Physiological Versatility of the Genus *Rhodocista*

Thi To Uyen Do[a,*], Van Nhi Tran[a], and Diethelm Kleiner[b]

[a] Photobiology Laboratory, National Center of Natural Science and Technology, Hanoi, Vietnam. E-mail: dothitouyen@yahoo.com
[b] Mikrobiologie, Universität Bayreuth, D-95440 Bayreuth, Germany

* Author for correspondence and reprint requests

Z. Naturforsch. **62c**, 571–575 (2007); received August 25, 2006/February 1, 2007

A new purple bacterium (strain T4), capable of heterotrophic aerobic and phototrophic anaerobic growth, was isolated from waste water of a noodle factory near Hanoi, Vietnam. A comparison of 16S rDNA sequences revealed its association with the genus *Rhodocista*. The isolate, tentatively named *"Rhodocista hanoiensis"*, forms cysts after growth on butyrate-containing plates at 42 °C. The vegetative cells form short (under aerobic conditions) or long curve-shaped rods. In contrast to other species of this genus T4 does not need any supplines (growth factors, not synthesized by the organisms). Comparative studies of T4 with *Rhodocista centenaria* (*Rhodospirillum centenum*) and *Rhodocista pekingensis* revealed a remarkable physiological versatility regarding nutrient spectra and survival properties of this genus.

Key words: Rhodocista hanoiensis, Cyst Formation, 16S rDNA

Introduction

Phototrophic proteobacteria are distinguished by their ability to carry out anoxygenic photosynthesis. They constitute a phylogenetically heterologous group, being found in three of the five proteobacteria classes together with heterotrophic genera (Kawasaki *et al.*, 1993). The spiral-shaped strains among them were formerly collected in the genus *Rhodospirillum* (Trüper and Imhoff, 1989). When, however, more isolates of this group had been analyzed biochemically and genetically – especially by sequencing of 16S rDNA – it became evident, that description of the genus *Rhodospirillum* had to be revised: new genera like *Rhodospira* (Pfennig *et al.*, 1997) or *Rhodocista* (Kawasaki *et al.*, 1992) were established. The first *Rhodocista* species, *R. centenaria*, had originally been described as *Rhodospirillum centenum* (Favinger *et al.*, 1989). So far two *Rhodocista* sp. have been described: *R. centenaria* and *R. pekingensis* (Zhang *et al.*, 2003). Based on the use of 16S rRNA-targeted oligonucleotide probes (Stoffels *et al.*, 2001) and the 16S rDNA sequences a distance matrix tree shows close phylogenetic relationships of *Rhodocista* with *Azospirillum* (see Fig. 1 and http://www.ncbi.nlm.nih.gov/blast/treeview/blast_tree_view.cgi?request=page&rid=1155726842–25814–85101723765.BLASTQ4&dbname=nr&queryID=lcl|1_25814&distmode=on&screenWidth=1024).

We here report:

(i) isolation and characterization of a novel bacterium of the *Rhodocista* group (strain T4), which differs considerably from the species mentioned above, and therefore is proposed to form a novel species, tentatively named *Rhodocista hanoiensis*, and

(ii) extensive comparative studies which document the interesting versatility and adaptability of the members of the genus *Rhodocista*.

Materials and Methods

Isolation and characterization of T4

Phototrophic bacteria were enriched from a waste water pond [depth 1.5 m, pH 7.6 ± 0.4, 28 °C, organic load (1200 ± 600) mg/l BOD (biological oxygen demand), 3–6 mg/l sulfide] in Laphu, Hoaiduc, HaTay province (Vietnam) by anaerobic growth in light in screw cap tubes or bottles filled with a medium containig (g/l): KH_2PO_4 (1.0); $MgCl_2 \cdot 6H_2O$ (0.5); $CaCl_2 \cdot 2H_2O$ (0.1); NH_4Cl (1); NaCl (1); $NaHCO_3$ (1); Na-acetate (3); 1 ml vitamin solution; 1 ml trace element solution (Trüper and Imhoff, 1989); at pH 6.8–7.2. Samples were diluted and streaked on agar plates which were incubated anaerobically (under N_2) at 30 °C in light from tungsten lamps. Pink-reddish colonies were selected and repeatedly streaked on plates and incubated anaerobically until they appeared pure as judged by microscopic inspection.

