**Chromatium tepidum** sp. nov., a Thermophilic Photosynthetic Bacterium of the Family Chromatiaceae†

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A new species belonging to the photosynthetic bacterial genus *Chromatium* is described. This new organism differs from all other *Chromatium* species in its thermophilic character and hot-spring habitat. In addition, the combination of its carotenoid pigments, physiological peculiarities, and deoxyribonucleic acid base composition clearly define this isolate as a new species of photosynthetic purple bacteria. The organism is a rod-shaped, gram-negative bacterium which produces bacteriochlorophyll **a** and grows photoautotrophically with sulfide as an electron donor at an optimum temperature of 48 to 50°C. No growth is observed below 34°C or above 57°C. Globules of elemental sulfur are produced from the oxidation of sulfide and are stored intracellularly. Acetate and pyruvate are the only organic compounds that are photoassimilated. The major carotenoids of the new organism are rhodovibrin and spirilloxanthin, and the deoxyribonucleic acid base composition is 61 mol% guanine plus cytosine. Based on these characteristics, I propose a new species, *Chromatium tepidum*; the specific epithet refers to the moderately thermophilic nature of this hot-spring photosynthetic bacterium.

**MATERIALS AND METHODS**

**Source of the organism.** Enrichment cultures were established from reddish mat material that was attached to the calcareous sinter of a small hot spring in the Upper Terrace region of Mammoth Hot Springs, Yellowstone National Park. These springs are rich in calcium carbonate and are mapped by Castenholz (2), and the Stygian Springs are indicated by this author.

**Media.** The new organism was isolated in a modified version of the standard liquid enrichment medium used for photosynthetic purple bacteria described by Pfenning (14). This medium contained (per liter of deionized water) 200 mg of MgSO₄ · 7H₂O, 50 mg of CaCl₂ · 2H₂O, 400 mg of NH₄Cl, 400 mg of NaCl, 0.5 g of KH₂PO₄, 1 g of sodium acetate, 1 ml of a trace element solution containing deionized water (1,000 ml), ethylenediaminetetraacetate (5.2 g), FeCl₃ · 4H₂O (1.5 g), ZnCl₂ (70 mg), MnCl₂ · 4H₂O (100 mg), H₂BO₃ (6 mg), CoCl₂ · 6H₂O (190 mg), CuCl₂ · 2H₂O (17 mg), NiCl₂ · 6H₂O (25 mg), Na₂MoO₄ · 2H₂O (188 mg), VO(SO₄)₂ · 2H₂O (30 mg), and Na₂WO₄ · 2H₂O (2 mg). The modified enrichment medium lacked yeast extract and vitamin B₁₂.

**Growth conditions.** Cultures were grown photoautotrophically in completely filled 17-ml to 1-liter tubes or in bottles that were incubated in a water bath or in a light cabinet at 48°C. Freshly inoculated vessels were always placed in darkness for 2 to 4 h before they were incubated photosynthetically at 1,500 to 2,000 lx of incandescent illumination.

**Absorption spectra.** Spectra of intact cells were recorded by suspending sulfur-free cells in 30% bovine serum albumin (10) and measuring absorption spectra with a Perkin-Elmer model 552A ultraviolet-visible double-beam spectrophotometer against 30% bovine serum albumin. Pigment extracts were obtained by extracting pellets for 30 min with acetone-methanol (7:2) in darkness at −20°C. Following centrifugation, the absorption spectra of the extracts were measured against acetone-methanol blanks.

**Electron microscopy.** Cells were fixed by the method of Ryter et al. (21), dehydrated in an ethanol series, and embedded in Spurr low-viscosity embedding medium. Sections were stained with uranyl acetate and lead citrate and examined with a Philips model 1300 electron microscope at 60 kV.

**DNA composition.** The deoxyribonucleic acid (DNA) base ratio of *C. tepidum* was kindly determined by Henry Burr, Wayne State University, Detroit, Mich., by using *Escherichia coli*, *Micrococcus luteus*, and chicken DNAs as standards. DNA from *C. tepidum* that was purified by the method of Marmur and Doty (12) was subjected to thermal denaturation, and the guanine-plus-cytosine content was calculated from the melting profile.

