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Staleya guttiformis gen. nov., sp. nov. and *Sulfitobacter brevis* sp. nov., α-3-*Proteobacteria* from hypersaline, heliothermal and meromictic antarctic Ekho Lake

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Two Gram-negative, aerobic, pointed and budding bacteria were isolated from various depths of hypersaline, heliothermal and meromictic Ekho Lake (Vestfold Hills, East Antarctica). 16S rRNA gene sequence comparisons show the isolates to be phylogenetically close to the genera *Sulfitobacter* and *Roseobacter*. Cells can be motile and contain storage granules. Sulfite addition does not stimulate growth. Isolate EL-38^T can produce bacteriochlorophyll *a* and has a weak requirement for sodium ions; polar lipids include phosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine and an unidentified amino lipid, but not diphosphatidylgycerol. The dominant fatty acid is 18:1ω7*c*; other characteristic fatty acids are 3-OH 10:0, 3-OH 14:1, 16:0, 18:0, 18:2 and 19:1. The DNA base composition is 55·0–56·3 mol% G+C. Isolate EL-162^T has an absolute requirement for sodium ions. Diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine and an unidentified amino lipid are present in the polar lipids. Dominant fatty acids of this isolate are 18:1ω7*c* and 18:1ω9*c* as well as 18:2 which is present as two isomers. Other characteristic fatty acids are 3-OH 10:0, 3-OH 14:1, 16:0 and 18:0. The G+C content is 57·9–58·1 mol%. Morphological, physiological and genotypic differences from related, thus far known genera support the description of *Staleya guttiformis* gen. nov. and sp. nov. with EL-38^T (= DSM 11458^T) as the type strain and of *Sulfitobacter brevis* sp. nov. with the type strain EL-162^T (= DSM 11443^T).

Keywords: *Staleya guttiformis* gen. nov., sp. nov., *Sulfitobacter brevis* sp. nov., Antarctica, α-*Proteobacteria*, bacteriochlorophyll *a*

INTRODUCTION

In the hypersaline Ekho Lake of East Antarctica (Vestfold Hills) salinity increases stepwise with depth, thus forming interface layers. Solar radiation penetrates these and is reflected from below the interfaces, thus resulting 'heliothermally' in locally higher temperatures. The lake has been described as being meromictic with the two lower water bodies stable throughout the year (Burton, 1981). Ekho Lake microbiology has been studied only recently, although the changing physical and chemical conditions at different depths offer quite a variety of microhabitats for micro-organisms. The ecology of bacterial isolates from other antarctic hypersaline lakes has been studied in Ekho Lake by James (1996). P. Hirsch, J. Siebert and H. R. Burton investigated the morphological diversity and bacterial numbers of this lake and isolated some 250 bacterial cultures from this source (unpublished). Among these were *Antarctobacter heliothermus* and *Roseovarius tolerans*, two new genera and species described by Labrenz *et al*. (1998, 1999). In the present paper we characterize and describe two additional new bacterial isolates from this lake.

Abbreviations: ASW, artificial seawater; bchl, bacteriochlorophyll.

The EMBL accession number for the 16S rRNA sequences are Y16427 (EL-38^T) and Y16425 (EL-162^T).

METHODS

Water sampling, enrichment conditions and isolations. The procedure of collecting water aseptically from specific depths and the sample treatment were described (Labrenz *et al.*, 1998). For the isolation of EL-38^T, PYGV agar plates (Staley, 1968) were prepared with Ekho Lake water (salinity 15‰), inoculated with a 1 m water sample taken on 20 January 1990 and incubated at 15 °C under dim light (4·1 μmol photons m^{-2} s^{-1}). For the enrichment of strain EL-162^T, 50 ml Ekho Lake water from 8 m depth was amended with a filter-sterilized solution of 12·5 mg Oxoid yeast extract and incubated as described above.

Bacterial strains. The following strains were used for comparisons: *Roseobacter denitrificans* (DSM 7001^T), *Roseobacter litoralis* (DSM 6996^T) and *Sulfitobacter pontiacus* (DSM 10014^T).

Media and culture conditions. Medium PYGV contained 0·25 g l^{-1} each of Bacto peptone, Bacto yeast extract and glucose as well as 20 ml l^{-1} of Hutner's Basal Mineral Salt Solution (HBM; Cohen-Bazire *et al.*, 1957) and 10 ml l^{-1} of Vitamin Solution no. 6 (van Ert & Staley, 1971). Media were solidified with 1·8 % (w/v) of Bacteriological agar (Gibco). Where needed, liquid and solid media were prepared with 25‰ (v/v) of artificial seawater (ASW; Lyman & Fleming, 1940). R2A Agar (Difco) + ASW was employed for BIOLOG tests. Bacteria for identification tests were incubated at 20 °C. Anaerobic cultivation in the light (4–21·5 μmol photons m^{-2} s^{-1}) occurred at 16 °C. Biomass for chemotaxonomic studies was grown aerated in liquid PYGV + ASW at 20 °C and harvested in the late exponential phase after 4–8 d.

Microscopy. Cellular morphology of exponential phase broth cultures was examined by phase-contrast light microscopy and by transmission electron microscopy as previously described (Labrenz *et al.*, 1998). For ultrathin sectioning, young cells were prepared as described previously (Labrenz *et al.*, 1999).

