

Nisaea denitrificans gen. nov., sp. nov. and *Nisaea nitritireducens* sp. nov., two novel members of the class *Alphaproteobacteria* from the Mediterranean Sea

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Two novel Gram-negative bacteria, designated strains DR41_21^T and DR41_18^T, were isolated from coastal, surface waters of the north-western Mediterranean Sea. The cells were motile, pleomorphic rods, 2.9 µm long and 0.9 µm wide and formed cream colonies on marine agar medium. The G + C content of the genomic DNA was 60 mol%. Phylogenetic analysis of 16S rRNA gene sequences positioned the isolates in the class *Alphaproteobacteria* within the family *Rhodospirillaceae*. The 16S rRNA gene sequence similarity of the two strains was 98.8% but DNA–DNA hybridization indicated only 55% relatedness. Strain DR41_21^T was able to denitrify and possessed *nirK* and *nosZ* genes, unlike strain DR41_18^T, which possessed only *nirK*. These isolates represent two novel species of a new genus, *Nisaea* gen. nov., for which the names *Nisaea denitrificans* sp. nov. and *Nisaea nitritireducens* sp. nov. are proposed. The type strain of *Nisaea denitrificans* is DR41_21^T (=DSM 18348^T=CIP 109265^T=OOB 129^T) and the type strain of *Nisaea nitritireducens* is DR41_18^T (=DSM 19540^T=CIP 109601^T=OOB 128^T).

Denitrification is a respiratory process in which nitrogen compounds are used as alternative electron acceptors for energy production when oxygen is limited. This stepwise reaction occurs in a wide variety of micro-organisms ranging from Archaea to Gram-positive bacteria and fungi (Tiedje, 1988). Denitrifying bacteria are more numerous

and active in the anaerobic layer of marine sediments than in the water column (Michotey *et al.*, 2000). However, activities have been reported within water-column particles (Michotey & Bonin, 1997). The eastern tropical Pacific Ocean, the eastern South Pacific Ocean and the Arabian Sea have been identified as major sites of water-column denitrification among the world's oceans (Anderson *et al.*, 1982; Bange *et al.*, 2000; Cline & Richards, 1972; Codispoti & Packard, 1980; Naqvi, 1994). This process can produce greenhouse gases, for example nitric and nitrous oxides, mainly in environments where denitrification is incomplete, such as in estuaries or in the bottom nepheloid layer (Bonin *et al.*, 2002). In this paper, we describe two novel strains, belonging to the family *Rhodospirillaceae*, isolated from the water column in the Mediterranean Sea and which are potentially involved in denitrifying processes.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of DR41_21^T and DR41_18^T are DQ665838 and DQ665839, respectively. The accession numbers for the partial sequences of the DR41_21^T *nirK* gene, the DR41_18^T *nirK* gene and the DR41_21^T *nosZ* gene are AM279765, AM279766 and AM279767, respectively.

An electron micrograph of a cell of strain DR41_21^T, the effects of pH, NaCl and temperature on the growth of the strain, the fatty acid and polar lipid compositions of strains DR41_18^T and DR41_21^T, neighbour-joining phylogenetic trees for their *nirK* and *nosZ* gene sequences and a graph of the growth of strain DR41_21^T under anaerobiosis with nitrate and nitrite or nitrate, nitrite and nitrous oxide as electron acceptors are available as supplementary material with the online version of this paper.

Samples were collected in February 2004 at the SOLA station located in the bay of Banyuls-sur-Mer (42° 29' N 3° 08' E) at a depth of 3 m. Dilution to extinction was

performed according to Schut *et al.* (1993). Seawater for the preparation of media was collected at the sampling site, filtered through a 0.22 µm polycarbonate membrane (Whatman) and then sterilized. Cultures were incubated in the dark at 20 °C for 4 weeks. Positive cultures were plated on seawater R2A agar (Difco) and incubated at 20 °C for 1 week. Colonies were picked and purified by means of at least three subcultures. Among these colonies, two isolates forming cream-coloured colonies were obtained and designated as strains DR41_21^T and DR41_18^T.

Microscopic observations (AX70; Olympus) showed that cells from isolates DR41_21^T and DR41_18^T were motile rods which were approximately 2.5 ± 0.6 µm long and 0.9 ± 0.2 µm wide. Cells were negatively stained for transmission electron microscopy (Raguénès *et al.*, 1997). Single polar flagella were observed for both strains (see Supplementary Fig. S1, available in IJSEM Online). The Ryu KOH reaction (Powers, 1995) led to immediate cell lysis, which was confirmed by light microscopy. This positive reaction indicated that both strains consisted of Gram-negative cells.