0939–5075/2007/0700–0571 $ 06.00

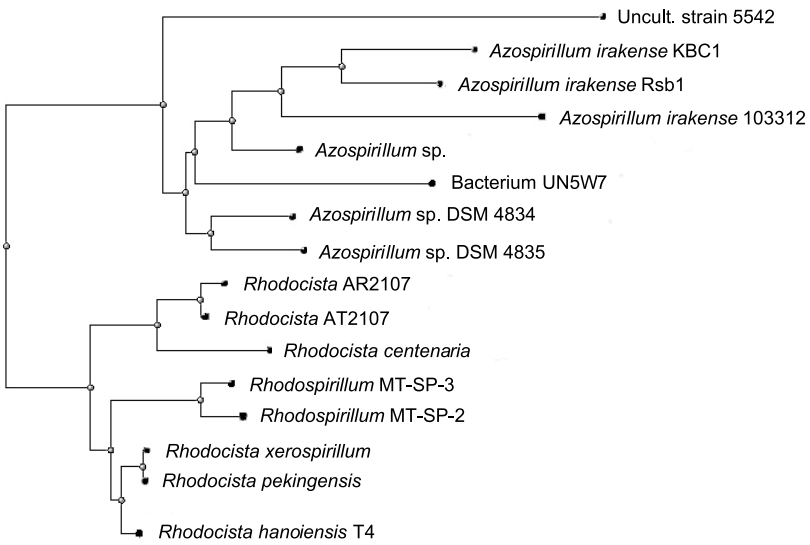

Fig. 1. Distance matrix tree showing the position of *Rhodocista* T4 (adapted from http://www.ncbi.nlm.nih.gov/blast/treeview/blast_tree_view.cgi?request=page&rid=1155726842–25814–85101723765.BLASTQ4&dbname=nr&queryID=lcl|1_25814&distmode=on&screenWidth=1024).

Purity of strains was also demonstrated by uniformity of growth on plates and of morphology of cells after growth on selective media (see below).

Expression of pigments as function of oxygen content in the gas phase was assessed by incubation under 0, 2.5, 5, 8, 10, 15 and 20% O_2 in the light.

DNA was isolated and purified according to standard techniques (Sambrook *et al.*, 1989). A fragment of 16S rDNA was amplified and sequenced using standard primers described by Wittke *et al.* (1997). The sequence was deposited in the EMBL gene bank and received the accession number AJ580798.

Comparative physiological characterization of Rhodocista centenaria, R. pekingensis and T4

Growth tests with different C (36.5 mM) or N (18 mM) sources (see Table I) were carried out at 30 °C in liquid cultures under aerobic heterotrophic (5 ml on a rotary shaker) or anaerobic phototrophic conditions (8 ml screw cap bottles without gas phase, illumination by a tungsten lamp) with the medium as described above, omitting NH_4Cl when testing N sources, and omitting acetate when testing C sources. When testing for vitamin requirement, the vitamin solution (Trüper and Imhoff, 1989) was omitted as well.

Miscellaneous techniques

Absorption spectra were recorded using intact cells grown under phototrophic conditions and resuspended in a 60% sucrose solution. Morphology and structure were observed by optical microscopy. Nitrogen fixation was measured by the acetylene reduction technique as described by Stewart *et al.* (1968).

Results

Characterization of Rhodocista T4

16S rDNA sequence alignments

From the enrichment cultures various strains of the genera *Rhodopseudomonas*, *Rhodospirillum* and *Rhodobacter* could be isolated as described. Our attention was drawn to an isolate capable of heterotrophic growth on benzoate, called strain T4. Repeated PCR of its 16S rDNA yielded a 1491 bp fragment, the sequence of which, according to a BLAST databank search, showed 99% similaritiy with a vitamin B_{12} – requiring unpublished strain (*"Rhodocista xerospirillum"*, acc. no. AM072288), 98% similaritiy with two other undescribed *Rhodocista* sp. (acc. nos. AJ401217, AJ401204) and *Rhodocista pekingensis* (acc. no. AF523824; Zhang *et al.*, 2003), and 97% similarity with *Rhodocista centenaria* (acc. no. D12701; Kawasaki *et al.*, 1992)

and un-characterized *Rhodocista* sp. (acc. nos. D12702, D12703).

Other properties of strain T4

Vegetative cells of T4 had a vibroid to rod shape with dimensions of $(0.7 \times 2)\ \mu$m under aerobic (Fig. 2a) and $(0.7 \times 4)\ \mu$m under anaerobic growth conditions (Fig. 2b).

In vivo absorption spectra of anaerobically cultivated cells showed maxima at 410, 480, 510, 590, 800 and 875 nm, indicative of the presence of bacteriochlorophyll a and spirilloxanthin-like pigments. A similar spectrum was reported for *R. pekingensis* (Zhang *et al.*, 2003).

Production of red pigments was O_2-dependent: the pigments appeared only in a gas phase containing less than 5% O_2.

Cyst formation of T4

Cyst formation was induced as described by Berleman and Bauer (2004), by aerobic maintenance for several days at 42 °C on plates containing NH_4Cl and butyrate as N and C sources, respectively. Their development closely followed the pattern as cited above: after 1 d some cells began to swell (Fig. 2c) and finally formed tetrads like *R. centenaria* (Figs. 2d, e). These cysts resumed vegetative growth when cultivated at optimum temperatures.

