***Antarctobacter heliothermus* gen. nov., sp. nov., a budding bacterium from hypersaline and heliothermal Ekho Lake**

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Four Gram-negative, aerobic, pointed and budding bacteria were isolated from various depths of the hypersaline, heliothermal and meromictic Ekho Lake (Vestfold Hills, East Antarctica). The cells contained storage granules and formed rosettes. Daughter cells may be motile. Growth required sodium ions. Nitrate was reduced to nitrite, and dissimilatory reduction of nitrite was possible. DNase and gelatinase were produced. Glutamate was metabolized with and without an additional source of combined nitrogen. The most abundant fatty acid was C_{18:1}; other fatty acids present in lower concentrations were C_{12:1} 3-OH, C_{16:1}, C_{16:0}, C_{18:0} and C_{19:0} cyc. The main polar lipids were phosphatidylglycerol and phosphatidylcholine. The DNA base composition was 62.3–62.8 mol% G+C. 16S rDNA sequence comparisons showed the isolates to be phylogenetically related to the genera *Sagittula* and *Roseobacter*. Morphological, physiological and genotypic differences to these and distinct characteristics supported the description of a new genus and a new species, *Antarctobacter heliothermus* gen. nov., sp. nov. The type strain is EL-219^T (= DSM 11445^T).

Keywords: *Antarctobacter* gen. nov., *Antarctobacter heliothermus* sp. nov., α -Proteobacteria, Antarctica, hypersaline lakes

INTRODUCTION

The ice-free area of the Vestfold Hills (East Antarctica) abounds with lakes and ponds of various sizes. Several of these lakes originated from fjords and, at one time, were open to the ocean. Thus, seawater determined their initial salt composition and concentration and, most likely, also the composition of their microbial communities. As the land rose about 5000–6000 years ago the fjords lost their connections to the ocean and became lakes. One such lake is Ekho Lake, which has since lost approximately 10 m of its water column due to evaporation; this and a minor influx of snow melt water have resulted in increased salinity. In addition, a vertical gradient of salinity exists; this was explained by Ferris *et al.* (9) as follows. The annual freezing of the lake water surface forms several metres of ice

cover. In this process, much salt is excluded as brine, which sinks downwards towards lower strata along the shore line. Consequently, zones of stepwise increasing salinity with depth were formed. In Ekho Lake, a summer salinity of 2–10‰ was measured between 0 and 1 m depth, whereas the salinity was 180‰ at the bottom depth of 42 m. At interfaces between zones of increasing depth, the salt concentration increased suddenly to the next higher level. Close to, and below, these interfaces temperature increases were measured. Thus, these interfaces appeared to act as one-way mirrors, allowing sun radiation to penetrate into the lower layers but preventing its escape due to downward deflection. The strata below such interfaces were significantly warmer than the upper ones: the lake is considered to be heliothermally heated. A similar situation has been described for Solar Lake (Sinai) (19, 21). Another consequence of separated water bodies with high salinity is meromixis. Wind energy does not turn over the lower strata in the summer, and during winter a turnover is prevented by a thick ice cover. The temperature of the lower layers of Ekho Lake remains

Abbreviations: ASW, artificial seawater; FAME, fatty acid methyl ester.

The EMBL accession numbers for the 16S rRNA sequences of strain EL-219^T and strain EL-172, another representative out of Ekho Lake, are Y11552 and Y11551, respectively.

at about +13 °C in winter. During the austral summer, intermediate strata reached as high as +19 °C (P. Hirsch & J. Siebert, unpublished). Because of these differences of environmental parameters at increasing depths, microbial communities of this hypersaline, heliothermal and meromictic Ekho Lake were studied.

To date, only a few bacterial species or genera have been isolated from similar Antarctic lakes; some of these were new. Nearby Organic Lake contained *Halomonas subglaciescola* (6, 10), 16 strains of the *Flavobacterium-Cytophaga-Flexibacter* group (4), *Flavobacterium gondwanense* and *Flavobacterium salegens* (5). Deep Lake contained the new taxon *Halorubrum* ('*Halobacterium*') *lacusprofundi* (15). Other new bacteria came from Ace Lake: *Carnobacterium funditum* and *Carnobacterium alterfunditum* (12), a spiral-shaped, psychrophilic and fermentative bacterium (13), a methanogenic and methylotrophic archaeon, *Methanococcoides burtonii* (14), and a psychrophilic spirochaete lacking a cell wall (11).

The presence of two *Halomonas* and two *Flavobacterium* species in various hypersaline lakes of the Vestfold Hills was studied by James *et al.* (24), who employed immunofluorescence of specific antibodies. These four serogroups were found during summer in those lakes which exceeded 61‰ of total salts. The two *Halomonas* spp. made up 40% of the total bacterial numbers, but *F. gondwanense* contributed only 10% of these numbers in Organic and Ekho Lake. The microbial communities of Ekho Lake remained largely unknown.

The morphological diversity, total bacterial numbers and c.f.u. in Ekho Lake were recently studied by P. Hirsch, J. Siebert & H. R. Burton (unpublished). These authors observed 135 prokaryotic morphotypes as well as 52 eukaryotic morphotypes. Such an unexpectedly high morphological diversity in Ekho Lake prompted us to isolate some 250 bacterial pure cultures from Ekho Lake, and a high taxonomic (genetic) diversity was expected. Additionally, the isolation of the major photosynthetic primary producer of Ekho Lake resulted in the description of a new green praseophyte, '*Tetraselmis antarctica*' (20). This publication describes four Ekho Lake bacterial isolates which represent a new genus and species.

