Roseicyclus mahoneyensis gen. nov., sp. nov., an aerobic phototrophic bacterium isolated from a meromictic lake

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Eight strains of Gram-negative bacteria able to form ring-like cells were isolated from Mahoney Lake, a meromictic lake in south-central British Columbia, Canada. All strains were pink–purple and contained bacteriochlorophyll *a* incorporated into the light-harvesting 1 and 2 and reaction-centre pigment–protein complexes. Growth did not occur anaerobically under illuminated conditions; these strains were obligately aerobic, prompting their designation as members of the aerobic phototrophic bacteria. Physiological characterization revealed that these isolates share a similar tolerance to high levels of salinity and pH, as would be expected of bacteria from a highly saline lake; however, the strains exhibited marked differences in their ability to utilize organic substrates for aerobic heterotrophic growth. 16S rRNA sequence analysis showed that the strains are closely related to members of the non-phototrophic genera *Octadecabacter* (92.0–92.9%) and *Ketogulonicigenium* (92.2–92.6%), as well as to aerobic phototrophs of the genera *Roseivivax* (92.2–92.9%) and *Roseovarius* (91.7–92.4%) within the 'Alphaproteobacteria'. The DNA G + C content was 66.2 mol%. The unusual light-harvesting complex 2, the distinct morphological features and physiological traits of these strains as well as the phylogenetic data support the proposal of the novel genus and species *Roseicyclus mahoneyensis* gen. nov., sp. nov., with ML6^T (= DSM 16097^T = VKM B-2346^T) as the type strain.

INTRODUCTION

Mahoney Lake is a meromictic saline lake located in the south-central region of British Columbia, Canada. Interesting features of this lake include a sharp chemical discontinuity at the chemocline and high concentrations of Na⁺, Ca²⁺, Mg²⁺ and SO₄²⁻, leading to Mahoney Lake's classification as a sodium sulfate-dominated lake (Northcote & Halsey, 1969; Hall & Northcote, 1986). The primary focus of most previous microbiological investigations of Mahoney Lake has been on the extremely dense population of the purple sulfur bacterium *Amoebobacter purpureus* (Overman *et al.*, 1991, 1994, 1996). However strains of the purple sulfur bacterium *Thiocapsa roseopersicina*, the purple non-sulfur bacterium *Rhodobacter*

capsulatus and the green sulfur bacteria *Chloroherpeton thalassium* and *Prosthecochloris aestuarii* have also been isolated from the chemocline (Overman *et al.*, 1991). In 1997, 33 aerobic bacteriochlorophyll (Bchl)-containing strains were isolated from the oxic mixolimnion, between the surface and a depth of 5 m. These strains included both purple non-sulfur bacteria and aerobic anoxygenic phototrophic bacteria, and exhibited a variety of interesting spectral absorption properties and morphologies (Yurkova *et al.*, 2002).

Aerobic bacteria that contain Bchl, commonly known as aerobic phototrophic bacteria (APB), are a relatively recently discovered and taxonomically diverse group. The primary distinguishing features of the APB are the presence of Bchl incorporated into light-harvesting (LH) and reaction-centre complexes, the relatively low level of photosynthetic units per cell, the inhibition of Bchl synthesis by light, the inability to grow phototrophically under anaerobic conditions, the high mid-point potential of the

Abbreviations: APB, aerobic phototrophic bacteria; Bchl, bacteriochlorophyll; LH, light-harvesting.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ML6^T is AJ 315682.

reaction-centre primary electron carrier and the abundance of carotenoid pigments (Yurkov & Beatty, 1998; Rathgeber *et al.*, 2004).

The first-reported member of the APB, *Erythrobacter longus*, is an orange-pigmented rod-shaped bacterium that was isolated from the Bay of Tokyo some 20 years ago (Shiba *et al.*, 1979; Shiba & Simidu, 1982). Since that time, other APB displaying a wide range of morphologies have been described. Members of the APB have been shown to produce typical rod shapes, elongated rods displaying thread-like cells and branching, ovoid cells and coccoid cells (Yurkov & Beatty, 1998); in addition, a highly pleomorphic member of this group, '*Citromicrobium bathyomarinum*' (Yurkov *et al.*, 1999), has been described. Although several vibrioid and spirillum-shaped genera and species have been described for the purple non-sulfur bacteria (Imhoff, 2001), no vibrioid species that are members of the APB have been identified. Here, we describe eight closely related vibrioid strains from Mahoney Lake as novel members of the APB.

