**Roseovarius tolerans gen. nov., sp. nov., a budding bacterium with variable bacteriochlorophyll a production from hypersaline Ekho Lake**

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Eight 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 daughter cells could be motile. Bacteriochlorophyll a was sometimes produced, but production was repressed by constant dim light. The strains tolerated a wide range of temperature, pH, concentrations of artificial seawater and NaCl, but had an absolute requirement for sodium ions. Glutamate was metabolized with and without an additional source of combined nitrogen. The dominant fatty acid was $C_{18:1}$; other characteristic fatty acids were $C_{18:2}$, $C_{12:0}$ 2-OH, $C_{12:1}$ 3-OH, $C_{16:1}$, $C_{15:0}$ and $C_{18:0}$. The main polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol and phosphatidylcholine. The DNA G+C base composition was 62-64 mole%. 16S rRNA gene sequence comparisons showed that the isolates were phylogenetically close to the genera *Antarctobacter*, *Marinosulfonomonas*, *Octadecabacter*, *Sagittula*, *Sulfitobacter* and *Roseobacter*. Morphological, physiological and genotypic differences to these previously described and distinct genera support the description of a new genus and a new species, **Roseovarius tolerans** gen. nov., sp. nov. The type strain is EL-172T (= DSM 11457T).

**Keywords:** *Roseovarius* gen. nov., *Roseovarius tolerans* sp. nov., α-Proteobacteria, bacteriochlorophyll a, Antarctica

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**INTRODUCTION**

The ice-free area of the Vestfold Hills (East Antarctica) contains several lakes of various sizes which originated from fjords about 5000–6000 years ago. When the land subsequently rose due to the melting of the ice, the links of some lakes with the ocean were lost. Their initial connections with the ocean partly explain their salt composition and salinity. This origin may also have influenced the structure and types of their microbial communities (Franzmann et al., 1987; James et al., 1994). One such lake is the hypersaline, heliothermal and meromictic Ekho Lake, which contains many different environments throughout its depth profile. For this reason, the diversity of bacterial morphotypes (MT), total bacterial numbers and c.f.u. were studied there during two Antarctic summers (P. Hirsch, J. Siebert & H. R. Burton, unpublished results). A total of 135 prokaryotic and 52 eukaryotic MT was discovered. This unexpectedly high morphological diversity prompted the isolation of some 250 bacterial cultures from different depths of Ekho Lake and the initiation of a study of their taxonomic (phenotypic and phylogenetic) diversity. The isolation of four Gram-negative, aerobic, pointed and budding bacteria resulted in the description of *Antarctobacter heliothermus* gen. nov., sp. nov. (Labrenz et al., 1998). Prokaryotic, photosynthetic primary producers such as cyanobacteria were almost completely absent from the lake, but some aerobic, eubacterial isolates contained bacteriochlorophyll a (bchl a). Also, several...
strains of eukaryotic photosynthetic primary pro-
ducers (praseophytes with four flagella) were obtained
in pure culture.

Obligately aerobic bchl a-containing bacteria have
been found in the genera Roseobacter (Shiba, 1991),
Roseococcus (Yurkov et al., 1994), Erythrobacter
(Shiba & Simidu, 1982), Erythromonas (Yurkov et al.,
1997), Sandaracinobacter (Yurkov et al., 1997) and
Porphyrobacter (Fuerst et al., 1993). This group covers
a wide range of organisms from different geographical
locations and with different physiological require-
ments. The relationship of bchl a-containing organ-
isms (both anoxygenic phototrophs and obligately
aerobic bchl a-containing bacteria) with other
bacteria (praseophytes with four flagella) were obtained
in pure culture.

METHODS

Water sampling, enrichment conditions and isolations.
Water samples were taken aseptically from every metre of
the Ekho Lake profile using a Kammerer sampler at the
42 m depth centre (Labrenz et al., 1998). Samples were
stored over ice until used for the inoculation of enrichments
or plates after returning to the Davis Station laboratory in
the Vestfold Hills. Characteristics of these water samples and
enrichment conditions are shown in Table I. Isolation of
pure cultures was achieved by several dilution transfers on
the corresponding agar media. Pure cultures were kept as
serial transfers on agar slants, or lyophilized, or deep-frozen
at −72 °C in the growth medium.

Bacterial strains. For comparisons, the following type strains
and other bacterial cultures were used: R. denitrificans
(DSM 7001T), R. litoralis (DSM 6996T), R. algicola (DSM
10251T), A. heliothermus EL-219 (DSM 11445T; Labrenz
et al., 1998), A. heliothermus EL-165 (DSM 11440), A.
heliothermus EL-54 and A. heliothermus EL-185.

