members of the genus *Arcobacter* have been isolated from various environments. *A. cryaerophilus*, *A. butzleri* and *A. skirrowii* have been recovered from livestock and humans (Neill *et al.*, 1985; Kiehlbauch *et al.*, 1991; Vandamme *et al.*, 1992) and are recognized as potential emerging human pathogens

(Mansfield & Forsythe, 2000). *A. nitrofigilis* was isolated from *Spartina alterniflora* roots in a salt marsh (McClung *et al.*, 1983). *A. halophilus* and *A. cibarius* were isolated from a hypersaline lagoon (Donachie *et al.*, 2005) and the skin of a broiler carcass (Houf *et al.*, 2005), respectively. In the present study, a novel strain affiliated with the genus *Arcobacter* was isolated and subjected to a polyphasic taxonomic analysis.

Seaweeds and a starfish were collected and added to a surface seawater sample (50 ml) taken from the vicinity of Dokdo, an island in the East Sea, Korea. The mixture was maintained at 4 °C for 15 days. The mixture was then homogenized by using a blender and an aliquot (50 µl) was spread onto MY medium (Bouchotroch *et al.*, 2001). The culture was incubated aerobically at 30 °C for 2 weeks. Strain CL-S1^T was isolated and subsequently purified on fresh marine agar 2216 (MA; Difco). Strain CL-S1^T grew well on MA or on saline blood agar [SBA; per litre distilled water: 40 g blood agar base (BBL), 50 ml sheep blood, 30 g NaCl] at 37 °C under either aerobic or microaerobic conditions. The novel strain was preserved in marine broth 2216 (MB; Difco) supplemented with 30% (v/v) glycerol at –80 °C.

For 16S rRNA gene amplification by PCR, DNA was extracted from a single colony by a boiling method (Englen & Kelley, 2000). The crude extracts served as the DNA template for PCRs, which included *Taq* DNA polymerase (Bioneer) and primers 27F and 1492R (Lane, 1991). The PCR product was purified by using the AccuPrep PCR

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *gyrA* gene sequences of strain CL-S1^T are EU512920 and FJ754218, respectively.

Neighbour-joining trees based on *gyrA* gene sequences and inferred amino acid sequences of the *gyrA* gene and transmission electron micrographs of negatively stained cells of strain CL-S1^T are available as supplementary material with the online version of this paper.

purification kit (Bioneer), and direct sequence determination of the purified 16S rRNA gene was performed by using sequencing primers (27F, 518F, 800R and 1492R (Lane, 1991; Anzai *et al.*, 1997) with an Applied Biosystems automated sequencer (ABI3730XL) at MacroGen (Seoul, Korea). For PCR amplification of the *gyrA* gene (encoding subunit A of DNA gyrase), genomic DNA was extracted from strain CL-S1^T and *A. halophilus* DSM 18005^T (Donachie *et al.*, 2005) by using the AccuPrep genomic DNA extraction kit. To amplify the *gyrA* gene, we first employed PCR by using two sets of degenerate primers (Abdelbaqi *et al.*, 2007). However, no PCR product was obtained from the genomic DNA of either strain CL-S1^T or *A. halophilus* DSM 18005^T in our study (data not shown). We designed new primers based on the alignment of the *gyrA* gene sequences of recognized *Arcobacter* strains in GenBank by using the Primer3 program (Rozen & Skaletsky, 2000). The following primers were used: forward primer, 5'-TGGACGTGCATTACCAGATG-3'; reverse primer, 5'-GCAACTTTTCTTTTCCACCT-3'. PCR amplifications were performed in a reaction mixture of 20 µl (total volume) containing approximately 30 ng genomic DNA, 125 µM each dNTP, 0.5 µM each primer, 1.5 U *Taq* DNA polymerase (Bioneer) and the buffer supplied with the enzyme. PCR amplifications were performed according to the following sequence: 95 °C (5 min), 30 cycles of 95 °C (30 s), annealing at 55 °C (30 s) and elongation at 72 °C (3 min) and an additional elongation at 72 °C (5 min). The PCR product was purified by using the AccuPrep PCR purification kit and was sequenced directly by using the newly designed primers as sequencing primers.