Physiological and biochemical characterization. All physiological tests were performed at 20 °C. Gram-staining was carried out on 24 h cultures (Skerman, 1967). Catalase production was detected with 5 % H_2O_2. Peroxidase and cytochrome oxidase activities were tested according to Drews (1974) and Kreisel & Schauer (1987). DNA hydrolysis was indicated by clear zones around colonies on Bacto DNase Test Agar (Difco) + ASW. Amylase activity and the relation to oxygen were studied according to Smibert & Krieg (1994). Motility was examined in hanging drop preparations. The ability to grow under various physical and chemical conditions was investigated in liquid media. Osmo-, NaCl-, temperature- and pH-tolerance were studied as by Labrenz *et al.* (1998, 1999) and evaluated by protein determination (Bradford, 1976) of triplicate cultures. The requirement(s) for vitamins were tested in six combinations, with each lacking one component of Vitamin Solution no. 6: biotin, thiamin.HCl, nicotinic acid, sodium pantothenate or vitamin B_{12}. In 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^+, Mg^{2+}, Ca^{2+}, Cl^- or SO_4^{2-} were studied in PYGV + ASW where Na^+ was exchanged with K^+, Mg^{2+} with Ca^{2+}, Cl^- with SO_4^{2-} and vice versa. Susceptibility to antibiotics was determined after four days with bioDiscs (bioMérieux). Aerobic nitrate reduction was tested after 14 d, anaerobic reduction to nitrite or N_2 after three weeks incubation. In both cases PYGV + ASW was employed with 5 mM $NaNO_3$. Anaerobic photolithoautotrophic or photoorganotrophic growth was examined in PYGV + ASW as described by Labrenz *et al.* (1998, 1999). Production of poly-β-hydroxybutyrate was followed in medium PHBA + ASW, which was PYGV modified to contain 20 ml HBM l^{-1}, 10 ml Vitamin Solution no. 6, 0·1 g Bacto yeast extract and 2·0 g succinate according to Smibert & Krieg (1994). Methyl red and Voges–Proskauer tests were performed in PYGV + ASW with 0·2 % (w/v) Bacto peptone and 0·2 % (w/v) glucose. H_2S and indole productions were tested with the Sulfide Indole Motility medium (Merck) + ASW. Indole was detected with Kóvacs reagent.

Table 1. Solid media for testing growth of the Ekho Lake strains and *S. pontiacus* DSM 10014^T on increasing initial sulfite concentrations (l^{-1})

All media contained (l^{-1}) 20 ml Hutner's basal medium, 10 ml vitamin solution no. 6, 0·25 g yeast extract, 15 g NaCl and 3 ml bromothymol blue (1 %).

Substance	Medium			
	1	2	3	4
Na-acetate	15 mM	10 mM	5 mM	5 mM
Na-sulfite	7·5 mM	15 mM	45 mM	60 mM
HEPES	8·0 g	1·0 g	—	—
Bacto peptone	0·25 g	0·25 g	0·10 g	0·10 g
Bacteriological agar	18 g	18 g	18 g	20 g

The aerobic dissimilation of 95 carbon sources with the BIOLOG System and carbon source utilization in a minimal medium were studied as described by Labrenz *et al.* (1998, 1999). Degradation of the following substrates was tested with the basal medium PYV + ASW, i.e. lacking glucose and containing (w/v): 0·2 % starch, 0·4 % gelatin, 1 % Tween 80 or 0·75 % alginate. Production of bchl *a* was followed in suspensions of cells grown in PYGV + ASW and analysed as described (Shiba & Simidu, 1982). Tests were performed with cells grown in constant light at 4–21·5 μmol photons m^{-2} s^{-1} or with dark-grown cells.

Growth with sulfite. The Ekho Lake strains and *S. pontiacus* DSM 10014 ^{T} were adapted to increasing sulfite concentrations on previously described, but modified media (Sorokin & Lysenko, 1993; Sorokin, 1995; Table 1). Adaptation started with solid sulfite medium 1 and ended on solid medium 4. Cultivation occurred for 8–16 d. In order to obtain high initial sulfite concentrations, the media were inoculated immediately after autoclaving. Growth was controlled on media without Na-sulfite. Growth was also determined in liquid medium. For this, sulfite-adapted cells were taken from solid medium 3 and inoculated into modified liquid medium 3 containing 0, 10, 20, 45 or 60 mM Na-sulfite; an indicator was not added. After 9 d incubation at 20 °C, protein contents were determined.

Chemotaxonomy. Analysis of fatty acid methyl esters (FAMEs) was performed with 20 mg freeze-dried biomass as previously described (Labrenz *et al.*, 1998). FAMEs

which could not be identified on the basis of their retention times were analysed by GC-MS using a model GCMS-QP2000 instrument (Shimadzu) under gas chromatographic conditions as described previously (Groth *et al.*, 1996). Respiratory lipoquinones and polar lipids were extracted from 100 mg freeze-dried material using a two-stage method and analysed as described before (Tindall, 1990a, b). Bacteriochlorophyll was detected initially in the lipid extracts, and was visible as a green band on the TLC plates. Diamino acids of cell walls were separated by one-dimensional TLC on cellulose using the solvent system of Rhuland *et al.* (1955).

Determination of DNA base composition. DNA G+C contents (mol %) were analysed using HPLC (Mesbah *et al.*, 1989). The HPLC apparatus (Pharmacia-LKB) was equipped with a Spherisorb ODS II C_{18} column (5 μm; 4 × 250 mm; Bischoff). *Escherichia coli* strain B type VIII-DNA (Sigma) and λ 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 Labelling and Detection kit from Boehringer Mannheim following manufacturer's instructions. DNA probes were prepared from EL-38^T, EL-162^T and *Sulfitobacter pontiacus*^{T}. Hybridization occurred against chromosomal DNA from Ekho Lake isolates and the type strains of *Sulfitobacter pontiacus* and *Roseobacter denitrificans*. The stringency of 70 % was calculated according to Sambrook *et al.* (1989).

