 doi:10.1111/j.1462-2920.2005.00815.x

Characterization of purple sulfur bacteria from the South Andros Black Hole cave system: highlights taxonomic problems for ecological studies among the genera *Allochromatium* and *Thiocapsa*

Rodney A. Herbert,[1]* Anthony Ranchou-Peyruse,[2] Robert Duran,[2] Rémy Guyoneaud[2] and Stephanie Schwabe[3]

[1]*Division of Environmental and Applied Biology, Biological Sciences Institute, University of Dundee, Dundee DD1 4HN, Scotland.*

[2]*Laboratoire d'Ecologie Moléculaire-Microbiologie, EA3525, IBEAS, Université de Pau, BP1155, 64013 Pau, France.*

[3]*Rob Palmer Blue Holes Foundation, 5 Longtitude Lane, Charleston, SC 29401, USA.*

Summary

A dense 1 m thick layer of phototrophic purple sulfur bacteria is present at the pycnocline (17.8 m depth) in the meromictic South Andros Black Hole cave system (Bahamas). Two purple sulfur bacteria present in samples collected from this layer have been identified as belonging to the family *Chromatiaceae*. One isolate (BH-1), pink coloured, is non-motile, non-gas vacuolated, 2–3 µm in diameter and surrounded by a capsule. The other isolates (BH-2 and BH-2.4), reddish-brown coloured, are small celled (4 µm × 2 µm), motile by means of a single polar flagellum. In both isolates (BH-1 and BH-2), the intracellular photosynthetic membranes are of the vesicular type and bacteriochlorophyll *a* and carotenoids of the normal spirilloxanthin series are present. Both isolates grow well in the presence of sulfide and carbon dioxide in the light. During photoautotrophic growth sulfur globules are stored intracellularily as intermediate oxidation products. According to the 16S rRNA gene sequence data the isolates belong to the genera *Thiocapsa* and *Allochromatium*. However, at the species level a number of inconsistencies exist between the phenotypic and phylogenetic data, highlighting taxonomic problems within these genera. These inconsistencies may have implications for microbiologists studying the ecology of anoxygenic phototrophs. For ecologists studying the functioning of an ecosystem it may not be particularily important to know whether a specific isolate belongs to one species or another. However, if one wants to study the role of different populations within a particular functional group then the species concept is important. This study demonstrates that further work is still required on the taxonomy of purple sulfur bacteria in order that microbial ecologists are able to accurately identify a population/species isolated from hitherto undescribed aquatic ecosystems.

Introduction

Phototrophic purple sulfur bacteria are widely distributed in nature where oxygen is either absent or present at low concentrations and opposing, yet overlapping, gradients of sulfide and light are simultaneously present. The largest and most significant developments of phototrophic sulfur bacteria occur in lacustrine environments (Takahashi and Ichimura, 1968; Caldwell and Tiedje, 1975; Guerrero *et al*., 1987; Pfennig and Trüper, 1989; Overmann *et al*., 1991; 1996). In stratified lakes phototrophic sulfur bacteria often form dense blooms during the summer months just below the chemocline and can contribute significantly to the primary production of these bodies of water (Wetzel, 1973; Biebl and Pfennig, 1979; Overmann *et al*., 1996). Sulfide required by the phototrophic bacteria as electron donor during anoxygenic photosynthesis is generated as an end-product of dissimilatory sulfate reduction by sulfate-reducing bacteria, which are dependent on a supply of organic matter as a source of energy and carbon. In contrast to the marine environment, microbial sulfate reduction in freshwater habitats is often limited by sulfate availability. However, where lakes develop in porous sedimentary rocks such as limestone or in close proximity to the sea, high levels of sulfate are commonly found either arising from gypsum dissolution or by seepage of seawater through fissures and fractures in the rock (Guerrero *et al*., 1987; Matsuyama, 1987).

Received 26 October 2004; accepted 22 February 2005. *For correspondence. E-mail r.a.herbert@dundee.ac.uk; Tel. (+44) 1382 3462; Fax (+44) 1382 3442 75. This paper is dedicated to Professor Norbert Pfennig on the occasion of his 80th birthday.

The rock platform on which the islands of the Bahamas sit is composed almost entirely of limestone (Sealey, 1994). Within the porous carbonate platform of these low lying islands some of the world's most spectacular cave systems have developed (Palmer, 1985). The most spectacular of these, with respect to scale, is the South Andros Black Hole. The almost circular entrance to this water-filled vertical cave system has a diameter of 300 ± 15 m and a depth of 47 m (Schwabe and Herbert, 2004). The water column comprises an upper brackish water mass (12‰ salinity) overlying a denser saline layer (35‰ salinity). At the pycnocline (17.8 m) a 1 m thick layer of phototrophic purple sulfur bacteria was present. Coincident with the microbial layer the water temperature increased sharply from 29°C to 36°C before returning to 29°C at 20 m depth. Similarly, the dissolved oxygen concentration which had an almost constant value of 6 mg l^{-1} in the upper brackish water mass decreased sharply at the pycnocline to < 1 mg l^{-1} while the pH decreased from pH 8.6 to pH 6.45. Sulfide which was absent from the upper water mass was present at a concentration of 30 μM in the phototrophic layer.

This present study describes the morphological and physiological characteristics of two purple sulfur bacteria present in the microbial layer. Their taxonomic position is discussed according to the current classification of anoxygenic phototrophs belonging to the family *Chromatiaceae*.

