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Nioella nitratireducens gen. nov., sp. nov., a novel member of the family Rhodobacteraceae isolated from Azorean Island

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Abstract A novel Gram-negative, non-spore forming, rod-shaped aerobic bacterium, designated SSW136^T, was isolated from a surface seawater sample collected at Espalamaca (in Faial Island), Azores. Growth was found to occur from 10 to 37 °C, pH 6.0–8.0, and with 2–11 % of NaCl. 16S rRNA gene sequence indicated that the strain SSW136^T belongs to the family Rhodobacteraceae. Strain SSW136^T exhibited 96.3, 95.9, 95.7 and 95.5 sequence similarity to the type strains *Oceanicola litoreus* M-M22^T, *Roseovarius aestuarii* SMK-122^T, *Marivita geojedonensis* DPG-138^T, and *Pseudoruegeria aquimaris* SW-255^T respectively. Neighbour-joining and maximum-parsimony phylogenetic trees based on 16S rRNA gene sequences revealed that strain SSW136^T was affiliated to the family Rhodobacteraceae and formed a separate branch. The G+C content was 63.5 mol%. The major respiratory quinone was found to be Q-10. The polar lipids of strain SSW136^T consisted of phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, two unidentified aminolipids and three unidentified phospholipids. The major fatty acids were $C_{18:1}$ ω7c (46.5 %), Cyclo-$C_{19:0}$ *ω8c* (16.0 %) and $C_{16:0}$ (12.8 %). On the basis of the morphological, genotypic, chemotaxonomic characteristics and low DNA–DNA relatedness, strain SSW136^T is proposed to represent a novel genus and novel species, *Nioella nitratireducens* gen. nov., sp. nov., in the family Rhodobacteraceae. The type strain is SSW136^T (=KCTC 32417^T = NCIM 5499^T).

The GenBank/EMBL/DDBJ accession number of the 16S rRNA gene sequence of the strain SSW136^T is KC534331.

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Introduction

The family Rhodobacteraceae, which belongs to the phylogenetic class Alphaproteobacteria and comprises of more than 100 recognized genera (http://www.bacterio.net/classifgenerafamilies.html#Rhodobacteraceae) was proposed by Garrity et al. (2005) with *Rhodobacter* as the type genus of this family. Around 55 % of the genera from the family Rhodobacteraceae consist of only one species, whereas *Paracoccus* being the largest, includes 40 species. During a comparative study on bacterial diversity from shallow water hydrothermal vent and non-vent regions of Espalamaca (Azorean Island), a strain designated SSW136^T, was isolated from the surface seawater of the non-vent region at Espalamaca. The main aim of the present investigation was to establish the precise taxonomic position of the strain SSW136^T using polyphasic approach.

Materials and methods

Isolation and maintenance of isolate

Surface seawater was collected from the Espalamaca region, Azores (38°33′N; 28°39′W) through an Indo-Portugal bilateral program during September 2010. To isolate lead (Pb) resistant bacteria, serial dilutions of the samples were spread plated on diluted nutrient agar (Peptone 1.25 g/L, Beef extract 0.75 g/L and 1.5 % Agar) amended with 1 mM $Pb(NO_3)_2$ prepared in 50 % seawater. After 3 days of incubation at 30 °C, strain SSW136^T was isolated and maintained on seawater nutrient agar (SWNA: Peptone 5.0 g/L, Beef extract 3.0 g/L, 1.5 % Agar prepared in 50 % seawater) at 4 °C for short-term maintenance and as a 15 % glycerol suspension at −80 °C for long-term maintenance.

