Rubritalea squalenifaciens sp. nov., a squalene-producing marine bacterium belonging to subdivision 1 of the phylum 'Verrucomicrobia'

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A taxonomic study was carried out to clarify the status of a Gram-negative, heterotrophic mesophile that was isolated from the marine sponge *Halichondria okadai*. The strain, designated HOact23^T, was a non-motile, rod-shaped (0.44–0.53 × 0.65–0.79 µm) bacterium. The strain produced squalene and a red–pink carotenoid pigment. The cell-wall peptidoglycan contained meso-diaminopimelic acid, glutamic acid and alanine. The G + C content of the genomic DNA was 52.4 mol%. The major fatty acids were iso-C_{14:0} (43.1 %), iso-C_{16:0} (20.6 %) and anteiso-C_{15:0} (18.1 %), and the major isoprenoid quinone was MK-9 (90.8 %). Based on 16S rRNA gene sequence data, the strain formed a distinct group within subdivision 1 in the phylum 'Verrucomicrobia'. It showed a range of phenotypic properties that distinguished it from its closest relative, *Rubritalea marina* Pol012^T (94.3 % 16S rRNA gene sequence similarity). On the basis of polyphasic taxonomic evidence, it was concluded that strain HOact23^T should be classified within a novel species in the genus *Rubritalea*. The name proposed for the taxon is *Rubritalea squalenifaciens* sp. nov., with the type strain HOact23^T (=MBIC08254^T = DSM 18772^T).

The genus *Rubritalea* was proposed as the first marine member of subdivision 1 of the phylum 'Verrucomicrobia' by Scheuermayer *et al.* (2006). These authors showed that various members of the 'Verrucomicrobia' from sponges and seawater remain uncultured, based on 16S rRNA gene sequences (Scheuermayer *et al.*, 2006). We have isolated the pink-coloured bacterium HOact23^T from the marine sponge *Halichondria okadai*, which is known as a producer of halichondrin B, an anticancer drug (Hart *et al.*, 2000). Strain HOact23^T is affiliated with subdivision 1 of the 'Verrucomicrobia' based on its 16S rRNA gene sequence. In this report, we present the characteristics of strain HOact23^T in comparison with other verrucomicrobia by using a polyphasic taxonomic approach.

Strain HOact23^T was isolated from a homogenate of the marine sponge *Halichondria okadai*, which had been

collected from the Miura peninsula (Kanagawa, Japan) and maintained in seawater at Kamaishi (Iwate, Japan) for 1 month. The homogenate was pretreated at 45 °C for 5 min and used for isolation on M5 agar (Mincer *et al.*, 2002). Pink-coloured colonies appeared after 4 weeks of incubation at 25 °C. M1 medium (Mincer *et al.*, 2002) was used for further cultivation of strain HOact23^T. Strain HOact23^T was incubated at 30 °C on plates and in liquid cultures shaken at 100 r.p.m., forming soft, red–pink colonies on M1 medium. Growth was observed between 15 and 37 °C, being optimal at 30 °C, but no growth occurred at 4 or 45 °C. Strain HOact23^T was not able to grow on M1 medium when incubated in an anaerobic pouch.

Strain HOact23^T showed a requirement for seawater. Growth was possible in a medium containing 0.5 % Bacto peptone (BD), 0.1 % Bacto yeast extract (BD), 0.2 % glucose and 1.5 % agar (Wako Chemicals) in artificial seawater with 1–4 % NaCl, while growth was inhibited in the absence of NaCl and with 5, 7 or 10 % NaCl. No growth was apparent in medium containing 0.5 % Bacto peptone (BD), 0.1 % Bacto yeast extract (BD), 0.2 % glucose and 1.5 % agar (Wako Chemicals) in distilled water with 0–10 % NaCl.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HOact23^T is AB277853.

Growth occurred between pH 7.5 and 8.5 but not at pH 5.5 or 9.5. Growth of strain HOact23^T was observed on minimal Ver100 medium (Scheuermayer *et al.*, 2006) containing nitrogen, phosphorus and vitamin solution no. 6 (Staley, 1968) with *N*-acetyl-D-glucosamine, D-galactose, D-glucose, lactose, melibiose, sucrose or xylose. No growth was apparent within 1 week at 30 °C with ribose, arabinose, fructose, rhamnose, sorbose, methanol, ethanol, mannitol, glycerol, pyruvic acid, tartaric acid, malic acid, citric acid, galacturonic acid, glutamate, alanine, isoleucine, glycine, lysine, leucine, proline, pectin, xylan, starch or chitin. Weak growth was observed with mannose and cellobiose. Utilization of polysaccharides was tested at 0.1 % (w/v); all other substances were tested at 10 mM.

