**Nereida ignava** gen. nov., sp. nov., a novel aerobic marine α-proteobacterium that is closely related to uncultured *Prionitis* (alg) gall symbionts

M. J. Pujalte,1,2 M. C. Macián,1,2 D. R. Arahal,2,3 W. Ludwig,4 K. H. Schleifer4 and E. Garay1,2,3

1 Instituto Cavanilles de Biodiversidad y Biología Evolutiva, Universitat de València, València, Spain
2,3 Departamento de Microbiología y Ecología and Colección Española de Cultivos Tipo (CECT), Facultad de Biología, Universitat de València, Campus de Burjassot, 46100 València, Spain
4 Lehrstuhl für Mikrobiologie, Technische Universität München, Am Hochanger 4, D-85350 Freising, Germany

A Gram-negative, slightly halophilic, non-pigmented, strictly aerobic, chemo-organotrophic bacterium was isolated from Mediterranean sea water off the Spanish coast near Valencia. This strain was poorly reactive, being unable to grow in most carbon sources analysed in minimal medium. However, good growth was observed when more complex media and longer incubation times were used. Phylogenetic analysis based on an almost complete 16S rRNA gene sequence placed strain 2SM4T within the *Roseobacter* group, in the vicinity of uncultured bacteria described as gall symbionts of several species of the red alga *Prionitis*. Sequence similarity values between strain 2SM4T and the closest neighbouring species were below 95.0%. The cellular fatty acid composition of the Mediterranean strain confirmed its position within the ‘Alphaproteobacteria’, sharing 18:1ω7c as the major cellular fatty acid. The phylogenetic distance from any taxon with a validly published name and also a number of distinguishing features support the designation of strain 2SM4T as representing a novel genus and species, for which the name *Nereida ignava* gen. nov., sp. nov. is proposed. The type strain is 2SM4T (≡ CECT 5292T = DSM 16309T = CIP 108404T = CCUG 49433T).

The *Roseobacter* group classified within the order ‘Rhodobacterales’ in the last release of the *Taxonomic Outline of the Prokaryotes* (Garrity et al., 2003) includes bacteria from a large number of marine environments (a Mediterranean coastal lagoon, an Atlantic Ocean central gyre, the Black Sea, the North Sea and Antarctic sea ice, among others) that have diverse types of metabolism (phototrophy, aerobic sulfite oxidation, organic sulfur compound degradation and lignin degradation) (Benlloch et al., 1995; Bowman et al., 1997; Gonzalez et al., 1997; Gosink & Staley, 1995; Wagner-Döbler et al., 2004). Thus it is clear that these bacteria play an important role in carbon, sulfur and nitrogen cycling (Gonzalez et al., 1999, 2000). The *Roseobacter* group also includes a number of environmental clones of uncertain biological significance. Several of these clones have been found consistently in geographically distant sites around the oceans of the world; to date, most remain uncultured and without cultured neighbours. The use of innovative culturing methods has allowed the cultivation of various α-proteobacteria, such as ‘Pelagibacter ubique’ of the SAR11 clade (Rappé et al., 2002).

Several bacteria belonging to the *Roseobacter* group have been described as epiphytes or symbionts of marine organisms such as bryozoans and algae (micro- and macroalgae). Nevertheless, the impact of such associations remains to be determined. In the red algal genus *Prionitis*, gall formation is known to be induced by bacteria phylogenetically related to the *Roseobacter* group (Ashen & Goff, 1996, 1998). It is well known that bacterial gall formation is species specific, and coevolution between the red algal hosts and their associated bacteria has been reported (Ashen & Goff, 2000). However, the physiological

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Nereida ignava* CECT 5292T is AJ748748.

Electron micrographs of strain 2SM4T and a table of differential oxidation profiles on Biolog GN plates are available as supplementary material in IJSEM Online.

Published online ahead of print on 4 October 2004 as DOI 10.1099/ijs.0.63442-0.
function of these galls has not been determined, and the bacteria have not been cultivated as pure cultures.

