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## Isolation and characterization of *Erythrobacter* sp. strains from the upper ocean

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**Abstract** Seven strains of marine aerobic anoxygenic phototrophs belonging to the genus *Erythrobacter* were isolated. The strains were characterized regarding their physiological and biochemical properties, 16S rDNA and *pufM* gene sequences, morphological features, substrate preference, as well as pigment and lipid composition. All strains had functional type-2 reaction centers containing bacteriochlorophyll, served by small, light-harvesting complex 1, and were photosynthetically competent. In addition, large pools of carotenoids were found, but only some of the accessory pigments transfer energy to the reaction centers. All of the isolates were facultative photoheterotrophs. They required an organic carbon substrate for growth; however, they are able to supplement a significant fraction of their

metabolic requirements with photosynthetically derived energy.

**Keywords** Aerobic anoxygenic phototrophs · Aerobic photosynthetic bacteria · Bacteriochlorophyll *a* · *Erythrobacter* · Photoheterotrophy

**Abbreviations** *BChl* Bacteriochlorophyll · *Chl* Chlorophyll · *Erb.* *Erythrobacter* · *Erm.* *Erythromicrobium* · *FAMEs* Fatty acid methyl esters · *IRFRR* Infrared fast repetition rate · *LH1*, *LH2* Light-harvesting complex 1 and 2, respectively · *Por.* *Porphyrobacter* · *PUFAs* Polyunsaturated fatty acids · *Rsb.* *Roseobacter* · *RubisCO* Ribulose-1,5-bisphosphate carboxylase/oxygenase ·  $\mu$  Growth rate ·  $\sigma_{470}$  Functional cross-section of the photosynthetic unit at 470 nm

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### Introduction

Photosynthetic organisms play a crucial role in the marine environment. Chlorophyll-containing eukaryotic and prokaryotic phytoplankton utilize light as their main energy source, evolve oxygen, and convert inorganic CO<sub>2</sub> into organic molecules. In addition to these dominant, oxygenic organisms, there exists a broad spectrum of anoxygenic photosynthetic bacteria that contain bacteriochlorophyll (BChl). These organisms evolved approximately 3 billion years ago (Des Marais 2000), long before the oxidation of Earth's atmosphere (Rye and Holland 1998). Most of these photosynthetic bacteria cannot sustain photoautotrophic metabolism under oxic conditions, although some can grow heterotrophically. In the present, oxygen-rich atmosphere, these organisms are confined to marginal ecological niches, such as anoxic zones of lakes, marine and freshwater sediments, and sulfide springs.

In the late 1970s, a new kind of BChl-*a*-containing bacteria was discovered in Tokyo Bay, Japan (Harashima et al. 1978; Shiba et al. 1979). In contrast to their close relatives, purple non-sulfur photosynthetic bacteria, these bacteria were obligate aerobes requiring oxygen for growth

(Shimada 1995; Yurkov and Beatty 1998; Imhoff 2001). Two of the first isolates (OCh 101 and OCh 114), later classified as *Erythrobacter* (*Erb.*) *longus* (Shiba and Shimidu 1982) and *Roseobacter* (*Rsb.*) *denitrificans* (Shiba 1991), established two major genera of marine photoheterotrophs. Subsequently, many strains of those bacteria were isolated from nutrient-rich coastal waters (Nishimura et al. 1994; Shiba et al. 1991; Yurkov et al. 1994). At present, all the reported marine isolates belong to the  $\alpha 3$  and  $\alpha 4$  *Proteobacteria*, forming four genera: *Erythrobacter* (Shiba and Shimidu 1982), *Roseobacter* (Shiba 1991), "*Citromicrobium*" (Yurkov et al. 1999), and *Roseibium* (Suzuki et al. 2000).

Until recently, aerobic anoxygenic photoheterotrophs were thought to occupy small, nutrient-rich ecological niches, and to be restricted to mostly heterotrophic metabolism. The discovery of a high level of bacterial phototrophy in the open upper ocean (Kolber et al. 2000) challenged this notion. Based on kinetic fluorescence measurements, BChl-*a*-containing bacteria were suggested to contribute 2–5% of the total photosynthetic electron transport fluxes in the upper ocean (Kolber et al. 2000). Microscopy studies indicated that they account for up to 8% of the total bacterial community in the open ocean (Kolber et al. 2001). *Erythrobacter* strains are frequently found in cultivated material (Shiba et al. 1991), and it was suggested that they might form a major fraction of marine photoheterotrophs (Kolber et al. 2001). However, this view is contradicted by a recent molecular study of oceanic bacterial communities, which showed that marine photoheterotrophs are much more diverse (Béjā et al. 2002). This is consistent with our recent culture studies in which, apart from *Erythrobacter* sp., also *Roseobacter*-like and "*Citromicrobium*"-like isolates have been found (Kobližek, unpublished data).

Little is known about the metabolism, physiology, and nutritional requirements of aerobic anoxygenic photoheterotrophs. Oceanic species appear to utilize dissolved organic matter as a source of organic carbon, and light energy to supplement heterotrophic metabolism. Their ability to fix CO<sub>2</sub> is controversial (Shimada 1995; Yurkov and Beatty 1998). To partially fill this knowledge gap, we characterized several marine isolates belonging to the genus *Erythrobacter* regarding their pigment and lipid composition, morphology, substrate preferences, vitamin requirements, antibiotic sensitivity, 16S rDNA and *pufM* sequences, spectroscopic properties, and photosynthetic ability.

## Material and methods

### Strain isolation

About 5  $\mu$ l of seawater was streaked onto 1.5% bacteriological agar plates supplemented with *f/2* media (Guillard and Ryther 1962). Plates were stored for 7 days in the dark to eliminate oxygenic photoautotrophs, and then exposed to a natural light/dark cycle, resulting in the production of small, pigmented colonies within 2 weeks. Under these conditions the plating yields ranged between 1 and 10 colonies per 1,000 cells applied. The type strains, *Erb. longus* (ATCC 33941) and *Erb. litoralis* (ATCC 700002), formed colonies under

these conditions. The colonies were resuspended in sterile *f/2* seawater medium [natural seawater enriched with  $9 \times 10^{-4}$  M NaNO<sub>3</sub>,  $3 \times 10^{-5}$  M NaH<sub>2</sub>PO<sub>4</sub>, trace metals ( $10^{-5}$  M sodium iron (III) ethylenediamine tetra-acetate,  $4 \times 10^{-8}$  M CuSO<sub>4</sub>,  $8 \times 10^{-8}$  M ZnSO<sub>4</sub>,  $4 \times 10^{-8}$  M CoCl<sub>2</sub>,  $9 \times 10^{-7}$  M MnCl<sub>2</sub>,  $3 \times 10^{-8}$  M Na<sub>2</sub>MoO<sub>4</sub>) and vitamins ( $2 \times 10^{-9}$  M biotin,  $3.7 \times 10^{-10}$  M B<sub>12</sub>)], and tested for the presence of BChl *a* using an infra-red fast repetition rate (IRFRR) fluorometer (Kolber et al. 1998). Samples exhibiting the BChl *a* fluorescence transient, indicative of bacterial photosynthetic electron transport, were repeatedly plated onto *f/2* agar plates until uniform orange or yellow colonies were obtained. The isolates were then grown in organic medium composed of *f/2* medium supplemented with 0.5 g peptone and 0.1 g yeast extract per liter.

The isolates could be maintained in liquid or on agar media at 4 °C for several months. For long-term storage, the cells were resuspended in 30% glycerol containing *f/2* medium, frozen in liquid nitrogen, and stored at –75 °C.

### Strain characterization

Whole-cell absorption spectra were recorded on an Aminco DW2000 spectrophotometer operated in the dual beam mode. The cells were resuspended in 70% glycerol in *f/2* medium. The spectra were recorded with a resolution of 0.33 nm, using 2-nm slit width. Because of high turbidity, the absorption spectra were corrected by subtracting the scattering profile, obtained from cells bleached with sodium hypochlorite. The same samples were also used for the measurement of BChl *a* fluorescence excitation spectra. The fluorescence excitation spectra were recorded using an Aminco AB2 spectrofluorometer at an emission wavelength of 875 nm, with a resolution of 1 nm, using 4 nm excitation and 16 nm emission slit-widths. The emission monochromator was protected against stray light by a Schott RG830 glass filter.