Further phenotypic testing of HOact23^T was done by growing the strain on M1 agar plates for 1 week at 30 °C. Using the API ZYM system (bioMérieux), tests for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl- β -glucosaminidase gave positive results. Using the API 20NE system (bioMérieux), β -glucosidase activity on aesculin, protease activity on gelatin, β -galactosidase activity on *p*-nitrophenyl β -D-galactopyranoside and oxidase activity were found. Reduction of nitrate to nitrite was detected. Biolog profiling with GN2 microplates was conducted by resuspending the cells in a modified Biolog medium containing 2.35 % NaCl, 1.06 % MgCl₂·6H₂O, 0.03 % Pluric F-68 (Sigma) and 0.01 % gellan gum (Sigma); strain HOact23^T was shown to oxidize *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, α -D-glucose, D-glucuronic acid, D-galactose, D-melibiose and D-trehalose. Observation of these Biolog tests was continued for 2 weeks. Other tests, including casein hydrolysis, aesculin hydrolysis and the production of indole, DNase, catalase, oxygenase, urease, β -galactosidase and amylase, were conducted as described by Smibert & Krieg (1994). H₂S production was observed with SIM and TSI agar (Eiken). Strain HOact23^T was able to grow in M1 medium supplemented with nalidixic acid, vancomycin, kanamycin, streptomycin, polymyxin B, fosfomycin, fradiomycin or aztreonam (each at 50 μ g ml⁻¹), but not with ampicillin, benzylpenicillin or carbenicillin (at 50 μ g ml⁻¹).

Transmission electron microscopy (TEM) of negatively stained whole-mount cells was carried out on cells that had been fixed with 2.5 % glutaraldehyde (GA), washed in distilled seawater, mounted on a collodion-coated copper grid and stained with 1 % uranyl acetate. Equal volumes of the liquid culture and fixative [5 % GA and 0.2 M sucrose in a 0.1 M cacodylate buffer at pH 7.2 (CdB)] with some drops of 2 % OsO₄ were mixed together for 1 h at room temperature for thin sections. The fixed cells were harvested by centrifugation at 1870 g for 15 min and rinsed three times in 0.1 M CdB and then embedded in Spurr's resin (Spurr, 1969), after being dehydrated in a graded ethanol series. Sections were cut with a diamond knife using a Reichert-Jung Ultracut-N ultramicrotome (Leica Microsystems) and

collected on Formvar-coated copper grids. These sections were double-stained with 2 % uranyl acetate and lead citrate (Reynolds, 1963). Both the whole-mount cells and thin sections were observed with an H-7000 transmission electron microscope (Hitachi) operated at 75 kV. Scanning electron microscopy (SEM) was conducted on a cell suspension mixed with an equal volume of fixative containing 5 % GA, 0.25 M sucrose and a drop of 2 % OsO₄ in 0.1 M CdB, which was mounted immediately on a poly-L-lysine-coated SEM glass plate. The cells were fixed for 1.5 h at room temperature. Each sample was rinsed in 0.1 M CdB with a graded reduction of sucrose and then dehydrated with a graded ethanol series. The sample was dried with tert-butanol and coated with platinum/palladium before being observed under an S-2500 scanning electron microscope (Hitachi) operated at 15 kV. Cells of strain HOact23^T appeared as rods, 0.65–0.79 μ m long and 0.44–0.53 μ m wide, as observed by optical microscopy (data not shown) and SEM observation (Fig. 1A). No flagella or prosthecae were apparent from TEM and SEM observations (Fig. 1A, B). Strain HOact23^T stained Gram-negative, and separate membranes could be detected by electron microscopy (arrows and arrowheads in Fig. 1C, D). Budding cells, which had been observed in *R. marina* Pol012^T (Scheuermayer *et al.*, 2006), were not found in observations of HOact23^T.