Results

From water samples collected from the 1 m thick microbial layer at a depth of 18 m in the South Andros Black Hole enrichment cultures for phototrophic bacteria were set up. The enrichment cultures were incubated in the light and samples periodically removed aseptically for microscopical examination. After 2–3 weeks of incubation the enrichment cultures began to appear pinkish red and phase-contrast microscopy showed the presence of non-motile spherical cells and motile rod-shaped cells, both of which accumulated elemental sulfur intracellularily. After repeated passage through deep agar dilution series pure cultures of purple sulfur bacteria were obtained from the highest dilutions. Isolate BH-1 had morphological characteristics similar to members of the genus *Thiocapsa*. Isolates BH-2 and BH-2.4 had a morphology resembling that of small-celled, motile member of the *Chromatiaceae.* Only strains BH-1 and BH-2 were further studied for phenotypic characterization.

Cell morphology and fine structure

Cells of isolate BH-1 were non-motile, spherical with a diameter of 2–3 μm, divided by binary fission, and were surrounded by a capsule. Bright-field microscopy showed the presence of sulfur globules within the cells but gas vesicles were absent irrespective of the growth conditions tested (light and temperature). Examination of ultrathin sections by electron microscopy showed the presence of an internal membrane system of the vesicular type. In contrast, cells of isolate BH-2 were motile with individual cells 3–4 μm long and between 1.8 and 2.5 μm in width. The cells were straight rods and electron microscopy of uranyl-acetate-stained cells showed the presence of a single polar flagellum. Older cultures showed a tendency to lose their motility and form dense clumps at the bottom of the vials. Electron microscopy of ultrathin sections revealed the presence of an internal membrane system of the vesicular type. There was no evidence to indicate, either from ultrastructure studies or incubation at low light intensities and/or low temperatures that isolate BH-2 produced gas vesicles.

Photosynthetic pigments

Dense populations of photoautotrophically grown cultures of isolate BH-1 were pale pink when the cells were full of elemental sulfur and became a progressively darker pink when they became sulfur depleted. The *in vivo* absorption spectrum of sulfur-depleted cells exhibited the typical absorption spectrum of bacteriochlorophyll *a* with maxima at 375, 590, 802 and 858 nm. Absorption maxima at 488, 515 and 550 nm were indicative of the presence of carotenoids of the normal spirilloxanthin series (Pfennig and Trüper, 1992) with spirilloxanthin as the dominant carotenoid (Schmidt *et al*., 1965). Quantitative high-performance liquid chromatography (HPLC) chromatography confirmed that spirilloxanthin accounted for 91% of the carotenoids together with a novel carotenoid tentatively identified as spirilloxanthin-2-ol (8%). Mass cultures of isolate BH-2 were pinkish-red when full of elemental sulfur becoming a darker red when depleted. *In vivo* absorption of cell suspensions is typical of bacteriochlorophyll *a* containing phototrophs (ë maxima 375, 592, 800 and 853 nm). As observed for isolate BH-1, the absorption maxima of 488, 515 and 550 nm indicate the presence of carotenoids of the normal spirilloxanthin series. Quantitative HPLC chromatography revealed rhodopin as major carotenoid (65%) and a substantial amount of spirilloxanthin (25%) relative to lycopene (3%).

Physiological characteristics

When grown under anoxic conditions in the light isolate BH-1 grew well photoautotrophically with sulfide, thiosulfate, molecular hydrogen and elemental sulfur as electron donors. In the presence of sulfide and bicarbonate low-molecular-weight organic compounds, principally tricar-

boxylic acid intermediates, acetate, propionate, butyrate, glycerol and fructose, were photoassimilated. Chemoautotrophic growth occurred under microoxic conditions with sulfide as electron donor. Neither respiratory nor fermentative metabolism was observed in the dark under anoxic conditions.

Growth occurred over a salinity range of 0–50‰ with an optimum at 20‰. The optimum temperature was 30–35°C with a doubling time for photoautotrophically growing cultures of 6.2 h. No growth was recorded at temperatures below 5°C or above 40°C. Vitamin B_{12} was not required for growth.

Isolate BH-2 equally grew well photoautotrophically with sulfide, thiosulfate, molecular hydrogen and elemental sulfur as electron donors. When this isolate was grown in media containing sulfide and bicarbonate a limited number of low-molecular-weight organic compounds, principally tricarboxylic acid intermediates, formate, acetate and propionate and lactate, were photoassimilated. Chemoautotrophic growth occurred under microoxic conditions in the dark using hydrogen sulfide as electron donor. Strain BH-2 did not exhibit fermentative nor respiratory metabolism under anoxic conditions in the dark. This bacterium had no obligate salt requirement for growth and grew over a salinity range from 0 to 50‰ with an optimum about 20‰. Optimum growth was recorded at 30–35°C with a doubling time for photoautotrophically grown cultures of 7.5 h. No growth was observed at temperatures below 5°C or above 40°C. Isolate BH-2 had no requirements for growth factors.

Genetic properties and relationships within the Chromatiaceae

The almost complete 16S rRNA gene sequences from isolates BH-1, BH-2 and BH-2.4 were determined and compared with available sequences within NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). The results obtained for strain BH-1 showed a high degree of similarity with 16S rRNA gene sequences belonging to *Thiocapsa* spp. strains 10 511, ML2 and CE2209 with similarities ranging form 98.4% to 99.2%. Indeed, the phylogenetic dendrogram presented in Fig. 1 clearly placed strain BH-1 into a cluster containing only *Thiocapsa* strains. The 16S rRNA gene sequences revealed that isolates BH-2 and BH-2.4 were related to *Allochromatium* spp. The phylogenetic dendrogram constructed (Fig. 1) indicated that both isolates formed a separate cluster together with species of the genera *Allochromatium* and *Thermochromatium*. Within this cluster, both isolates could be considered as a separate lineage and thus form a new species within the genus *Allochromatium*. According to the calculated similarities, isolate BH-2 was closely related to *Allochromatium vinosum* DSM 180^T and *Allochromatium minutissimum* DSM 1376^T with similarity values of 97.3% and 97.1% respectively. Strain BH-2 was more distantly related with *Allochromatium warmingii* DSM172^T and *Thermochromatium tepidum* DSM 3771^T with similarity values of 94.2% and 93.7% respectively. From the constructed dendrogram it appeared that isolates BH-2 and BH-2.4 were only distantly related to the marine species of the genera *Marichromatium* and *Isochromatium*.

