***Roseivivax halodurans* gen. nov., sp. nov. and *Roseivivax halotolerans* sp. nov., aerobic bacteriochlorophyll-containing bacteria isolated from a saline lake**

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Phenotypic and phylogenetic studies were performed with two strains (OCh 239^T and OCh 210^T, T = type strain) of aerobic bacteriochlorophyll-containing bacteria isolated from the charophytes and the epiphytes on the stromatolites, respectively, of a saline lake located on the west coast of Australia. Both strains were chemoheterotrophic, Gram-negative and motile rods with subpolar flagella. Catalase and oxidase were produced. ONPG reaction was positive. Cells utilized D-glucose, acetate, butyrate, citrate, DL-lactate, DL-malate, pyruvate, succinate, L-aspartate and L-glutamate. Acids were produced from D-fructose and D-glucose. Bacteriochlorophyll *a* was synthesized under aerobic conditions. Strain OCh 239^T had nitrate reductase and phosphatase. Acids were produced from L-arabinose, D-galactose, lactose, maltose, D-ribose and sucrose. The strain could grow in 0–20.0% (w/v) NaCl. Strain OCh 210^T had urease. Hydrolysis of gelatin was positive. Acids were produced from D-xylose. The strain could grow in 0.5–20.0% (w/v) NaCl. The results of 16S rRNA sequence comparisons revealed that strains OCh 239^T and OCh 210^T formed a new cluster within the α -3 group of the α subclass of the class *Proteobacteria*. The similarity value of the 16S rRNA sequences between strains OCh 239^T and OCh 210^T was 95.8%. Therefore, it was concluded that these two strains should be placed in a new genus, *Roseivivax* gen. nov., as the new species *Roseivivax halodurans* sp. nov. and *Roseivivax halotolerans* sp. nov. The type species of the genus is *Roseivivax halodurans*. The type strains of *Roseivivax halodurans* and *Roseivivax halotolerans* are OCh 239^T (= JCM 10272^T) and OCh 210^T (= JCM 10271^T), respectively.

Keywords: *Roseivivax halodurans*, *Roseivivax halotolerans*, aerobic bacteriochlorophyll-containing bacteria, saline lake, 16S rRNA

INTRODUCTION

The first paper on aerobic bacteriochlorophyll-containing bacteria was published by Sato in 1978. Afterwards, aerobic bacteriochlorophyll-containing bacteria were isolated from various sources (Shiba *et al.*, 1979) and identified (Nishimura *et al.*, 1981). Recently, new aerobic photosynthetic bacteria have been isolated from a hot spring (Hanada *et al.*, 1997)

The GenBank/EMBL/DBJ accession numbers for the 16S rRNA gene sequences of strains OCh 239^T and OCh 210^T are D85829 and D85831, respectively.

and deep-ocean hydrothermal vents (Yurkov & Beatty, 1998). Based on the phylogenetic analysis, it has been shown that these and newly isolated organisms belong to the α subclass of the class *Proteobacteria*. *Acidiphilium* (Wakao *et al.*, 1993), *Roseococcus* (Yurkov *et al.*, 1994), *Craurococcus* (Saito *et al.*, 1998) and *Paracraurococcus* (Saito *et al.*, 1998) are located in the α -1 group. *Methylobacterium* (Green & Bousfield, 1983) and *Rhizobium* sp. BTAi 1 (Evans *et al.*, 1990) are located in the α -2 group. *Roseobacter* (Shiba, 1991) is a member of the α -3 group. *Erythrobacter* (Shiba & Simidu, 1982), *Erythromicrobium* (Yurkov *et al.*, 1994), *Erythromonas* (Yurkov *et al.*,

Fig. 1. Electron micrographs of negatively stained cells of strains OCh 239^T (top) and OCh 210^T (bottom). Bars, 1 µm.