Media and culture conditions. Medium PYGV contained
(1−1) 0.25 g each of Bacto peptone, Bacto yeast extract
and glucose, as well as 20 ml Hutton's basal salt solution
(HBM; Cohen-Bazire et al., 1957) and 10 ml Vitamin Solution no. 6
(Staley, 1968; Van Ert & Staley, 1971). Media were solidified
with 1.8% (w/v) bacteriological agar (Gibco). Where
needed, liquid and solid media were prepared to contain
final concentrations of 25 or 40%, (v/v) artificial seawater
(ASW; Lyman & Fleming, 1940). Before autoclaving,
medium PYGV containing ASW was adjusted to pH 8.5
the final pH after autoclaving was 7.2. R2A agar (Difco) plus
ASW was used to culture isolates for BIOLOG tests.

Water cultures for identification tests were incubated at
20 °C. Anaerobic cultivation in the light (at 4.0–21.5 μmol
photon m−2 s−1) occurred at 16 °C. Biomass for chem-
taxonomic studies was grown in aerated liquid PYGV+ASW
at 20 °C and harvested in the late exponential phase after 4–8 d.

Microscopy. Cell morphologies and aggregation behaviour
were examined by phase-contrast light microscopy of young,
liquid-grown cells on 2% water-agar-coated slides (Pfennig
et al., 1986). Cells for transmission electron microscopy
were washed with 0.5 M ammonium acetate, nega-
tively stained with 1% phosphotungstic acid (PTA) and
observed with a Philips EM 300 electron microscope at
80 kV.

For ultrathin sectioning, young cells were fixed with glutar-
alddehyde (3%) for 3 h. After washing in Sorensen buffer
(0.15 M, pH 7.5), post-fixation was carried out with OsO4

Table 1. Characteristics of the original 1989/1990 Ekho Lake samples from which the isolates were obtained and the
enrichment conditions

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Depth (m)</th>
<th>Salinity (%)</th>
<th>Temperature (°C)</th>
<th>pH</th>
<th>Enrichment conditions</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Medium</td>
</tr>
<tr>
<td>EL-171</td>
<td>6</td>
<td>63</td>
<td>12-9</td>
<td>8.30</td>
<td>PYGV*</td>
</tr>
<tr>
<td>EL-78</td>
<td>6</td>
<td>65</td>
<td>14-8</td>
<td>8.22</td>
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<tr>
<td>EL-164</td>
<td>9</td>
<td>72</td>
<td>14-6</td>
<td>8.16</td>
<td>50 ml sample + 25 mg YE†</td>
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<tr>
<td>EL-172</td>
<td>10</td>
<td>73</td>
<td>14-9</td>
<td>8.16</td>
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</tr>
<tr>
<td>EL-83</td>
<td>14</td>
<td>73</td>
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<td>75</td>
<td>16-0</td>
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<td>82</td>
<td>17-0</td>
<td>7.66</td>
<td>Sabouraud dextrose agar‡</td>
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<tr>
<td>EL-222</td>
<td>22</td>
<td>142</td>
<td>15-5</td>
<td>6.98</td>
<td>PYGV + 130% ASW</td>
</tr>
</tbody>
</table>

* Medium PYGV (Staley, 1968), prepared with Ekho Lake water of 72%o salinity.
† YE, Bacto yeast extract.
‡ Prepared with Ekho Lake water of 72%o salinity.

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(1%) in the same buffer for 3 h. Agar-embedded cells were then dehydrated by a graded series of ethanol, followed by embedding by ultratome (Spurr) and polymerization at 70°C for 8 h. Ultrathin sections were cut with a Reichert Ultratome S with a diamond knife. Sections were post-stained with uranyl acetate and lead citrate.

