Corynebacterium maris sp. nov., a marine bacterium isolated from the mucus of the coral *Fungia granulosa*

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A bacterial strain, designated Coryn-1^T, was isolated from mucus of the coral *Fungia granulosa* (northern Red Sea, Gulf of Eilat, Israel) by growth and enrichment of micro-organisms in agar spheres and subsequent plating. The bacterium was found to be a Gram-positive, non-motile, halotolerant, heterotrophic coccobacillus. Comparative 16S rRNA gene sequence analyses showed that strain Coryn-1^T belonged to the genus *Corynebacterium*, exhibiting the highest levels of similarity (94 %) with the 16S rRNA gene sequence of *Corynebacterium halotolerans* YIM 70093^T. The novel strain grew well at 0.5–4.0 % salinity, at pH 7.2–9.0 and at 30–37 °C. The major cellular fatty acids were oleic acid (C_{18:1}ω9c; 58 %), palmitic acid (C_{16:0}; 30 %) and tuberculostearic acid (10-methyl-C_{18:0}; 12 %). The DNA G + C content was 66.6 mol%. On the basis of its phenotypic properties and phylogenetic distinctiveness, strain Coryn-1^T represents a novel species, for which the name *Corynebacterium maris* sp. nov. is proposed. The type strain is Coryn-1^T (=DSM 45190^T=LMG 24561^T).

The genus *Corynebacterium* (phylum *Actinobacteria*) was originally described in 1896 as a primarily pathogenic species that showed morphological similarity to the diphtheroid bacillus (Barksdale, 1970; Ventura *et al.*, 2007). The genus *Corynebacterium* includes both aerobic and facultatively anaerobic, asporogenous Gram-positive species (Collins & Cummins, 1986). Some species synthesize tuberculostearic acid and/or short-chain and structurally distinctive mycolic acids (Collins *et al.*, 1982), while some lack mycolic acids (Collins *et al.*, 1988, 2004). Consequently, for several decades, the genus comprised an extremely diverse collection of morphologically similar Gram-positive micro-organisms, including pathogenic and non-pathogenic soil bacteria (Collins & Cummins, 1986). Chemotaxonomic studies and comparative phylogenetic analyses have defined the border of the genus *Corynebacterium*, clearly demonstrating that species assigned to this genus form a monophyletic association and, together with other chemotype IV and mycolic acid-containing taxa (including the genera *Dietzia*, *Gordonia*, *Millisia*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Segniliparus*, *Skermania*, *Tsukamurella* and *Williamsia*),

form a natural suprageneric group (Pascual *et al.*, 1995; Ruimy *et al.*, 1995). *Corynebacterium* strains exhibit considerable heterogeneity in mycolic acid content as well as in DNA G + C content, which ranges from 46 to 74 mol% (Ruimy *et al.*, 1995). Currently, there are over 90 recognized species of the genus *Corynebacterium* (Euzéby, 2007), including many novel species isolated from human clinical samples (Otsuka *et al.*, 2005; Riegel *et al.*, 2006; Renaud *et al.*, 2007), wild animals (Collins *et al.*, 2004; Fernández-Garayzabal *et al.*, 2004; Goyache *et al.*, 2003), saline soil (Chen *et al.*, 2004), food (Brennan *et al.*, 2001) and even from cosmetic dye (Yassin *et al.*, 2003).

Scleractinian corals make up the backbone of coral reefs and are the most diverse of all marine ecosystems. These corals harbour large, diverse and specific populations of micro-organisms, including viruses, bacteria, archaea, algae, fungi and protozoa that have apparently co-evolved with them (Rosenberg *et al.*, 2007). Studies have revealed a dynamic microbial biota living in the mucus, on the surface and in the tissues of many coral species but still little is known of their function, metabolic capabilities and their potential benefit to the coral host. Recently, the development of high-throughput culturing techniques and the application of single-cell isolation methods have improved the isolation of micro-organisms from their natural habitats (Giovannoni *et al.*, 2007). In the present

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Coryn-1^T is FJ423600.

An extended phylogenetic tree based on 16S rRNA gene sequences is available as supplementary material with the online version of this paper.

study, we used phenotypic, chemical and genetic methodologies to facilitate the characterization of a *Corynebacterium*-like organism recovered from mucus of the coral *Fungia granulosa* from the Red Sea.