16S rRNA gene sequence determination and analysis of sequence data. 16S rRNA gene fragments were generated by PCR as described by Hudson *et al.* (1993). A large fragment of the 16S rRNA gene was amplified from DNA by PCR 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 Ekho Lake strains, preliminary searches in the EMBL database were performed with the program FASTA (Pearson & Lipman, 1988). 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 (Devereux *et al.*, 1984). 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 (1969) parameters. A phylogenetic tree was constructed according to the neighbour-joining method (Saitou & Nei, 1987) with the program NEIGHBOR. For this analysis, the following additional taxa, listed by genera and accession number, were included: *Rhodovulum euryhalinum* (D13479), *Rhodovulum sulfidophilum* (U55277), *Rhodovulum adriaticum* (D16418), *Rhodobacter blasticus* (D16429), *Rhodobacter capsulatus* (D16428), *Rhodobacter sphaeroides* (D16425), *Rhodobacter veldkampii* (D16421), *Paracoccus denitrificans* (X69159) and *Paracoccus kocurii* (D32241). The stability of the groupings was estimated by bootstrap analysis (500 replications) using the programs of the PHYLIP package (Felsenstein, 1989).

RESULTS

Isolations

EL-38^T was isolated from a 1 m Ekho Lake water sample, which had a salinity of 10‰, a temperature of 5 °C, and a pH of 8·09. EL-162^T came from an 8 m water sample with a salinity of 55‰, a temperature of 11·5 °C and a pH of 8·3. The pure cultures were kept on PYGV slants with the appropriate concentration of ASW, corresponding to that of the original sample.

Morphology and motility

Both isolates were Gram-negative short rods with one or both cell poles narrower (Fig. 1). Cells of EL-38^T were more pointed (Fig. 1a, c) and those of EL-162^T often more coccoid (Fig. 1b, d). The isolates frequently formed rosettes (Table 2) and often contained poly-β-hydroxybutyrate granules. Motility was observed in both strains and electron microscopy revealed a flagellum in strain EL-38^T, but the point of insertion could not be determined. Flagella were not detected in strain EL-162^T.

Culture and growth characteristics

Aerobic growth of both strains was visible after 3–5 d on PYGV + 25‰ ASW and at 20 °C. Colonies were in both cases circular, smooth, convex, beige or yellowish-brown and had a diameter of 1–3 mm (Table 2). Older colonies of EL-38^T changed from beige to pink.

Strain EL-38^T grew between < 4 and < 32 °C and at pH values of 5·5 to > 9·5. Optimal growth occurred at pH values of 7·0–8·5. Its osmotolerance ranged from < 10 to 130–150‰ ASW, with an optimum between 10 and 40‰. NaCl was found to be tolerated from < 1·0 to 2·5–4·0 %, with an optimum of 1·0 % NaCl. The temperature range for growth of EL-162^T was between < 3 and 33·5 °C, and growth occurred between pH values of 5·5 and 9·5, with an optimum between 7·5 and 8·0. The osmotolerance of EL-162^T ranged from < 10 to > 150‰ ASW, and was optimal between 10 and 80‰. The NaCl tolerance range was < 1·0 to 8·0 %, with an optimum of 1·0–2·0 % NaCl. Other growth characteristics are shown in Table 2.

EL-38^T had a weak and EL-162^T an absolute requirement for Na^+; the other cations and anions could all be replaced as indicated in Methods. Both EL-isolates had a requirement for pantothenate, a weak requirement for biotin and nicotinic acid, but none for vitamin B_{12}. Both strains also required thiamin, but EL-38^T only weakly.

16S rRNA gene sequence determinations and phylogenetic analyses

The almost complete 16S rRNA gene sequences of strains EL-38^T and EL-162^T were determined. Sequence searches of the EMBL database revealed that the newly determined sequences were related to the α-

Table 2. Differential characteristics of *Staleya guttiformis* EL-38^T and *Sulfitobacter brevis* EL-162^T with closely related *α-Proteobacteria*

Strains (data from this study, except where reference is given): 1, *Staleya guttiformis* EL-38^T; 2, *Sulfitobacter brevis* EL-162^T; 3, *Sulfitobacter pontiacus* DSM 10014^T (Sorokin, 1995); 4, *Roseobacter litoralis* DSM 6996^T (Shiba, 1991); 5, *Roseobacter denitrificans* DSM 7001^T (Shiba, 1991); 6, *Octadecabacter antarcticus* 307^T (Gosink *et al*., 1997); 7, *Octadecabacter arcticus* 238^T (Gosink *et al*., 1997); 8, *Marinosulfonomonas methylotropha* PSCH4^T (Holmes *et al*., 1997); 9, *Sagittula stellata* ATCC 700073^T (Gonzalez *et al*., 1997); 10, *Antarctobacter heliothermus* DSM 11445^T (Labrenz *et al*., 1998); 11, *Roseovarius tolerans* DSM 11457^T (Labrenz *et al*., 1999); 12, *Ruegeria algicola* ATCC 51440^T (Lafay *et al*., 1995); 13, *Roseobacter gallaeciensis* CIP 105210^T (Ruiz-Ponte *et al*., 1998). v, Variable; w, weak; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
Morphology:													
Rosettes formed	+	+	+	−	−	−	−	+	+	+	−	−	ND
Bud formation	+	+	+	−	−	−	−	ND	ND	+	+	−	ND
Physiology:													
Oxidase	+	+	+	+	+	−	−	+	+	+	+	+	+
Bchl *a*	v	−	−	+	+	−	−	−	−	−	+	−	−
Growth at 37 °C	−	−	−	−	−	−	−	+	+	+	+	+	+
Gelatin hydrolysis	−	−	−	+	+	−	−	ND	−	+	−	+	−
C-source: citrate	−	+	−	+	+	−	v	+	+	+	−	+	−
C-source: butyrate	−	+	+	−	−	−	−	ND	+	+	w	−*	+
C-source: methanol	−	−	ND	−	−	−	−	+	+	−	−	−	ND
Pantothenate requirement	+	+	ND	−	−	−	+	ND	−	−	−	−	−
Fatty acids (%):			†‡	§	§							§	
3-OH 10:0	5·9	4·5	3·6	1·9	4·2	2	4	ND	−	−	−	−	ND
3-OH 12:1	−	−	−	−	−	−	−	ND	3·6	3·1	3·6	−	ND
3-OH 14:1	2·1	2·6	2·0	3·9	−	−	−	ND	−	−	−	−	ND
16:1ω7*c*	−	−	1·2	−	1·4	12	8	ND	−	0·8	0·8	−	ND
16:0	3·9	5·8	8·1	1·1	1·6	6	6	ND	8·6	2·5	6·2	1·6	ND
18:2‖	−	3·2	−	−	−	−	−	ND	−	−	−	−	ND
18:2¶	5·3	7·8	−	1·4	7·1	−	−	ND	−	−	10·6	1·6	ND
18:1ω9*c*	−	18·9	−	−	−	−	−	ND	−	−	−	−	ND
18:1ω7*c*	79·7	50·0	79·1	88·8	84·6	77#	75#	ND	**	83·2	70·2	91·5	ND
18:0	0·7	0·9	−	1·3	1·2	−	−	ND	6·8	1·0	0·8	2·2	ND
cyclo 19:0	−	−	−	−	−	−	−	ND	1·8	2·4	−	−	ND
19:1	1·4	−	−	−	−	−	−	ND	−	−	−	−	ND
Polar lipids:			‡	§	§							§	
Diphosphatidylglycerol	−	+	+	+	+	ND	ND	ND	ND	−	+	+	ND
Phosphatidylethanolamine	+	+	+	−	−	ND	ND	ND	ND	−	+	+	ND
Phosphatidylcholine	+	+	+	−	+	ND	ND	ND	ND	+	+	+	ND
G+C content (mol%)	55–56	58	62–63	56–59	60	56	57	57	65	62–64	63	64–65§	58