The DNA base composition of isolate BH-2 was 65.7 mol percentage G+C. This value lies within the range 61.3–66.3% mol G+C reported for strains of *A. vinosum* but is lower than that reported for *Marichromatium gracile* (68.9–70.4% mol G+C) and higher than that for *Isochromatium buderi* (62.2–62.8 mol percentage G+C) (Pfennig and Trüper, 1992; Imhoff *et al*., 1998). DNA–DNA hybridization analysis revealed that isolate BH-2 and *A. vinosum* DSM 180^T had 87.6% DNA–DNA similarity.

Discussion

The water column of the South Andros Black Hole cave system is characterized by a sharp pynocline at 17.8 m depth which is coincident with the development of a 1 m thick bloom of purple sulfur bacteria. While there are many reports in the scientific literature of mass blooms of purple sulfur bacteria developing in stratified lakes they usually develop at shallower depths (5–10 m) where light intensities are higher (Trüper and Genovese, 1968; Guerrero *et al*., 1987; Pfennig, 1989). In the South Andros Black Hole the water column has a high transparency to visible light wavelengths and sufficient light between 450 and 550 nm is evidently able to reach these greater depths where it can be utilized by the resident populations of purple sulfur bacteria. Isolates BH-1 and BH-2 contain carotenoids of the normal spirilloxanthin series which strongly absorb light between 480 and 550 nm. These data imply that both bacteria are well adapted to the light wavelengths that penetrate to 17.8 m depth in this cave system.

With respect to morphological and physiological traits (Table 1) as well as phylogenetic relationships (Fig. 1), isolate BH-1 is clearly a strain belonging to the genus *Thiocapsa*. Whether it is a member of the species *Thiocapsa roseopersicina* has yet to be determined as it is only distantly related to the type strain of the species *T. roseopersicina* (DSMZ 217^T) isolated from a wastewater treatment plant in Hardenberg (Germany). According to 16S rRNA gene sequence comparisons and the phylogenetic dendrogram constructed strain BH-1 is closely related to *Thiocapsa* strains ML2 (Coolen and Overmann, 1998) and CE2209 (Guyoneaud *et al*., 1996): this latter isolate is considered to be a *Thiocapsa* sp. (Guyoneaud *et al*., 1998). These strains were isolated from brackish ecosystems but are phylogenetically distinct from