METHODS

Water sampling, enrichment conditions and isolations. Water samples were taken aseptically at each metre of the depth profile using a Kammerer sampler at the 42-m-deep centre of Ekho Lake (20). Samples were stored on ice until used for the inoculation of enrichments or plates after return to the Davis Station laboratory (Vestfold Hills). Characteristics of these water samples are shown in Table 1. Enrichments were set up in three different ways. (I) A 5 ml Ekho Lake water sample was inoculated into 10 ml sterile medium '398' and incubated for 4 weeks at 15 °C under dim light (4.1 µmol photons m⁻² s⁻¹). Medium '398' had the following composition: Hutner's Basal Salts (HBM; 2, 20 ml; Bacto yeast extract (Difco), 1.0 g; disodium D,L-

malate, 1.0 g; Ekho Lake water (salinity 98‰), 980 ml; final pH after autoclaving 8.6. (II) Ekho Lake water samples (50 ml) were amended with 1 ml of a filter-sterilized solution of 12.5 mg Bacto yeast extract in lake water and incubated at 15 °C and at 4.1 µmol photons m⁻² s⁻¹. (III) Ekho Lake water samples (0.5 ml) were spread directly on agar plates of medium PYGV (41) which had been prepared with Ekho Lake water of 10‰ salinity. Incubation was at 15 °C and in the dark. Pure cultures were isolated by several dilution transfers on the corresponding agar media; they were kept as serial transfers on agar slants, lyophilized or deep-frozen at -72 °C.

Bacterial strains. Type strains used for comparisons were of the following species: *Roseobacter denitrificans* (DSM 7001^T), *Roseobacter litoralis* (DSM 6996^T) and *Roseobacter algicola* (DSM 10251^T).

Media and culture conditions. Medium PYGV contained (l⁻¹): 0.25 g each of Bacto peptone, Bacto yeast extract and glucose as well as 20 ml HBM and 10 ml Vitamin Solution no. 6 (45). Solid media contained 1.8% (w/v) Bacteriological agar (Gibco). Where needed, liquid and solid media were prepared with 25‰ (v/v) artificial seawater (ASW; 28) (strains EL-219^T, EL-185, EL-165, *R. algicola*, *R. denitrificans* and *R. litoralis*) or, in case of EL-54, with 40‰ ASW. Before autoclaving, medium PYGV containing ASW was adjusted to a pH of 8.5; the final pH after autoclaving was 7.2. R2A agar (Difco) was prepared with the corresponding concentration of ASW and was employed to culture the strains for BIOLOG tests. Bacterial cultures for identification tests were incubated at 20 °C; the anaerobic cultivation of isolates (at 4–21.5 µmol photons m⁻² s⁻¹) occurred at 16 °C. Biomass for chemotaxonomic studies was grown in liquid PYGV+ASW at 20 °C that was bubbled with filter-sterilized air and harvested in the late-exponential phase after 4–8 d.

Microscopy. Cellular morphology was examined by phase-contrast light microscopy of exponential phase broth cultures employing 2% water-agar-coated slides (31). For transmission electron microscopy, cells were washed with 0.5 M ammonium acetate, negatively stained with 1% (w/v) phosphotungstic acid and observed with a Philips EM 300 electron microscope at 80 kV.

Physiological and biochemical characterization. All physiological tests were performed at 20 °C. Gram-staining occurred with 24 h cultures (39). Catalase production was detected with 5% H₂O₂. Peroxidase and cytochrome oxidase activity was tested as described previously (7, 26). DNA hydrolysis was indicated by clear zones around colonies on Bacto DNase Test agar (Difco)+ASW. Amylase and the relation to oxygen were studied as described previously (40). Cellulase was tested with bacterial suspensions with 4-methylumbelliferyl β-D-glucoside (Sigma) under UV light (366 nm). Motility was examined in hanging-drop preparations. Additionally, EL-219^T was examined for the presence of flagella by electron microscopy after 1, 2, 3, 4 and 8 d incubation in broth. The ability to grow under various physical and chemical conditions was investigated in liquid media and evaluated by determining the protein content (1) of triplicate suspensions: osmotolerance in PYGV prepared with 0, 10, 20–100, 130 and 150‰ ASW was determined after 14 d incubation. The concentration of 150‰ ASW was achieved by adding NaCl to 130‰ ASW. NaCl tolerance was determined in PYGV prepared with 0, 10–40, 60, 80, 100, 130 and 150‰ NaCl after 14 d incubation. The ability to grow at pH 5.3, 5.9, 6.2, 6.9, 7.8, 8.3

Table 1. Characteristics of the original 1989/90 Ekho Lake samples from which the isolates were obtained, and enrichment conditions

Depth (m)	Salinity (‰)	Temp. (°C)	pH	Isolate	Enrichment method*
5	60	+12.9	8.3	EL-185	I
7	62	+12.9	8.2	EL-219 ^r	II
14	75	+16.0	8.0	EL-54	III
16	76	+16.4	7.8	EL-165	II

* See Methods.

