Amorphus orientalis sp. nov., an exopolysaccharide-producing bacterium isolated from salt mine sediment

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A Gram-negative, moderately halophilic, non-motile, aerobic bacterium, designated strain YIM D10^T, was isolated from a salt mine sediment sample from Yunnan, south-west China. The strain grew optimally in the presence of 3–8 % NaCl and at 28 °C and pH 7.5. The polar lipid profile of strain YIM D10^T comprised diphosphatidylglycerol, an unknown phospholipid and two unknown aminolipids. The major cellular fatty acids were C_{18:1}ω7c (30.5 %), C_{19:0} cyclo ω8c (29.3 %) and C_{18:0} (13.2 %). The respiratory quinone was ubiquinone 10 (Q-10). The genomic DNA G + C content was 65.4 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain YIM D10^T was closely related to *Amorphus coralli* RS.Sph.026^T (96.8 % gene sequence similarity). Results confirmed the placement of isolate YIM D10^T within the genus *Amorphus*. However, DNA–DNA hybridization between strain YIM D10^T and the type strain of the only recognized species of the genus *Amorphus*, *A. coralli* RS.Sph.026^T, was 16.7 %, showing clearly that the isolate constitutes a new genospecies. Strain YIM D10^T could be clearly differentiated from *A. coralli* and other phylogenetic neighbours on the basis of some phenotypic, genotypic and chemotaxonomic features. Therefore, strain YIM D10^T is considered to represent a novel species of the genus *Amorphus*, for which the name *Amorphus orientalis* sp. nov. is proposed; the type strain is YIM D10^T (=DSM 21202^T=CCTCC AA 208035^T).

Taxonomically, the majority of the Gram-negative halophilic bacteria belong to the class *Gammaproteobacteria*, but they can also be found in other bacterial groups (Ventosa *et al.*, 1998), such as the genera *Salipiger* (Martínez-Cánovas *et al.*, 2004) and *Palleronia* (Martínez-Checa *et al.*, 2005) of the class *Alphaproteobacteria*. The genus *Amorphus* was proposed by Zeevi Ben Yosef *et al.* (2008) to accommodate a Gram-negative, non-motile, halotolerant bacterium with cells that differed morphologically. At the time of writing, the genus comprised only one recognized species, *Amorphus coralli*, which was isolated from mucus of the coral *Fungia granulosa* collected from the northern Red Sea. The major fatty acids in this species were C_{18:1}ω7c and C_{19:0} cyclo ω8c

and the DNA G + C content of the type strain was 67.1 mol%. The aim of this study was to determine the exact taxonomic position of strain YIM D10^T by using a polyphasic approach.

During an investigation of the cultured microbial diversity of a salt mine in Yunnan, south-west China, strain YIM D10^T was isolated from a sediment sample collected from the Fenggang salt mine using a standard dilution-plating technique on marine agar 2216 (MA, pH 7.2; Difco) incubated at 28 °C for 3 weeks. Strain YIM D10^T was isolated and subsequently purified four times both on MA and trypticase soy agar (TSA; Difco) supplemented with 3 % (w/v) NaCl at 28 °C. Strain YIM D10^T was maintained on TSA supplemented with 3 % NaCl at 28 °C (referred to henceforth as ‘maintenance medium’) and stored as 20 % (v/v) glycerol suspensions at –80 °C. For phenotypic characterization and fatty acid profile comparison, *A. coralli* RS.Sph.026^T was grown on TSA supplemented with 3 % (w/v) NaCl and used as a reference strain.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM D10^T is FJ998414.

A transmission electron micrograph of cells of strain YIM D10^T is available as a supplementary figure with the online version of this paper.

