Robiginitalea myxolifaciens sp. nov., a novel myxol-producing bacterium isolated from marine sediment, and emended description of the genus *Robiginitalea*

Hao Do Manh,[†] Yoshihide Matsuo, Atsuko Katsuta, Satoru Matsuda, Yoshikazu Shizuri and Hiroaki Kasai

Marine Biotechnology Institute, 3-75-1 Heita, Kamaishi, Iwate 026-0001, Japan

Correspondence

Hiroaki Kasai

hkasai@kitasato-u.ac.jp

A marine, Gram-negative, deep-orange-coloured, rod/coccoid-shaped, strictly aerobic, non-motile, oxidase- and catalase-positive bacterial strain, designated YM6-073^T, was isolated from sediment collected in Okinawa, Japan. Strain YM6-073^T produced the carotenoid (3*R*,2'*S*)-myxol. A phylogenetic analysis based on 16S rRNA gene sequences revealed that strain YM6-073^T belonged to a distinct lineage in the family *Flavobacteriaceae*, the closest relative being *Robiginitalea biformata* HTCC2501^T (93.7 % sequence similarity). Strain YM6-073^T could be distinguished from other members of the family *Flavobacteriaceae* by means of a number of phenotypic characteristics. The DNA G + C content was 50.1 mol%. The predominant cellular fatty acids were iso-C_{15:0}, iso-C_{15:1}ω10c, iso-C_{17:0} 3-OH and C_{15:0}, and menaquinone-6 was the only respiratory quinone. DNA–DNA hybridization between strain YM6-073^T and *R. biformata* DSM 15991^T showed less than 10 % DNA–DNA relatedness. On the basis of phenotypic, genotypic and phylogenetic features, strain YM6-073^T represents a novel species of the genus *Robiginitalea*, for which the name *Robiginitalea myxolifaciens* sp. nov. is proposed. The type strain is YM6-073^T (=MBIC 06409^T=KCTC 22216^T=DSM 21019^T).

The family *Flavobacteriaceae* (Bernardet *et al.*, 2002; Bernardet & Nakagawa, 2006) is one of the largest phylogenetic groups within the phylum *Bacteroidetes* (Garrity & Holt, 2001). Marine members of the family form a well-defined clade (Bowman, 2006) and are among the most abundant microbes in marine environments such as marine sediments (Llobet-Brossa *et al.*, 1998). We have extensively isolated and characterized novel strains from marine sediment in order to identify microbes that might produce useful compounds. In this study, we describe one of these strains as a novel species belonging to the genus *Robiginitalea* (Cho & Giovannoni, 2004) within the family *Flavobacteriaceae*. This strain has been characterized as a producer of the monocyclic carotenoid (3*R*,2'*S*)-myxol (Shindo *et al.*, 2007).

[†]Present address: Vietnamese Academy of Science and Technology, No. 246 Da Nang Street, Hai Phong City, Vietnam.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YM6-073^T is AB270585.

Detailed compositions of the isolation media and a more detailed phylogenetic tree are presented as supplementary material available with the online version of this paper.

Strain YM6-073^T was isolated from a sediment sample collected on the shore of Cape Maeda (Onna Village, Okinawa Prefecture, Japan) in July 2003. The sample (0.5–1.0 ml) was homogenized with a glass rod in 5 ml sterile seawater. A 50 µl sample of the homogenate was plated onto HSV agar (for composition, see Supplementary Table S1 available in IJSEM Online) and incubated at 25 °C for 30 days. A deep orange-coloured colony was picked from the agar and subcultured for 7–10 days on marine broth 2216 (MB; Difco) solidified with 1.5 % agar. All of the subsequent investigations were performed on cells grown on marine agar 2216 (MA; Difco) or in MB for 48 h at 30 °C unless otherwise stated.