and 9.0 and at temperatures of 3.0, 8.5, 16.0, 20.0, 26.0, 33.5 and 43.5 °C was also tested. Requirement(s) for the following vitamins was tested in six combinations, each lacking one vitamin of Vitamin Solution no. 6: biotin, thiamin hydrochloride, nicotinic acid, sodium pantothenate or vitamin B₁₂. For these experiments casein hydrolysate (vitamin-free; Merck) was used instead of Bacto peptone and Bacto yeast extract. Triplicate results were recorded after three serial transfers in the corresponding test media. Requirements for Na⁺, K⁺, Mg²⁺, Ca²⁺, Cl⁻ or SO₄²⁻ were studied in PYGV + ASW, where Na⁺ was exchanged with K⁺, Mg²⁺ with Ca²⁺, Cl⁻ with SO₄²⁻ and vice versa. Identical growth response indicated independence of these cations or anions. Susceptibility to antibiotics was determined with bioDiscs (bioMérieux) and the results were recorded after 4 d. The aerobic reduction of nitrate was tested after 14 d; the anaerobic reduction of nitrate to nitrite or molecular nitrogen was examined after 3 weeks incubation. In both cases, PYGV + ASW which contained 5 mM NaNO₃ was employed. Anaerobic growth was examined in modified PYGV + ASW, which contained (l⁻¹) 0.1 g glucose, 0.1 g acetate, 0.1 g glutamate and 0.5 g Tris. The medium was saturated with N₂ for 20 min, and 20 ml was distributed to 100 ml type-III serum bottles. For photolithoautotrophic or photoorganotrophic growth tests, the cultures were incubated under an atmosphere of H₂/CO₂ (80:20; 0.9 bar) and at light intensities of 4–21.5 µmol photons m⁻² s⁻¹. Production of poly-β-hydroxybutyrate was determined in PYGV + ASW which was modified to contain (l⁻¹) 20 ml HBM, 10 ml Vitamin Solution no. 6, 0.1 g Bacto yeast extract and 2.0 g succinate as previously described (40). Methyl red and Voges–Proskauer tests were performed in PYGV + ASW containing 0.2% (w/v) Bacto peptone and 0.2% (w/v) glucose. H₂S and indole production was tested with the sulfide indole motility medium (Merck) + ASW. Indole was detected with 1 ml Kovács' reagent.

To study the aerobic dissimilation of 95 carbon sources with the BIOLOG system, the Microlog software, a Tandon computer and a microplate photometer (Molecular Devices) were used (Biolog). Suspensions were obtained from growth on R2A agar prepared with the individually required concentration of ASW after 3–4 d at 20 °C. The cells were suspended in the necessary ASW to a transmission of 53–59% and inoculated into microplate wells. Test evaluation was performed after 4, 24 and 48 h. Additionally, carbon source utilization was studied in a minimal medium containing (l⁻¹): HBM, 20 ml; Vitamin Solution no. 6, 10 ml; Bacto yeast extract, 0.02 g; and NH₄NO₃, 0.25 g. The following carbon sources were tested at 0.2% (w/v): acetate, pyruvate, malate, citrate, succinate, butyrate, glutamate, α-

D-glucose, methanol and methanesulfonic acid. Degradation of the following substrates was tested with the basal medium PYV + ASW (i.e. lacking glucose): 0.2% (w/v) starch, 0.4% gelatin, 1% Tween 80 or 0.75% alginate. Production of bacteriochlorophyll *a* was tested with suspensions of cells grown in PYGV + ASW and analysed as described previously (38). Methanolic extracts were examined with a Beckman DU-600 spectrophotometer and by TLC (27).

Chemotaxonomy. Analysis of fatty acid methyl esters (FAMES) was performed on 20 mg freeze-dried cells employing methods which allowed selective hydrolysis of ester- and amide-linked fatty acids. FAMES were analysed by GC (GC-14A; Shimadzu) using a 0.2 mm × 25 m non-polar capillary column and flame-ionization detector. The chromatography conditions were: injector and detector port temperature 300 °C, inlet pressure 80 kPa, split ratio 50:1, injection volume 1 µl and a temperature program of 130–310 °C at a rate of 4 °C min⁻¹. Hydrogen was the carrier gas. The fatty acids were identified by converting retention times to equivalent chain-length data (35). Saturated C₁₀–C₃₀ FAMES were used as standards. Respiratory lipoquinones and polar lipids were extracted and analysed from 100 mg freeze-dried cells using the two-stage method as previously described (43, 44). Purified cell wall preparations were obtained by the methods of Schleifer & Kandler (36). Amino acids and peptides of cell wall hydrolysates were analysed by two-dimensional ascending TLC on cellulose plates using solvent systems described previously (36). Diamino acids were recognized by one-dimensional TLC on cellulose using a solvent system of Rhuland *et al.* (32).

Determination of DNA base composition. DNA G+C contents were analysed using HPLC (29). The HPLC apparatus (Pharmacia-LKB) was equipped with a Spherisorb ODS II C₁₈ column (5 µm; 4 × 250 mm; Bischoff, Leonberg). *Escherichia coli* Strain B Type VIII-DNA (Sigma) and lambda phage DNA from *E. coli* host strain GM 119 (Sigma) were used as standards.

DNA–DNA hybridization. Dot-blot hybridization experiments were carried out with the DIG DNA Labeling and Detection kit from Boehringer Mannheim following the manufacturer's instructions. Gene probes were made from EL-219^r and hybridized against chromosomal DNA of EL-185, EL-165, EL-54 and *R. denitrificans*. The stringency of 70 and 75% was calculated according to Sambrook *et al.* (34).

