Amorphus coralli gen. nov., sp. nov., a marine bacterium isolated from coral mucus, belonging to the order *Rhizobiales*

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A bacterial strain, designated RS.Sph.026^T, was isolated from mucus of the coral *Fungia granulosa* collected from the northern Red Sea (Gulf of Eilat, Israel). The bacterium was found to be Gram-negative, non-motile, halotolerant and heterotrophic. Comparative 16S rRNA gene sequence analyses showed that strain RS.Sph.026^T belonged to the order *Rhizobiales*, with the highest levels of 16S RNA gene sequence similarity with *Rhodobium orientis* (92 %). Strain RS.Sph.026^T grew optimally at a salinity of 3–4 %, pH 7.5–8 and 25–30 °C. The major cellular fatty acids were *cis*-7-octadecenoic acid ($C_{18:1}\omega7c$; 57.2 %) and $C_{19:0}$ cyclo $\omega8c$ (15.5 %). The DNA G+C content of strain RS.Sph.026^T was 67.1 mol%. On the basis of its phenotypic properties and phylogenetic distinctiveness, strain RS.Sph.026^T represents a novel genus and species in the order *Rhizobiales*, for which the name *Amorphus coralli* gen. nov., sp. nov. is proposed. The type strain is RS.Sph.026^T (=LMG 24307^T=DSM 19760^T).

The order *Rhizobiales*, the nomenclature of which was suggested in the most recent edition of *Bergey's Manual of Systematic Bacteriology*, is the biggest group within subgroup 2 of the *Alphaproteobacteria* (Garrity & Holt, 2001). According to *Bergey's Manual* (Garrity & Holt, 2001), the *Alphaproteobacteria* are divided into six orders. The order *Rhizobiales* is divided into ten families, based on 16S rRNA gene phylogenetic analyses (Garrity & Holt, 2001). Among the six orders of the *Alphaproteobacteria*, marine bacteria are mainly distributed in three orders: '*Rhodobacterales*', '*Sphingomonadales*' and *Caulobacterales* (Jannasch & Jones, 1960; Shiba *et al.*, 1991; Giovannoni & Rappé, 2000). Only a few members of the order *Rhizobiales*, such as the genus *Rhodobium*, including *Rhodobium orientis* and *Rhodobium marinum* (Hiraishi *et al.*, 1995), and the genera *Roseibium* (Suzuki *et al.*, 2000) and *Aurantimonas* (Denner *et al.*, 2003), have been isolated from marine environments (Cho & Giovannoni, 2003).

Coral reefs are the most diverse of all marine ecosystems, although most of this diversity remains uncharacterized. Recently, culture-independent techniques have revolutionized our knowledge of planktonic marine bacteria (reviewed by Giovannoni & Rappé, 2000), although they have not been employed systematically to study bacteria living on coral reefs (Rohwer *et al.*, 2001; Frias-Lopez *et al.*, 2002). Corals that make up the backbone of these reefs harbour a diverse array of bacterial associates (reviewed by Brown & Bythell, 2005). To date, very little is known of the metabolic capabilities of these bacteria, their function on the coral surface and their potential benefit to the coral host or symbiotic algae.

Strain RS.Sph.026^T was isolated from the mucus of the coral *Fungia granulosa* using the double encapsulation method developed by A. Kushmaro (unpublished results.) This bacterium exhibited the closest 16S rRNA gene similarity (92 %) to the marine, budding, phototrophic bacterium *Rbi. orientis* (Hiraishi *et al.*, 1995). In this paper, we have compared the phenotypic, genotypic and phylogenetic characteristics of strain RS.Sph.026^T with those of *Rbi. orientis* (GenBank no. D30792).