Antibiotic sensitivity was tested in the organic medium with individual antibiotics added at concentrations 50 mg/l if not stated otherwise. Growth was assayed after 4–5 days. For the simple organic carbon-source growth tests, an artificial medium was derived from the *f/2* medium. It contained: 0.4 M NaCl, 20 mM MgSO<sub>4</sub>, 10 mM CaCl<sub>2</sub>, 10 mM KCl, 20 mM MgCl<sub>2</sub>, 1.7 mM KBr, 1 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>,  $10^{-4}$  M NaH<sub>2</sub>PO<sub>4</sub>,  $2 \times 10^{-4}$  M H<sub>3</sub>BO<sub>3</sub>,  $10^{-8}$  M Na<sub>2</sub>SeO<sub>3</sub>, pH 8.0. The trace metal mix and vitamins were the same as in *f/2* media. For the growth test, the medium was enriched with 1 mM of the individual carbon sources.

Respiration was measured using a Clark oxygen electrode placed in a temperature-stabilized measuring chamber at 23 °C. The electrode signal was recorded using a custom made potentiostat and a nanoamperometer.

Carbon dioxide fixation was assayed at a BChl *a* concentration of 30 nM, at 25 °C. The bacterial suspension was labeled with 37 kBq per ml NaH<sup>14</sup>CO<sub>3</sub> (New England Nuclear, USA) and divided into 2-ml aliquots placed in glass scintillation vials. The vials were exposed to varying light intensities produced by a white halogen lamp attenuated by a set of neutral density filters. Incubations were terminated by addition of 30  $\mu$ l 36% HCl (final pH ~1), and samples were evaporated to dryness and resuspended in 2 ml 30 mM Tris buffer (pH 7.7) with 15 ml scintillation cocktail (Ready Safe, Beckman). The incorporated radioactivity was determined with a Beckman LS6K-IC scintillation counter and corrected for blank counts.

Cells were counted using epifluorescence microscopy with acridine orange staining. To determine organic carbon and nitrogen content, bacterial cells were collected onto pre-combusted glass-fiber filters, washed, dried in a dessicator, and analyzed with an automated Perkin Elmer model 2400 CHN elemental analyzer.

### Pigment analysis

BChl *a* concentration was determined spectroscopically in methanol with an Aminco DW2000 spectrophotometer using the absorption coefficient  $\epsilon_{771} = 54.8 \text{ mM}^{-1} \text{ cm}^{-1}$  (Permentier et al. 2000). Total carotenoid content was determined in the same extracts, using an absorption coefficient of  $\epsilon_{460} = 128 \text{ mM}^{-1} \text{ cm}^{-1}$  (Yurkov et al. 1993).

Pigment composition was assayed by HPLC. The cell pellets were placed in 3 ml acetone, ground using a hand-held glass/glass tissue homogenizer, and extracted for 1–2 h (0 °C, dark). Samples (200  $\mu$ l) of a mixture of 0.3 ml water plus 1.0 ml extract were injected onto a Varian 9012 HPLC system equipped with a Varian 9300 autosampler, a Timberline column heater (26 °C), and Spherisorb 5  $\mu$ m ODS2 analytical (4.6 $\times$ 250 mm) column and corresponding guard cartridge. Pigments were detected with a ThermoSeparation UV 2000 detector ( $\lambda=360$  nm). A ternary solvent system was employed for HPLC pigment analysis: solvent A (methanol:0.5 M ammonium acetate, 80/20, v/v), solvent B (acetonitrile:water, 85/15, v/v), and solvent C (ethyl acetate). The flow rate was held constant at 1 ml  $\text{min}^{-1}$ . The linear gradient used for pigment separation was a modified version of the method of Wright et al. (1991): 0.0' (100% A), 10.0' (100% B), 11.0' (78% B, 22% C), 27.5' (10% B, 90% C), 29.0' (100% B), and 30.0' (100% B). Solvents A and B contained 0.01% 2,6-di-tert-butyl-p-cresol (0.01% BHT, w/w; Sigma-Aldrich) to prevent the formation of allomerized BChl *a* and bacteriopheophytin *a* derivatives (Bidigare and Trees 2000).

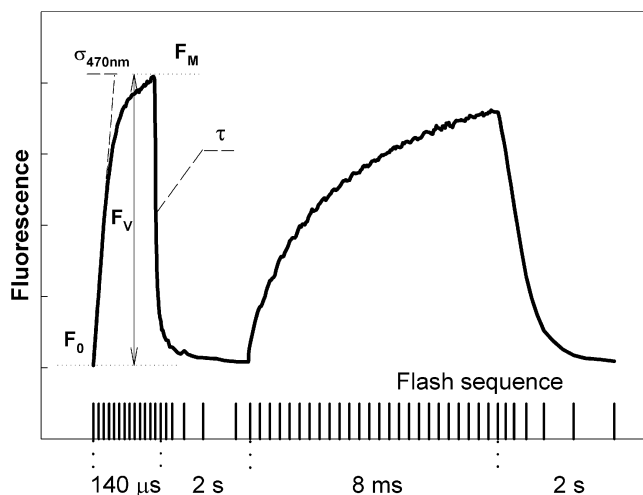
The individual peaks were identified by an absorption UV/VIS diode array detector and by comparison of the retention times with those of authentic standards ( $\beta,\beta$ -carotene, zeaxanthin, BChl *a*, and BPhe *a*) and with extracts prepared from the type strain for *Erb. longus* (ATCC 33941). HPLC peak identities were confirmed by “off-line” UV/VIS and LC/MS analyses of corresponding fractions. Mass spectra of selected carotenoid fractions were obtained using an Agilent Technologies 1100 series HPLC (0.7 ml methanol  $\text{min}^{-1}$ , operated in column by-pass mode) interfaced with model SL quadrupole MSD (APCI, fragmentor setting of 30 V). The relative pigment abundances in the extracts were estimated as percentages of total peak area at 360 nm ( $\%A_{360}$ ).

#### Fatty acid analysis

Cells grown on the organic medium were pelleted, combined with 10 ml 5%  $\text{H}_2\text{SO}_4$  in methanol, and divided equally among three 5-ml reaction vials. A total of 200  $\mu$ l C19:0 fatty acid recovery standard was added to the three vials. The sample vials were vortexed and placed in a 100 °C dry-bath for 2 h. After cooling, the vial contents were transferred to a 20-ml vial with methanol and hexane rinses totaling 1.5 ml. An additional 1.5 ml hexane and 3 ml  $\text{H}_2\text{O}$  were placed in the vial and mixed vigorously for 1 min. Fatty acid methyl esters (FAMES) were partitioned into the hexane layer, which was separated from the methanol/water layer via centrifugation for 5 min at 700 $\times$ g. The hexane layer was pipetted off and two additional 1.5-ml hexane extractions were done. The hexane extract was evaporated to  $\sim$ 1 ml under a  $\text{N}_2$  stream and passed through a  $\text{Na}_2\text{SO}_4$  column to remove residual water. The sample was evaporated to 500  $\mu$ l under a  $\text{N}_2$  stream and a 50- $\mu$ l aliquot was removed for GC/MS analysis. The GC/MS system consisted of a Hewlett Packard 6890 Plus GC interfaced to a HP 5973 Mass Selective Detector. An HP 7683 Autoinjector was used to introduce 0.5  $\mu$ l sample directly onto a J and W DB-5 column (60 m, 0.32 mm I.D., 0.25 mm film thickness) through a cooled on-column inlet. The initial GC oven temperature was 50 °C (1-min hold) followed by a ramp of 20 °C  $\text{min}^{-1}$  to 180 °C (0-min hold), a 2 °C  $\text{min}^{-1}$  ramp to 280 °C (0-min hold), and a 10 °C  $\text{min}^{-1}$  ramp to 320 °C (10-min hold). FAMES were identified either through the use of authentic standards (Alltech, Sigma-Aldrich) or by their mass spectra. FAMES were quantified using a C23 normal alkane as both an internal standard and as part of a FAME standard mixture.