**Physiological and biochemical characterization.** All physiological tests were performed at 20°C. Gram-staining was done with 24 h cultures (Skerman, 1967). Catalase production was detected with 5% H2O2. Peroxidase and cytochrome oxidase activities were tested as previously described (Drews, 1974; Kreis & Schauer, 1987). DNA hydrolysis was indicated by clear zones around colonies on Bacto DNA Test Agar (Difco) plus ASW. Amylase activity and the relation to oxygen were studied as previously described (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. Osmotolerance was studied in PYGV prepared with 0, 10, 20, 100, 130, and 150% ASW after 14 d incubation. Since a concentration of 150% ASW did not easily dissolve, 20% NaCl was added to 130% ASW to reach the required salinity. NaCl tolerance was determined in PYGV with 0, 1-10, 60, 80, 100, 130, and 150% NaCl after 14 d incubation. The ability to grow between pH 5.3 and 9.0, and at temperatures between 30 and 43.5°C, was also tested in PYGV + ASW medium and evaluated by protein determination (Bradford, 1976) of triplicate cultures. The requirement(s) for the following vitamins were tested in six combinations, each lacking one vitamin of Vitamin Solution no. 6: biotin, thiamin hydrochloride, nicotinic acid, sodium pantothenate or vitamin B12. In these experiments casein hydrolysate (vitamin-free poly-P-hydroxybutyrate was followed in PHBA medium, versa. Susceptibility to antibiotics was determined with bioDiscs (bioMérieux) and the results were recorded after 3 weeks of incubation. In both cases, after 14 d and the anaerobic reduction of nitrate to nitrite or SO4- were tested with the sulfide indole motility medium (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++, Ca++, Cl- or SO4- were studied in PYGV + ASW where Na+ was exchanged with K+, Mg++ with Ca++, Cl- with SO4- and vice versa. Susceptibility to antibiotics was determined with bioDiscs (bioMérieux) and the results were recorded after 4 d. The aerobic reduction of nitrate to nitrite was tested after 14 d and the anaerobic reduction of nitrate to nitrite or N2 was assessed after 3 weeks of incubation. In both cases, PYGV + ASW containing 5 mM NaN3 was used. Anaerobic growth was examined in PYGV + ASW which was modified to contain (l-1) 0.1 g glucose, 0.1 g acetate, 0.1 g glutamate and 0.5 g Tris pure (Biomol). The medium was saturated with N2 for 20 min, and 20 ml was distributed in 100 ml serum bottles. To test for photolithoheterotrophic or photoorganotrophic growth, cultures were incubated under an atmosphere of H2/CO2 (80:20; 0.9 bar) and at light intensities of 40-215 µmol photons m-2 s-1. Production of poly-β-hydroxybutyrate was followed in PHBA medium, which was PYGV + ASW modified to contain (l-1) 20 ml HBM, 10 ml Vitamin Solution no. 6, 0.1 g Bacto yeast extract and 2.0 g sucinate as described previously (Smibert & Krieg, 1994). 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 were tested with the sulfide indole motility medium (Merck) plus ASW. Indole was detected with Kovács reagent.

To study the aerobic dissimilation of 95 carbon sources with the BIOLOG system, Microlog software, a Tandon computer and a microplate photometer ( Molecular Devices) were used. Standard suspensions were prepared from growth on R2A agar after 3-4 d at 20°C and test evaluation was after 4, 24 and 48 h; the results were reproducible. Additionally, C source utilization was studied in a minimal medium containing (l-1) 20 ml HBM, 10 ml Vitamin Solution no. 6, 0.02 g Bacto yeast extract and 0.25 g NH4NO3. The following C 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 bchl a was followed in suspensions of cells grown in PYGV + ASW and analysed as described (Shiba & Simidu, 1982). Methanolic extracts were also studied with a Beckman DU-600 spectrophotometer and by TLC as previously described (Lafay et al., 1995). Tests were performed with cells grown in constant light at 40-215 µmol photons m-2 s-1 or with cells grown in the dark.

**Chemotaxonomy.** Fatty acid methyl esters were analysed in 20 mg freeze-dried biomass and using methods which allowed selective hydrolysis of ester- and amide-linked fatty acids. Fatty acid methyl esters were analysed in two different ways. The first method was GC (GC-14A; Shimadzu) using a 0.2 mm x 25 m non-polar capillary column and flame-ionization detection. The running conditions were as follows: injector and detector port temperature, 300°C; inlet pressure, 80 kPa; split ratio, 50:1; injection volume, 1 µl; and a temperature programme 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 (Sasser, 1990). Saturated C16-C20 fatty acid methyl esters were used as standards. Fatty acid methyl esters that could not be identified on the basis of their retention times were analysed by GC-MS using a model GCMS-QP2000 instrument (Shimadzu) under previously described GC conditions (Groth et al., 1996). Respiratory lipoquinones and polar lipids were extracted from 100 mg freeze-dried material using the two-stage method and analysed as previously described (Tindall, 1990a, b). Diamino acids of cell walls were separated by one-dimensional TLC on cellulose plates using the solvent system of Rhuland et al. (1955).