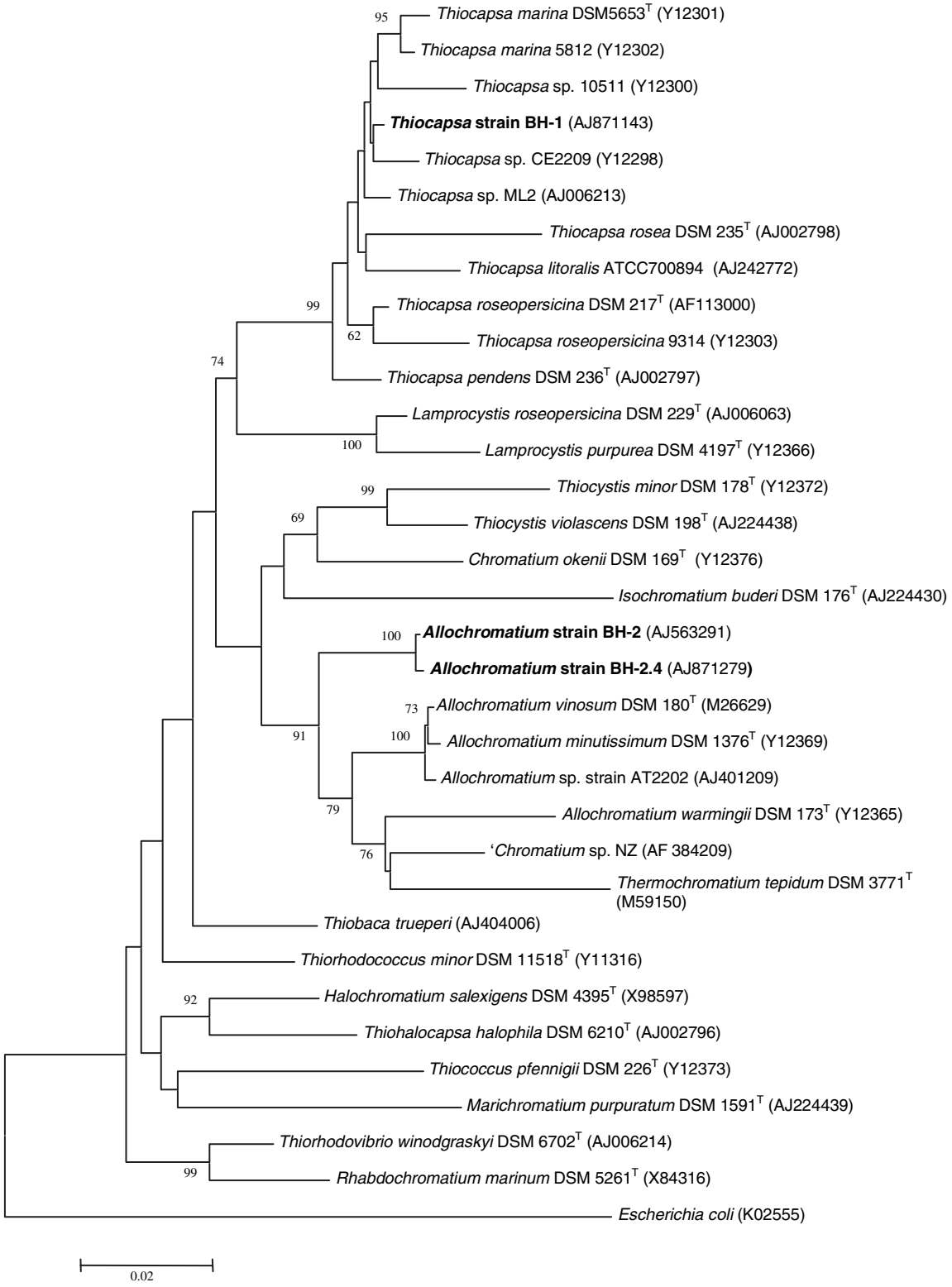

Fig. 1. Phylogenetic tree showing the relationship of strains BH-2, BH-2.4 and BH-1 with members of the family *Chromatiaceae*. The accession numbers of all strains are indicated in the figure. Bar represents 0.02 substitutions/base.

Table 1. Major properties of some *Thiocapsa* strains and strain BH-1.

	Thiocapsa marina	*Thiocapsa roseopersicina*	*Thiocapsa litoralis*	*Thiocapsa* sp.	*Thiocapsa* sp.
Strain	DSMZ 5653^T	DSMZ 217^T	ATCC700894	CE2209	BH-1
Cell shape	Coccus	Coccus	Coccus	Coccus	Coccus
Cell diameter (μm)	1.5–3.0	1.2–3.0	1.5–2.5	2.0–3.0	1.5–2.0
			Rose-red	Rose-red	
Colour of cell suspensions	Purple-red	Rose-red	Rose-red	Rose-red	Rose-red
Major carotenoid	Ok	Sp	Sp	Sp	Sp
Mol% G+C of DNA	62.7–63.2	65.3	64.0	64.0	65.3
Vitamin requirement	–	–	B_{12}	–	–
Sulfate assimilation	–	+	+	+	+
NaCl optimum (%)	1–2	0	1	2	2
Substrates used					
Hydrogen	+	+	ND	+	+
Thiosulfate	+	+	+	+	+
Acetate	+	+	+	+	+
Butyrate	–	–	+	–	(+)
Fumarate	+	+	+	+	+
Succinate	+	+	+	+	+
Glycerol	+	+	–	+	+
Fructose	+	+	+	+	+
Glucose	–	–	+	–	–

Ok, Okenone; Sp, spirilloxanthin; +, utilized; –, not utilized; +/–, utilized by some strains; ND, not determined.

Thiocapsa litoralis (Puchkova *et al*., 2000). The traditional taxonomic system for the family *Chromatiaceae* was not phylogenetically oriented. Phylogenetic dendrogram based on 16S rDNA sequences (Fig. 1) revealed that the existence of the genus *Thiocapsa* is coherent and justified with a bootstrap value of 99%. However, within this genus, the relationships between different existing species and strains remain uncertain with very low bootstraps values. Only the cluster with the two strains belonging to the species *Thiocapsa marina* (Caumette *et al*., 2004) has a significant bootstrap value of 95%. However, as shown in Table 1, strain BH-1 shares many of the characteristics of the *Thiocapsa* species (Guyoneaud *et al*., 1998) in terms of morphology, carotenoid content and physiology (use of glycerol and fructose as substrates, chemoautotrophy). These anoxygenic phototrophs have been found either in freshwater or marine ecosystems such as stratified lakes (Guerrero *et al*., 1987; Overmann *et al*., 1991), sediments (Caumette, 1986; Guyoneaud *et al*., 1996) or microbial mats (Van Gemerden *et al*., 1989). A number of *Thiocapsa* strains, isolated from very diverse environments, have been described to be members of the species *T. roseopersicina* (Caumette, 1986; Guerrero *et al*., 1987; Van Gemerden *et al*., 1989; Overmann *et al*., 1991; Guyoneaud *et al*., 1996; 1998) on the basis of phenotypic data. Moreover, *T. roseopersicina* has been used by several investigators as a model organism for physiological studies on purple sulfur bacteria (see Van Gemerden and Mas, 1995 for review) and more recently by Pringault and colleagues (1999) and Jonkers and colleagues (1998). The taxonomic position of the different strains used in these studies has not been well defined. Thus, many *Thiocapsa* strains, isolated from diverse environments and sharing many phenotypic characteristics, have been identified as different species. This means it is now difficult to clearly identify a new isolate that shares many of the traits of *T. roseopersicina*. Further studies are required to clarify the taxonomy of this genus using a polyphasic approach based on phenotypic traits and genomic data rather than one based on 16S rRNA gene sequences.

Isolates BH-2 and BH-2.4 are typical small, rod-shaped members of the *Chromatiaceae* (Pfennig and Trüper, 1989; 1992). Both isolates grow optimally in the presence of 2% w/v NaCl and must therefore be considered as slightly halophilic bacteria that do not require NaCl for growth. Phylogenetic analysis has revealed that isolates BH-2 and BH-2.4 were related to species in the genera *Allochromatium* and *Thermochromatium* which are all considered to be freshwater species according to the taxonomic scheme of Imhoff and colleagues (1998) and Imhoff (2001). Moreover, strains isolated from the Black Hole cave system exhibit several phenotypic traits (NaCl tolerance, carotenoid composition, substrate utilization) that separate them from the species *A. vinosum*, *A. minutissimum, A. warmingii* and *T. tepidum*. These features, in addition to phylogenetic relatedness based on 16S rRNA gene comparisons, could justifiably lead to the description of strain BH-2 as representative of a new species within the genus *Allochromatium* (see Table 2). The constructed phylogenetic dendrogram revealed strains BH-2 and BH-2.4 as a separate grouping within the cluster of *Allochromatium* species. The similarity value between *A. vinosum* (DSM180^T) and BH-2 (97.2%) is clearly less than the similarity value between the type strains of *A. minutissi-*

Table 2. Major properties of some *Allochromatium* strains and strain BH-2.

