Eionea nigra gen. nov., sp. nov., a gammaproteobacterium from the Mediterranean Sea

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A novel aerobic, Gram-negative bacterial strain, designated 17X/A02/237^T, was isolated from waters of the coastal north-western Mediterranean Sea. Cells were motile straight rods and formed dark-grey colonies on marine agar medium. Strain 17X/A02/237^T contained ubiquinone Q-8 and its major fatty acids were C_{16:1}ω7c and/or iso-C_{15:0} 2-OH, C_{18:1}ω7c, C_{16:0}, C_{18:0} and C_{10:0} 3-OH. The G + C content of the genomic DNA was 47.5 mol%. Phylogenetic analysis of the 16S rRNA gene sequence placed the strain in the class *Gammaproteobacteria*. Based on 16S rRNA gene sequence data, as well as physiological and biochemical characteristics, this isolate represents a novel species of a new genus, for which the name of *Eionea nigra* gen. nov., sp. nov. is proposed. The type strain is 17X/A02/237^T (=DSM 19752^T=CIP 109759^T=MOLA 288^T).

Bugula simplex and *Bugula neritina* are marine bryozoans that produce bryostatins (Lim & Haygood, 2004; Haygood & Davidson, 1997). These compounds confer a chemical defence to the larvae but are also of biotechnological interest, having potential as anticancer drugs (Hale *et al.*, 2002; Winegarden *et al.*, 2003). These bryozoans harbour bacterial symbionts that may play a role in the production of bryostatins (Lim & Haygood, 2004). Although these symbionts have not been cultured or fully characterized, molecular analysis has led to the identification of two *Candidatus* species, '*Candidatus* Endobugula sertula' and '*Candidatus* Endobugula glebosa' (Haygood & Davidson, 1997; Lim & Haygood, 2004), which are affiliated to the class *Gammaproteobacteria*. Their closest described relative is the type strain of *Teredinibacter turnerae* (Distel *et al.*, 2002) with a 16S rRNA gene sequence similarity value of 92 %.

In this study, a strain, designated 17X/A02/237^T, was isolated from seawater. On the basis of 16S rRNA gene sequence similarities, the closest relative was '*Candidatus* Endobugula glebosa' (94 % similarity) and the closest relative with a validly published name was the type strain of *T. turnerae* (92 % similarity). Thus, the taxonomic position

and characteristics of the novel isolate were analysed. On the basis of the results, the name *Eionea nigra* gen. nov., sp. nov. is proposed to accommodate strain 17X/A02/237^T.

Samples were collected in October 2002 in the bay of Banyuls-sur-Mer (42° 29' N 3° 08' E). Subsamples were spread on marine agar plates (MA 2216; Difco) and incubated at 25 °C for 2 weeks. Colonies were picked and purified after three stages of subculturing. Among these colonies, an isolate forming dark-grey-coloured colonies was obtained and referenced as strain 17X/A02/237^T (Agogué *et al.*, 2005).

Microscopic observations (Olympus AX70) showed that cells of strain 17X/A02/237^T were motile rods. Negatively stained cells (Raguénès *et al.*, 1997) were $1.4 \pm 0.1 \times 0.5 \pm 0.05$ µm in size and a polar flagellum was observed. The Ryu KOH reaction (Powers, 1995) led to immediate cell lysis, which was confirmed by microscopy (Olympus AX70). This positive reaction indicated that the strain was Gram-negative.

The isolate was routinely grown in marine broth medium (MB 2216; Difco). To determine the salinity tolerance range of 17X/A02/237^T, MB 2216, prepared according to the manufacturer's instructions, containing various NaCl concentrations was used. Growth was determined at

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 17X/A02/237^T is AY576771.

Table 1. Differential characteristics of strain 17X/A02/237^T and related species

Strains: 1, 17X/A02/237^T; 2, *Teredinibacter turnerae* DSM 15152^T (Distel *et al.*, 2002); 3, *Saccharophagus degradans* DSM 17024^T (Ekborg *et al.*, 2005; González & Weiner, 2000); 4, *Dasania marina* JCM 13441^T (Lee *et al.*, 2007); 5, *Haliea salexigens* DSM 19537^T (Urios *et al.*, 2008b); 6, *Melitea salexigens* DSM 19753^T (Urios *et al.*, 2008a). +, Positive; –, negative; (+), weakly positive; ND, not determined. API ZYM data for *T. turnerae* and *S. degradans* are from this study.

