A purple sulfur bacterium from a high-altitude lake in the Colombian Andes

MARÍA TERESA NÚÑEZ-CARDONA^{1*}, JOHN CHARLES DONATO RONDON², COLIN S. REYNOLDS³ and JORDI MAS⁴

¹ Universidad Autónoma Metropolitana-Xochimilco, DEHA (Laboratorio de Ecología Microbiana),

Calzada de Hueso 1100, Col. Villa Quietud, 04960, Distrito Federal, México

² Universidad Nacional de Colombia, Departamento de Biologia.

Av. (Cra.) 30 No 45-03, Ciudad Universitaria, Bogotá, Colombia

³ CEH Lancaster, Library Avenue, GB-LA1 4AP Lancaster, UK

⁴ Department of Genetics and Microbiology, Autonomous University of Barcelona,

E-08193 Bellaterra, Spain

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As a systematic survey of lakes in Colombian Andes, we collected water samples from the Cumbal lake, located at the Western Cordillera at an elevation of 3424 m and recorded simultaneous basic field measurements of water temperature, pH, nutrient and oxygen. Microscopic examination of water drawn from the deeper layers revealed the presence of phototrophic bacteria. From the water samples collected at 18 m depth, a purple sulfur bacterium (strain Bk18) was isolated. Light microscopy revealed spherical cells with intracellular sulfur granules, which are characteristic of the Chromatiaceae. Analysis of the photosynthetic pigments showed the presence of bacteriochlorophyll *a* with absorption peaks at 373, 800, 853 and 895 nm, respectively. The presence of normal spirilloxanthin carotenoid series was also detected. Gas-chromatography analysis revealed that the following major fatty acids were present: C18:1 (47.58%), C16:1 (27.67%), and C16:0 (12.41%), while the minor fatty acids included C12:0, C13:0, C14:0, C17:0, aC17:0 and C20:1. The morphological and chemotaxonomic properties of the strain Bk18 suggest that this could be placed within the genus *Thiocapsa*.

Key words: Chromatiaceae, purple sulfur bacteria, fatty acids, spirilloxanthin, Thiocapsa.

INTRODUCTION

Some meromictic lakes show high physical stability of water masses, with clearly separated compartments permitting relative constant vertical stratification in bacterial population (Bosshard *et al.*, 2000). It has been reported that bacterioplankton growth in oligotrophic, mesotrophic and eutrophic lakes is limited by inorganic and organic C alone or in combination (Vrede, 2005). In the case of anoxyphotobacteria like purple sulfur bacteria, anoxic conditions with sulphide and light are essential for their development (Karr *et al.*, 2003). In stratified lakes these bacteria often

form dense blooms during the summer below the chemocline where they contribute to the primary production (Herbert *et al.*, 2005), and as part of the bacterioplankton they provide important food resources for zooplankton (Vrede, 2005).

Numerous studies have revealed the presence of purple sulfur bacteria in a wide variety of freshwater ecosystems (Rees *et al.*, 2002). Nevertheless, knowledge of tropical high mountain lakes bacterioplankton and the factors that participate in its ecology is relativately limited.

Molecular techniques have been used to detect purple sulfur bacteria but there are few cases of them being isolated into pure cultures (Karr *et al.*, 2003; Tonolla *et al.*, 2003) and these are necessary to obtain information about their structure, physiology and

^{*} Corresponding author: tel.: +52 548 37003, fax: +52 548 37469, e-mail: mtnunez@correo.xoc.uam.mx

function in the ecosystems (Gray & Head, 1999; To-nolla *et al.*, 2003).

Considering the meagre knowledge on phototrophic bacteria in tropical mountain lakes and the factors that influence their ecology (van Gemerden & Mas, 1995), we sought to clarify the identity and biological characteristics of a purple sulfur bacterium isolated from a high mountain lake, using – in particular– analyses of its pigment and the fatty acid contents following the procedures of Friggard *et al.* (1996) and Bustillos-Guzmán *et al.* (2000). Water samples from the Cumbal lake were collected on August (summer) and simultaneously field measurements of water temperature, pH, oxygen and nutrient content were recorded.