* May also be positive (Labrenz *et al*., 1999).

† Grown on Marine Broth 2216 (Difco).

‡ Data from the present study.

§ Data from Labrenz *et al*. (1999).

‖ First isomer, which could not be further identified.

¶ Second isomer, which could not be further identified.

Unclear if 18:1ω7*c*, 18:1ω9*t* or 18:1ω12*t*.

** Major fatty acid, but not quantified.

subclass of the *Proteobacteria* (data not shown). Both EL-strains displayed highest 16S rRNA gene sequence relatedness (approx. 96·5–98%) with *Sulfitobacter pontiacus* (Sorokin, 1995). Also high sequence relatedness was observed with *Roseobacter denitrificans* and *Roseobacter litoralis*. Other species belonging to the α-subclass of *Proteobacteria* examined showed lower levels of relatedness. An unrooted tree depicting the phylogenetic relationships of the unknown Ekho Lake bacteria and their closest relatives is shown in Fig. 2. The results of treeing analyses showed that the Ekho Lake strain EL-162^T phylogenetically clustered

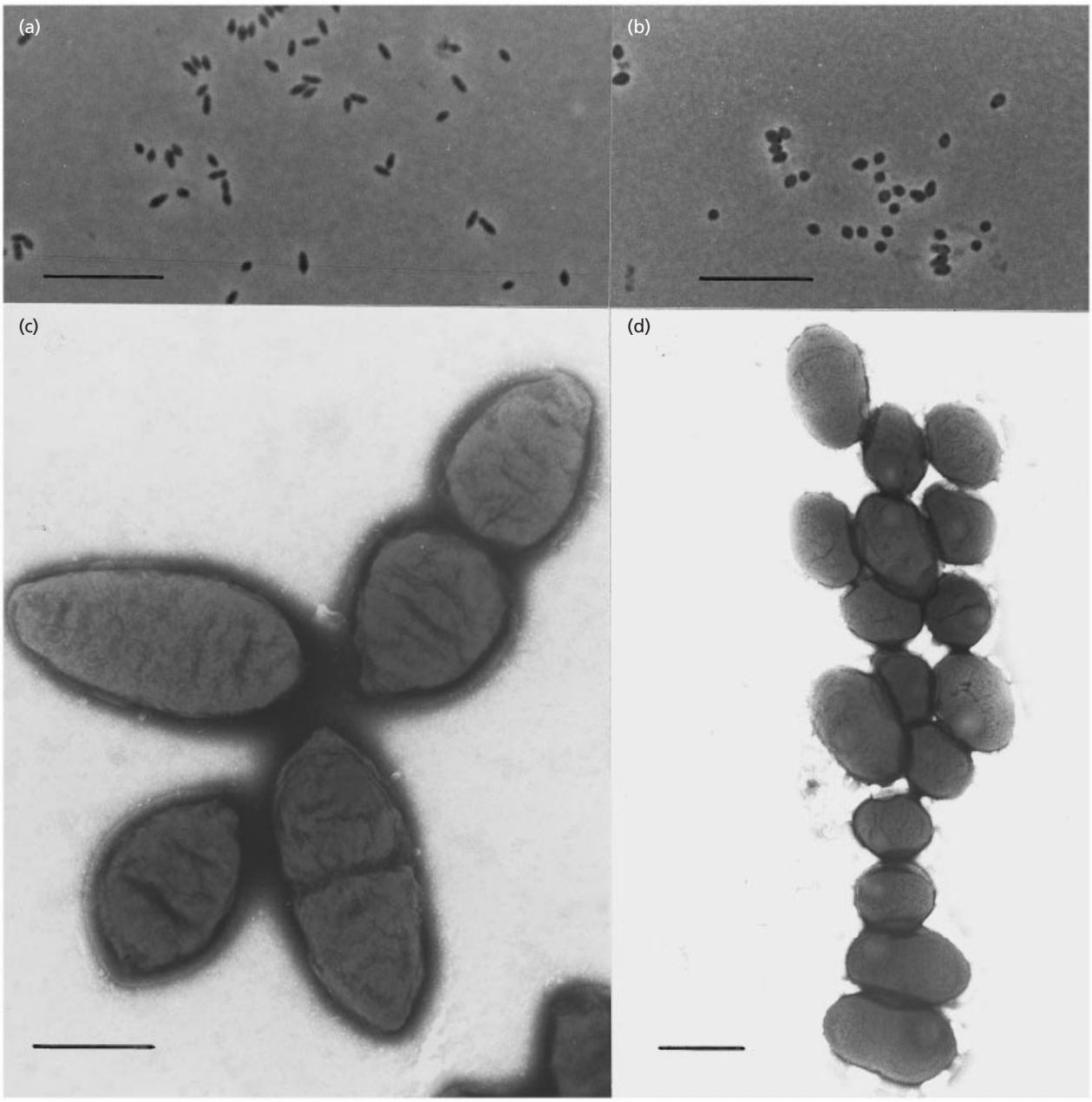

Fig. 1. Phase-contrast light micrograph of *Staleya guttiformis* EL-38^T (a) and *Sulfitobacter brevis* EL-162^T (b) on an agar-coated slide (Pfennig & Wagener, 1986). Bars, 15 μm (a) and 10 μm (b). Electron micrograph of PTA negatively stained cells of strain EL-38^T (c) and EL-162^T (d). Bars, 1 μm.

with *Sulfitobacter pontiacus*, while EL-38^T formed a distinct subline.

Chemotaxonomic characteristics

The peptidogylcan of both isolates contained *meso*-diaminopimelic acid. The only respiratory lipoquinones detected were ubiquinones, with ubiquinone 10 as the dominant isoprenologue. The presence of ubiquinone 10 as the dominant respiratory lipoquinone is characteristic of members of the α-subclass of the *Proteobacteria*. The predominant fatty acid was 18:1ω7*c* (Table 2), accounting for 50–80 % of the total fatty acids, a feature characteristic of several major phyletic groups within the α-subclass of the *Proteobacteria*. The presence of 3-OH 10:0 and a compound provisionally identified as 3-OH 14:1, the latter being exclusively (presumptively) amide bound is indicative of the fact that the novel isolates belonged within the same phyletic group as members of the genera *Roseobacter* or *Ruegeria* (Ushino *et al.*, 1998). In previous work the compound presumptively identified here as 3-

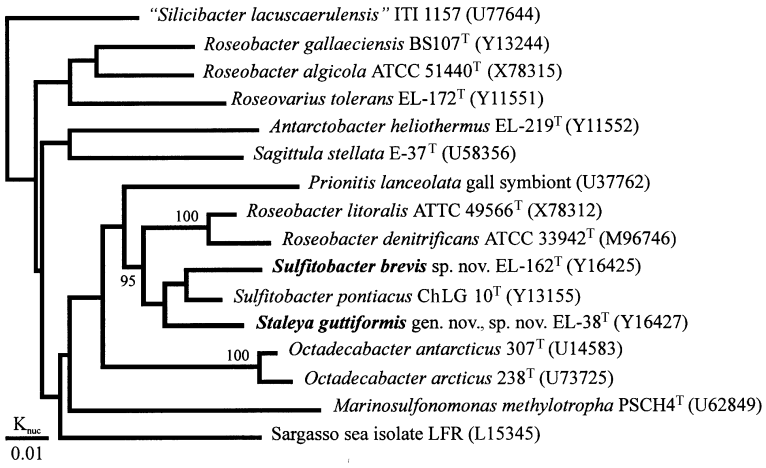

Fig. 2. Unrooted tree showing phylogenetic relationships of Ekho Lake strains with *Roseobacter* spp., *S. pontiacus* and closely related *Proteobacteria*. Additional sequences were used to perform the phylogenetic analyses (see Methods). The tree was constructed using the neighbour-joining method (K_{nuc} values). It was based on a comparison of 1320 nucleotides. Bootstrap values, expressed as a percentage of 500 replications, are given at branching points; only those above 90 % are shown.