METHODS

Strains and cultivation. Strains ML6^T, ML16 and ML18 were isolated and cultivated on a rich organic medium, medium N1 (Yurkova *et al.*, 2002) (containing 15 g Na₂SO₄ l⁻¹), and strains ML33, ML38, ML39, ML40 and ML44 were isolated and cultivated on Medium N4 (containing 50 g Na₂SO₄ l⁻¹) (Yurkova *et al.*, 2002). These media were used for all of the following experiments unless otherwise noted. Strains were kept on agar plates of the above media at 4 °C for up to 2 months for short-term storage. Long-term storage was achieved by freezing (at -70 °C) a thick cell suspension in the above-mentioned media supplemented with 30 % glycerol as a cryoprotectant.

Morphological and cytological tests. The cell size, shape and motility of late-exponential-phase cultures were determined by using phase-contrast light microscopy (Zeiss Axioskop 2 equipment). Electron-microscopic negative stains were performed by staining cells with 1.0 % aqueous uranyl acetate. For thin sections, the bacteria were embedded in Epon (Canemco) after fixation with 2.5 % glutaraldehyde and 1.0 % osmium tetroxide as described by Kellenberger *et al.* (1958).

Physiological and biochemical tests. The utilization of soluble organic substrates, Tweens, gelatin and starch, the ability to grow at different pH levels, the ability to ferment sugars and reduce nitrate, the ability to produce catalase and oxidase and antibiotic sensitivity were determined as previously described (Yurkov & van Gemerden, 1993; Yurkov *et al.*, 1994). Growth at different salinities was tested in tubes containing the above-described media with NaCl or Na₂SO₄ at concentrations of 0, 0.5, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0 or 14.0 %, incubated aerobically on a test-tube rotor at 30 °C. Photoheterotrophic growth under anaerobic conditions was tested in screw-capped test tubes and in agar deeps containing the above-described media, a basal medium, designed for purple sulfur bacteria (Imhoff, 1988), containing H₂S or Na₂S₂O₃ and CO₂ with or without acetate, or a basal medium for purple non-sulfur bacteria (Imhoff, 1988) containing acetate, malate or succinate as the sole source of organic carbon. Tubes were incubated at 30 °C with tungsten-filament lamp illumination of about 30 µE m⁻² s⁻¹.

Pigment analysis. Spectral absorption measurements were performed on strains grown under aerobic conditions in liquid culture.

Cell samples were collected by centrifugation and resuspended in 125 µl 10 mM Tris/HCl buffer (pH 7.8) and added to 375 µl 30 % BSA solution (ICN Biomedicals) to reduce light-scattering. Absorption spectra were recorded using a Hitachi U-2010 spectrophotometer.

DNA G+C content determination. The DNA G+C content was determined by using HPLC (with Shimadzu apparatus) (Tamaoka & Komagata, 1984) of nucleotides obtained according to Mesbah & Whitman (1989).

16S rRNA gene sequence analysis. Extraction of genomic DNA, PCR-mediated amplification of the 16S rRNA gene sequences and direct sequencing of the purified PCR products were carried out as described by Rainey *et al.* (1996). The sequence reaction mixtures were electrophoresed using a model 373A automatic DNA sequencer (Applied Biosystems). The partial 16S rRNA gene sequences were aligned with published sequences obtained from the EMBL nucleotide sequence database and the Ribosomal Database Project, using the ae2 editor (Maidak *et al.*, 1996), so that similarity values could be determined.

RESULTS AND DISCUSSION

Isolation

The strains examined in this taxonomic description were isolated from the meromictic Mahoney Lake in south-central British Columbia, Canada, in October 1997. Details of the isolation and enumeration of these bacteria were given by Yurkova *et al.* (2002).

Culture properties

All strains formed small, ~2 mm-diameter pink-purple to purple colonies on the surface of agar media. In liquid media under aerobic conditions, cultures appear pink-purple after 24 h, becoming more purple with age.

Growth did not occur anaerobically in either light or dark conditions, and light was not required for growth under aerobic conditions, which led us to designate them as APB (Yurkov & Beatty, 1998).

Morphology and cytology

Morphology was examined in exponential-phase cells grown in rich organic medium (described above) under aerobic conditions. Strains ML6^T, ML16, ML18, ML33, ML38, ML39, ML40 and ML44 shared a similar morphology, which varied from elongated rods to slightly curved rods to vibrioid, almost cyclical cells (Fig. 1a). Elongated rods were 0.6 × 2.6 µm in size, while vibrioid cells were 0.6–0.7 × 2.7–3.0 µm. This morphology is similar to that of the purple non-sulfur bacterium *Rhodocyclus purpureus* (Pfennig, 1978), except that our isolates had pointed ends rather than flat to rounded ends.