The almost-complete 16S rRNA gene sequence of strain CL-S1^T (1426 bp) was obtained along with partial *gyrA* gene sequences (approximately 60% coverage) of strain CL-S1^T (1410 bp) and *A. halophilus* DSM 18005^T (1368 bp). These sequences were compared with sequences available in GenBank by using the BLASTN algorithm (Altschul *et al.*, 1990). The 16S rRNA gene sequence of strain CL-S1^T was aligned via the jPHYDIT program (Jeon *et al.*, 2005) with sequences of the type strains of recognized *Arcobacter* species and phylogenetically related taxa obtained from GenBank and the Ribosomal Database Project II database (Cole *et al.*, 2007). Accurate multiple alignment was made manually according to the 16S rRNA gene secondary-structure information implemented in the jPHYDIT program. For the *gyrA* gene sequences of strain CL-S1^T, *A. halophilus* DSM 18005^T, other type strains of recognized *Arcobacter* species and phylogenetically related taxa obtained from GenBank, multiple sequence alignment was performed by using the T-COFFEE program (Notredame *et al.*, 2000). Phylogenetic trees were obtained according to 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, with 1000 replications for the neighbour-joining and maximum-parsimony methods and 100 replications for the maximum-likelihood method. Phylogenetic analyses were carried out by using MEGA 3 (Kumar *et al.*, 2004) and PAUP 4.0 (Swofford, 1998). Likelihood parameters were estimated by using the hierarchical ratio tests in MODELTEST version 3.04 (Posada & Crandall, 1998). Levels of genomic DNA relatedness between strains CL-S1^T and *A. halophilus* DSM 18005^T were determined by dot-blot hybridization as described by Choi *et al.* (2006). The experiment was repeated on different days.

Morphological, physiological and biochemical analyses were performed in duplicate. *A. halophilus* DSM 18005^T was used as a reference strain for all tests. Unless specified otherwise, phenotypic characteristics of strains CL-S1^T and *A. halophilus* DSM 18005^T were based on cultures grown on SBA at 37 °C under both aerobic and microaerobic conditions. Gram-staining was performed as described by Smibert & Krieg (1994). Cell shape and size and presence of flagella were determined by using transmission electron microscopy (EX2; JEOL) with cells grown under both aerobic and microaerobic conditions for 1 and 7 days. Cell motility was observed by phase-contrast microscopy (BX50; Olympus). Anaerobic and microaerobic growth were checked on SBA, MA and trypticase soy agar (TSA; Difco) supplemented with 3% NaCl; growth was tested by using GasPak Anaerobe and GasPak Campy Container systems (BBL) at 37 °C for 15 days. Anaerobic growth was further tested on a synthetic growth agar (Teske *et al.*, 1996) in the presence of 10% CO₂ and 90% N₂ at 35 °C for 15 days. The temperature range for growth was assessed based on colony formation on SBA incubated aerobically at 5–40 °C (increments of 5 °C) and at 37 °C. The tolerance of strain CL-S1^T to NaCl was determined based on the formation of visible single colonies on TSA with different concentrations of NaCl (1–15% in increments of 1% as well as 0.5, 1.5, 2.5 and 3.5%, w/v) at 37 °C aerobically. The pH range (pH 4.0, 4.5, 5.0, 5.9, 7.0, 7.5, 8.0, 8.5, 9.4 and 10.3) for growth in MB was determined by assessing changes in OD₆₀₀ over an incubation period of 6 days at 37 °C; prior to autoclaving the medium, its pH was adjusted by using 1 M NaOH and 1 M HCl solutions. After autoclaving and cooling, the pH in the test medium increased by 0.1–0.4 pH units (for pH 4.0–8.0 and 9.4) or decreased by 0.7 pH units (for pH 10.3). Thus, the pH was measured in subsamples of each medium, before inoculation with bacterial cells.