#### IRFRR fluorometry

The photosynthetic ability of the bacterial isolates was assessed using an IRFRR fluorometer (Kolber et al. 1998, 2000) equipped with a large-area avalanche photodiode detector (Advanced Photonix, 630-70-72-631, 16-mm diameter) protected by RG695 glass and 880-nm interference (50-nm half-width) filters. The instrument generates a train of short (0.6  $\mu$ s) blue (470 nm) or infrared



**Fig. 1** The infrared fast repetition rate (IRFRR) transient used for the detection of bacteriochlorophyll *a* (BChl *a*)-containing reaction centers and the derivation of the main biophysical parameters. The fluorescence transient is elicited by a series of subsaturating flashlets (indicated by ticks on the x-axis).  $F_0$  Initial fluorescence level, measured at the onset of the excitation sequence;  $F_M$  saturation level of the fluorescence transient, reached within about 100  $\mu$ s of the excitation protocol. Effective absorption cross-section of the bacterial photosynthetic units  $\sigma_{470}$  is determined from the initial slope of the fluorescence rise

(795 nm) flashlets in the microsecond to millisecond timescale. Electron transport elicited by these light pulses induced transient changes in the BChl *a* fluorescence emission at 880 nm originating from the functional bacterial reaction centers (Fig. 1). The background illumination was provided with the same set of blue LEDs. The fluorescence parameters  $F_V/F_M$ ,  $\sigma_{470}$  (functional cross-section of photosynthetic units) and  $p$  (connectivity) were determined as described previously (Kolber et al. 1998). The photosynthetic electron transport rate  $ETR$  was estimated as  $ETR = I \sigma_{470} (F_M - F_S) / (F_M - F_0)$ , where  $I$  is light intensity,  $F_M$  is the maximum and  $F_0$  the minimum BChl *a* fluorescence yield in the dark and  $F_S$  is the fluorescence yield upon illumination. The maximum electron transport  $ETR_{MAX}$  was determined by fitting experimental data points obtained at varying light intensities using the formula:

$$ETR = I \sigma_{470nm} ETR_{MAX} / (I \sigma_{470nm} + ETR_{MAX})$$

#### Phylogenetic analysis

16S rRNA genes were amplified directly from cells using a eubacterial primer, SSEub27F (Giovanonni et al. 1988), and a universal primer, SS1492R (Lane 1991). Primers for *pufL* used in the study were (5'-CTKTTTCGACTTCTGGGTSGG-3'), *pufMr* (5'-CATSGTCCAGCGCCAGAA-3') (a modification of the primer reported in Nagashima et al. 1997). PCR products were verified by agarose gel electrophoresis. Fragments containing 16S rDNA were extracted from the gel using the Ultrafree-DA spin kit (Millipore) and directly sequenced. DNA was sequenced by the dideoxy termination reaction with labeled SSEub27F and the universal SS1390R (Raskin et al. 1994) primers using the Excel II LC kit (Epicenter). PCR fragments containing *pufL-M* were extracted from agarose gels using the GENECLEAN spin kit (BIO101). DNA fragments were cloned into the pCR2.1 vector using the original TA cloning kit (Invitrogen). The labeled fragments were sequenced using either a model DNA4000 or DNA4200 automated DNA sequencers (LiCor, Lincoln, Neb., USA).

Nucleotide sequences were aligned using the ClustalW program and edited manually. The phylogenetic trees were constructed us-

**Table 1** Locations where the bacterial isolates were collected

Strain	Location	Temperature (°C)	Date
AT8	NE Pacific, 48°15'N, 128°19'W, surface	16	July 21, 2000
BA13	NE Pacific 48°33'N 129°53'W, 22 m	16	July 30, 2000
COL13	French Mediterranean coast, 42°28'N 3°11'E, surface	23	September 04, 2000
MG3	SE Atlantic/W Indian 41°06'S 19°25'E, surface	16	December 1, 2000
MG22	SE Atlantic/W Indian 45°37'S 20°15'E, surface	8	November 30, 2000
NAP1	NW Atlantic 39°36'N 72°27'W, surface	12	April 16, 2000
NJ3Y	New Jersey coast 40°N 74°W, surface	11	December 18, 1999

ing the nucleotide sequences and the maximum-likelihood method using TREE-PUZZLE package (v. 5.0, Schmidt et al. 2002). The GenBank sequence accession numbers for the 16S rDNA are as follows: M59062 (*Erb. longus*), AB013354 (*Erb. litoralis*), AF118020 (*Erb. citreus*), Y16267 (*Citromicrobium bathyomarinum*), AB033327 (*Por. neustonensis*) AB013355 (*Erm. ramosum*), AB024289 (*Erythromonas ursincola*), AB021493 (*Por. sanguineus*=*Agrobacterium sanguineum*), AY326257 (MG3), AY326258 (NJ3Y), AY326259 (NAP1). The accession numbers for the *pufM* sequences are as follows: D50648 (*Erb. longus*), AB010981 (*Erb. litoralis*), AB010981 (*Por. neustonensis*) AB010873 (*Erm. ramosum*), AB031016 (*Blastomonas* "*Erythromonas*" *ursincola*), AB011074 (*Por. sanguineus*), AY326260 (NAP1), AY326261 (COL13), AY326262 (MG3), AY326263 (MG22), AY326264 (NJ3Y), AY326265 (BA13).

## Results

### Isolation

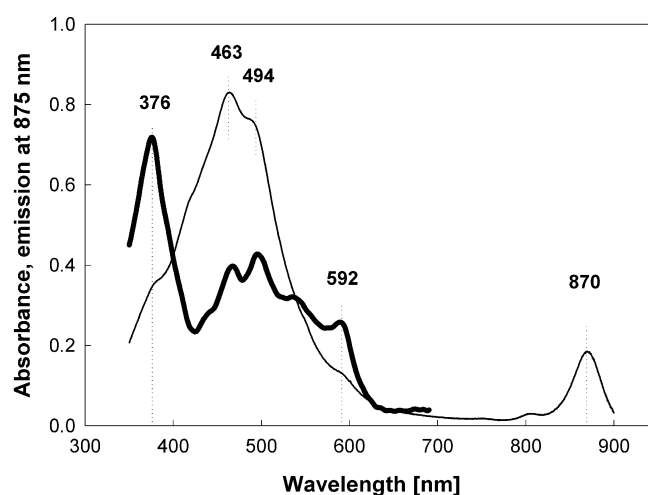
Seven strains containing functional bacterial photosynthetic units were isolated from various marine environments in the Atlantic, Pacific, and Indian Oceans and the Mediterranean Sea (Table 1). Six of these were collected from surface waters, with the exception of strain AT8, which was collected from a depth of 22 m (Chl *a* and BChl *a* maximum). Sea surface temperatures in the sampling locations ranged from 8 °C (MG22 isolate) to 23 °C (COL13 isolate) (Table 1). Strain NJ3Y was isolated from coastal waters, COL13 from water collected on a beach, and the remaining strains from open-ocean waters. The isolation procedure yielded also other BChl-*a*-containing isolates related to *Roseobacter* and *Citromicrobium* genera. However, only *Erythrobacter* isolates are described here.

