Nautella italica gen. nov., sp. nov., isolated from a marine electroactive biofilm

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Five isolates obtained from a marine electroactive biofilm grown on a stainless steel cathode were investigated by using a polyphasic taxonomic approach. Analyses of whole-cell fatty acid methyl esters and 16S rRNA gene sequences showed that the isolates belonged to the *Roseobacter* lineage of the class *Alphaproteobacteria*. Both phenotypic and genotypic analyses demonstrated that the five new isolates constituted a single species that did not represent a recognized member of the *Roseobacter* lineage. Therefore the five isolates represent a novel genus and species, for which the name *Nautella italica* gen. nov., sp. nov. is proposed. The type strain is LMG 24365^T (=CCUG 55857^T). The DNA G+C content of the type strain is 61 mol%.

The *Roseobacter* lineage of the class *Alphaproteobacteria* is one of the most predominant marine bacterial groups, representing up to 20 % of coastal, and up to 15 % of mixed-layer, bacterioplankton populations (González & Moran, 1997; González *et al.*, 2000; Selje *et al.*, 2004; Suzuki *et al.*, 2001). Members of the *Roseobacter* lineage are found in almost every marine environment, i.e. from coastal seawater to open-ocean seawater and from sea ice to sea-floor sediments (e.g. Brinkmeyer *et al.*, 2003; González & Moran, 1997; Inagaki *et al.*, 2003; Selje *et al.*, 2004). They exhibit a diverse range of metabolic and physiological characteristics, including aerobic anoxygenic phototrophy (Shiba, 1991), sulphur transformations (Moran *et al.*, 2003), carbon monoxide oxidation (King, 2003; Moran *et al.*, 2004) and degradation of aromatic compounds (Buchan *et al.*, 2000; González *et al.*, 1996). Several studies have indicated an important role for roseobacters in the formation of marine biofilms (Dang & Lovell, 2000) in which they use the production of antibiotics and secondary metabolites to outcompete other bacteria (Martens *et al.*, 2007).

The present study was part of an analysis of the microbial diversity of a marine electroactive biofilm (EAB) which was grown on a stainless steel cathode exposed to natural

seawater at the ISMAR–CNR Marine Station, located in the port of Genoa, Italy (Faimali *et al.*, 2008; Vandecandelaere *et al.*, 2008). The biofilm was removed from the stainless steel cathode by sonication (3200; Branson) for 90 s in a sterile plastic tube containing 30 ml 0.85 % NaCl solution. Diluted cell suspensions (10^{-1} to 10^{-6}) were inoculated onto marine agar 2216 (MA; Difco) and incubated aerobically at 20 °C for 3 days. Pure cultures were obtained from initial isolation plates and were inoculated onto MA; pure cultures were stored at –80 °C using Microbank vials.

All isolates were investigated by analysing the whole-cell fatty acid methyl esters as described by Mergaert *et al.* (2001). Comparison of the fatty acid methyl ester profiles of the five isolates with those available in a commercial (MIS) database led to the tentative identification of these strains as members of the genus *Paracoccus*, with low to moderate identification scores (0.192–0.361). The predominant fatty acids were as follows: C_{18:1}ω7c (74.5 ± 3.6 %), C_{16:0} 2-OH (5.5 ± 0.7 %), 11-methyl C_{18:1}ω7c (4.5 ± 2.5 %), an unknown fatty acid with an equivalent chain-length of 11.799 (3.6 ± 0.4 %), C_{10:0} 3-OH (3.2 ± 0.4 %), C_{18:0} 12-OH (2.6 ± 0.6 %), C_{12:0} 3-OH (2.4 ± 0.3 %), C_{16:0} (1.8 ± 0.3 %) and C_{18:0} (1.4 ± 0.3 %); the remaining fatty acids constituted <1.0 % (Table 1).

DNA was extracted as described by Pitcher *et al.* (1989). Almost-complete 16S rRNA gene sequences (1425–1432 bp) were obtained for the five isolates (LMG 24364, LMG 24365^T, R-28717, R-28753 and R-25532) using the

Abbreviation: EAB, electroactive biofilm.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains LMG 24365^T, LMG 24364, R-25532, R-28717 and R-28753 are AM904562, AM904563, AM944520, AM944521 and AM944522, respectively.

Table 1. Fatty acid content (%) of strains LMG 24365^T and LMG 24364 and the type strains of related species

Taxa: 1, strains LMG 24365^T and LMG 24364; 2, *Phaeobacter inhibens*; 3, *Phaeobacter gallaeciensis*; 4, *Phaeobacter daeponensis*; 5, *Leisingera methylohalidivorans*; 6, *Ruegeria atlantica*. –, Fatty acids constituting <1 %; ND, no data available.