Catalase and oxidase tests were performed according to the protocols described by Smibert & Krieg (1994). Hydrolysis of urea was determined as described by Owen *et al.* (1985). Nitrate reductase activity was determined according to Hansen & Sørheim (1991). H₂S production and hydrolysis of hippurate were determined as described by Skirrow & Benjamin (1980). Hydrolysis of indoxyl acetate was determined according to Popovic-Uroic *et al.* (1990). Alkaline phosphatase activity was determined as described by Reddy *et al.* (2007).

Fatty acid methyl esters in whole cells of strain CL-S1^T grown on SBA at 37 °C microaerobically for 1 day were analysed by GC according to the instructions of the Microbial Identification System (MIDI) at the Korean Culture Center of Microorganisms (KCCM; Seoul, Korea). The DNA G+C content was determined by HPLC (HP 100; Hewlett Packard) of deoxyribonucleosides as described by Mesbah *et al.* (1989), after DNA extraction had been performed according to the method proposed by Marmur (1961). Lambda phage DNA was used as a standard, and genomic DNA of *A. halophilus* DSM 18005^T (35 mol%; Donachie *et al.*, 2005) and *Pseudomonas jinjuensis* KACC 10760^T (66.9 mol%; Kwon *et al.*, 2003) was used as references.

Phylogenetic analyses based on 16S rRNA gene sequences revealed that strain CL-S1^T fell within the cluster comprising *Arcobacter* species (Fig. 1). The closest relative of strain CL-S1^T was *A. halophilus* LA31B^T (96.1% 16S rRNA gene sequence similarity). Levels of 16S rRNA gene sequence similarity between strain CL-S1^T and the type strains of other *Arcobacter* species were 92.4–94.3%. In phylogenetic trees constructed with the three algorithms, strain CL-S1^T formed a tight clade with *A. halophilus* LA31B^T (Fig. 1), showing high bootstrap values (neighbour-joining, 99%; maximum-likelihood, 85%; maximum-parsimony, 93%). Phylogenetic analyses based on *gyrA* gene sequences gave results similar to those based on 16S rRNA gene sequences (Supplementary Fig. S1a available in IJSEM Online). Strain CL-S1^T was affiliated with the genus *Arcobacter* and was related most closely to the type strain of *A. halophilus* (88.3% *gyrA* gene sequence similarity) followed by other members of the genus *Arcobacter* (81.2–84.0% *gyrA* gene sequence similarity). The level of *gyrA* gene sequence divergence between the type strain of *A. halophilus* and strain CL-S1^T (11.7%) was similar to the range of sequence divergences between the six recognized *Arcobacter* species (10.2–18.6%; Supplementary Fig. S1a). The level of DNA–DNA relatedness between strain CL-S1^T and *A. halophilus* DSM 18005^T was 44%, indicating that they represent genomically distinct species. Taken together, the above results indicate

that strain CL-S1^T represents a novel species of the genus *Arcobacter*.

Cells of strain CL-S1^T were Gram-negative and motile with a single polar flagellum (Supplementary Fig. S2a). Cells were slightly curved rods (Supplementary Fig. S2b) in cultures grown for 1 and 7 days. Cell shape and size did not differ between cells grown under aerobic or microaerobic conditions. All phenotypic tests with cells of strain CL-S1^T grown under microaerobic conditions gave results consistent with those grown under aerobic conditions except hydrolysis of indoxyl acetate. Indoxyl acetate was hydrolysed by cells grown under microaerobic conditions, but not by those grown under aerobic conditions. Other phenotypic characteristics of strain CL-S1^T are given in the species description below and in Table 1. The fatty acid profile of strain CL-S1^T is given in Table 2.

