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: $C_{18:1}\omega7c$ (74.5±3.6 %), $C_{16:0}$ 2-OH (5.5±0.7 %), 11-methyl $C_{18:1}\omega7c$ (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
$C_{18:1}\omega7c$	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 $C_{18:1}\omega7c$	4.5±2.5	7.45	6.60	16.6	6.86	30.40

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 $NH_4H_2PO_4$ (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)

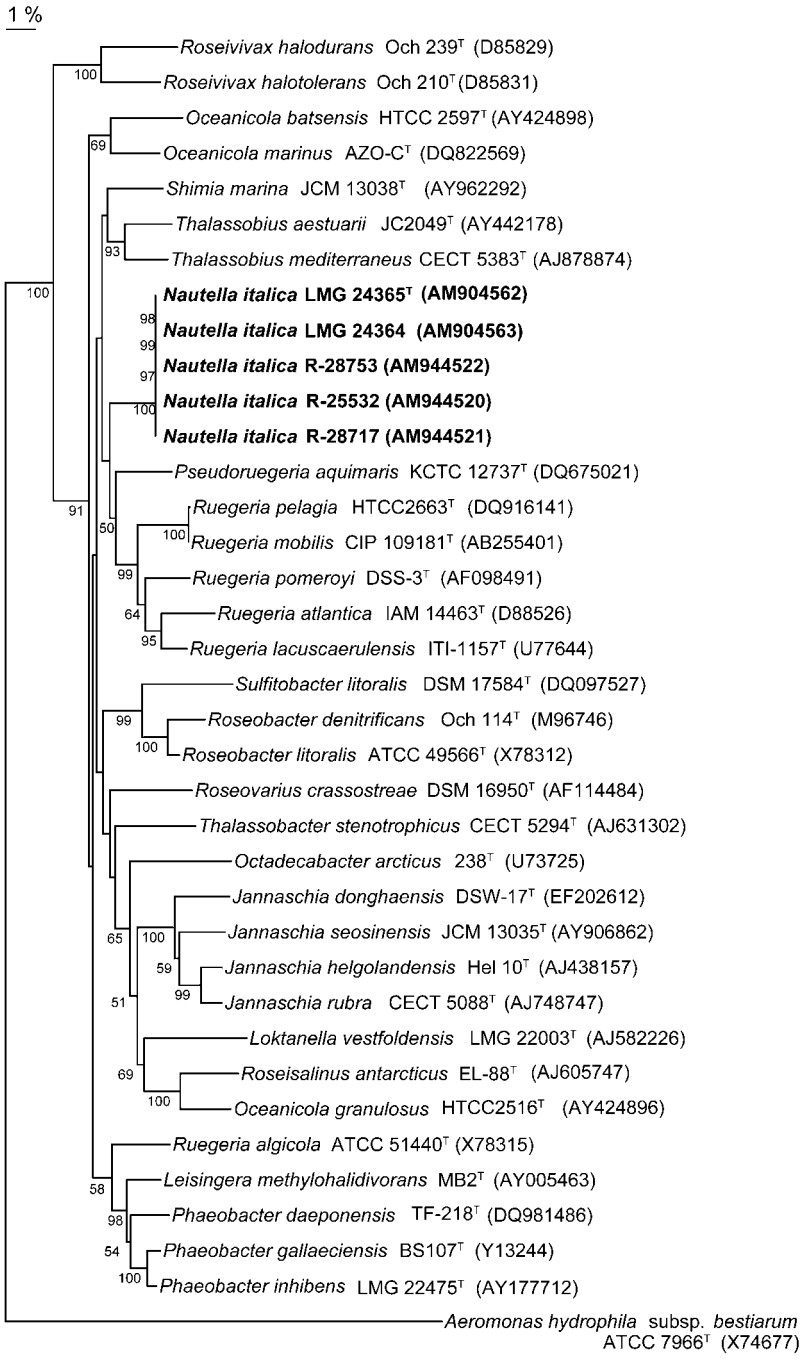

Fig. 1. Neighbour-joining dendrogram, based on 16S rRNA gene sequences, showing the phylogenetic positions of strains LMG 24365^T, LMG 24364, R-25532, R-28717 and R-28753 and their closest neighbours. Bar, 1 % sequence divergence.

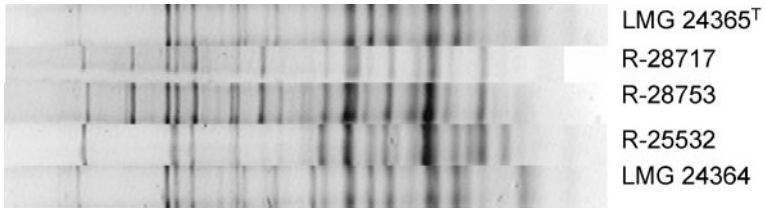

Fig. 2. BOX-PCR fingerprints of strains LMG 24365^T, LMG 24364, R-25532, R-28717 and R-28753.

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*.

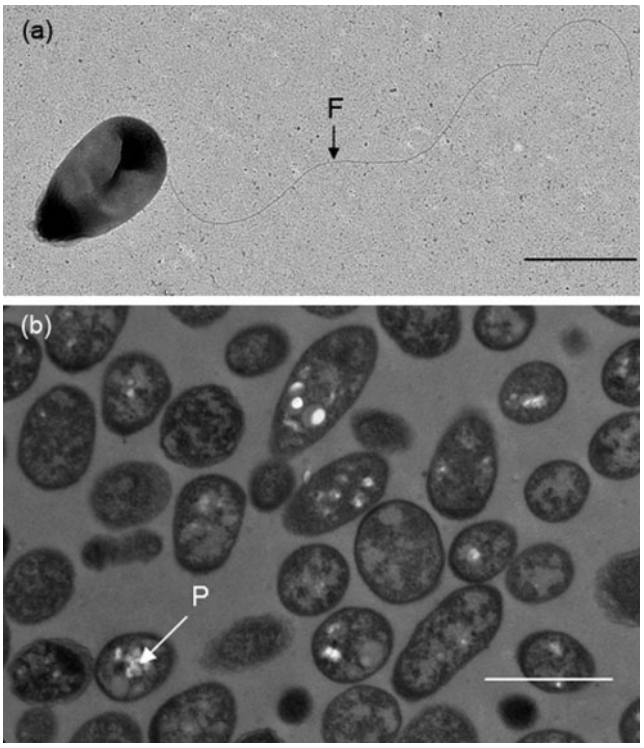

Fig. 3. Electron micrographs of cells of strain LMG 24365^T, showing (a) a polar flagellum (F) and (b) poly-β-hydroxybutyrate (P) inclusion bodies. Bars, 1 μm.

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 × 1.5–2.1 μm) 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 μg), erythromycin (15 μg) and streptomycin (25 μg), moderately susceptible to tetracycline (30 μg) and resistant to gentamicin (30 μg), vancomycin (30 μg), trimethoprim (1.25 μg) and clindamycin (2 μg). The predominant fatty acids are $C_{18:1}\omega7c$, $C_{16:0}$ 2-OH, 11-methyl $C_{18:1}\omega7c$, 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±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 *Pecten maximus*, (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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