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Phaeobacter caeruleus sp. nov., a blue-coloured, colony-forming bacterium isolated from a marine electroactive biofilm

Ilse Vandecandelaere,[1] Eveline Segaert,[1] Alfonso Mollica,[2] Marco Faimali[2] and Peter Vandamme[1]

Correspondence
Ilse Vandecandelaere
Ilse.vandecandelaere@UGent.be

[1]Laboratorium voor Microbiologie, Vakgroep Biochemie, Fysiologie en Microbiologie, Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium

[2]Instituto di Scienze Marine – Consiglio Nazionale delle Ricerche (ISMAR-CNR), Via de Marini 6, 16149 Genoa, Italy

Three isolates (LMG 24369^T, LMG 24370 and R-26156) obtained from a marine electroactive biofilm that was grown on a cathodically polarized electrode were investigated by using a polyphasic taxonomic approach. Whole-cell fatty acid methyl ester and 16S rRNA gene sequence analyses indicated that the isolates were members of the genus *Phaeobacter*, class *Alphaproteobacteria*. Genotypic and phenotypic analyses demonstrated that the three isolates represent a novel species of the genus *Phaeobacter*, for which the name *Phaeobacter caeruleus* sp. nov. is proposed. The type strain is LMG 24369^T (=CCUG 55859^T). The DNA G+C content of strain LMG 24369^T is 63.6 mol%.

The genus *Phaeobacter* was erected by Martens *et al.* (2006) to accommodate a novel species (*Phaeobacter inhibens*) plus *Phaeobacter gallaeciensis*, which was previously classified within the genus *Roseobacter*. *Phaeobacter inhibens* was isolated from the German Wadden Sea and produces an antibiotic, tropodithietic acid, which exhibits inhibitory effects against various marine bacteria and algae (Brinkhoff *et al.*, 2004; Martens *et al.*, 2007). *Phaeobacter gallaeciensis*, the type species of the genus, was obtained from larval cultures and collectors of the scallop *Pecten maximus* (Ruiz-Ponte *et al.*, 1998). Other species of the genus have since been described: *Phaeobacter daeponensis* was isolated from a tidal flat at Daepo Beach (Yellow Sea, Korea) (Yoon *et al.*, 2007) and *Phaeobacter arcticus*, a psychrophilic species, was isolated from marine sediments of the Arctic Ocean (Zhang *et al.*, 2008).

The present study describes novel *Phaeobacter*-like strains that were isolated during the course of analysis of the microbial diversity of a marine electroactive biofilm, grown on a cathodically polarized stainless-steel cathode exposed to natural seawater at the ISMAR-CNR Marine Station, Genoa, Italy (Vandecandelaere *et al.*, 2008a). The biofilm was removed from the stainless-steel cathode by sonication (Branson 3200) (90 s) in a sterile plastic tube containing 30 ml 0.85 % NaCl solution. Diluted cell suspensions (10^{-1}–10^{-6}) were inoculated on marine agar 2216 (MA; Difco) and incubated aerobically at 20 °C for several days. Pure cultures were obtained and the isolates were stored at –80 °C by using MicroBank vials (Pro-Lab Diagnostics).

Three isolates (LMG 24369^T, LMG 24370 and R-26156) were tentatively identified based on whole-cell fatty acid methyl ester (FAME) analysis (Mergaert *et al.*, 2001) as representing *Paracoccus denitrificans* (mean ± SD identification score of 0.688 ± 0.034) by comparison of the FAME profiles obtained with a commercial database (MIS; MIDI, Inc.). The major fatty acids of the three novel strains were $C_{18:1}\omega7c$ (81.5 ± 0.8 % of the total fatty acids; mean ± SD), $C_{16:0}$ (4.2 ± 0.5 %), an unknown fatty acid with an equivalent chain-length (ECL) value of 11.799 (2.9 ± 0.4 %), $C_{10:0}$ 3-OH (2.8 ± 0.4 %), $C_{16:0}$ 2-OH (2.4 ± 0.4 %), $C_{12:0}$ 3-OH (2.4 ± 0.2 %), 11-methyl $C_{18:1}\omega7c$ (1.3 ± 0.4 %) and $C_{18:0}$ (1.0 ± 0.1 %); the remaining fatty acids constituted only minor fractions (<1 %) of the total (Table 1). FAME analysis demonstrated that the isolates were related, as the standard deviations of the FAME percentages were very low (typically 0.5 %).