16S rRNA sequence determination and analysis of sequence data. 16S rRNA gene fragments were generated by PCR as previously described (23). A large fragment of the 16S rRNA gene was amplified from DNA using universal primers pA (positions 8–28, *E. coli* numbering) and pH* (1542–1522). The amplified product was purified by using a QIAquick PCR Purification kit (Qiagen) and sequenced directly using primers to conserved regions of the rRNA. Sequencing was performed using a PRISM Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 373A; Applied Biosystems). To establish the closest relatives of the four Ekho Lake strains, preliminary searches in the EMBL database were performed with the program FASTA (30). Sequences closely related to those of the Ekho Lake strains were retrieved from the EMBL database and aligned with the newly determined sequences using the program PILEUP (3). The rRNA alignment was corrected manually and approximately 100 bases at the 5' end of the molecule were omitted from further analysis because of alignment uncertainties due to the highly

variable region V1. Percentage sequence similarities were calculated and corrected for substitution rates by using Jukes & Cantor parameters (25). A phylogenetic tree was constructed according to the neighbour-joining method (33) with the program NEIGHBOR. For this analysis, the following additional taxa, listed by genera and accession number, were included: *Rhodomicrobium vannielii* (M34127), '*Methylosulfonomonas methylovora*' (U62893), *Rhodobium marinum* (D30790), *Rhodobium orientis* (D30792), *Rhodospirillum salexigens* (M59070), *Rhodovulum euryhalinum* (D13479), *Rhodovulum sulfidophilum* (U55277), *Rhodovulum adriaticum* (D16418), *Rhodobacter blasticus* (D16429), *Rhodobacter capsulatus* (D16428), *Rhodobacter sphaeroides* (D16425), *Rhodobacter veldkampii* (D16421), *Rhodospirillum rubrum* (D30778), *Rhodospirillum photometricum* (D30777), *Rhodospirillum fulvum* (D14433), *Rhodospirillum molisichianum* (M59067), *Rhodospirillum salinarum* (M59069), *Rhodospirillum sodomense* (M59072), *Rhodopila globiformis* (M59066), *Rhodoplanes roseus* (D25313), *Blastochloris viridis* (D25314), *Rhodopseudomonas palustris* (D25312) and *Rhodopseudomonas acidophila* (M34128). The stability of the groupings was estimated by bootstrap analysis (500 replications) using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE of the PHYLIP package (8).

RESULTS

Isolation of bacteria, morphology and motility

Three different enrichment procedures yielded four pure cultures with similar morphology; they were obtained from 5, 7, 14 and 16 m Ekho Lake samples (Table 1). These isolates are referred to as EL-219^T, EL-54, EL-165 and EL-185. They all were Gram-negative rods with one or both cell poles narrower, and of variable length (Fig. 1a, b). Cells had a tendency to form rosettes (Table 2; Fig. 1c). Cell growth appeared to be monopolar since one cell end was usually narrower and shorter, possibly indicating a budding process (18). In such cases, the 'daughter cell' was often positioned at an angle to the 'mother cell' (Fig. 1c). All four isolates contained electron-transparent poly- β -hydroxybutyrate granules. Their cell sizes were very similar; a peculiar characteristic of laboratory-cultured cells, as well as those from Ekho Lake samples, was the frequent formation of elongated cells of up to 33 μ m in length (Table 2). Motility was observed in all strains except EL-219^T. Electron microscopy revealed one to three subpolar flagella in strains EL-54 and EL-185 (Fig. 2).

16S rRNA sequence determinations and phylogenetic analyses

Partial 16S rRNA gene sequences of the four Ekho Lake isolates were determined by direct sequence analysis of PCR-amplified products. Based on a comparative analysis of a continuous stretch of approximately 800 bases, the isolates formed a distinct group, exhibiting 100% 16S rRNA sequence similarity among each other. The almost complete 16S rRNA gene sequence (> 1400 bases) of strain EL-219^T as a representative was determined. For comparison, Ekho Lake strain EL-172 (a representative of another

hybridization group) was also almost completely sequenced. Sequence searches of the EMBL database revealed the newly determined sequences were related to the α -subclass of *Proteobacteria* (data not shown). Strain EL-219^T displayed the highest 16S rRNA sequence relatedness (approximately 92–94%) with species of the genus *Roseobacter* (viz. *R. algicola*, *R. litoralis* and *R. denitrificans*), the latter was previously named *Erythrobacter* sp. Och 114 (37), *Sagittula stellata* (16), *Sulfitobacter pontiacus* (42), *Octadecabacter antarcticus* (17), a Sargasso sea isolate and the Ekho Lake strain EL-172. Other proteobacterial species examined showed significantly lower levels of relatedness. An unrooted tree depicting the phylogenetic relationships of the Ekho Lake strains and their close relatives is shown in Fig. 3. The results of treeing analyses confirmed that the Ekho Lake strain EL-219^T was phylogenetically most closely related to *Roseobacter* species and their close relatives. Strain EL-219^T formed a distinct subline within this grouping and clustered with another strain of Ekho Lake (EL-172). However, bootstrap resampling showed the relationship between Ekho Lake strains EL-219^T and EL-172 was supported in only 63% of trees. Furthermore, although bootstrap resampling showed that the bacterium from Ekho Lake was associated with the above-mentioned *Roseobacter* cluster (Fig. 3; bootstrap value = 100), the unknown bacterium did not possess a particularly significant phylogenetic affinity with any species within this clade.