Isolates were grown in marine broth 2216 medium (MB; Difco). For the determination of salinity ranges, MB was prepared according to the composition provided by the manufacturer but with the appropriate NaCl concentration. To test the pH ranges for growth of the novel isolates, MES, PIPES, AMPSO or MOPS (Sigma) was added to MB to achieve the required pH. Cultures were incubated at 30 °C under aerobic conditions. The methods used to determine the growth parameters for strains DR41_18^T and DR41_21^T were as reported by Wery *et al.* (2001b). Growth was observed at 15–44 °C, the optimum temperature being 30 °C (see Supplementary Fig. S2 in IJSEM Online). The strains grew at NaCl concentrations ranging from 0 to 60 g l⁻¹, the optimum concentration being 20 g l⁻¹ (Supplementary Fig. S2). Growth occurred at pH 5.0–9.0; there was a clear optimum at pH 6.0. Growth decreased by 45 % at pH 5.0 relative to the value obtained at pH 6.0, whereas a relative decrease of only 20 % was observed at pH 7.0 (Supplementary Fig. S2). Both strains exhibited the same growth curves.

The ability of isolates DR41_21^T and DR41_18^T to use various substrates was investigated using Biolog GN2 MicroPlates (De Groote *et al.*, 1999): the assessments were performed according to the manufacturer's instructions except that the incubation periods were made hourly over a 24 h period. The results for both strains were as follows: positive reactions were observed for fructose, glucose, raffinose, acetate, γ -hydroxybutyrate and propionate and weakly positive reactions were noted for mannitol, rhamnose, trehalose, xylitol and glycerol.

The enzymic activities of the two isolates were investigated using the API ZYM system (bioMérieux) according to the manufacturer's instructions. Positive reactions were obtained for alkaline phosphatase, acid phosphatase and leucine arylamidase for both strains.

Fatty acid methyl ester composition determinations were carried out by the identification service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany) (see Supplementary Fig. S3 in IJSEM Online). The fatty acid compositions for isolates DR41_18^T and DR41_21^T were 18:1 ω 7c (67.6 and 69.1 %, respectively), 16:1 ω 7c (14.1 and 13.9 %, respectively), 16:0 (10.6 and 11.3 %, respectively), 16:0 3-OH (1.0 and 1.1 %, respectively), 12:0 ALDE (1.1 and 1.0 %, respectively), 15:0 (not detected and 1.0 %, respectively), 17:0 (1.0 and 1.0 %, respectively) and 18:0 3-OH (1.0 % and not detected, respectively). The large amounts of 18:1 ω 7c and, to some extent, 16:1 ω 7c and 16:0, were comparable with those found in *Azospirillum oryzae* and *Azospirillum lipoferum*. In contrast, 19:0 ω 8c cyclo, 14:0 and 18:0 3-OH were not found in strain DR41_21^T and fatty acids 16:0 3-OH and 17:0 were 4–8-fold less represented than previously found for *A. oryzae* and *A. lipoferum* (Xie & Yokota, 2005).

Analyses of respiratory quinones and polar lipids were carried out by the identification service of the DSMZ and Dr Brian Tindall (DSMZ). Both strains contained a ubiquinone (Q-10) system. The polar lipids were as follows: phosphatidylglycerol and an aminophospholipid running close to phosphatidylethanolamine (both strains), two undefined phospholipids for strain DR41_18^T and two undefined aminolipids for strain DR41_21^T (see Supplementary Fig. S4 in IJSEM Online). Comparisons of the lipid compositions and the polar lipids are presented in Table 1.

Table 1. Comparison of lipid compositions and polar lipid profiles of strains DR41_21^T and DR41_18^T

Lipid	DR41_21 ^T	DR41_18 ^T
Fatty acids		
16:0 3-OH	1.1	1.0
18:0 3-OH	–	1.0
15:0	1.0	–
16:0	11.3	10.6
17:0	1.0	1.0
18:0	–	1.0
18:1 ω 7c	69.1	67.6
16:1 ω 7c/iso-15 2-OH	13.9	14.1
10-Methyl 19:0	1.0	–
19:0 ω 8c cyclo	–	1.8
12:0 ALDE	1.0	1.1
Polar lipids		
Phosphatidylglycerol	+	+
Unknown aminophospholipid 1	+	+
Unknown aminophospholipid 2	+	+
Unknown phospholipid 1	–	+
Unknown phospholipid 2	–	+
Unknown aminolipid 1	+	–
Unknown aminolipid 2	+	–