Growth on MA was investigated at 4, 15, 20, 25, 30, 37, 42 and 45 °C. Tolerance of NaCl was evaluated on a medium containing the following (l⁻¹): 5 g Bacto peptone (Difco), 1 g Bacto yeast extract (Difco) and 0–10 % (w/v) NaCl (using 1 % increments). The pH range for growth was studied on MA supplemented with 25 mM ACES (pH 6.5), TAPSO (pH 7.6), TAPS (pH 8.5) or CHES (pH 9.5). Gram staining was performed according to the method of Murray *et al.* (1994). Cell morphology and flagellar motility were observed by using phase-contrast microscopy

(Eclipse E600; Nikon) examination of a 48 h MB culture. Gliding motility was observed by using the same method, according to the minimal standards for describing novel taxa in the family *Flavobacteriaceae* (Bernardet *et al.*, 2002). Oxidase activity was tested using oxidase test paper (Eiken). Catalase activity was tested using a 3 % H₂O₂ solution. Degradation of alginate (1 %, w/v), agar (1.5 %, w/v), aesculin (0.1 %, w/v), starch (0.2 %, w/v), casein (2 %, w/v), gelatin (1 %, w/v), cellulose (carboxymethyl-cellulose at 1 %, w/v, and filter paper), chitin (1 %, w/v), DNA (0.4 %, w/v) and urea (2 %, w/v) were determined according to the methods of Smibert & Krieg (1994). Chitinase activity was tested by using the plate method of O'Brien & Colwell (1987). Other biochemical characteristics were examined by using the API 20NE commercial system (bioMérieux) incubated at 30 °C for 24 h. For the determination of nutritional features, strain YM6-073^T was grown on MA at 30 °C for 72 h and suspended in a basal medium that consisted of artificial seawater (Tropic Marin) containing 0.01 % yeast extract (Difco). A 20 µl aliquot of the suspension was transferred to each well of a 96-well plate containing 500 µl basal medium supplemented with various carbon sources at 0.2 %. Plates were incubated at 30 °C for 1 week on a Küner orbital shaker (300 r.p.m.). Susceptibility to antibiotics was examined as described by Ivanova *et al.* (2001).

Phenotypic properties of YM6-073^T are given in the species description, while those characteristics that serve to differentiate the strain from *Robiginitalea biformata* DSM 15991^T are listed in Table 1. No flexirubin-type pigments could be detected with the simple KOH test detailed in the

minimal standards for describing novel taxa of the family *Flavobacteriaceae* (Bernardet *et al.*, 2002). The deep-orange-coloured pigment of strain YM6-073^T has been identified as a mixture of carotenoids, among which (3*R*,3'*R*)-zeaxanthin and (3*R*,2'*S*)-myxol were predominant (Shindo *et al.*, 2007). The respiratory quinone system and the cellular fatty acid composition were determined by using LC-MS as described previously (Katsuta *et al.*, 2005). For direct comparisons, the fatty acid composition and respiratory quinone content of *R. biformata* DSM 15991^T were determined in parallel. For both strain YM6-073^T and *R. biformata* DSM 15991^T, MK-6 was found to be the only respiratory quinone. The predominant cellular fatty acids in strain YM6-073^T were iso-C_{15:0} (34.9 %), iso-C_{15:1}ω10*c* (29.8 %), iso-C_{17:0} 3-OH (17.8 %) and C_{15:0} (9.5 %), constituting 92.0 % of the total fatty acid content. The fatty acid profile of *R. biformata* DSM 15991^T was very similar (Table 2).

The genomic DNA of strain YM6-073^T was extracted by using a Wizard genomic DNA purification kit (Promega) from cells grown in MB. The DNA G+C content was determined using the HPLC method of Mesbah & Whitman (1989) and was found to be 50.1 mol%.

