Porphyrobacter tepidarius sp. nov., a Moderately Thermophilic Aerobic Photosynthetic Bacterium Isolated from a Hot Spring

SATOSHI HANADA,1 YOSHIE KAWASE,1 AKIRA HIRAISHI,2 SHINICHI TAKAICHI,3 KATSUMI MATSUURA,1 KEIZO SHIMADA,1 AND KENJI V. P. NAGASHIMA1

Department of Biology, Tokyo Metropolitan University, Hachioji 192-03,1 Department of Ecological Engineering, Toyohashi University of Technology, Toyohashi 441,2 and Biological Laboratory, Nippon Medical School, Kawasaki 211,3 Japan

A new thermophilic bacterium, strain OT3T (T = type strain), was isolated from a brackish hot spring. Strain OT3T is an obligate aerobe that synthesizes bacteriochlorophyll a and has a photosynthetic apparatus. This isolate is a thermophilic bacterium with an optimal growth temperature of 40 to 48°C. The cells are nonmotile, ovoid to short rods. An analysis of 16S rRNA sequences revealed that the new strain forms a coherent cluster with members of the α-4 group of the α subclass of the Proteobacteria, which contains the genera Erythrobacter, Erythromicrobiurn, and Porphyrobacter. The closest relative is Porphyrobacter neustonensis, with a 16S rRNA sequence similarity of 96.8%. The in vivo absorption spectrum has maxima at 460, 494, 596, 800, and 870 nm. The main carotenoids are OH-B-carotene sulfate derivatives, xanthoxanthin, and bacteriorubixanthin. Growth occurs with glucose, acetate, glutamate, butyrate, Casamino Acids, and yeast extract as sole energy sources. The pigment composition and nutritional profile of the new isolate are similar to the pigment composition and nutritional profile of P. neustonensis. Although there are marked differences in cell morphology between the new isolate and the budding bacterium P. neustonensis, the results of phenotypic and genotypic comparisons suggest that the new isolate is closely related to P. neustonensis. Consequently, we assign the new isolate to the genus Porphyrobacter and propose the name Porphyrobacter tepidarius sp. nov. for it; the type strain of P. tepidarius is strain OT3 (= DSM 10595).

Anoxygenic photosynthetic bacteria, in general, synthesize bacteriochlorophylls under anaerobic conditions. Several obligately aerobic bacteria, however, produce photosynthetic pigments only under aerobic conditions. These bacteria hardly grow anaerobically in the light, but contain photochemical reaction centers and light-harvesting systems involving bacteriochlorophyll a and carotenoids. The group that contains these photosynthetic aerobic bacteria includes the marine species Erythrobacter longus (20), Erythrobacter litoralis (29, 32), Roseobacter denitrificans (19), and Roseobacter litoralis (19) and the freshwater species Porphyrobacter neustonensis (4), Erythromicrobiurn ramosum (31, 32), and Roseococcus thiosulfatophilum (30, 32). In addition, it has been reported that some strains belonging to the genera Bradyrhizobium, Acidiphilium, and Methylobacterium, which are considered nonphotosynthetic aerobic bacteria, synthesize bacteriochlorophyll a (1, 25, 27). Recently, it was reported by Wakao et al. that species of the genus Acidiphilium contain Zn-bacteriochlorophyll a instead of Mg-bacteriochlorophyll a as the major photosynthetic pigment (26).

Phylogenetic analyses based on 16S rRNA sequences have revealed that all photosynthetic aerobic bacteria belong to the α subclass of the Proteobacteria (9, 28, 32). In the α subclass, three genera of photosynthetic aerobic bacteria, the genera Erythrobacter, Erythromicrobiurn, and Porphyrobacter, are distant from the other taxa and are classified in a distinct group, the α-4 group. The remainder of the genera, the genera Roseobacter, Roseococcus, Bradyrhizobium, Acidiphilium, and Methylobacterium, belong to the α-1, α-2, and α-3 groups along with the purple photosynthetic bacteria.

Almost all of the photosynthetic bacteria in the Proteobacteria are mesophilic organisms; there are only a few exceptions. The purple sulfur bacterium Chromatium tepidum grows optima-ly at 48 to 50°C, and Rhodocista centenaria (formerly Rhodospirillum centenum) [2], [12] and Rhodopseudomonas strain G1 (17) are able to grow at temperatures up to 47°C. All of the previously described aerobic photosynthetic bacteria are mesophiles and have optimal growth temperatures between 25 and 30°C (3, 21).