Culture and growth characteristics

Visible aerobic growth appeared after 3–5 d on medium PYGV + 25‰ (or 40‰) ASW and at 20 °C. Colonies were circular, smooth, convex, 1–4 mm in diameter and brownish-yellow. On R2A agar + ASW, the colonies were brownish-red. Optimal growth occurred between 16 and 26 °C and pH values of 6.9–7.8. The four isolates had an absolute requirement for Na⁺; the pairwise replacement of the other cations and anions (see Methods) did not influence normal growth yields. Likewise, *R. denitrificans* did not need Cl[−] or SO₄^{2−} under our growth conditions. The EL isolates had a requirement for thiamin, nicotinic acid and possibly biotin, but neither pantothenate nor vitamin B₁₂ were required, as was also the case with *R. denitrificans*. Their osmotolerance ranged from < 10 to > 150‰ ASW with an optimum between 10 and 40‰; however, strain EL-54 had an optimum between 40 and 70‰ ASW. The NaCl tolerance of EL-219^T and EL-54 ranged from < 10 to 100‰ with an optimum of 20–60‰ NaCl, and that of EL-165 and EL-185 from < 10–20 to 40–60‰ NaCl with an optimum at 20‰.

Physiological and biochemical characteristics

All four strains exhibited cytochrome oxidase, peroxidase and catalase activity. They did not produce acetoin or acids from glucose. They were susceptible to

Fig. 1. Phase-contrast light micrographs of strains EL-219^T (a, b) and EL-165 (c) on agar-coated slides. Note differences in morphology and size of EL-219^T cells due to either growth for 12 d on PYGV + 67‰ agar (a), or growth for 56 d on PYGV + 25‰ NaCl agar (b). Cells of EL-165 (c) were cultivated for 29 d on PYGV + 67‰ ASW. Bar, 10 µm.

Table 2. Morphological characteristics of the EL strains, *Sagittula stellata*, *R. algicola*, *R. denitrificans*, *R. litoralis* and '*Marinosulfonomonas methylotropha*'

Organism*	Cell shape	Cell budding†	Storage granules‡	Rosettes	Cell size (mean length) (µm)	Flagella; motility§	Colony colour on PYGV
EL-219 ^T	Rods, 1–2 poles pointed	+	PHBA	+	0.8–1.2 × 2.5–10.9 (5.5)	—	Brownish-yellow
EL-54	Rods, 1–2 poles pointed	+	PHBA	+	0.8–1.2 × 2.0–11.0 (3.8)	1–3; sp	Brownish-yellow
EL-165	Long rods, 1–2 poles pointed	+	PHBA	+	0.8–1.8 × 2.5–33.6 (7.7)	v	Brownish-yellow
EL-185	Rods, 1–2 poles pointed	+	PHBA	+	0.8–1.5 × 3.5–13.8 (6.8)	1–2; sp	Brownish-yellow
<i>Sagittula stellata</i> ^a	Rods, 1 pole narrow; holdfasts; fibrils	ND	PHBA	+	0.9 × 2.3	+ / —	Cream-coloured ^a
<i>R. algicola</i> ^b	Ovoid rods	ND	PHBA¶	—	0.8–1.5 × 2.0–3.0	1–2; sp	Brownish-beige ^b
<i>R. denitrificans</i> ^c	Ovoid or rods	ND	ND	—	0.6–0.9 × 1.2–2.0	1–3; sp	Red ^b
<i>R. litoralis</i> ^c	Ovoid or rods	ND	ND	—	0.6–0.9 × 1.0–2.0	1–3; sp	Red ^b
' <i>M. methylotropha</i> ' ^d	Rods	ND	Polyphosphates	+	0.5 × 2.5	—	Semi-translucent ^c

ND, Not determined.

* Data from reference: *a*, (16); *b*, (27); *c*, (37); *d*, (22).

† Daughter cells substantially smaller than mother cells and often positioned at an angle.

‡ PHBA, poly-β-hydroxybutyrate.

§ sp, subpolar to lateral; v, variable.

|| *a*, On Marine agar; *b*, present data supplement published diagnoses; *c*, on methanesulfonic acid/Phytigel (1 %, w/v) medium.

¶ Present data supplement published diagnoses.

chloramphenicol (30 µg), penicillin G (10 U), streptomycin (10 µg), tetracycline (30 µg) and polymyxin B (300 U). All strains reduced nitrate to nitrite. Nitrite was not reduced aerobically, and anaerobic reduction to N₂ was variable. H₂S or indole was not produced. The following carbon sources were utilized in the presence of available nitrogen: succinate, butyrate, glutamate, acetate, pyruvate, malate, citrate and α-D-glucose. Not utilized were methanol and methanesulfonic acid. In the absence of other nitrogen sources, glutamate was utilized as a carbon and nitrogen source. Without any added nitrogen source there was slight growth with acetate, butyrate, pyruvate, malate or succinate. The strains hydrolysed gelatin and DNA,

but not Tween 80 or alginate. Other hydrolysis data and differences in the metabolism of carbon compounds as revealed with the BIOLOG system are shown in Table 3.