Samples of mucus from healthy corals of *F. granulosa* were collected from the Red Sea (Gulf of Eilat), from depths of 10–15 m, in front of the Inter-University Institute for Marine Science, Eilat, Israel (29° 51′ N 34° 94′ E). Bacteriological peel loops were used to collect the coral surface microlayer *in situ* and inserted into air-dry, sterile, 50 ml polypropylene centrifuge tubes, sealed underwater. The tubes were brought to the surface and immediately placed on ice. Initial cultures of strain RS.Sph.026^T were obtained by using the recently developed agar sphere culturing technique, described by Kushmaro and others (unpublished results) and in patent application nos WO 2004/022698 A2 and EP1556480. This novel encapsulation technology for the isolation and culture of previously 'uncultivable' micro-organisms includes the collection of an environmental sample, estimation of the bacterial number and dilution of the sample in order to entrap approximately one bacterium in one agar sphere (1–2 mm

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain RS.Sph.026^T is DQ097300.

in diameter). The diluted samples were mixed with warm autoclaved agar and a sphere was made by dripping agar droplets into cold mineral oil. The sphere size can be modulated by the nozzle diameter and drip rate. Coating of the agar spheres with a polymeric membrane was carried out by inserting them into a polymer solution (polysulfone) followed by transfer into a polymerization medium. The polymeric membrane coating allows an exchange of chemicals between the sphere and the environment, but restricts the movement of cells and enables their incubation in the environment (Kushmaro and others, unpublished results). Domestication of the novel bacterial strain, RS.Sph.026^T, was achieved by repeated transfers through agar spheres and subsequent plating on 100 % marine agar 2216 (MA-100 %; HiMedia Laboratories) enrichment plates.

Liquid cultures of strain RS.Sph.026^T in Leibovitz L-15 medium, supplemented with 3 % heat-inactivated fetal calf serum (medium and supplements purchased from Biological Industries, Israel), were spread and purified as single colonies on MA-100 % and incubated at 22 °C. The strains were maintained as viable cultures on MA-100 % agar plates at 4 °C and were also stored as 25 % (v/v) glycerol suspensions at −80 °C.

Strain RS.Sph.026^T was a Gram-negative, budding bacterium with a variety of amorphous morphologies, with cells ranging in size from 0.5 to 3 μm (Fig. 1a). Strain RS.Sph.026^T differed from *Rbi. orientis* and *Rbi. marinum* in the following phenotypic characteristics: cell shape, cell diameter, rosette formation, motility and salinity requirement (see Table 1). All of these differences may be the result of the unique habitat of this bacterium. Living corals are often described as being slick with mucous secretions. Secreted mucus, whether still closely associated with the surface of a coral, or not, invariably becomes enriched with microbes, in comparison with the overlying water column (Coffroth, 1990). There is mounting evidence that the bacterial community that develops on the surface of the coral is distinctly different from that of the water column overlying it (Cooney *et al.*, 2002; Frias-Lopez *et al.*, 2002). In addition, as the mucus environment differs in viscosity (Brown & Bythell, 2005) from the surrounding water environment, it is possible that these differences dictate the observed differences in morphology. Moreover, it should be mentioned that the amorphous cell shapes we observed could be cells that are unable to divide normally because of

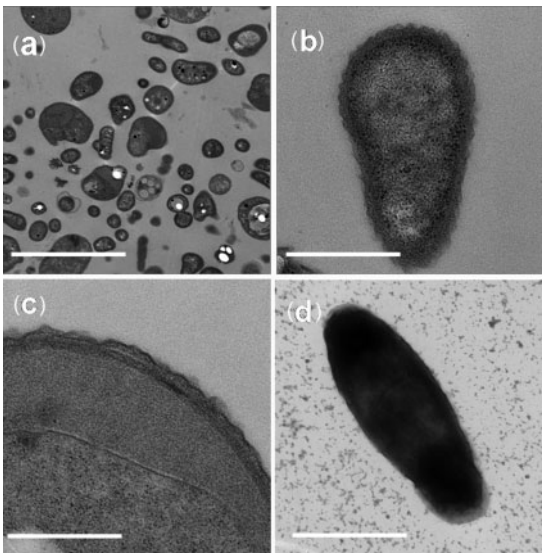

Fig. 1. Transmission electron micrographs of cells of strain RS.Sph.026^T 'domesticated' on marine agar. (a) Amorphous shapes, (b) cell arrangement and (c) lamellar arrangement of the intracytoplasmatic membrane. (d) Negatively stained cell showing no flagella. Bars, 5000 nm (a), 500 nm (b) and (c) and 1000 nm (d).