1997), *Porphyrobacter* (Fuerst *et al.*, 1993) and *Sandarinobacter* (Yurkov *et al.*, 1997) comprise the α -4 group.

Shiba *et al.* (1991) isolated aerobic bacteriochlorophyll-containing bacteria from the east and west coasts of Australia. These organisms have been divided into four groups (Group I–IV) on the basis of colony colour, absorption spectrum type of bacteriochlorophyll and cell morphology (Nishimura *et al.*, 1994). Based on the results of the DNA–DNA hybridization (Nishimura *et al.*, 1994), each group (except Group III) has been divided into several genotype groups. Group II (19 strains) is divided into four genotype groups and the others (strains OCh 215, 231, 210, 239 and 263). In the present study, we investigated the

phenotypic characteristics and the phylogenetic situation of strains OCh 239^T (T = type strain) and OCh 210^T in Group II. Based on the results of this study, we propose the new genus *Roseivivax* with two new species, *Roseivivax halodurans* and *Roseivivax halotolerans*.

METHODS

Bacterial strains. Strains OCh 239^T and OCh 210^T were previously isolated from the charophytes and the epiphytes on the stromatolites, respectively, at Lake Clifton (isolated saline lake located in 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 (per litre) 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. Strains OCh 239^T and OCh 210^T were 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 as a final concentration. Chromosomal DNA was purified according to standard procedures (Sambrook *et al.*, 1989).

Amplification of 16S rRNA gene. Amplification of 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 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 the Prep-A-Gene DNA Purification System (Bio-Rad).

Sequencing and analysis of sequence data. Sequencing was carried out as described previously (Suzuki & Yamasato, 1994). The sequences that were determined and the sequences of reference bacterial species were aligned using the program CLUSTAL W version 1.7 (Thompson *et al.*, 1994). The alignment was checked manually. Phylogenetic analysis was performed using the PHYLIP (phylogeny inference package) version 3.57c (Felsenstein, 1995). A distance matrix was calculated with DNADIST using the Kimura 2-parameter model, and a phylogenetic tree was reconstructed using NEIGHBOR. The stability of the clusters was ascertained by performing a bootstrap analysis (1000 replications) with DNABOOT, DNADIST, NEIGHBOR and CONSENSE.

RESULTS

Colony and cell morphology

Colonies of strains OCh 239^T and OCh 210^T were circular, smooth, slightly convex, entire, glistening, opaque and pink. Electron micrographs of negatively stained cells showed that they were rods with subpolar flagella (Fig. 1). Cells of strain OCh 239^T were 0.5–1.0 × 1.0–5.0 µm, and cells of strain OCh 210^T were 0.5–1.0 × 1.0–3.0 µm.

Physiological and biochemical characteristics

Strains OCh 239^T and OCh 210^T 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 239^T and OCh 210^T are shown in Table 1. These two strains had catalase and oxidase. Voges–Proskauer test was negative. ONPG reaction was positive. Both strains produced indole, but did not generate H₂S. Starch, Tween 80 or alginate were not hydrolysed. Both strains utilized D-glucose, acetate, butyrate, citrate, DL-lactate, DL-malate, pyruvate, succinate, L-aspartate and

L-glutamate, but did not utilize fumarate, glycolate, ethanol or methanol. Acids were produced from D-fructose and D-glucose. Both strains were resistant to penicillin and tetracycline, but were sensitive to chloramphenicol and streptomycin.

Strain OCh 239^T had nitrate reductase and phosphatase, but did not have urease. Gelatin was not hydrolysed. Acids were produced from L-arabinose, D-galactose, lactose, maltose, D-ribose and sucrose, but were not produced from D-xylose. The strain could grow in media supplemented with 0–20.0 % (w/v) NaCl.

Strain OCh 210^T had urease, but did not have nitrate reductase or phosphatase. Gelatin was hydrolysed. Acids were produced from D-xylose, but were not produced from L-arabinose, D-galactose, lactose, maltose, D-ribose or sucrose. The strain required NaCl for growth, and could grow in 0.5–20.0 % (w/v) NaCl.