### General characterization

The cells exhibited a varied morphology but were most frequently ovoid or rod-shaped, 0.5–0.7×1–4 μm. In organic-rich liquid media, the cells tended to form irregular, rapidly sedimenting "clumps" or aggregates. All strains formed circular, smooth orange-pink (yellow for NJ3Y) colonies on agar. Large amounts of carotenoids (Table 2) were responsible for their color. The bacteriochlorophyll content ranged from 10<sup>-20</sup> to 10<sup>-19</sup> mol BChl *a* per cell (Table 2), which is more than an order of magnitude lower than that found in purple non-sulfur bacteria (Göbel 1978). Correspondingly, the cellular carbon per BChl *a* ratios ranged from 700:1 to 1500:1 (w:w), which is more

**Table 2** Biochemical and biophysical characteristics of strains NAP1, MG3, and NJ3Y. The strains were grown at room temperature in organic media under a natural light/dark cycle

	Strains		
	NAP1	MG3	NJ3Y
Major carotenoid	Erythroanthin sulfate		Zeaxanthin
BChl <i>a</i> /cell (10 <sup>-21</sup> mol)	30–70	25–60	5–40
Carotenoids/cell (10 <sup>-21</sup> mol)	220–260	340–370	380–410
C/cell (10 <sup>-15</sup> g)	25–45	50–80	25–45
Growth optimum (°C)	32.5	25	30
μ <sub>25°C</sub> (days <sup>-1</sup> )	4.7	2.4	3.0
μ <sub>max</sub> (days <sup>-1</sup> )	6.8	2.4	4.0
C:N ratio (mol/mol)	5.4–6.0	4.9–5.5	4.3–4.8
$F_v/F_M$			0.80–0.85
σ <sub>470</sub> (Å <sup>2</sup> )			40–45
<i>p</i>			0–0.3
$ETR_{MAX}$ (electrons s <sup>-1</sup> )			60–120



**Fig. 2** In vivo absorption (*thin line*) and fluorescence excitation (*thick line*) spectra of the NAP1 isolate with marked positions of primary BChl *a* (376, 592, 870 nm) and carotenoid (463, 494 nm) absorption and excitation peaks. The vertical scale of the excitation spectrum is expanded about four times relative to the absorption spectrum

than ten times higher than the C:Chl *a* ratios reported for marine phytoplankton. BChl *a* was synthesized exclusively in the dark.

**Table 3** Comparative characteristics of the isolates and other species of aerobic anoxygenic photoheterotrophs. Strain NAP1 is considered representative for all the open ocean isolates of group 1 (AT8, BA13, MG22, NAP1). + Antibiotic sensitivity, – antibiotic

Strain	NAP1	COL13	NJ3Y	MG3	<i>Erb. litoralis</i>	<i>Erb. longus</i>	„ <i>Cit. bathyomarinum</i> “	<i>Erm. ramosum</i>	<i>S. sibiricus</i>	<i>Rsb. denitrificans</i>
Environment	Open ocean	Marine coast	Marine coast	Open ocean	Marine coast	Marine coast	Deep ocean	Fresh water	Fresh water	Marine coast
Subclass of <i>Proteobacteria</i>	$\alpha$ -4	$\alpha$ -4	$\alpha$ -4	$\alpha$ -4	$\alpha$ -4	$\alpha$ -4	$\alpha$ -4	$\alpha$ -4	$\alpha$ -4	$\alpha$ -3
Color	Orange	Orange	Yellow	Orange	Orange	Orange	Yellow	Orange	Yellow	Pink
In vivo BChl <i>a</i> Abs. max.										
LH1	870	870	869	865	871	866	867	868	867	870
LH2	–	–	–	–	–	–	–	798,832	–	806
Chloramphenicol	+	+	+	+	+	+	+	+	+	+
Erythromycin	+	+	+	+	+	+	n.d.	+	–	+
Nalidixic acid	–	–	–	–	–	–	n.d.	+	+	+
Penicillin G	+	+	+	+	+	+	–	–	–	+
Polymyxin B	–	–	–	–	–	–	+	+	–	+
Streptomycin	–	–	–	–	–	–	–	–	+	+
Tetracycline	+	+	+	+	+	+	+	+	–	+

**Table 4** Substrate utilization among the studied strains. None of the strains is capable of growth on arabinose, citrate, glycolate, malate, sorbitol, sucrose, or tartrate. High organic medium corresponds to 5 g peptone and 1 g yeast extract per liter

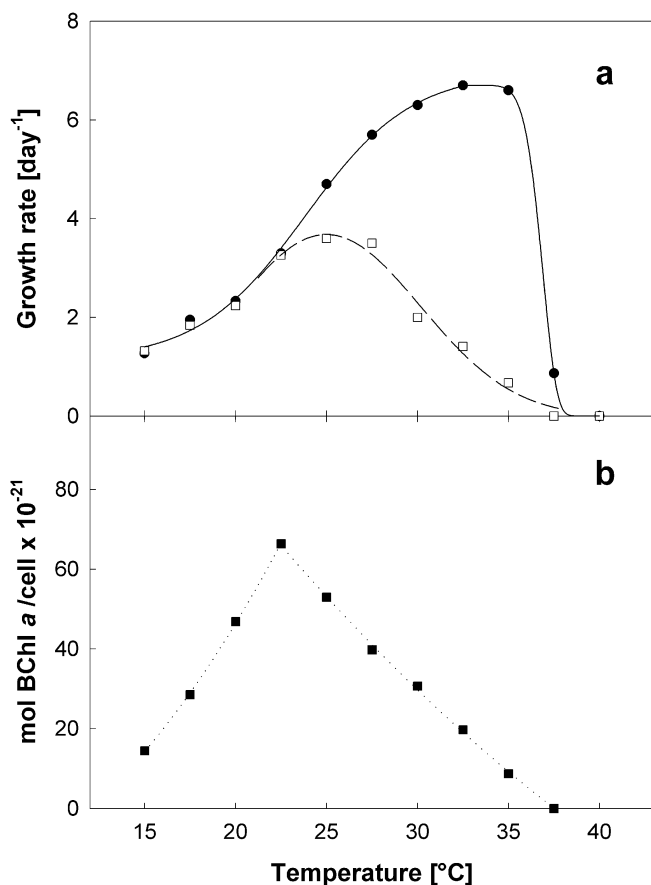
Nutrient	Strains						
	AT8	BA13	COL13	MG3	MG22	NAP1	NJ3Y
Acetate	++	++	+	+	++	++	+
Butyrate	++	+++	++	++	+++	+++	+++
Fructose	+	–	–	–	+	+	–
Fumarate	–	–	–	–	–	–	++
Glucose	+++	++	+++	++	++	+++	++
Glutamate	+++	+++	+++	+++	+++	+++	+
Glutamine	–	–	–	–	–	+	–
Glycerol	+	–	–	++	–	–	+
Lactate	+	–	–	–	–	+	–
Pyruvate	+++	++	++	+	+++	+++	+++
Succinate	–	–	–	–	–	–	+++
High organic medium	++	++	++	–	+++	+++	+++

All the isolates exhibited relatively uniform in vivo absorption spectra (Fig. 2), with the infrared  $Q_Y$  band of BChl *a* light-harvesting 1 (LH1) antenna centered between 865 and 871 nm, depending upon the specific strain (Table 3). Light-harvesting complex 2 (LH2), frequently found in purple photosynthetic bacteria and *Roseobacter* species, was absent, resulting in a small functional cross-section of the photosynthetic units. At 470 nm, the calculated cross-sections  $\sigma_{470}$  averaged between 40 and 45 Å<sup>2</sup>; this is five to seven times lower than values typically measured in oxygenic photoautotrophs at the same wavelength (Gorbunov et al. 2000). Carotenoids were responsible for the major absorption bands between 350 and 600 nm, with peaks at 460–464 nm and 494–497 nm. The absorption bands of cytochromes could be distinguished in the in vivo absorption spectra by means of second derivative analysis (not shown). The positions of the  $\gamma$  (Soret) band at 408–416 nm and the  $\alpha$  band at 550–551 nm indicated the presence

of cytochrome *c* type. The  $Q_X$  and the Soret ( $B_Y$ ) absorption bands of BChl *a* overlapped with carotenoids absorption, and their peaks at 592 nm and 376 nm were better resolved in the BChl *a* fluorescence excitation spectrum (Fig. 2).