In the BIOLOG system, the isolates metabolized *N*-acetyl-D-galactosamine, *N*-acetylglucosamine, adonitol, D-arabitol, *i*-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, α-D-glucose, α-lactose, α-D-lactose-lactulose, maltose, D-mannitol, D-mannose, D-melibiose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, acetic acid, citric acid, itaconic acid, propionic acid, glucuronamide, L-alanyl-glycine and uridine. They did not metabolize α-

Fig. 2. Electron micrograph of phosphotungstic acid negatively stained cells and flagella of strain EL-54 after cultivation for 3 d in liquid medium PYGV + 75‰ ASW. The cell without a flagellum shows monopolar growth. Bar, 2 µm.

Fig. 3. Unrooted tree showing phylogenetic relationships of strain EL-219^T with EL-172 and closely related proteobacteria. The tree was constructed using the neighbour-joining method and K_{nuc} values; it was based on a comparison of approximately 1400 nucleotides. Bootstrap values, expressed as a percentage of 500 replications, are given at branching points; only those above 90% are shown.

cyclodextrin, dextrin, glycogen, Tween 80, β -methyl-D-glucoside, methyl pyruvate, *cis*-aconitic acid, mono-methylsuccinate, formic acid, D-galactonic acid lactone, D-galacturonic acid, α -hydroxybutyric acid, β -hydroxybutyric acid, γ -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α -ketobutyric acid, α -ketoglutaric acid, α -ketovaleric acid, DL-lactic acid, sebamic acid, succinic acid, bromosuccinic acid, succinamic acid, D-alanine, L-aspartic acid, glycyl-L-glutamic acid, glycyl-L-aspartic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, D-serine, L-pyroglutamic acid, DL-carnitine, γ -aminobutyric acid, urocanic acid, phenylethylamine, putrescine, 2-aminoethanol, glycerol, glucose 6-phosphate or DL- α -glycerophosphate.

Strains EL-54 and EL-165 grew microaerobically, but they did not grow photolithoautotrophically under anaerobic conditions with H_2/CO_2 (80:20) in the gas phase or photoorganotrophically. Bacteriochlorophyll

a was never found in cell suspensions or in methanolic extracts. Absorption spectra of methanolic extracts were very similar to those of *R. algicola* (27) grown on PYGV + 25‰ ASW. The G + C content ranged from 62.3 to 62.8 mol%. Gene probes from EL-219^T hybridized with DNA from the other three Ekho Lake strains but not with DNA of *R. denitrificans*.

Chemotaxonomic parameters

The peptidoglycan of all four isolates contained *m*-diaminopimelic acid, and the peptidoglycan of strain EL-185 was of the directly cross-linked type A1 γ (36). The ubiquinone of the four strains was Q-10. Polar lipid patterns and fatty acid compositions of the strains grown on PYGV + ASW are listed in Table 4 and compared to some other members of the α -*Proteobacteria*. Their fatty acid profile was characterized by the predominance of C_{18:1}.

Table 3. Differences in physiological properties of the EL strains

+, Positive; —, negative; v, variable; w, weak; ND, not determined.

Character	EL-219 ^T	EL-54	EL-165	EL-185
BIOLOG data				
Tween 40	—	+	—	—
L-Arabinose	—	+	—	v
Cellobiose	—	+	—	—
m-Inositol	—	+	—	—
Psicose	—	—	—	+
Xylitol	v	+	v	v
D-Gluconic acid	—	+	—	—
D-Glucosaminic acid	—	+	v	—
D-Glucuronic acid	—	+	—	—
Malonic acid	—	+	—	—
Quinic acid	—	v	—	—
D-Saccharic acid	—	+	—	v
Alaninamide	—	+	—	v
L-Alanine	—	v	—	v
L-Asparagine	—	+	—	—
L-Glutamic acid	—	v	—	—
L-Serine	—	v	—	—
Inosine	—	+	+	—
Thymidine	—	v	—	v
2,3-Butanediol	—	v	—	—
Glucose 1-phosphate	—	v	—	—
Hydrolysis of:				
Starch	—	—	+	w
β-D-Glucoside	—	ND	—	—

DISCUSSION

The three very different enrichment procedures used for Ekho Lake samples yielded four nearly identical bacterial isolates, although these came from 5–16 m depth. Apart from the salinity (60–76‰), the original samples were fairly similar, and thus it may be possible that the isolates were widely distributed in the upper layers of the lake. DNA of all four isolates hybridized with gene probes of strain EL-219^T (75% stringency), and sequencing of 16S rDNA confirmed the isolates were genealogically homogeneous and members of a distinct taxon within the α -3-subclass of the *Proteobacteria*. The four isolates displayed very similar morphologies (Table 2). Strain EL-219^T did not exhibit directed motility but rather a tumbling and twitching of daughter cells. There were only minor differences in biochemical and physiological activities such as in the hydrolysis of starch or in the metabolism of carbon compounds as revealed by the BIOLOG system (Table 3). The strains were also identical in the battery of chemotaxonomic tests employed: they all contained ubiquinones as the sole respiratory quinones, indicating that the organisms were members of the α -, β - or γ -subclasses of the *Proteobacteria*. Generally, Q-10 is

the major respiratory quinone only in members of the α -subclass of the *Proteobacteria*, although some organisms, such as *Legionella* spp. in the γ -subclass of *Proteobacteria*, also produce Q-10, which is accompanied by significant quantities of other, long-chain homologues. The fatty acid pattern of the EL strains was dominated by the presence of a C_{18:1} fatty acid. This predominance, in combination with the presence of Q-10, is again typical of members of the α -subclass of *Proteobacteria*. The only indication of the presence of non-ester-linked fatty acids (potentially amide-linked fatty acids) was found among the 3-hydroxy fatty acids. The polar lipid composition of all strains indicated that the organisms contained phospholipids as the major polar lipids. Taken together, the sum of respiratory lipoquinones, fatty acids and polar lipid data indicated that the strains examined belonged to a group of organisms (at the genus or family rank) within the α -subclass of the *Proteobacteria*.