**Determination of DNA G+C composition.** DNA G+C contents were analysed by HPLC (Mesbah et al., 1989). The HPLC equipment (Pharmacia-LKB) had a Spherisorb ODS II C18 column (5 µm; 4 x 250 mm; Bischoff). Escherichia coli strain B type VIII DNA (Sigma) and phage lambda 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 according to the manufacturer's instructions. DNA probes were prepared from EL-90, EL-164, EL-171, EL-172, EL-222 and A. heliothermus EL-219. Hybridization occurred against chromosomal DNA from the Ekh Lake strains, A. heliothermus and against R. denitrificans. The stringency of 70 and 75% 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 previously described (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+ (positions 1542-1522). The amplified product was purified with a QIAquick PCR Purification kit.
(Qiagen) and sequenced directly using primers to conserved regions of the rRNA. Sequencing was performed using a PRISM Tag DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 373A; Applied Biosystems). To establish the closest relatives of the eight Ekho Lake strains, preliminary searches in the EMBL database were performed with the FASTA program (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 PILEUP program (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 using the parameters of Jukes & Cantor (1969). A phylogenetic tree was constructed according to the neighbour-joining method (Saitou & Nei, 1987) with the NEIGHBOR program. For this analysis, the following additional taxa, listed by genera and accession number, were included: Rhodomicrobium vannielli (M34127), "Methyllosulfonomonas methylavora" (U62893), Rhodobium marinus (D30790), Rhodobium orientis (D30792), Rhodospirillum salexigens (M59070), Rhodopseudomonas eurythrix (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 mollischianum (M59067), Rhodospirillum salinarum (M59069), Rhodospirillum sodo-mense (M59072), Rhodopila globiformis (M59066), Rhodo- planes roseus (D25313), Blastochloris viridis (D25314), Rhdospseudomonas palustris (D25312) and Rhodospseudomonas acidophila (M34128). The stability of the groupings was estimated by bootstrap analysis (500 replications) using the programs of the PHYLIP package (Felsenstein, 1989).

RESULTS

Isolation of bacteria, morphology and motility

Six different enrichment procedures yielded pure cultures with similar morphology; eight of these were obtained from Ekho Lake samples from 6, 9, 10, 14, 16 and 22 m (Table 1). These isolates are referred to as EL-52, EL-78, EL-83, EL-90, EL-164, EL-171, EL-172T and EL-222. They were all Gram-negative rods with one or both cell poles pointed (Figs 1, 2b). Motility was observed in PHBA medium. Small daughter cells showed predominantly tumbling motion and only rarely a directed movement; flagella were not found. Cell growth appeared to be monopolar since one cell end was usually narrower and shorter, which indicated a budding process (Fig. 2a, b; Hirsch, 1974). All eight isolates contained electron-transparent poly-β-hydroxybutyrate granules. Their cell sizes varied (0.7–1.0 × 1.1–2.2 μm).

Culture and growth characteristics

Aerobic growth was visible after 3–5 d at 20 °C on medium PYGV + 25% (or 40%) ASW or on R2A agar with ASW. Colonies were circular, smooth, convex, 1–2 mm in diameter and red (EL-78, EL-172T), pink (EL-83), beige to light red (EL-90, EL-171), beige or whitish-beige (EL-52, EL-164, EL-222). Osmotolerance ranged from below 10 to over 33.5 °C and at pH values of 6.9 to > 9.0. The eight isolates had an absolute requirement for Na⁺; the other cations and anions tested could all be replaced as indicated in Methods. The EL isolates had a weak requirement for thiamin, nicotinic acid and possibly biotin. Pantothenate was not required and vitamin B₁₂ stimulated most isolates except for EL-164 and EL-222. Osmotolerance ranged from below 10 to over 150% ASW, with an optimum between 10 and 130% ASW. The NaCl tolerance was < 1.0 to 10.0%, with an optimum between 1.0 and 8.4% NaCl.

Physiological and biochemical characteristics

Phenotypic characteristics that differentiated EL-172T, a representative strain of this group, from A. helioter-mus, R. algicola, R. dentificrans, R. litoralis or from Sulfitobacter pontiacus (Sorokin, 1995) are listed in Table 2. All eight strains of the unknown bacterium exhibited peroxidase, catalase and a weak cytochrome oxidase activity. They did not produce acetoin or acids from glucose. They were susceptible to chloramphenicol (30 μg) and streptomycin (10 μg), but not to polymyxin B (300 U). Strains EL-164 and EL-222

![Fig. 1. Phase-contrast light micrograph of strain EL-172T on an agar-coated slide (Pfennig & Wagener, 1986). Bar, 10 μm.](image-url)

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Fig. 2. (a) Cell of EL-171 shows monopolar growth. Bar, 1 μm. (b) Electron micrograph of cells of strain EL-90 negatively stained with PTA. Bar, 2 μm.

