***Rubrimonas cliftonensis* gen. nov., sp. nov., an aerobic bacteriochlorophyll-containing bacterium isolated from a saline lake**

Tomonori Suzuki,¹ Yasutaka Muroga,¹ Manabu Takahama,¹
Tsuneo Shiba² and Yukimasa Nishimura¹

Author for correspondence: Tomonori Suzuki. Tel: +81 471 24 1501. Fax: +81 471 23 9767.
e-mail: chijun@rs.noda.sut.ac.jp

¹ Department of Applied Biological Science, Science University of Tokyo, 2641, Yamazaki, Noda, Chiba 278-8510, Japan

² Department of Food Science and Technology, National Fisheries University, 2-7-1, Nagata-Honmachi, Shimonoseki, Yamaguchi 759-6595, Japan

Phenotypic and phylogenetic studies were performed with two strains (OCh 317^T and OCh 318; T = type strain) of aerobic chemoheterotrophic bacteriochlorophyll-containing bacteria isolated from water of a saline lake located on the west coast of Australia. Both strains were Gram-negative, short rods and were motile by means of polar flagella. Catalase, oxidase, nitrate reductase, phosphatase and urease were produced. The cells utilized D-glucose, citrate, glycolate, pyruvate and ethanol. Acids were produced from L-arabinose, D-fructose, D-galactose, D-glucose, D-ribose and D-xylose. The strains could grow in media containing 0.5–7.5% NaCl. Bacteriochlorophyll *a* was synthesized under aerobic conditions. The results of 16S rRNA gene sequence comparisons revealed that strain OCh 317^T represented a new lineage in the α -3 group of the class *Proteobacteria*. Strains OCh 317^T and OCh 318 were identified as strains of the same species because of their very similar phenotypic characteristics and their previously described high DNA–DNA homology. Therefore, it was concluded that the two strains should be assigned to a new genus and species, for which the name *Rubrimonas cliftonensis* is proposed. The type strain is OCh 317^T (= JCM 10189^T).

Keywords: *Rubrimonas cliftonensis*, aerobic bacteriochlorophyll-containing bacteria, saline lake, 16S rRNA

INTRODUCTION

Photosynthetic bacteria generally synthesize bacteriochlorophylls under anaerobic conditions. However, in 1978, Sato reported on aerobic methylotrophs containing bacteriochlorophyll *a*. Also, Shiba *et al.* (1979) isolated aerobic marine bacteria which contained bacteriochlorophyll *a*, but did not utilize methanol. An aerobic, radiation-resistant methylotroph has also been shown to contain bacteriochlorophyll *a* (Nishimura *et al.*, 1981). In addition to the above, it has been reported that aerobic bacteriochlorophyll-containing bacteria have been isolated from various sources. Recently, new aerobic photosynthetic bacteria have been isolated from a hot spring (Hanada *et al.*, 1997) and deep-ocean hydrothermal vents (Yurkov & Beatty, 1998). Based on phylogenetic analysis, it has

been shown that these organisms belong to the α subclass of the *Proteobacteria*: the α -1 group comprises *Acidiphilium* (Wakao *et al.*, 1993), *Roseococcus* (Yurkov *et al.*, 1994), *Craurococcus* (Saito *et al.*, 1998) and *Paracraurococcus* (Saito *et al.*, 1998); the α -2 group comprises *Methylobacterium* (Green & Bousfield, 1983) and *Rhizobium* sp. BTAi 1 (Evans *et al.*, 1990); the α -3 group comprises *Roseobacter* (Shiba, 1991); and the α -4 group comprises *Erythrobacter* (Shiba & Simidu, 1982), *Erythromicrobium* (Yurkov *et al.*, 1994), *Erythromonas* (Yurkov *et al.*, 1997), *Porphyrrobacter* (Fuerst *et al.*, 1993) and *Sandaracinobacter* (Yurkov *et al.*, 1997).