Several chemotaxonomic and phenotypic characteristics could be used to differentiate strain CL-S1^T from recognized *Arcobacter* species. The inability to grow under anaerobic conditions differentiated strain CL-S1^T from *A. nitrofigilis*, *A. skirrowii*, *A. cibarius* and *A. halophilus* (Table 1). Strain CL-S1^T could be distinguished from its phylogenetically closest relative, *A. halophilus*, by its ability to grow at 10 °C aerobically (this test was performed simultaneously for strain CL-S1^T and *A. halophilus* DSM 18005^T in this study; not shown). Strain CL-S1^T could be distinguished from *A. nitrofigilis* by its inability to produce catalase (Table 1) and from *A. butzleri* by the absence of C_{16:1}ω7t and C_{14:1}ω6c (Table 2). Strain CL-S1^T could be distinguished from *A. skirrowii* by the absence of C_{16:1}ω7 (Table 2) and its inability to produce catalase and haemolysis (Table 1). Strain CL-S1^T could be distinguished from *A. cibarius* by its ability to produce nitrate reductase and ability to grow in air at 37 °C and in the presence of 2–4% NaCl (Table 1). Strain CL-S1^T could be distinguished from *A. cryaerophilus* by the absence of C_{14:1}ω6c (Table 2) and its ability to grow in the presence of 4% NaCl (Table 1).

In conclusion, the phylogenetic, chemotaxonomic and phenotypic data obtained in this study indicate that strain

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the relationship between strain CL-S1^T and members of the genus *Arcobacter*. Bootstrap values (expressed as percentages of 1000 replications) >80% are shown at nodes. Solid circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-likelihood and maximum-parsimony methods. The sequence of *Nautilia lithotrophica* 525^T was used as an outgroup. Bar, 0.02 nucleotide substitutions per site.

Table 1. Differential characteristics between strain CL-S1^T and recognized *Arcobacter* species

Taxa: 1, strain CL-S1^T; 2, *A. halophilus* (data from Donachie *et al.*, 2005); 3, *A. nitrofigilis*; 4, *A. butzleri*; 5, *A. skirrowii*; 6, *A. cibarius* (Houf *et al.*, 2005); 7, *A. cryaerophilus*. For taxa 3–5 and 7, phenotypic data and DNA G + C content data were from Vandamme *et al.* (1992) and Vandamme *et al.* (2005b), respectively. Prior to phenotypic tests, cells were grown under microaerobic conditions at 37 °C for taxa 1, 3–5 and 7, and at 18–22 °C for taxon 2 (temperature data were not available for taxon 6; Houf *et al.*, 2005). Unless indicated otherwise, all phenotypic tests were performed under microaerobic conditions at 37 °C. All strains were positive for oxidase and hydrolysis of indoxyl acetate. All strains were negative for Gram staining, H₂S production, hydrolysis of hippurate and alkaline phosphatase. +, Positive; –, negative; v, variable; w, weakly positive.

Characteristic	1	2	3	4	5	6	7
Anaerobic growth	–	+	+	+ / v*	+	w	+ / v*
Catalase	–	–†	+	+ / v*	+	v†	+ / v*
Urease	–	–†	– / +‡	–	–	–†	–
Nitrate reductase	+	+†	+	+ / v*	+	–†	v
Haemolysis	–	–†	–	v	+	–†	– / v*
Growth in:							
Air at 37 °C	+	+	v	+	+	–	v
2 % (w/v) NaCl	+	+†	+‡	+*	+*	–†	v*
4 % (w/v) NaCl	+	+†	+‡	v*	+*	–†	–*
DNA G + C content (mol%)	28	35	28–29	28–31	29–30	26.8–27.3	28–30

*Temperature for cultivation of cells was not available (Atabay *et al.*, 1998); for the anaerobic growth test, a temperature of 25 °C was used for taxon 4 (Atabay *et al.*, 1998) and, for taxon 7, the result was from On *et al.* (1996).