Morphological and physiological tests were performed as follows. Gram staining was carried out by using the standard Gram reaction combined with the KOH lysis test method (Gregersen, 1978). Motility was observed via the hanging-drop method (Suzuki *et al.*, 2001) and staining of flagella was carried out according to Blenden & Goldberg (1965) and observed by light microscopy (BH-2; Olympus). Cell morphology was observed by transmission electron microscopy. Growth at various salt concentrations was determined on TSA supplemented with NaCl at 0, 0.5, 1, 2, 3, 5, 7, 10, 12, 15, 20, 25 and 30 % (w/v). Growth at different pH values (pH 5–10, in increments of 0.5) and temperatures (4, 10, 15, 20, 25, 28, 37, 42, 45, 50 and 55 °C) was determined on maintenance medium. Growth under anaerobic conditions was determined after incubation in an anaerobic jar (GasPak Anaerobic systems; BBL) on maintenance medium. Photosynthetic and carotenoid pigments were analysed by using a methanol/acetone mixture (1:1, v/v) and a scanning UV/visible spectrophotometer (Biospec-1601; Shimadzu). For exopolysaccharide recovery, cells were incubated and centrifuged at the stationary growth phase; supernatants were treated and analysed as reported by Manca *et al.* (1996). Catalase and oxidase activities were determined by using 3 % (v/v) hydrogen peroxide and Kovacs' reagent (Kovacs, 1956), respectively. L-Phenylalanine deamination was examined using the method of Richard & Kiredjian (1995). Citrate utilization was tested on Simmons' citrate agar (Sigma). H₂S production was determined on Kligler iron agar (Difco). Methyl red and Voges–Proskauer tests were performed as described by Smibert & Krieg (1994). Nitrate reduction, hydrolysis of aesculin and gelatin, acid production from glucose, indole production, and arginine dihydrolase, urease, β -galactosidase and other enzyme activities were tested using the API 20NE and API ZYM kits (bioMérieux) according to the manufacturer's instructions. For all of these tests, cell suspensions were supplemented with 5 % (w/v) NaCl. Carbon source utilization was tested by using the Biolog GN2 system. Antibiotic resistance was determined by the disc diffusion method using commercial antibiotic-impregnated discs.

Isoprenoid quinones were extracted by using the method of Collins *et al.* (1977) and analysed by HPLC as described by Tamaoka *et al.* (1983). Polar lipids were extracted according to the procedures described by Minnikin *et al.* (1979) and identified by two-dimensional TLC after spraying with the appropriate detection reagents (Collins & Jones, 1980). Biomass for quantitative fatty acid analysis was obtained by scraping cells from plates of maintenance medium that had been incubated for 5 days at 28 °C. Analysis of the cellular fatty acid pattern followed the method described by Sasser (1990) using the Microbial Identification System (MIDI). The G+C content of the genomic DNA was determined by HPLC according to Mesbah *et al.* (1989), after DNA extraction by the method of Cui *et al.* (2001); the genomic DNA of *Escherichia coli* DH5 α was used as a standard.

The 16S rRNA gene was amplified and sequenced as described by Cui *et al.* (2001). The almost-complete 16S rRNA gene sequence (1385 nt) of strain YIM D10^T was obtained and compared with available 16S rRNA gene sequences in GenBank by using BLAST searches (Altschul *et al.*, 1990). Alignments and similarities were obtained by the CLUSTAL_X program (Thompson *et al.*, 1997). The phylogenetic tree was constructed by using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods with MEGA4 (Tamura *et al.*, 2007). Bootstrap analysis was used to evaluate tree topology of the neighbour-joining data by means of 1000 resamplings (Felsenstein, 1985).

Strain YIM D10^T was a Gram-negative, non-motile and aerobic bacterium; cells were rod-shaped structures of 1.0–2.5 μ m in length that differed morphologically (see Supplementary Fig. S1, available in IJSEM Online). The isolate was moderately halophilic, showing optimal growth

Table 1. Differential characteristics of strain YIM D10^T and its phylogenetically closest neighbour

Strains: 1, YIM D10^T; 2, *A. coralli* RS.Sph.026^T. Data from this study unless otherwise indicated. Both strains were Gram-negative and positive for catalase, alkaline phosphatase, butyrate esterase (C4), caprylate esterase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, α -chymotrypsin, trypsin, naphthol-AS-BI-phosphohydrolase, acid phosphatase, arginine dihydrolase, urease, and utilization of L-arabinose, α -cyclodextrin, dextrin, α -D-glucose, maltose, D-mannose, β -hydroxybutyric acid, succinic acid mono-methyl ester and succinic acid. +, Positive; –, negative.