Characteristic	1	2	3	4	5	6
Sampling environment	Seawater	<i>Lyrodus pedicellatus</i>	<i>Spartina alterniflora</i>	Marine sediment	Seawater	Seawater
DNA G + C content (mol%)	47.5	49–51	45.8	37	61.4	57.2
Growth temperature (°C)						
Range	10–44	15–40	4–37	4–30	10–37	15–37
Optimum	30	30	30	17–22	25	30
Growth salinity (g l ⁻¹):						
Range	7–70	6–36	7–70	10–90	7–70	7–70
Optimum	35	18	25	35	42	28
Growth pH:						
Range	5.0–9.0	6.0–10.5	4.5–10.0	7–10	5–9	6–10
Optimum	8.0	8.5	7.5	7–8	8	8
Major quinone	Q-8	ND	ND	Q-8	Q-8	Q-8
API ZYM tests:						
Alkaline phosphatase	+	+	+	+	+	+
Valine arylamidase	–	(+)	(+)	ND	+	–
N-Acetyl-β-glucosaminidase	–	–	–	–	–	(+)
Esterase (C4)	(+)	–	–	–	–	–
Lipase (C8)	–	+	–	–	(+)	(+)
Naphthol-AS-B1-phosphohydrolase	(+)	+	(+)	ND	+	+
Utilization of:						
Cellobiose	–	+	ND	–	–	(+)
Fructose	(+)	+	+	–	–	+
Galactose	–	ND	+	–	–	–
Glucose	+	+	+	–	–	+
Lactose	–	ND	+	–	–	(+)
Maltose	–	ND	+	–	–	+
Mannose	–	ND	+	–	(+)	+
Raffinose	–	ND	ND	–	–	+
Sucrose	–	+	+	–	–	+
Glycerol	–	ND	ND	–	+	–
Mannitol	+	ND	ND	–	–	–
Acetate	–	+	ND	ND	–	(+)
Aspartate	–	ND	ND	ND	+	–
Glutamate	–	+	ND	ND	(+)	(+)
β-Hydroxybutyrate	–	ND	ND	ND	+	–
Pyruvate	–	+	ND	ND	+	–
Citrate	+	ND	ND	ND	–	–
Succinate	–	+	ND	ND	+	–
Alanine	–	ND	ND	ND	–	(+)
Leucine	–	ND	ND	ND	+	–
Proline	+	ND	ND	ND	+	–
Tween 80	–	ND	+	ND	+	–

different pH levels by adjusting MB with MES, PIPES, AMPSO or MOPS buffers (Sigma). Cultures were incubated at 30 °C under aerobic conditions. Methods for the determination of growth parameters were used as described by Wery *et al.* (2001b). Growth was observed at 10–44 °C, with optimum growth at 30 °C; growth rate values were 9 % lower at 25 °C and 25 % lower at 37 °C. The strain grew at

NaCl concentrations ranging from 7 to 70 g l⁻¹, with optimum growth at 35 g l⁻¹; growth decreased by 40 % at 42 g l⁻¹ and 8 % at 28 g l⁻¹. Growth occurred at pH 5–9, with optimum growth at pH 8; growth decreased by 90 % at pH 9 compared with growth at pH 8 and a growth variation of only 15 % occurred between pH 8 and pH 6. To investigate the possibility of growth under anaerobic

Table 2. Cellular fatty acid composition (%) of strain 17X/A02/237^T and related species

Strains: 1, 17X/A02/237^T; 2, *Saccharophagus degradans* DSM 17024^T (González & Weiner, 2000); 3, *Dasania marina* JCM 13441^T (Lee *et al.*, 2007); 4, *Haliae salexigens* DSM 19537^T (Urios *et al.*, 2008b); 5, *Melitea salexigens* DSM 19753^T (Urios *et al.*, 2008a). tr, Trace; —, not detected.

Fatty acid	1	2	3	4	5
C _{9:0}	—	—	—	—	1.9
C _{10:0}	—	5	1.4	—	—
C _{11:0}	—	—	—	1.0	0.7
C _{12:0}	—	—	—	1.6	0.3
C _{13:0}	—	—	—	1.3	0.4
C _{14:0}	—	15	5.9	1.3	0.8
C _{15:0}	—	—	—	4.5	5.3
C _{16:0}	17.8	37	18.4	2.0	3.9
C _{17:0}	1.3	—	—	9.3	13
C _{18:0}	6.8	—	1.8	—	0.5
iso-C _{11:0}	—	—	—	—	0.9
iso-C _{13:0}	—	—	—	—	1.0
iso-C _{15:0}	—	—	—	—	2.0
iso-C _{17:0}	—	—	—	—	1.3
C _{15:1} ω6c	—	—	—	5.8	—
C _{17:1} ω6c	—	—	—	2.7	0.6
C _{17:1} ω8c	—	—	—	23.9	34.1
C _{18:1} ω7c	29.0	tr	4.1	17.5	11.4
C _{18:1} ω9t	—	tr	—	—	—
C _{18:1} ω12c	—	tr	—	—	—
C _{9:0} 3-OH	—	—	—	—	0.3
C _{10:0} 3-OH	6.7	11	10.4	1.8	1.7
C _{11:0} 3-OH	—	—	—	3.3	4.6
C _{12:0} 3-OH	—	—	—	1.1	—
C _{12:1} 3-OH	—	6	—	—	0.5
C _{16:0} 3-OH	1.0	—	—	—	—
iso-C _{11:0} 3-OH	—	—	—	3.3	4.3
C _{16:1} ω7c and/or iso-C _{15:0} 2-OH	36.8	—	45.3	21.2	9.0
ECL 11.799*	—	—	11.2	—	—

*ECL, Equivalent chain-length.

conditions, cultures were grown in anaerobic jars on MA 2216 plates under optimal temperature, pH and salinity conditions. No growth was observed after 10 days and, thus, the strain was considered to be strictly aerobic.