Genomic DNA was extracted as described by Wery *et al.* (2001a). The DNA G+C content was determined by thermal denaturation using the method of Marmur & Doty (1962) and the conditions described by Raguénès *et al.* (1997). The G+C contents of the genomic DNA of strains DR41_21^T and DR41_18^T were 60.1±1.3 mol% and 60.2±1.7 mol%, respectively. The 16S rRNA genes were amplified and sequenced as described by Agogué *et al.* (2005) and the sequences were compared with those available in GenBank by using BLAST (Altschul *et al.*, 1997). The sequences were analysed as described by Urios *et al.* (2006). Strains DR41_21^T and DR41_18^T (similarity value, 98.8%) were found to be phylogenetically affiliated to the family *Rhodospirillaceae* in the class *Alphaproteobacteria* (Fig. 1). The closest relative was *A. oryzae* IAM 15130^T, having a similarity value of only 89%. DNA–DNA hybridization was performed by the DSMZ identification service: the results indicated 55±0.5% DNA–DNA relatedness.

Due to the known metabolic capabilities of the closest relatives of strains DR41_18^T and DR41_21^T, possible involvement in the nitrogen cycle was investigated. To amplify the *narG*, *nirS*, *nirK*, *nosZ*, *nifH* and *amoA* genes, the following published primers were used: na3F-narG5'R (for *narG*; Goregues *et al.*, 2005), cd3F-cd4R (for *nirS*;

Michotey *et al.*, 2000), nirKCF-nirKCR (for *nirK*; Goregues *et al.*, 2005), nosZ1211F-nosZ1897R (for *nosZ*; Rosch *et al.*, 2002), nifHF-nifHR (for *nifH*; Zehr & McReynolds, 1989), AmoA-1F-AmoA-2R (for *amoA*; Rotthauwe *et al.*, 1997). PCR amplification was carried out with 20 µl reaction mixture (20 mM Tris/HCl, 50 mM KCl, 1.5 mM MgCl₂) containing 0.2 mM each deoxyribonucleoside triphosphate, 160 pmol each primer and 0.5 U *Taq* polymerase (Promega). For PCRs, 50 ng template DNA was used in the reaction. Amplification was performed with a mini-cycler (MJ Research) for 30 cycles. PCR cycles were performed as described previously (Goregues *et al.*, 2005; Michotey *et al.*, 2000; Rosch *et al.*, 2002; Rotthauwe *et al.*, 1997; Zehr & McReynolds, 1989). Amplification products were analysed by means of electrophoresis on a 1% (w/v) agarose gel (Roche Diagnostics). Each PCR band was sequenced by MWG Biotech (Germany). The sequences were aligned with the same region of the sequence from the closest relative, using the BLASTN facility (<http://www.ncbi.nlm.nih.gov/BLAST/>) and sequences available in the GenBank database. Sequence alignment was achieved using CLUSTAL W (Thompson *et al.*, 1994). The phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis with 1000 replicates was used to check the robustness of the trees. Finally, the trees

