Rubritalea halochordaticola sp. nov., a carotenoid-producing verrucomicrobial species isolated from a marine chordate

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A Gram-negative-staining, obligately aerobic, non-motile, rod-shaped and chemoheterotrophic bacterium, designated strain MN1-1006^T, was isolated from an ascidian (sea squirt) sample, and was studied using a polyphasic taxonomic approach. Phylogenetic analyses based on 16S rRNA gene sequences indicated that the new isolate shared approximately 93–99% sequence similarity with recognized species of the genus *Rubritalea* within the phylum ‘*Verrucomicrobia*’. DNA–DNA hybridization values between strain MN1-1006^T and *Rubritalea squalenifaciens* HOact23^T and *Rubritalea sabuli* YM29-052^T were 57% and 14.5%, respectively. Strain MN1-1006^T produced carotenoid compounds that rendered the cell biomass a reddish pink colour. The strain also contained squalene. The cell-wall peptidoglycan of the novel strain contained muramic acid and meso-diaminopimelic acid. The DNA G+C content of strain MN1-1006^T was 51.4 mol%. The major cellular fatty acids were iso-C_{14:0}, iso-C_{16:0} and anteiso-C_{15:0}. The major isoprenoid quinone was MK-9. On the basis of these data, it was concluded that strain MN1-1006^T represents a novel species of the genus *Rubritalea*, for which the name *Rubritalea halochordaticola* sp. nov. is proposed. The type strain is MN1-1006^T (=KCTC 23186^T=NBRC 107102^T).

The phylum ‘*Verrucomicrobia*’ (Hedlund *et al.*, 1997) represents a primary lineage within the bacterial phylogenetic tree and contains a number of environmental species as well as a small number of cultured species. The genus *Rubritalea* (Scheuermayer *et al.*, 2006) is a member of this phylum and consists of several marine species such as *Rubritalea marina* (Scheuermayer *et al.*, 2006), *Rubritalea squalenifaciens* (Kasai *et al.*, 2007), *Rubritalea spongiae* (Yoon *et al.*, 2007), *Rubritalea tangerina* (Yoon *et al.*, 2007) and *Rubritalea sabuli* (Yoon *et al.*, 2008). These species were isolated from marine invertebrates and marine sediments and appeared as reddish pink-coloured colonies following their culture with various types of seawater media containing polysaccharides, amino acids, vitamins

and antibiotics. The four latter species were found to be squalene- and carotenoid-producing bacteria. Carotenoid-producing isolates are potential genetic resources for isoprenoid pigments of industrial and nutritional interest. Carotenoids are an abundant group of isoprenoid pigments synthesized by all photosynthetic organisms and some non-photosynthetic bacteria and fungi. Carotenoids such as lycopene also act as natural antioxidants that could help to prevent some types of human cancer and degenerative diseases (Fraser & Bramley, 2004). Moreover, the colourant and aromatic properties of some carotenoids and derived compounds have an important industrial value (Bartley & Scolnik, 1995; Sandmann, 2001; Fraser & Bramley, 2004). Recently, a novel acyl glyco-carotenoid, diapolycopenedioic acid xylosyl ester, possessing anti-oxidative activities was discovered in *R. squalenifaciens* HOact23^T (Shindo *et al.*, 2007). In this study, we attempted to elucidate the phylogenetic position of a novel carotenoid-producing bacterium, strain MN1-1006^T isolated from a marine ascidian, using a polyphasic taxonomic approach including 16S rRNA gene sequence analysis. Based on these data, it is proposed that the new isolate represents a novel species of the genus *Rubritalea* within the phylum ‘*Verrucomicrobia*’.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Rubritalea halochordaticola* MN1-1006^T is AB543683.

Strain MN1-1006^T was isolated from an ascidian sample collected by scuba diving near the arch-like configuration of Himezu Port, Sado Island, Niigata Prefecture, Japan, in December 2006. The tissue sample (0.5–1.0 cm³) was homogenized with a glass rod in 5 ml sterile seawater. A 50 µl sample of the homogenate was applied to the surface of an agar plate containing medium M1 (Mincer *et al.*, 2002). Strain MN1-1006^T appeared after incubation for 30 days at 25 °C. The reddish pink-pigmented colony was purified on M1 agar after being cultivated for 7–10 days.