	Allochromatium warmingii	*Allochromatium minutissimum*	*Allochromatium vinosum*	*Allochromatium vinosum*	*Allochromatium vinosum*
Strain	DSMZ173^T	DSMZ1376^T	DSMZ180^T	ATCC35206	BH-2
Cell shape	Rod	Rod	Rod	Rod	Rod
Cell diameter (μm)	3.5–4.0	1.0–1.2	2.0–2.5	2.0	1.8–2.5
Colour of cell suspensions	Purple-violet	Brown-red	Brown-red	Brown-red	Rose-red
Carotenoid series	R^{al}	Sp	Sp	Sp	Sp
Mol% G+C of DNA	55.1	63.7	64.3	64.7	65.7
Vitamin B_{12} requirement	+	–	–	–	–
Sulfate assimilation	–	+	+	+	+
NaCl optimum (%)	0	0	0	2.5–4.5	2
Substrates used					
Thiosulfate	–	+	+	+	+
Formate	–	–	–	–	(+)
Acetate	+	+	+	+	+
Butyrate	–	–	–	+	–
Lactate	–	+	–	+	+

R^{al}, rhodopinal; Sp, spirilloxanthin; +, utilized; –, not utilized; +/–, utilized by some strains; ND, not determined.

mum and *A. vinosum* (99.8%). However, DNA–DNA hybridization analysis has shown that strain BH-2 and the type strain of *A. vinosum* (DSM180^T) are the same species with a high DNA–DNA similarity value of 87.6%. Thus, strain BH-2 could be considered either as an atypical strain of the species *A. vinosum* or a subspecies of *A. vinosum.* Similarly, on the basis of phylogenetic relationships there is a need to reconsider the taxonomic position of *A. minutissimum* (DSMZ 1376^T) and redesignate it as a strain of *A. vinosum*. Indeed, genetic studies, based on riboprinting and large restriction fragments analysis using pulse-field gel electrophoresis, have already demonstrated the close relationship between the type strains of both species *A. vinosum* (DSMZ 180T) and *A. minutissimum* (DSMZ 1376^T) (Mas-Castellà *et al*., 1996; Pavon and Gaju, 1997). *Thermochromatium tepidum* (a thermophilic species) is included in a cluster containing *Allochromatium* species and strains. Nevertheless, strong phenotypic differences (thermophily) permit its classification in a separate genus. The current definition of a species is: 'A species is a category that circumscribes a genomically coherent group of individual isolates/strains sharing a high degree of similarity in independent features, comparatively tested under highly standardized conditions' (Rosselló-Mora and Amann, 2001). So according to the data presented here *A. warmingii* is clearly a separate species within the genus *Allochromatium* (Table 2) as already demonstrated by oligonucleotide cataloguing (Fowler *et al*., 1984). In contrast, several strains (BH-2, AT2202 and DSMZ1276^T), included in the phylogenetic analysis presented in Fig. 1, occupy an uncertain taxonomic position related to *A. vinosum* DSM180^T. Thus, in the case of the genus *Allochromatium*, more work needs to be done to clarify the taxonomic relationship between available strains.

In conclusion, the results presented in this study have revealed a number of ambiguities and inconsistencies in the current classification of the *Chromatiaceae*. A lack of congruence currently exists between the modern taxonomic schemes, mainly based on DNA analysis (Guyoneaud *et al*., 1998; Imhoff *et al*., 1998; Imhoff, 2001) and those based on phenotypic characteristics (Pfennig and Trüper, 1989). Recently, studies involving 16S rRNA gene sequencing were undertaken to reassess and reorganize the taxonomy of purple sulfur bacteria and these have clearly established that the *Chromatiaceae* form a coherent phylogenetic group. However, as more sequence data become available, it is evident that the resolving power of 16S rRNA sequences is limited when closely related organisms are involved (Grimont, 1988; Stackebrandt and Goebel, 1994). In order to resolve the issues discussed earlier a more detailed investigation of this group is required using DNA–DNA hybridization as recommended by Imhoff and Caumette (2004). DNA–DNA hybridization would be the best way to establish the genetic relationships within this family although DNA–DNA homology 'has not been widely used in studies of anoxygenic phototrophic bacteria' (Imhoff and Caumette, 2004) and especially within the *Chromatiaceae*. Nevertheless, this technique only establishes direct genome comparisons, is time-consuming, expensive and difficult to apply to a large number of strains. Other techniques such as random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) or sequence data on functional genes such as *puf genes* should be developed in parallel so that species 'could be identifiable by readily available methods' (Mougel *et al*., 2002; Stackebrandt *et al*., 2002).

It must be remembered that phenotypic characteristics play a significant role in the decision regarding species

delineation. Phenotypic traits are generally the first information that are obtained on pure strains (and even before they are pure) during ecological studies. For example, when strain BH-1 was first isolated in pure culture its phenotypic traits suggested that it was a strain of *T. roseopersicina.* More detailed studies described in this article, now indicate that this may not be the case. In contrast, when strain BH-2 was characterized (including 16S rRNA gene sequencing) the data suggested that it was a new species within the genus *Allochromatium*. Subsequent, DNA–DNA hybridization studies have demonstrated that this is not the case. Taxonomists should not forget that the species is the basis for ecological studies as they define populations and then communities. Equally, ecologists need to make greater efforts to describe their isolates in order to enable taxonomists to do their work. Increasingly microbial ecologists are employing 16S rRNA gene sequences to characterize microbial communities, determine microbial biodiversity and follow changes in population dynamics in natural environments. Data from these studies are analysed by comparison with those held in central databases and are thus highly dependent on the availability of reliable and accurate taxonomic data. As a consequence there is a need to develop reliable, unambiguous, readily available and 'easy to use' identification schemes which microbial ecologists can use with confidence.