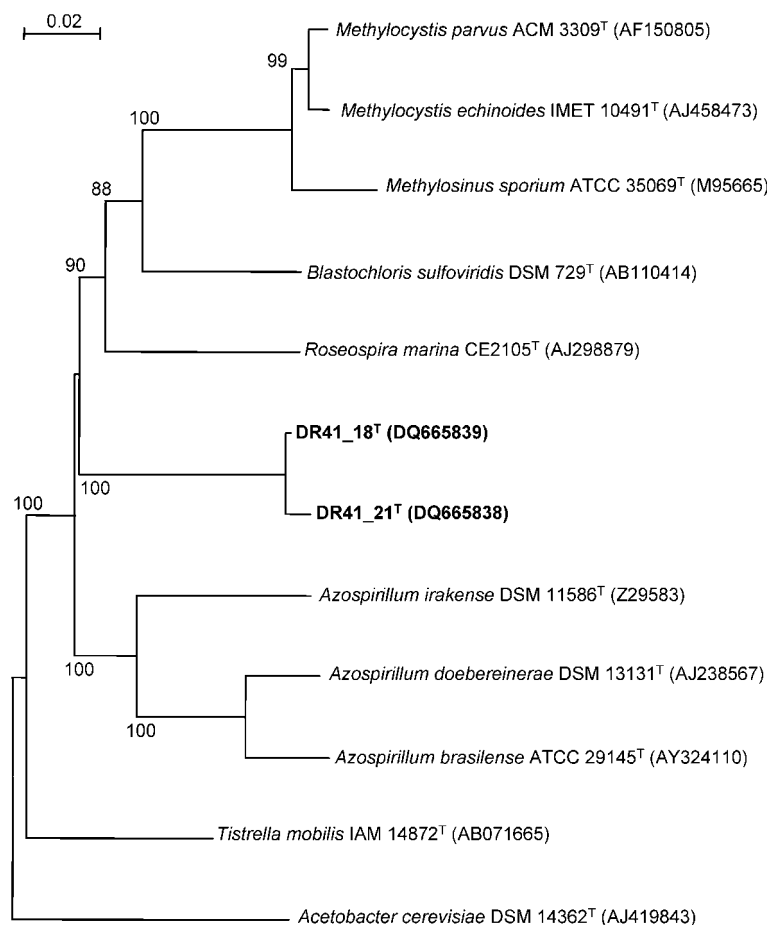


Fig. 1. Phylogenetic tree, based on 16S rRNA gene sequences, showing the positions of strains DR41_18^T and DR41_21^T. GenBank accession numbers and type strains are indicated. The tree corresponds to an unrooted tree obtained with the neighbour-joining algorithm (Kimura corrections). Bootstrap percentages (based on 1000 replications) are shown at branch points. *Acetobacter cerevisiae* was used as an outgroup. Bar, 0.02 changes per nucleotide position.

were plotted using the NJPLOT program (Perrière & Gouy, 1996).

Of the genes tested, only two relating to denitrification were successfully amplified using PCRs: *nirK*, coding for one of the nitrite reductases found in denitrifying bacteria, and *nosZ*, coding for the nitrous oxide reductase. The sequence analyses of these PCR products confirmed their affiliation to both *nirK* and *nosZ* genes. The closest *nirK* representatives in GenBank corresponded to an environmental clone (GenBank accession no. DQ337782; sequence similarity of 80.6%) and the closest representative from cultivated strains corresponded to the alpha-proteobacterium *Rhodobacter sphaeroides* (U62291; sequence similarity of 73.4%) (see Supplementary Fig. S5 in IJSEM Online). The closest *nosZ* representatives in GenBank corresponded to alphaproteobacteria: *Silicibacter pomeroyi* (CP000032; 20.36% sequence similarity); *Azospirillum brasilense* sp7 (AF361791; 24.1% sequence similarity) and *Rhodobacter* sp. *denitrificans* (F125260; 26.8% sequence similarity) (see Supplementary Fig. S6).

Anaerobic growth experiments were performed in MB supplemented with 0.15 mM nitrate and 0.035 mM nitrite, with or without 3.4 mM nitrous oxide. The kinetics of nitrate, nitrite and nitrous oxide changes were followed during growth (see Supplementary Fig. S7 in IJSEM Online). Assays for NO_3^- , NO_2^- and N_2O concentrations were carried out as described elsewhere (Goregues *et al.*, 2005; Tréguer & Le Corre, 1975). Physiological experiments confirmed the molecular results. Growth experiments revealed that strain DR41_21^T was able to utilize nitrate, nitrite and nitrous oxide as electron acceptors under anaerobiosis.

Some characteristics of strains DR41_21^T and DR41_18^T were quite similar to those of their closest relatives, e.g. pleomorphic motile cells, the pH and temperature ranges for growth and some of the major fatty acids present. Nevertheless, strains DR41_21^T and DR41_18^T were able to grow in a wider range of salinities than any related strain (Table 2) and had different fatty acid contents (Table 1).

The DNA G+C contents of strains DR41_21^T and DR41_18^T were 6–10 mol% lower than those of their relatives. The most closely related strain shared only 89% 16S rRNA gene sequence similarity. The closest *nirK* and *nosZ* gene sequences were from taxa other than *Azospirillum* species. Strains DR41_21^T and DR41_18^T differed from each other in terms of their lipid profiles, polar lipid compositions and nitrogen-cycle genes and showed a DNA–DNA relatedness of only 55%.

On the basis of the phenotypic and genotypic differences between strains DR41_21^T and DR41_18^T and their closest recognized relatives, we propose that strains DR41_21^T and DR41_18^T should be assigned to two novel species of a new genus belonging to the family *Rhodospirillaceae*. In view of its marine origin and denitrification ability, the name *Nisaea denitrificans* gen. nov., sp. nov. is proposed for strain DR41_21^T. As strain DR41_18^T possesses only *nosZ* (encoding a nitrite reductase), the name *Nisaea nitritireducens* sp. nov. is proposed.

Description of *Nisaea* gen. nov.

Nisaea (Nis'ae.a. L. fem. n. *Nisaea* nymph of the sea, referring to the marine origin).