Comparative 16S rDNA gene sequencing showed also that the unknown bacteria from Ekho Lake, as exemplified by EL-219^T, were members of the α -subclass of *Proteobacteria* and were specifically associated with the *Roseobacter* supercluster of organisms, which included '*Marinosulfonomonas*' (22), *Prionitis lanceolata* gall symbiont, *Octadecabacter*, *Roseobacter*, *Sagittula*, *Sulfitobacter* and an unknown Sargasso sea isolate. This cluster was supported by a bootstrap value of 100%. Sequence divergence values of > 5% showed that the unknown bacteria were genetically distinct from all currently recognized members of the *Proteobacteria*. Furthermore, bootstrap resampling showed that EL-219^T from Ekho Lake did not possess a particularly significant phylogenetic affinity with any individual species within the above-mentioned genera (Fig. 3). The association between Ekho Lake strains EL-219^T and EL-172 was recovered in only 63% of trees (500 replicates). It is evident from the treeing analyses that the genus *Roseobacter* as currently recognized is not monophyletic, with several taxa (e.g. *Prionitis lanceolata* gall symbiont, *Sulfitobacter*) interdispersed with species of this genus. Based on sequence divergence values combined with tree topology considerations, it is evident that the Ekho Lake strain EL-219^T merits a similar taxonomic rank (i.e. genus) as that of *Sagittula*, *Prionitis lanceolata* gall symbiont, *Sulfitobacter* and *Octadecabacter*. In view of the phylogenetic separation of the unknown bacteria and their phenotypic distinctiveness (Table 5), we conclude that the four Ekho Lake isolates are representatives of a new and distinct taxon for which we formally propose the name *Antarctobacter heliothermus* gen. nov., sp. nov.

Description of *Antarctobacter* gen. nov.

Antarctobacter [Ant.arc.to.bac'ter. *Antarctica* M.L. name of the South Pole continent, derived from Gr. pref. *anti* against (on the other side), Gr. adj. *arcticos* northern; Gr. n. *baktron* a rod or staff; M.L. masc. n.

Table 4. Chemotaxonomic characteristics of the EL strains, *Sagittula stellata*, *R. algicola*, *R. denitrificans*, *R. litoralis*, *O. arcticus* and *O. antarcticus*

For the FAME composition data, values less than 1% and unidentified FAMEs are not shown. All polar lipid data are from the present study; strains were cultivated on PYGV + ASW. ND, Not determined.

Characteristic	EL-219 ^T	EL-54	EL-165	EL-185	<i>S. stellata</i> [*]	<i>R. algicola</i> ^{†‡}	<i>R. denitrificans</i> [§]	<i>R. litoralis</i> ^{†§}	<i>O. arcticus</i>	<i>O. antarcticus</i>
FAME composition (%)										
C _{10:0} 3-OH							4.2	1.9	4.0	2.0
C _{12:1} 3-OH	3.1	3.1	3.0	3.1	3.6					
C _{14:0} 2-OH								3.9		
C _{16:1}	0.8	1.1	0.8	0.6			1.4		8.0	12.0
C _{16:0}	2.5	2.7	2.5	2.3	8.6	1.6	1.6	1.1	6.0	6.0
C _{18:2}						1.6	7.1	1.4		
C _{18:1}	83.2	81.9	81.4	84.0	¶	91.5	84.6	88.8	75.0	77.0
C _{18:0}	1.0	0.7	1.1	1.4	6.8	2.2	1.2	1.3		
C _{19:0} cyc	2.4	2.5	2.7	1.9	1.8					
Polar lipids										
Phosphatidylglycerol	+	+	+	+	ND	+	+	+	ND	ND
Phosphatidylcholine	+	+	+	+	ND	+	+	—	ND	ND
Diphosphatidylglycerol	—	—	—	—	ND	+	+	+	ND	ND
Phosphatidylethanolamine	—	—	—	—	ND	+	—	—	ND	ND
Unknown aminolipid	+	+	+	+	ND	+	+	+	ND	ND
Unknown phospholipid(s)	+	+	+	+	ND	—	+	+	ND	ND

* Data from reference (16).

† Grown on Marine broth.

‡ Data from the present study.

§ Present data supplement published diagnoses.

|| Data from reference (17).

¶ Major fatty acid, but not quantified.

Table 5. Comparison of isolate EL-219^T and the other related strains with *Sagittula stellata* (16), *R. algicola* (27), *R. denitrificans* (37), *R. litoralis* (37), *Sulfitobacter pontiacus* (42), *O. arcticus* (17), *O. antarcticus* (17) and '*Marinosulfonomonas methylophila*' (22)

+, Positive; —, negative; ND, not determined; w, weak reaction.