Phylogenetic analysis

The 16S rRNA gene sequences of strains OCh 239^T and OCh 210^T were determined, and aligned with the other available 16S rRNA sequences of the strains that belong to the α-3 group of the α subclass of the class *Proteobacteria*. A comparison of the 16S rRNA

Table 1. Differential characteristics of isolates and reference strains *Roseobacter denitrificans* OCh114^T and *Roseobacter litoralis* OCh149^T

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

Character	Och239 ^T	Och210 ^T	OCh114 ^T	OCh149 ^T
Nitrate reductase	+	–	+	–
Phosphatase	+	–	+	–
Urease	–	+	–	–
Hydrolysis of:				
Gelatin	–	+	+	+
Tween 80	–	–	+	+
Utilization of:				
Butyrate	+	+	–	+
Fumarate	–	–	+	+
Glycolate	–	–	+	+
L-Aspartate	+	+	–	–
Acid production from:				
L-Arabinose	+	–	w	w
D-Fructose	+	w	w	–
D-Galactose	+	–	–	–
D-Glucose	+	w	–	–
Lactose	+	–	–	–
Maltose	+	–	–	–
D-Ribose	+	–	–	–
Sucrose	+	–	–	–
D-Xylose	–	w	–	w
Growth in the presence of:				
0% NaCl	w	–	–	–
20.0% NaCl	w	+	–	–

Fig. 2. Unrooted phylogenetic tree derived from the analysis of the 16S rRNA sequences of *Roseivivax halodurans* OCh 239^T, *Roseivivax halotolerans* OCh 210^T and other members of the α subclass of the *Proteobacteria*. The numbers at the nodes indicate the levels of bootstrap support based on 1000 resamplings.

sequences revealed that strains OCh 239^T and OCh 210^T belonged to the α -3 group of the α subclass of the class *Proteobacteria*, forming a distinct cluster (Fig. 2). The cluster neighboured on *Roseobacter algicola* and *Roseobacter gallaeciensis*. The similarity values of the 16S rRNA sequences of strains OCh 239^T and OCh 210^T to *Roseobacter algicola* and *Roseobacter gallaeciensis* were 91.8–93.3%. The similarity value of the 16S rRNA sequences between strains OCh 239^T and OCh 210^T was 95.8%.

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, absorption spectrum type of bacteriochlorophyll and cell morphology (Nishimura *et al.*, 1994). These phenotypic groups have been investigated for their chemotaxonomic characteristics and by DNA–DNA hybridization. Strains OCh 239^T and OCh 210^T have been included in Group II, but did not belong to any of the genotype groups. To define

the phylogenetic positions of these strains, we performed comparative 16S rRNA sequence analysis. In consequence, it was revealed that strains OCh 239^T and OCh 210^T formed a new distinct cluster moderately related to *Roseobacter algicola*, *Roseobacter gallaeciensis*, *Sagittula stellata*, *Octadecabacter arcticus* and *Sulfitobacter pontiacus*. The cluster was distantly related to other taxa at the generic level (93.3% 16S rRNA sequence similarity or less). The phylogenetic analysis supports the proposal of the new genus *Roseivivax* for strains OCh 239^T and OCh 210^T.

Strain OCh 239^T was phylogenetically distant from strain OCh 210^T (95.8% 16S rRNA sequence similarity). Phenotypic characteristics of strain OCh 239^T were very different from those of strain OCh 210^T, i.e. nitrate reduction, urease, phosphatase, hydrolysis of gelatin and acid production from carbon sources. These results indicate that strains OCh 239^T and OCh 210^T are different species.

In conclusion, we propose that strains OCh 239^T and OCh 210^T should be described as *Roseivivax halodurans* gen. nov., sp. nov. and *Roseivivax halotolerans* sp. nov., respectively.