DNA was extracted according to Pitcher *et al.* (1989). The three isolates were investigated by repetitive DNA-PCR (rep-PCR) fingerprinting by using the BOX-A1R-primer (5′-CTACGGCAAGGCGACGCTGACG-3′) (Rademaker *et al.*, 2000; Versalovic *et al.*, 1994) to determine their

Abbreviation: FAME, fatty acid methyl ester.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains LMG 24369^T and LMG 24370 are AM943630 and AM943631, respectively.

Figures showing the BOX-PCR profiles of strains LMG 24369^T, LMG 24370 and R-26156 and the dark-blue colony colour of *Phaeobacter caeruleus* sp. nov. are available with the online version of this paper.

Table 1. Profile of major fatty acids of strains LMG 24369^T, LMG 24370 and R-26156 and their nearest phylogenetic neighbours

Taxa: 1, strains LMG 24369^T, LMG 24370 and R-26156; 2, *Phaeobacter daeponensis* TF-218^T (data from Yoon *et al.*, 2007); 3, *Phaeobacter gallaeciensis* (Martens *et al.*, 2006; Ruiz-Ponte *et al.*, 1998); 4, *Phaeobacter inhibens* (Martens *et al.*, 2006); 5, *Phaeobacter arcticus* 20188^T (Zhang *et al.*, 2008); 6, *L. methylohalidivorans* (Martens *et al.*, 2006; Schaefer *et al.*, 2002); 7, *L. aquimarina* LMG 24366^T (Vandecandelaere *et al.*, 2008b). Values are percentages of the total fatty acids. −, Other fatty acids that constitute only minor fractions of the total (<1 %).

Fatty acid	1	2	3	4	5	6	7
Straight-chain							
$C_{12:0}$	−	1.2	−	−	−	−	−
$C_{16:0}$	4.2±0.5*	8.6	6.3	5.2	9.69	3.2	3.5
$C_{18:0}$	1.0±0.1	2.4	1.3	2.0	−	−	−
Unsaturated							
$C_{18:1}\omega7c$	81.5±0.8	57.7	74.5	70.8	44.63	77.5	71.6
Hydroxy							
$C_{10:0}$ 3-OH	2.8±0.4	1.7	1.9	1.8	6.75	1.8	2.0
$C_{12:0}$ 3-OH	2.4±0.2	2.6	1.6	2.0	−	2.1	2.1
$C_{16:0}$ 2-OH	2.4±0.4	5.6	2.7	2.8	3.95	4.7	4.2
11-Methyl $C_{18:1}\omega7c$	1.3±0.4	16.6	7.8	11.8	18.10	4.7	−
Unknown fatty acid (ECL 11.799)	2.9±0.4	2.3	2.6	2.7	10.88	3.1	2.7

*Values are mean ± SD.

genetic diversity. The DNA profiles demonstrated that the isolates represent three genetically distinct strains (see Supplementary Fig. S1 in IJSEM Online).

Strains LMG 24369^T and LMG 24370 were chosen for further genotypic (i.e. 16S rRNA gene sequence analysis, determination of DNA G+C content and determination of levels of DNA–DNA relatedness) and phenotypic investigation on the basis that their BOX-PCR profiles were the most distinct.

An almost-complete 16S rRNA gene sequence was obtained for strain LMG 24369^T (1422 bp) by using the universal primers pA (5′-AGAGTTTGATCCTGGCTCAG-3′) and pH (5′-AAGGAGGTGATCCAGCCGCA-3′) (Edwards *et al.*, 1989) as described by Mergaert *et al.* (2001). A partial 16S rRNA gene sequence (522 bp) was obtained for strain LMG 24370 by using the universal primers pA and pD (5′-GTATTACCGCGGCTGCTG-3′) as described by Coenye *et al.* (1999) (data not shown). The FASTA software program was used to find the most similar sequences in public databases. These sequences were aligned by using CLUSTAL_X (Thompson *et al.*, 1997) and were edited by using BioEdit (Hall, 1999) and FORCON (Raes & Van De Peer, 1999), separately. A neighbour-joining dendrogram was constructed (Saitou & Nei, 1987) by using TREECON software (Van De Peer & De Wachter, 1994) (Fig. 1). The tree topology was confirmed by maximum-likelihood and maximum-parsimony analyses (data not shown).

Numerical analysis demonstrated that strains LMG 24369^T and LMG 24370 shared 99.1 % 16S rRNA gene sequence similarity. In contrast, relatively low levels of 16S rRNA gene sequence similarity were obtained between strain LMG 24369^T and its nearest phylogenetic neighbours, namely the type strains of *Phaeobacter daeponensis* (98.4 %), *Leisingera aquimarina* (97.8 %), *Phaeobacter gallaeciensis* (97.6 %), *Phaeobacter inhibens* (97.5 %), *Leisingera methylohalidivorans* (97.2 %) and *Phaeobacter arcticus* (95.8 %), suggesting that the three isolates may represent a novel species of the genus *Phaeobacter* (Stackebrandt & Ebers, 2006) (Fig. 1).