The temperature range and pH range for growth were determined by incubating the isolates on 1/2 strength R2A agar (Difco) with 75 % artificial seawater (Lyman & Fleming, 1940). The NaCl concentration for growth was determined on 1/2 strength R2A agar containing 0–10 % (w/v) NaCl. Gram-staining was performed as described by Murray *et al.* (1994). Cell morphology was observed using light microscopy (BX60; Olympus). Growth under anaerobic conditions was determined after 2 weeks of incubation in an AnaeroPack (Mitsubishi Gas Chemical Co., Inc.) on 1/2 strength R2A agar (Difco) with 75 % artificial seawater. Catalase activity was determined by bubble formation in a 3 % H₂O₂ solution. Oxidase activity was determined by cytochrome oxidase paper (Nissui Pharmaceutical Co., Ltd). API 20NE and API ZYM strips (bioMérieux) were used to determine the physiological and biochemical characteristics of the novel strain. All suspension media for the API test strips were supplemented with 0.85 % (w/v) NaCl solution (final concentration). API 20NE and API ZYM strips were read after 24 h incubation at 30 °C and 4 h incubation at 37 °C, respectively. The substrate oxidation range of strain MN1-1006^T was determined using Biolog MicroPlates.

Determination of the respiratory quinone system and cellular fatty acid composition were carried out as described previously (Katsuta *et al.*, 2005). For the analysis of cellular fatty acid composition, bacterial strains investigated in the present and previous studies were grown on marine broth (Difco) for a week at 30 °C. DNA was extracted and purified from cells grown on 1/2 strength R2A agar with 75 % artificial seawater according to the method of Marmur (1961). The DNA base composition was determined by using the HPLC method of Mesbah *et al.* (1989).

DNA–DNA hybridization tests were performed with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989). The hybridization temperature was set at 46 °C. Hybridization was performed with five replicates. Of the values obtained, the highest and lowest value for each sample was excluded and the means of the remaining three values are quoted as the final DNA–DNA relatedness values.

Cell walls were prepared according to the methods described by Schleifer & Kandler (1972). The amino acids

in an acid hydrolysate of the cell walls were identified by TLC (Harper & Davis, 1979) and by HPLC, as their phenylthiocarbamoyl derivatives, with HPLC apparatus (LC-10AD; Shimadzu) equipped with a Wakopak WS-PTC column (Wako Pure Chemical Industries) (Yokota *et al.*, 1993).

An approximately 1500 bp fragment of the 16S rRNA gene was amplified from the extracted DNA by using bacterial universal primers specific to the 16S rRNA gene: 27F and 1492R (*Escherichia coli* numbering system; Weisburg *et al.*, 1991). To ascertain the phylogenetic position of the new isolate, the 16S rRNA gene sequence of strain MN1-1006^T was compared with sequences obtained from GenBank (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov>). Multiple alignments of the sequences were performed using CLUSTAL_X (version 1.83) (Thompson *et al.*, 1997). Alignment gaps and ambiguous bases were not taken into consideration when the 1404 bases of the 16S rRNA gene nucleotides were compared. Aligned sequences were analysed with MEGA3.1 software (Kumar *et al.*, 2004). Distances were calculated by using the Kimura two-parameter model (Kimura, 1983). Clustering with the neighbour-joining method (Saitou & Nei, 1987) was determined by using bootstrap values based on 1000 replications (Felsenstein, 1985). The similarity values were calculated using the same software.

Cells of strain MN1-1006^T grown on 1/2 strength R2A agar were rod-shaped, ranging from 0.5 to 0.8 µm in width and from 1.0 to 1.5 µm in length. Cell motility due to flagella or gliding was not observed. Cell division occurred by binary fission only.

On the basis of 16S rRNA gene sequence analysis, strain MN1-1006^T was found to be closely related to the genus *Rubritalea* within the phylum 'Verrucomicrobia' (Fig. 1). Comparative phylogenetic analysis of the 16S rRNA gene sequences also showed that the sequence of strain MN1-1006^T had a high similarity of >99 % to the marine representatives *R. squalenifaciens* HOact23^T and *R. sabuli* YM29-052^T, followed by *R. marina* Pol012^T (93.5 %), *R. tangerina* YM27-005^T (93.3 %) and *R. spongiae* YM21-132^T (93.3 %). All other cultivated species of the family *Verrucomicrobiaceae* with validly published names were more distantly related.

The mean DNA–DNA hybridization values between strain MN1-1006^T and *R. squalenifaciens* HOact23^T and *R. sabuli* YM29-052^T were 57 % and 14.5 %, respectively (Table 1). These results strongly suggested that strain MN1-1006^T could represent a separate and independent species (Wayne *et al.*, 1987).

Analysis of the reddish pink pigments produced by strain MN1-1006^T was performed using HPLC/photodiode array detection (HPLC/PAD)/atmospheric pressure chemical ionization (APCI)-MS (Thermo Finnigan) of crude acetone extract from frozen cells. The pigment peaks detected by HPLC/PAD/APCI-MS showed a UV-visible spectrum with

Fig. 1. Neighbour-joining tree showing the phylogenetic relationship of strain MN1-1006^T and all recognized cultivated species of the genus *Rubritalea* within the phylum 'Verrucomicrobia' based on 16S rRNA gene sequences. Numbers at nodes are percentage bootstrap values derived from 1000 replications. The sequence of *Oritatus terrae* PB90-1^T was used as an outgroup. Bar, 2% estimated difference in nucleotide sequence.

absorption maxima at 494 and 522 nm. This spectrum was similar to that of the novel carotenoid produced by *R. squalenifaciens* HOact23^T (Shindo *et al.*, 2007). However, the molecular-related ion peak of the carotenoid could not be identified in the MS spectrum because of the presence of many peaks from other components in the extract. Squalene was also detected in the new isolate.