Description of *Roseivivax* gen. nov.

Roseivivax (Ro.se.i.vi'vax. M.L. adj. *roseus* rose-coloured, pink; L. adj. *vivax* living; M.L. masc. n. *Roseivivax* pink living organism).

Cells are aerobic and Gram-negative rods that are motile by means of subpolar 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 acids are C18:1 (Nishimura *et al.*, 1994). The type species is *Roseivivax halodurans*.

Description of *Roseivivax halodurans* sp. nov.

Roseivivax halodurans (ha.lo.du'rans. Gr. n. *hals* salt; L. pres. part. *durans* enduring; M.L. part. adj. *halodurans* salt-enduring).

Colonies are circular, smooth, slightly convex, entire, glistening, opaque and pink. Cells are 0.5–1.0 $\times$ 1.0–5.0 μ m. Optimum growth occurs at pH 7.5–8.0 and at 27–30 °C. Growth occurs in the presence of 0–20.0% (w/v) NaCl. Cells have nitrate reductase and phosphatase, but do not have urease. Voges–Proskauer test is negative. ONPG reaction is positive. Cells produce indole, but do not generate H₂S. Hydrolysis of alginate, gelatin, starch and Tween 80 is negative. Cells utilize D-glucose, acetate, butyrate, citrate, DL-lactate, DL-malate, pyruvate, succinate, L-aspartate and L-glutamate, but do not utilize fumarate, glycolate, ethanol or methanol. Acids are produced from L-arabinose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-ribose and sucrose, but are not produced from D-xylose. Cells are resistant to penicillin and

tetracycline, but are sensitive to chloramphenicol and streptomycin. The absorption spectrum of the membrane fraction in the near-IR region has maxima at 803 and 873 nm (Nishimura *et al.*, 1994). The source of the strain is the charophytes of the saline lake. The G + C content of the DNA is 64.4 mol % (Nishimura *et al.*, 1994). The type strain is strain OCh 239^T (= JCM 10272^T).

Description of *Roseivivax halotolerans* sp. nov.

Roseivivax halotolerans (ha.lo.to'le.rans. Gr. n. *hals* salt; L. pres. part. *tolerans* tolerating; M. L. part. adj. *halotolerans* salt-tolerating).

Colonies are circular, smooth, slightly convex, entire, glistening, opaque and pink. Cells are 0.5–1.0 × 1.0–5.0 µm. Optimum growth occurs at pH 7.5–8.0 and at 27–30 °C. Growth occurs in the presence of 0.5–20.0 % (w/v) NaCl. No growth occurs in the absence of NaCl. Cells have urease, but do not have nitrate reductase or phosphatase. Voges–Proskauer test is negative. ONPG reaction is positive. Cells produce indole, but do not generate H₂S. Hydrolysis of gelatin is positive, but hydrolysis of alginate, starch and Tween 80 is negative. Cells utilize D-glucose, acetate, butyrate, citrate, DL-lactate, DL-malate, pyruvate, succinate, L-aspartate and L-glutamate, but do not utilize fumarate, glycolate, ethanol or methanol. Acids are produced from D-fructose, D-glucose and D-xylose, but are not produced from L-arabinose, D-galactose, lactose, maltose, D-ribose or sucrose. Cells are resistant to penicillin and tetracycline, but are sensitive to chloramphenicol and streptomycin. The absorption spectrum of the membrane fraction in the near-IR region has maxima at 805 and 871 nm (Nishimura *et al.*, 1994). The source of the strain is the epiphytes on the stromatolites of the saline lake. The G + C content of the DNA is 59.7 mol % (Nishimura *et al.*, 1994). The type strain is strain OCh 210^T (= JCM 10271^T).

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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., Feischman, 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. Seattle: University of Washington.
- 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 radiora*. *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* 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. *FEMS 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, positions-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. & 7 other authors (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.