Nishimura *et al.* (1994) have reported that aerobic bacteriochlorophyll-containing bacteria isolated from the east and west coasts of Australia (Shiba *et al.*, 1991) can be divided into four groups (GI, GII, GIII and GIV) on the basis of colony colour, bacteriochlorophyll absorption spectra and cell morphology. DNA–DNA hybridization results from the four

The DDBJ accession number for the 16S rRNA gene sequence of strain OCh 317^T is D85834.

groups have shown the following: GI (14 strains) is divided into three genotype groups with the exception of one strain; GII (19 strains) is divided into four genotype groups with the exception of five strains; the DNA homology value between strains of GIII (two strains) is 83%; and the DNA homology value between strains of GIV (two strains) is 11%; and the DNA homology values between the isolated strains and the reference organisms (*Erythrobacter longus*, *Roseobacter litoralis* and *Roseobacter denitrificans*) are very low, with the exception of one GIV strain. In the present study, the phenotypic characters of the GIII strains (OCh 317^T and OCh 318; T = type strain) were investigated and the 16S rRNA gene of strain OCh 317^T was analysed. Based on the results of these investigations, a new genus and species, *Rubrimonas cliftonensis*, is proposed.

METHODS

Bacterial strains. Strains OCh 317^T and OCh 318 were isolated from water of Lake Clifton (a saline lake located on the west coast of Australia) by Shiba *et al.* (1991). The strains were cultivated on PPES-II medium (Taga, 1968) containing 2.0 g Bacto peptone, 1.0 g Proteose peptone No. 3, 1.0 g Bacto soytone, 1.0 g Bacto yeast extract, 0.1 g Fe(III)-EDTA, 1000 ml artificial sea water and 15.0 g agar (if needed). The artificial sea water contained (l⁻¹) 30.0 g NaCl, 0.7 g KCl, 10.8 g MgCl₂·6H₂O, 5.4 g MgSO₄·7H₂O and 1.0 g CaCl₂·2H₂O. The pH was adjusted to 7.8 with 10% (w/v) NaOH. *Roseobacter litoralis* OCh 149^T and *Roseobacter denitrificans* OCh 114^T were used as references for physiological and biochemical characterization.

Electron microscopy. Cells were stained with 1% (w/v) aqueous uranyl acetate and examined under a JEOL model JEM-1200 EX electron microscope at an accelerating voltage of 80 kV.

Physiological and biochemical characteristics. Physiological and biochemical characteristics were examined according to the methods of Shiba & Simidu (1982).

Preparation of chromosomal DNA. Strain OCh 317^T was grown in PPES-II broth at 27 °C with shaking. The cells were suspended in 0.1 M saline/EDTA (0.15 M NaCl, 0.1 M EDTA; pH 8.5), and then lysed at 60 °C for 10 min with 0.5% SDS (final concentration). Chromosomal DNA was purified according to standard procedures (Sambrook *et al.*, 1989).

Amplification of 16S rRNA gene. Amplification of the 16S rRNA gene was performed on a Quick Thermo Personal QTP-1 (Nippon Genetics) in 100 µl reaction volume containing 100 ng chromosomal DNA, 10 µl 10 × Ex Taq buffer (Takara Shuzo), 200 µM each dNTP, 1 µM each primer and 2.5 U Takara Ex Taq (Takara Shuzo). The primers were 5' AGTTTGATCCTGGCTC 3' [*Escherichia coli* numbering system (Brosius *et al.*, 1978); positions 10–25] and 5' AAGGAGGTGATCCAGCC 3' (positions 1525–1541). Amplification conditions were as described previously (Suzuki & Yamasato, 1994). The amplified DNA fragments were purified by gel electrophoresis on 1% Agarose S (Nippon Gene), and recovered with glass powder using Prep-A-Gene DNA Purification Systems (Bio-Rad).

Sequencing and analysis of sequence data. Sequencing was carried out as previously described (Suzuki & Yamasato,

1994). The determined sequence and the sequences of reference bacterial species were aligned with the CLUSTAL W program version 1.7 (Thompson *et al.*, 1994). The alignment was checked manually. Phylogenetic analysis was performed with PHYLIP version 3.57c (Felsenstein, 1995). A distance matrix was calculated with DNADIST using the Kimura two-parameter method and a phylogenetic tree was reconstructed using NEIGHBOR. The stability of the clusters was ascertained by performing bootstrap analysis (1000 replications) with DNABOOT, DNADIST, NEIGHBOR and CONSENSE.

RESULTS

Colony and cell morphology

Colonies were circular, smooth, slightly convex, entire, glistening, opaque and pink. An electron micrograph of negatively stained cells showed that they were short rods with polar flagella (Fig. 1). Cells were 1.0–1.5 × 1.2–2.0 µm and divided by binary fission.