Table 1. Differential characteristics of strain RS.Sph.026^T (*Amorphus coralli* gen. nov., sp. nov.) and recognized species of the genus *Rhodobium*

Taxa: 1, strain RS.Sph.026^T; 2, *Rbi. orientis* (data from Hiraishi *et al.*, 1995); 3, *Rbi. marinum* (Hiraishi *et al.*, 1995). +, Positive (in most strains); −, negative (in most strains); +/−, variable (in different strains); (+), weak; ND, not determined. All taxa show the same internal membrane system and all are positive for oxidation of succinic acid, glucose and sorbitol and negative for oxidation of capric acid.

Characteristic	1	2	3
Cell shape	Amorphous	Rod	Rod
Cell diameter (μm)	0.5–3	0.7–0.9	0.7–0.9
Rosette formation	−	+/−	−
Motility	−	+	+
Colour of cultures	Cream (in aggregates)	Pink to red	Pink to red
Bacteriochlorophyll	ND	*a*	*a*
Salt requirement	0–4 %	4–5 %	1–5 %
Optimal pH	7.5–8	7.0–7.5	6.9–7.1
Optimal temperature (°C)	25–30	30–35	25–30
Aerobic dark growth	+	+	(+)
Denitrification	−	+	−
Fermentation of fructose	−	−	+
Oxidation of:			
Citrate	(+)	−	+/−
Acetic acid	−	+	+
D-Fructose	−	+	+
Glycerol	−	−	+/−
Lactose	−	+	+/−
Maltose	+	ND	ND
Mannitol	+	+/−	+
Mannose	+	ND	ND
Sorbitol	+	+	+

the lack of essential elements in the growth medium used (MA-100 %). Such abnormal dividing cells have been demonstrated with *Escherichia coli* that were thymine limited (Zaritsky *et al.*, 1999; Zaritsky & Woldringh, 2003).

Strain RS.Sph.026^T grew slowly and formed aggregates in liquid culture. On plates of MA-100 %, colonies could be detected only at 3 days after plating; hence the generation time was approximately 4–6 h. Colonies on MA-100 % were cream in colour, convex, round and glossy and 0.5–1 mm in diameter. No photosynthetic or carotenoid pigments were detected in cells extracted from cultures grown on MA-100 % using a methanol/acetone mixture (1 : 1, v/v) and a scanning UV/visible spectrophotometer (Biospec-1601; Shimadzu). No differences in growth were detected between light and dark conditions. This heterotrophic characteristic differentiates strain RS.Sph.026^T from its closest relatives *Rbi. orientis* and *Rbi. marinum* (see Table 1), which are able to grow anaerobically by photosynthesis and contain bacteriochlorophyll *a* and carotenoids (Hiraishi *et al.*, 1995). In spite of the lack of pigment, strain RS.Sph.026^T possessed a lamellar arrangement of the intracytoplasmatic membrane (Fig. 1b, c), a trait that resembles the lamellar arrangement of the closest related species, *Rbi. orientis* (Hiraishi *et al.*, 1995). The detection of a lamellar membrane in strain RS.Sph.026^T might imply that this bacterium had 'lost' its photosynthetic pigments due to the fact that its habitat is coral mucus, which is a rich environment that includes dissolved organic matter and sugars (see the review by Brown & Bythell, 2005).

Biochemical tests were carried out by using API 20NE strips (micromethod for the identification of Gram-negative rods; bioMérieux) following the manufacturer's instructions. Carbon oxidation was analysed using Biolog GN microwell plates. Pure cultures (4–6 colonies) from MA-100 % plates were suspended in 20 ml of the Biolog medium supplied (salinity of the medium was not adjusted due to the fact that this bacterium could grow in 0–4 % NaCl; see results below). The suspension was then distributed into Biolog GN plates containing 96 microwells, with a different carbon source in each and tetrazolium violet as an indicator of metabolic activity. The plates were incubated for 48 h at 30 °C. Wells that showed a change of colour to purple were marked as positive for metabolic activity.