Morphological, physiological and biochemical characterization

Gram staining was carried out using the standard Gram staining method, and counter checked by KOH test (Cerny 1978). Motility was determined by hanging drop method (Collee et al. 2008) under ×1,000 magnification and confirmed by motility agar test. Spore staining was performed using Schaeffer and Fulton's spore stain kit (K006, Himedia, Mumbai). Morphological characteristics of strain SSW136^T were observed by light microscopy (Olympus BX-60) and scanning electron microscopy (Hitachi TM3000). Growth at temperature range of 4, 10, 20, 25, 30, 37, 40 and 50 °C was tested on sea water nutrient broth (SWNB, the compositions are same as SWNA excluding agar). The pH range for growth was determined in SWNB adjusted to pH 4–10 (1 unit increment) by using acetate (pH 4–5), carbonate (pH 9–10) (Lee et al. 2012) and phosphate (pH 6–8) buffer systems (Wang et al. 2012). For pH and temperature experiments, cell densities for growth were measured at 600 nm using a spectrophotometer (Cary 300) after 2 days incubation. NaCl tolerance tests were examined with different NaCl concentrations from 0 to 15 % (1 % increment) on nutrient agar (NA) prepared with distilled water. Catalase activity was detected by the production of bubbles after the addition of a drop of 3 % H_2O_2 (Smibert and Krieg 1994). Oxidase activity was determined by the oxidation of tetramethyl-*p*-phenylenediamine. Nitrate reduction, Methyl red, Voges–Proskauer tests, determination of indole and H_2S production, were assessed using Hi25^{TM} Enterobacteriaceae identification kit (Himedia). Utilization of various carbon sources was determined using API50CH strips (bioMérieux) according to the manufacturer's instructions with inoculation medium API CHB/E amended with marine cations supplement (MCS, Farmer and Hickman-Brenner 2006). Hydrolysis of urea and DNA were determined on Urea agar base (M112, Himedia) and DNase test agar (M1041, Himedia) respectively. Xylan, starch and carboxymethyl cellulose hydrolysis were determined as per the methods provided by Khandeparker et al. (2011). Hydrolysis of casein (1 %), gelatin (1 %), Tweens 20 and 80 (1 %), Tributyrin (1 %), xanthine (0.4 %) and alginate (1 %) (Smibert and Krieg 1994) were tested on SWNA medium. API ZYM kit (bioMérieux) was used to analyse the various substrate utilization according to manufacturer's protocol.

Antibiotic susceptibility tests were performed on SWNA media using antibiotic discs (Himedia) containing the following concentrations (μg per disc

unless indicated): ampicillin (25), kanamycin (30), streptomycin (300), chloramphenicol (25), tetracycline (10), ciprofloxacin (30), ceftazidime (30), lincomycin (10), novobiocin (30), neomycin (30), vancomycin (5), amoxycillin (30), cefadroxyl (30), tobramycin (10), chlortetracycline (30), rifampicin (15), amikacin (30), norfloxacin (10), penicillin-G (2 U), polymyxin-B (50 U) and bacitracin (8 U).

Phylogenetic analyses

Genomic DNA of strain SSW136^T was isolated using DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. The 16S rRNA gene was PCR amplified using universal eubacterial primers 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) (Lane 1991). The PCR products were purified using PCR cleanup kit (Sigma) as per the method provided by the manufacturer. Sequencing of the amplified 16S rRNA gene was performed by using the automated 3130xl DNA analyzer (Applied Biosystems). The sequence alignment was carried out using Clustal W sequence alignment program (Thompson et al. 1994). The 16S rRNA gene sequences of the neighbouring taxa were obtained from the GenBank database. Phylogenetic trees were established with neighbour-joining (Saitou and Nei 1987) and maximum-parsimony (Fitch 1971) algorithms using MEGA 5 software (Tamura et al. 2011) with bootstrap values based on 1,000 replications (Felsenstein 1985).

DNA–DNA hybridization was performed between strain SSW136^T and *Oceanicola litoreus* M-M22^T by the method proposed by Ezaki et al. (1989). Hybridization was performed with five replications for each sample. The highest and lowest values obtained in each sample were excluded and the means of the remaining three values are quoted as DNA–DNA relatedness values.

Chemotaxonomy

Cell biomass of the strain SSW136^T for the analysis of polar lipids, isoprenoid quinones and DNA extraction was obtained from the cultures grown in LB broth (M1245, Himedia) prepared in 50 % seawater for 2 days at 30 °C. For the analysis of whole cell fatty acids, strain SSW136^T and *Oceanicola litoreus* M-M22^T were harvested from MA plates after cultivation for 5 days at 30 °C. Fatty acids were saponified, methylated and extracted using the standard procedure of Sherlock microbial identification system (MIDI 6.2B). The fatty acids were analysed by Gas Chromatograph (Hewlett Packard 6890) and identified using TSBA 6.0 database (Sasser 1990). Polar lipids were extracted and examined according to the procedures described by (Collins and Jones 1980). Briefly, polar lipids were extracted from freeze-dried cells and separated by two dimensional silica gel thin layer chromatography (Merck). The first direction was developed in chloroform/methanol/water (65:25:3.8, by vol.) and the second direction was developed in chloroform/methanol/acetic acid/water (40:7.5:6:1.8, by vol.). Total lipid material and specific functional groups were detected using molybdophosphoric acid (total lipids), molybdenum blue spray reagent (phosphate), ninhydrin (free amino groups), periodate-Schiff (α-glycols), and α-naphthol reagent (sugars). Isoprenoid quinones were determined as depicted by Minnikin et al. (1984). The DNA G+C content of strain SSW136^T was determined by reverse-phase HPLC of nucleosides according to Mesbah et al. (1989).