A new aerobic photosynthetic bacterium was isolated from a brackish hot spring. This organism was an obligate aerobe that synthesized bacteriochlorophyll a and was able to grow at temperatures up to 50°C, and optimal growth occurred at 40 to 48°C. The new isolate is the first thermophile among the aerobic photosynthetic bacteria. In this study, we describe morphological, physiological, and genetic characteristics of the new isolate and propose that this strain represents a new species in the genus Porphyrobacter, Porphyrobacter tepidarius.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strain OT3T (T = type strain) was isolated from the Usami hot spring in Shizuoka Prefecture, Japan. The strain was isolated by using PE medium (6) supplemented with 1.5% agar. This medium contained (per liter) 0.5 g of sodium glutamate, 0.5 g of sodium succinate, 0.5 g of sodium acetate, 0.5 g of yeast extract (Difco Laboratories, Detroit, Mich.), 0.5 g of Casamino Acids (Difco Laboratories), 0.5 g of Na2SO4 • 5H2O, 0.18 g of KH2PO4, 0.38 g of KH2PO4, 0.39 g of K2HPO4, 0.5 g of (NH4)2SO4, 1 ml of a vitamin mixture, and 5 ml of a basal salt solution. The pH of the medium was adjusted to 7.5 with NaOH. The vitamin mixture contained (per 100 ml) 100 mg of nicotinic acid, 100 mg of thiamine hydrochloride, 5 mg of biotin, 50 mg of p-aminobenzoic acid, 1 mg of vitamin B6, 50 mg of calcium pantothenate, 50 mg of pyridoxine hydrochloride, and 50 mg of folic acid. The basal salt solution contained (per liter) 1.11 g of FeSO4 • 7H2O, 24.65 g of MgSO4 • 7H2O, 2.94 g of CaCl2 • 2H2O, 23.4 g of NaCl, 111 mg of MgSO4 • 7H2O, 28.8 mg of ZnSO4 • 7H2O, 29.2 mg of Co(NO3)2 • 6H2O, 25.2 mg of CuSO4 • 5H2O, 24.2 mg of Na2MoO4 • 2H2O, 31.0 mg of H3BO3, and 4.53 g of trisodium EDTA.

Enrichment cultures were established by using the same medium (pH 7.5), and the cultures were incubated in 30-ml L-shaped tubes shaken vigorously in the dark at 45°C.
The mesophilic marine bacterium *Erythrobacter longus* Och 101T was received from T. Shibata (Ocean Research Institute, University of Tokyo, Otsuchi, Japan). The marine organism *Erythrobacter litoralis* DSM 8590T and the mesophilic freshwater organisms *Erythromicrobium rumosum* DSM 8310T and *P. neustonensis* DSM 9432T from the DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) were also used in comparative studies. These mesophilic species were grown in PE medium with or without 2% NaCl and with vigorous agitation in the dark at 30°C.

Morphological and physiological tests. The size and shape of the cells were determined by phase-contrast microscopy and electron microscopy. Motility was determined by observing 24-h-old cells in liquid PE medium. For nutritional tests, we used 3-ml portions of filtered basic medium containing one of the eight vitamins tested (nicotinic acid, thiamine hydrochloride, biotin, p-aminobenzoic acid, vitamin B₆, calcium pantothenate, pyridoxine hydrochloride, and folic acid). Vitamin-free Casamino Acids (Difco Laboratories) was used in this test as an energy source at a concentration of 0.2%. The final reading was obtained after two serial transfers. Susceptibility to antibiotics was detected in liquid PE medium containing antibiotics at 100 μg/ml. Pigments were extracted with chloroform-methanol (3:1, vol/vol) and were analyzed by reverse-phase thin-layer chromatography (C18-silica gel; Whatman International Ltd., Maidstone, England) and by high-performance liquid chromatography (HPLC) by using a μBondapak C18 column (Waters, Nikon Millipore Ltd., Tokyo, Japan) and methanol as the developing solvent or the mobile phase (22).

**Genetic properties.** Genomic DNA was purified by the method of Marmur (15). The guanine-plus-cytosine (G+C) content was determined by HPLC of nucleoside PI hydrolysates of genomic DNA (10). 16S rRNA-specific DNA was amplified by PCR (8, 11) and was sequenced directly with a Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, San Jose, Calif.) and a model ABI 373A DNA sequencer. The 16S rRNA sequences were aligned by using Clustal W, version 1.5 (24). Phylogenetic trees were constructed with the MEGA program (13).

