Porphyrobacter neustonensis gen. nov., sp. nov., an Aerobic Bacteriochlorophyll-Synthesizing Budding Bacterium from Fresh Water

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Four strains of orange- or red-pigmented bacteria isolated from freshwater surfaces were shown to synthesize bacteriochlorophyll under aerobic conditions. These strains shared unusual morphological features, such as acellular stalks, crateriformlike structures, and buds, with bacteria in the order *Planctomycetales*. However, comparisons of 16S rRNA sequences showed them to be members of the α -4 subdivision of the class *Proteobacteria* and most closely related to the marine aerobic bacteriochlorophyll-synthesizing bacterium *Erythrobacter longus*. They also differ from members of the *Planctomycetales* phenotypically in their synthesis of bacteriochlorophyll and possession of a peptidoglycan cell wall. They can be distinguished from *E. longus* on the basis of their 16S rRNA sequence, the G+C content of their DNA, cellular fatty acid composition, and carbon substrate spectrum. A new genus, *Porphyrobacter*, with a single species, *P. neustonensis* gen. nov., sp. nov., is proposed for these strains. The type strain is ACM 2844.

Classical anoxygenic photosynthetic bacteria typically exhibit repression of bacteriochlorophyll synthesis under aerobic conditions. Strains of several bacterial genera have however been shown to synthesize bacteriochlorophyll under aerobic conditions. Such bacteria include *Erythrobacter longus* (12, 41), "*Erythrobacter sibiricus*" (61), *Roseobacter litoralis* and *Roseobacter denitrificans* (formerly *Erythrobacter* strain OCh 114) (40), *Methylobacterium* species (34), *Rhodospirillum centenum* (60), mutants of *Rhodobacter sphaeroides* (19, 44), and the stem-nodulating *Rhizobium* strain BTAi 1 (6). The hot-spring mat bacterium *Heliothrix oregonensis* may also synthesize bacteriochlorophyll aerobically under in situ conditions in nature, although this bacterium has not been grown in pure culture (29, 30). Microorganisms of this physiological type have been referred to as the aerobic photosynthetic bacteria, with exclusion of the cyanobacteria being understood (39). Some are capable of photophosphorylation and may even possess photosynthetic reaction centers, although they appear not to be phototrophic (10, 24, 26, 38, 42, 52).

We have isolated bacteria capable of synthesizing bacteriochlorophyll under aerobic conditions from fresh water. These bacteria thus bear superficial resemblance to *Erythrobacter* species including the marine *E. longus* and the freshwater "*E. sibiricus*." However, our freshwater strains also share some morphological and ultrastructural features with a distinctive group of budding bacteria distant from the class *Proteobacteria*, the planctomycetes. They display budding reproduction in a similar manner to that seen in the planctomycetes, produce some form of noncellular multi-fibrillar stalk or fascicle, and possess negative stain-collecting pits in the cell surface, termed crateriform structures, thought to be specific to planctomycetes (36). The phylogenetic possibility thus arose that these bacteria might be related to a quite distinct phylum of eubacteria distant from the *Proteobacteria* and furthermore one in which bacterio-

chlorophyll had never been found. With the exception of *H. oregonensis*, all of the named aerobic photosynthetic bacteria appear to be well-defined members of the α subclass of the *Proteobacteria*. It is known from 16S rRNA oligonucleotide cataloguing that *E. longus* is a member of the α subclass of the *Proteobacteria*, which includes many of the purple nonsulfur bacteria in addition to many nonphotosynthetic bacteria (59). *E. longus* represents the α -4 subgroup as its only genus and species.

The phylogenetic relationships of the bacteriochlorophyll-synthesizing methylophilic strains are complex and still relatively unexplored. On the basis of 16S rRNA sequencing, bacteriochlorophyll-synthesizing *Methylobacterium* strains (such as *M. extorquens* AM1) are not closely related to either the α -4 subclass or *Roseobacter* spp. (56).

We present here evidence from ultrastructural, nutritional phenotype, bacteriochlorophyll spectral, and cellular fatty acid analyses and 16S rRNA sequencing which supports the proteobacterial nature of the Australian freshwater budding aerobic bacteriochlorophyll-synthesizing bacteria and their separation from previously described aerobic bacteriochlorophyll synthesizers within the *Proteobacteria*. These bacteria constitute a novel distinct group within the α -4 subdivision of the *Proteobacteria*, and the name *Porphyrobacter neustonensis* is proposed, establishing a new genus with one species in the α subdivision of the *Proteobacteria*.

MATERIALS AND METHODS

Culture media. The following culture media were used during isolation and in cultural and nutritional studies. (i) Peptone-yeast extract agar consisted of (per liter of distilled water) 10 g of peptone, 5 g of yeast extract, 5 g of NaCl, and 15 g of agar; the pH was adjusted to 7.2, and the medium was sterilized by autoclaving at 121°C for 20 min. (ii) Casitone-yeast extract agar consisted of (per liter of distilled water) 5.0 g of casitone, 1.0 g of yeast extract, and 15 g of agar; the pH was adjusted to 7.2, and the medium was sterilized by autoclaving at 121°C for 15 min. (iii) Lake water agar (LWA)

