Coraliomargarita akajimensis gen. nov., sp. nov., a novel member of the phylum 'Verrucomicrobia' isolated from seawater in Japan

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An obligately aerobic, Gram-negative, non-spore-forming, non-motile, spherical bacterium, designated strain 04OKA010-24^T, was isolated from seawater surrounding the hard coral *Galaxea fascicularis* L., collected at Majanohama, Akajima, Japan, and was subjected to a polyphasic taxonomic study. Phylogenetic analyses based on the 16S rRNA gene sequence indicated that the new strain represented a member of the phylum 'Verrucomicrobia' and shared 84–95 % sequence similarity with cultivated strains of 'Verrucomicrobia' subdivision 4. Amino acid analysis of the cell-wall hydrolysate indicated the absence of muramic acid and diaminopimelic acid, which suggested that the strain did not contain peptidoglycan in the cell wall. The G + C content of the DNA was 53.9 mol%. MK-7 was the major menaquinone and C_{14:0}, C_{18:1ω9c} and C_{18:0} were the major fatty acids. On the basis of these data, it was concluded that strain 04OKA010-24^T represents a novel species in a new genus in subdivision 4 of the phylum 'Verrucomicrobia', for which the name *Coraliomargarita akajimensis* gen. nov., sp. nov. is proposed. The type strain of *Coraliomargarita akajimensis* is 04OKA010-24^T (=MBIC06463^T = IAM 15411^T = KCTC 12865^T).

Molecular phylogenetic approaches based on 16S rRNA gene sequence analysis have revealed that members of the phylum 'Verrucomicrobia' (Hedlund *et al.*, 1997; Hugenholtz *et al.*, 1998), a phylum represented by very few cultivated strains, are a globally distributed, abundant group of bacteria that are increasingly recognized as being of environmental significance (Sangwan *et al.*, 2005). The phylum 'Verrucomicrobia' has been informally classified into subdivisions 1–5 by Hugenholtz *et al.* (1998), and has also been classified into subdivisions 1–6 by Vandekerckhove *et al.* (2000). At the time of writing, only three of these subdivisions are recognized by *Bergey's Manual of Systematic Bacteriology* (Garrrity & Holt, 2001), all three corresponding to the rank of family: *Verrucomicrobiaceae* (subdivision 1), *Opitutaceae* (subdivision 4) and *Xiphinematobacteriaceae* (subdivision 2) (Sangwan *et al.*, 2005). Subdivision 4 comprises just a few recognized representatives, such as *Opitutus terrae* PB90-1^T and related strains (Janssen *et al.*, 1997; Chin *et al.*, 1999, 2001), which have been isolated from soil

environments, and the marine bacteria '*Fucophilus fucoida-nolyticus*' SI-1234 (Sakai *et al.*, 2003) and *Alterococcus agarolyticus* ADT3^T (Shieh & Jean, 1998), which were isolated from thermal springs. *A. agarolyticus* was misclassified as a member of the class *Gammaproteobacteria* (Sangwan *et al.*, 2004).

A specimen of the hard coral *Galaxea fascicularis* L. was collected from 3–5 m below the surface of the sea at Majanohama, Akajima, Japan, in March 2004 together with the seawater around the coral, and these were kept in a 500-ml plastic bottle for 2–5 h. Strain 04OKA010-24^T was isolated from the seawater in the sample bottle. The seawater was diluted 1 : 10 with filtered, autoclaved seawater and used for isolation on 1 : 10 diluted marine agar [3.74 g marine broth 2216 (Difco), 750 ml filtered seawater, 250 ml distilled water, 15 g agar]. The phylogenetic position of strain 04OKA010-24^T was investigated by using a polyphasic taxonomic approach including 16S rRNA gene sequence analysis, together with physiological, biochemical and chemotaxonomic analyses. Based on these data, it is proposed that the isolate represents a novel species of a new genus in the phylum 'Verrucomicrobia'.

The temperature range and pH range for growth were determined by incubating the isolate in marine agar 2216

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 04OKA010-24^T is AB266750.

Fig. 1. Transmission electron micrograph of negatively stained cells of strain 04OKA010-24^T. Bar, 500 nm.