Physiological and biochemical characteristics

Strains OCh 317^T and OCh 318 grew chemoheterotrophically under aerobic conditions, but could not grow phototrophically under anaerobic conditions in the light. They synthesized bacteriochlorophyll *a* under aerobic conditions. Optimum growth occurred at pH 7.5–8.0 and at 27–30 °C. The physiological and biochemical properties of strains OCh 317^T and OCh 318 are shown in Table 1. These two strains had catalase, oxidase, nitrate reductase, phosphatase and urease activities. The Voges–Proskauer test and ONPG reaction were negative. Both strains produced indole, but not H₂S. The strains did not hydrolyse alginate, gelatin, starch or Tween 80. The two strains utilized D-glucose, citrate, glycolate, pyruvate and ethanol, but

Fig. 1. Electron micrograph of a negatively stained cell of strain OCh 317^T. Bar, 1 µm.

Table 1. Differential characteristics of isolates and reference strains

–, Negative; +, positive; w, weakly positive.

Character	OCh 317 ^T	OCh 318	OCh 114 ^{T*}	OCh 149 ^{T†}
Nitrate reductase	w	w	+	–
Phosphatase	+	+	+	–
Urease	+	+	–	–
ONPG reaction	–	–	+	+
Hydrolysis of:				
Gelatin	–	–	+	+
Tween 80	–	–	+	+
Utilization of:				
Acetate	–	–	w	+
Butyrate	w	–	–	+
Fumarate	–	–	+	+
DL-Lactate	–	–	+	+
DL-Malate	–	–	+	+
Succinate	–	–	+	+
Ethanol	+	+	–	–
L-Aspartate	+	–	–	–
L-Glutamate	–	–	+	+
Acid production from:				
D-Fructose	+	w	w	–
D-Galactose	+	+	–	–
D-Glucose	+	+	–	–
Maltose	w	–	–	–
D-Ribose	+	+	–	–
D-Xylose	+	+	–	w
Susceptibility to tetracycline	+	+	–	–

* *Roseobacter denitrificans*.

† *Roseobacter litoralis*.

did not utilize acetate, fumarate, DL-lactate, DL-malate, succinate, methanol or L-glutamate. Acids were produced from L-arabinose, D-fructose, D-galactose, D-glucose, D-ribose and D-xylose, but were not produced from lactose or sucrose. The strains required NaCl for growth and could grow in media containing 0.5–7.5% (w/v) NaCl. They were resistant to penicillin, but were sensitive to chloramphenicol, streptomycin and tetracycline. Butyrate and L-aspartate were utilized by strain OCh 317^T, but not by strain OCh 318. Strain OCh 317^T produced acid from maltose, but strain OCh 318 did not.

Phylogenetic analysis

The 16S rRNA gene sequence of strain OCh 317^T was determined and aligned with other available 16S rRNA sequences of strains belonging to the α subclass of the *Proteobacteria*. A comparison of the 16S rRNA sequences in which a phylogenetic tree was reconstructed (Fig. 2) revealed that strain OCh 317^T belonged to the α -3 group of the class *Proteobacteria*, forming a separate line of descent. The 16S rRNA sequence similarity values between strain OCh 317^T

and the other strains belonging to the α -3 group of the class *Proteobacteria* were low: *Rhodobacter veldkampii*, 89.8%; *Rhodovulum sulfidophilum*, 89.6%; and *Rhodobacter sphaeroides*, 89.1%.

DISCUSSION

Aerobic bacteriochlorophyll-containing bacteria isolated from the east and west coasts of Australia (Shiba *et al.*, 1991) have been divided into four groups on the basis of colony colour, bacteriochlorophyll absorption spectra and cell morphology (Nishimura *et al.*, 1994). Chemotaxonomic characteristics and DNA–DNA hybridization of GIII (OCh 317^T and OCh 318) strains have been investigated; however, the taxonomic position of the strains is not clear. To solve this problem, phylogenetic analysis based on 16S rRNA gene sequences was carried out. The 16S rRNA gene sequence of strain OCh 317^T was determined and compared with sequences of strains belonging to the α subclass of the class *Proteobacteria*. In consequence, it was revealed that strain OCh 317^T was only distantly related to the other genera studied. The sequence similarity value of strain OCh 317^T to the most related strain,

Fig. 2. Unrooted phylogenetic tree derived from analysis of the 16S rRNA gene sequences of *Rubrimonas cliftonensis* OCh 317^T and other members of the α subclass of the class *Proteobacteria*. The numbers at the nodes indicate the levels of bootstrap support based on 1000 resamplings.