Motile, Gram-negative rods growing optimally at 30 °C, pH 6.0 and 20 g l⁻¹ NaCl. The major fatty acids are 18:1 ω 7c (69.1%), 16:1 ω 7c (13.9%) and 16:0 (11.3%). Possess Q-10 and contain the polar lipid phosphatidylglycerol. Phylogenetically affiliated with the class *Alphaproteobacteria* within the family *Rhodospirillaceae*. The type species is *Nisaea denitrificans*.

Description of *Nisaea denitrificans* sp. nov.

Nisaea denitrificans [de.ni.tri.fi'cans. N.L. part. adj. *denitrificans* (from N.L. v. *denitrifico*) denitrifying].

The main characteristics are as those given in the genus description. In addition, cells are rods of $2.5 \pm 0.6 \mu\text{m}$ long

Table 2. Characteristics that distinguish the novel strains from related genera

Taxa: 1, *Azospirillum doebereineriae* GSF 71^T (Eckert *et al.*, 2001); *A. lipoferum* ATCC 29707^T (Sly *et al.*, 1999); *A. brasilense* ATCC 29145^T (Sly *et al.*, 1999); *Azospirillum halopraeferens* DSM 3675^T (Reinhold *et al.*, 1987); *A. oryzae* IAM 15130^T (Xie & Yokota, 2005); *Azospirillum melinis* TMCY 0552^T (Peng *et al.*, 2006); *Azospirillum canadense* LMG 23617^T (Mehnaz *et al.*, 2007); 2, *Roseospira marina* CE210^T (Guyoneaud *et al.*, 2002); 3, strains DR41_18^T and DR41_21^T.

Characteristic	1	2	3
Sampling environment	Soil	Marine environment	Water column
DNA G+C content (mol%)	66.8–70.7	65–72.3	60.1
Cell width (μm)	0.7–3.0	1.5–6.0	2–3 \times 0.9
Motility	+ or –	+	+
Growth conditions:			
Temp. range (optimum) (°C)	5–41 (30–41)	(30–35)	15–44 (30)
Salinity range (optimum) (g l ⁻¹)	0–<30 (0–2.5)	0.2–15 (0.5–7)	0–60 (20)
pH range (optimum)	3–8 (6–7.2)	5.3–8.5 (6.7–7)	5–9 (6)

and 0.9 ± 0.2 μm wide and have a single polar flagellum. Cream colonies are formed on marine agar medium. Growth occurs at 15–44 °C (optimum, 30 °C), at pH 5.0–9.0 (optimum, pH 6.0) and at salinities in the range 0–60 g l⁻¹ (optimum, 20 g l⁻¹). Positive reactions with Biolog GN2 plates are obtained for fructose, glucose, raffinose, acetate, γ -hydroxybutyrate and propionate. Positive reactions with API ZYM are obtained for alkaline phosphatase, acid phosphatase and leucine arylamidase. Oxidase- and catalase-positive. The major fatty acids are 18:1 ω 7c (69.1%), 16:1 ω 7c (13.9%), 16:0 (11.3%), 16:0 3-OH (1.1%), 12:0 ALDE (1.0%), 15:0 (1.0%) and 17:0 (1.0%).

The type strain, DR41_21^T (=DSM 18348^T=CIP 109265^T=OOB 129^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 the type strain is 60 mol%.

Description of *Nisaea nitritireducens* sp. nov.

Nisaea nitritireducens [ni.tri.ti.re.duc'ens. N.L. *nitris* -itis nitrite; L. part. adj. *reducens* leading back, bringing back and (in chemistry) converting to a different oxidation state; N.L. part. adj. *nitritireducens* reducing nitrite].

The main characteristics are as those given in the genus description. In addition, cells are rods of 2.5 ± 0.6 μm long and 0.9 ± 0.2 μm wide with a single polar flagellum. Cream colonies are formed on marine agar medium. Positive reactions with Biolog GN2 plates are obtained for fructose, glucose, raffinose, acetate, γ -hydroxybutyrate and propionate. Positive reactions with API ZYM are obtained for alkaline phosphatase, acid phosphatase and leucine arylamidase. Oxidase- and catalase-positive. The major fatty acids are 18:1 ω 7c (67.6%), 16:1 ω 7c (14.1%), 16:0 (10.6%), 16:0 3-OH (1.0%), 12:0 ALDE (1.1%) and 17:0 (1.0%).

The type strain, DR41_18^T (=DSM 19540^T=CIP 109601^T=OOB 128^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 the type strain is 60 mol%.

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