**Results.** Isolation. Strain OT3T was isolated from bacterial mats in the Usami hot spring (Shizukuoka Prefecture, Japan). This spring was brackish. The temperature at the sampling site was 42.7°C, and the pH was 5.8. The bacterial mat mainly consisted of a dark green layer of thermophilic filamentous cyanobacteria.

PE medium supplemented with 1.5% agar was used for isolation (6). The bacterial mats that had been collected were directly used for inoculation by dragging them over the surface of the agar with a watchmaker’s forceps, and the preparations were incubated at 45°C with illumination (approximately 30 W/m²). Orange colonies of strain OT3T formed on the agar within a few days.
FIG. 1. Electron micrograph of negatively stained cells of strain OT3T, showing binary fission. No sign of flagella was detected. Bar = 1 μm.

Morphology and ultrastructure. Cells of strain OT3T grown in PE medium were nonmotile, ovoid to short rods that were 0.5 to 0.7 μm wide and 0.8 to 1.4 μm long (Fig. 1). An electron micrograph of negatively stained cells revealed that they had no flagella and divided by binary fission.

No type of intracytoplasmic membranes was observed in ultrathin sections of strain OT3T (Fig. 2). No storage materials were seen in the cells.

Growth conditions and temperature relationships. Strain OT3T grew chemoheterotrophically under aerobic conditions, but was not able to grow photoheterotrophically under anaerobic conditions in the light. It exhibited no anaerobic respiration when nitrate, dimethyl sulfoxide, or trimethylamine N-oxide was the electron acceptor.

Optimal growth of strain OT3T occurred at temperatures between 40 and 48°C, with a generation time of approximately 1.8 h (Fig. 3a). The strain also grew at temperatures up to 50°C (generation time, approximately 5 h), but not at temperatures above 53°C. None of the other species belonging to the α-4 group examined grew at 40°C or above.

Strain OT3T grew at pH values between 6.5 and 8.5, and the generation times within this pH range were similar. The strain grew more slowly at pH 6.0 or 9.0 and did not grow at pH values below 5.5 or above 9.5. The new isolate was a freshwater bacterium, but it grew in the presence of up to 1.3% NaCl (Fig. 3b). *P. neustonensis* and *Erythromicrobium ramosum* had similar growth responses to NaCl; these bacteria were able to grow in the presence of 1.5 and 1.0% NaCl, respectively. The ranges of NaCl concentrations for growth are summarized in Table 1.

Physiological and biochemical characteristics. The results of the nutritional and biochemical tests performed with strain OT3T are shown in Table 1. Strain OT3T utilized glucose, acetate, glutamate, butyrate, Casamino Acids, and yeast extract as sole sources of carbon and energy for growth. The following carbon substrates were not used: fructose, pyruvate, citrate, lactate, malate, succinate, methanol, and ethanol. Tween 80 and starch were hydrolyzed, but gelatin was not hydrolyzed. Biotin was required as a growth factor. The new strain was sensitive to penicillin G (20 U) and chloramphenicol (100 μg/ml) and resistant to streptomycin (50 μg/ml).

Photosynthetic pigments. The colonies and liquid cultures of strain OT3T were orange when the organism grew chemoheterotrophically in PE medium. Ultrasonically disrupted cells in buffer had absorption maxima at 460, 494, 596, 800, and 870 nm (Fig. 4), which reflected the presence of carotenoids and bacteriochlorophyll a. This in vivo spectrum resembled the spectrum of the mesophilic aerobic photosynthetic bacterium *P. neustonensis* (4). Absorption peaks in the near-infrared region of strain OT3T indicated that the strain contained light-harvesting complex I (B870) together with the photochemical reaction centers, but another peripheral light-harvesting complex (LH II) was not present, as in *P. neustonensis* and *Erythrobacter* species (4, 20, 32).

The photochemical activity of the strain OT3T membrane (ultrasonically disrupted cells in MOPS-potassium-magnesium buffer) was determined by examining light-induced absorption changes in the pigments. Reversible photobleaching of bacteriochlorophyll a (540 nm) and photooxidation of soluble cytochrome c (550 - 540 nm) were observed by flash excitation.
These observations indicate that strain OT3T has a photosynthetic electron transport system. The reaction center genes in strain OT3T were amplified by PCR by using primers designed for well-conserved sequences at both ends of the reaction center genes of purple photosynthetic bacteria (16). The sequence of the PCR product of strain OT3T was similar to the sequence of the products of the purple photosynthetic bacteria.