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). Sterile bacteriological quadloops were carried into these waters to collect the coral surface microlayer *in situ*. Near the coral, a sterile 15 ml polypropylene tube was opened upside down and mucus was collected from the coral surface using quadloops (three in every tube). Before sealing and while the tube was still in an upside-down position, compressed air was added and the vial was sealed to exclude seawater. The tubes were brought to the surface and immediately placed on ice. An initial culture of strain Coryn-1^T was isolated through the use of a recently developed agar-sphere culturing technique (patent applications WO 2004/022698 A2 and EP1556480). This encapsulation technology for isolating and culturing previously uncultivable micro-organisms includes collecting an environmental sample, estimating the bacterial number and diluting the sample in order to entrap approximately one bacterium per agar sphere. Dripping the diluted samples mixed with warm autoclaved agar into cold mineral oil leads to the formation of spheres, the size of which (1–2 mm in diameter) can be modulated by the nozzle diameter and drip rate. The agar spheres are coated with a polymeric membrane by inserting the spheres into a polymer solution (polysulfone) and transferring them to a polymerization medium. The polymeric membrane allows the exchange of chemicals between the sphere and the environment but restricts the movement of cells, thus enabling bacterial incubation in environmental conditions. Enrichment of strain Coryn-1^T was achieved by repeated transfer through agar spheres incubated in proximity to the coral and subsequent plating on 100 % marine agar 2216 (MA-100 %; HiMedia Laboratories) plates. Liquid Luria-Bertani (LB) or marine broth cultures of the strain were spread onto LB or MA-100 % plates and purified as single colonies after 48–72 h of incubation at 30 °C. Haemolytic activity of the strain was tested on tryptic soy blood agar containing 5 % defibrinated sheep blood (Hy-Laboratories). The strain was maintained as viable cultures on the plates at 4 °C and stored as 20 % (v/v) glycerol suspensions at –80 °C.

Coryn-1^T cells were Gram-positive, aerobic, non-spore-forming coccobacilli approximately 0.5–0.8 µm in width and 0.8–1.5 µm in length (Fig. 1). The isolate was non-motile and grew well on marine agar, LB agar and nutrient agar at 30 °C, forming small colonies approximately 1 mm and 2.5 mm in diameter after 48 and 72 h incubation, respectively, yellowish to yellow after 72 h, circular, convex, smooth, opaque and non-haemolytic. Strain Coryn-1^T proliferated well on liquid LB broth or LB agar at 26–37 °C (optimum 35 °C). The strain grew about 1.5-times faster (doubling time of around 54 min) at 35 °C

Fig. 1. Transmission electron microscopy images of cells of strain Coryn-1^T showing (a) ring compaction of the chromosome, as seen in initial stage of division; (b) DNA ring in different stages of segregation; (c) cells after division; (d) attached diplo cells after division. Bars, 0.2 µm (a); 0.5 µm (b, c); 1 µm (d).

than at 26 °C (doubling time around 82 min), as determined from growth curves. No growth was observed at 42 °C in liquid LB broth or at 45 °C on LB agar plates. No differences in growth were detected between light and dark conditions. For pH tolerance experiments (on LB agar medium with 0.5 % NaCl), citric acid monohydrate/Na₂HPO₄ was used as the buffer for pH 5.0–7.8 and glycine + NaCl/NaOH was used for pH 8.6–12.0. The isolate grew well at pH 7.2–9.0 (optimum pH 7.2). The salinity range for growth was determined both by using a sea-salt mixture (Instant Ocean) at concentrations of 0–10 % (w/v), with 0.5 % sucrose as a carbon source, 0.005 % yeast extract as a vitamin source and 1.5 % bacteriological agar, and on LB agar plates with NaCl concentrations ranging between 0.5 and 25 % (w/v). Prolific growth occurred at between 0 and 10 % salinity, with optimal growth being observed with 0.5–4.0 % NaCl or sea-salt mixture. The strain did not grow following incubation for 2 weeks at 30 °C on LB agar plates in an anaerobic cell (Gas Pak EZ; BD).