Results and discussion

Morphological observations of 2 days old colonies grown in SWNA media were observed to be punctiform, white, convex and circular in shape. Strain SSW136^T was found to be a Gram negative, non-motile, rod shaped (Supplementary Fig. S1) and non-spore forming bacterium. The temperature range for growth was determined to be 10–37 °C, with optimal growth at 30 °C. The pH range for growth was determined to be 6–8, with optimal pH 6. Growth of strain SSW136^T was observed at NaCl concentrations between 2 and 11 %, with an optimum of 8 %. Strain SSW136^T was found to be susceptible to all the tested antibiotics. Strain SSW136^T was distinguishable from the four recognized relative species (different genera) by differences in several phenotypic characteristics represented in Table 1.

The major isoprenoid quinone was determined to be Q-10. Polar lipids of the strain SSW136^T consisted of phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, two unidentified aminolipids and three unidentified phospholipids (Supplementary

Table 1 Characteristics that differentiate strain SSW136^T and closest type strains in the family Rhodobacteraceae

Characteristic	1	2	3	4	5
Colony colour†	Wh	GY	C	GY	GY
Cell size (>10 μm)	+	−	−	−	−
Motility	−	−	+	−	−
Growth at 4 °C	−	+	−	−	−
Growth at 40 °C	−	+	−	+	+
Growth at 10 % NaCl	+	−	−	−	−
Nitrate reduction	+	−	+	+	−
Hydrolysis of					
Casein	−	−	+	+	−
Gelatin	+	+	−	−	−
Tween 20	−	+	−	+	−
Tween 80	−	+	−	+	−
Aesculin	+	+	−	+	+
Urea	−	+	−	ND	−
API ZYM results*					
Lucine arylamidase	+	+	−	+	+
Acid phosphatase	+	−	−	+	+
Naphthol-AS-BI-phosphohydrolase	−	−	−	+	W
α-galactosidase	+	−	−	−	−
β-galactosidase	W	−	−	−	+
α-glucosidase	W	−	−	−	−
N-Acetyl-β-glucosaminidase	−	−	−	−	+
Major polar lipids	PC, PG, PE, PL, AL	PC, PG, PE, AL, L	ND	PC, PG, PE, AL	PG, DPG, PE, PL, GL
DNA G+C content (mol%)	63.5	67.6	58.6	59.9	67.0

Strains 1 SSW136^T; *2 Oceanicola litoreus* M-M22^T; *3 Roseovarius aestuarii* SMK-122^T; *4 Marivita geojedonensis* DPG-138^T; *5 Pseudoruegeria aquimaris* SW-255^T

Data for column 2 (except *asterisk) were taken from Park et al. 2013; Data for columns 3, 4, 5 were taken from Yoon et al. (Yoon et al. 2007, Yoon et al. 2008 and Yoon et al. 2013). Data for column 1 and asterisk (*) shown in column 2 were obtained from this study. All five taxa are positive for catalase, oxidase, alkaline phosphatase, esterase (C4) and esterase lipase (C8). All are negative for Gram-staining, starch hydrolysis, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, β-glucuronidase, β-glucosidase, α-mannosidase and α-fucosidase

+ positive, − negative, *W* weak, *ND* no data available, † *Wh* white; *C* cream; *GY* greyish yellow

Polar lipid: *AL* unidentified aminolipid, *PL* unidentified phospholipid, *GL* unidentified glycolipid, *DPG* diphosphatidylglycerol, *PC* phosphatidylcholine, *PE* phosphatidylethanolamine, *PG* phosphatidylglycerol, *L* unidentified lipid

Fig. S2). Cellular fatty acid analysis revealed that $C_{18:1}$ ω7c (46.5 %), Cyclo-$C_{19:0}$ *ω8c* (16.0 %) and $C_{16:0}$ (12.8 %) are the major components (Table 2) observed in a 5 days old culture. Whereas, in a 2 days old culture $C_{18:1}$ ω7c (62.6 %) and $C_{16:0}$ (20.0 %) are dominant (Supplementary Table 1). The proportion of Cyclo-$C_{19:0}$ *ω8c* was found to be lower (2.9 %) at 2 days and to be higher with longer incubation of 5 days (16.0 %). At the same time, the proportion of major fatty acid $C_{18:1}$ ω7c was reduced nearly 16 % as the cultures got older. It may be estimated that there is difference between the growth phases of strain SSW136^T and *Oceanicola litoreus* M-M22^T. Comparative analysis with *Oceanicola litoreus* M-M22^T revealed that Methyl-$C_{19:0}$ *ω7c*, $C_{12:0}$ 3-OH and $C_{16:0}$ 2-OH were detected only in strain SSW136^T. Further, strain SSW136^T could be distinguished from its closest relative *Oceanicola litoreus* M-M22^T by the differences in proportions of fatty acids (Table 2). The chemotaxonomic properties of strain SSW136^T i.e.,