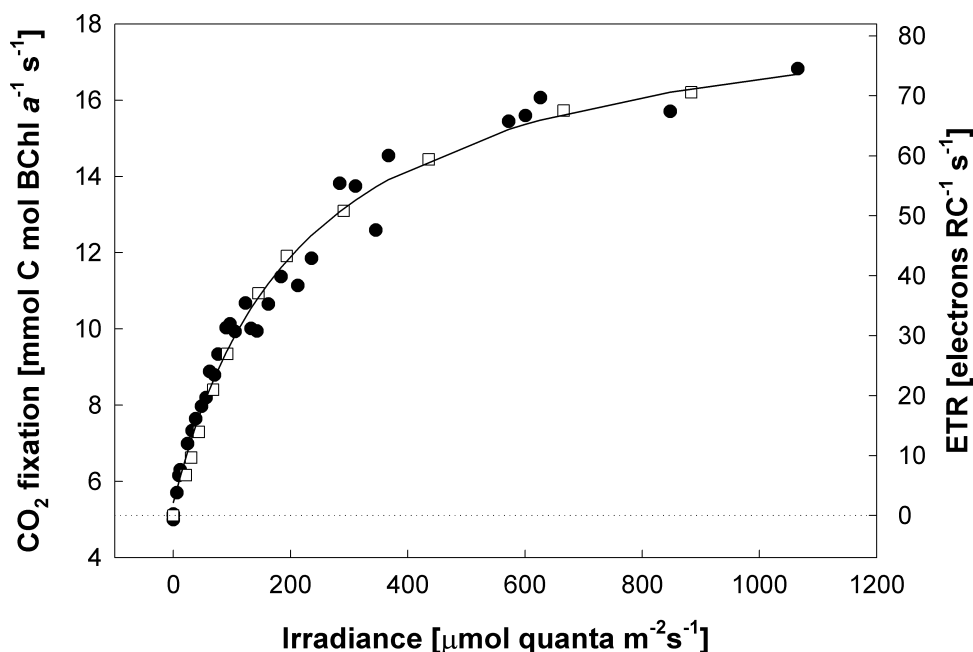
#### Nutrition and growth

All isolates were capable of growth at room temperature (20 °C) in the organic medium under oxic conditions. No growth was observed in anaerobiosis. Acetate, butyrate, glucose, and pyruvate were utilized as simple carbon sources for growth. Utilization of other carbon sources (e.g., glycerol, fumarate, succinate, lactate, and glutamine) varied among the strains (Table 4). No growth occurred in artificial medium devoid of an added organic carbon source. Optimal growth was typically obtained using standard



**Fig. 3** Temperature effects on cell (solid line) and pigment (dashed line) specific growth rates of the NAP1 isolate (a). Growth was assayed in batch cultures (organic medium) exposed to a 12/12-hour light/dark cycle with irradiance of  $50 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$ . Growth was followed by measurements of optical density at 650 nm (●) and BChl *a* content (□). BChl *a* content per cell (■) was determined on day 7 of the experiment (b)

**Fig. 4** CO<sub>2</sub> fixation activity of the NAP1 isolate and IRFRR fluorescence-based estimates of the electron transport rates (□). The carbon fixation activity was assayed as the NaH<sup>14</sup>CO<sub>3</sub> incorporation rate at 25 °C under varying irradiance (●). Continuous line Numerical fit of carbon fixation data to a rectangular hyperbola function

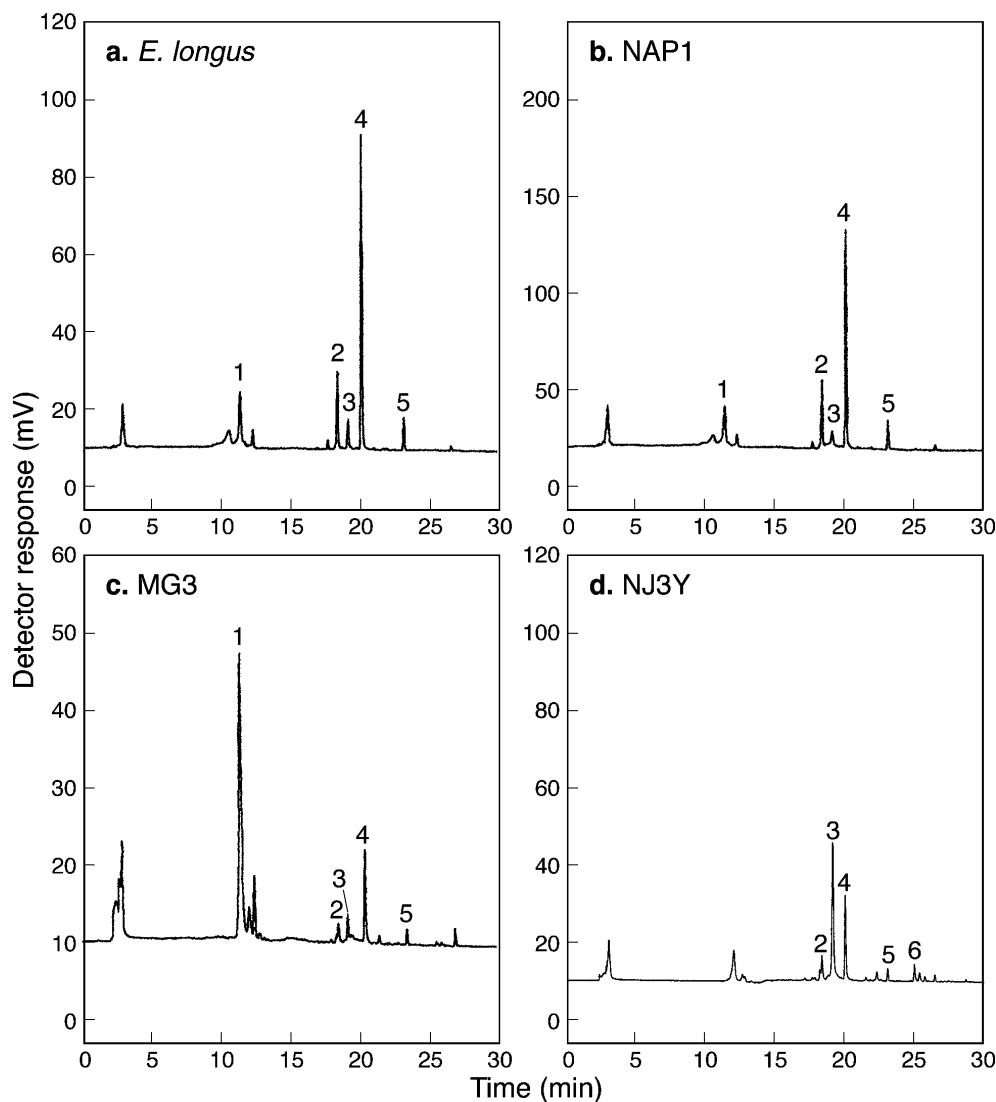


peptone+yeast extract media. All the strains, except MG3, were capable of growth on high organics medium (5 g peptone and 1 g yeast extract per liter), but both growth and BChl *a* accumulation optima were frequently found at lower concentrations of the organics (not shown). Ammonium and urea could be utilized as nitrogen sources while nitrite and nitrate were not utilized. No growth was detected under diazotrophic conditions. Glutamate, leucine, and isoleucine could serve as combined sources of both carbon and nitrogen. All of the isolates required vitamin B<sub>12</sub> and biotin for growth.

Specific growth rates were tested in the organic medium at 25 °C with NAP1, NJ3Y and MG3 strains and ranged from 2.4 days<sup>-1</sup> for MG3 to 4.7 days<sup>-1</sup> for NAP1 (see also Table 2). The optimal growth temperature for NAP1 was 32.5 °C, but the synthesis of BChl *a* was inhibited at temperatures higher than 30 °C (Fig. 3); the temperature optimum for BChl *a* accumulation was 22.5 °C. Optimal growth temperatures were lower in the NJ3Y (30 °C) and MG3 isolates (25 °C), which, unlike NAP1, did not exhibit a sharp temperature dependence of cellular BChl *a* content. The  $Q_{10}$  values for growth ranged from 1.8 for NJ3Y to 2.6 for NAP1.