Table 2. Comparison of isolate EL-172T with literature data for A. heliothermus EL-219T and the type strains of R. algicola, R. denitrificans, R. litoralis, Sulfitobacter pontiacus and Sagittula stellata

ND, Not determined; w, weak reaction.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>EL-172T</th>
<th>A. heliothermus</th>
<th>R. algicola</th>
<th>R. denitrificans</th>
<th>R. litoralis</th>
<th>Sulfitobacter pontiacus</th>
<th>Sagittula stellata</th>
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<tbody>
<tr>
<td>Cell shape: rods</td>
<td>+</td>
<td>+</td>
<td>Ovoid</td>
<td>Ovoid</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rosettes formed</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Colony colour</td>
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<td>Brown-beige</td>
<td>Red</td>
<td>Red</td>
<td>Colourless</td>
<td>Cream</td>
</tr>
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<td>Bchl a</td>
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<td>Temperature optimum (°C)</td>
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<td>16-20</td>
<td>25-30</td>
<td>20-30</td>
<td>20-30</td>
<td>22-25</td>
<td>30</td>
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<tr>
<td>NaCl optimum (%)</td>
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<td>20</td>
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<td>G+C content (mol%)</td>
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<td>64.9-65.0</td>
<td>59.1-60.1</td>
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<td>ND</td>
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<tr>
<td>Unknown phospholipid</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Not determined, but requires NaCl (Gonzalez et al., 1997).
† Positive in the present study.
were not susceptible to penicillin G (10 U), and EL-83, EL-90 and EL-222 were not susceptible to tetracycline (30 μg). The eight strains were not capable of assimilatory or dissimilatory nitrate reduction. Neither sulfide nor indole were produced. Only EL-52 hydrolysed Tween 80, and EL-83 had DNase activity. None of the strains hydrolysed gelatin or starch. In the presence of available nitrogen, they utilized succinate, butyrate, glutamate, acetate, pyruvate and malate, but not methanol, methanesulfonic acid, citrate or α-D-glucose. In the absence of other N sources, glutamate was utilized as a sole C and N source. Slight growth was observed with acetate, pyruvate, malate, succinate and possibly butyrate without any added nitrogen source.

Differences in the metabolism of carbon compounds as revealed with the BIOLOG system are shown in Table 3. The eight test strains, *A. heliothermus* and the *Roseobacter* species metabolized N-acetyl-D-galactosamine, N-acetylglucosamine, adonitol, D-arabitol, L-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, pionic acid, glucuronamide, L-alanyl-glycine and uridine. However, they did not metabolize α-cyclodextrin, dextrin, methyl β-D-glucoside, methyl pyruvate, monomethylsucinate, formic acid, D-galactonic acid lactone, D-galacturonic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, p-hydroxyphenylactic acid, α-ketobutyric acid, α-ketoglutaric acid, α-ketovaleric acid, D-lactic acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, D-alanine, L-alanine, glycyl-L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-tyrosine, L-carnitine, γ-aminobutyric acid, urocanic acid, phenylethylamine, putrescine, 2-aminoethanol, glycerol, glucose-6-phosphate or DL-α-glycerophosphate. They did not grow anaerobically and photolithoautotrophically with H₂/CO₂ (80:20) in the gas phase, and they did not grow photoorganotrophically. Strain EL-222 grew microaerophilically.

Bchl a was found in cell suspensions or methanolic extracts of EL-78, EL-83, EL-171 and EL-172 when grown in the dark. Under identical conditions, it was not detected in suspensions or methanolic extracts of EL-52, EL-90, EL-164, or EL-222. Even concentrated extracts of these strains did not give any indication of

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**Table 3. Differences in the utilization of carbon sources of strain EL-172 to the type strains of A. heliothermus, R. algicola, R. denitrificans and R. litoralis as detected with the BIOLOG system in the present study**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>EL-172</th>
<th>A. heliothermus</th>
<th>R. algicola</th>
<th>R. denitrificans</th>
<th>R. litoralis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogen</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tween 40</td>
<td>v</td>
<td>-</td>
<td>+</td>
<td>v</td>
<td>+</td>
</tr>
<tr>
<td>Tween 80</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>v</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>v</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Psicose</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Xylitol</td>
<td>-</td>
<td>v</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>cis-Aconitic acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>v</td>
<td>+</td>
</tr>
<tr>
<td>D-Glucoronic acid</td>
<td>-</td>
<td>-</td>
<td>v</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-Glycolic acid</td>
<td>v</td>
<td>-</td>
<td>v</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-Glucuronic acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Malonic acid</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Quinic acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-Saccharic acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alanine amide</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>v</td>
<td>+</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>v</td>
<td>-</td>
<td>+</td>
<td>v</td>
<td>+</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>v</td>
<td>+</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glycyl-L-aspartic acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-Serine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Serine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Inosine</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Thymidine</td>
<td>v</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2,3-Butanediol</td>
<td>-</td>
<td>-</td>
<td>v</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Glucose-1-phosphate</td>
<td>-</td>
<td>-</td>
<td>v</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
bchl a when TLC was applied. Absorbances that were characteristic of the presence of bchl a were found in vivo with a large peak at 877–879 nm and smaller peaks at 799–802 and 589–591 nm (Fig. 3). They were similar to the maxima found in bchl a-containing anoxygenic phototrophs (Biebl & Drews, 1969), but differed from the maxima of the bchl a-containing R. denitrificans (Fig. 3) or R. litoralis (Shiba, 1991). Other features, which are very likely due to the presence of carotenoids, were not characterized further. The absorption spectra of methanolic extracts showed a large peak at 767–769 nm and smaller ones at 605–607 and 699–700 nm and around 350 nm. Unlike the situation in R. denitrificans, the production of bchl a by EL strains was repressed in constant dim light.