Microscopic examination of water drawn from one of the microaerophilous layers revealed the presence of gas-vacuolate purple sulfur bacteria. Their occurrence is of added interest because previous understanding of the ecology of the Chromatiaceae is that they are found normally in the microaerophilous, oligophotic layers of small, stably-stratified water bodies, having been recorded more frequently at temperate latitudes. We may address the possibility that microbial species, which are able to disperse their propagules relatively freely, have the ability to occupy habitats in remote locations, provided that they are physiologically adapted and suited to the new conditions.

MATERIALS AND METHODS

Physical and chemical properties of the Cumbal lake

The Cumbal lake is of glacial volcanic origin, located at the Western Cordillera of southern Colombia (1° 07' N, 77° 58' W), at an elevation of 3424 m a.s.l. Geologically, the mountain range comprises quaternary massifs with igneous metamorphic rocks, volcanic relieves and lava flows, having an associated series of ash-flow tuffs (INGEOMINAS, 1988). The watershed of the Cumbal lake has an area of 1071 hectares; the lake is 226 hectares in surface and has a maximum depth of 26 m.

The physical environment is remarkably constant in comparison with those encountered in temperate zones. Temporal variability is mainly due to wind and rain events (Donato-Rondon, 2001). Vertical profiles of temperature, dissolved oxygen, conductivity and pH were measured using YSI-57 oxygen meter, YSI-33 conductivity meter and Scott-Gerade pH meter, respectively. Concentrations of nitrite, ammonium, sulfate, soluble reactive phosphorus (SRP), total phosphorus (TP) and iron were also recorded in samples collected at 18 m depth. Prior to the analyses, at the Water Analysis Laboratory of the Environmental Engineering Department of the Universidad Nacional de Colombia, the sub-samples were stored in darkness at 4°C. Analytical techniques followed APHA *et al.* (1998).

Source and culture of microorganisms

Purple sulfur bacteria were recovered from depths corresponding to microaerophilous hypoxic layers within the photic zone. Preliminary microscopic inspection revealed the presence of intracellular sulfur globules and enabled us to ascribe the main component species to the family Chromatiaceae. Test tubes containing a Chromatiaceae-specific culture medium were inoculated with sub-samples of raw lake water. Based on Pfennig & Trüper (1989), the basal culture medium used comprised of 1.0 g KH₂PO₄, 0.5 g NH₄ Cl, 0.40 g MgCl· 6H₂O, 0.05 g CaCl₂· 6H₂O, 1.0 ml trace element solution SL12, 1.0 ml vitamin B_{12} (2.0 mg l⁻¹ distilled water), 30.0 ml sodium bicarbonate solution (5% in distilled water), 6.0 ml Na₂S·9H₂O (6% in distilled water), and distilled water (~950 ml) up to 1 l. Test tubes were inoculated and kept at room temperature under constant illumination by a 60-W incandescent light bulb placed 20-25 cm above the cultures (van Niel, 1971). Isolation and purification of cultures were carried out once the cultures had acquired a visible red, pink or brown coloration. A deepagar technique sealed with oil-paraffin was used to purify cultures (Pfennig & Trüper, 1981). Colonies were isolated after 15-20 days of incubation at 28-30°C with light as energy source.

Growth conditions of cultures

Batch cultures were necessary for pigment and fatty acid analysis. Isolates of strain Bk18 were grown in glass flasks containing 300-1000 ml liquid medium (Pfennig & Trüper, 1989) to which a solution of sodium sulfide neutralized with hydrochloric acid was added for constant growth. All cultures were exposed to similar temperatures (22-24°C) and illuminated constantly with an incandescent lamp of 2,000 lux. Substantial cultures were raised after five days of growth under the conditions described above.

Cellular characterization

For cellular characterization, phase-contrast micrographs were prepared by immobilizing bacterial cells on agar-coated cells and viewed under an optical microscope. Fresh preparations were also observed for organism motility, cellular inclusions and to assess culture purity. After this, cultures judged to be pure were submitted to analysis of pigment and fatty acid content.

Pigment analysis

In vivo absorption spectra of bacterial pigments (bacteriochlorophylls and carotenes) were determined in 10-ml aliquots of culture, after centrifugation at 5,000 rpm for 20 min. Cell concentrates were suspended in 5 ml of culture medium (without sodium sulfide) and pigment absorption was measured with the opaque side of the cuvettes facing the nearest point of the spectrophotometer photocell and using culture medium as blank (Nuñez-Cardona, 1999; Nuñez-Cardona, 2003).