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was a modification of the LWA medium employed by Franzmann and Skerman (8) and consisted of 15 g of agar per liter of filtered lake water from University Lake, University of Queensland campus, Brisbane, Australia, sterilized by autoclaving at 121°C for 15 min. (iv) Staley peptone-yeast extract-glucose agar was the PYG medium of Staley (49), with vitamin solution and Hutner's mineral salts made up according to Staley (50). Marine PYA is a modification of Staley PYG medium with 0.25 g of acetate liter⁻¹ in place of glucose and made up in artificial seawater (20). (v) BMMV basal medium for carbon substrate utilization consisted of (per liter of distilled water) 20 ml of Hutner's basal salts (50), 10 ml of Staley's vitamin solution (50), 0.25 g of (NH₄)₂SO₄, and 2.0 g of KH₂PO₄; the pH was adjusted to 7.0 to 7.1 before autoclaving. (vi) SM agar consisted of (per liter of artificial seawater of Lyman and Fleming [20]) 5 g of Soytone (Difco) and 1 g of yeast extract; the pH was adjusted to 7.2, and the medium was sterilized by autoclaving at 121°C for 20 min. (vii) Schmidt medium III agar as described by Schmidt (37) was used with 1.5% agar.

Origin of strains. The marine strains *E. longus* OCh 101^T and *Roseobacter denitrificans* OCh 114^T were received from Tsuneo Shiba (University of Tokyo, Otsuchi, Japan). Three of the freshwater strains (ACM 2844^T, ACM 2847, and ACM 2721, deposited in the Australian Collection of Microorganisms, Department of Microbiology, University of Queensland) were isolated from University Lake, a eutrophic freshwater pond on the campus of the University of Queensland, Brisbane; a fourth strain, ACM 2508, was isolated from a swimming pool in a previous study (47). For isolation of the first three strains, sampling of the surface microlayers of the littoral zone of University Lake was performed by floating a sterile autoclaved hydrophilic Nuclepore polycarbonate membrane filter (47-mm diameter, 0.4-µm pore size) onto the water surface for a few seconds, retrieving the filter with sterile flat forceps into 10 ml of sterile University Lake water, and spreading dilutions onto either peptone-yeast extract agar or LWA and incubating plates at 28°C. Colonies were selected for restreaking onto peptone-yeast extract agar or casitone-yeast extract agar. ACM 2844^T was derived from a peptone-yeast extract agar plate, and ACM 2847 and ACM 2721 were derived from LWA. ACM 2508 had been isolated by Sly and Hargreaves (47), using a Millipore *Pseudomonas* count water tester (Millipore Corporation, Bedford, Mass.).

Biochemical and nutritional tests. Motility on Staley PYG and casitone-yeast extract media was examined in wet mounts from 3-day cultures. Colony color was examined on casitone-yeast extract agar plates. Acid production from the fermentation of glucose was determined using Hayward's modification of the Hugh and Leifson medium (13) and gram-negative nonfermentative (GNMF) medium (32). Oxidase activity was determined by the method of Kovacs (17), and catalase production was determined with 3% H₂O₂. The ability to hydrolyze Tween 80 was examined using the medium of Sierra (43). Gelatin and starch hydrolysis were tested as described by Skerman (45). Chitinase activity was tested by using solubilized chitin in an agar basal medium, and casein hydrolysis was examined on milk agar plates. The production of aminopeptidase was examined by using L-alanine *p*-nitroanilide-impregnated disks. The ability to grow at 10, 20, 28, and 37°C was tested by using microtiter trays containing Staley PYG broth. The ability to grow anaerobically in the light was tested by the following protocol. An inoculum was prepared from 3-day 30°C-incubated Staley PYG plates by suspending growth in PYG broth to visible

turbidity. Kimax tubes containing 4 ml of PYG broth were inoculated with 0.2 ml of inoculum and incubated at room temperature in an anaerobic hood with a hydrogen-CO₂-N₂ atmosphere under incandescent tungsten lamp illumination. Tubes were also incubated anaerobically under dark conditions in a metal container and aerobically in the dark at the same temperature. Growth was determined by *A*₆₀₀ against a distilled water blank. For antibiotic sensitivity tests, inoculum was spread onto 20-ml PYG agar plates by using sterile glass spreaders, antibiotic disks soaked in 500 µg of penicillin ml⁻¹, 10 µg of cycloserine ml⁻¹, or 100 µg of vancomycin ml⁻¹ were placed onto the agar surface, and the plates were incubated at 30°C for 4 days before inhibition zone diameters were read.

The ability to use various carbon substrates as sole carbon and energy sources was examined by two different methods. For most substrates, microtiter trays were used and filter-sterilized carbon substrates were added to a final concentration of 0.1% in BMMV medium. For inoculum preparation, growth from a 3-day culture grown on Staley PYG medium was washed twice and diluted to the absence of visible turbidity in sterile Milli-Q water. For most substrates, BMMV basal liquid medium was used as the growth medium in microtiter trays, and 10 µl of inoculum was added to each well. For lactate, butyrate, and glycolate only, BMMV basal medium with 1.5% agar was used in plates and inoculated with 10 µl of inoculum as a patch with no more than four patches per plate. Plates were incubated at 28°C for 7 days, and the results were read at 5 and 7 days. Growth was recorded as positive if microcolonies formed on the test substrate and not on a control plate consisting of distilled water plus BMMV agar.

DNA base composition analyses. DNA was extracted and purified by the method of Marmur (21). The DNA base composition was determined by using thermal denaturation and second-derivative plots (46).