A 1465 bp fragment of the 16S rRNA gene was amplified from the extracted DNA by using a bacterial universal primer pair, 27F and 1492R, (*Escherichia coli* numbering system; Weisburg *et al.*, 1991) specific to the 16S rRNA gene. To ascertain the phylogenetic position of strain YM6-073^T, the 16S rRNA gene sequence was compared with related sequences obtained from Ribosomal Database Project II (Cole *et al.*, 2007). A neighbour-joining phylogenetic tree was constructed by using MEGA, version

Table 1. Differential characteristics of strain YM6-073^T with respect to *R. biformata* DSM 15991^T

Strains: 1, YM6-073^T (this study); 2, *R. biformata* DSM 15991^T (Cho & Giovannoni, 2004). +, Positive; –, negative.

Characteristic	1	2
Pigmentation	Deep orange	Orange
Growth at/with:		
10 °C	–	+
0.25 % NaCl	–	+
8 % NaCl	–	+
Susceptibility to:		
Nalidixic acid	–	+
Streptomycin	+	–
Degradation of aesculin	+	–
Utilization of:		
D-Galactose	+	–
Glycerol	+	–
D-Sorbitol	+	–
L-Glutamate	–	+
L-Serine	–	+
Sodium succinate	–	+
DNA G+C content (mol%)	50.1	55–56

Table 2. Fatty acid profiles of strain YM6-073^T and *R. biformata* DSM 15991^T

Strains: 1, YM6-073^T; 2, *R. biformata* DSM 15991^T. All data are from this study. Data are reported as percentages of the total fatty acids. Fatty acids amounting to less than 1 % in both species are not listed. ND, Not detected.

Fatty acid	1	2
Saturated		
C _{15:0}	9.5	10.5
Branched saturated		
iso-C _{15:0}	34.9	35.3
anteiso-C _{15:0}	ND	4.3
Monounsaturated		
C _{15:1}	ND	1.4
C _{16:1} ω7 <i>c</i>	2.0	2.1
Branched monounsaturated		
iso-C _{15:1} ω10 <i>c</i>	29.8	25.8
iso-C _{17:1}	1.6	ND
Hydroxy		
iso-C _{15:0} 3-OH	3.2	2.5
iso-C _{17:0} 3-OH	17.8	16.0

3.1 (Kumar *et al.*, 2004). The tree topology was estimated by means of a bootstrap analysis (Felsenstein, 1993) with 1000 resamplings of the dataset. Strain YM6-073^T showed the highest 16S rRNA gene sequence similarity (93.7%) with the type strain of *R. biformata* and showed 89.0–91.6% similarity with respect to members of the genera *Arenibacter*, *Costertonia*, *Flagellimonas*, *Maribacter*, *Muricauda*, *Zeaxanthinibacter* and *Zobellia* (Fig. 1). A tree based on the maximum-likelihood method and constructed using PhyML (Guindon *et al.*, 2005) showed essentially the same topology (data not shown). Strain YM6-073^T also showed 93.6–94.9% sequence similarity with respect to several uncultured organisms and environmental clones from marine sediment (see Supplementary Fig. S1 available in IJSEM Online).

DNA–DNA hybridization between strain YM6-073^T and *R. biformata* DSM 15991^T was performed with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989) with five replications. The temperature was set at 44.7 °C. The highest and lowest values obtained were excluded and the mean of the remaining three values was quoted as the DNA–DNA relatedness value. Strain YM6-073^T and *R. biformata* DSM 15991^T shared approximately 8.5% DNA–DNA relatedness.

On the basis of the results of the 16S rRNA gene sequence-based phylogenetic analysis and the above-mentioned phenotypic data, strain YM6-073^T represents a novel species within the genus *Robiginitalea*, for which the name *Robiginitalea myxolifaciens* sp. nov. is proposed. Additional

Fig. 1. Unrooted neighbour-joining phylogenetic tree based on the 16S rRNA gene sequences of strain YM6-073^T and representative members of related genera in the family *Flavobacteriaceae*. Only bootstrap percentages greater than 50% (based on 1000 replications) are indicated at nodes. GenBank accession numbers are shown in parentheses. Bar, 0.01 substitutions per nucleotide position.

data obtained in this study also make emendation of the genus description necessary.