OH 14:1 was incorrectly labelled 2-OH 14:0 (Labrenz *et al*., 1998, 1999). The polar lipids of the two novel isolates were not identical, with phosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine, together with an unidentified amino lipid being present in both strains. Strain EL-38^T differed from strain EL-162^T in that it did not produce diphosphatidylglycerol. This pattern was similar to that of *Sulfitobacter pontiacus*, but significantly different from members of the genus *Roseobacter*, which do not synthesize phosphatidylethanolamine.

Physiological and biochemical characteristics

Phenotypic and biochemical characteristics which differentiated EL-38^T and EL-162^T from possibly related type strains of *Sulfitobacter pontiacus*, *Roseobacter litoralis* or *Roseobacter denitrificans*, are listed in Table 2. In addition, both strains exhibited peroxidase and cytochrome oxidase activity and produced catalase, but EL-38^T only weakly. They did not produce acetoin or acids from glucose. Neither sulfide nor indole were produced. They were susceptible to chloramphenicol (30 μg), streptomycin (10 μg), polymyxin B (300 U), penicillin G (10 U) or tetracycline (30 μg). EL-38^T was also susceptible to nalidixic acid (30 μg), but EL-162^T was not. The two strains were not able to reduce nitrate anaerobically or nitrite aerobically. Neither of the strains hydrolysed alginate or starch. In the presence of available nitrogen, they utilized succinate, glutamate, acetate, pyruvate or malate, but not methanol or methanesulfonic acid. α-D-Glucose was only weakly utilized. In the BIOLOG system, the two EL-strains metabolized acetic acid, itaconic acid, propionic acid, and weakly i-erythritol. They did not metabolize α-cyclodextrin, dextrin, glycogen, methylpyruvate, monomethylsuccinate, *cis*-aconitic acid, 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-ketovaleric acid, DL-lactic acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, D-alanine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, L-threonine, DL-carnitine, γ-aminobutyric acid, urocanic acid, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol or glycerol. Differences in the metabolism of carbon compounds as revealed with the BIOLOG system are shown in Table 3. They did not grow anaerobically and photolithoautotrophically with H_2/CO_2 (80:20) in the gas phase, and they did not grow photo-organotrophically.