Fatty acid	1	2	3	4	5	6
Straight-chain fatty acids						
C _{16:0}	1.8 ± 0.3	3.83	3.79	8.6	5.08	6.25
C _{18:0}	1.4 ± 0.3	3.14	2.57	2.4	–	2.28
Unsaturated fatty acids						
C _{14:1}	ND	2.19	2.14	ND	2.27	ND
C _{18:1ω7c}	74.5 ± 3.6	73.77	75.51	57.7	70.27	45.53
Hydroxy fatty acids						
C _{10:0} 3-OH	3.2 ± 0.4	1.71	1.86	1.7	1.83	–
C _{12:0} 3-OH	2.4 ± 0.3	1.59	1.27	2.6	2.27	8.67
C _{16:0} 2-OH	5.5 ± 0.7	3.10	3.90	5.6	6.97	10.44
C _{18:0} 12-OH	2.6 ± 0.6	ND	ND	ND	ND	ND
11-Methyl	4.5 ± 2.5	7.45	6.60	16.6	6.86	30.40
C _{18:1ω7c}						

universal primers pA (5'-AGAGTTTGATCCTGGCTCAG-3') and pH (5'-AAGGAGGTGATCCAGCCGCA-3') (Edwards *et al.*, 1989) as described previously (Mergaert *et al.*, 2001). The most similar sequences in the public databases were found using FASTA. The sequences were aligned using CLUSTAL_X (Thompson *et al.*, 1997) and edited using BioEdit (Hall, 1999) and ForCon (Raes & Van De Peer, 1999).

Numerical analysis showed that isolates LMG 24365^T, LMG 24364, R-28753, R-28717 and R-25532 shared 100 % 16S rRNA gene sequence similarity. The recognized species of the genus *Phaeobacter* [*Phaeobacter inhibens*, *Phaeobacter daeponensis* and *Phaeobacter gallaeciensis* (at the time of writing)] were found to be their closest phylogenetic neighbours, although the 16S rRNA gene sequence similarities were relatively low (96.6–97.2 %, but mainly <97 %). A neighbour-joining dendrogram was constructed (Saitou & Nei, 1987) using TREECON (Van De Peer & De Wachter, 1994) (Fig. 1) and the tree topology was confirmed by maximum-parsimony and maximum-likelihood analyses (data not shown).

DNA–DNA hybridization experiments were performed with isolates LMG 24365^T, LMG 24364 and representatives of their closest neighbours, using photobiotin-labelled probes in microplate wells, as described by Ezaki *et al.* (1989), and a HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. The hybridization temperature was 45 °C and reciprocal reactions were performed for every pair of strains. The DNA–DNA hybridization values between strains LMG 24365^T and LMG 24364 were high, i.e. >70 % (88 ± 5 %). The DNA–DNA hybridization values between strain LMG 24365^T and

the type strains of *P. gallaeciensis*, *P. daeponensis* and *P. inhibens* were low, ranging from 7 ± 2 % to 17 ± 0 %. These data demonstrate that the five new isolates constitute a novel species, which is best allocated to a novel genus within the *Roseobacter* lineage of the class *Alphaproteobacteria* (Fig. 1).

The genetic diversity of the five isolates was examined by repetitive extragenic palindromic DNA-PCR fingerprinting using the BOX-A1R primer (5'-CTACGGCAAGGCGA-CGCTGACG-3') (Rademaker & De Bruijn, 1997; Versalovic *et al.*, 1994). Differences between the BOX-PCR fingerprints of all five isolates were observed, indicating the presence of five genetically distinct strains (Fig. 2).

DNA was subsequently enzymically degraded into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was then separated using a Water Breeze HPLC system and an Xbridge Shield RP18 column thermostabilized at 37 °C. The solvent was 0.02 M NH₄H₂PO₄ (pH 4.0) with 1.5 % acetonitrile. Non-methylated lambda phage (Sigma) and *Escherichia coli* LMG 2093 DNA samples were used as the calibration reference and control, respectively. The DNA G+C contents of strains LMG 24365^T and LMG 24364 were 61.0 ± 0.8 %.

The following morphological, physiological and biochemical characteristics were determined for strains LMG 24365^T and LMG 24364. Colony morphology (i.e. determination of the surface, edge and transparency features) was examined microscopically (× 40 magnification) after 4 days incubation at 20 °C on MA. Cells were tested for their Gram reaction, catalase activity and oxidase activity. Growth was verified on nutrient agar, trypticase soy agar, R2A agar and peptone/yeast extract/glucose agar (Tan & Rüger, 1999). The optimal salinity was determined using R2A agar supplemented with 1–20 % NaCl; plates were incubated for 2 weeks at 20 °C. The optimal growth temperature was determined on MA incubated at 4–45 °C for 2 weeks. The effect of pH on growth was tested at pH 5.0–10.0 (using increments of 0.5 pH units) in marine broth 2216 (Difco) incubated at 20 °C for 7 days. Growth was checked by measuring optical density at 600 nm.