Spectroscopy. In vivo spectra were determined with TSM buffer (44) from 3-day-old aerobically grown plate cultures. The cell suspension was sonicated with 10 30-s bursts with a Branson Sonifier cell disrupter 200. After removal of cell debris, the supernatant was examined spectrophotometrically on a Cary 2300 spectrophotometer.

In vitro spectra were determined by using methanol extracts of 3-day cultures grown aerobically on Staley PYG broth stirred at 250 rpm with a magnetic stirrer; for bacteriophageophytin spectra, samples were acidified by the addition of 0.25 ml of 1 M HCl to 3 ml of methanol extract. Spectra were recorded with a Hitachi 150-20 spectrophotometer.

Cell wall analysis. Cultures were grown on Staley PYG agar for 13 days at 30°C and then washed in 0.1 M Tris buffer (pH 8) and stored as a washed pellet at -20°C. Frozen pellets were resuspended in TSM buffer (pH 7.7) and sonicated as described above. Sonicates were centrifuged at 12,000 × *g* for 10 min, and the cell wall pellets were stored at -20°C. Before analysis, cell pellets were washed three times with double-distilled water and freeze-dried.

Freeze-dried pellets of the crude cell wall preparations were hydrolyzed with 4N HCl at 115°C for 4 h in vacuo. Pelleted cell wall material was analyzed according to the method of Agabian and Unger (1), modified to use the hydrolysis procedure mentioned above. The procedure was adapted for use on a Dionex D500 amino acid analyzer using the sodium citrate buffer systems recommended by the Dionex manufacturer for protein hydrolysates.

16S rRNA sequencing. Sequence data were obtained both

by using the reverse transcriptase (RT) method (5, 18) and by sequencing of 16S rDNA polymerase chain reaction (PCR) products after amplification of 16S rDNA (58), using primers in the vicinity of positions 343, 516, 690, 787, 907, 1100, 1392, and 1500 (according to the *Escherichia coli* map numbering of Brosius et al. [3]).

Amplification of the 16S rDNA was performed by PCR, using conserved regions 10-30 and 1520-1540 as targets for the amplification primers. Template DNA was prepared by heating a cell suspension to 95°C for 10 min (33). PCR reaction products were purified by phenol and chloroform extraction and then precipitated with ethanol. After resuspension in water they were separated by electrophoresis on 0.6% agarose, and the DNA was recovered by freeze-squeeze elution (53). After concentration by ethanol precipitation, the double-stranded products were used directly for sequencing (4).

Sequences were obtained for the freshwater bacteriochlorophyll-synthesizing isolates ACM 2844^T, ACM 2721, ACM 2847 and ACM 2508 and for the marine bacteriochlorophyll-synthesizing reference strains *E. longus* OCh 101^T and *Roseobacter denitrificans* OCh 114^T. Sequences from ACM 2844^T, ACM 2721, *E. longus*, and *Roseobacter denitrificans* were aligned with reference sequences from members of the class *Proteobacteria* (27). Unambiguously alignable regions were compared, and evolutionary distances were calculated by using DNADIST (utilizing the Kimura algorithm) from the PHYLIP v3.2 phylogenetic analysis software package (7); such distances were then used to generate a phylogenetic tree, employing the FITCH treeing algorithm in the same package. Sequence positions are referred to according to the numbering system for *E. coli* of Brosius et al. (3).

Cellular fatty acid analysis. Cells for analysis were grown on Staley PYG medium (ACM 2508, ACM 2721, and ACM 2844^T), casitone-yeast extract agar (ACM 2847), or SM agar (*E. longus* OCh 101^T and *Roseobacter denitrificans* OCh 114^T) for 2 days at 28°C aerobically in the dark. Cells were harvested after 2 days by suspension in 2 ml of water and stored at -20°C for further processing. Saponification of cells and methylation of fatty acids were performed according to the method of Moss et al. (22), with the modification that fatty acid methyl esters (FAMES) were extracted in hexane-methyl *t*-butyl ether (1:1). Analysis of FAMES was performed by gas chromatography via the HP 5898A Microbial Identification System (MIS; Microbial ID Inc., Newark, Del.) using for calibration the chemical standards recommended by the system manufacturers and the biological reference extracts of *Xanthomonas maltophilia*. The diunsaturated nature of 18:2 acids was established, and the identity of other FAMES was confirmed by gas chromatography-mass spectroscopy (GC-MS).

Electron microscopy. Negative staining was performed on cells washed from agar plates with membrane-filtered distilled water. Cells were negatively stained on carbon-coated nitrocellulose-filmed grids with filtered 1% uranyl acetate directly or after a drop of suspension was first mixed with a drop of a bacitracin solution (50 µg ml⁻¹). Cells for thin sectioning were fixed in filtered 3% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.0) at 0°C for 1 h, enrobed in agarose, and cut into cubes. Agarose cubes were washed in cacodylate buffer before postfixation in 1% osmium tetroxide in cacodylate (1 h, 0°C); this was followed by en bloc treatment with 1% aqueous uranyl acetate for 15 min and dehydration in ethanol before embedding in LR White resin, sectioning with an LKB ultramicrotome, and staining of sections with Reynold's lead citrate and uranyl acetate.

FIG. 1. Electron micrograph of ACM 2844^T, displaying pleomorphism and budding swarmer cells. Cells were grown on Schmidt medium III agar for 2 days at 28°C and negatively stained with 1% uranyl acetate. Bar = 1 µm.

Sections were examined with a Hitachi H-800 transmission electron microscope.