Experimental procedures

Source of isolates

Water samples were collected using sterile 500 ml O_2-free N_2 gas-filled Duran bottles (Schott Glass Gmbh, Germany) by a SCUBA diver from the microbial layer in the South Andros Black Hole at a depth of 18 m on 28 June 1999.

Enrichment and isolation of the dominant phototrophic bacteria

Aliquot volumes (1 ml) of water collected from the dense microbial layer at 17.8 m depth were used to inoculate enrichment cultures for anoxygenic phototrophic bacteria. The enrichment medium used was prepared according to the method of Pfennig and Trüper (1992) and dispensed into 50 ml of Wheaton vials (Alltech Associates, Deerfield, Illinois). The enrichment cultures were incubated at 30°C and light intensity of 500 lux from a tungsten lamp (16 h light and 8 h dark). At selected time intervals samples were removed for microscopical examination using an Olympus phase contrast microscope. When significant numbers of bacterial cells with the characteristic morphology of purple sulfur bacteria had developed in the enrichment cultures 1 ml samples were aseptically removed and transferred to deep-agar dilution tubes in order to obtain pure cultures. Pure cultures were obtained by repeated passage through agar dilution series according to the method of Pfennig and Trüper (1992). The purity of the cultures was checked by both microscopy and growth tests in Difco AC medium supplemented with 3% w/v NaCl, 0.05% w/v sodium thiosulfate and 0.05% w/v glucose and incubated at 25°C in the dark both aerobically and anaerobically.

Cell morphology and ultrastructure

The morphology and motility of living cells was determined by phase-contrast microscopy using an Olympus BH2 photomicroscope. The flagella pattern was determined by transmission electron microscopy after negative staining with 3% w/v uranyl acetate. For determination of the intracytoplasmic membrane structure, cells were fixed by the method of Ryter and Kellenberger (1958) and ultrathin sections prepared as described by Glazer and colleagues (1971). The sections were examined using a JEOL 1200 EX transmission electron microscope.

Photopigment composition

In vivo absorption spectra of living cells were determined using a Unicam Helios α scanning spectrophotometer of a suspension of the cell pellets prepared in a sucrose solution (Pfennig and Trüper, 1992). Quantitative HPLC analysis of the photopigments was carried out according to the method of Takaichi and colleagues (2001).

Physiological tests

The ability of the isolates to utilize organic carbon sources and different electron donors was tested by growing the isolates in the basal mineral salt medium supplemented with 1 mM $Na_2S{\cdot}9H_2O$. Except where indicated in Table 1 the final substrate concentration used was 5 mM. The tests were performed in triplicate using completely filled 20 ml volume screw cap tubes (Corning Life Sciences). The optical density of the culture was measured at 650 nm, after depletion of elemental sulfur, using a Bausch and Lomb Spectronic 20 spectrophotometer.

The capacity of growth under oxic, microoxic and anoxic conditions in the dark, either chemolithoautotrophically or chemoorganotrophically, was tested according to the method of Kämpf and Pfennig (1980).

Determination of the optimum salt concentration, temperature and pH for growth of the isolates was similarly tested in the basal mineral salt medium (Pfennig and Trüper (1992) using screw top tubes (Corning Life Sciences) according to the method of Guyoneaud and colleagues (1997).

16S rRNA gene analysis, genomic G+C content and DNA–DNA hybridization

Isolation of genomic DNA, amplification of 16S rDNA by polymerase chain reaction (PCR), sequencing and phylogenetic analysis of the two isolates were carried out according to the protocols described by Guyoneaud and colleagues (2002). Sequences were aligned using the CLUSTAL W program. The phylogenetic dendrogram presented in Fig. 1 was

constructed from the calculated similarity coefficients based on 1330 nucleotides. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.0 (Kumar *et al*., 2004). The distance matrix was calculated on the basis of the algorithm of Kimura's 2-parameter model (Kimura, 1980).

The G+C content of the DNA was determined by HPLC according to the methods of Mesbah and colleagues (1989) and Tamaoka and Komagata (1984). For DNA–DNA hybridization studies, DNA was isolated as described by Cashion and colleagues (1977). DNA–DNA hybridization was carried out as described by De Ley and colleagues (1970) with the modifications of Huss and colleagues (1983) and Escara and Hutton (1980) using a Gilford System model 2600 spectrometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the TRANSFER.BAS program by Jahnke (1992).

Allochromatium isolate BH-2 has been deposited with the German Collection of Microorganisms in Braunschweig, Germany (DSMZ No. 15591) and with the National Collection of Marine and Industrial Bacteria, UK (NCIMB 13948) and *Thiocapsa* isolate BH-1 with the National Collection of Marine and Industrial Bacteria (NCIMB 13950).

Acknowledgements

The authors would like to thank Professor Shinichi Takaichi, Biological Laboratory, Nippon Medical School, Nakahara, Kawasaki 211-0063, Japan for carrying out the quantitative photopigment analysis.

References

Biebl, H., and Pfennig, N. (1979) Anaerobic CO_2 uptake by phototrophic bacteria. A review. *Arch Hydrobiol Beih (Ergebn Limnol)* **12:** 48–58.

Caldwell, D.E., and Tiedje, J.M. (1975) The structure of anaerobic bacterial communities in the hypolimnia of several Michigan Lakes. *Can J Microbiol* **21:** 377–385.

Cashion, P., Holder-Franklin M.A., McCully J., and Franklin M. (1977) A rapid method for base ratio determination of bacterial DNA. *Anal Biochem* **81:** 461–466.