Rhodobacter veldkampii, was 89.8%. Therefore, strain OCh 317^T should be a new taxon in the α -3 group.

The DNA–DNA homology value between strain OCh 318, also included in GIII, and strain OCh 317^T has been previously reported as 83% (Nishimura *et al.*, 1994). In terms of physiological and biochemical characteristics, strain OCh 318 was similar to strain OCh 317^T (Table 1). These results show that strains OCh 317^T and OCh 318 belong to same species.

Thus, it is proposed that a new genus should be created for strains OCh 317^T and OCh 318, *Rubrimonas cliftonensis* gen. nov., sp. nov.

Description of *Rubrimonas* gen. nov.

Rubrimonas (Ru.bri.mo'nas. L. adj. *ruber* reddish; Gr. n. *monas* monad, unit; M.L. fem. n. *Rubrimonas* reddish monad).

Cells are aerobic, Gram-negative, short rods that are motile by means of polar flagella. Catalase and oxidase are produced. Chemoheterotrophic. Bacteriochlorophyll *a* is synthesized under aerobic conditions. The ubiquinone system is Q-10 (Nishimura *et al.*,

1994). The major cellular fatty acid is C_{18:1} (Nishimura *et al.*, 1994). The type species is *Rubrimonas cliftonensis*.

Description of *Rubrimonas cliftonensis* sp. nov.

Rubrimonas cliftonensis (clif.to.nen'sis. M. L. adj. *cliftonensis* referring to Lake Clifton, Australia, the source of the type strain).

Colonies are circular, smooth, slightly convex, entire, glistening, opaque and pink. Cells are 1.0–1.5 × 1.2–2.0 µm and divide by binary fission. Optimum growth occurs at pH 7.5–8.0 and at 27–30 °C. Growth occurs in the presence of 0.5–7.5% (w/v) NaCl. No growth occurs in the absence of NaCl. Nitrate reductase, phosphatase and urease are produced. Voges–Proskauer test and ONPG reaction are negative. Cells produce indole, but not H₂S. Does not hydrolyse alginate, gelatin, starch or Tween 80. Cells utilize D-glucose, citrate, glycolate, pyruvate and ethanol, but not acetate, fumarate, DL-lactate, DL-malate, succinate, methanol or L-glutamate. Acids are produced from L-arabinose, D-fructose, D-galactose, D-glucose, D-ribose and D-xylose, but not from lactose or sucrose. Cells are resistant to penicillin, but are sensitive to chloramphenicol, streptomycin and tetracycline. The absorption spectrum of the membrane fraction in the near-IR region has maxima at 806 and 871 nm. The habitat of the strains is saline lake water. The DNA G+C content is 74.0–74.8 mol% (Nishimura *et al.*, 1994). The type strain is strain OCh 317^T (=JCM 10189^T).

ACKNOWLEDGEMENTS

We thank Isamu Mutoh (Department of Applied Biological Science, Science University of Tokyo, Noda, Japan) for his help with electron microscopy.

REFERENCES

- Brosius, J., Palmer, M. L., Kennedy, P. J. & Noller, H. F. (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc Natl Acad Sci USA* **75**, 4801–4805.
- Evans, W. R., Fleischman, D. E., Calvert, H. E., Pyati, P. V., Alter, G. M. & Rao, N. S. S. (1990). Bacteriochlorophyll and photosynthetic reaction centers in *Rhizobium* strain BTAi 1. *Appl Environ Microbiol* **56**, 3445–3449.
- Felsenstein, J. (1995). PHYLIP (phylogeny inference package), version 3.57c. University of Washington, Seattle, WA, USA.
- Fuerst, J. A., Hawkins, J. A., Holmes, A., Sly, L. I., Moore, C. J. & Stackebrandt, E. (1993). *Porphyrobacter neustonensis* gen. nov., sp. nov., an aerobic bacteriochlorophyll-synthesizing budding bacterium from fresh water. *Int J Syst Bacteriol* **43**, 125–134.
- Green, P. N. & Bousfield, I. J. (1983). Emendation of *Methylobacterium* Patt, Cole, and Hanson 1976; *Methylobacterium rhodinum* (Heumann 1962) comb. nov. corrig.; and *Methylobacterium radiotolerans* (Ito and Iizuka 1971) comb. nov. corrig.; and *Methylobacterium mesophilicum* (Austin and Goodfellow 1979) comb. nov. *Int J Syst Bacteriol* **33**, 875–877.
- Hanada, S., Kawase, Y., Hiraishi, A., Takaichi, S., Matsuura, K., Shimada, K. & Nagashima, K. V. P. (1997). *Porphyrobacter*