Nucleic acid sequence accession numbers. The 16S rDNA nucleotide sequence data reported in this paper for *Porphyrobacter* strain ACM 2721, *Roseobacter denitrificans* OCh 114^T, *P. neustonensis* ACM 2844^T, and *E. longus* OCh 101^T have been submitted to GenBank and assigned accession numbers L01783 to L01786.

RESULTS

Morphology. Four strains of orange- to red-pigmented budding bacteria were isolated either from Nuclepore filter-derived samples of the surface microlayer of a subtropical freshwater pond, University Lake (strains ACM 2844^T, ACM 2847, and ACM 2721) or from the surface of a freshwater swimming pool (ACM 2508 [47]). Cells of these strains are pleomorphic, although they predominantly exhibit rod morphology. On certain media at least, coccoid and ovoid cells can be produced, especially by ACM 2844^T (Fig. 1). Budding occurs in all strains and is predominantly polar in ACM 2844^T, ACM 2721, and ACM 2508 but may be predominantly lateral in ACM 2847. All strains were motile when grown on Staley PYG agar, and, with the exception of ACM 2847, on casitone-yeast extract agar. Cells of all strains possessed subpolar or peritrichous flagella under permissive conditions.

The ultrastructure of the isolates is notable for several features shared with planctomycetes, namely budding, crateriform structures (circular pits visible on the surface of negatively stained cells), and noncellular multifibrillar stalk-like (fascicle) structures (Fig. 1 to 4). Budding is often of a type found in planctomycetes, in which the daughter cell appears as a small spherical form (Fig. 1 and 3). Stalklike structures can be ropelike multifibrillar structures of twisted fibers, or, as in strain ACM 2721 (Fig. 2), dramatically cross-striated ribbonlike structures with distinct holdfastlike tips. The same strain may display both structures. Such stalklike structures are found only on some cells in a preparation. Crateriform structures are most apparent on some cells and buds of ACM 2847; those on the bud in Fig. 3 are 9 to 11 nm in outer diameter (4- to 6-nm inner diameter

FIG. 2. Electron micrograph of ACM 2721 cell possessing a noncellular fibrillar striated stalk (arrow) with holdfast (H). Cells were grown for 2 days on Staley PYG Noble agar and stained with uranyl acetate. Bar = 0.5 μ m. Inset shows an enlarged view of stalk; bar = 100 nm.

of the electron-dense core). This can be compared with the diameter of 12 nm recorded for crateriform structures of planctomycete strain ICPB 4232, a morphotype IV planctomycete (54).

Thin sections of ACM 2844^T indicate the presence of an electron-dense cell wall layer presumably representing peptidoglycan in the mother cell but the absence of an analogous electron-dense layer in the bud (Fig. 4).

Cell wall composition. Analysis of crude cell wall material isolated from all four isolates, ACM 2844^T, ACM 2847, ACM 2721, and ACM 2508, indicated that muramic acid and diaminopimelic acid were both present, consistent with the presence of peptidoglycan in the cell wall and the occurrence of an electron-dense layer in thin sections of the cell wall in ACM 2844^T. Strain ACM 2844^T possessed 0.0645 μ mol of muramic acid mg^{-1} and 0.139 μ mol of diaminopimelic acid mg^{-1} , and strain ACM 2508 possessed 0.0563 μ mol of muramic acid mg^{-1} and 0.106 μ mol of diaminopimelic acid mg^{-1} , while strains ACM 2847 and ACM 2721 possessed intermediate values for each compound per milligram (dry weight) of freeze-dried cell wall preparation. These data are consistent with gram-negative murein (15) and thus with thin-section data suggesting an electron-dense peptidoglycan cell wall layer (23).

Biochemical and nutritional characteristics. All of our isolates produce orange or red colonies, the intensity of pigmentation depending on the growth medium, casitone-yeast extract agar being the optimum for pigment production. The following carbon substrates were utilized as sole sources of carbon and energy by all four isolates: galactose, glucose, maltose, mannose, sucrose, xylose, arginine, glutamine, and proline. The following carbon substrates were used by none of the strains: arabinose, fucose, lactose,

FIG. 3. Electron micrograph of ACM 2847 showing a bud with crateriform structures (arrows) over its surface. Cells were grown on Staley PYG agar for 2 days at 28°C and negatively stained with uranyl acetate. Bar = 100 nm.

FIG. 4. Electron micrograph of a thin-sectioned cell of ACM 2844^T grown on Schmidt III agar for 3 days at 28°C, showing electron-dense layer (arrow) of wall present in mother cell only. Bar = 0.2 μm.

TABLE 1. Biochemical and physiological characteristics which vary among *Porphyrobacter* strains

Characteristic	Result for strain:			
	ACM 2508	ACM 2721	ACM 2844	ACM 2847
Assimilation of:				
Arabinogalactan	+	+	-	-
Cellobiose	+	-	-	-
Fructose	+	+	-	-
Melezitose	-	-	-	+
Raffinose	+	+	-	+
Trehalose	-	-	+	-
Mannitol	-	-	+	-
Alanine	+	+	+	-
Asparagine	+	+	+	-
Histidine	-	-	+	-
Hydroxyproline	+	+	-	-
Isoleucine	+	+	-	+
Ornithine	+	+	+	-
Serine	-	-	+	-
Threonine	-	-	+	-
Fumarate	-	-	-	+
DL-Hydroxybutyrate	-	+	-	-
Pyruvate	-	-	+	-
Succinate	-	-	+	-
Methylamine	+	-	-	-
Hydrolysis of:				
Casein	-	+	-	-
Esculin	-	+	+	-
Colony color ^a	o	r	o	o
Mol% G+C	66.3	65.7	66.4	66.4