Caumette, P. (1986) Phototrophic sulfur bacteria and sulfate-reducing bacteria causing red waters in a shallow brackish coastal lagoon (Prévost Lagoon, France). *FEMS Microbiol Ecol* **38:** 113–124.

Caumette, P., Guyoneaud, R., Imhoff, J.F., Süling, J., and Gorlenko, V. (2004) *Thiocapsa marina* sp. nov., a novel, okenone-containing, purple sulfur bacterium isolated from brackish coastal and marine environments. *Int J Syst Evol Microbiol* **54:** 1031–1036.

Coolen, M.J., and Overmann, J. (1998) Analysis of subfossil molecular remains of purple sulfur bacteria in a lake sediment. *Appl Environ Microbiol* **64:** 4513–4521.

De Ley, J., Cattoir, H., and Reynaerts, A. (1970) The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12:** 133–142.

Escara, J.F., and Hutton, J.R. (1980) Thermal stability and renaturation of DNA in dimethylsulphoxide solutions: acceleration of renaturation rate. *Biopolymers* **19:** 1315–1327.

Fowler, V.J., Pfennig, N., Schubert, W., and Stackebrandt, E. (1984) Towards a phylogeny of phototrophic purple bacteria – 16S rRNA oligonucleotide cataloguing of 11 species of *Chromatiaceae*. *Arch Microbiol* **139:** 382–547.

Glazer, A.N., Cohen-Bazire, G., and Stanier, R.Y. (1971) Characterization of phycoerythrin from a *Cryptomonas* species. *Arch Microbiol* **80:** 1–18.

Grimont, P. (1988) Use of DNA reassociation in bacterial classification. *Can J Microbiol* **34:** 541–546.

Guerrero, R., Pedros-Alio, C., Esteve, I., and Mas, J. (1987) Communities of phototrophic sulfur bacteria of lakes of the Spanish Mediterranean Region. *Acta Academiae Aboensis* **4:** 125–151.

Guyoneaud, R., Matheron, R., Baulaigue, R., Podeur, K., Hirschler, A., and Caumette, P. (1996) Anoxygenic phototrophic bacteria in eutrophic coastal lagoons of the French Mediterranean and Atlantic coasts (Prévost Lagoon, Arcachon Bay, Certes fishponds). *Hydrobiologia* **329:** 16–23.

Guyoneaud, R., Matheron, R., Liesack, W., Imhoff, J.F., and Caumette, P. (1997) *Thiorhodococcus minus*, gen.nov., sp.nov., a new purple sulphur bacterium isolated from coastal lagoon sediments. *Arch Microbiol* **168:** 16–23.

Guyoneaud, R., Süling, J., Petri, R., Matheron, R., Caumette, P., Pfennig, N., and Imhoff, J.F. (1998) Taxonomic rearrangements of the genera *Thiocapsa and Amoebobacter* on the basis of 16S rDNA sequence analyses, and description of *Thiolamprovum* gen.nov. *Int J Syst Bacteriol* **48:** 957–964.

Guyoneaud, R., Mouné, S., Eatock, C., Bothorel, V., Hirschler-Réa, A., Willison, J., *et al.* (2002) Characterisation of three spiral-shaped purple nonsulfur bacteria isolated from coastal lagoon sediments, saline sulfur springs and microbial mats: emended description of the genus *Roseospira* and description of *Roseospira marina* sp. nov., *Roseospira navarrensis* sp. nov. and *Roseospira thiosulfatophila* sp. nov. *Arch Microbiol* **178:** 315–324.

Huss, V.A.R., Festl, H., and Schleifer, K.H. (1983) Studies on the spectrometric determination of DNA hybridization from renaturation rates. *J Syst Appl Microbiol* **4:** 184–192.

Imhoff, J.F. (2001) True marine and halophilic bacteria. *Arch Microbiol* **176:** 243–254.

Imhoff, J.F., and Caumette, P. (2004) Recommended standards for the description of new species of anoxygenic phototrophic bacteria. *Int J Syst Evol Microbiol* **54:** 1415–1421.

Imhoff, J.F., Süling, J., and Petri, R. (1998) Phylogenetic relationships among the *Chromatiaceae*, their taxonomic reclassification and description of the new genera *Allochromatium*, *Halochromatium*, *Isochromatium*, *Marichromatium*, *Thiococcus*, *Thiohalocapsa* and *Thermochromatium*. *Int J Syst Bacteriol* **48:** 1129–1143.

Jahnke, K.-D. (1992) Basic computer program for evaluation of spectroscopic DNA renaturation data from GILFORD System 2800 spectrometer on a PC/XT/AT type personal computer. *J Microbiol Meth* **15:** 61–73.

Jonkers, H.M., De Bruin, S., and Van Gemerden, H. (1998) Turnover of dimethylsulfoniopropionate (DMSP) by the purple sulfur bacterium *Thiocapsa roseopersicina* M11: ecological implications. *FEMS Microbiol Ecol* **27:** 281–289.

Kämpf, C., and Pfennig, N. (1980) Capacity for *Chromati-*

aceae for chemotrophic growth. Specific respiration rates of *Thiocystis violacea* and *Chromatium vinosum*. *Arch Microbiol* **127:** 125–135.

Kimura, M. (1980) A simple model for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16:** 111–120.

Kumar, S., Tamura, K., and Nei, M. (2004) MEGA3: integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* **5:** 150–163.

Mas-Castellà, J., Guerrero, R., and De Jonckheere, J.F. (1996) High degree of similarity between *Chromatium vinosum* and *Chromatium minutissimum* as revealed by riboprinting. *Int J Syst Bacteriol* **46:** 922–925.