Isolate OT3T was found to contain bacteriochlorophyll a (phytlyl ester) based on the retention time on HPLC and the absorption spectrum. The major carotenoids were a group of highly polar carotenoids, nostoxanthin, and bacteriorubixanthin (approximately 63, 18, and 12% of the total carotenoids, respectively). The highly polar carotenoids of strain OT3T were somewhat different from those of carotene, and hydroxy-P-carotene, as determined by comparison with the carotenoids of Erythrobacter longus. However, the carotenoid sulfates were somewhat different from those of Erythrobacter species and Erythromicrobium ramosum, in which erythroxanthin sulfate is the major component (23). The new isolate contained little zeaxantin, which was observed in Erythrobacter species and was a major carotenoid in Erythromicrobium ramosum. The carotenoid profile of P. neustonensis was similar to that of the new isolate.

**Genetic properties.** The DNA base composition of the new isolate as determined by HPLC was 65.0 mol% G+C. This value was similar to the values obtained for the freshwater species belonging to the α-4 group (Table 1).

The nucleotide sequence of the 16S rRNA gene enzymatically amplified from strain OT3T was determined by direct automated sequencing. Table 2 shows the evolutionary distances for representative members of the α subclass of the Proteobacteria as determined by the neighbor-joining method (18). A phylogenetic tree (Fig. 5) was constructed on the basis of the distance matrix data by using the sequence of Escherichia coli (γ subclass of the Proteobacteria) as the outgroup reference sequence. The new bacterium, strain OT3T, belonged to the α-4 group of the α subclass, and Erythrobacter longus, Erythrobacter literarius, P. neustonensis, and Erythromicrobium ramosum were its phylogenetic neighbors. Within the phylogenetic group of aerobic photosynthetic bacteria, strain OT3T was comparatively distant from marine Erythrobacter species and formed a coherent cluster with the freshwater species P. neustonensis and Erythromicrobium ramosum (bootstrap confidence value, 100%). The new isolate was 96.8% similar to P. neustonensis and 96.6% similar to Erythromicrobium ramosum.

**DISCUSSION**

Strain OT3T, which was isolated from a hot spring, was an obligately aerobic bacterium which contained a certain amount of bacteriochlorophyll a along with light-harvesting systems and photochemical reaction centers and showed photochemical activity. The new isolate grew at temperatures up to 50°C.

<table>
<thead>
<tr>
<th>Species</th>
<th>Evolutionary distance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porphyrobacter neustonensis</td>
<td>0.033</td>
</tr>
<tr>
<td>Erythromicrobium ramosum</td>
<td>0.034</td>
</tr>
<tr>
<td>Erythrobacter longus</td>
<td>0.061</td>
</tr>
<tr>
<td>Erythrobacter literarius</td>
<td>0.032</td>
</tr>
<tr>
<td>Acidithiobacillus angustum</td>
<td>0.178</td>
</tr>
<tr>
<td>Rhodopila globiformis</td>
<td>0.017</td>
</tr>
<tr>
<td>Roseococcus thiiosulfatophilus</td>
<td>0.198</td>
</tr>
<tr>
<td>Rhodobacterium vanniellii</td>
<td>0.148</td>
</tr>
<tr>
<td>Rhodopseudomonas palustris</td>
<td>0.155</td>
</tr>
<tr>
<td>Methyllobacterium etonquoris</td>
<td>0.149</td>
</tr>
<tr>
<td>Roseobacter denitrificans</td>
<td>0.157</td>
</tr>
<tr>
<td>Rhodovulum sulfidophilum</td>
<td>0.140</td>
</tr>
<tr>
<td>Rhodobacter capsulatus</td>
<td>0.151</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>0.202</td>
</tr>
</tbody>
</table>

* See reference 18.
and optimal growth occurred at 40 to 48°C. Thus, to our knowledge, the new isolate is the first thermophile among the aerobic photosynthetic bacteria, because the upper temperature limits for growth of the previously described strains of aerobic photosynthetic bacteria are less than 40°C and their optimal growth temperatures are between 25 and 30°C (3, 21).

The results of the 16S rRNA sequence comparison indicated that strain OT3 belongs to the α-4 group in the α subclass of the Proteobacteria. The new isolate is, however, different in cell morphology and motility from the other members of the α-4 group. While Erythrobacter species and Erithromicrobium ramosum are rod shaped and P. neustonensis is a pleomorphic budding bacterium, cells of strain OT3 are ovoid to short rods (phenotypic characteristics for the members of the α-4 group are summarized in Table 3). The new isolate is nonmotile and has no flagella, while all of the other members of the group have flagella and are motile. These phenotypic and genetic features suggest that strain OT3 represents a new taxon in the α-4 group.