^a o, orange; r, red.

lyxose, *N*-acetylglucosamine, rhamnose, ribose, adonitol, arabinol, dulcitol, erythritol, sorbitol, aspartate, cysteine, glutamate, leucine, lysine, methionine, phenylalanine, tryptophan, tyrosine, valine, acetate, citrate, benzoate, caproate, caprylate, formate, lactate, butyrate, glycolate, *m*-hydroxybenzoate, *p*-hydroxybenzoate, L- α -aminobutyrate, 4-amino-*n*-butyrate, malate, phthalate, tartrate, ethanol, methanol, propan-2-ol, propan-1,2-diol, carnosine, and inulin. Results for carbon substrates for which utilization abilities varied with the strain are given in Table 1. Of the C-1 compounds tested, only methylamine was utilized and only by one strain (ACM 2508). Chitin, gelatin, starch, and DNA were not hydrolyzed, but all the strains hydrolyzed Tween 80. Results for casein and esculin were variable (Table 1). All strains were fermentative as shown by acid production in sealed tubes if GNNF medium was used but were inactive if Hayward's modification of Hugh and Leifson medium was used. None of the strains produced gas from glucose in either Hayward's modification of Hugh and Leifson medium or GNNF medium. All strains were catalase positive and oxidase negative. None of the strains reduced nitrate. All strains grew between 10 and 37°C and grew on glucose-mineral salts without added vitamins, on 0.1% peptone medium, and peptone-yeast extract agar. Other characters in which the strains varied are noted in Table 1. However, none of the strains grew anaerobically in the light or the dark in the same medium (Staley PYG broth) supporting growth in aerobic dark conditions. All four strains were sensitive to vancomycin (9- to 15-mm-diameter inhibition zones after 4 days of incubation) and resistant to cycloserine. Strains ACM 2844^T and ACM 2508 were sensitive to penicillin,

while strains ACM 2847 and ACM 2721 displayed only partial inhibition with penicillin.

Photosynthetic pigments. All strains produced bacteriochlorophyll *a* (Bchl *a*) when grown aerobically in the dark on Staley PYG medium, but production of this pigment was repressed on richer media such as casitone-yeast extract agar. Both in vivo and in vitro absorption spectra (Fig. 5 and 6) for extracts of all four isolates (ACM 2844^T, ACM 2847, ACM 2721, and ACM 2508) displayed absorbance maxima characteristic for Bchl *a*. In vivo absorption spectroscopy of cell-free sonicates of the strains displayed peaks at 799 to 806

FIG. 5. In vivo absorption spectrum of sonicated cell extract of strain ACM 2721 showing characteristic Bchl *a* peaks at 799 and 806 nm.

FIG. 6. In vitro absorption spectrum of methanol extract of ACM 2844^T (continuous line) showing characteristic BChl *a* peak at 768.8 nm and methanol extract of ACM 2844^T cells acidified with HCl (dashed line) showing characteristic bacteriopheophytin peak at 748 nm.

nm and 868 to 871 nm, characteristic for BChl *a*-protein complexes (28). Spectra for ACM 2844^T, ACM 2847, and ACM 2721 also displayed Soret bands at 376 nm, also characteristic of BChl *a* (28). An in vivo spectrum for ACM 2721 is shown in Fig. 5. BChl *a* presence was confirmed for all four isolates by in vitro spectra of methanol extracts of cells, which displayed a major peak at 768 to 769 nm (Fig. 6). A characteristic bacteriopheophytin peak at 748 nm was demonstrated by all strains upon acidification of a methanol extract (Fig. 6 displays a spectrum for the type strain).

Cellular fatty acids. As can be seen in Table 2, the dominant fatty acid of our freshwater isolates and the marine reference strains *E. longus* OCh 101^T and *Roseobacter denitrificans* OCh 114^T was octadecenoic acid (18:1). ACM 2721 had a second major fatty acid, heptadecenoic acid (17:1). *Roseobacter denitrificans* was distinguished by the extremely high level of 18:1, with very few other detectable products. *E. longus* and strain ACM 2508 contained high

FIG. 7. Unrooted phylogenetic tree derived from unambiguously alignable positions of complete 16S rRNA sequences derived in this study for *P. neustonensis* ACM 2844^T, *Porphyrobacter* strain ACM 2721, *E. longus* OCh 101^T, *Roseobacter denitrificans* OCh 114^T, data base-derived sequences for 12 reference *Proteobacteria* species, and the proteobacterial sequence SAR83, retrieved from marine biomass (2). *E. coli* was used as an outlier sequence to root the tree.

levels of octadecadienoic acid (18:2), which is rare in prokaryotes. *E. longus* also contained significant amounts of 16:0 and 17:1. Our isolates were similar to *E. longus* but could be distinguished by the higher levels of 3-hydroxy-iso-undecanoic (3OH11:0) and 2-hydroxytetradecanoic acid (2OH14:0) and lower levels of hexadecanoic acid (16:0). Three of our isolates contained some level of octadecadienoic acid (18:2), which was not detectable in strain ACM 2721.