Emended description of the genus *Robiginitalea* Cho & Giovannoni 2004

The description of the genus is as given by Cho & Giovannoni (2004), with the following amendments. MK-6 is the only respiratory quinone. The predominant fatty acids are iso-C_{15:0}, iso-C_{15:1}ω10c, iso-C_{17:0} 3-OH and C_{15:0}. The DNA G+C content is 50–56 mol%.

Description of *Robiginitalea myxolifaciens* sp. nov.

Robiginitalea myxolifaciens [my.xo.li.fa'ci.ens. N.L. n. *myxol*-olis myxol; L. part. adj. *faciens* (from L. v. *facio*) producing; N.L. part. adj. *myxolifaciens* myxol-producing].

Cells are straight rods (0.9–3.5 µm long and 0.3–0.65 µm wide) during the exponential growth phase and coccoid (0.48–1.30 µm in diameter) in the stationary phase and are devoid of flagellar and gliding motility. Gram-negative. Colonies on 1/10-strength MA are rhizoid, convex and deep orange in colour. Carotenoid pigments are produced, but flexirubin pigments are not produced. Obligatory aerobic and chemoheterotrophic. Growth occurs at 15.0–42.0 °C (optimally at 30.0 °C) and at pH 6.5–9.5 (optimally at pH 7.6–8.5). Optimal growth occurs in the presence of 3–4 % (w/v) NaCl; growth is inhibited in the absence of NaCl and in the presence of >7 % (w/v) NaCl. Nitrate is not reduced. Oxidase, catalase and β-galactosidase activities are present. Glucose, galactose, maltose, sucrose, fructose, raffinose, sorbitol, trehalose, mannose, lactose, cellobiose, sorbose, melibiose, ornithine monohydrochloride and glycerol are utilized as sole carbon sources, but rhamnose, ethanol, lysine, serine, isoleucine, leucine, glycine, glutamate, sodium citrate, sodium lactate, sodium succinate, sodium pyruvate, sodium gluconate and itaconic acid are not. Starch and aesculin are hydrolysed, but gelatin, DNA, carboxymethylcellulose, crystalline cellulose (filter paper), urea, chitin, agar, casein and alginate are not. Benzylpenicillin, rifampicin, erythromycin and tetracycline affect growth, but nalidixic acid, kanamycin, gentamicin and cycloheximide do not. The predominant cellular fatty acids (>9 %) are iso-C_{15:0}, iso-C_{15:1}ω10c, iso-C_{17:0} 3-OH and C_{15:0}. The DNA G+C content of the type strain (YM6-073^T) is 50.1 mol% (HPLC method).

The type strain, YM6-073^T (=MBIC 06409^T=KCTC 22216^T=DSM 21019^T), was isolated from marine sediment in Okinawa, Japan.

Acknowledgements

We thank Dr J. Euzéby for his recommendations about etymology and we are grateful to Dr K. Adachi for her help with chemotaxonomic analyses. We appreciate the technical assistance of Yukiko Itazawa, Ayako Matsuzaki and Tomomi Haga. This research was supported by the New Energy and Industrial Technology

Development Organization (NEDO) and the Japan International Cooperation Agency (JICA).