The results of the Biolog and API 20NE tests for carbon source utilization shown in Table 1 showed that strain RS.Sph.026^T can metabolize the following sugars: arabinose, glucose, maltose, mannitol, mannose and sorbitol. Some of these sugars are found in abundance in coral mucus (see Meikle *et al.*, 1987; Brown & Bythell, 2005). Strain RS.Sph.026^T did not utilize fructose, lactose or acetic acid, substances that are utilized by *Rbi. orientis* and *Rbi. marinum* (see Table 1). Strain RS.Sph.026^T also utilized succinic acid, butyric acid, xylitol, Tween 40, putrescine, propionic acid, phenylalanine, ornithine, histidine and butanediol.

For electron microscopy, purified colonies from MA-100 % agar were fixed with 3 % glutaraldehyde in cacodylate buffer, then washed and gently mixed with 3 % bacteriological agar, treated with osmium, dehydrated, embedded in Araldite epoxy resin and sectioned at 70–80 nm. The resulting sections were stained with uranyl acetate and lead citrate and examined by using a JEM-1230 transmission electron microscope at 80 kV excitation voltage. Negative staining was performed on exponentially growing cells that were centrifuged, washed twice with 1 % PBS at pH 8 and placed in 1.5 % glutaraldehyde. The cells were stained with 1 % uranyl acetate and examined by using a JEM-1230 transmission electron microscope at 80 kV excitation voltage. No flagella were detected on negatively stained cells (Fig. 1d). The temperature range for growth, tested at 4–37 °C on MA-100 %, was 22–37 °C (optimum, 25–30 °C). The pH range for growth examined at pH 4.0–10.0 at the optimum growth temperature (25–30 °C) on MA-100 % agar plates was 6–10 (optimum, 7.5–8). The salinity range and optimum salinity for growth determined using a sea-salt mixture (Instant Ocean) for preparing artificial seawater at concentrations of 0–14 % (w/v) was 0–4 %, with no growth occurring on media containing 5 % sea-salt mixture (optimum 3–4 %). The strain did not grow after incubation for 2 weeks on MA-100 % in an anaerobic cell (BD Gas Pak EZ).

For analysis of the cellular fatty acids, cells were grown on MA-100 % for 7 days and then transferred to tryptic soy agar at 28 °C before extraction of the fatty acids. The microbial fatty acid profile was analysed using the MIDI/Hewlett Packard microbial identification system (Analytical Services), which uses GC profiles of fatty acid methyl esters. In total, seven kinds of fatty acids, containing 18–20 carbon atoms, were observed (Table 2). The measurable fatty acids were as follows: $C_{18:1}\omega7c$, $C_{18:0}$, $C_{19:0}$ cyclo $\omega8c$, $C_{20:1}\omega7c$, 11-methyl $C_{18:1}\omega7c$, $C_{16:0}$ and $C_{18:0}$ 3-OH. The most abundant fatty acid was *cis*-7-octadecenoic acid ($C_{18:1}\omega7c$; 57.2 %). The major fatty acid is similar to that found in *Rbi. marinum* (Hiraishi *et al.*, 1995), but four other abundant fatty acids, $C_{19:0}$ cyclo $\omega8c$, $C_{20:1}\omega7c$, 11-methyl $C_{18:1}\omega7c$ and $C_{18:0}$ 3-OH are not present in *Rbi. marinum* (Table 2; Hiraishi *et al.*, 1995).

Table 2. Fatty acid profiles of strain RS.Sph.026^T (*Amorphus coralli* gen. nov., sp. nov.) and *Rbi. marinum*

Values are percentages of the total fatty acids. ND, Not detected.