Transmission electron microscopy (using Zeiss 10C equipment) of negatively stained cells showed the pointed regions at the poles to be electron dense (Fig. 1b), while electron microscopy of thin sections indicated that these polar zones are actually due to an enlarged periplasmic space found in

Fig. 1. (a) Phase-contrast microscopy of strain ML6^T showing cyclical shaped cells. (b) Negatively stained cells with polar dark colouration of pointed ends. (c, d) Ultrathin sections of cells show polar periplasmic modifications [confirming polar staining in (b); indicated by arrows]. Bars: (a), 5 µm; (b), 0.5 µm; (c, d), 0.25 µm.

both rod-shaped and vibrioid cells (Fig. 1c, d). The appearance is similar to that seen in *Rhodospirillum tenue* (Pfennig, 1969), although the significance of these polar, periplasmic structures is unclear. Polar localization of proteins critical for cell division, chromosome partitioning and cell-cycle control in *Escherichia coli*, *Bacillus subtilis* and *Caulobacter crescentus* have been described. Bacterial polarity seems to play a critical role in cell structure and life cycles (Lybarger & Maddock, 2001).

The above-mentioned strains are all non-motile. Cell division in strain ML6^T occurs by way of symmetric and asymmetric constrictions.

The Gram-negative structure of the cell wall was confirmed by the electron-microscopic thin sections, but intracytoplasmic membrane formations, of the type usually found in true anoxygenic phototrophs, were not observed, which is typical of the APB (Fig. 1c, d). Cells contained electron-clear inclusions presumably due to storage of poly-β-hydroxyalkanoates.

The unusual morphological characteristics of these strains, ranging from rod shapes through vibrioid shapes to cyclical shapes, as well as the pointed periplasmic space, have not been previously reported in the APB (Yurkov & Beatty, 1998). Thus, these isolates are an exciting new addition to this already morphologically diverse group.

Photosynthetic apparatus

Absorption spectra for the representative strain ML6^T are shown in Fig. 2. As for all the strains in the present study, the spectra showed *in vivo* Bchl *a* peaks at 805–806 nm and at 870–871 nm. The 870–871 nm peak is indicative of the LH1 complex, and the peak at 805–806 nm is indicative of a peripheral LH2 complex. This unusual organization of the

Fig. 2. Absorption spectra of intact cells of strain ML6^T grown aerobically in the dark (solid line), showing peaks at 805 and 870 nm corresponding to Bchl incorporated into the LH2 and LH1 complexes, respectively. These peaks are reduced or absent in cells grown aerobically in the presence of continuous illumination (broken line).

photosynthetic apparatus, where the LH2 complex has only one peak at approximately 805 nm, has been found in only two genera of the APB so far, namely *Roseobacter* (Shiba,

1991) and *Rubrimonas* (Suzuki *et al.*, 1999), both of which have morphological and physiological characteristics quite distinct from those of our isolates. The presence of a

Table 1. Comparative physiological characteristics of the aerobic phototrophic strains isolated from Mahoney Lake and close phylogenetic relatives

Symbols: +, substrate is utilized, substrate is hydrolysed or antibiotic-sensitive; ++, substrate is utilized for very good growth; –, substrate is not utilized, substrate is not hydrolysed or antibiotic-resistant; W, very weak growth; NG, no growth; NA, not available. Reference strains were *Roseobacter litoralis* (Och 149^T), *Roseivivax halodurans* (Och 239^T) and *Roseovarius tolerans* (EL-172^T). None of the strains shown utilized methanol and all were sensitive to chloramphenicol.