Table 2 Cellular fatty acid compositions of strain SSW136^T and closest type strain *Oceanicola litoreus* M-M22^T

Fatty acids	1	2
$C_{16:0}$	12.8	7.1
$C_{17:0}$	–	1.7
$C_{18:0}$	2.1	11.5
$C_{10:0}$ 3-OH	3.3	4.3
$C_{12:0}$ 3-OH	3.5	–
$C_{16:0}$ 2-OH	3.7	–
$C_{18:1}\omega7c$	46.5	65.4
Methyl-$C_{18:1}\omega7c$	8.4	–
Cyclo-$C_{19:0}$ $\omega8c$	16.0	5.0
Summed feature 3	–	1.1

Strains 1 SSW136^T; *2 Oceanicola litoreus* M-M22^T. Data were obtained from this study. Fatty acids that represented ≥1.0 % are given. Summed feature 3 contained $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH

ubiquinone Q-10, the presence of phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol and the large proportion of unsaturated fatty acid $C_{18:1}$ ω7c are similar to those described for phylogenetically related genera of the order Rhodobacterales, class Alphaproteobacteria (Romanenko et al. 2011). However, strain SSW136^T differed from its closest relatives by some unidentified phospholipids and aminolipids, and the percentage variations of fatty acids (Table 2). The DNA G+C content of the strain SSW136^T was determined to be 63.5 mol% which matched the values reported for related type strains (58.6–67.6 mol%).

The nearly complete 16S rRNA gene sequence of the strain SSW136^T (1,411 nt) has highest similarity values with the type strains of the family Rhodobacteraceae. EzTaxon-e (Kim et al. 2012) showed closest similarities to *Oceanicola litoreus* M-M22^T (96.3 %) *Roseovarius aestuarii* SMK-122^T (95.9 %), *Marivita geojedonensis* DPG-138^T (95.7 %), *Pseudoruegeria aquimaris* SW-255^T (95.5 %), *Marivita cryptomonadis* CL-SK44^T (95.4 %), *Tropicimonas sediminicola* M97^T (95.4 %), *Roseibacterium elongatum* DSM 19469^T (95.4 %), *Celeribacter neptunius* H 14^T (95.3 %), *Jannaschia cystaugens* CFPB-A9^T (95.2 %), *Roseovarius tolerans* EL-172^T (95.2 %), *Roseisalinus antarcticus* EL-88^T (95.2 %) *Roseovarius nubinhibens* ISM^T (95.2 %), *Jannaschia donghaensis* DSW-17^T (95.1 %), *Roseovarius lutimaris* 112^T (95.1 %) and *Thioclava pacifica* DSM 10166^T (95.0 %). The remaining type strains of the Rhodobacteraceae members had similarity of <95 %.

Various phylogenetic tree algorithms (neighbour-joining and maximum-parsimony) based on 16S rRNA gene sequences (Fig. 1 and Supplementary Fig. S3) revealed a close phylogenetic relationship between strain SSW136^T and members of the family Rhodobacteraceae by forming a separate branch within the type species of closely related genera. In addition, strain SSW136^T exhibited DNA–DNA relatedness value of 14.3 ± 4.5 % to *Oceanicola litoreus* M-M22^T. This information confirms that the strain SSW136^T represents a novel genus and species within the *Roseobacter* lineage of the Alphaproteobacteria, for which the name *Nioella nitratireducens* gen. nov., sp. nov. is proposed.

Description of *Nioella* gen. nov.

Nioella (N.L. fem. dim. n. *Nioella*, arbitrary name after NIO, the National Institute of Oceanography, where the taxonomic study of this taxon was conducted).

Cells are Gram-negative, aerobic, non-spore forming, rod shaped bacteria that are positive for catalase and oxidase. Sodium ions are necessary for growth. Predominant quinone is Q-10. The polar lipids comprise phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, two unidentified aminolipids and three unidentified phospholipids. Major fatty acids are $C_{18:1}$ ω7c, Cyclo-$C_{19:0}$ *ω8c* and $C_{16:0}$. The DNA G+C content of the type strain of the type species is 63.5 mol%. Based on 16S rRNA gene sequence analysis, the genus represents a separate branch within the class Alphaproteobacteria, closely related to the genera *Oceanicola*, *Roseovarius*, *Marivita* and *Pseudoruegeria*. The type species is *Nioella nitratireducens*.