References

- Bernardet, J.-F. & Nakagawa, Y. (2006). An introduction to the family Flavobacteriaceae. In *The Prokaryotes, a Handbook on the Biology of Bacteria*, 3rd edn, vol. 7, pp. 455–480. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer & E. Stackebrandt. New York: Springer.
- Bernardet, J.-F., Nakagawa, Y. & Holmes, B. (2002). Proposed minimal standards for describing new taxa of the family Flavobacteriaceae and emended description of the family. *Int J Syst Evol Microbiol* 52, 1049–1070.
- Bowman, J. P. (2006). The marine clade of the family Flavobacteriaceae: the genera *Aequorivita*, *Arenibacter*, *Cellulophaga*, *Croceibacter*, *Formosa*, *Gelidibacter*, *Gillisia*, *Maribacter*, *Mesonina*, *Muricauda*, *Polaribacter*, *Psychroflexus*, *Psychroserpens*, *Robiginitalea*, *Salagentibacter*, *Tenacibaculum*, *Ulvibacter*, *Vitellibacter*, and *Zobellia*. In *The Prokaryotes, a Handbook on the Biology of Bacteria*, 3rd edn, vol. 7, pp. 677–694. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer & E. Stackebrandt. New York: Springer.
- Cho, J.-C. & Giovannoni, S. J. (2004). *Robiginitalea biformata* gen. nov., sp. nov., a novel marine bacterium in the family Flavobacteriaceae with a higher G+C content. *Int J Syst Evol Microbiol* 54, 1101–1106.
- Cole, J. R., Chai, B., Farris, R. J., Wang, Q., Kulam-Syed-Mohideen, A. S., McGarrell, D. M., Bandela, A. M., Cardenas, E., Garrity, G. M., Tiedje, J. M. & other authors (2007). The ribosomal database project (RDP-II): introducing *myRDP* space and quality controlled public data. *Nucleic Acids Res* 35 (Database issue), D169–D172.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* 39, 224–229.
- Felsenstein, J. (1993). PHYLIP (phylogeny inference package), version 3.5. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, USA.
- Garrity, G. M. & Holt, J. G. (2001). The road map to the *Manual*. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 1, pp. 119–166. Edited by D. R. Boone, R. W. Castenholz & G. M. Garrity. New York: Springer.
- Guindon, S., Lethiec, F., Duroux, P. & Gascuel, O. (2005). PHYML Online—a web server for fast maximum likelihood-based phylogenetic inference. *Nucleic Acids Res* 33 (Web Server issue), W557–W559.
- Ivanova, E. P., Nedashkovskaya, O. I., Chun, J., Lysenko, A. M., Frolova, G. M., Svetashev, V. I., Vysotskii, M. V., Mikhailov, V. V., Huq, A. & Colwell, R. R. (2001). *Arenibacter* gen. nov., new genus of the family Flavobacteriaceae and description of a new species, *Arenibacter latericius* sp. nov. *Int J Syst Evol Microbiol* 51, 1987–1995.
- Katsuta, A., Adachi, K., Matsuda, S., Shizuri, Y. & Kasai, H. (2005). *Ferrimonas marina* sp. nov. *Int J Syst Evol Microbiol* 55, 1851–1855.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* 5, 150–163.
- Llobet-Brossa, E., Rosselló-Mora, R. & Amann, R. (1998). Microbial community composition of Wadden Sea sediments as revealed by fluorescence *in situ* hybridization. *Appl Environ Microbiol* 64, 2691–2696.
- Mesbah, M. & Whitman, W. B. (1989). Measurement of deoxyguanosine/thymidine ratios in complex mixtures by high-performance liquid chromatography for determination of the mole percentage guanine + cytosine of DNA. *J Chromatogr* 479, 297–306.

Murray, R. G. E., Doetsch, R. N. & Robinow, F. (1994). Determinative and cytological light microscopy. In *Methods for General and Molecular Bacteriology*, pp. 21–41. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.

O'Brien, M. & Colwell, R. R. (1987). A rapid test for chitinase activity that uses 4-methylumbelliferyl-*N*-acetyl- β -D-glucosaminide. *Appl Environ Microbiol* **53**, 1718–1720.

Shindo, K., Kikuta, K., Suzuki, A., Katsuta, A., Kasai, H., Yasumoto-Hirose, M., Matsuo, Y., Misawa, N. & Takaichi, S. (2007). Rare

carotenoids, (3*R*)-saproxanthin and (3*R*,2'*S*)-myxol, isolated from novel marine bacteria (*Flavobacteriaceae*) and their antioxidative activities. *Appl Microbiol Biotechnol* **74**, 1350–1357.

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.

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.