Comparative studies of Rhodocista strains

Survival at non-growing conditions

In a comparative set of experiments the three strains were plated on agar plates containing NH_4Cl and butyrate, and incubated at 42 °C for 4 months, so that the plates dried completely. Cells were scraped off, transferred into LB (Luria-Bertani) medium and incubated aerobically. Growth was visible after 2 d (*R. pekingensis* and T4) or after 5 d (*R. centenaria*). In another experiment vegetative cells were incubated in liquid ammo-

Table I. C and N spectra of *Rhodocista* strains. Aerobic growth in liquid cultures (5 ml on a rotary shaker) was conducted with cells pre-grown in an ammonium/acetate medium and washed twice with water to remove all nutrients. Anaerobic growth in liquid cultures (8 ml screw cap bottles without gas phase) was conducted with cells treated as above. Vitamins were supplied as required (see Table II). Results in square brackets are from Favinger *et al.* (1989) (*R. centenaria*, Rce), Stadtwald-Demchick *et al.* (1990) (Rce), and Zhang *et al.* (2003) (*R. pekingensis,* Rpk).

Medium		Aerobic growth of			Anaerobic growth of		
N source	C source	Rce	Rpk	T4	Rce	Rpk	T4
LB medium	LB medium	+	+	+	+	+	+
NH_4^+	Glucose	+	+	+	–	– [–]	+
NH_4^+	Fructose	+	+	+	[+]		+
NH_4^+	Sucrose	+	+	+	[+]	[–]	+
NH_4^+	Formiate	+	–	+	–	– [–]	–
NH_4^+	Acetate	+	+	+	+ [+]	+ [+]	+
NH_4^+	Butyrate	+	+	+	+ [+]	+ [+]	+
NH_4^+	Benzoate	+	+	+	[+]	[–]	+
NH_4^+	Citrate	+	+	+	[+]	[–]	+
NH_4^+	Methanol	–	+	+			
NH_4^+	Ethanol	+	+	+	[+]	[–]	+
Arginine	Arginine	+	+	+	+ [+]	[–]	+
Glutamate	Glutamate	+	+	+	+ [+]	[+]	+
Methylamine	Methylamine	+	+	+	–		–
NO_3^-	Formiate	+	+	+			
NO_3^-	Acetate	+	+	+	+		+
NO_3^-	Benzoate	+	+	+			
NO_3^-	Fructose				–		+
NO_3^-	Glycerol				+		+
NO_3^-	Sucrose				+		–
NO_3^-	Glucose				+		+
N_2	Acetate				[+]	[+]	+
NH_4^+ + 2% NaCl	Acetate	+	+	+			
NH_4^+ + 4% NaCl	Acetate	–	+ [–]	+			
NH_4^+ + 6% NaCl	Acetate	–	–	–			

+, Growth after 5 d; –, no growth after 5 d.

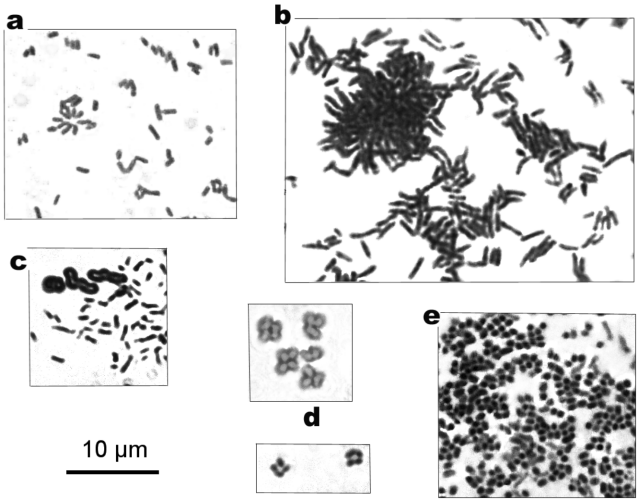

Fig. 2. Light microscopic appearance of *Rhodocista* T4 (stained with carbol fuchsin) after growth at different conditions: (a) after aerobic cultivation for 3 d in liquid ammonium/acetate medium (30 °C); (b) after anaerobic cultivation for 3 d in liquid ammonium/acetate medium (30 °C); (c) after 1 d on ammonium/butyrate plate (42 °C); (d) after 2 and 3 d on ammonium/butyrate plate (42 °C); (e) after 3 d or more on ammonium/butyrate plate (42 °C).

nium butyrate medium and supplied with 10% NaCl. Incubation at 37 °C gradually resulted in complete loss of water within 4 months. The cultures additionally were dried at 45 °C for one week. After resuspension in water, aliquots were transferred to LB medium and incubated. Growth of all strains was visible after 5 d. Microscopic inspections showed that cultures were pure and showed the typical characteristics of the original strains.

