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 C₁₈:₁; other characteristic fatty acids were C₁₈:₂, C₁₂:₀ 2-OH, C₁₂:₁ 3-OH, C₁₆:₀ and C₁₈:₀. The main polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol and phosphatidyldicholine. 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

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bacterial numbers and c.f.u. were studied there during two Antarctic summers (P. Hirsch, J. Siebert & H. R. Burton, unpublished results).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 producers (praseophytes with four flagella) were obtained in pure culture.

Obliquely aerobic bchl a-containing bacteria have been found in the genera Roseobacter (Shiba, 1991), Roseococcus (Yukrov et al., 1994), Erythrobacter (Shiba & Simidu, 1982), Erythromonas (Yukrov et al., 1997), Sandaracinobacter (Yukrov 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 requirements. The relationship of bchl a-containing organisms (both anoxic and aerobic) with organisms which do not contain bchl a has led to speculations about the evolutionary origin of the class of Proteobacteria [which include purple non-sulfur bacteria (Woese, 1987)] together with differing interpretations of the role of bchl a as a taxonomic marker. In the genus Roseobacter, for example, both Roseobacter denitrificans and Roseobacter litoralis (Shiba, 1991) produce bchl a, whereas Roseobacter algicola (Lafay et al., 1995) does not. Yukrov et al. (1997) considered the presence of bchl a to be of such importance that, despite practically identical 16S rDNA sequence similarity (99.8%), they considered Erythromonas ursincola and Blastomonas natatoria as members of different genera.

The present publication describes eight closely related bacterial isolates from Ekho Lake which can produce bchl a under strictly aerobic conditions and represent a new genus and species.

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 (l-1) 0.25 g each of Bacto peptone, Bacto yeast extract and glucose, as well as 20 ml Hutner'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 (Difco). 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. Bacterial cultures for identification tests were incubated at 20 °C. Anaerobic cultivation in the light (at 4.0-21-5 pmol photons m-2 s-1) occurred at 16 °C. Biomass for chemotaxonomic 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 & Wagener, 1986). Cells for transmission electron microscopy were washed with 0.5 M ammonium acetate, negatively 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 glutaraldehyde (3%) for 3 h. After washing in Sörensen buffer (0.15 M, pH 7.5), post-fixation was carried out with OsO4.
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; Kreisel & Schauer, 1987). DNA hydrolysis was indicated by clear zones around colonies on Bacto DNase 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 prepared with 0, 1, 2, 3, 4, 6, 8, 10, 12 and 15% NaCl after 14 d incubation. The ability to grow between pH 5.5 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, intensities of 4.0–21.5 pmol photons m–2 s–1). Production of chl 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 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 × 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-C40 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 × 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. heliottermus EL-219. Hybridization occurred against chromosomal DNA from the Eglo Lake strains, A. heliottermus 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 16S (positions 8–28, E. coli numbering) and pH1 (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.

Phylogenetic trees were 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: Rhodobacter sphaeroides (D 16418), Rhodobacter blasticus (D16425), Rhodospirillum rubrum (D 30778), Rhodospirillum socaldi (D30779), Rhodospirillum salinarum (M59067), Rhodospirillum molischianum (M59069), Rhodospirillum salineticum MN (M59072), Rhodospirillum euryhalinum (U55277), Rhodovulum adriaticum (D 16418), Rhodobacter capsulatus (M59069), Rhodobacter veldkampii (D16421), Rhodospirillum rubrum (D 30778), Rhodospirillum photometricum (D30777), Rhodospirillum fulvum (D14433), Rhodospirillum molischianum (M59067), Rhodospirillum salinarum (M59069), Rhodospirillum sdomense (M59072), Rhodopila globiformis (M59066), Rhodopseudomonas palustris (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 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-172 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 predominately 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 \times 1.1-2.2 \mu m]\.

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-172), pink (EL-83), beige to light red (EL-90, EL-171), beige or whitish-beige (EL-52, EL-164, EL-222). The osmotic range for growth was below 3 and below 43.5 °C. Optimal growth occurred between 8.5 and 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 8.0% NaCl.

Physiological and biochemical characteristics

Phenotypic characteristics that differentiated EL-172, a representative strain of this group, from A. heliothermus, R. algicola, R. dentriticins, R. littoralis 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...
Roseovarius gen. nov.