Degradation of casein and chitin (Reichenbach & Dworkin, 1981), DNA [using DNA agar (Difco) containing 0.01 % toluidine blue (Merck)], starch and L-tyrosine (Barrow & Feltham, 1993) was tested after 5 days incubation at 20 °C. To determine their lipolytic activity, the isolates were inoculated onto Sierra's medium and incubated for 10 days at 20 °C (Sierra, 1957).

Susceptibility to the following antibiotics (from Oxoid) was tested on MA plates, using the diffusion disc method: cefoxitin (30 µg), gentamicin (10 µg), erythromycin (15 µg), streptomycin (25 µg), tetracycline (30 µg), vancomycin (30 µg), trimethoprim (1.25 µg) and clindamycin (2 µg). Results were read after 5 days incubation at 20 °C.

Biochemical characteristics were determined using commercial microtest galleries (API ZYM and API 20NE)

Fig. 1. Neighbour-joining dendrogram, based on 16S rRNA gene sequences, showing the phylogenetic positions of strains LMG 24365^T, LMG 24364, R-25532, R-28717 and R-28753 and their closest neighbours. Bar, 1 % sequence divergence.

Fig. 2. BOX-PCR fingerprints of strains LMG 24365^T, LMG 24364, R-25532, R-28717 and R-28753.

according to the manufacturer's instructions (bioMérieux). API ZYM was used to determine the enzymic activities of the isolates investigated; the results were read after 4 h incubation at 20 °C. The API 20NE results were read after 48 h incubation at 20 °C.

The cell morphology of strain LMG 24365^T was determined using transmission electron microscopy. Cells were negatively stained with 2% uranyl acetate. Ultrathin sections were prepared and analysed as described by Mast *et al.* (2005) (Fig. 3).

The results of the phenotypic tests are summarized in Table 2. In conclusion, we have demonstrated that the five isolates represent genetically distinct strains constituting a novel species within a novel genus, for which we propose the name *Nautella italica* gen. nov., sp. nov.

Description of *Nautella* gen. nov.

Nautella (Naut.el.la. L. n. *nauta* seaman; L. dim. suf. *-ella*; N.L. fem. n. *Nautella* the small seaman, referring to the marine habitat of this novel bacterial genus).

Gram-negative, motile rods that are moderately halophilic and strictly aerobic. Catalase- and oxidase-positive. The temperature range for growth is 4–45 °C. The type species is *Nautella italica*.

Fig. 3. Electron micrographs of cells of strain LMG 24365^T, showing (a) a polar flagellum (F) and (b) poly- β -hydroxybutyrate (P) inclusion bodies. Bars, 1 μ m.

Description of *Nautella italica* sp. nov.

Nautella italica (i.ta'li.ca. L. fem. adj. *italica* from Italy, where this species was first isolated).

Cells are Gram-negative rods (0.7–0.11 $\times$ 1.5–2.1 μ m) that are motile by means of a single polar flagellum. Poly- β -hydroxybutyrate inclusion bodies are observed (Fig. 3). Colonies are beige, round and 1–2 mm in diameter after 3 days incubation on MA at 20 °C. Colonies have a smooth surface and are convex with entire margins. Growth occurs after 2 days at 20 °C on MA, but not on trypticase soy agar, R2A agar, nutrient agar or peptone/yeast extract/glucose agar. The temperature range for growth is 4–45 °C (growth at 4 °C is weak). The optimal growth temperature is 20–28 °C. Moderately halophilic (1–5% NaCl); only weak growth occurs in the presence of 7% NaCl and no growth occurs in the absence of NaCl. The optimal salinity is 2–3% NaCl. The pH range for growth is 5.5–9.0 and the optimal pH for growth is 6.5–8.0. Catalase- and oxidase-positive. Degrades Tween 80 and aesculin (API 20NE), but not tyrosine, DNA, starch, chitin, gelatin (API 20NE) or casein. Activity was recorded for leucine arylamidase, alkaline phosphatase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and α -glucosidase. Weak enzymic activity was observed for esterase lipase (C8), valine arylamidase, α -galactosidase and β -glucosidase. Variable results were obtained for β -glucuronidase. No activity was detected for esterase (C4), *N*-acetyl- β -glucosamidase, α -mannosidase, lipase (C14), cystine arylamidase, trypsin, α -chymotrypsin, arginine hydrolase, urease or α -fucosidase. Nitrate is not reduced to nitrite (API 20NE). Indole is not produced and glucose is not fermented (API 20NE). No assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, maltose, potassium gluconate, capric acid, adipic acid, malate, citrate or phenylacetic acid (API 20NE) is detected. Susceptible to cefoxitin (30 μ g), erythromycin (15 μ g) and streptomycin (25 μ g), moderately susceptible to tetracycline (30 μ g) and resistant to gentamicin (30 μ g), vancomycin (30 μ g), trimethoprim (1.25 μ g) and clindamycin (2 μ g). The predominant fatty acids are C_{18:1} ω 7c, C_{16:0} 2-OH, 11-methyl C_{18:1} ω 7c, an unknown fatty acid with an equivalent chain-length of 11.799, C_{10:0} 3-OH, C_{18:0} 12-OH, C_{12:0} 3-OH, C_{16:0} and C_{18:0}. The DNA G + C content is 61.0 $\pm$ 0.8 mol%.