**RESULTS**

**Isolation and culture.** During a survey of alkaline hot springs for photosynthetic purple bacteria, thin reddish mats were observed in certain sulfide-rich springs in the Upper Terrace region of Mammoth Hot Springs, Yellowstone National Park. These springs are rich in calcium carbonate and...
contain sulfide of geochemical origin at concentrations of about 2 mg/liter (2). The thin mats were firmly embedded in carbonaceous sinter from the springs and were composed of rod-shaped bacteria, most of which contained bright refractile globules that strongly resembled the sulfur globules of purple sulfur bacteria. Material collected from one such spring (44°C), inoculated into a sulfide-acetate enrichment medium, and incubated photosynthetically at 52°C yielded a bright red-pigmented culture within 72 h. The spring which was sampled smelled of sulfide and was devoid of cyanobacteria. Other rod-shaped bacteria that were free of refractile globules also were present in the mat sample. The primary enrichment culture was observed microscopically, and a 5% inoculum was transferred to fresh medium. The predominant organism was a highly motile rod-shaped bacterium containing sulfur globules, which displayed a distinct phobophototactic response (24) when the microscope field was darkened. The major contaminant was a long thin rod-shaped organism that was devoid of sulfur globules. The transferred enrichment grew within 24 h and was kept at 4°C for 2 weeks before pure culture isolation was performed.

A pure culture of the new organism was eventually obtained following successive applications of the agar shake culture technique (17). Bacto-Agar (Difco Laboratories) was the solidifying agent used. By the third shake culture series the only remaining contaminant was the long thin rod-shaped organism mentioned above. In shake cultures prepared thereafter the long thin rod-shaped organism was present in low numbers until shake tubes were prepared by using agar that had been washed with deionized water, followed by washes in ethanol and then acetone. The washes converted the slightly yellow agar preparation into a much whiter crystalline powder. Media prepared from this "purified" agar readily yielded pure cultures of the thermophilic phototroph following two shake tube applications. Apparently, crude agar preparations were supplying the contaminant with needed nutrients. Liquid cultures of the new isolate, previously referred to as Chromatium sp. strain MC⁴ (T = type strain) (8), were grown in the modified enrichment medium described in Materials and Methods, and samples of log-phase cells were frozen at −80°C in growth medium containing 10% glycerol; frozen cultures remained viable for more than 1 year.

Morphology. C. tepidum consists of rod-shaped cells which measure 1.2 by 2.8 to 3.2 μm and occasionally form short chains of two to four cells (Fig. 1). Cells in young cultures (Fig. 1A) contain two or more sulfur globules, while cells in stationary phase (Fig. 1B) are generally sulfur free. Although motile cells are occasionally observed, pure cultures of the new organism have not been observed to be as highly motile as the original enrichment cultures. Electron microscopy of thin sections of C. tepidum (Fig. 2) revealed features that are typical of Chromatium species. The intracellular membranes of C. tepidum are of the vesicular (chromatophore) type (Fig. 2). Large holes in the cell periphery are frequently observed, and these presumably represent the locations of elemental sulfur or poly-β-hydroxybutyrate granules before the embedding and dehydration process; large amounts of poly-β-hydroxybutyrate are produced by cells of C. tepidum that are grown photoheterotrophically on acetate. A fibrous material arranged in clumps (Fig. 2) was observed in the cytoplasm of thin sections of C. tepidum, and these structures appeared to be loosely attached via membrane connections to the cytoplasmic membrane; their function, if any, is unknown.

The cell wall of C. tepidum is morphologically distinct. Outside what appears to be a typical lipopolysaccharide layer, a dense, darkly staining area of unknown chemical composition is present (Fig. 2). Although chemical assays of the cell wall of C. tepidum have not been performed, the

![Fig. 1. Photomicrographs of C. tepidum. (A) Cells from mid-logarithmic growth filled with elemental sulfur (arrow). (B) Sulfur-free cells from stationary-phase cultures. The cells were grown photoheterotrophically with sulfide, acetate, and CO₂ at 48°C. Bar = 3 μm.](image-url)
organism is highly susceptible to penicillin and therefore presumably contains peptidoglycan. However, the thickness of this outer layer is not typical of peptidoglycan in gram-negative bacteria and the layer may consist of other polysaccharide material. In addition to penicillin, which inhibits the growth of *C. tepidum* at a concentration of 10 μg/ml, the cell wall inhibitors vancomycin and cycloserine also inhibit the growth of *C. tepidum* at the same concentration. The protein synthesis inhibitors chloramphenicol and oxytetracycline are growth inhibitory at a concentration of 10 μg/ml as well.