Characteristic	EL-219 ^T	<i>S. stellata</i>	<i>R. algicola</i>	<i>R. denitrificans</i>	<i>R. litoralis</i>	<i>S. pontiacus</i>	<i>O. arcticus</i>	<i>O. antarcticus</i>	' <i>M. methylophila</i> '
Cell shape	Rods	Rods	Ovoid	Ovoid	Ovoid	Rods	Rods	Rods	Rods
Rosettes formed	+	+	—	—	—	+	—	—	+
Colony colour	Brown-yellow	Cream	Brown-beige	Pink	Pink	Colourless	White	White	Translucent
Gas vesicles	—	—	—	—	—	—	+	+	—
Motility	+ / —	+*	+	+	+	+	—	—	—
Maximum length (µm)	10.9–33.6	2.3	2–3	2	2	2–5	4.4	4.8	2.5
Minimum temp. (°C)	3–8.5	< 4	10	< 2	2	4	< 4	< 4	20
Maximum temp. (°C)	33.5–43.5	> 41	> 37	> 30	> 30	35	> 15	10–15	37
Nicotinic acid requirement	+	—	—	+	+	ND	+	ND	ND
Biotin requirement	w	—	+	+	+	ND	—	ND	ND
Dissimilatory NO ₃ [−] reduction	+	—	—	+	—	ND	—	—	ND
Assimilatory NO ₃ [−] reduction	+	—	—	+†	ND	ND	—	ND	+
Carbon source: methanol	—	+	—	—	—	—	—	—	+
Carbon source: butyrate	+	+	—‡	—	—	+	—	—	ND
Cellulase activity	—	+	ND	ND	ND	ND	ND	ND	ND
Gelatine hydrolysis	+	—	+	+	+	—	—	—	ND
Bacteriochlorophyll <i>a</i>	—	—	—	+	+	—	—	—	—
G + C content (mol%)	62.3–62.8	65	64†	59.6	56.3–58.1	62.1	57	56	57

* Motility was assumed since flagella were found in suspension, but motile cells were not seen.

† Data from the present study.

‡ Positive in the present study.

bacter; M.L. masc. n. *Antarctobacter* a rod-shaped bacterium from Antarctica].

Gram-negative rods, multiplying by monopolar growth, i.e. by a budding process. Daughter cells may be motile. Cell sizes vary due to age and growth conditions: $0.8\text{--}1.2 \times 2.0\text{--}3.6 \mu\text{m}$. The cells may contain poly- β -hydroxybutyrate; rosettes are formed. Do not form spores. Colonies smooth, convex and brownish to yellowish on medium PYGV with ASW, or brownish-red on medium R2A with ASW. The temperature range for growth is $< 3\text{--}43.5^\circ\text{C}$. The cells have an absolute requirement for Na^+ , and grow in the range of < 10 to $< 100\%$ NaCl. They grow between < 10 and $> 150\%$ ASW. The pH tolerance range is $5.3\text{--}> 9$. Strictly aerobic, non-fermentative heterotrophs. The cells exhibit cytochrome oxidase, peroxidase and catalase activities. No growth on glucose anaerobically in the absence of nitrate. They do not grow photoautotrophically with H_2/CO_2 (80:20) or photoorganotrophically with acetate or glutamate; they do not contain bacteriochlorophyll *a*. The peptidoglycan contains *m*-diaminopimelic acid and is of the directly cross-linked type A1 γ . The respiratory quinone is Q-10. The following polar lipids are present: phosphatidylglycerol, phosphatidylcholine as well as unknown phospholipids and an aminolipid. The dominant fatty acid is $\text{C}_{18:1}$; another characteristic fatty acid is $\text{C}_{12:1}$ 3-OH. The G+C content is 62.3–62.8 mol %. Origin: water samples from Ekho Lake, Antarctica (Vestfold Hills). The type species for the genus is *Antarctobacter heliothermus*.

Description of *Antarctobacter heliothermus* sp. nov.

Antarctobacter heliothermus (he.li.o.ther'mus. Gr. n. *helios* Sun; Gr. adj. *thermos* hot; M.L. masc. adj. *heliothermus* heated by the Sun, referring to the heliothermal waters of Ekho Lake, from where the species was isolated).

Rods with a mean length of $3.8\text{--}7.7 \mu\text{m}$. One or both cell poles nearly pointed. Daughter cells may be motile with one to three subpolar flagella. Optimal growth occurs at $16\text{--}26^\circ\text{C}$ with salt concentrations of 20–60% NaCl or 10–70% ASW. The optimum pH is 6.9–7.8. Requires thiamin and nicotinic acid but not pantothenate or vitamin B_{12} ; they are stimulated by biotin. Nitrate is reduced to nitrite and dissimilatory reduction of nitrite is variable. H_2S and indole are not produced. Hydrolyses gelatin and DNA, but not Tween 80 and alginate. Hydrolysis of starch is variable. Cellulase activity was not detected. Growth occurs on acetate, pyruvate, malate, citrate, succinate, butyrate, glutamate or α -D-glucose. Glutamate is used with and without an additional source of combined nitrogen. In the absence of any added nitrogen compounds, the cells grow slightly on acetate, pyruvate, succinate, malate or butyrate. Methanol or methanesulfonic acid are not used. Susceptible to chloramphenicol, penicillin G, streptomycin, tetracycline and polymyxin B. Further description as for the genus. The type strain

EL-219^T was deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ 11445^T). Additional isolates are EL-165 (DSMZ 11440), EL-54 and EL-185.

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