The type strain, LMG 24365^T (=CCUG 55857^T), and four additional strains (LMG 24364, R-25532, R-28717 and R-28753) were obtained from a marine electroactive biofilm in Genoa, Italy.

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Table 2. Summary of the phenotypic characteristics of strains LMG 24365^T and LMG 24364 and the type species of the genera *Leisingera*, *Ruegeria* and *Phaeobacter*

Strains: 1, LMG 24365^T and LMG 24364; 2, *P. inhibens* LMG 22475^T (Martens *et al.*, 2006); 3, *P. gallaeciensis* LMG 23163^T (Martens *et al.*, 2006; Ruiz-Ponte *et al.*, 1998); 4, *P. daeponensis* LMG 24139^T (Yoon *et al.*, 2007); 5, *Leisingera methylohalidivorans* LMG 23656^T (Schaefer *et al.*, 2002); 6, *Ruegeria atlantica* LMG 23161^T (Martens *et al.*, 2006; Rüger & Höfle, 1992; Uchino *et al.*, 1998). +, Positive; –, negative; w, weak activity; M, moderately susceptible; ND, no data available. All strains were susceptible to streptomycin (25 µg).

Characteristic	1	2	3	4	5	6
Origin	Marine EAB (Genoa, Italy)	Tidal mud flat, (Wadden Sea, Germany)	Scallop <i>Pecten maximus</i> , (Coruña, Spain)	Tidal flat, (Yellow Sea, Korea)	Tidal pool (USA)	Marine sediments (NW Atlantic Ocean)
Colony colour	Beige	Dark brown	Brown	Yellowish white	Unpigmented	Beige
Growth at:						
4 °C	w	+	–	+	+	–
40 °C	+	–	–	+	–	–
45 °C	+	–	–	–	–	–
Growth with NaCl at:						
1 %	+	+	+	+	–	–
2 %	+	+	+	+	+	–
7 %	w	+	+	+	–	+
10 %	–	–	+	–	–	–
Growth on:						
Nutrient agar	–	–*	ND	w*	–*	ND
Trypticase soy agar	–	w*	ND	w*	w*	ND
Degradation of:						
L-Tyrosine	–	+	–*	+	–*	ND
DNA	–	–*	–	–*	ND	–
Tween 80	+	+	–	–	ND	–
Aesculin	+	–*	–	–	ND	+
Gelatin	–	–	–	–	–*	–
Reduction of nitrate to nitrite	–	–	–	+	–	+
Susceptible to:						
Erythromycin (15 µg)	+	M*	+	M*	+	M*
Tetracycline (30 µg)	M	M*	ND	–	+	–*
Gentamicin (30 µg)	–	–*	+	+	M*	–*
Vancomycin (30 µg)	–	–	ND	+	–*	+
Enzymic activity						
Alkaline phosphatase	+	–*	ND	+	–	ND
Esterase (C4)	–	–*	ND	+	–	–
Esterase lipase (C8)	w	–*	ND	+	–	–
Valine arylamidase	w	–*	ND	–	w	w
Acid phosphatase	+	w*	ND	+	–	–
Naphthol-AS-BI- phosphohydrolase	+	–*	ND	–	w	–
α-Galactosidase	w	–*	ND	–	ND	ND
β-Glucuronidase	v	–*	ND	–	ND	ND
α-Glucosidase	+	w*	ND	–	–	w
β-Glucosidase	w	–*	ND	–	ND	ND
DNA G + C content (mol%)	61.0 ± 0.8	55.7	58	64.9	60.5	55–58

*Data from this study.

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