Utilization of C and N sources

The spectrum of C and N sources for the *Rhodocista* strains, especially T4 is rather wide (Table I): apart from many mono- and dicarboxylic acids, the organisms can use a number of carbohydrates and benzoate as C and most amino acids as N sources. Like other phototrophs, T4 is able to fix N_2 anaerobically. In contrast to other *Rhodocista,* T4 does not require any supplines like vitamins (Table II).

Miscellaneous

Apart from vitamin requirement other characteristic differences are listed in Table II.

Discussion

The 16S rDNA sequence data as well as the physiological characteristics and ability of cyst formation demonstrate, that T4 is a novel species belonging to the genus *Rhodocista*. The physiological comparison reveals a remarkable versatility as to C and N source utilization of this genus. *Rhodocista* species are methylotrophs, being able to aerobically use methanol, formiate and methylamine as C sources. Furthermore because of their ability to form cysts, all strains can survive severe drought and salt stress.

Acknowledgements

T. T. U. D. is grateful for a DAAD grant. We thank Drs. Bauer (Bloomington, IN, USA) and Liu (Beijing, China) for strains of *R. centenaria* and *R. pekingensis*, respectively.

Characteristic	Rce	Rpk	T4
Vitamin requirement	biotin + B_{12}	thiamin + B_{12}	none
Optimum growth temperature [°C]	39–45	31–42	32–35
Cell diameter × length	1 × 2–3	0.6–0.8 × 0.8–1.5	0.6–0.8 × 2–4
pH Optimum	6.8	7.0	7.2–7.5

Table II. Comparison of miscellaneous physiological properties of *R. centenaria* (Rce), *R. pekingensis* (Rpk) and strain T4.

Berleman J. E. and Bauer C. E. (2004), Characterization of cyst cell formation in the purple photosynthetic bacterium *Rhodospirillum centenaria*. Microbiology **150**, 383–390.

Favinger J., Stadtwald R., and Gest H. (1989), *Rhodospirillum centenum*, sp. nov., a thermotolerant cyst-forming anoxygenic photosynthetic bacterium. Antonie van Leeuwenhoek **55**, 291–296.

Kawasaki H., Hoshino Y., Kuraishi H., and Yamasato K. (1992), *Rhodocista centenaria* gen. nov. sp. nov., a cyst-forming anoxygenic phototrophic bacterium and its phylogenetic position in the proteobacteria alpha group. J. Gen. Appl. Microbiol. **38**, 541–551.

Kawasaki H., Hoshino Y., and Yamasato K. (1993), Phylogenetic diversity of phototrophic purple non-sulfur bacteria in the proteobacteria alpha group. FEMS Microbiol. Lett. **112**, 61–66.

Pfennig N., Lunsdorf H., Suling J., and Imhoff J. F. (1997), *Rhodospira trueperi* gen. nov., spec. nov., a new phototrophic proteobacterium of the alpha group. Arch. Microbiol. **168**, 39–45.

Sambrook J., Fritsch E. F., and Maniatis T. (1989), Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Stadtwald-Demchick R., Turner R. F., and Gest H. (1990), Physiological properties of the thermotolerant photosynthetic bacterium, *Rhodospirillum centenum*. FEMS Microbiol. Lett. **67**, 139–141.

Stewart W. D. P., Fitzgerald G. P., and Burris R. H. (1968), Acetylene reduction by nitrogen-fixing blue-green algae. Arch. Mikrobiol. **62**, 336–348.

Stoffels M., Castellanos T., and Hartmann A. (2001), Design and application of new 16S rRNA-targeted oligonucleotide probes for the *Azospirillum-Skermanella-Rhodocista*-cluster. System. Appl. Microbiol. **24**, 83–97.

Trüper H. G. and Imhoff J. F. (1989), The genus *Rhodospirillum*. In: Bergey's Manual of Systematic Bacteriology, 8th ed. (Staley J. T., Bryant M. P., Pfennig N., and Holt J. G., eds.). Williams & Wilkins, Baltimore, pp. 1662–1666.

Wittke R., Ludwig W., Peiffer S., and Kleiner D. (1997), Isolation and characterization of *Burkholderia norimbergensis* sp. nov., a mildly alkaliphilic sulfur oxidizer. System. Appl. Microbiol. **20**, 549–553.

Zhang D., Zhang W., Yang H., Huang Z., and Liu S. J. (2003), *Rhodocista pekingensis* sp. nov., a cyst-forming phototrophic bacterium from a municipal wastewater. Int. J. Syst. Evol. Microbiol. **53**, 1111–1114.