Strain Coryn-1^T was characterized biochemically using the API ZYM and API Coryne systems (bioMérieux), according to the manufacturer's instructions. The tests were read after 4 and 48 h incubation, respectively, at 37 °C. The enzymic profile obtained with API ZYM strips revealed positive reactions for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase and α-glucosidase activities. All of the other enzyme tests were deemed negative using this kit. Using the API Coryne

system, positive results were obtained for pyrazinamidase, pyrrolidonyl arylamidase, alkaline phosphatase, α -glucosidase and gelatin hydrolysis activities. Strain Coryn-1^T was catalase- and oxidase-positive and no fermentation of sugars was detected. Support for the distinctiveness of the novel isolate also came from its phenotypic characteristics in comparison with its closest phylogenetic relative, *Corynebacterium halotolerans* YIM 70093^T (Table 1). A carbon-source utilization profile was obtained using the GN MicroPlate system (Biolog) in duplicate. Pure cultures (4–6 colonies) were removed from LB plates and suspended in 20 ml sterile 0.85 % NaCl. The suspension was then distributed into the 96-well plates, each well containing a different carbon source in each in addition to tetrazolium violet, which turns from colourless to purple in the presence of respiring cells. The plates were incubated at 30 °C for 48 h and changes in absorbance (A_{590}) were determined with an ELx808 microplate reader (BioTek Instruments). Accordingly, strain Coryn-1^T was shown to metabolize the following carbon compounds as sole energy sources: maltose, lactulose, β -hydroxybutyric acid, α -ketovaleric acid, Tween 40, phenylethylamine, *N*-acetyl-D-galactosamine, malonic acid, L-threonine, L-glutamic acid, L-fucose, L-alanyl glycine, inosine and, less efficiently, raffinose, D-arabitol, L-asparagine and citric acid.

Antimicrobial susceptibility was tested by the agar disc-diffusion method using commercial discs (Oxoid). The inhibition zone of each antibiotic was measured for the strain grown on marine agar for 48 h at 30 °C. Strain Coryn-1^T was sensitive to sulfamethoxazole/trimethoprim, tetracycline, chloramphenicol, erythromycin, ampicillin and meticillin and was resistant to nalidixic acid.

For electron microscopy, purified cultures of the bacterium were prepared with LB broth, washed and gently mixed with 0.5 % NaCl, fixed in Karnovsky's formaldehyde-glutaraldehyde fixative (Karnovsky, 1965), treated with osmium tetroxide, dehydrated, embedded in araldite epoxy resin and sectioned into 70–80 nm slices. The resulting sections were stained with uranyl acetate and lead citrate and examined using a JEM-1230 transmission electron microscope (JEOL) at 80 kV excitation. The coccobacilli of strain Coryn-1^T contained a thick peptidoglycan layer and displayed a diplo-cellular form (Fig. 1a and b) as a result of incomplete separation after cell division. The chromosomal DNA was tightly packed into a ring (Fig. 1c and d), and resembled that described for *Deinococcus radiodurans*, where such an arrangement is thought to prevent any pieces of DNA that have been broken by radiation from floating into the cytoplasm (Levin-Zaidman *et al.*, 2003).

Table 1. Characteristics that differentiate *Corynebacterium maris* sp. nov. from its nearest phylogenetic relatives

Strains: 1, *Corynebacterium maris* sp. nov. Coryn-1^T; 2, *C. halotolerans* YIM 70093^T (data from Chen *et al.*, 2004); 3, *Corynebacterium casei* LMG S-19264^T (Brennan *et al.*, 2001); 4, *Corynebacterium lipophiloflavum* DSM 44291^T (Funke *et al.*, 1997); 5, *C. pilosum* ATCC 29592^T (Yanagawa & Honda, 1978). All strains were positive for catalase activity. All strains were negative for β -galactosidase, β -glucosidase and *N*-acetyl- β -glucosaminidase (except *C. pilosum*, not determined) and for acid production from sucrose, mannitol, xylose and lactose. +, Positive; –, negative; ND, not determined.

Characteristic	1	2	3	4	5
Cell shape	Coccobacilli	Diphtheroid and irregular rods	Irregularly shaped rods	Club-shaped	Piliated rods
Anaerobic growth	–	–	+	–	+
Nitrate reduction	–	+	+	–	+
Enzyme activities					
Alkaline phosphatase	+	–	+	+	–
Esterase	+	–	+	+	ND
Esterase lipase	+	–	+	+	ND
Lipase	+	+	–	+	ND
Leucine arylamidase	+	–	+	+	ND
β -Glucuronidase	–	+	–	–	ND
α -Glucosidase	+	–	–	–	ND
Pyrazinamidase	+	–	+	+	ND
Pyrrolidonyl arylamidase	+	–	+	–	ND
Urease	–	+	–	+	+
Oxidase	+	–	–	ND	–
Hydrolysis of gelatin	+	+	–	ND	–
Fermentation of:					
Glucose	–	+	+	–	+
Maltose	–	–	–	–	+
Ribose	–	–	+	ND	ND
DNA G + C content (mol%)	66.6	63	51	65	59.8