16S rRNA sequencing and phylogenetic analysis. Figure 7 shows the phylogenetic tree, displaying the relationship of two of our isolates, ACM 2844^T and ACM 2721, relative to certain members of the α subclass of the *Proteobacteria*. The dendrogram was derived from analysis of the almost

TABLE 2. Fatty acid composition of *Porphyrobacter* strains, *E. longus*, and *Roseobacter denitrificans*

Fatty acid	% of total fatty acids					
	<i>Porphyrobacter</i> strain ACM 2508	<i>Porphyrobacter</i> strain ACM 2721	<i>Porphyrobacter</i> strain ACM 2844	<i>Porphyrobacter</i> strain ACM 2847	<i>E. longus</i>	<i>Roseobacter</i> <i>denitrificans</i>
n10:0	1.2	0.9	1.1	1.3	ND ^a	1.4
3-OH i11:0	2.4	1.0	1.2	1.6	0.3	ND
n14:0	ND	ND	ND	0.6	ND	ND
2-OH n14:0	4.5	1.8	2.7	3.1	0.4	ND
n15:0	0.8	ND	ND	0.5	2.7	ND
2-OH n15:0	ND	1.9	ND	ND	1.4	ND
n16:0	4.0	2.7	2.6	5.0	9.4	3.7
3-OH i16:0	ND	ND	0.7	ND	ND	0.7
n16:1	1.9	1.8	0.9	2.3	0.7	ND
n17:0	ND	1.6	ND	ND	11.0	ND
i17:0	ND	ND	ND	ND	1.6	ND
n17:1	ND	32.0	ND	5.5	5.5	ND
n18:0	1.2	ND	ND	ND	0.4	3.4
n18:1	71.7	47.0	84.0	74.9	57.4	89.5
n18:2	13.8	ND	6.1	2.3	14.6	ND
11,12cy19:0	1.5	ND	ND	ND	ND	ND

^a ND, not detected.

complete 16S rRNA sequences generated in this study for ACM 2844^T, ACM 2721, *E. longus* OCh 101^T, and *Roseobacter denitrificans* OCh 114^T. Evolutionary distance values used for tree generation are shown in Table 3. rRNA and rDNA sequencing gave complete agreement.

A total of 1,168 positions were used for phylogenetic comparison. Signature sequences and secondary structural features at the stems from positions 180 to 220 and 455 to 480 show clearly that our isolates are related to members of the α subdivision of the class *Proteobacteria* (35, 48, 59). Strains ACM 2844^T and ACM 2721 both showed a closer relationship to *E. longus* (evolutionary distance, less than 0.035 [Table 3]; the similarity of complete sequence of ACM 2844^T to *E. longus* is 92.6%) than to any other member of the data set, and these strains together with *E. longus* form an isolated group (α -4) within the α subclass (59). They show no close relationship to other bacteriochlorophyll-synthesizing or budding bacteria, including *Roseobacter denitrificans* (synonymous with *Erythrobacter* strain OCh 114), and are also, as expected for a member of the *Proteobacteria*, very distant from the representative planctomycete *Pirellula staley*. Partial sequences for strains ACM 2508 and ACM 2847 showed greater than 98% sequence similarity to ACM 2844^T and hence were not included in the phylogenetic analysis. Our isolates could be distinguished from *E. longus* on the basis of signature sequences at positions 738 to 747 and 1254 to 1280, which were conserved not only in the four study strains, ACM 2844^T, ACM 2847, ACM 2721, and ACM 2508, but also in 10 other isolates of similar bacteria from south-east Queensland (unpublished data). These freshwater strains tended to cluster together (data not shown) to the exclusion of *E. longus*.

DISCUSSION

On the basis of distinctive ultrastructural features combined with 16S rRNA sequences, DNA base composition, cellular fatty acid composition, and nutritional data, all our freshwater isolates of aerobic bacteriochlorophyll-synthesizing bacteria are proposed to be members of a new genus, *Porphyrobacter*, with type species *P. neustonensis*, comprising the three strains ACM 2844^T, ACM 2847, and ACM 2508 (ACM 2721 is an allied but distinct strain). The freshwater *P. neustonensis* is a close relative of the marine *E. longus*, and these genera form the α -4 group of the α subclass of the *Proteobacteria*. However, they may be distinguished readily on the basis of 16S rRNA sequences, and the consistent genotypic and phenotypic separation of the two taxa warrants their placement in separate genera. Since there is no standard phylogenetic distance defining taxonomic ranks, other data must be used for assigning these strains to separate taxa of appropriate rank. On the basis of the G+C content of their genomic DNA, the genera are easily separated, since *E. longus* OCh101^T has a G+C content of 57.4 mol% (40), while the *Porphyrobacter* type strain has a G+C content of 66.4 mol%. They share the trait of aerobic bacteriochlorophyll synthesis but can be distinguished on the basis of chemotaxonomic and phenotypic data. More similar on the basis of G+C content is the freshwater aerobic bacteriochlorophyll synthesizer "*E. sibiricus*," but no 16S rRNA sequence data exist for that species and thus its phylogenetic relationship to *E. longus* has not been established. In addition, it differs from *P. neustonensis* in oxidase and catalase reactions as well as in its in vivo photosynthetic pigment spectrum and requirement for vitamins (61). When compared with members of the α