Bacteriochlorophyll *a* was present in dark-grown cell suspensions of EL-38^T, but not in EL-162^T. Characteristic absorbances were found with a larger peak at 861–862 nm and smaller ones at 800–802 and 590–592 nm (Fig. 3). These differed from the maxima of bchl *a*-containing *Roseobacter denitrificans* and *Roseobacter litoralis* (Shiba, 1991) or *Roseovarius tolerans* (Labrenz *et al*., 1999). Other features, such as carotenoids, were not characterized further. The bchl *a* production by EL-38^T was very low in constant dim light, with only a very small peak at 865 nm after 2 weeks. Unlike *Roseobacter denitrificans*, vesicular structures of intracytoplasmatic membrane systems (Harashima *et al*., 1982) were never found in ultrathin sections of aerobically and dark grown cells of EL-38^T.

Growth with sulfite

Unlike *Sulfitobacter pontiacus* (Sorokin, 1995), the addition of sulfite did not stimulate growth of EL-38^T or EL-162^T in liquid media. The *Sulfitobacter pontiacus* type strain grew at initial sulfite concentrations of 0, 10, 20 and to a lower degree at 45 or even 60 mM. Here the protein content was significantly higher with 10 mM sulfite than in sulfite-free media. EL-162^T grew at concentrations of 0, 10, 20 and to a lesser extent at 45 mM, but not at 60 mM sulfite. Growth of EL-38^T occurred at 0, 10 and less well at 20 mM, but not at 45 or 60 mM sulfite. On autoclaved, unbuffered solid

Table 3. Differences of strains EL-38^T and EL-162^T in the metabolism of carbon compounds as revealed by the BIOLOG system

+, Positive; −, negative; w, weak.

Substrate	EL-38^T	EL-162^T
Tween 40	+	w
Tween 80	−	+
N-Acetyl-D-galactosamine	−	+
N-Acetylglucosamine	−	+
Adonitol	−	+
L-Arabinose	−	+
D-Arabitol	−	+
Cellobiose	−	w
D-Fructose	w	+
L-Fucose	w	+
D-Galactose	−	+
Gentiobiose	−	+
α-D-Glucose	w	+
meso-Inositol	−	w
α-Lactose	w	+
D-Glucosaminic acid	−	w
α-D-Lactose-lactulose	w	+
Maltose	−	+
D-Mannitol	−	w
D-Mannose	−	+
D-Melibiose	w	+
Methyl β-D-glucoside	−	w
Psicose	−	w
D-Raffinose	−	+
L-Rhamnose	w	+
D-Sorbitol	−	+
Sucrose	w	+
D-Trehalose	w	+
Turanose	−	+
Xylitol	−	w
Citric acid	−	+
D-Gluconic acid	−	w
D-Glucuronic acid	−	w
α-Ketoglutaric acid	−	w
Malonic acid	−	w
Quinic acid	−	w
D-Saccharic acid	−	w
Glucuronamide	−	+
Alaninamide	−	w
L-Alanylglycine	−	+
D-Serine	−	w
L-Serine	−	w
Inosine	−	+
Uridine	−	w
Thymidine	−	w
DL-α-Glycerolphosphate	−	w
Glucose 1-phosphate	−	w
Glucose 6-phosphate	−	w

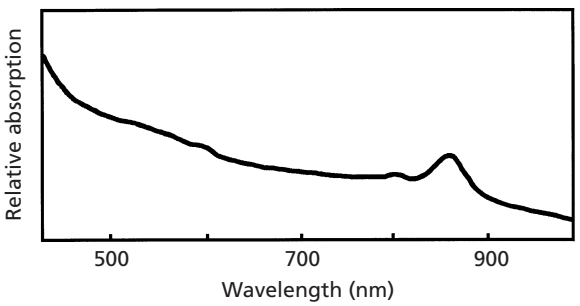

Fig. 3. *In vivo* absorption spectra of 8-month-old strain EL-38^T cells grown aerobically on PYGV+25‰ ASW agar in the dark.

medium 3 or 4 (Table 1) autoxidation of Na-sulfite was visible after 18 h, followed by a bromothymol blue indicator colour change from blue to yellow. Bacterial growth of Ekho Lake strains and *Sulfitobacter pontiacus* was indicated by (i) colony formation and (ii) blue zones around the colonies, which showed the pH to be > 6·0. Acetate and peptone assimilation could be reasons for this locally increased pH. On agar media, all strains grew at initial sulfite concentrations up to 45 mM, but only *Sulfitobacter pontiacus* grew at 60 mM sulfite.

DNA base composition

The base ratios of the isolates are shown in Table 2. Those of *Sulfitobacter pontiacus* differed from the mol% G+C of the other tw. EL isolates.

Dot-blot hybridizations

At 70% stringency, DNA probes from EL-38^T, EL-162^T and *Sulfitobacter pontiacus* DSM 10014^T hybridized only with their own chromosomal DNA.