Matsuyama, M. (1987) A large phototrophic bacterium densely populating the O_2–H_2S interface of lake Kaiike on Kamikoshi Island, Southwest Japan. *Acta Acadaemiae Aboensis* **47:** 29–43.

Mesbah, M., Premachandran, U., and Whitman, W. (1989) Precise measurement of the G+C content of deoxyribonucleic acid by high performance liquid chromatography. *Int J Syst Bacteriol* **39:** 159–167.

Mougel, C., Thioulouze, J., Perriere, G., and Nesme, X. (2002) A mathematical method for determining genome divergence and species delineation using AFLP. *Int J Syst Evol Microbiol* **52:** 573–586.

Overmann, J., Beatty, J.T., Hall, K.J., Pfennig, N., and Northcote, T.G. (1991) Characterisation of a dense, purple sulphur bacterial layer in a meromictic salt lake. *Limnol Oceanog* **36:** 846–859.

Overmann, J., Beatty, T.J., Krause, H.R., and Hall, K.J. (1996) The sulfur cycle in a meromictic salt lake. *Limnol Oceanog* **41:** 147–156.

Palmer, R.J. (1985) *The Blue Holes of the Bahamas*. London, UK: Jonathan Cape.

Pavon, V., and Gaju, N. (1997) Genomic relationship of five species of the genus *Chromatium* by analysis of large restriction fragments (macrorestriction analysis) using pulse-field gel electrophoresis. *Int J Syst Bacteriol* **47:** 586–589.

Pfennig, N. (1989) Ecology of phototrophic purple and green sulphur bacteria. In *Autotrophic Bacteria.* Schlegel, H.G., and Bowien, B. (eds). New York, USA: Springer-Verlag, pp. 97–116.

Pfennig, N., and Trüper, H.G. (1989) Family *Chromatiaceae*. In *Bergey's Manual of Determinative Bacteriology*, Vol. 3. Staley, J.T., Bryant, M.P., Pfennig, N., and Holt, J.G. (eds). Baltimore, MD, USA: Williams & Wilkins, pp. 1637–1653.

Pfennig, N., and Trüper, H.G. (1992) The family *Chromatiaceae*. In *The Prokaryotes*. Balows, A., Trüper, H.G., Dworkin, M., Harder, W., and Schleifer, K.H. (eds). New York, USA: Springer-Verlag, pp. 3200–3221.

Pringault, O., de Wit, R., and Kuhl, M. (1999) A microsensor study of the interaction between purple sulfur and green sulfur bacteria in experimental benthic gradients. *Microb Ecol* **37:** 173–184.

Puchkova, N.N., Imhoff, J.F., and Gorlenko, V.L. (2000) *Thiocapsa litoralis* sp.nov., a new purple sulfur bacterium from microbial mats from the White Sea. *Int J Syst Evol Microbiol* **50:** 1441–1447.

Rosselló-Mora, R., and Amann, R. (2001) The species concept for prokaryotes. *FEMS Microbiol Rev* **25:** 39–67.

Ryter, A., and Kellenberger, E. (1958) Etude au microscope électronique de plasmas contenant de l'acide désoxyribonucléique. 1 Les nucléotides des bactéries en croissance active. *Z Naturforsch* **13:** 597–605.

Schmidt, K., Pfennig, N., and Liaaen-Jensen. S. (1965) Carotenoids of *Thiorhodaceae*. IV The carotenoid composition of 25 pure isolates. *Arch Microbiol* **52:** 132–146.

Schwabe, S., and Herbert, R.A. (2004) Black Holes of the Bahamas: what are they and why they are black. *Quatern Int* **121:** 3–11.

Sealey, N.E. (1994) *Bahamian Landscapes: An Introduction to the Geography of the Bahamas*, 2nd edn. Miami, USA: Media Publishing.

Stackebrandt, E., and Goebel, B.M. (1994) Taxonomic note: a place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44:** 846–849.

Stackebrandt, E., Frederiksen, W., Garrity, G.M., Grimont, A.D., Kämpfer, P., Maiden, M.C.J., *et al.* (2002) Report of the *ad hoc* committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* **52:** 1043–1047.

Takahashi, M., and Ichimura, S. (1968) Vertical distribution and organic matter production of photosynthetic sulfur bacteria in Japanese lakes. *Limnol Oceanog* **31:** 644–655.

Takaichi, S., Maoka, T., and Masamoto, K. (2001) Myxoxantophyll in *Synechocystis* sp. PCC 6803 is myxol 2′-dimethyl-fucoside, (3R,2′S)-myxol 2′-(2-4-di-Omethyl-a-L-fucoside), not rhamnoside. *Plant Cell Physiol* **42:** 756–762.

Tamaoka, J., and Komagata, K. (1984) Determination of DNA base composition by reversed-phase high performance liquid chromatography. *FEMS Microbiol Lett* **25:** 125–128.

Trüper, H.G., and Genovese, S. (1968) Characterisation of photosynthetic sulphur bacteria causing red water in Lake Faro (Messina, Sicily). *Limnol Oceanog* **13:** 225–232.

Van Gemerden, H., and Mas, J. (1995) Ecology of phototrophic purple sulfur bacteria. In *Anoxygenic Photosynthetic Bacteria*. Blankenship, R.E., Madigan, M.T., and Bauer, C.E. (eds). Dordrecht, Germany: Kluwer Academic Publishers, pp. 50–85.

Van Gemerden, H., De Wit, R., Tughan, C.S., and Herbert, R.A. (1989) Development of mass blooms of *Thiocapsa roseopersicina* on sheltered beaches on the Orkney Islands. *FEMS Microbiol Ecol* **62:** 111–118.

Wetzel, R.G. (1973) Productivity investigations of interconnected lakes. 1. The eight lakes of the Oliver and Walters chain, North-Eastern Indiana. *Hydrobiol Stu* **3:** 91–143.