**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><em>A. heliothermus</em></th>
<th><em>R. algicola</em></th>
<th><em>R. denitrificans</em></th>
<th><em>R. litoralis</em></th>
<th><em>Sulfitobacter pontiacus</em></th>
<th><em>Sagittula stellata</em></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>
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<td>Rosettes formed</td>
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<tr>
<td>Colony colour</td>
<td>Red</td>
<td>Brown-yellow</td>
<td>Brown-beige</td>
<td>Red</td>
<td>Red</td>
<td>Colourless</td>
<td>Cream</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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<td>NaCl optimum (%)</td>
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<td>20</td>
<td>ND</td>
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<td>pH optimum</td>
<td>6.2-90</td>
<td>70-80</td>
<td>7.5</td>
<td>70-80</td>
<td>70-80</td>
<td>7-3-7.5</td>
<td>7.5</td>
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<td>Indole production</td>
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<tr>
<td>Nicotine acid requirement</td>
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<td>+</td>
<td>+</td>
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<td>ND</td>
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<td>Vitamin B₆ requirement</td>
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<td>-</td>
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<td>C₆₀ fatty acid</td>
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<td>G+C content (mol%)</td>
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<td>62.6-62.8</td>
<td>64.0-65.0</td>
<td>59.1-60.1</td>
<td>58.3-58.1</td>
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<td>Diposphatidylglycerol</td>
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<td>+</td>
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<tr>
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<td>+</td>
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<td>Unknown phospholipid</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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</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, D-erythritol, D-fructose, D-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, pro-
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 \textit{R. denitrificans} (Fig. 3) or \textit{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 \textit{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-172$^T$. Methanolic extracts of all four bchl-producing EL strains had identical absorption spectra.

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

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, phosphatidylinositol, diphasphatidylglycerol 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 C\textsubscript{18:1} and by the presence, in lower proportions, of the double-unsaturated fatty acid C\textsubscript{18:2}$\omega$ and of C\textsubscript{12:0} 2-OH and C\textsubscript{12:1} 3-OH.

### Dot-blot hybridizations

DNA probes from EL-90, EL-164, EL-171, EL-172$^T$ 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 \textit{A. heliothermus}, or with \textit{R. denitrificans} strain DSM 7001$^T$. DNA probes from \textit{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
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-172T 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 a. Unlike \( R. \) denitrificans, bchl a production was repressed by constant dim light; \textit{in vivo} characteristic absorption bands were measured at 799–802 and 877–879 nm.

**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 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-172T 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 a. Unlike \( R. \) denitrificans, bchl a 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 a by two strains which had been bchl a-free during most of the cultivation time was observed. Possibly the bchl a genes of these EL strains were silent, but conditions for their induction were not detected. These results indicated likewise that \textit{E. ursincola} and \textit{B. natatoria} (see Introduction) could be members of at least the same genus. The data presented here show that the presence of bchl a 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 α-subclass of the Proteobacteria (Labrenz et al., 1998).

**Fig. 4.** Unrooted tree showing phylogenetic relationships of strain EL-172T with \( R. \) algicola, \( A. \) heliothermus and closely related Proteobacteria. Additional sequences (listed in Methods) were used to perform the phylogenetic analyses. The tree was constructed using the neighbour-joining method \((K_{\text{neu}}\text{ values})\). It was based on a comparison of 1320 nucleotides. Bootstrap values, expressed as a percentage of 500 replications, are given at branching points; those below 90% are not shown.
C\textsubscript{18:3} is the characteristic fatty acid within the \(\alpha\)-subclass of the Proteobacteria and C\textsubscript{18:2}, 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\textsubscript{12:0} 2-OH and C\textsubscript{12:1} 3-OH for the eight isolates, or C\textsubscript{10:0} 3-OH and C\textsubscript{14:0} 2-OH for \textit{R. denitrificans} and \textit{R. litoralis}. When grown on Bacto Marine broth, \textit{R. algicola} did not contain any of the characteristic fatty acids observed at lower proportions in \textit{R. denitrificans} and \textit{R. litoralis}. However, the polar lipid and the utilization patterns of carbon sources of the eight strains and \textit{R. algicola} (as detected in the BIOLOG system; Table 3) were nearly identical; these patterns were quite different from those of \textit{R. denitrificans}, \textit{R. litoralis} or \textit{A. heliothermus}. In addition, the G+C content of \textit{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 \textit{R. denitrificans} and \textit{R. litoralis}. On the other hand, \textit{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\textsuperscript{3}, were members of the \(\alpha\)-subclass of Proteobacteria and were specifically associated with the Roseobacter cluster of organisms which also included Antarctobacter and 'Marinosulfonomonas' (Holmes et al., 1997), the Prionitis gall sambiont, 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 \textit{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 treeing 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 of being a monophyletic group, the genus 