Bacterial pigments were also extracted from whole cells with an acetone-methanol (7:2) mixture. Aliquots (10 ml) of culture were centrifuged at 5,000 rpm for 20 min and solvents were added. Extraction was carried out overnight, in the dark at 4°C. Scans were obtained using a Shimadzu UV160A spectrophotometer prior to centrifugation, with an acetone: methanol (7:2) mixture as blank (Stal *et al.*, 1984; Núñez-Cardona, 2003).

Fatty acid analysis

Lipids were extracted from cells of three separate cultures of strain Bk18, *Thiocapsa rosea* (DSMZ 235) reclassified by Guyoneaud *et al.* (1998) and *Allochromatium vinosum* DSMZ 185 (Imhoff *et al.*, 1998). Three samples from each culture, and 0.5 g of freezedried cells were used for extraction by saponification, followed by diazomethane derivation (Sigma N-nitrous-methylurea) and evaporation with nitrogen gas.

Lipid extracts were suspended in 0.04-1.0 ml of *n*hexane and 2.0 ml were injected into the silica capillary column (15 m×0.25 mm I.D.) of a gas chromatograph with cross-linked methyl silicone (HP-1 Hewlett-Packard) as stationary phase. In turn, the column was inserted into a HP-5890 gas chromatograph, equipped with a flame ionization detector. The column was programmed for operation between 175 °C and 300 °C for 15 min. Injector and detector temperatures were 275 °C and 300 °C, respectively. Identification of fatty acids was accomplished comparing the retention times with standard mixtures of fatty acid methyl esters (Nuñez-Cardona, 1999).

Fatty acids were submitted to gas chromatography and mass spectrometry. An HP-5890 gas chromatograph was attached to an HP-5989X quadripolar mass spectrometer via a dimethyl-polysiloxinate column TRB1 (30 m). The injector and detector temperatures were both at 225 °C. Two ramps were used, one to 240 °C at 10 °C min⁻¹ and other one to 270 °C at 40 °C min⁻¹. The injection mode was split-less, *ca* 1:50.

RESULTS

Environmental parameters

Maximum, minimum and mean values of various physico-chemical environmental variables and nutrient content from water samples collected at 18 m depth are shown in Table 1. Maximum nutrient values and minimal oxygen concentrations from the deep layer of the lake are noted.

A slight thermal layering with oxyclines located at 12, 16, and 20 m was recorded. This difference resulted in a hypoxic environment in the deep layers of the lake with a slight increase in conductivity (Fig. 1).

Microbial diversity of the lake and phenotypic characteristics of strain Bk18

Strain Bk18 was grown from cells isolated from the samples collected at 18 m depth of the Cumbal lake. Other microorganisms recovered from the same deep layers included species of the algal genera *Euglena*,

TABLE 1. Minimum, mean and maximum values of physicochemical variables and nutrients concentrations recorded at 18 m depth of the Cumbal lake (in summer)

	рН [H ⁺]	Alkalinity (mg l ⁻¹)	Iron (mg l ⁻¹)	Ammonium (µmol l ⁻¹)	Nitrites (µmol l ⁻¹)	SRP (µmol l ⁻¹)	TP (µmol l ⁻¹)	DO (mg l ⁻¹)	Magnesium (µeq l ⁻¹)	T (°C)
Min.	6.90	34.00	0.10	5.55	0.16	1.05	1.26	0.50	342.1	12.5
Mean	7.04	34.62	0.11	10.25	0.25	1.18	1.51	2.60	356.2	12.9
Max.	7.10	36.00	0.13	22.2	0.44	1.58	2.10	4.16	370.2	13.5



FIG. 1. Vertical variation of physico-chemical variables in the Cumbal lake during the sampling period (summer).

Peridinium, Oocystis, Chlorella, Cryptomonas, Aulacoseira and *Staurastrum*. Cells of strain Bk18 obtained from deep agar dilutions series were inoculated with a well growth pink-colored batch culture. After incubation for three weeks in the light, pink colonies were isolated. Pure cultures were obtained using isolated colonies as inocula for further deep agar dilution series.