The production of a red pigment by strain HOact23^T was apparent, this pigment being accumulated in the cells. This pigment was extracted by acetone from frozen cells and analysed with an HPLC/PAD (photodiode array detection)/APCI (atmospheric pressure chemical ionization)-MS (mass spectrometry) system (TermoFinnigan). Two components were detected in the acetone extract from the cells. One, which was not a pigment, was identified to be squalene by comparing the retention time and MS data showing the (M+H)⁺ mass at *m/z* 411 with an authentic sample. Strain HOact23^T produced squalene at 15 μ g (mg dry weight cells)⁻¹ by liquid cultivation in M1 medium. The other component was the pigment, showing an absorption spectrum characteristic of a carotenoid. The major carotenoid produced by HOact23^T was purified by the chromatographic method and analysed by APCI-MS and UV-Vis spectrometry. The APCI-MS data showed the (M-H)⁻ mass at *m/z* 801, and the relative molecular mass was determined to be 802. The absorption maxima of the UV-Vis data were at 312, 470, 490 and 518 nm. Quinones MK-9 (90.8 %), MK-8 (6.9 %) and MK-10 (2.3 %) were detected. The G+C content of the genomic DNA was 52.6 mol%. *meso*-Diaminopimelic acid, glutamic acid and alanine were detected as amino acids in the peptidoglycan fraction. The major cellular fatty acids were iso-C_{14:0} (43.1 % of the total), iso-C_{16:0} (20.6 %), anteiso-C_{15:0} (18.1 %) and C_{16:1} ω 7c (7 %).

A 16S rRNA gene fragment was amplified by using a forward primer corresponding to positions 8–27 and reverse primer corresponding to 1492–1510 (*Escherichia coli* numbering system; Weisburg *et al.*, 1991). The 1424 bp nucleotide

Fig. 1. Electron micrographs of cells of strain HOact23^T. (A) Scanning electron micrograph of cells of strain HOact23^T growing on an agar medium. (B) Transmission electron micrograph of negatively stained whole cells of HOact23^T. (C, D) Transmission electron micrographs of thin sections of cells of strain HOact23^T. Bars, 1 µm (A–C) and 500 nm (D).

Fig. 2. Phylogenetic analysis of 16S rRNA gene sequences of members of subdivision 1 of the phylum 'Verrucomicrobia'. The tree was generated by the neighbour-joining method. Cultured strains are indicated in bold. Numbers at nodes indicate percentages of occurrence in 1000 bootstrapped trees. Bar, 2 substitutions per 100 nucleotides.

sequence was used to search for phylogenetically related bacteria using BLAST (Altschul *et al.*, 1990) and the DDBJ database. The sequence was also compared to 16S rRNA gene sequence data stored in RDPII (Cole *et al.*, 2005) by using the Sequence Match tool. Both results suggested that the closest described species was *R. marina*. The 1424 bp nucleotide sequence of the 16S rRNA gene was used for a phylogenetic analysis, which was performed by using the MEGA 3.1 program (Kumar *et al.*, 2004) after multiple alignment of the data by CLUSTAL_X (Thompson *et al.*, 1997). Distances (distance options according to the Kimura two-parameter model) and clustering by the neighbour-joining and maximum-parsimony methods were determined by using bootstrap values based on 1000 replications. Alignment gaps and unidentified base positions were not taken into consideration for these calculations. As shown in Fig. 2, strain HOact23^T revealed a sequence similarity of 94.3 % to its closest cultured relative, *Rubritalea marina* Pol012^T, and 94.7 % sequence similarity to the closest environmental clone. It is concluded that strain HOact23^T is affiliated to verrucomicrobial subdivision 1.

On the basis of phenotypic, chemotaxonomic and phylogenetic characteristics (Table 1), it is concluded that strain HOact23^T belongs to a novel species of the genus *Rubritalea*, for which we propose the name *Rubritalea squalenifaciens* sp. nov.

Description of *Rubritalea squalenifaciens* sp. nov.

Rubritalea squalenifaciens (squa.le.ni.fa'ci.ens. N.L. n. *squalenum* squalene; L. part. adj. *faciens* producing, from L. v. *facio* to produce; N.L. part. adj. *squalenifaciens* squalene-producing).