Characteristic	1	2
Origin	Salt mine	Red Sea*
Cell size (μ m)	1.0–2.5	0.5–3.0*
Temperature range for growth (°C)	15–37	22–37*
pH range for growth	6.5–8.5	6.0–10.0*
Nitrate reduction	–	+
Hydrolysis of:		
Aesculin	–	+
Gelatin	+	–
Substrates utilized (Biolog GN2)		
Acetic acid	+	–
D-Arabinose	–	+
L-Asparagine	–	+
Cellobiose	–	+
D-Fructose	–	+
Glycerol	+	–
DL-Lactic acid	–	+
D-Mannitol	+	–
Sucrose	+	–
Enzyme activity (API ZYM)		
α -Galactosidase	+	–
Myristate lipase (C14)	+	–
DNA G+C content (mol%)	65.4	67.1*

*Data from Ze'evi Ben Yosef *et al.* (2008).

Table 2. Cellular fatty acid composition of strain YIM D10^T and its phylogenetically closest neighbour

Strains: 1, YIM D10^T; 2, *A. coralli* RS.Sph.026^T. Data for both strains were from this study. Values are percentages of the total fatty acids. —, Not detected.

Fatty acid	1	2
C _{9:0}	0.2	—
C _{12:0}	0.3	—
C _{14:0}	0.4	—
C _{16:0}	3.2	9.2
C _{17:0}	2.3	—
C _{18:0}	13.2	10.1
C _{19:0}	0.7	—
iso-C _{17:1}	1.1	—
C _{18:0} 3-OH	0.6	—
C _{17:1} ω7c	0.7	—
C _{18:1} ω7c	30.5	43.4
C _{20:1} ω7c	8.6	15.4
C _{20:2} ω6,9c	1.2	—
11-methyl-C _{18:1} ω7c	7.9	—
C _{19:0} cyclo ω8c	29.3	21.9

at 3–8 % (w/v) NaCl, and was able to grow in media with 0.5–12 % (w/v) NaCl. Cells produced exopolysaccharide. No photosynthetic or carotenoid pigment was detected. Other phenotypic characteristics of strain YIM D10^T are given in Table 1 and the species description.

The cellular fatty acid profiles of strain YIM D10^T (Table 2) and *A. coralli* RS.Sph.026^T were analysed in this study. The major fatty acids of strain YIM D10^T were similar to those of *A. coralli* RS.Sph.026^T in that C_{18:1}ω7c, C_{20:1}ω7c, C_{19:0} cyclo ω8c and C_{18:0} were the predominant fatty acids, but there were also differences in the proportions of some fatty acids, particularly 11-methyl C_{18:1}ω7c, C_{20:1}ω7c and

C_{16:0}. The major polar lipids of strain YIM D10^T were diphosphatidylglycerol, one unknown phospholipid and two unknown aminolipids. The genomic DNA G+C content was 65.4 mol%.

Strain YIM D10^T was affiliated phylogenetically to the genus *Amorphus*. In the neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, strain YIM D10^T joined the lineage comprising the type strain of *A. coralli* at a bootstrap value of 100 % (Fig. 1). The relationship between strain YIM D10^T and *A. coralli* RS.Sph.026^T was maintained in a tree based on the maximum-parsimony algorithm (Fig. 1). Strain YIM D10^T exhibited 16S rRNA gene sequence similarities of 96.8 % to *A. coralli* RS.Sph.026^T and similarities of less than 93.4 % to strains of other species included in the phylogenetic analysis. DNA–DNA hybridization experiments between strain YIM D10^T and *A. coralli* RS.Sph.026^T were performed using the optical renaturation method (De Ley *et al.*, 1970; Huß *et al.*, 1983; Jahnke, 1992). The hybridization value of the two strains was 16.7 %.