Cells of *Escherichia coli* DH5 α (as control) and strain Coryn-1^T were grown for 24 h (37 °C) and 48 h (30 °C), respectively, to stationary phase, collected by centrifugation, washed with saline (0.85 %) and diluted to a titre of 10⁷–10⁸ cells ml⁻¹ in the same saline buffer. A suspension (5 ml) of these cells was irradiated with 254 nm light from a UV lamp (UV 722; Trojan Technologies). The dose rate was measured using a UV digital radiometer (IL1400A; International Light Technologies) and shown to be 0.7 mW cm⁻², yielding a total dose of 7.5 mJ cm⁻² in 10 s. Comparison of the survival rates of *E. coli* and strain Coryn-1^T revealed the higher viability of strain Coryn-1^T cells under these conditions: the colony-forming ability of *E. coli* cells decreased by five orders of magnitude after the first 10 s, whereas strain Coryn-1^T cells lost only 50 % of viability in the same time and their viability was decreased by five orders of magnitude after 90 s.

For the analysis of cellular fatty acids, cells of strain Coryn-1^T were grown on tryptic soy agar at 28 °C. The cellular fatty acid profile was analysed using the MIDI/Hewlett Packard microbial identification system (Analytical Services), which uses GC profiles of fatty acid methyl esters. The major cellular fatty acids that were detected corresponded to oleic acid C_{18:1} ω 9c (58 %), palmitic acid C_{16:0} (30 %) and tuberculostearic acid 10-methyl C_{18:0} (12 %). The predominant cellular fatty acids of *C. halotolerans* (Chen *et al.*, 2004), a species closely related to strain Coryn-1^T (94 % 16S rRNA gene sequence similarity), are C_{16:0} (42 %), C_{18:1} ω 9c (29 %) and 10-methyl C_{18:0} (7 %). Mycolic acid analysis was performed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen Identification Service. Mycolic acids were examined as trimethylsilylated derivatives by high-temperature GC with a microbial identification system apparatus equipped with a HT5 column (Klatte *et al.*, 1994), which revealed the presence of short-chain mycolic acids (C₃₀–C₃₆) in the following proportions: C₃₀ (6 %), C₃₂ (27 %), C₃₄ (47 %) and C₃₆ (20 %).

Genomic DNA was extracted from the bacterial cultures using a PowerSoil purification kit (Mo Bio Laboratories), according to the manufacturer's instructions. Genomic DNA was eluted with 20–40 μ l elution buffer or double-distilled water and stored at -20 °C. 16S rRNA gene sequence fragments were amplified by PCR with a Mastercycler gradient thermocycler (Eppendorf) using 16S rRNA primers for bacteria (forward primer, 8F, 5'-GG-ATCCAGACTTTGAT(C/T)(A/C)TGGCTCAG-3'; reverse primer, 1512R, 5'-GTGAAGCTTACGG(C/T)TAGCTTGTACGACTT-3'), as described by Felske *et al.* (1997) with the modification that the 8F primer was shortened at the 5' end. The resulting 16S rRNA gene sequences were compared with those in the GenBank database using the basic local alignment search tool BLAST (<http://www.ncbi.nlm.nih.gov/blast/blast.cgi>) and aligned with representative corynebacterial strains using CLUSTAL W in the MEGA package (Kumar *et al.*, 2004). The phylogenetic tree (Fig. 2) was constructed by the neighbour-joining method (Saitou & Nei, 1987),