Cells are Gram-negative, non-motile, rod-shaped and red-pink in colour. Able to grow with *N*-acetyl-D-glucosamine, D-galactose, D-glucose, lactose, melibiose, sucrose or xylose as the sole carbon source under aerobic conditions. Does not grow anaerobically. The DNA G + C content is 52.6 mol%. The major cellular fatty acids are iso-C_{14:0}, iso-C_{16:0}, anteiso-C_{15:0} and C_{16:1}ω7*c*. Menaquinones MK-8, MK-9 and MK-10 are present. The peptidoglycan in the cell wall

Table 1. Characteristics that differentiate strain HOact23^T from *Rubritalea marina* Pol012^T

Data for *R. marina* Pol012^T were taken from Scheuermayer *et al.* (2006). +, Positive; –, negative; ND, no data available. Both strains were isolated from species of sponge.

Characteristic	Strain HOact23 ^T	<i>R. marina</i> Pol012 ^T
Isolation source	<i>Halichondria okadai</i>	<i>Axinella polypoides</i>
Cell shape [size (μm)]	Rod [0.44–0.53 × 0.65–0.79]	Coccoid [0.5–1.1], rod [0.6–1.8 × 0.4–1.0]
Catalase	+	–
Temperature range (°C)	15–37	8–30
pH range	7.5–8.5	6.8–8.2
Growth with:		
<i>N</i> -Acetyl-D-glucosamine	+	ND
D-Galactose	+	–
Sucrose	+	–
Raffinose	+	–
Pyruvate	–	+
Pectin	–	+
Oxidation of:		
Acetic acid	–	+
Dextrin	–	+
D-Cellobiose	–	+
D-Glucuronic acid	+	–
D-Mannose	–	+
Formic acid	–	+
<i>N</i> -Acetyl-D-galactosamine	+	–
<i>N</i> -Acetyl-D-glucosamine	+	–
D-Trehalose	+	–
Major cellular fatty acids	i-C _{14:0} , i-C _{16:0} , a-C _{15:0} , C _{16:1} ω7 <i>c</i>	C _{16:0} , i-C _{14:0} , C _{16:1} ω7 <i>c</i> and/or i-C _{15:0} 2-OH, a-C _{15:0} , C _{15:0}
Major quinones	MK-8, MK-9, MK-10	MK-8, MK-9
DNA G + C content (mol%)	52.6	50.9

contains *meso*-diaminopimelic acid, glutamic acid and alanine. Positive for catalase, oxidase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl- β -glucosaminidase. Produces acid from *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, D-glucose, D-glucuronic acid, D-galactose, D-melibiose and D-trehalose. Grows in seawater medium containing 1–4% NaCl, at pH 7.5–8.5 and temperatures of 15–37 °C.

The type strain, HOact23^T (= MBIC08254^T = DSM 18772^T), was isolated from the marine sponge *Halichondria okadai*.

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References

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Cole, J. R., Chai, B., Farris, R. J., Wang, Q., Kulam, S. A., McGarrell, D. M., Garrity, G. M. & Tiedje, J. M. (2005). The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Res* **33**, D294–D296.
- Hart, J. B., Lill, R. E., Hickford, S. J. H., Blunt, J. W. & Munro, M. H. G. (2000). The halichondrins: chemistry, biology, supply and delivery. In *Drugs from the Sea*, pp. 134–153. Edited by N. Fusetani. Basel: Karger.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Mincer, T. J., Jensen, P. R., Kauffman, C. A. & Fenical, W. (2002). Widespread and persistent populations of a major new actinomycete taxon in ocean sediments. *Appl Environ Microbiol* **68**, 5005–5011.
- Reynolds, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J Cell Biol* **17**, 208–212.
- Scheuermayer, M., Gulder, T. A. M., Bringmann, G. & Hentschel, U. (2006). *Rubritalea marina* gen. nov., sp. nov., a marine representative of the phylum ‘Verrucomicrobia’, isolated from a sponge (Porifera). *Int J Syst Evol Microbiol* **56**, 2119–2124.
- Smibert, R. M. & Krieg, N. R. (1994). Phenotypic characterization. In *Methods for General and Molecular Bacteriology*, pp. 607–654. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.
- Spurr, A. R. (1969). A low-viscosity epoxy resin embedding medium for electron microscopy. *J Ultrastruct Res* **26**, 31–42.
- Staley, J. T. (1968). *Prosthecomicrobium* and *Ancalomicrobium*: new prosthecate freshwater bacteria. *J Bacteriol* **95**, 1921–1942.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.