Fatty acid	Strain RS.Sph.026^T	*Rbi. marinum*
$C_{18:1}\omega7c$	57.2	69
$C_{18:0}$	6.5	14.1
$C_{19:0}$ cyclo $\omega8c$	15.5	ND
$C_{20:1}\omega7c$	11	ND
11-methyl $C_{18:1}\omega7c$	8.5	ND
$C_{16:0}$	0.63	1.9
$C_{18:0}$ 3-OH	0.79	ND

Genomic DNA was extracted using a PowerSoil purification kit (Mo Bio Laboratories), according to the manufacturer's instructions. Genomic DNA was eluted by using 20–40 μl of elution buffer or double distilled water and stored at −20 °C. Total DNA was amplified with a Mastercycler gradient thermocycler (Eppendorf) by PCR using 16S rRNA primers for bacteria [forward primer, 8F (5′-GGATCCAGACTTTGAT(C/T)(A/C)TGGCTCAG-3′) and reverse primer, 1512R (5′-GTG-AAGCTTACGG(C/T)TAGCTTGTTACGACTT-3′), taken and modified (8F primer was shortened from 5′-end) according to Felske *et al.* (1997). The resulting 16S rRNA gene sequences were compared with those in the GenBank database using the basic local alignment search tool BLAST network service (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) and were aligned with sequences of representative species from the order *Rhizobiales* using CLUSTAL W with the MEGA package (Kumar *et al.*, 2004). The phylogenetic tree (Fig. 2) was constructed by using the neighbour-joining method (Saitou & Nei, 1987) with the MEGA package (Kumar *et al.*, 2004). Bootstrap resampling analysis (Felsenstein, 1985) for 100 replicates was performed to estimate the confidence levels of the tree topologies.

The 16S rRNA gene sequence of strain RS.Sph.026^T (1438 bases) demonstrated a 92 % similarity to the sequence of *Rbi. orientis* (D30792), a marine, budding, phototrophic bacterium (Hiraishi *et al.*, 1995). In the phylogenetic tree, strain RS.Sph.026^T clustered within members of the order *Rhizobiales*, but had the highest similarity of only 92 % to *Rbi. orientis*, *Rhodobium gokarnense* (Srinivas *et al.*, 2007) and *Rhodobium pfennigi* (Caumette *et al.*, 2007; not included in phylogenetic tree due to the short sequence). Furthermore, the relationship of strain RS.Sph.026^T to the genus *Rhodobium* is represented by the relatively low level (60 %) of bootstrap confidence that supported this node (see Fig. 2). For the determination of the DNA G+C content, the genomic DNA of strain RS.Sph.026^T was prepared according to a modified version of the procedure of Wilson (1987). The G+C content of the DNA was determined in three independent analyses using the HPLC technique (Mesbah *et al.*, 1989) and was performed at the BCCM/LMG. The DNA G+C content of strain