DISCUSSION

Sequencing of 16S rRNA genes confirmed that the two Ekho Lake isolates were members of distinct taxa within the α-3 subclass of the *Proteobacteria*. The combination of respiratory lipoquinone, fatty acid and polar lipid data also indicated that these strains belonged to this group of organisms (above the species rank) within the α-subclass of the *Proteobacteria* (Labrenz *et al.*, 1998). Comparative 16S rRNA gene sequencing showed that the two bacteria from Ekho Lake were specifically associated with the *Sulfitobacter–Roseobacter* cluster of organisms, but not with *Ruegeria algicola* (Lafay *et al.*, 1995) or *Roseobacter gallaeciensis* (Ruiz-Ponte *et al.*, 1998). It is evident from treeing analyses that the genus *Roseobacter*, as currently recognized, is interdispersed with several other taxa. This is also reflected in the inclusion of *R. algicola* in the genus *Ruegeria* by Uchino *et al.* (1998).

The chemotaxonomic data indicated that members of the genus *Roseobacter* (i.e. *Roseobacter litoralis* and

Roseobacter denitrificans) had a distinctive polar lipid composition, in which both species had phosphatidylglycerol, diphosphatidylglycerol and an unidentified amino lipid as the major components. In contrast, strains EL-162^T, EL-38^T and *Sulfitobacter pontiacus* all synthesized phosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine, together with the same unidentified amino lipid. This polar lipid composition serves to distinguish these two groups from one another, and clearly shows that the isolates EL-162^T and EL-38^T do not belong to the genus *Roseobacter*.

Sequence divergence values of 2 % for strain EL-162^T and 3·5 % for strain EL-38^T indicated that both strains were phylogenetically closely related to *Sulfitobacter pontiacus*. Morphologically, both antarctic strains were more similar to *Sulfitobacter pontiacus* than to *Roseobacter* strains: their cells multiplied by monopolar growth (a budding process) and formed rosettes (Table 2, Fig. 1). The ability to oxidize sulfite and thereby to increase growth characterizes *Sulfitobacter pontiacus* (Sorokin, 1995). Although EL-162^T and the type strain of *Sulfitobacter pontiacus* tolerated and grew in liquid media with initial sulfite concentrations of 20 mM, sulfite oxidation or a stimulation of growth could not be demonstrated with EL-162^T or EL-38^T. The chemotaxonomic data indicated that the type strain of *Sulfitobacter pontiacus* and the two antarctic isolates were very similar. The polar lipids were qualitatively identical in *Sulfitobacter pontiacus* and strain EL-162^T, but strain EL-38^T did not synthesize diphosphatidylglycerol, which was present in the other two organisms. Also, there were some differences in the fatty acid composition. In particular, strain EL-162^T synthesized two isomers of 18:2 together with 18:1ω9*c* and 18:1ω7*c*, whereas strain EL-38^T synthesized only one isomer of 18:2 and 18:1ω7*c*, while *Sulfitobacter pontiacus* only synthesized 18:1ω7*c* (Table 2). Examination of the G+C base content indicated that the value for EL-38^T was about 5 mol% lower than that for *Sulfitobacter pontiacus*, while the value for strain EL-162^T was about 3 mol% lower. The significance of differences in the G+C base content in an evolutionary context has been outlined by De Ley (1967).

The two EL-strains showed distinct differences in their metabolism of carbon compounds, as detected with the BIOLOG system (Table 3). Other differences concerned bchl *a*, which was present in light- or dark-grown cells of EL-38^T, as shown by *in vivo* absorption bands at 800–802 and 861–862 nm (Yurkov & Beatty, 1998). But these peaks were clearly absent from EL-162^T, from the *Roseobacter* spp. and *Sulfitobacter pontiacus*. The importance of bchl *a* for taxonomic separation of these aerobic bacteria has already been questioned (Labrenz *et al*., 1999).

In conclusion, organisms of the *Sulfitobacter–Roseobacter* cluster appear to be phylogenetically quite closely related (Fig. 3), but phenotypically they are very diverse (Table 2). On the basis of the chemical data presented here we consider that the genus *Roseobacter* (containing only *Roseobacter litoralis* and *Roseobacter denitrificans*) is taxonomically distinct from *Sulfitobacter pontiacus* and the two strains EL-38^T and EL-162^T. This is so despite the relatively 'high' degree of 16S rDNA sequence similarity. That the genera *Sulfitobacter* and *Roseobacter* share a common ancestor is evident by the 16S rDNA sequence data and the similarities in chemotaxonomic data. However, it should be noted that the creation of taxa based solely on arbitarily determined sequence similarity levels (Fry *et al*., 1991; Devereux *et al*., 1990) does not take into account the phenotypic diversity of prokaryotes, which is also an indirect measure of their genotypic diversity. The potential stability in prokaryotic taxonomy is better served by using a wide range of 'reliable' markers, by which taxa are defined (a polythetic system), rather than using one dominant marker (a monothetic system), a problem which has been discussed previously (Tindall, 1994). Although the present data show that strains EL-38^T and EL-162^T do not belong in the genus *Roseobacter*, there are three alternatives: (i) To unite the EL-strains with members of the genus *Sulfitobacter*. However, 16S rRNA gene sequence divergence between the two antarctic isolates was nearly 4 %, dot-blot hybridization was not observed at 70 % stringency, and significant physiological and chemotaxonomic differences from *Sulfitobacter pontiacus* were found (Table 2). (ii) To split both Ekho Lake strains into two new genera. EL-162^T has a sequence divergence from *Sulfitobacter pontiacus* of only 2 %, its sulfite tolerance was fairly high, and physiological properties were quite similar to those of *Sulfitobacter pontiacus*. (iii) To create a new genus for EL-38^T, and place strain EL-162^T in the genus *Sulfitobacter* as a new species. In our opinion, this last possibility would be suitable, because strain EL-38^T does not fulfil all criteria for inclusion in the genus *Sulfitobacter*. It could be placed intermediately between *Roseobacter* (Shiba, 1991) and *Sulfitobacter* (Sorokin, 1995), but as a representative of a new genus and species. We propose for it the name *Staleya guttiformis* gen. nov., sp. nov.