(MA; Difco). The following buffers were used for pH tests in MA: MES (pH 5.5), ACES (pH 6.5 and 7.0), TAPSO (pH 7.6), TAPS (pH 8.5) and CHES (pH 9.0 and 9.5). Gram staining was performed as described by Murray *et al.* (1994). Cell morphology was observed by using light microscopy (BX60; Olympus) and transmission electron

microscopy (TEM). For TEM observation, cells were mounted on Formvar-coated copper grids and negatively stained with 2% (w/v) aqueous uranyl acetate. Grids were observed in an H-7000 transmission electron microscope (Hitachi) operated at 75 kV. In the course of TEM, cells of various sizes were observed. Cells were coccoid and generally 0.5–1.2 µm in diameter. In the stationary growth phase, smaller cells (0.5–0.6 µm in diameter) were predominantly observed. The cells were non-motile and no flagella were seen by electron microscopy (Fig. 1). Growth under anaerobic conditions was determined after 2 weeks incubation in an AnaeroPack (Mitsubishi Gas Chemical Co., Inc.) on MA. Catalase activity was determined by bubble formation in a 3% H₂O₂ solution. Oxidase activity was determined by use of cytochrome oxidase paper (Eiken Chemical Co., Ltd). API 20E and API 50CH strips (bioMérieux) were used to determine the physiological and biochemical characteristics of strain 04OKA010-24^T. These were read after incubation at 30 °C for 48 and 72 h, respectively. Determination of the respiratory quinone system and cellular fatty acid composition was carried out as described by Katsuta *et al.* (2005). DNA was prepared by using Genomic tips (Qiagen) from cells grown in DSM medium no. 607, and the DNA base composition was determined by using the HPLC method of Mesbah *et al.* (1989). Cell walls were prepared by the methods described by Schleifer & Kandler (1972), and 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 a model LC-10AD HPLC apparatus (Shimadzu) equipped with a Wakopak WS-PTC column

Fig. 2. Phylogenetic tree showing the position of strain 04OKA010-24^T in relation to representative 16S rRNA gene sequences that include the currently known phylogenetic diversity within subdivision 4 of the phylum 'Verrucomicrobia'. The tree, which was rooted using recognized representatives and *Escherichia coli* ATCC 11775^T as the outgroup, was generated by the neighbour-joining method. Numbers at nodes indicate the occurrence of the strain in 1000 bootstrapped trees; only values greater than 40% are shown. Bar, 2% sequence divergence.

(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 27F and 1492R (Weisburg *et al.*, 1991) specific to the 16S rRNA gene. To ascertain the phylogenetic position of the new isolate, the 16S rRNA gene sequence of strain 04OKA010-24^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 by using CLUSTAL_X (version 1.83) (Thompson *et al.*, 1997). Alignment gaps and ambiguous bases were not taken into consideration when 1213 bases of the 16S rRNA gene nucleotides were compared. Phylogenetic relationships were analysed by using the same software. Distances were calculated by using the two-parameter model of Kimura (1980). Clustering with the neighbour-joining method (Saitou & Nei, 1987) was determined by using bootstrap values based on 1000 replications (Felsenstein, 1985).

Sequence similarity values were calculated via MEGA3 (Kumar *et al.*, 2004).

On the basis of 16S rRNA gene sequence analysis, strain 04OKA010-24^T was found to be related most closely to the as yet undescribed marine bacterium '*Fucophilus fucoidanolyticus*' SI-1234 (94.8 % similarity). The most closely related type strains of species with validly published names were *Alterococcus agarolyticus* ADT3^T (87.6 % similarity) and *Opiritatus terrae* PB90-1^T (83.9 %). Based on levels of 16S rRNA gene sequence divergence, strain 04OKA010-24^T is thus determined to be related to the genera *Alterococcus* and *Opiritatus*, which are members of '*Verrucomicrobia*' subdivision 4. These results indicate that strain 04OKA010-24^T belongs to the phylum '*Verrucomicrobia*' within subdivision 4 (Fig. 2).

Strain 04OKA010-24^T also showed distinct phenotypic features that discriminated it from recognized members

Table 1. Characteristics that differentiate strain 04OKA010-24^T from representatives of other genera of subdivision 4 in the phylum '*Verrucomicrobia*'

Strains: 1, 04OKA010-24^T; 2, *Alterococcus agarolyticus* BCRC 9135^T (data from Shieh & Jean, 1998); 3, *Opiritatus terrae* DSM 11246^T (Chin *et al.*, 2001). +, Positive; W, weakly positive; –, negative; ND, no data.

Characteristic	1	2	3
Isolation source	Seawater	Hot springs	Rice paddy soil
Cell diameter (µm)	0.5–1.2	0.8–0.9	0.4–0.6
Flagella	–	+	+
Motility	–	+	+
O ₂ requirement	Obligately aerobic	Facultatively anaerobic	Obligately anaerobic
Growth in 3 % (w/v) NaCl	+	–	+
Catalase	–	+	–
Oxidase	+	+	–
Nitrate reduction	–	ND	+
Temperature range for growth (°C)	20–30	38–58	10–37
pH range for growth	7.0–9.0	7.0–8.5	5.5–9.0
Hydrolysis of:			
Agar	–	+	ND
DNA	W	–	ND
Starch	–	W	+
Urea	+	ND	–
Acid production from:			
D-Arabinose	–	–	+
Cellobiose	–	+	+
Galactose	W	+	+
Glucose	–	+	+
Lactose	–	+	+
Mannitol	+	–	+
Mannose	+	W	+
Melibiose	–	–	+
Xylose	–	+	–
DNA G+C content (mol%)	53.9	65.5–67.0	74
Major quinone	MK-7	ND	ND

of 'Verrucomicrobia' subdivision 4 (Table 1): cells lacked flagella and were non-motile (Fig. 1).