The isolates exhibited light-enhanced CO<sub>2</sub> fixation, with a simultaneous decrease in respiration rates down to about 30% of the dark value (not shown). A photosynthesis-irradiance curve for carbon incorporation was measured for the NAP1 isolate grown in the organic medium. In parallel, electron transport rates were estimated using IRFRR fluorometry (Fig. 4). This isolate exhibited light-saturated photosynthetic activity of  $17 \text{mmol}(\text{CO}_2) \text{mol}(\text{BChl } a)^{-1}\text{s}^{-1}$ , whereas dark carbon fixation was  $\sim 5 \text{mmol}(\text{CO}_2) \text{mol}(\text{BChl } a)^{-1}\text{s}^{-1}$ . Assuming 36 BChl *a* molecules per reaction center, and electron transport rates of up to 70 electrons per reaction center per second (Fig. 4), a ratio of one CO<sub>2</sub> molecule fixed per 170 electrons transported was es-

**Fig. 5** Reverse-phase HPLC chromatograms (360 nm) for acetone extracts prepared from whole cell pellets of **a** *Erythrobacter longus* ATCC 33941, **b** NAP1, **c** MG3, and **d** NJ3Y. Peak identities: 1 erythro-xanthin sulfate, 2 bacteriorubixanthinal, 3 zeaxanthin, 4 bacteriochlorophyll *a*, 5 bacteriopheo-phytin *a*, and 6  $\beta,\beta$ -carotene



timated. This is an efficiency about one order of magnitude less than that measured in oxygenic photoautotrophs (Falkowski and Raven 1997).

All of the isolates, as well as the type strains (*Erb. longus* and *Erb. litoralis*), were sensitive to amikacin, ampicillin, cephalirin, chloramphenicol, cloxacillin, erythromycin, fusidic acid, gentamycin, kanamycin, linkomycin, neomycin, novobiocin, penicillin G (20 kU/l), rifampicin (10 mg/l), spectinomycin (20 mg/l), tetracycline, and vancomycin. Isolates were resistant to amphotericin B (2.5 mg/l), cycloheximide, dihydrostreptomycin, geneticin, nalidixic acid, nystatin, paromomycin, polymyxin B, and streptomycin. Resistance towards streptomycin and dihydrostreptomycin seems to be typical for this bacterial group (see Table 3), and (dihydro)streptomycin-containing (50 mg/l) plates might be used as selective media for isolation or maintenance of these strains.

#### Pigment distribution

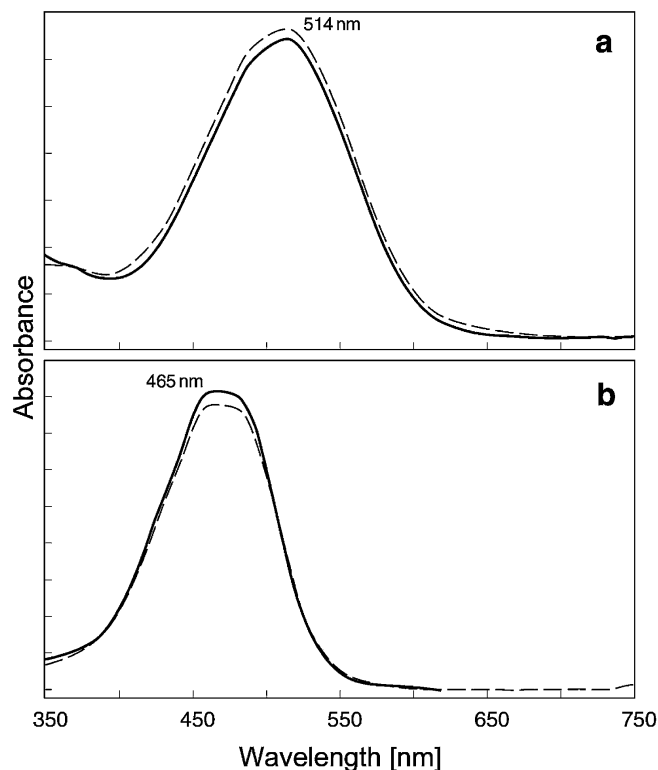
The major pigments identified in our isolates were erythro-xanthin sulfate, bacteriorubixanthinal, zeaxanthin, BChl *a*, bacteriopheo-phytin *a*, and  $\beta,\beta$ -carotene (Fig. 5). BChl *a* contains a phytol side chain. The retention times and absorption properties of these pigments are summarized in Table 5. The observed mean absorption maxima ( $\lambda_{\max}$ ) and carotenoid band ratios  $\%(\text{III/I})\text{I}$  (Ke et al. 1970) are consistent with published values. The uncertainty of measured  $\lambda_{\max}$  values was  $\pm 1$  nm, except in the case of weakly absorbing pigment fractions where the uncertainty was  $\pm 2$  nm. The differences observed between measured and published  $\lambda_{\max}$  values can be attributed to uncertainties associated with the absorption measurements, solvent effects, and/or the presence of minor co-eluting pigments. The absorption spectra of erythro-xanthin sulfate and bacteriorubixanthinal isolated from the type strain *Erb. longus* are nearly identical to those obtained for isolate AT8 (Fig. 6).

The identities of erythro-xanthin sulfate and bacteriorubixanthinal were confirmed by LC/MS analysis. For the

**Table 5** Retention times and mean absorption properties (in HPLC eluant) of the major pigments detected in *Erythrobacter longus* (ATCC 33941) and isolates NAP1, MG3, and NJ3Y. Peak numbers correspond to those indicated in Fig. 5. Solvents and

carotenoid band ratios from the literature data: 1 solvent=methanol+water (4:1) containing 40 mM NH<sub>4</sub>OH, %(III/II)=0; 2 solvent=methanol, %(III/II)=0; 3 solvent=acetone, %(III/II)=33; 4, 5 solvent=diethyl ether; 6 solvent=acetone, %(III/II)=21

Peak no.	$R_t$ (min)	Pigment identification	Observed $\lambda_{max}$ (nm)	Published $\lambda_{max}$ (nm)	Reference
1	11.4	Erythroanthin sulfate	465	469	Takaichi et al. (1991)
2	18.4	Bacteriorubixanthinal	513	510	Takaichi et al. (1988)
3	19.1	Zeaxanthin	(428), 454, 482	(428), 454, 481	Jeffrey et al. (1997)
4	20.4	Bacteriochlorophyll <i>a</i>	359, 580, 771	358, 577, 773	Scheer (1991)
5	23.4	Bacteriopheophytin <i>a</i>	358, 525, 750	357, 525, 749	Scheer (1991)
6	25.4	$\beta,\beta$ -carotene	(426), 454, 478	(426), 454, 480	Jeffrey et al. (1997)



**Fig. 6** Absorption spectra (in HPLC eluant) of **a** bacteriorubixanthinal and **b** erythroanthin sulfate isolated from *Erb. longus* ATCC 33941 (solid lines) and strain AT8 (dashed lines)

HPLC fraction collected at ~11 min, major ions were observed at  $m/z$  679 (50%), 599 (17%) and 597 (100%), corresponding to the pseudomolecular ion  $[M+H]^+$  and fragment ions  $[M+H-SO_3]^+$  and  $[M+H-H_2SO_3]^+$  of erythroanthin sulfate, respectively (Takaichi et al. 1991). For the HPLC fraction collected at ~18 min, major ions were observed at  $m/z$  597 (100%), 579 (14%) and 565 (9%), corresponding to the pseudomolecular ion  $[M+H]^+$  and fragment ions  $[M+H-H_2O]^+$  and  $[M+H-HOCH_3]^+$  of bacteriorubixanthinal, respectively (Takaichi et al. 1988).

An “unknown” polar carotenoid ( $R_t=12.0$  min,  $\lambda_{max}=455, 480$  nm) was present in the NJ3Y extract (Fig. 1D). This pigment may be structurally related to caloxanthin

sulfate ( $\lambda_{max}=450, 475$  nm), a minor carotenoid found in *Erb. longus* (Takaichi et al. 1991).