- tepidarius* sp. nov., a moderately thermophilic aerobic photosynthetic bacterium isolated from a hot spring. *Int J Syst Bacteriol* **47**, 408–413.
- Nishimura, Y., Shimadzu, M. & Iizuka, H. (1981). Bacteriochlorophyll formation in radiation-resistant *Pseudomonas radiosa*. *J Gen Appl Microbiol* **27**, 427–430.
- Nishimura, Y., Muroga, Y., Saito, S., Shiba, T., Takamiya, K. & Shioi, Y. (1994). DNA relatedness and chemotaxonomic feature of aerobic bacteriochlorophyll-containing bacteria isolated from coasts of Australia. *J Gen Appl Microbiol* **40**, 287–296.
- Saito, S., Suzuki, T. & Nishimura, Y. (1998). Proposal of *Craurococcus roseus* gen. nov., sp. nov. and *Paracraurococcus ruber* gen. nov., sp. nov., novel aerobic bacteriochlorophyll *a*-containing bacteria from soil. *Int J Syst Bacteriol* **48**, 1043–1047.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sato, K. (1978). Bacteriochlorophyll formation by facultative methylotrophs, *Protaminobacter ruber* and *Pseudomonas* AM1. *FEBS Lett* **85**, 207–210.
- Shiba, T. (1991). *Roseobacter litoralis* gen. nov., sp. nov. and *Roseobacter denitrificans* sp. nov., aerobic pink-pigmented bacteria which contain bacteriochlorophyll *a*. *Syst Appl Microbiol* **14**, 140–145.
- Shiba, T. & Simidu, U. (1982). *Erythrobacter longus* gen. nov., sp. nov., an aerobic bacterium which contains bacteriochlorophyll *a*. *Int J Syst Bacteriol* **32**, 211–217.
- Shiba, T., Simidu, U. & Taga, N. (1979). Distribution of aerobic bacteria which contain bacteriochlorophyll *a*. *Appl Environ Microbiol* **38**, 43–45.
- Shiba, T., Shioi, Y., Takamiya, K., Sutton, D. C. & Wilkinson, C. R. (1991). Distribution and physiology of aerobic bacteria containing bacteriochlorophyll *a* on the east and west coasts of Australia. *Appl Environ Microbiol* **57**, 295–300.
- Suzuki, T. & Yamasato, K. (1994). Phylogeny of spore-forming lactic acid bacteria based on 16S rRNA gene sequences. *FEMS Microbiol Lett* **115**, 13–18.
- Taga, N. (1968). Some ecological aspects of marine bacteria in the kuroshio current. *Bull Misaki Mar Biol Inst Kyoto Univ* **12**, 56–76.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL w: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Wakao, N., Shiba, T., Hiraishi, A., Ito, M. & Sakurai, Y. (1993). Distribution of bacteriochlorophyll *a* in species of the genus *Acidiphilium*. *Curr Microbiol* **27**, 277–279.
- Yurkov, V. & Beatty, J. T. (1998). Isolation of aerobic anoxygenic photosynthetic bacteria from black smoker plume waters of the Juan de Fuca Ridge in the Pacific Ocean. *Appl Environ Microbiol* **64**, 337–341.
- Yurkov, V., Stackebrandt, E., Holmes, A., Fuerst, J. A., Hugenholtz, P., Golecki, J., Gad'on, N., Gorlenko, V. M., Kompantseva, E. I. & Drews, G. (1994). Phylogenetic positions of novel aerobic, bacteriochlorophyll *a*-containing bacteria and description of *Roseococcus thiosulfatophilus* gen. nov., sp. nov., *Erythromicrobium ramosum* gen. nov., sp. nov., and *Erythrobacter litoralis* sp. nov. *Int J Syst Bacteriol* **44**, 427–434.
- Yurkov, V., Stackebrandt, E., Buss, O., Vermeglio, A., Gorlenko, V. & Beatty, J. T. (1997). Reorganization of the genus *Erythromicrobium*: description of '*Erythromicrobium sibiricum*' as *Sandaracinobacter sibiricus* gen. nov., sp. nov., and of '*Erythromicrobium ursincola*' as *Erythromonas ursincola* gen. nov., sp. nov. *Int J Syst Bacteriol* **47**, 1172–1178.