The combination of phylogenetically coherent clustering and the cellular fatty acid profiles indicated that strain YIM D10^T was a member of the genus *Amorphus*. However, it could be differentiated readily from *A. coralli* based on several phenotypic characteristics (Table 1): the ability to grow at 15 °C (Zeevi Ben Yosef *et al.*, 2008); a different pH range for growth (i.e. 6.5–8.5 and 6.0–10.0 for strain YIM D10^T and *A. coralli* RS.Sph.026^T, respectively); nitrate reduction; hydrolysis of aesculin and gelatin; and utilization of D-arabinose, cellobiose, D-fructose, D-mannitol, sucrose, acetic acid, D- and L-lactic acid, L-asparagine and glycerol. Based on the phenotypic and genotypic differences between strain YIM D10^T and its closest relative, strain YIM D10^T represents a novel species of the genus *Amorphus*, for which the name *Amorphus orientalis* sp. nov. is proposed.

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain YIM D10^T and *A. coralli* RS.Sph.026^T and members of related genera. Bootstrap values (%; based on 1000 replications) >50 % are shown at branching points. Filled circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony method. Bar, 0.01 substitutions per nucleotide position.

Description of *Amorphus orientalis* sp. nov.

Amorphus orientalis (o.ri.en.ta'lis. L. masc. adj. *orientalis* eastern, bacterium inhabiting the east).

Colonies are cream-white, smooth, circular, convex and opaque with entire edges. Cells are Gram-negative, catalase-positive, oxidase-negative, non-motile, rod-shaped structures of 1.0–2.5 µm in length that differ morphologically. Temperature range for growth is 15–37 °C; optimum is 28 °C. pH range for growth is 6.5–8.5; optimum is 7.5. Growth occurs at NaCl concentrations of 0.5–12 % (w/v). Positive for urease, arginine dihydrolase and gelatin hydrolysis. Negative for aesculin hydrolysis, nitrate reduction, glucose acidification, β -galactosidase, methyl red and Voges-Proskauer tests, indole and H₂S production, and L-phenylalanine deaminase activity. The following carbon sources are utilized: L-arabinose, α -cyclodextrin, citrate, dextrin, α -D-glucose, maltose, mannitol, D-mannose, sucrose, succinic acid monomethyl ester, acetic acid, citric acid, β -hydroxybutyric acid, succinic acid and glycerol. The following carbon sources are not utilized: adonitol, D-arabitol, D-fructose, L-fructose, D-galactose, *myo*-inositol, D-psicose, L-rhamnose, D-sorbitol, xylitol, formic acid, D- and L-lactic acids, bromosuccinic acid, L-alaninamide, D- and L-alanine, L-alanyl glycine, L-asparagine, L-glutamic acid, L-proline, α -hydroxybutyric acid, γ -hydroxybutyric acid, L-aspartic acid, L-ornithine, glycogen, Tweens 40 and 80, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, cellobiose, *i*-erythritol, gentiobiose, α -D-lactose, lactulose, melibiose, methyl β -D-glucoside, raffinose, trehalose, turanose, pyruvic acid methyl ester, *cis*-aconitic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, *p*-hydroxyphenylacetic acid, itaconic acid, α -ketobutyric acid, α -ketoglutaric acid, α -ketovaleric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinamic acid, glucuronamide, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-phenylalanine, L-pyroglutamic acid, D-serine, L-serine, L-threonine, D- and L-carnitine, γ -aminobutyric acid, urocanic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, D- and L- α -glycerol phosphate, α -D-glucose 6-phosphate and D-glucose 1-phosphate. Positive for the following enzyme activities: alkaline and acid phosphatases, butyrate esterase (C4), caprylate esterase (C8), myristate lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, α -chymotrypsin, trypsin, naphthol-AS-BI-phosphohydrolase and α -galactosidase. Negative for the following enzyme activities: β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. Sensitive to (µg per disc) novobiocin (30), gentamicin (10) and penicillin (10), but resistant to ampicillin (10), erythromycin (15), chloramphenicol (30), norfloxacin (10) and streptomycin (10).

The type strain, YIM D10^T (=DSM 21202^T=CCTCC AA 208035^T), was isolated from a salt sediment sample

collected from the Fenggang salt mine in Yunnan, southwest China. The genomic DNA G + C content of the type strain is 65.4 mol%.

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