using the MEGA package. Bootstrap resampling analysis (Felsenstein, 1985) with 100 replicates was performed to estimate the confidence levels of tree topologies. Sequence database searches revealed that strain Coryn-1^T was most closely related to the actinobacteria, with highest sequence similarities with species of the genus *Corynebacterium* (data not shown). A 16S rRNA gene sequence (1468 bases) of strain Coryn-1^T demonstrated 94 % similarity to that of *C. halotolerans* YIM 70093^T, which was isolated from saline soil in west China (Chen *et al.*, 2004) (Fig. 2). No sequence similarity of more than 97 % was obtained with any member of the genus *Corynebacterium*. The genus *Corynebacterium* embraces a very diverse range of organisms, although phylogenetic analyses clearly demonstrate that the species form a monophyletic association (Ruimy *et al.*, 1995). The phylogenetic tree (Fig. 2) showed that strain Coryn-1^T clustered with *C. halotolerans* YIM 70093^T as its nearest relative into a separate subline (see also the extended phylogenetic tree, Supplementary Fig. S1, available in IJSEM Online, constructed with 67 *Corynebacterium* strains obtained from the Ribosomal Database Project, release 10). The clustering of strain Coryn-1^T with *C. halotolerans* YIM 70093^T was supported by bootstrap resampling values of 78 % (Fig. 2) and 93 % (see Supplementary Fig. S1). Strain Coryn-1^T, *C. halotolerans* YIM 70093^T, *Corynebacterium pilosum* ATCC 29592^T and *Corynebacterium lipophiloflavum* CCUG 37336^T formed a distinct, small subcluster, and maximal score sequence similarities between strain Coryn-1^T and the other three strains were 2267, 2232 and 2102, respectively, as detected by the Align two sequences (bl2seq) program (<http://www.ncbi.nlm.nih.gov/staff/tao/URLAPI/bl2seq.html>). The results of the treeing analysis, together with sequence divergence values of 6 % with *C. halotolerans* YIM 70093^T or more with other members of the genus, therefore unequivocally demonstrated that strain Coryn-1^T represents a novel species.

For determination of the DNA G+C content, genomic DNA of strain Coryn-1^T was prepared according to a modified version of the procedure of Wilson (1987). The G+C content of the DNA sample was determined in three independent analyses using the HPLC technique (Mesbah *et al.*, 1989) and was performed by the BCCM/LMG Bacteria Collection Identification Service. The DNA G+C content of strain Coryn-1^T was found to be 66.6 mol%.

On the basis of the phenotypic characterization and the phylogenetic analysis, strain Coryn-1^T should be classified in a novel species in the genus *Corynebacterium*, for which the name *Corynebacterium maris* sp. nov. is proposed.

Description of *Corynebacterium maris* sp. nov.

Corynebacterium maris (ma'ris. L. gen. n. *maris* of the sea).

Cells are Gram-positive, non-motile, aerobic (catalase- and oxidase-positive), non-spore-forming coccobacilli approximately 0.5–0.8 μ m in width and 0.8–1.5 μ m in length. The species is non-haemolytic and forms small colonies (approximately 1 mm and 2.5 mm in diameter after 48

Fig. 2. Neighbour-joining 16S rRNA gene sequence phylogenetic tree, based on a comparison of ~1265 bases, showing the relationship between *C. maris* sp. nov. and 29 representative strains of the genus *Corynebacterium*. Bootstrap values (>50%) based on 100 replications are shown at branch nodes. Bar, 1 substitution per 100 nucleotide positions.

and 72 h, respectively, incubation at 30 °C) that are yellowish to yellow, circular, convex, smooth and opaque. Grows well at 0.5–4.0% salinity, at pH 7.2–9.0 and at 30–37 °C. Alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, α -glucosidase, pyrazinamidase, pyrrolidonyl arylamidase and gelatin hydrolysis activities are detected. No activity is observed for reduction of nitrates, valine and cystine arylamidases, trypsin, α -chymotrypsin, acid phosphatase, naphthol-AS-BI phosphohydrolase, α - and β -galactosidases, β -glucuronidase, β -glucosidase, urease, *N*-acetyl- β -glucosaminidase, α -mannosidase or α -fucosidase. The strain utilizes the following carbon compounds as sole energy sources: maltose, lactulose, β -hydroxybutyric acid, α -ketovaleric acid, Tween 40, phenylethylamine, *N*-acetyl-D-galactosamine, malonic acid, L-threonine, L-glutamic acid, L-fucose, L-alanyl glycine, inosine and, less efficiently, raffinose, D-arabitol, L-asparagine and citric acid, as determined with the Biolog GN system. Long-chain fatty acids are of the straight-chain saturated and mono-unsaturated types, with C_{16:0}, C_{18:1 ω 9c} and tuberculoearic acid (10-methyl C_{18:0}) predominating. Mycolic acids (C₃₀–C₃₆) are present.

The type strain, Coryn-1^T (=DSM 45190^T=LMG 24561^T), was isolated from the mucus of the coral *Fungia granulosa*, Gulf of Eilat, Red Sea. The DNA G + C content of the type strain is 66.6 mol%.

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