DNA was enzymically degraded into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was then separated by using a Water Breeze HPLC system and 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 were used as the calibration reference and control, respectively. The DNA G+C content of strain LMG 24369^T was 63.6 mol%.

DNA–DNA hybridization experiments were performed to further elucidate the taxonomic position of the novel strains and were carried out with photobiotin-labelled probes in microplate wells, as described by Ezaki *et al.* (1989), by using an 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 nearest phylogenetic neighbours of the new isolates were incorporated in the experiments, although *Phaeobacter arcticus* JCM 14644^T was excluded as it showed only 95.8 % 16S rRNA gene sequence similarity to strain LMG 24369^T.

Levels of DNA–DNA relatedness between strains LMG 24369^T and LMG 24370 were high (98±2 %; mean ± SD), indicating that the three new isolates represent a single

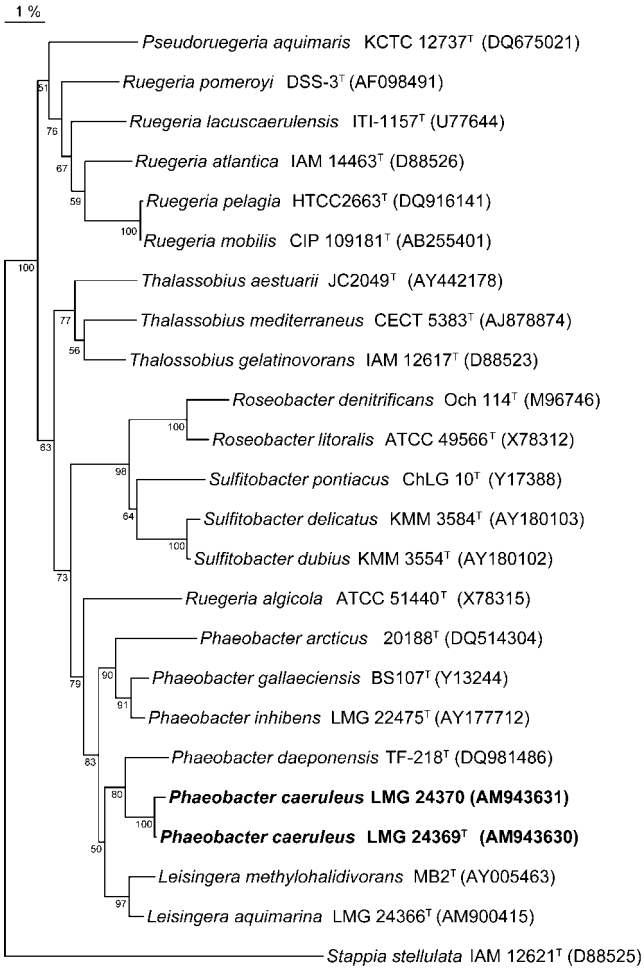

Fig. 1. Neighbour-joining dendrogram based on 16S rRNA gene sequences showing the position of strains LMG 24369^T and LMG 24370 and their nearest phylogenetic neighbours. Bootstrap values above 50 % (based on 1000 replicates) are shown at nodes. Bar, 1 % sequence divergence.

species. Levels of DNA–DNA relatedness between strain LMG 24369^T and the type strains of its closest phylogenetic neighbours, *Phaeobacter inhibens* LMG 22475^T, *Phaeobacter daeponensis* LMG 24139^T, *Phaeobacter gallaeciensis* LMG 23163^T, *Ruegeria atlantica* LMG 23161^T, *Ruegeria lacuscaerulensis* LMG 23162^T, *Ruegeria pomeroyi* LMG 23168^T, *L. aquimarina* LMG 24366^T and *L. methylohalidovorans* LMG 23656^T, were much lower than 70 % (25 ± 3, 28 ± 1, 40 ± 5, 23 ± 2, 29 ± 5, 18 ± 4, 35 ± 15 and 55 ± 1 %, respectively), indicating that strains LMG 24369^T, LMG 24370 and R-26156 represent a novel species.

The following morphological, physiological and biochemical characteristics were evaluated for strains LMG 24369^T and LMG 24370. Colony morphology was described after 4 days incubation at 20 °C on MA. Cells were tested for their Gram reaction and the presence of catalase and oxidase activity.