Characteristic	Mahoney Lake strains								Reference strains		
	ML6 ^T	ML16	ML18	ML33	ML38	ML39	ML40	ML44	Och 149 ^T	Och 239 ^T	EL-172 ^T
Growth at:											
4 °C	–	–	–	–	+	–	–	–	+		+
10 °C	+	–	–	+	+	+	+	+	+		++
28 °C	++	++	++	++	++	++	++	++	+	+	++
37 °C	+	–	–	+	+	+	+	+	–		+
45 °C	–	–	–	–	–	–	–	–	–		–
Growth at pH:											
5.5	–	–	–	–	–	–	–	–	–		–
6.0	++	++	++	++	++	++	++	++	–		–
7.0	++	++	++	++	++	++	++	++	–		+
8.0	++	++	++	++	++	++	++	++	+	+	+
9.5	++	++	++	++	++	++	++	++	+		NA
10.0	++	++	++	++	++	++	++	++	–		NA
11.0	++	++	++	+	+	+	+	+	–		NA
Utilization of:											
Acetate	+	+	+	–	–	–	W	–	+	+	+
Pyruvate	+	+	+	–	+	–	W	W	+	+	+
Glutamate	+	+	+	–	+	–	–	W	+	+	+
Butyrate	–	+	–	–	–	–	–	–	–	+	+
Citrate	+	–	+	W	+	+	+	+	+	+	–
Malate	+	+	+	–	+	–	–	–	+	+	+
Succinate	+	+	+	–	+	–	–	W	+	+	+
Lactate	+	+	+	–	+	–	W	+	NA	+	–
Formate	–	–	–	–	–	–	–	–	NA	NA	NA
Fructose	++	++	++	–	+	+	+	+	NA	+	+
Glucose	++	++	++	–	+	+	–	+	+	+	–
Ethanol	–	–	–	–	–	–	–	–	NA	–	NA
Yeast extract	++	++	++	+	++	++	++	++	+	+	+
Hydrolysis of:											
Starch	+	+	NG	–	–	–	–	–	–	NA	–
Gelatin	+	+	+	+	+	+	+	+	+	–	–
Tween 60	–	–	–	–	+	NG	+	+	+	NA	NA
Antibiotic sensitivity											
Penicillin G	–	–	–	–	–	–	–	–	+	–	+
Streptomycin	+	+	–	–	–	–	–	–	+	+	+
Polymixin B	+	+	+	+	+	+	+	+	+	NA	–
Tetracycline	+	+	+	+	+	+	+	–	NA	–	+
Ampicillin	–	–	–	–	–	–	–	–	NA	NA	NA
Kanamycin	+	+	–	–	–	–	–	–	NA	NA	NA
Nalidixic acid	–	–	–	–	–	–	–	–	NA	NA	NA

*Only optimum temperature and pH have been published (Suzuki *et al.*, 1999).

These isolates lack absorption peaks in both the 805 and 870 nm regions when grown aerobically in the presence of continuous illumination (as shown for strain ML6^T in Fig. 2). This trait further confirms the identification of these strains as members of the APB. All strains show similar carotenoid peaks at approximately 408 and 484 nm.

A variety of physiological characteristics are presented in Table 1. All of the isolates produce both catalase and cytochrome *c* oxidase.

All strains tolerate a wide range of pH values and grow between pH 6.0 and 11.0, the latter being the highest value tested.

The isolates described in this paper cannot be repeatedly transferred and cultivated on minimal media in the absence of yeast extract. This indicates that they require an unknown growth factor present in yeast extract. Other strains from Mahoney Lake have been shown to be dependent on vitamin B₁₂ and/or biotin (Yurkova *et al.*, 2002); however, the addition of these vitamins to minimal media was not sufficient to allow the successive cultivation of these isolates.

antibiotics (Table 1). Again, this trait does not appear to be a useful taxonomic marker, as highly similar, highly related strains show markedly different responses to the antibiotics tested.

On the basis of the analysis of almost-complete 16S rRNA gene sequences (>1430 nucleotides), strain ML6^T is a member of the α -3 group of the *Proteobacteria*, within the *Roseobacter* clade (Fig. 3). On the basis of the analysis of the algorithm of De Soete (1983), neighbour-joining and maximum-likelihood, strain ML6^T, along with the purple non-sulfur strain ML42 (Yurkova *et al.*, 2002), define a novel lineage. As bootstrap values are at this level very low, the branching point may change when novel sequences are included. The phylogenetic distance between ML6^T and ML42 (96.9%) is like that which is generally found for well-separated species. However, physiological differences clearly place them in different genera (Yurkova *et al.*, 2002). Strain ML6^T shares less than 93.0% 16S rRNA gene sequence similarity with its phylogenetically closest taxonomically characterized relatives, i.e. members of the non-phototrophic genera *Octadecabacter* (92.0–92.9%) and *Ketogulonicigenium* (92.2–92.6%) and the APB

species *Roseivivax halodurans* (92.2–92.9%) and *Roseovarius tolerans* (91.7–92.4%).

Concluding remarks

The presence of Bchl *a* incorporated into the reaction centre, the LH1 and LH2 complexes, the inability to grow photoheterotrophically under anaerobic conditions, the strong inhibition of Bchl synthesis by light and the absence of an intracytoplasmic membrane system prompt us to conclude that all of the novel strains described in this work are indeed members of the APB.