subclass outside the α -4 cluster, *Porphyrobacter* strains are phenotypically most similar to some *Rhodospseudomonas* species, in that they bud without forming hyphae, sometimes form rosettes, synthesize BChl *a*, and possess DNA with a G+C content within the range indicated for the latter genus (55). The exact mode of budding, intracellular membrane type, and photosynthetic physiology differ, however, and phylogenetically they are distinct, as shown in the present study. *Porphyrobacter* strains are not closely related to the other described strain of *Erythrobacter*, OCh 114, which was recently assigned as the type strain of *Roseobacter denitrificans* (40). Most of the published physiological data pertaining to the photosynthetic abilities and apparatus of *Erythrobacter* species refer to this strain rather than to *E. longus* (e.g., references 10, 11, 14, and 42). Sequence data generated in the present study show *Roseobacter denitrificans* to branch separately from the cluster of *E. longus* and *Porphyrobacter* strains; it branches more closely to the marine 16S rRNA sequence SAR 83, known only from 16S rDNA sequence retrieval via cloning from bacterioplankton genomic DNA of Sargasso Sea seawater, confirming phylogenetic results of the original study that revealed the SAR 83 sequence (2).

Although *Porphyrobacter* strains possess ultrastructural characters such as crateriform structures and multifibrillar stalklike or fasciclelike structures resembling those characteristic of budding eubacteria in the order *Planctomycetales*, they are phylogenetically only remotely related to the planctomycetes. Correlated with this absence of evolutionary relationship are the differences in the chemistry of the cell wall and the sensitivity to antibiotics directed against the synthesis of peptidoglycan (9, 16). Thus, the occurrence of noncellular stalklike structures and of crateriform structures combined with budding reproduction in *Porphyrobacter* strains appears to be an interesting example of phenotypic convergence, perhaps reflecting habitat similarities, rather than a reflection of phylogenetic relationship. The utility of 16S rRNA sequencing employed here as a tool to resolve several possibilities of relationship is clearly demonstrated by the solution of the problem posed by superficial phenotypic resemblance to planctomycetes.

Cellular fatty acids. The strains examined for cellular fatty acids could be separated into three groups on the basis of the resulting profiles. These groups correlated well with the phylogenetic 16S rRNA sequence analysis. *Roseobacter denitrificans* (formerly *Erythrobacter* sp. strain OCh 114) was characterized by an extremely high proportion (89% of total fatty acids) of octadecenoic acid (18:1) and very few other detectable fatty acids. Those strains which clustered together as the α -4 subclass in the phylogenetic analysis again showed a close relationship to each other, forming a second and third group with similar profiles. The second group comprised the marine strain *E. longus*, and the third group comprised the freshwater *Porphyrobacter* strains ACM 2844^T, ACM 2847, and ACM 2508. These groups could be distinguished on the basis of the minor components of their fatty acid profiles. One of the *Porphyrobacter* strains classified on the basis of 16S rRNA sequencing, ACM 2721, could not be definitively placed either with the other *Porphyrobacter* strains or with *E. longus* on the basis of fatty acid profile. It lacked 18:2 and possessed a second major fatty acid, heptadecenoic acid (17:1), found only at low levels or not at all in the other strains. Other aerobic bacteriochlorophyll-synthesizing proteobacteria, such as *Methylobacterium* species, were not included in this study. However, published results for such strains (57) demonstrate

TABLE 3. Matrix of evolutionary distances between 16S rRNA sequence pairs from *Porphyrobacter* strains and other members of the α subclass of the *Proteobacteria*

Species or strain	Evolutionary distance														
	<i>P. neustonensis</i> ACM 2844	<i>Porphyro- bacter</i> strain ACM 2721	<i>E. longus</i>	<i>H. baltica</i>	Sar83	<i>R. denitri- ficans</i>	<i>R. sphaer- oides</i>	<i>R. capsu- latus</i>	<i>R. ful- vum</i>	<i>R. cent- enum</i>	<i>H. vul- gare</i>	<i>R. palus- tris</i>	<i>M. organo- philum</i>	<i>M. extor- quens</i>	<i>A. tun- faciens</i>
<i>Porphyrobacter</i> strain ACM 2721	0.0164														
<i>Erythrobacter longus</i>	0.0333	0.0346													
<i>Hirschia baltica</i>	0.1496	0.1490	0.1455												
SAR83	0.1363	0.1339	0.1378	0.1354	0.0671										
<i>Roseobacter denitrificans</i>	0.1542	0.1553	0.1446	0.1435	0.0809	0.0796									
<i>Rhodobacter sphaeroides</i>	0.1345	0.1310	0.1308	0.1442	0.0860	0.1766	0.1547	0.1515							
<i>Rhodobacter capsulatus</i>	0.1532	0.1473	0.1433	0.1454	0.0950	0.1607	0.1375	0.1214	0.1100						
<i>Rhodospirillum rubrum</i>	0.1359	0.1332	0.1385	0.1817	0.1500	0.1766	0.1547	0.1515	0.1100						
<i>Rhodospirillum centenum</i>	0.1426	0.1369	0.1418	0.1851	0.1500	0.1684	0.1375	0.1214	0.1100						
<i>Hyphomicrobium vulgare</i>	0.1163	0.1148	0.1240	0.1633	0.1278	0.1346	0.1178	0.1117	0.1239	0.1264					
<i>Rhodopseudomonas palustris</i>	0.1260	0.1224	0.1275	0.1690	0.1463	0.1674	0.1296	0.1274	0.1287	0.1315	0.1039				
<i>Methylobacterium organophilum</i>	0.1136	0.1131	0.1183	0.1693	0.1335	0.1510	0.1163	0.1244	0.1213	0.1177	0.1007	0.0835			
<i>Methylobacterium extorquens</i>	0.1127	0.1122	0.1195	0.1650	0.1326	0.1468	0.1144	0.1212	0.1235	0.1230	0.0988	0.0916	0.0194		
<i>Agrobacterium tumefaciens</i>	0.1167	0.1090	0.1133	0.1475	0.1303	0.1456	0.1143	0.1244	0.1245	0.1386	0.1037	0.1191	0.0923	0.0953	
<i>Rhodospirillum rubrum</i>	0.1136	0.1059	0.1173	0.1379	0.1262	0.1477	0.1204	0.1189	0.1245	0.1386	0.0987	0.1118	0.0942	0.0963	0.0512
<i>Ehrlichia risticii</i>	0.1857	0.1872	0.1876	0.2276	0.2046	0.2088	0.1975	0.2033	0.1987	0.2137	0.1981	0.1919	0.1965	0.1945	0.2022