Morphology, pigment content and fatty-acid composition of the cultured strain

Individual cells of strain Bk18 were spherical, measured $1.5-2.5 \ \mu m$ in diameter, and were non-motile. The strain developed single cells or diplococcoid aggregates; moreover, the presence of gas vesicles and elemental sulfur became evident inside the cells.

The colour of cell suspensions of strain Bk18 was pink; spectra of whole cells exhibited *in vivo* absorption maxima at 374, 513, 550, 587, 800, 853, and 892 nm (Fig. 2). These peaks are indicative of the presence of bacteriochlorophyll *a* (374, 587, 800, 853 and 892 nm) and carotenes related to spirilloxanthins. According to Imhoff (1995) the bacteriochlorophyll *a* shows peaks at 375, 590, 800-810, 830-890 nm. Spectrophotometric analysis of the extracts in acetonemethanol mixture (7:2) showed absorption maxima at 365, 495, 529 and 771 nm. The first and last peaks correspond to bacteriochlorophyll *a*. The peaks at 495 and 529 nm confirm the presence of pigments of the spirilloxanthin series (Zülling, 1985; Eichler & Pfennig, 1991).

The fatty-acid composition of the strain Bk18 and the culture collection strains is shown in Table 2. To-



FIG. 2. Absorption spectrum from whole cells of the strain Bk18.

Fatty acid	Chemical name	Bk18	DSMZ235	DSMZ183 1.25	
C12:0	Dodecanoic or Lauric	1.83	2.04		
C13:0	Tridecanoic	0.29	0.45	0.00	
C14:0	Tetradecanoic or Myristic	0.33	0.37	0.79	
C16:1	Hexadecenoic or Palmitoleic	27.67	26.86	31.77	
C16:0	Hexadecanoic or Palmitate	12.41	12.87	13.62	
aC17:0	14-methyl-hexadecanoic	2.31	2.29	0.00	
C17:0	Heptadecanoic	0.30	0.40	0.27	
C18:1	Octadecenoic or Oleic	47.58	49.65	47.14	
C18:0	Octadecanoic or Stearic	0.68	0.35	0.00	
C20:1	Eicosenoic or Gadoleic	0.54	0.69	0.43	
	Total (relative abundance %)	93.94	95.96	95.27	

TABLE 2. Mean fatty acid concentration (%) of the strains Bk18, DSMZ235* and DSMZ183 analyzed by gas chromatography

* Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures)

tal fatty acids extracted amounted to 93.94% of the total lipid composition. It was possible to identify at least ten fatty acids at mean concentrations greater than 0.1% of the total extracted fatty acids. The principal fatty acids identified from strain Bk18 included C18:1 (octadecenoic), C16:1 (hexadecenoic) and C16:0 (hexadecanoic), which contributed 47.58%, 27.67% and 12.41% of the total, respectively. Minor fatty acids contributed a further 6.28% of the total; they included dodecanoic C12:0 (1.83%), tridecanoic C13:0 (0.29%), tetradecanoic C14:0 (0.33%), 14-methyl-hexadecanoic aC17:0 (2.31%), heptadecanoic C17:0 (0.3%), octadecanoic C18:0 (0.68%) and eicosenoic C20:1 (0.54%). All these fatty acids were found in strain DSMZ235 and C13:0, aC17:0 and C18:0 fatty acids were not detected in strain DSMZ183.

DISCUSSION

The Cumbal lake showed a thermal and chemical layering with hypoxic deep layers, high content of ammonium, phosphates and iron. Microscopic examinations of water drawn from the deeper microaerophilous layers in this lake revealed the presence of gas vacuolated purple sulfur bacteria which we were able to isolate and brought into pure culture (strain Bk18), and it was characterized using its phenotypic and chemotaxonomic properties. The microorganism isolated has been shown to be a purple sulfur bacterium. The investigation of its properties in the field and in culture as strain Bk18 (spherical cells, intracellular sulfur inclusions, spirilloxanthin pigment series, and fatty acid composition), indicates that it should be a member of the genus *Thiocapsa*. Prior to 1998, this might have been ascribed to the genus *Amoebobacter*. However, Guyoneaud *et al.* (1998) proposed the reclassification of *Thiocapsa* and *Amoebobacter*, removing the dependence of generic separation on the presence or absence of gas vacuoles, which is a variable and, therefore, inappropriate as a diagnostic.