**Pigments.** *C. tepidum* produces bacteriochlorophyll *a* as its sole bacteriochlorophyll. Spectra of intact cells suspended in 30% bovine serum albumin are shown in Fig. 3. Prominent peaks are observed at 858, 808, and 599 nm. Acetone-methanol extracts (Fig. 4) produce peaks at 770 and 596 nm, which are typical of bacteriochlorophyll *a* (4). The esterifying alcohol of the bacteriochlorophyll *a* of *C. tepidum* is phytol, based on high-pressure liquid chromatography-mass spectrometer analyses kindly performed by Hans Brockmann, Universität Bielefeld.

The carotenoids of *C. tepidum* are mostly carotenoids of the normal spirilloxanthin series (22); however, the proportions of the various components are not typical of any known *Chromatium* species. The major carotenoid, as kindly determined by Karin Schmidt, Universität Göttingen, is rhodovibrin (or a rhodovibrinlike compound), and this pigment makes up nearly 50% of the total carotenoid pool. Other major carotenoids include spirilloxanthin (20%), rhodopin (15%), anhydrorhodovibrin (12%), lycopene (3%), and demethylated spirilloxanthin (3%). In addition, a small amount (~2%) of glycosidic carotenoids of unknown chemical structure is produced by *C. tepidum*.

**Physiology and biochemistry.** *C. tepidum* is an obligately phototrophic bacterium. This organism requires substrate levels of sulfide for growth under any nutritional conditions and grows photoautotrophically in mineral media containing HCO₃⁻ and CO₂ as sole carbon sources. No vitamins, including vitamin B₁₂, are required for growth. The sulfide tolerance of strain MC³ is fairly high; cultures routinely grow in the presence of 2 to 3 mM sulfide, and the upper limit is near 4 mM sulfide. Acetate and pyruvate are photoassimilated (in the presence of sulfide) and substantially increase cell yields. No other organic compounds tested (various organic and amino acids, fatty acids, sugars, and complex...
substrates, such as yeast extract and Casamino Acids) stimulate the growth of *C. tepidum*. The only nitrogen sources utilized by *C. tepidum* are ammonia, urea, and glutamine; \( \text{N}_2 \) does not support growth as a sole nitrogen source when it is tested at either 37 or 48°C.

The sulfide requirement of *C. tepidum* does not simply reflect a need for a biosynthetic sulfur source. No growth of *C. tepidum* is obtained in growth tests when sulfide is replaced with either cysteine (2.5 to 5 mM), thiosulfate (2 to 8 mM), or sulfate (0.5 to 2 mM) in a medium containing acetate as the carbon source. Hence, sulfide probably serves a dual role in the metabolism of *C. tepidum* (as a photosynthetic electron donor and as a biosynthetic sulfur source). Hydrogen and thiosulfate do not serve as electron donors in place of sulfide, nor do they stimulate the growth of *C. tepidum* above the level achieved on sulfide alone. When the gradient technique of Kämpf and Pfennig (7) was used, no dark growth of *C. tepidum* was obtained in a medium containing sulfide and acetate as energy sources.

A major difference between recognized species of *Chromatium* and *C. tepidum* concerns the temperature requirements for growth. Reflecting its hot spring origin, *C. tepidum* grows optimally at temperatures of 48 to 50°C, but it also grows at reasonable rates at temperatures as low as 37°C and as high as 55°C (Fig. 5). The minimum generation time at the optimum growth temperature is about 3.5 h (8). The mass doubling times at 55 and 38°C are 23 and 10 h, respectively. Slow growth of *C. tepidum* occurs at 56 to 57°C; however, cells grown at this temperature are considerably smaller than cells grown at 48°C and produce long chains, suggesting that the organism is experiencing problems with cell division. The minimum growth temperature for *C. tepidum* has not been determined precisely, although cultures incubated photosynthetically at 33 to 34°C did not develop, while cultures at 37°C did. Fully grown cultures of *C. tepidum* remain viable for days at room temperature if they are left in an unopened, anaerobic state; cultures exposed to oxygen lose viability more quickly. Cells of *C. tepidum* withstand freezing at −80°C if they are suspended in fresh growth medium containing 10% glycerol.