Biochemical and physiological characteristics of strain 04OKA010-24^T further supported the phylogenetic results. The major quinone system was menaquinone MK-7. The G + C content of the DNA of strain 04OKA010-24^T was 53.9 mol%. Major fatty acids of strain 04OKA010-24^T were C_{14:0} (24.2 %), C_{18:1ω9c} (23.5 %) and C_{18:0} (15.6 %). The cell wall was prepared by disrupting cells, followed by heating with 3 % SDS, washing and centrifugation. Amino acid analysis of the cell-wall hydrolysate indicated the absence of muramic acid and diaminopimelic acid, suggesting that the cell wall did not contain peptidoglycan.

Strain 04OKA010-24^T was obligately aerobic, was isolated from seawater and was able to tolerate 5 % NaCl; by contrast, the other members of 'Verrucomicrobia' subdivision 4, originating from rice paddy soil and hot springs, could only tolerate up to 1–3 % NaCl. Strain 04OKA010-24^T could also be distinguished on the basis of the following characteristics: catalase, oxidase, nitrate reduction, temperature range for growth and hydrolysis of DNA and urea. Some differential characteristics were noted from the API 50CH results, including acid production from galactose, mannitol and mannose.

Based on the results of the phylogenetic analysis and its biochemical and physiological properties, strain 04OKA010-24^T was considered to represent a novel species in a new genus belonging to subdivision 4 of the phylum 'Verrucomicrobia'. We propose the name *Coralimargarita akajimensis* gen. nov., sp. nov. for this organism.

Description of *Coralimargarita* gen. nov.

Coralimargarita (Co.ra'li.o.mar.ga.ri'ta. Gr. n. *koralion* coral; L. fem. n. *margarita* a pearl; N.L. fem. n. *Coralio-margarita* coral pearl, referring to a white-colony-forming, coccoid micro-organism isolated from seawater in a sample bottle of hard coral).

Cells are Gram-negative, obligately aerobic cocci. Cells lack flagella and are non-motile. No spores are formed. Catalase-negative, but oxidase-positive. Nitrate is not reduced. The major respiratory quinone is MK-7. The G + C content of the genomic DNA of the type strain of the type species is 53.9 mol%. Predominant cellular fatty acids are C_{14:0}, C_{18:1ω9c} and C_{18:0}. The type species is *Coralimargarita akajimensis*.

Description of *Coralimargarita akajimensis* sp. nov.

Coralimargarita akajimensis (a.ka.ji.men'sis. N.L. fem. adj. *akajimensis* pertaining to Akajima, an island in Okinawa, from where the type strain was isolated).

Main characteristics are as given for the genus. In addition, cells are 0.5–1.2 µm in diameter. Neither cellular gliding

movement nor swarming growth is observed. Colonies grown on half-strength R2A agar medium with 75 % artificial seawater are circular, convex and white. The optimum temperature for growth is 20–30 °C; no growth occurs at 4 or 45 °C. The pH range for growth is 7.0–9.0. NaCl is required for growth and can be tolerated at up to 5 % (w/v). Urea and DNA are hydrolysed but agar, casein, aesculin and gelatin are not. Reactions for acetoin, ONPG and tryptophan deaminase are positive, but reactions for citrate utilization, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, hydrogen sulfide and indole production are negative. Acid is produced from glycerol, galactose, fructose, mannose, mannitol, sorbitol, trehalose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol and 5-ketogluconate, but not from erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, methyl β-D-xylopyranoside, glucose, sorbose, rhamnose, dulcitol, inositol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, gluconate or 2-ketogluconate. The usual components of bacterial cell walls such as muramic acid and diaminopimelic acid are not detected. Major fatty acid components (> 2.0 % of the total) are iso-C_{14:0} (8.2 %), C_{14:0} (24.2 %), anteiso-C_{15:0} (2.9 %), C_{16:0} (3.3 %), C_{18:1ω9c} (23.5 %), C_{18:0} (15.5 %), C_{19:0} (2.8 %) and C_{21:0} (6.9 %).

The type strain, 04OKA010-24^T (= MBIC06463^T = IAM 15411^T = KCTC 12865^T), was isolated from seawater in a sampling bottle of the hard coral *Galaxea fascicularis* L., collected at Majanohama, Akajima, Japan.

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