Growth was examined on nutrient agar (NA), trypticase soy agar (TSA), R2A (Difco) and peptone/yeast extract/glucose agar (PYG) (Tan & Rüger, 1999). Optimal salinity and optimal temperature for growth were determined by using R2A supplemented with 1–20 % NaCl, incubated for 2 weeks at 20 °C, and MA incubated at 4–45 °C for 2 weeks, respectively. The effect of pH on growth was analysed by using marine broth growth medium 2216 (Difco) with a pH ranging from 5.0 to 10.0 (at intervals of 0.5 pH units), with incubation at 20 °C for 7 days. Growth was detected by spectrometric measurements at 600 nm.

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

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

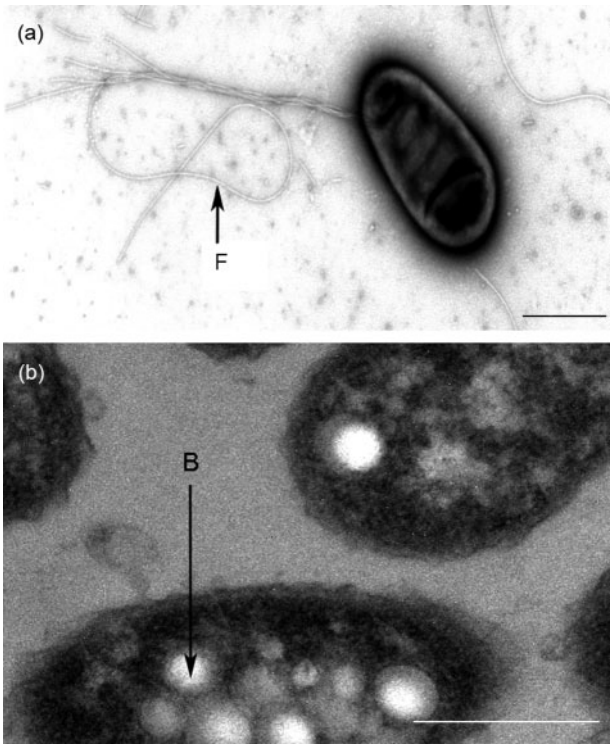

Fig. 2. Electron micrographs of cells of strain LMG 24369^T in exponential growth phase showing flagella (F) and poly-β-hydroxybutyrate inclusion bodies (B). Bars, 1 μm.

Biochemical characteristics in the commercial API ZYM and API 20NE (bioMérieux) microtest galleries were determined for strains LMG 24369^T, LMG 24370 and R-26156 according to the manufacturer's instructions. API ZYM reactions were read after 4 h incubation at 20 °C whereas API 20NE reactions were read after 48 h incubation at 20 °C.

The cell morphology of strain LMG 24369^T was determined by 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). Poly-β-hydroxybutyrate inclusion bodies were observed in cells of the novel strain (Fig. 2). Colonies were blue after 3 days incubation on MA at 20 °C (see

Table 2. Differential phenotypic characteristics between strains LMG 24369^T and LMG 24370 and their nearest phylogenetic neighbours

Strains: 1, LMG 24369^T and LMG 24370; 2, *Phaeobacter inhibens* LMG 22475^T (data from Martens *et al.*, 2006); 3, *Phaeobacter gallaeciensis* LMG 23163^T (Ruiz-Ponte *et al.*, 1998; Martens *et al.*, 2006); 4, *Phaeobacter daeponensis* LMG 24139^T (Yoon *et al.*, 2007); 5, *Phaeobacter arcticus* JCM 14644^T (Zhang *et al.*, 2008); 6, *L. methylohalidivorans* LMG 23656^T (Martens *et al.*, 2006; Schaefer *et al.*, 2002; Vandecandelaere *et al.*, 2008b); 7, *L. aquimarina* LMG 24366^T (Vandecandelaere *et al.*, 2008b). +, Positive; −, negative; W, weak activity; I, intermediately susceptible to an antibiotic; ND, no data available.