\textit{Roseobacter} should be restricted to the species \textit{R. litoralis} and \textit{R. denitrificans}. However, \textit{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 distinctive, not only from the more distantly related species (i.e. \textit{R. litoralis}, \textit{R. denitrificans}), but they can also be distinguished from more closely related species (i.e. \textit{R. algicola}). Thus, it is concluded that the eight Ekho Lake isolates are representatives of a new and distinctive taxon, for which the name \textit{Roseovarius tolerans} gen. nov., sp. nov. is proposed.

### Description of \textit{Roseovarius tolerans} gen. nov.

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

Gram-negative rods with one or both cell poles pointed, multiplying by monopolar growth, i.e. by a budding process. Daughter cells may be motile. The cells may contain poly-\(\beta\)-hydroxybutyrate. They do not form spores. On PYGV + ASW or R2A + ASW media, colonies are smooth, convex and variously coloured. Bchl \(a\) may be produced. The temperature range for growth is \(< 3\) to 43.5\(^\circ\)C. The cells have an absolute requirement for Na\(^+\); 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 7.9. Strictly aerobic, non-fermentative heterotrophs. No growth is observed on glucose anaerobically in the absence of nitrate. Cells do not grow photoautotrophically with H\(_2\)/CO\(_2\) (80:20) or photoorganotrophically with acetate or glutamate. The cells exhibit peroxidase, catalase and weak cytochrome oxidase activity. The following polar lipids are present: diphasphatidylglycerol, phosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, as well as an unknown phospholipid and an aminolipid. The dominant fatty acid is C\textsubscript{16:1} \(\omega\)-9; other characteristic fatty acids are C\textsubscript{18:2}, C\textsubscript{12:0} 2-OH, C\textsubscript{12:1} 3-OH, C\textsubscript{16:1} \(\omega\)-9 and C\textsubscript{18:0}. 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 \textit{Roseovarius tolerans}.

### \textit{Roseovarius tolerans} sp. nov.

\textit{Roseovarius tolerans} (tol’e.rans. L. part. adj. tolerans bearing/tolerating, as the bacterium endures stress conditions).

Cell sizes vary (0.7–1.0 x 1.1–2.2 \(\mu\)m), with a mean size of 0.74–0.83 x 1.34–1.94 \(\mu\)m. 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\(^\circ\)C with salt concentrations of 1.0–8.0\% NaCl or 10–130\% ASW. The optimum pH is 5.9–9. Bchl \(a\) may be produced with a large in \textit{vitro} absorption band at 877–879 nm and smaller ones at 589–591 and 799–802 nm. The production of bchl \(a\) is repressed by constant dim light. The isolates have a weak requirement for thiamin and nicotinic acid. The requirement for vitamin B\(_{12}\) is variable, and cells are
stimulated by biotin but do not need pantotenate. 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. Nitrate is not reduced, H₂S and indole are not produced. The G+C content is 62.2–63.8 mol%. Chemotaxonomic properties and other characteristics are as for the genus. The type strain, *Roseovarius tolerans* EL-172ᵀ, has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen under accession number DSM 11457ᵀ. Additional isolates of *Roseovarius tolerans* are EL-222 (= DSM 11463), EL-52, EL-78, EL-83, EL-90, EL-164 and EL-171.

ACKNOWLEDGEMENTS

We gratefully acknowledge the skilful technical assistance of B. Hoffmann, M. Beebe, R. Emcke and J. Buschdorf. J. Siebert (Hamburg) helped with the Antarctic field work and H. Völker (Kiel) with electron microscopy. We especially want to thank the Australian Antarctic Division (Kingston, Tasmania) for supporting two summer visits to Davis Station and the Vestfold Hills (Antarctica). H. R. Burton (Australian Antarctic Division) and Professor T. A. McMeekin (Univ. of Tasmania, Hobart) supported this research with much practical help and generous hospitality. We thank the Deutsche Forschungsgemeinschaft (DFG) for grants Hi 68/16-3, Hi 68/19-3 and Hi 68/25-1 and 25-2, and the European Union for grants CT 93-0194 and CT 93-0119.

REFERENCES


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