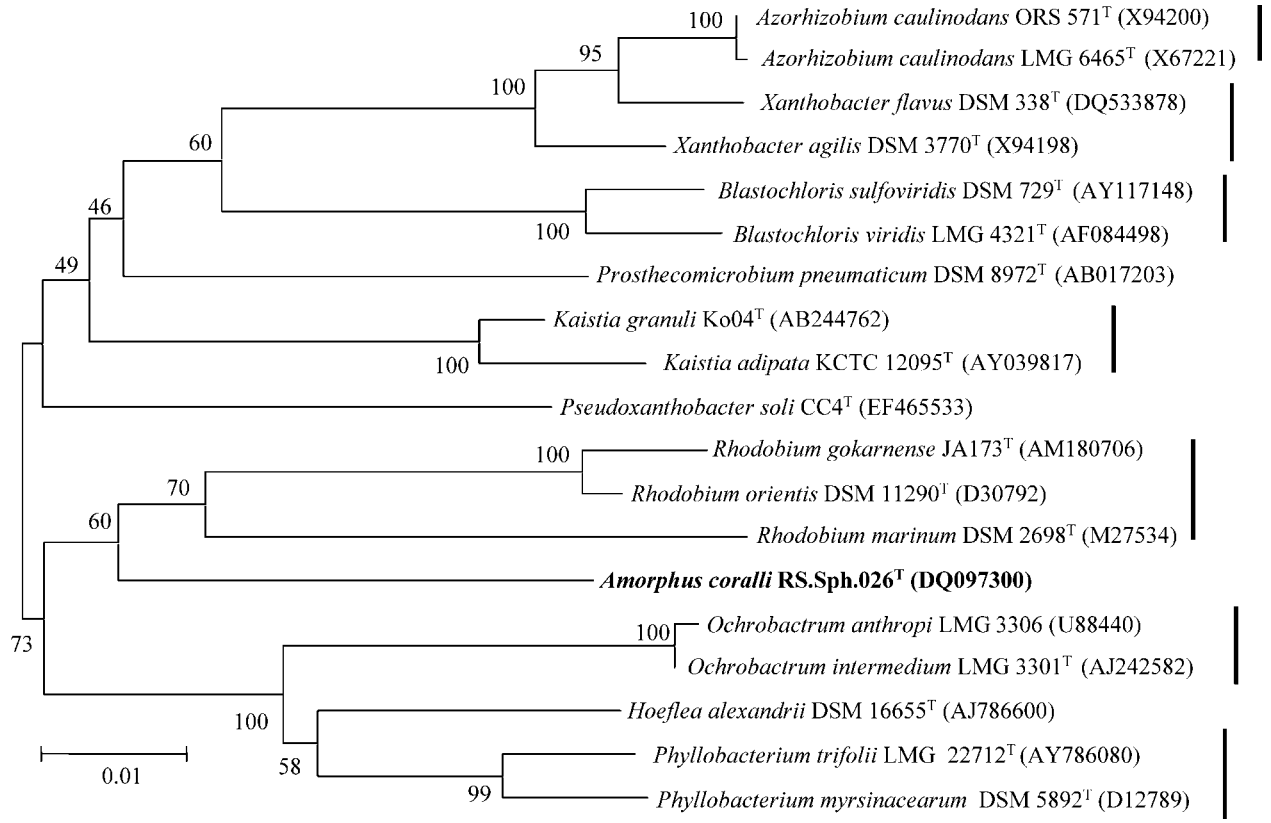

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain RS.Sph.026^T and 18 representative species from the order *Rhizobiales*. Bootstrap percentages based on 100 replications are given at branch points. GenBank numbers are given in parentheses. Vertical lines indicate the genera. Bar, 0.01 substitutions per nucleotide position.

RS.Sph.026^T was found to be 67.1 mol%. Based on the phenotypic characterization and the phylogenetic analysis, strain RS.Sph.026^T should be classified as representing a novel genus and species of the order *Rhizobiales*, for which the name *Amorphus coralli* gen. nov., sp. nov. is proposed.

Description of *Amorphus* gen. nov.

Amorphus [A.mor′phus. N.L. masc. n. *Amorphus* (from Gr. adj. *amorphos* without form, shapeless), a bacterium without defined shape].

Gram-negative, heterotrophic, non-motile, budding bacteria with morphologically different shaped structures of 0.5–3 μm. Cells have lamellar arrangements of the intracytoplasmatic membranes, but no photosynthetic pigments. Fermentative growth and denitrification do not occur. The type species is *Amorphus coralli*.

Description of *Amorphus coralli* sp. nov.

Amorphus coralli (co.ral′li. L. gen. n. *coralli* of coral, from which the organism was isolated).

Exhibits the following properties in addition to those given in the genus description. Optimal growth occurs at 25–30 °C, pH 7.5–8 and in NaCl concentrations of 3–4 %. The following carbon sources are utilized: glucose, arabinose, mannose, maltose, mannitol, mannose, sorbitol, succinic acid, butyric acid, xylitol, Tween 40, putrescine, propionic acid, phenylalanine, ornithine, histidine and butanediol. Fatty acids are $C_{18:1}\omega7c$, $C_{18:0}$, $C_{19:0}$ cyclo $\omega8c$, $C_{20:1}\omega7c$, 11-methyl $C_{18:1}\omega7c$, $C_{16:0}$ and $C_{18:0}$ 3-OH. The G+C content of the DNA of the type strain is 67.1 mol% (T_m).

The type strain, RS.Sph.026^T (=LMG 24307^T=DSM 19760^T), was isolated from mucus of the coral *F. granulosa*, collected from the Gulf of Eilat, Red Sea.

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