Fatty acids C14:0, C16:0, C16:1, C18:0, C18:1, and C20:1 have been reported as components in Thiocapsa roseopersicina (= Thiocapsa floridiana) by Tacks and Holt (1971) and in Amoebobacter roseus DSMZ235 linked to phospholipids (Macarrón, 1998), corresponding to 96.78%; in A. roseus DSMZ235, C18:1 accounted for 44.17%, and in strain Bk18 it corresponded to 47.58% of the total fatty acid composition. Octadecenoic acid is one of the most widely distributed among living organisms and it has several isomers. Two of them i.e. C18:1 (9), or oleic acid, and C18:1 (11), or vaccenic acid, are commonly found in living organisms (Auran & Schmidt, 1976). Fatty-acid analysis, using gas chromatography and mass spectrometry, failed to resolve the identity of the C18:1 isomer, because the retention time of the standard used was insufficient to separate it.

Mass spectrometry with an adjustment level of 99% and identification by means of the used library revealed that the isomers are C18:1 (9) and C18:1 (11), although the possibility of being a combination of these two isomers along with C18:1 (8) and C18:1 (12) is not discarded. This possibility agrees with Macarrón (1998), who reported the presence of the isomers C18:1 ω 9, C18:1 ω 7 *cis* and *trans*, and C18:1 ω 5 linked to glycerophospholipids of different strains from the Chromatiaceae family. From these isomers, the second one constitutes a part of the known "sum-

med feature 7", which is composed of one or more of the following fatty acids: C18:1 ω 7c, C18:1 ω 9t, or C18:1 ω 12t. This denomination results from the difficulty in separating them by gas chromatography when the Microbial Identification System (MIDI) is employed (Mergaert *et al.*, 1996; Vauterin *et al.*, 1996).

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The presence of *Thiocapsa* in a deep oligophotic layer of the Cumbal lake is closely allied to the chemical stratification within the microaerophilous deep layer and its high content of ammonium, phosphates and iron.

Purple sulfur bacteria operate towards the lower limit of light (just above the green sulfur bacteria), harvesting energy from extremely modest photon fluxes and using hydrogen sulfide as a donor of electrons. Purple sulfur bacteria with gas vesicles in cells have been detected just below the chemocline of the alpine Cadagno lake (Tonolla et al., 2003) and Mahoney lake (Yurkova et al., 2002). In the first one, they usually were found with non sulfur bacteria (Tonolla et al., 2003) where gas vacuolated cells and presumably Chromatium contribute to the biomass in the chemocline (Bosshard et al., 2000). In the stratified Mahoney lake, dense populations of Amoebobacter purpureus have been observed where it was proposed to be the major route for transfer of phosphorous (Yurkova et al., 2002). The presence of gas vesicles in cells of the genera Thiocapsa and Amoebobacter is a means for positioning themselves at depths where the conditions for their growth are optimal (Karr et al., 2003). In general, purple sulfur bacteria store elemental sulfur in a protein vesicle within the cell.

Although this is a first record for Colombia, we cannot assume that it will be proved unique and that other locations may be revealed in more widespread and more thorough limnological regional investigations. At present, however, we are unable to point to another site close to the Cumbal lake with which propagules might be exchanged. On the other hand, the pragmatic deduction that this particular lake offers a suitable habitat for Thiocapsa cannot be disputed. Indeed, the microenvironmental characteristics noted in this study resemble those of other habitats of purple sulfur bacteria reported from well structured, stratified lakes with redoxclines, in other, widely separated regions of the world: in Sinai (Cohen et al., 1977), Tasmania (Croome & Tyler, 1984), parts of Spain (Vicente & Miracle, 1988; Camacho & Vicente, 1998) and even on the Antarctic continent (Vincent, 1981; Karr et al., 2003).

The occurrence of that we eventually identified as a species of the genus *Thiocapsa* (Anoxyphotobacteria, Chromatiaceae) in deep layers of the Cumbal lake plainly suggests that the environmental conditions are sufficiently similar to habitats in which purple sulfur bacteria are usually known to thrive. We consider that our findings have significant, wider implications in the interpretation of species dispersal, global distribution and participation in community assembly.

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