The predominant cellular fatty acids of the novel strain were iso-C_{14:0} (54.7%), iso-C_{16:0} (30.1%) and anteiso-C_{15:0} (9.3%), which were similar to those found for other members of the genus *Rubritalea* (Table 2). However, strain MN1-1006^T could be distinguished from the other species in the genus *Rubritalea* by the different proportions of fatty acids C_{15:0}, C_{15:1ω6c} and C_{16:1ω7c}. In addition, strain MN1-1006^T could be distinguished from the other species of the genus *Rubritalea* by the physiological and biochemical properties given in Table 3. The cell walls of the new isolate were prepared by disrupting cells, followed by heating with 3% SDS, washing and centrifugation. Amino acid analysis of the cell-wall hydrolysate indicated the presence of muramic acid and *meso*-diaminopimelic acid as the diagnostic diamino acids of the cell-wall peptidoglycan of strain MN1-1006^T.

Based on the results of this polyphasic taxonomic study, strain MN1-1006^T warrants description as a novel species

of the genus *Rubritalea*. The name *Rubritalea halochordaticola* sp. nov. is proposed.

Description of *Rubritalea halochordaticola* sp. nov.

Rubritalea halochordaticola [ha.lo.chor.da.ti'co.la. Gr. n. *hals halos* sea; N.L. pl. n. *Chordata* scientific name of an animal phylum; L. suff. *-cola* (from L. n. *incola*) inhabitant; N.L. n. *halochordaticola* inhabitant of marine chordates].

Table 2. Comparison of the cellular fatty acid contents of strain MN1-1006^T and type strains of related species of the genus *Rubritalea*

Strains: 1, MN1-1006^T; 2, *R. sabuli* YM29-052^T (data from Yoon *et al.*, 2008); 3, *R. marina* Pol012^T (Scheuermayer *et al.*, 2006); 4, *R. squalenifaciens* HOact23^T (Kasai *et al.*, 2007); 5, *R. spongiae* YM21-132^T (Yoon *et al.*, 2007); 6, *R. tangerina* YM27-005^T (Yoon *et al.*, 2007). Values are percentages of total fatty acids; values <1% are not shown. ND, Not determined; —, not detected; tr, trace.

Fatty acid	1	2	3	4	5	6
Saturated						
C _{14:0}	tr	tr	ND	tr	tr	2.9
C _{15:0}	—	1.0	4.5	1.9	2.6	1.4
C _{16:0}	1.9	2.5	23.0	4.4	7.3	23.1
C _{17:0}	—	—	ND	tr	2.8	1.1
C _{18:0}	—	—	ND	tr	tr	2.4
Unsaturated						
C _{15:1ω6c}	—	2.1	ND	1.0	2.0	tr
C _{16:1ω7c}	2.0	7.0	21.5	7.0	11.8	12.7
C _{18:1ω7c}	—	—	ND	—	—	1.5
Branched						
iso-C _{14:0}	54.7	49.4	22.0	43.1	40.6	35.5
iso-C _{16:0}	30.1	29.1	ND	20.6	16.1	12.2
anteiso-C _{15:0}	9.3	5.1	6.0	18.1	12.9	4.7
anteiso-C _{17:0}	tr	1.3	ND	1.6	2.0	—

Table 1. DNA–DNA hybridization values between strain MN1-1006^T and type strains of related members of the genus *Rubritalea*

Strain	DNA–DNA relatedness values (%) with labelled probes of:		
	1	2	3
1. MN1-1006 ^T	—	58	12
2. <i>R. squalenifaciens</i> HOact23 ^T	56	—	23
3. <i>R. sabuli</i> YM29-052 ^T	17	28	—

Table 3. Physiological and biochemical characteristics of strain MN1-1006^T compared with those of related type strains of species of the genus *Rubritalea*

Strains: 1, MN1-1006^T; 2, *R. sabuli* YM29-052^T (data from Yoon *et al.*, 2008); 3, *R. marina* Pol012^T (Scheuermayer *et al.*, 2006); 4, *R. squalenifaciens* HOact23^T (Kasai *et al.*, 2007); 5, *R. spongiae* YM21-132^T (Yoon *et al.*, 2007); 6, *R. tangerina* YM27-005^T (Yoon *et al.*, 2007).