Three different pigment signatures were observed in the bacterial isolates examined in this study. The major group (*Erb. longus*, AT8, BA13, COL13, MG22 and NAP1) was characterized by relatively high amounts of BChl *a* ( $\%A_{360}=37-51$ ) relative to erythroanthin sulfate ( $\%A_{360}=12-19$ ) and bacteriorubixanthinal ( $\%A_{360}=9-13$ ). Two strains differ from this major group. Strain MG3 was distinguished by its relatively high level of erythroanthin sulfate ( $\%A_{360}=59$ ) relative to BChl *a* ( $\%A_{360}=10$ ) and bacteriorubixanthinal ( $\%A_{360}=2$ ). In strain NJ3Y erythroanthin sulfate was absent; however, the strain contained a high level of zeaxanthin ( $\%A_{360}=39$ ) relative to the other isolates ( $\%A_{360}=4-7$ ).

#### Fatty acid distributions

The distribution of fatty acids in the isolates was similar (Table 6) to that found in *Erb. longus* ATCC 33941 (Urakami and Komagata 1988), with the major fatty acids consisting of 11-octadecenoic acid (18:1, n-7) and 6,9-octadecadienoic acid (18:2, n-9). These two compounds accounted for 61–84% of the total fatty acids contained in each sample. The high concentration of 18:2 (n-9), 16–26%, is surprising since bacteria usually do not contain polyunsaturated fatty acids (PUFAs). Another PUFA, 18:2 (n-6), was also present in each sample, although at less than 2% of the total fatty acids. Typical for bacterial cultures, fatty acids longer than C20 were not observed. Only two hydroxy-fatty acids, 2-hydroxytetra-decanoic acid and 2-hydroxyhexadecanoic acid, were detected, with a combined abundance of 7–18%. Significant amounts, 1–9%, of the branched fatty acid 2-hexyl-cyclo-propaneoctanoic acid, were measured in each sample. At closer examination, AT8, NAP1, COL13, MG3, and NJ3Y were more similar to each other than to the type strain *Erb. longus* (Table 6), which contained more of the primary saturated fatty acids (C15:0, C16:0, C17:0 and C18:0) and less 11-octadecenoic acid. In addition, the abundance of 2-hexyl-cyclopropane-octanoic acid in strains AT8, NAP1, COL13, MG3, and NJ3Y (9% of the total fatty acids present) was less than half of that found in *Erb. longus*. In two strains, NJ3Y and MG3, the relative content of 16:1 monounsatu-

**Table 6** FAME composition (% total) of *Erb. longus* (ATCC 33941) and strains AT8, NAP1, COL13, MG3, and NJ3Y. With the exception of C20:0, long-chain saturated (C21:0, C22:0, C23:0, C24:0 and C28:0) and polyunsaturated (C20:4, C20:5, C20:3, C22:4, C22:5 and C22:6) FAMES were below the limit of detection in all the bacterial isolates examined. *b.d.* Below detection

			<i>Erb. longus</i>	AT8	NAP1	COL13	MG3	NJ3Y
<b>Saturated fatty acids</b>								
C12:0	Lauric	Dodecanoic acid	b.d.	0.04	0.05	0.01	0.02	0.05
C13:0		Tridecanoic acid	0.02	0.08	0.10	0.02	0.02	<0.01
C14:0	Myristic	Tetradecanoic acid	0.07	0.49	0.59	0.28	0.10	0.25
C15:0		Pentadecanoic acid	2.09	1.09	1.16	0.77	0.61	1.58
C16:0	Palmitic	Hexadecanoic acid	4.87	3.16	3.42	2.78	0.34	4.51
C17:0	Margaric	Heptadecanoic acid	8.08	2.15	2.38	1.32	<0.01	0.80
C18:0	Stearic	Octadecanoic acid	0.74	0.37	0.49	0.27	0.22	0.32
C20:0	Arachidic	Eicosanoic acid	0.09	0.02	0.05	0.01	0.29	b.d.
Sum			16.0	7.39	8.25	5.46	1.61	7.50
<b>Monounsaturated fatty acids</b>								
C16:1 total			0.44	0.42	0.40	0.37	3.86	1.58
C18:1 (n-9)	Oleic	9-Octadecenoic acid	0.11	0.12	0.11	0.04	0.07	1.09
C18:1 (n-7)	<i>cis</i> -Vaccenic	11-Octadecenoic acid	40.6	52.1	52.9	59.5	68.7	47.2
C19:1 (n-9)		10-Nonadecenoic acid	4.96	1.89	2.60	1.22	0.16	0.01
Sum			46.1	54.5	56.0	61.1	72.8	49.9
<b>Polyunsaturated fatty acids</b>								
C16:2 total			b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
C18:2 (n-6)	Linoleic	9,12-Octadecadienoic acid	0.18	0.78	0.74	1.74	0.34	0.77
C18:2 (n-9)		6,9-Octadecadienoic acid	20.5	26.3	24.5	17.6	15.9	23.2
C18:3 total			b.d.	b.d.	b.d.	b.d.	b.d.	b.d.
Sum			20.7	27.0	25.2	19.3	16.2	24.0
<b>Other fatty acids</b>								
		2-Hydroxytetradecanoic acid	5.86	3.31	3.14	7.57	6.50	17.6
		2-Hydroxyhexadecanoic acid	2.19	3.88	3.94	3.85	0.60	0.16
		2-Hexyl-cyclopropanoic acid	9.22	3.86	3.40	2.68	2.32	0.91
Total			100	100	100	100	100	100

rated acids was 2–4%, over four times the amount seen in any of the other samples. In MG3, the increased amount of 16:1 corresponded with a dramatic decrease in hexadecanoic acid.

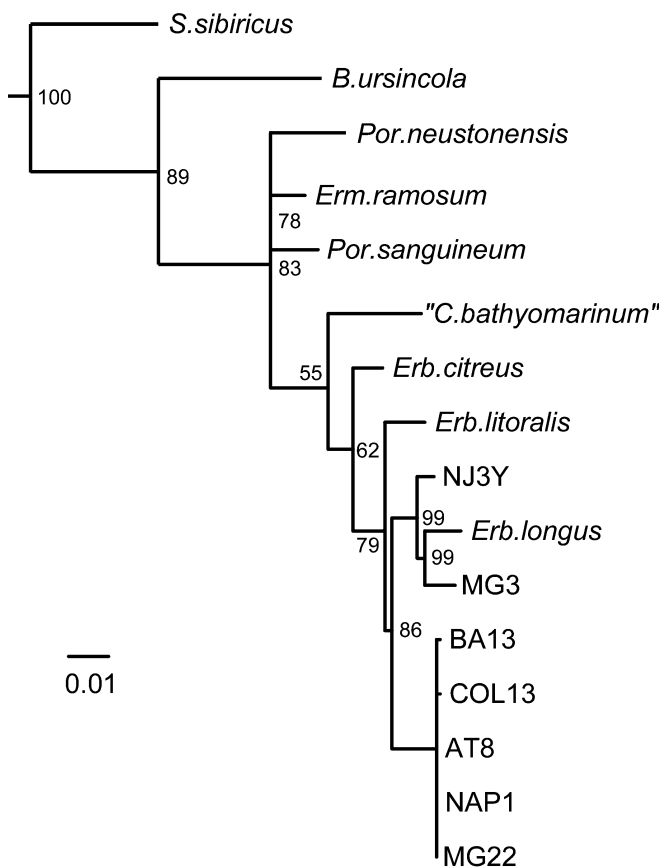
#### Phylogenetic analysis

Phylogenetic analysis was done using partial sequences of 16S rDNA (1115–1195 bp). Calculated genetic distances confirmed a close genetic relationship of all the isolates with the earlier described members (Shiba and Shimidu 1982; Yurkov et al. 1994) of the *Erythrobacter* genus in the  $\alpha$ -4 subclass of *Proteobacteria*. Isolates AT8, BA13, COL13, MG22, and NAP1 had identical (~99.9%) 16S rDNA sequences and were phylogenetically placed between *Erb. litoralis* and *Erb. longus* (Fig. 7). MG3 and NJ3Y isolates diverged from this main group and were more closely related to *Erb. longus*. An independent phylogenetic tree was constructed using sequences of *pufM*, which encodes the M subunit of the bacterial reaction center. The pattern of the *pufM* tree (Fig. 8) constructed for isolates BA13, COL13, MG3, MG22, NAP1, and NJ3Y was similar to that based on 16S rDNA sequences. Again,