Within the α-4 group, the closest relatives of the new isolate are P. neustonensis and Erithromicrobium ramosum, with rRNA sequence similarities of 96.8 and 96.6%, respectively (Table 2 and Fig. 5). Because these values are apparently too high to warrant placement of strain OT3 in a different genus, this strain should be classified in the genus Porphyrobacter or the genus Erithromicrobium as a new species. The results of the physiological comparison suggest that the new isolate is more closely related to P. neustonensis than to Erithromicrobium ramosum. Strain OT3 contains a single light-harvesting complex (B870) like P. neustonensis, in contrast to Erithromicrobium ramosum, which has another peripheral complex (B803-836) in addition to B870 (31, 33). The carotenoid compositions of the new strain and P. neustonensis are similar, and both of these bacteria contain little zeaxanthin, which occurs in Erithromicrobium ramosum as a major carotenoid. The profiles for utilization of energy sources of P. neustonensis and strain OT3 are rather restricted, whereas Erithromicrobium ramosum is able to use various organic acids, which suggests that the closed tricarboxylic acid cycle and a glyoxalate shunt are present. P. neustonensis and strain OT3 are also sensitive to penicillin G and hydrolyze Tween 80, unlike Erithromicrobium ramosum.

The evidence presented above suggests that the new isolate belongs to the genus Porphyrobacter. Although the genus Porphyrobacter and the new isolate are morphologically quite different, a new genus should not be created for the new isolate.
soley on basis of morphological differences. Consequently, we propose the name Porphyrobacter tepidarius sp. nov. for the thermophilic bacterium that was isolated from a hot spring.

Porphyrobacter tepidarius sp. nov. Porphyrobacter tepidarius (tep.id.i.ar. tis. L. n. tepidarium, a warm bath fed by natural thermal water; M.L. adj. tepidarius, warm bathing). Cells are nonmotile, ovoid or short rods (0.5 to 0.7 by 1.4 μm). Gram negative. Cells divide by binary fission. No intracytoplasmic membrane. No storage material. Colonies and liquid cultures are orange due to the presence of carotenoids and bacteriochlorophyll a. The in vivo absorption spectrum has maxima at 460, 494, 596, 800, and 870 nm. The main carotenoids are carotenoid sulfates, nostoxanthin, and bacteriorubixanthin. Thermophilic, optimal growth occurs at 40 to 48°C. Aerobic. Chemoheterotrophic. Freshwater species, but growth also occurs with glucose, acetate, glutamate, butyrate, Casamino Acids, or yeast extract as a sole energy source. Does not utilize methanol, ethanol, pyruvate, malate, or succinate. Starch and Tween 80 are hydrolyzed, but gelatin is not hydrolyzed. Susceptible to penicillin G and chloramphenicol. Resistant to streptomycin. On the basis of the results of a 16s rRNA sequence comparison, the bacterium belongs to the genus Eiythrobacter and the family Eiythrobacteraceae. The closest relative is Porphyrobacter nauticus (as determined by HPLC).

Cells are nonmotile, ovoid or short rods (0.5 to 0.7 by 0.8 μm). Cells occur in cell aggregates by active gliding movement. Int. J. Syst. Bacteriol. 41:119-130.

We thank Isao Uemura (Tokyo Metropolitan University, Hachioji, Tokyo, Japan) for providing bacterial strains. We are indebted to Shigeaki Kikuchi, Japan) for providing Porphyrobacter tepidarius sp. nov. for his help with electron microscopy. We are indebted to Akihiko Tabuchi, Japan) for kindly providing bacterial strains. We thank Isao Uemura (Tokyo Metropolitan University, Hachioji, Tokyo, Japan) for providing Eiythrobacter ramosum for the accession of 16s rRNA amplified by polymerase chain reaction without DNA purification. J. Bacteriol. 32:211-217.

Habitat: cyanobacterial mats in brackish water of a hot spring in Shizuoka Prefecture, Japan. The type strain is OT3, which has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) for the accession of 16s rRNA gene segment. J. Bacteriol. 165:63-65.

ACKNOWLEDGMENTS

We thank Isao Uemura (Tokyo Metropolitan University, Hachioji, Japan) for his help with electron microscopy. We are indebted to Tszuo Shiba (Ocean Research Institute, University of Tokyo, Otsu, Shiga, Japan) for providing Eyrzyhrobacter longus. We also thank Khurshed A. Malik (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) for kindly providing bacterial strains. This work was supported in part by grants from the Ministry of Education, Science, and Culture of Japan.

REFERENCES