The colony colours of strains EL-83 and EL-171 were white-beige to beige during the first 6 years of cultivation, indicating that these strains did not produce bchl during this period. After this time, the colony colours of EL-83 became pinkish, and thereafter, this strain produced bchl a with a large peak at 878 nm, as is the situation in EL-78 and EL-172T. Methanolic extracts of all four bchl-producing EL strains had identical absorption spectra.

Unlike R. denitrificans (Harashima et al., 1982), vesicular structures of intracytoplasmic membrane systems were never found in ultrathin sections of aerobically and dark grown cells of EL-172T.

The DNA G+C base ratios were 62·2–63·8 mol%.

### Chemotaxonomic characteristics

The peptidoglycan of all eight isolates contained m-diaminopimelic acid. The ubiquinone was Q-10. Polar lipids found in all cases were phosphatidylglycerol, phosphatidylcholine, diphosphatidylglycerol and phosphatidylethanolamine. Additionally, cells contained an unidentified aminolipid as well as one phospholipid. The fatty acid composition of the strains grown on PYGV + ASW is shown in Table 4. Their fatty acid profile was characterized by the predominance of C18:1 and by the presence, in lower proportions, of the double-unsaturated fatty acid C18:2 and of C12:0 2-OH and C12:1 3-OH.

### Dot-blot hybridizations

DNA probes from EL-90, EL-164, EL-171, EL-172T or EL-222 hybridized at 75% stringency with chromosomal DNA from the other seven Ekho Lake strains but not with DNA of the four strains of A. heliothermus, or with R. denitrificans strain DSM 7001T. DNA probes from A. heliothermus did not hybridize with chromosomal DNA from any of the eight Ekho Lake strains discussed here.

### 16S rRNA sequence determinations and phylogenetic analyses

Partial 16S rRNA gene sequences of the eight Ekho Lake isolates were determined by direct sequence analysis of PCR amplified products. Based on a

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**Table 4. Cellular fatty acid composition of EL-172T, A. heliothermus EL-219T, and the type strains of R. algicola, R. denitrificans and R. litoralis**

<table>
<thead>
<tr>
<th>Organism</th>
<th>C10:0 3-OH</th>
<th>C12:0 2-OH</th>
<th>C12:1 3-OH</th>
<th>C14:0 2-OH</th>
<th>C16:1</th>
<th>C16:0</th>
<th>C18:2</th>
<th>C18:1</th>
<th>C18:0</th>
<th>C19:0 cy</th>
</tr>
</thead>
<tbody>
<tr>
<td>EL-172T†</td>
<td>--</td>
<td>2.4</td>
<td>3.6</td>
<td>--</td>
<td>0.8</td>
<td>6.2</td>
<td>10.6</td>
<td>70.2</td>
<td>0.8</td>
<td>--</td>
</tr>
<tr>
<td>A. heliothermus†</td>
<td>--</td>
<td>--</td>
<td>3.1</td>
<td>--</td>
<td>0.8</td>
<td>2.5</td>
<td>--</td>
<td>83.2</td>
<td>1.0</td>
<td>2.4</td>
</tr>
<tr>
<td>R. algicola§</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>1.6</td>
<td>1.6</td>
<td>84.6</td>
<td>1.2</td>
<td>--</td>
</tr>
<tr>
<td>R. denitrificans†</td>
<td>4.2</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>14</td>
<td>1.6</td>
<td>7.1</td>
<td>84.6</td>
<td>1.2</td>
<td>--</td>
</tr>
<tr>
<td>R. denitrificans§</td>
<td>--</td>
<td>1.8</td>
<td>--</td>
<td>2.9</td>
<td>--</td>
<td>2.2</td>
<td>--</td>
<td>87.7</td>
<td>2.3</td>
<td>--</td>
</tr>
<tr>
<td>R. litoralis§</td>
<td>1.9</td>
<td>--</td>
<td>3.9</td>
<td>--</td>
<td>1.1</td>
<td>1.4</td>
<td>88.8</td>
<td>1.3</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