**DNA base composition.** The DNA of *C. tepidum* strain MC\(^1\) contains 61 mol% guanine plus cytosine, as determined by thermal denaturation.

**Ecology.** It is likely that the natural habitat of *C. tepidum* is warm to moderately hot (up to ~60°C) neutral to alkaline hot springs containing sulfide. Although strain MC\(^1\) is the only strain to be studied in any detail, a second strain of an organism that strongly resembles *C. tepidum* was isolated from a New Mexico hot spring (8). The New Mexico isolate differs from strain MC\(^1\) in that the cells are slightly more narrow and cultures are considerably less sulfide tolerant. Of possible significance in the latter regard is the fact that the New Mexico strain originated from filamentous cyanobacterial mat material and therefore may represent a less sulfide-tolerant variant of *C. tepidum*. Like *C. tepidum*, however, the New Mexico strain grows at 50°C, oxidizes sulfide, and deposits elemental sulfur intracellularly.

**DISCUSSION**

Most of the properties of the thermophilic photosynthetic bacterium described in this paper readily identify it as a member of the genus *Chromatium*, including (i) the rod-shaped cells, (ii) bacteriochlorophyll \( \alpha \), as the sole bacteriochlorophyll, (iii) carotenoids of the normal spirilloxanthin series, (iv) intracytoplasmic membranes of the vesicular type, and (v) photoautotrophic growth with sulfide as the electron donor and accumulation of elemental sulfur intracellularly. When the properties of *C. tepidum* are compared with those of other small-celled species of *Chromatium*, however, there are several differences (Table 1). The most obvious difference relates to the optimum temperature for growth. *C. tepidum* grows optimally at 48°C, whereas all other *Chromatium* species grow optimally at temperatures between 25 and 35°C (16). Therefore, the thermophilic character alone makes *C. tepidum* unique among species of the *Chromatiaceae*. It should be pointed out that some of the extremely halophilic *Ectothiorhodospira* species isolated from African saline lakes by Imhoff and Trüper also show a rather high optimum temperature. For example, *Ectothiorhodospira halochloris* grows optimally at 45 to 48°C (5). However, the optimum growth temperature of *E. halochloris* is very close to its maximum growth temperature (~50°C); *C. tepidum* is the only purple sulfur bacterium that is capable of growth at temperatures above 50°C. On the other hand, the thermophilic property of *C. tepidum* is not extreme enough to suggest that the organism is an archaebacterium. Indeed, the antibiotic susceptibility of *C. tepidum* indicates it is not an archaeabacterium, and a preliminary analysis of the 16S ribosomal ribonucleic acid sequences clearly identified *C. tepidum* as a eu bacterium (Carl Woese, personal communication).

The color of mass suspensions of *C. tepidum* resembles the colors of suspensions of *Chromatium vinosum* and *Chromatium gracile* (i.e., brownish red), but cultures of *C. tepidum* are distinctly more red than cultures of the other two species. This may be due to the predominance in *C. tepidum* of rhodovibrin, a carotenoid that is essentially absent from *C. vinosum* and *C. gracile* (22). Rhodovibrin is not a common carotenoid in photosynthetic bacteria. It is found in trace amounts in a variety of purple bacterial species, but in substantial amounts only in the nonsulfur purple bacterium *Rhodospirillum photometricum* and in certain strains of *Rhodopseudomonas palustris* and *Rhodospirillum rubrum* (22). *C. tepidum* is morphologically similar
to the small-celled Chromatium species Chromatium minus, Chromatium purpuratum, and Chromatium violascens, but clearly differs from these species in carotenoid content and guanine-plus-cytosine base ratio (Table 1), as well as in thermal characteristics.