Characteristic	1*	2	3	4	5	6	7
Origin	Marine EAB, Genoa, Italy	Tidal mudflat, Germany	Scallop *Pecten maximus*, Spain	Tidal flat, Korea	Sediment, Arctic Ocean	Tidal pool, USA	Marine EAB, Genoa, Italy
Colony colour	Blue	Dark brown	Brown	Yellowish white	Yellow	Unpigmented	Dark beige–pink
Growth at:							
4 °C	+	+	−	+	+	+	+
40 °C	+	−	−	+	−	−	−
45 °C	+	−	−	−	−	−	−
Growth in NaCl at:							
1 %	W	+	+	+	−	−	W
7 %	−	+	+	+	+	−	W
10 %	−	−	+	−	−	−	−
Growth on:							
R2A	W	ND	ND	ND	ND	−	−
NA	−	−*	ND	W*	ND	−	−
TSA	−	W*	ND	W*	ND	W	−
Reduction of NO_3 to NO_2	+	−	−	+	−	−	−
Degradation of:							
Tween 80	+	+	−	−	ND	ND	−
Tyrosine	+	+	−*	+	ND	−	−
Susceptible to:							
Erythromycin (15 μg)	I	I*	+	I*	ND	+	+
Gentamicin (30 μg)	+	−*	+	+	ND	I	−
Tetracycline (30 μg)	−	−*	ND	−	ND	+	+
Vancomycin (30 μg)	−	−*	ND	+*	ND	−	−
Enzymic activity:*							
Acid phosphatase	W	W	ND	+	−	−	−
Alkaline phosphatase	W	−	ND	+	+	−	W
Esterase (C4)	−	−	ND	+	−	−	−
Esterase lipase (C8)	−	−	ND	+	+	−	W
α-Glucosidase	−	W	ND	−	−	−	−
Naphthol-AS-BI-phosphohydrolase	+	−	ND	−	−	W	W
Valine arylamidase	−	−	ND	−	−	W	−
DNA G+C content (mol%)	63.6	55.7	58.0	64.9	59.6	60.5	61.4

*Data from the present study.

Supplementary Fig S2 in IJSEM Online). The colony surface showed a concentric pattern of dark and bright blue circles. The colony colour became darker with longer incubation times.

The results of the phenotypic analyses are summarized in Table 2. Strains LMG 24369^T and LMG 24370 could be discriminated clearly from recognized species of the genus *Phaeobacter*. On the basis of phylogenetic, genomic and phenotypic data, we conclude that strains LMG 24369^T, LMG 24370 and R-26156 represent a novel species of the genus *Phaeobacter*, for which the name *Phaeobacter caeruleus* sp. nov. is proposed.

Description of *Phaeobacter caeruleus* sp. nov.

Phaeobacter caeruleus (cae.ru′le.us. L. masc. adj. *caeruleus* dark blue coloured, referring to the colony colour of the isolates).

Cells are Gram-negative rods (0.9 × 1.8 μm) with bundles of polar flagella. Inclusion bodies are observed (Fig. 2). Colonies are round, 2 mm in diameter and blue after 3 days incubation on MA at 20 °C (see Supplementary Fig S2 in IJSEM Online). The colony surface shows a concentric pattern of dark and bright blue circles. Colony colour becomes darker as incubation time extends. Growth occurs on MA after 2 days at 20 °C. Weak growth is observed on R2A after 5 days incubation at 20 °C but no growth occurs on NA, PYG or TSA. The temperature range for growth is 4–45 °C (optimal, 20–28 °C) and NaCl range for growth is 2–5 % (optimal, 3–4 %). The pH range for growth is 6.0–9.0 (optimal, pH 6.5–8.0). Catalase- and oxidase-positive. Degrades tyrosine, DNA, Tween 80 and aesculin (API 20NE) but not starch, casein or chitin. Degradation of gelatin is strain-dependent. Positive for leucine arylamidase and naphthol-AS-BI-phosphohydrolase and weakly positive for alkaline phosphatase and acid phosphatase. Negative for the presence of esterase (C4), esterase lipase (C8), valine arylamidase, α-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosamidase, α-mannosidase, lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, arginine hydrolase, urease and α-fucosidase (API ZYM). Nitrate is reduced to nitrite (API 20NE). Indole is not produced (API 20NE) and glucose is not fermented (API 20NE). Unable to assimilate D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, maltose, potassium gluconate, capric acid, adipic acid, malate, citrate or phenylacetic acid (API 20NE). Susceptible to gentamicin (30 μg), streptomycin (25 μg) and cefoxitin (30 μg) and intermediately susceptible to erythromycin (15 μg). Resistant to tetracycline (30 μg), vancomycin (30 μg), trimethoprim (1.25 μg) and clindamycin (2 μg). The major fatty acids are $C_{18:1}\omega7c$, $C_{16:0}$, an unknown fatty acid with an ECL value of 11.799, $C_{10:0}$ 3-OH, $C_{16:0}$ 2-OH, $C_{12:0}$ 3-OH, 11-methyl $C_{18:1}\omega7c$ and $C_{18:0}$; the remaining fatty acids comprise only minor fractions of the total.

The type strain, LMG 24369^T (=CCUG 55859^T), was isolated from a marine electroactive biofilm (Genoa, Italy). The DNA G+C content of strain LMG 24369^T is 63.6 mol%. Strains LMG 24370 and R-26156, isolated from the same source, are additional reference strains of the species.

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