†Temperature for the tests was not available.

‡Temperature for both cultivation of cells and performing the tests was 25 °C (On *et al.*, 1996).

CL-S1^T represents a novel species of the genus *Arcobacter*, for which we propose the name *Arcobacter marinus* sp. nov.

Description of *Arcobacter marinus* sp. nov.

Arcobacter marinus (ma.ri'nus. L. masc. adj. *marinus* referring to the sea, from where the type strain was isolated).

Cells grow well under both aerobic and microaerobic conditions. Cells are slightly curved rods, approximately 0.5–1.7 µm long and 0.1–0.3 µm wide. Cells are Gram-negative and motile with a single polar flagellum. After 3 days on MA plates at 37 °C, colonies are circular, convex, creamy white and approximately 1 mm in diameter. After 3 days on SBA plates at 37 °C, colonies are irregular,

Table 2. Cellular fatty acid composition of strain CL-S1^T and recognized *Arcobacter* species

Taxa: 1, strain CL-S1^T; 2, *A. halophilus*; 3, *A. nitrofigilis*; 4, *A. butzleri*; 5, *A. skirrowii*; 6, *A. cryaerophilus*. Data for taxa 2 and 3–6 are from Donachie *et al.* (2005) and Vandamme *et al.* (1992), respectively. All strains were cultivated at 37 °C on blood agar supplemented with 0.5 % NaCl (final concentration) except strains CL-S1^T and *A. halophilus* LA31B^T, for which blood agar supplemented with 3.5 % NaCl (final concentration) was used. Values are percentage of total fatty acids. ND, Not detected or <1 %. Data are not available for *A. cibarius*.

Fatty acid	1	2	3	4	5	6
C _{12:0}	6.6	8.2	7.2	6.8	9.1	3.7–7.7
C _{14:0}	5.0	5.0	4.8	3.2	2.4	1.7–1.8
C _{16:0}	26.2	19.3	32.0	19.6	22.2	17.8–18.7
C _{18:0}	1.6	ND	ND	ND	ND	ND
C _{14:1} ω6c	ND	ND	ND	4.9	1.4	9.2
C _{16:1} ω7c	28.4*	37.9	30.9	19.2	22.8	19.0–45.2
C _{16:1} ω6t	ND	ND	ND	13.8	7.8	ND–18.8
C _{18:1} ω9c	2.1	ND	ND	ND	ND	ND
C _{18:1} ω7c	22.3	21.7	12.8	11.6	19.7	10.6–10.8
C _{12:0} 3-OH	2.9	2.7	5.5	ND	ND	ND
iso-C _{16:1} I, C _{14:0} 3-OH and/or C _{12:0} alde	2.0	2.1	5.3†	17.4†	9.2†	9.4–14.3†

*Reported as summed feature 3 (iso-C_{15:0} 2-OH and/or C_{16:1}ω7c).

†Reported as C_{14:0} 3-OH (Vandamme *et al.*, 1992).

umbonate, grey and approximately 4 mm in diameter. Growth occurs at 10–40 °C (optimum 30–37 °C) and at pH 5.9–8.5 (optimum pH 7.0). Growth occurs at NaCl concentrations of 1–9 % (w/v) (optimum 3–5 %). Oxidase is produced, but catalase, urease, alkaline phosphatase and H₂S are not. Nitrate is reduced. Indoxyl acetate is hydrolysed but hippurate is not. Haemolysis is not observed. The dominant fatty acids are iso-C_{15:0} 2-OH and/or C_{16:1}ω7c, C_{16:0} and C_{18:1}ω7c. The DNA G+C content of the type strain is 28 mol%.

The type strain, CL-S1^T (=KCCM 90072^T =JCM 15502^T), was isolated from seawater to which seaweeds and a starfish were added.

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