no close similarities in fatty acid profiles for *Porphyrobacter* and *Methylobacterium* spp. Given the rarity of diunsaturated fatty acids in bacteria, the 18:2 compound may prove to be a useful marker for the α -4 group of the *Proteobacteria*; that is, *Erythrobacter* and *Porphyrobacter* spp. 18:2 fatty acids have also been found in *Hirschia*, *Gemmobacter*, *Blastobacter*, and *Campylobacter* spp. (22, 31, 35) but not at such high levels. Its absence from ACM 2721 suggests limits to its usefulness for identifying members of the α -4 subclass, however. It should be noted that salt concentration can affect bacterial fatty acid content (51), and this limits the use of this type of data to infer evolutionary relationships. There is no growth medium common to both strains to allow culture under identical salt conditions.

Photosynthetic pigments. Like several other members of the α -subdivision of the *Proteobacteria*, including the aerobic photosynthetic bacteria *E. longus* and *Roseobacter denitrificans*, *Porphyrobacter* strains synthesize BChl *a* when grown aerobically. In vitro spectra displaying ca. 770-nm peaks are consistent with the presence of BChl *a*, as is the bacteriopheophytin peak at 748 nm observed on acidification (25). The presence of peaks in the region of 800 and 870 nm in the in vivo spectra of sonicated extracts of *Porphyrobacter* strains is consistent with the presence of BChl *a*-protein complexes (28), and these peaks are similar but not identical to those found in in vivo (sucrose suspensions) spectra of cell suspensions of *E. longus* (41), which exhibited a major peak at 863 to 867 nm, and differ from those found for "*E. sibericus*," which possessed a major peak at 863 nm, a minor peak at 875 nm, and a shoulder at 817 nm (61).

Conclusions. Our four isolates cannot be assigned to any of the previously described genera of aerobic bacteriochlorophyll-synthesizing bacteria. Despite some ultrastructural similarities with members of the *Planctomycetales*, our strains are clearly members of the class *Proteobacteria* and are phylogenetically distant, as is this class as a whole, from the planctomycetes. 16S rRNA sequence data indicate that these bacteria share a position within the α -4 subclass of the *Proteobacteria* with *E. longus*. However, they are distinguishable in their 16S rRNA sequences from *E. longus* and differ from *E. longus* in DNA base composition and the following characteristics: *Porphyrobacter* strains do not require vitamins for growth, in contrast to the biotin-requiring *E. longus*; cannot utilize acetate, glutamate, or butyrate or hydrolyze gelatin; and are all cytochrome *c* oxidase negative. There are slight quantitative differences in cellular fatty acids between *Porphyrobacter* strains and *E. longus*, namely consistently higher amounts of 3-hydroxy iso undecanoic acid (3-OH i11:0) and 2-hydroxytetradecanoic acid (2-OH n14:0) and lower amounts of hexadecanoic acid (16:0) in *Porphyrobacter* strains and detectable though small amounts of n10:0 in *Porphyrobacter* strains.

Description of *Porphyrobacter* gen. nov. *Porphyrobacter* (Por.phy.ro.bac.ter. Gr. adj. *porphyreos*, purple; Gr. n. *bacter*, rod; M.L. masc. n. *Porphyrobacter*, porphyrin-producing rod, referring to bacteriochlorophyll production). Gram-negative pleomorphic motile rods or cocci, reproducing by polar growth or budding. Capable of producing multifibrillar stalklike fascicle structures and crateriform structures on the cell surface. Aerobic. Chemoheterotrophic. Synthesizes BChl *a* on low-nutrient media under aerobic and semiaerobic conditions. DNA base composition is 65.7 to 66.4 mol% G+C (by thermal denaturation). Member of the α -4 group of the α subclass of *Proteobacteria*. The type species is *P. neustonensis*.

Description of *Porphyrobacter neustonensis* sp. nov. *Porphy-*

robacter neustonensis (neu.sto.nen'sis M.L. masc. adj. derived from Gr. n. *neustos*, swimming [floating], referring to occurrence of the bacterium as a member of the neuston [organisms floating at the air-water interface surface layer of a body of water]). Colonies pigmented orange or red. Octadecenoic acid (18:1) is the major cellular fatty acid. Utilizes glucose, galactose, maltose, mannose, sucrose, xylose, arginine, glutamine, and proline as sole carbon sources; does not utilize acetate, glutamate, or butyrate or require vitamins. Lives at the air-water interface of freshwater subtropical pond. G+C content of 65.7 to 66.4 mol%. Type strain, ACM 2844.

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