C. tepidum shares the following interesting physiological characteristic with large-celled species of Chromatium (Chromatium okenii, Chromatium weissei, and Chromatium buderi) and with Thiospirillum jenense: the only organic compounds photoassimilated are acetate and pyruvate. According to the classification of Trüper (25), C. tepidum should therefore be included with the species listed above in the nutritionally restricted Chromatiaceae I group. By contrast, the members of the Chromatiaceae II group (C. vinosum, C. violascens, C. gracile, C. purpuratum, and others) photoassimilate, in addition to acetate and pyruvate, C-4 citric acid cycle intermediates, fatty acids with longer chains than acetate, and certain sugars (25) (Table 1). Representatives of the Chromatiaceae I group also lack an assimilatory sulfate reduction pathway and are unable to grow under any nutritional conditions in darkness. These properties are also observed in C. tepidum (Table 1) and serve to further separate this species from the remaining small-celled Chromatium species.

The observations of Castenholz (2) suggest that the thermophilic chromatia are widely distributed in high-sulfide neutral-pH springs in the Mammoth Hot Springs region, but are generally absent from the springs dominated by stands of "Heliothrix". Interestingly, however, the upper temperature limit for photosynthetic purple bacteria in general is about 56°C (20), the same as the upper temperature limit of C. tepidum. Perhaps this is the upper temperature limit for photosynthetic purple bacteria in general.

Based on the novel assemblage of properties shown by pure cultures of the thermophilic purple sulfur bacterium described above, this organism, provisionally assigned to the genus Chromatium and referred to previously (8) as Chromatium sp. strain MC², is proposed as a new species of the genus Chromatium, Chromatium tepidum.

Description of Chromatium tepidum. Chromatium tepidum (tep.f.dum. L. neut adj. tepidum lukewarm) cells are rod shaped and 1 to 2 μm wide by 2.8 to 3.2 μm long. Gram negative, occasionally motile. Photosynthetic intracytoplasmic membrane system of the vesicular type.

Pigments. Absorption spectra of intact cells show peaks at 858, 808, and 599 nm, which are typical of bacteriochlorophyll a. The esterifying alcohol of bacteriochlorophyll a is phytol. Carotenoids of the normal spirilloxanthin series are present; rhodovibrin and spirilloxanthin predominate.

Physiology. Obligately phototrophic. Growth occurs photoautotrophically in mineral media supplemented with...
sulfide as electron donor. Sulfur is formed as an intermediate product, which is further oxidized to sulfate. Hydrogen and thiosulfate are not utilized as electron donors. Acetate and pyruvate are photoassimilated in the presence of sulfide; citric acid cycle intermediates, fatty acids other than acetate, and sugars are not utilized. Ammonia, urea, and glutamine serve as nitrogen sources. No growth factors are required. Optimum pH: 7. Optimum growth temperature, 48 to 50°C; no growth below 34°C or above 57°C. NaCl is not required for growth and is growth inhibitory at concentrations above 1% (wt/vol). Catalase negative.

Storage material. Poly-β-hydroxybutyrate is a storage material.

DNA base composition. The DNA contains 61.5 mol% guanine plus cytosine, as determined by thermal denaturation.

Habitat. The habitat of C. tepidum: sulfide-containing hot springs of neutral to alkaline pH at temperatures below 60°C.

Type strain. The type strain is strain MC, which was isolated from Stygian Springs, located in the Upper Terraces of Mammoth Hot Springs, Yellowstone National Park. This strain has been deposited with the American Type Culture Collection as strain ATCC 43061T.

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ADDENDUM IN PROOF

Near infra-red spectroscopy of membrane preparations from cells of C. tepidum shows a distinct absorption peak at 910 nm due to an unusual antennae form of bacteriochlorophyll a (André Verméglio, personal communication). In addition, “Heliothix” has been given formal taxonomic standing with the description of Heliothrix oregonensis, gen. nov. sp. nov. (B. K. Pierson, S. J. Giovannoni, D. A. Stahl, and R. W. Castenholz. 1985. Arch. Microbiol. 142:164-167).

LITERATURE CITED