* Values less than 1% not shown. OH, Hydroxyl; cy, cyclopropane.
† Cells grown on PYGV + ASW.
‡ Sometimes present at around 1%.
§ Cells grown on Bacto Marine broth (Difco).
Apart from differences in the sample salinity (63–142‰), the properties of these original samples were rather similar (Table 1). It is, therefore, possible that this new species, represented by the eight isolates, is widely distributed in different layers of the lake. Chromosomal DNA of all eight isolates hybridized with DNA probes of strains EL-90, EL-164, EL-171, EL-172 and EL-222 (at 75% stringency), and sequencing of 16S rRNA genes confirmed that the isolates were genetically very similar and that they were all members of a distinct taxon within the α-3-subclass of the Proteobacteria. The eight isolates displayed very similar morphologies, but there were some minor physiological differences. All strains tolerated wide ranges of temperature, ASW, NaCl and pH. Four of the eight strains produced bchl \(\alpha\). Unlike \(R.\) denitrificans, bchl \(\alpha\) production was repressed by constant dim light; \textit{in vivo} characteristic absorption bands were measured at 799–802 and 877–879 nm.

Production of bchl \(\alpha\) by two strains which had been bchl \(\alpha\)-free during most of the cultivation time was observed. Possibly the bchl \(\alpha\) genes of these EL strains were silent, but conditions for their induction were not detected. These results indicated likewise that \(E.\) ursincola and \(B.\) natatoria (see Introduction) could be members of at least the same genus. The data presented here show that the presence of bchl \(\alpha\) production may not be a very significant characteristic for taxonomic purposes.

The eight EL strains were nearly identical based on the results of the chemotaxonomic tests used. Taken together, the combination of respiratory lipoquinones, fatty acids and polar lipid data indicated that these strains belonged to a group of organisms (at the genus or family rank) within the \(\alpha\)-subclass of the Proteobacteria (Labrenz et al., 1998).

### DISCUSSION

The six different enrichment procedures used for Ekho Lake samples yielded eight nearly identical bacterial isolates, although these came from depths of 6–22 m. Apart from differences in the sample salinity (63–142‰), the properties of these original samples were rather similar (Table 1). It is, therefore, possible that...
C<sub>18:1</sub> is the characteristic fatty acid within the α-subclass of the Proteobacteria and C<sub>18:2</sub>, in most cases, was characteristic of the eight isolates and Roseobacter spp. The strains were differentiated by fatty acids present in lower proportions, such as C<sub>12:0</sub> 2-OH and C<sub>12:1</sub> 3-OH for the eight isolates, or C<sub>10:0</sub> 3-OH and C<sub>14:0</sub> 2-OH for R. denitrificans and R. litoralis. When grown on Bacto Marine broth, R. algicola did not contain any of the characteristic fatty acids observed at lower proportions in R. denitrificans and R. litoralis. However, the polar lipid and the utilization patterns of carbon sources of the eight strains and R. algicola (as detected in the BIOLOG system; Table 3) were nearly identical; these patterns were quite different from those of R. denitrificans, R. litoralis or A. heliothermus. In addition, the G+C content of R. algicola (64.0–65.0 mol%) was more similar to that of the eight strains (62.2–63.8 mol%) than to that of the other Roseobacter species (56.3–60.1 mol%). Comparative 16S rRNA gene sequencing, chemotaxonomic, biochemical and physiological studies clearly show the close relationship of R. denitrificans and R. litoralis. On the other hand, R. algicola appears to be more related to, albeit different from, the eight isolates, as shown by physiological (Table 2) and fatty acid data (Table 4).