EL-162^T is phylogenetically more closely related to *Sulfitobacter pontiacus*. In addition, both of these bacteria had the same polar lipid pattern; they utilized butyrate and did not produce bchl *a* (Table 2). Both organisms grew well in liquid media at initial sulfite concentrations of 20 mM. Based on these observations we consider strain EL-162^T to be a new species of the genus *Sulfitobacter* and propose the name *Sulfitobacter brevis* sp. nov.

Description of *Staleya* gen. nov.

Staleya (Sta′ley.a. M.L. n. *Staleya* after the American microbiologist J. T. Staley in recognition of his work on budding and appendaged bacteria and his contributions to polar microbiology).

Gram-negative rods with one or both cell poles

pointed, multiplying by monopolar growth, i.e. by a budding process. Cells may be motile, may contain poly-β-hydroxybutyrate and do not form spores. On medium PYGV + ASW colonies are smooth, convex and beige to pink. Bchl *a* may be produced. The temperature range for growth is from <4 to 32 °C. The cells have a weak requirement for Na^+, growing in the range of $<1{\cdot}0$ to 4·0 % NaCl. In the presence of ASW they grow between <10 and <150‰. The pH tolerance range is 5·3–6·5 to >9. Strictly aerobic, non-fermentative heterotrophs. No growth on glucose anaerobically in the absence of nitrate. They do not grow photoautotrophically with H_2/CO_2 (80:20) or photo-organotrophically with acetate or glutamate. The cells exhibit peroxidase, cytochrome oxidase and weak catalase activity. Polar lipids present are: phosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, as well as an unknown aminolipid. Dominant fatty acid is 18:1ω7*c*; other characteristic fatty acids are 3-OH 10:0, 3-OH 14:1, 16:0, 18:0, 18:2 and 19:1. Major respiratory quinone is Q-10. Origin: water sample from Ekho Lake, Antarctica. The type species of the genus is *Staleya guttiformis*.

Description of *Staleya guttiformis* sp. nov.

Staleya guttiformis (gut.ti.for′mis. L. fem. n. *gutta* the drop; M.L. n. *guttiformis* drop-shaped).

Cell sizes vary from 1·0–1·5 × 1·5–8·9 μm, with a mean of 1·1 × 1·8 μm. On medium PYGV + ASW, colonies are smooth, convex and beige, later pink. Optimal growth occurs at 12–20 °C with 1·0 % NaCl or 10–40‰ ASW. The optimum pH is 7·0–8·5. Bchl *a* may be produced in older cells with a larger *in vivo* absorption band at 861–862 nm and small ones at 590–592 and 800–802. Production of bchl *a* is not totally repressed by constant dim light. *S. guttiformis* has an absolute requirement for pantothenate and a weak one for thiamin, biotin and nicotinic acid. Tween 80 and DNA are hydrolysed, but not alginate, gelatin or starch. Growth occurs on acetate, pyruvate, malate, succinate or glutamate, but not on citrate, butyrate, methanesulfonic acid or methanol. α-D-Glucose is only weakly utilized. Cells are susceptible to chloramphenicol, streptomycin, penicillin G, tetracycline, polymyxin B or nalidixic acid. Nitrate is aerobically reduced to nitrite. H_2S and indole are not produced. Methyl red- and Voges–Proskauer-negative. The G + C content is 55·0–56·3 mol %. Chemotaxonomic properties and other characteristics are as described for the genus. The type strain of *Staleya guttiformis* (EL-38^T) has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen as DSM 11458^T.

Description of *Sulfitobacter brevis* sp. nov.

Sulfitobacter brevis (bre′vis. L. adj. *brevis* short, referring to the short cells).

Gram-negative short rods with one or both cell poles pointed, multiplying by monopolar growth, i.e. by a budding process. Cell sizes vary from 0·8–1·0 × 1·1–1·5 μm, with a mean size of 0·9 × 1·3 μm. Rosettes may be formed. Cells may be motile and contain poly-β-hydroxybutyrate; do not form spores. On PYGV + ASW the colonies are smooth, convex and yellowish-brown. The cells have an absolute requirement for Na^+. Strictly aerobic, non-fermentative heterotrophs. Do not grow on glucose anaerobically in the absence of nitrate. Do not grow photoautotrophically with H_2/CO_2 (80:20) or photo-organotrophically with acetate or glutamate. The cells exhibit peroxidase, catalase and cytochrome oxidase activity. Optimal growth occurs between 3 and 26 °C with 1·0–2·0 % NaCl or 10–80‰ ASW. The optimum pH is 7·5–8·0. Bchl *a* is not produced. Cells have a requirement for pantothenate and thiamin, a weak requirement for biotin and nicotinic acid, but none for vitamin B_{12}. Tween 80 is hydrolysed, but not alginate, gelatin, DNA or starch. Growth occurs on acetate, pyruvate, malate, succinate, citrate, butyrate or glutamate, but not on methanesulfonic acid or methanol. α-D-Glucose is utilized weakly. Cells are susceptible to chloramphenicol, streptomycin, penicillin G, polymyxin B and tetracycline, but not to nalidixic acid. Nitrate is not reduced, H_2S and indole are not produced. Methyl red- and Voges–Proskauer-negative. Polar lipids present are: diphosphatidylgycerol, phosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine and an unknown aminolipid. Dominant fatty acids are 18:1ω7*c* and 18:1ω9*c* and 18:2 is present as two isomers. Other characteristic fatty acids are 3-OH 10:0, 3-OH 14:1, 16:0 and 18:0. Major respiratory quinone is Q-10. The G + C content is 57·9–58·1 mol %. Origin: water sample from Ekho Lake, Antarctica. The type strain of *Sulfitobacter brevis* (EL-162^T) has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen as DSM 11443^T.

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