Description of *Nioella nitratireducens* sp. nov.

Nioella nitratireducens (N.L. n. *nitratum*, nitrate; L. v. *reducens*, bringing back to a state or condition; N.L. part. adj. *nitratireducens*, reducing nitrate).

Cells are aerobic, Gram-staining negative, non-motile and small rods-long rod shaped (0.6–0.9 × 2.4–13.5 μm). Colonies on SWNA are punctiform, white, convex, circular and opaque after incubation at 30 °C for 48 h. Optimal growth temperature is 30 °C; growth occurs between 10 and 37 °C but not at 40 °C.

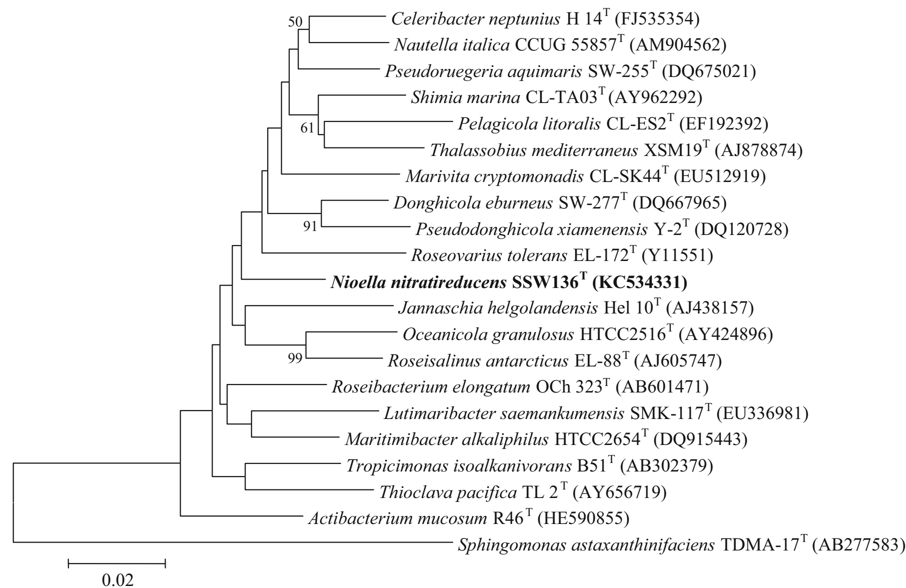

Fig. 1 Neighbour-joining phylogenetic tree showing the relationship of strain SSW136^{T} and phylogenetically related species of the family Rhodobacteraceae based on 16S rRNA gene sequences. *Numbers* at the nodes indicate percentage bootstrap values above 50 (1,000 replicates). *Bar* 0.02 substitutions per nucleotide position. *Sphingomonas astaxanthinifaciens* (AB277583) was used as an out group

Optimal pH for growth is 6.0; growth occurs at pH 6.0–8.0. Optimum NaCl concentration for growth is 8 % (w/v); growth occurs in the presence of 2–11 % of NaCl (w/v). Mg^{2+} ions are not required for growth. Catalase and oxidase are positive. Nitrate is reduced to nitrite. Gelatin and aesculin are hydrolyzed but Tweens 20, and 80, casein, starch, agar, alginate, xylan, CMC, DNA, urea and xanthine are not. Acid is produced from erythritol, L-arabinose, D-xylose, D-adonitol, D-galactose, D-glucose, D-fructose, D-mannose (weak), D-mannitol, D-sorbitol, D-cellobiose, D-maltose, D-lactose (weak), D-turanose, D-fucose and D-arabitol. In assays with the API ZYM system, alkaline phosphatase, esterase (C 4), esterase lipase (C 8), leucine arylamidase, acid phosphatase, α-galactosidase, β-galactosidase (weak) and α-glucosidase (weak) activities are present, but lipase (C 14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are absent. The main respiratory quinone is Q-10. The major fatty acids (>10 %) are $C_{18:1}$ ω7c, Cyclo-$C_{19:0}$ *ω*8*c* and $C_{16:0}$. The major polar lipids are phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, two unidentified aminolipids and three unidentified phospholipids. The DNA G+C content of the type strain is 63.5 mol%.

The GenBank/EMBL/DDBJ accession number of the 16S rRNA gene sequence of strain SSW136^{T} is KC534331. The type strain, SSW136^{T} (= KCTC 32417^{T} = NCIM 5499^{T}), was isolated from coastal surface seawater at the Espalamaca, Azores, Portugal.

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