The phylogenetic analysis revealed that these strains have identical rRNA gene sequences and constitute a distinct branch closely related to the chemotrophic genera *Octadecabacter* and *Ketogulonicigenium*, as well as to phototrophs of the genera *Roseivivax* and *Roseovarius*.

Morphological, physiological and biochemical properties allow us to differentiate the novel strains easily from their close phylogenetic neighbours in the genera *Octadecabacter* and *Ketogulonicigenium*. Members of the genus *Octadecabacter* are obligate psychrophiles and form rod-shaped cells containing gas vacuoles (Gosink *et al.*, 1997), whereas *Ketogulonicigenium* species are facultatively anaerobic, ovoid rods that exhibit relatively narrow pH, temperature and salinity ranges (Urbance *et al.*, 2001). Neither *Octadecabacter* nor *Ketogulonicigenium* species form the vibrioid or cyclical cells characteristic of our Mahoney Lake isolates, and neither contain Bchl *a* or carotenoid pigments, although all species of *Ketogulonicigenium* produce an unidentified, water-soluble, brown pigment.

Additionally, the strains represented by ML6^T differ significantly from their closest phototrophic relatives, members of the genera *Roseivivax* and *Roseovarius*, in terms of their cellular morphology and photosynthetic LH apparatus. Although both *Roseivivax* and *Roseovarius* were isolated from a similar habitat (i.e. a saline lake) and exhibit broad tolerance to saline conditions similar to that found in the Mahoney Lake isolates, they do not produce a peripheral LH2 complex and form normal motile rods (Suzuki *et al.*, 1999; Labrenz *et al.*, 1999).

On the basis of these important taxonomic markers and the low 16S rRNA gene sequence similarity (<93.0%) between the novel isolates and their closest phylogenetic relatives, we propose the novel genus *Roseicyclus*, with *Roseicyclus mahoneyensis* as the type species.

Description of *Roseicyclus* gen. nov.

Roseicyclus (Ro.se.i.cyc'lus. L. adj. *roseus* rose, pink; L. masc. n. *cyclus* cycle; N.L. masc. n. *Roseicyclus* pink cyclic bacterium).

Cells are Gram-negative and are ovoid to elongated rods, vibrioid or almost cyclical. Produce pointed enlarged areas of the periplasm located at the poles. Non-motile. Divide by

means of symmetric or asymmetric constrictions. Cultures are pink–purple to purple due to the production of carotenoids and Bchl *a*. Produce both LH1 complex, with an absorption peak at 870–871 nm, and an interesting LH2 complex with one absorption peak at 805–806 nm. No growth occurs anaerobically in the light. Obligately aerobic; no fermentation or dissimilatory denitrification observed. The habitat of the first isolated strains is a saline, Na₂SO₄-dominated lake. Member of the 'Alphaproteobacteria'. The type species is *Roseicyclus mahoneyensis*.

Description of *Roseicyclus mahoneyensis* sp. nov.

Roseicyclus mahoneyensis (ma.ho.ney.en'sis. N.L. masc. adj. *mahoneyensis* from Mahoney Lake, where the species was originally isolated).

Shows the following properties in addition to those given for the genus. Cells are elongated rods (0.6 × 2.6 µm) or vibrioid cells (0.6–0.7 × 2.7–3.0 µm). Bchl gives *in vivo* absorption spectrum peaks at 805–806 and 870–871 nm. Aerobic organoheterotroph and facultative photoheterotroph. Best substrate for growth is yeast extract; growth also occurs on acetate, pyruvate, glutamate, butyrate, citrate, malate, succinate, lactate, fructose and glucose, depending on the strain. Strains differ in their ability to hydrolyse starch and Tween 60; all strains hydrolyse gelatin. Optimum temperature for growth is 30 °C, with growth occurring at temperatures as low as 4 °C and as high as 37 °C, depending on the strain. Absolute requirement for saline conditions, with growth occurring over a wide range of NaCl and Na₂SO₄ concentrations, from 0.5 to 10%. Growth occurs over a wide range of pH values, from pH 6.0 to 11.0. May or may not be resistant to a variety of antibiotics including penicillin G, streptomycin, tetracycline, ampicillin, kanamycin and nalidixic acid, depending on the strain. Requires an unidentified growth factor present in yeast extract. The DNA G + C content is 66.2 mol%.

The habitat of the first isolated strains is the meromictic saline Mahoney Lake in south-central British Columbia, Canada. The type strain is ML6^T (=DSM 16097^T = VKM B-2346^T).

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