Characteristic	1	2	3	4	5	6
Source	Sea squirt	Marine sediment	Marine sponge	Marine sponge	Marine sponge	Sea hare
Cell shape	Rod	Coccoid or rod	Coccoid or rod	Rod	Coccoid or rod	Coccoid or rod
Colony colour	Reddish pink	Pink	Red	Reddish pink	Reddish pink	Reddish orange
O ₂ requirement	Obligately aerobic	Obligately aerobic	Aerobic	Obligately aerobic	Obligately aerobic	Facultatively anaerobic
pH range	6.5–9.0	6.5–8.5	6.8–8.2	7.5–8.5	6.5–8.0	6.5–8.5
Temperature range (°C)	20–37	10–37	8–30	15–37	4–37	15–37
NaCl range (%)	1–7	0–8	1.4–3.8	1–4	1–7	0–9
Production of:						
Catalase	+	+	–	+	+	–
Oxidase	+	–	+	+	+	+
Nitrate reduction	+	–	+	+	+	–
Oxidation of:						
Acetic acid	–	+	+	–	+	–
Dextrin	+	+	+	–	–	–
Cellobiose	–	+	+	–	–	–
D-Glucuronic acid	+	+	–	+	–	–
D-Mannose	–	+	+	–	–	+
D-Mannitol	–	–	–	–	+	–
Melibiose	–	+	+	+	+	–
Formic acid	–	–	+	–	–	–
N-Acetyl-D-galactosamine	+	+	–	+	–	–
N-Acetyl-D-glucosamine	+	+	–	+	–	–
D-Sorbitol	–	–	–	–	+	–
Trehalose	+	+	–	+	–	–
L-Fructose	+	+	–	–	+	–
L-Fucose	–	–	–	–	+	+
D-Glucose	+	+	+	+	+	–
<i>cis</i> -Aconitic acid	–	–	–	–	+	+
Citric acid	–	–	–	–	–	+
α-Ketobutyric acid	–	–	–	–	+	–
α-Ketoglutaric acid	–	–	–	–	+	–
Succinic acid	–	+	–	–	–	+
Inosine	–	–	–	–	+	–
Glucose 1-phosphate	–	–	–	–	+	+
Glucose 6-phosphate	–	–	–	–	–	+
Enzyme activity of:						
Esterase (C4)	+	–	+	+	–	–
Esterase lipase (C8)	–	–	+	+	–	–
Leucine arylaminase	+	+	+	+	–	+
Trypsin	–	–	+	–	–	–
Chymotrypsin	+	–	–	+	–	–
N-Acetyl-β-glucosaminidase	+	+	–	+	–	+
α-Fucosidase	–	–	–	–	+	–
DNA G + C content (mol%)	51.4	47.7	50.9	52.6	48	50.3
Major quinone	MK-9	MK-8, MK-9	MK-8, MK-9	MK-8, MK-9, MK-10	MK-8, MK-9	MK-8, MK-9

Cells are Gram-negative-staining, non-motile, obligately aerobic and rod-shaped. Cells are 0.5–0.8 µm in width and 1.0–1.5 µm in length. Neither cellular gliding movement nor swarming growth is observed. Colonies grown on 1/2 strength R2A agar (Difco) with 75 % artificial seawater are circular, convex and reddish pink. The temperature range for growth is 20–37 °C. Optimal growth occurs at 30–37 °C; no growth occurs at 4 °C or 45 °C. The pH range for growth is 6.5–9.0. NaCl not required for growth; tolerates concentrations of up to 7 % (w/v) NaCl. Catalase- and oxidase-positive. Nitrate is reduced. Tests for alkaline phosphatase, esterase (C4), leucine arylamidase, chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase and *N*-acetyl-β-glucosaminidase are positive, but tests for esterase lipase (C8), lipase (C4), valine arylamidase, cystine arylamidase, trypsin, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase and α-fucosidase are negative. Dextrin, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, L-arabinose, D-fructose, D-galactose, gentiobiose, D-glucose, lactose, lactulose, maltose, methyl β-D-glucoside, trehalose, furanose, methylpyruvate, D-glucuronic acid, alaninamide, L-alanine, L-alanyl glycine, L-glutamic acid, L-serine and L-threonine are oxidized. The major respiratory menaquinone is MK-9. The cell-wall peptidoglycan contains muramic acid and *meso*-diaminopimelic acid. Major fatty acid components (>1.0 %) include iso-C_{14:0}, anteiso-C_{15:0}, iso-C_{16:0}, C_{16:1ω7c} and C_{16:0}.

The type strain, MN1-1006^T (=KCTC 23186^T=NBRC 107102^T), was isolated from a marine ascidian from Sado Island, Japan. The G+C content of the genomic DNA of the type strain is 51.4 mol%.

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