MG3 and NJ3Y diverged from the main group represented by strains BA13, COL13, MG22 and NAP1. The genetic distances calculated from *pufM* sequences were obviously larger than those from 16S rDNA. Hence, strains with identical 16S rDNA sequences displayed about 98% similarity in *pufM*. Unlike the 16S rDNA analysis, however, *Erythrobacter* strains (including our isolates) in the *pufM* tree did not form a separated clade, but rather clustered with the other representatives, both marine and freshwater, of *Erythrobacter*/*Erythromicrobium*/*Porphyrobacter* lineage within the  $\alpha$ -4 subclass of *Proteobacteria*.

#### Discussion

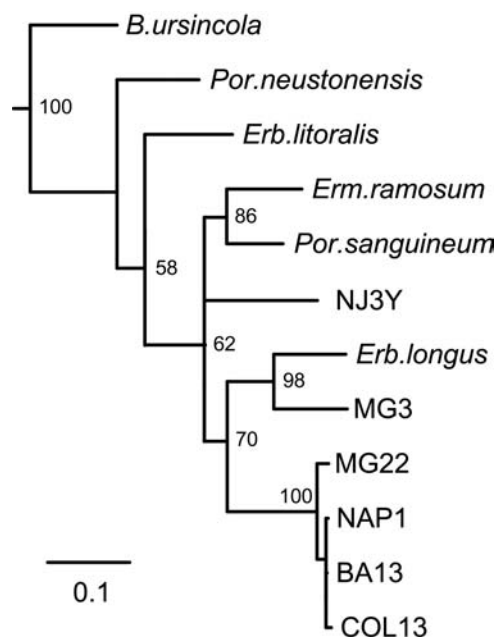
All seven *Erythrobacter* isolates are strict aerobes and require a source of organic carbon (such as acetate, butyrate, glucose, glutamate, leucine, or pyruvate) for growth. They utilize ammonium, urea, and amino acids as nitrogen sources, but they are incapable of utilizing nitrate or of diazotrophic growth. The inability to utilize nitrate is relatively common in marine heterotrophic prokaryotes, which frequently access the nitrogen in dissolved organic matter (Zehr and Ward 2002).



**Fig. 7** 16S rDNA phylogenetic tree constructed by the maximum-likelihood method using the TREE-PUZZLE package. Support for the internal branches of the quartet puzzling tree topology is shown in percent. *Scale bar* 0.01-bp substitutions per nucleotide position

All the isolates contain type-2 bacterial reaction centers composed of the reaction center and LH1 antenna. LH2 complex is absent. The functionality of the reaction centers was verified by IRFRR measurements. The maximum electron transport rates are on the order of 100 electrons per second (see Table 2).

All of the isolates exhibit a complex carotenoid composition, containing polar, sulfated carotenoids, such as erythroanthin sulfate, and non-polar carotenoids, such as zeaxanthin and bacteriorubixanthin. Based on the excitation spectrum, anoxygenic photosynthesis is driven by light absorption in the 350–420 nm (BChl *a*), 420–575 nm (zeaxanthin and bacteriorubixanthin) and 575–900 nm (BChl *a*) regions of the spectrum. This is consistent with the previously reported absorption spectrum of isolated photosynthetic complexes of *Erb. longus* (Shimada et al. 1985). In contrast to the excitation spectrum, the absorption spectrum is dominated by a large absorption band that corresponds to erythroanthin sulfate, overlaying absorption bands of zeaxanthin and bacteriorubixanthin. A comparison of the absorption and excitation spectra (Fig. 2) reveals that only a small fraction of the light absorbed by carotenoids is transferred to the reaction centers. These results suggest that the strongly polar erythroanthin sulfate is not bound to the photosynthetic com-



**Fig. 8** *pufM* dendrogram constructed by the maximum-likelihood method using the TREE-PUZZLE package. Support for the internal branches of the quartet puzzling tree topology is shown in percent. *Scale bar* represents 0.1-bp substitutions per nucleotide position

plexes and does not serve as a light-harvesting pigment for photosynthesis (see also Yurkov et al. 1993). The NJ3Y isolate differs from the other strains in that it does not contain erythroanthin sulfate, and its major carotenoid is zeaxanthin. The relatively low yield of excitation transfer (about 15%) indicates that only a small fraction of the cellular zeaxanthin in NJ3Y is involved in photosynthesis.

The lipid composition of the isolates displays some unique features. Similar to non-sulfur photosynthetic bacteria (Kenyon 1978; Urakami and Komagata 1988), the major fatty acid was *cis*-vaccenic acid (18:1, *n*-7). Characteristic is the large amount (~ 20%) of PUFAs, especially 6,9-octadecadienoic acid (18:2, *n*-9). The high concentration of 18:2 (*n*-9) is surprising since bacteria usually do not contain PUFAs. Earlier reports failed to detect any PUFAs in *Erb. longus* (Urakami and Komagata 1988) or in any other aerobic BChl-*a*-containing strains (Nishimura et al. 1994). In contrast, Fuerst et al. reported the presence of 18:2 fatty acids in the freshwater strain *Por. neustonensis* as well as in the *Erb. longus* type strain (Fuerst et al. 1993). While 18:2 PUFAs are frequently found in oxygenic photosynthetic organisms, cyanobacteria, and photosynthetic eukaryotes, they are absent in anaerobic photosynthetic bacteria (Kenyon 1978).

Based on their spectral characteristics, pigment and lipid composition, nutrient requirements, and 16S rDNA sequence analyses, isolates AT8, BA13, COL13, MG22 and NAP1 appear to be closely related. MG3 and NJ3Y clearly diverge strains from this main group.

All the isolates are photoheterotrophs, capable of utilizing light as a source of energy, but they require organic

carbon for growth. They display light-mediated CO<sub>2</sub> fixation, although carbon fixation activity is relatively low, more than one order of magnitude less efficient than in oxygenic photoautotrophs. The isolates lack RubisCO (R. Tabita, Ohio State University, personal communication), which suggests an alternative pathway for carbon fixation. The significant (3.5-fold) enhancement of carboxylation in the light (Fig. 4) indicates that photosynthesis provides the major portion of energy required for carboxylation.

More important is the ability of anoxygenic photoheterotrophs to substitute respiratory carbon with photosynthetically driven ATP production, as indicated by the strong suppression of respiration under irradiance. Assuming an average irradiance of 200 μmol quanta m<sup>-2</sup>s<sup>-1</sup>, the photosynthetic electron transport rates approach 30 electrons per second per reaction center (see Fig. 4). With one and half protons translocated across the membrane per electron transported, and three protons required for production of one ATP, a photosynthetic flux of 15 ATP molecules per reaction center per second can be maintained. Containing about 1,000 reaction centers (36 BChl *a* per photosynthetic unit, Table 2) and exposed to 12 h of sunlight, the cell can derive about 10<sup>-15</sup> mol ATP per day from cyclic photophosphorylation.

Aerobic anoxygenic photoheterotrophs account for a significant fraction of the marine microbial community in oligotrophic waters. Cell counts carried out in these regions indicate that BChl-*a*-containing bacteria contribute up to 8% of the total bacteria, with BChl *a*/Chl *a* values ranging from 0.8 to 10% (Kolber et al. 2000, 2001). Thus, their relative abundance and photoheterotrophic metabolism suggest a specific role for these bacteria in the marine carbon cycle.

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