The ability of strain 17X/A02/237^T to utilize different substrates was investigated using Biolog GN2 MicroPlates (Tang *et al.*, 1998) according to the manufacturer's instructions. Positive reactions were obtained with D-glucose, D-mannitol, citrate and L-proline. Weakly positive reactions were observed for erythritol and D-fructose. A comparison of substrate utilization by strain 17X/A02/237^T and its closest relatives is presented in Table 1.

Enzymic activities were investigated using the API ZYM system (bioMérieux) according to the manufacturer's instructions. Strain 17X/A02/237^T was positive for alkaline phosphatase activity, whereas esterase (C4) and naphthol-AS-B1-phosphohydrolase activities were weakly positive (Table 1).

Fatty acid methyl ester analysis was performed by the DSMZ Identification Service (Braunschweig, Germany). Biomass was harvested from colonies cultivated on MA plates under optimal conditions. The fatty acid composition of strain 17X/A02/237^T was (%): C_{16:1}ω7c and/or iso-C_{15:0} 2-OH (36.8), C_{18:1}ω7c (29.0), C_{16:0} (17.8), C_{18:0} (6.8), C_{10:0} 3-OH (6.7), C_{17:0} (1.3) and C_{16:0} 3-OH (1.0). Of all the strains studied (Table 2), C_{16:0} 3-OH was only detected in strain 17X/A02/237^T, C_{14:0} was found in all strains except for strain 17X/A02/237^T and C_{18:0} and C_{18:1}ω7c were present in significantly higher amounts in strain 17X/A02/237^T than in the other strains.

Analysis of respiratory quinones and polar lipids was carried out by the DSMZ Identification Service (Braunschweig, Germany). Strain 17X/A02/237^T contained ubiquinone-8 (97 %) and ubiquinone-7 (3 %).

Genomic DNA was extracted as described by Wery *et al.* (2001a). The DNA G+C content was determined by

Fig. 1. Phylogenetic tree showing the position of strain 17X/A02/237^T. Accession numbers and type strains are indicated. The tree corresponds to an unrooted tree obtained by the neighbour-joining algorithm (Kimura corrections). Bootstrap values >50% (based on 1000 resamplings) are shown at nodes. Bar, 0.01 substitutions per nucleotide position.

thermal denaturation using the method of Marmur & Doty (1962) under conditions described by Raguénès *et al.* (1997). The G+C content of the genomic DNA of strain 17X/A02/237^T was 47.5 ± 0.5 mol%. The 16S rRNA gene was amplified and sequenced as described by Agogué *et al.* (2005). The sequence was analysed as described by Urios *et al.* (2006). Strain 17X/A02/237^T was phylogenetically affiliated to the class *Gammaproteobacteria* (Fig. 1). Its closest relatives were *T. turnerae* DSM 15152^T and *Saccharophagus degradans* DSM 17024^T, with similarity values of 92 and 91 %, respectively.

Strain 17X/A02/237^T could be distinguished from its nearest phylogenetic relatives by differences in their phenotypic properties (Table 1), fatty acid composition (Table 2) and 16S rRNA gene sequences. Based on these differences, strain 17X/A02/237^T represents a novel species in a new genus, for which the name *Eionea nigra* gen. nov., sp. nov. is proposed.

Description of *Eionea* gen. nov.

Eionea (Ei.on'e.a. N.L. fem. n. *Eionea* a nymph of the sea, referring to the marine origin of the type strain).

Cells are motile Gram-negative rods. The two dominant fatty acids are C_{16:1}ω7c and/or iso-C_{15:0} 2-OH and C_{18:1}ω7c. The major ubiquinone is ubiquinone Q-8. Phylogenetically affiliated to the class *Gammaproteobacteria*. The type species is *Eionea nigra*.

Description of *Eionea nigra* sp. nov.

Eionea nigra (ni'gra. L. adj. *nigra* black, referring to the colour of the colonies of the type strain).

Forms dark-grey colonies on MA medium. Cells are motile Gram-negative rods, $1.4 \pm 0.1 \times 0.5 \pm 0.5$ μm, with a single polar flagellum. Growth occurs at 10–44 °C (optimum 30 °C), at pH 5–9 (optimum pH 8) and in 7–70 g NaCl l⁻¹ (optimum 35 g l⁻¹). Positive reactions with Biolog

GN2 plates were obtained for D-glucose, D-mannitol, citrate and L-proline. In API ZYM tests, alkaline phosphatase activity is positive and esterase (C4) and naphthol-AS-B1-phosphohydrolase activities are weakly positive. Oxidase-negative and catalase-positive. The major fatty acids are C_{16:1}ω7c and/or iso-C_{15:0} 2-OH, C_{18:1}ω7c, C_{16:0}, C_{18:0} and C_{10:0} 3-OH.

The type strain, 17X/A02/237^T (=DSM 19752^T =CIP 109759^T =MOLA 288^T), was isolated from a water column in the bay of Banyuls-sur-Mer (42° 29' N 3° 08' E). The DNA G+C content of strain 17X/A02/237^T is 47.5 mol%.

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