Comparative 16S rRNA gene sequencing showed that the eight bacteria from Ekho Lake, as exemplified by EL-172<sup>T</sup>, were members of the α-subclass of Proteobacteria and were specifically associated with the Roseobacter cluster of organisms which also included Antarcctobacter and Marinosulfonomonas (Holmes et al., 1997), the Prionitis gall symbiont, Octadecabacter (Gosink et al., 1997), Roseobacter, Sagittula (Gonzalez et al., 1997), Sulfitobacter (Sorokin, 1995), and an uncharacterized Sargasso Sea isolate. Sequence divergence values of >5% (4-7%) to R. algicola showed that the eight isolates were phylogenetically distinct from all currently recognized members of the Proteobacteria. Furthermore, bootstrap resampling showed the organisms from Ekho Lake did not possess a particularly significant phylogenetic affinity with any individual species within the above-mentioned Roseobacter cluster (Fig. 4). It is evident from the treering analyses that the genus Roseobacter, as currently recognized, is interdispersed with several other taxa. Based on the chemotaxonomic and biochemical data presented here it is concluded that, to fulfill the criteria from all currently recognized members of the genus Roseobacter should be restricted to the species R. litoralis and R. denitrificans. However, R. algicola should be a member of a new and different taxon, to be described in the future. On the basis of the genotypic (16S rDNA sequence) and phenotypic (biochemical, chemotaxonomic, physiological) data presented here (Table 2), the eight Antarctic strains are distinct, not only from the more distantly related species (i.e. R. litoralis, R. denitrificans), but they can also be distinguished from more closely related species (i.e. R. algicola). Thus, it is concluded that the eight Ekho Lake isolates are representatives of a new and distinctive taxon, for which the name Roseovarius tolerans gen. nov., sp. nov. is proposed.

**Description of Roseovarius gen. nov.**

Roseovarius (Ro.se.o.va’ri.us. L. adj. roseus rose-coloured; L. adj. varius diverse, varied; M.L. masc. n Roseovarius the varying rose-coloured one).

Gram-negative rods with one or both cell poles pointed, multiply by monopolar growth, i.e. by a budding process. Daughter cells may be motile. The cells may contain poly-β-hydroxybutyrate. They do not form spores. On PYGV+ASW or R2A+ASW media, colonies are smooth, convex and variously coloured. Bchl <i>a</i> may be produced. The temperature range for growth is <3 to 43.5 °C. The cells have an absolute requirement for Na<sup>+</sup>; they grow in <1 to 100% NaCl. In the presence of ASW, they grow at <10 to >150%. The pH tolerance range is 5.3 to >9. Strictly aerobic, non-fermentative heterotrophs. No growth is observed on glucose anaerobically in the absence of nitrate. Cells do not grow photoautotrophically with H<sub>2</sub>/CO<sub>2</sub> (80:20) or photoorganotrophically with acetate or glutamate. The cells exhibit peroxidase, catalase and weak cytochrome oxidase activity. The following polar lipids are present: diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, as well as an unknown phospholipid and an aminolipid. The dominant fatty acid is C<sub>18:1ω7</sub>; other characteristic fatty acids are C<sub>18:2ω6</sub>, C<sub>12:0</sub> 2-OH, C<sub>12:1ω9</sub> 3-OH, C<sub>16:1ω7</sub>, and C<sub>18:0ω6</sub>. The major respiratory quinone is Q-10. The strains were originally obtained from water samples from Ekho Lake, Antarctica (Vestfold Hills). The type species for the genus is Roseovarius tolerans.

**Roseovarius tolerans sp. nov.**

Roseovarius tolerans (tol‘er.ans. L. part. adj. tolerans bearing/tolerating, as the bacterium endures stressful conditions).

Cell sizes vary (0.7–1.0 × 1.1–2.2 μm), with a mean size of 0.74–0.83 × 1.34–1.94 μm. On PYGV+ASW or R2A+ASW media, colonies of individual strains are smooth and convex and red, pinkish, beige to red, beige or whitish-beige. Optimal growth occurs at 8–33.5 °C with salt concentrations of 10–80% NaCl or 10–130% ASW. The optimum pH is 5–9 to 9–10. Bchl <i>a</i> may be produced with a large in <i>vivo</i> absorption band at 877–879 nm and smaller ones at 589–591 and 799–802 nm. The production of bchl <i>a</i> is repressed by constant dim light. The isolates have a weak requirement for thiamin and nicotinic acid. The requirement for vitamin B<sub>12</sub> is variable, and cells are
stimulated by biotin but do not need pantothenate. Alginate, gelatin and starch are not hydrolysed, Tween 80 and DNA are only hydrolysed by some strains. Growth occurs on acetate, pyruvate, malate, succinate, butyrate or glutamate, but not on citrate, methanesulfonic acid, methanol or α-d-glucose. In the absence of any added nitrogen compounds, the cells grow slightly on acetate, pyruvate, succinate, malate and butyrate. Glutamate is used with and without an additional source of combined nitrogen. Cells are susceptible to chloramphenicol, streptomycin, penicillin G and tetracycline, but not to polymyxin B. Growth occurs on acetate, pyruvate, malate, succinate, butyrate or glutamate, but not on citrate, methanesulfonic acid, methanol or α-D-glucose. In the absence under accession number DSM 11457\textsuperscript{T}. Additional isolates of Roseovarius tolerans are EL-222 (= DSM 11463), EL-52, EL-78, EL-83, EL-90, EL-164 and EL-171.

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**REFERENCES**