The present study reports on one bacterium, strain 2SM4^T, isolated from Mediterranean sea water, that is phylogenetically related, in terms of 16S rRNA gene sequences, to the gall symbionts. Strain 2SM4^T was obtained from sea water surrounding cultivated oysters 2–3 miles off the Mediterranean coast near Valencia (Spain). In a previous phenotypic study, it clustered in phenon 40 (Ortigosa et al., 1994) together with other unreactive marine bacteria, and remained unidentified.

Cultures were maintained on marine agar (MA; Difco) slants at room temperature and as suspensions in marine broth 2216 (MB; Difco) plus 10 % (v/v) glycerol at −80 °C. They were routinely grown at 24–26 °C on MA or MB. Most of the phenotypic characterization methods were performed as previously described (Macian et al., 2001). The organism was unable to ferment sugars under anaerobic conditions, as determined on anaerobic Hugh & Leifson O/F medium (Difco) with half-strength artificial sea water (ASW) (400 mM NaCl, 100 mM MgSO_4·7H_2O, 20 mM KCl and 20 mM CaCl_2·H_2O; Baumann & Baumann, 1981). Strain 2SM4^T exhibited catalase and oxidase activities. It did not reduce nitrate to nitrite in nitrate broth and it was also unable to grow in the denitrification medium of Baumann & Baumann, 1981]. The BMA was supplemented with 0-1 g yeast extract 1^-1 since the organism was originally reported as being unable to grow on minimal medium (Ortigosa et al., 1994). Carbohydrates were added at 2 g l^-1 whereas the remaining compounds were added at 1 g l^-1. Cells were incubated for 30 days. Positive control plates were prepared with 5 g yeast extract 1^-1 whereas negative control plates consisted of BMA plus 0-1 g yeast extract 1^-1. Growth was scored as negative when growth was equal to, or less than, that in the negative control plates. In general, strain 2SM4^T utilized few substrates and was slowly reactive, since all positive results were recorded after 14 days incubation; even then, the amount of cell mass obtained was scarce in many instances. Detailed results of the nutritional tests are listed in the species description.

The substrate-oxidation profile was obtained by using the Biolog system according to the recommendations of the manufacturer, except for the preparation of the inoculum. Strain 2SM4^T was grown for 2–3 days on MA, since it did not grow on Biolog Universal growth medium plus blood, and the inoculating fluid was half-strength ASW. The plate was incubated at 28 °C for 2 days. Positive oxidation was obtained only with maltose; borderline reactions were observed with adonitol, lactulose, d-mannitol, d-raffinose, turanose, γ-hydroxybutyric acid, L-ketovaleric acid, L-alaninamide, DL-carnitine, inosine and 2-aminoethanol. This behaviour is in agreement with the slow growth and narrow utilization pattern of strain 2SM4^T with regard to sole carbon and energy sources in BMA.

Cell morphology and motility were examined by using optical, scanning electron and transmission electron microscopy. Cells grown on MA for 2–3 days were suspended in half-strength ASW and observed on wet mounts by using phase-contrast microscopy (using Leica MBRD apparatus). In parallel, cell suspension was adsorbed onto Isopore (Millipore) membrane filters, dehydrated with a graded series of ethanol [50, 80 and 100 % (v/v) ethanol], critical-point dried with CO_2 (Autosamdry-814; Tousimis) and sputter-coated (using Bio-Rad apparatus) with a gold–palladium film to a thickness of approximately 10 nm. Samples were examined in a Hitachi S-4100 field emission scanning microscope with 7–15 mm working distance and at an acceleration voltage of 10 kV. Pictures were stored digitally and processed using EMIP 3.0. A drop of cell suspension was deposited on a grid and examined in a JEOL JEM-1010 transmission electron microscope at 60 kV after negative staining with 2 % (w/v) phosphotungstic acid. Young (48 h) pure cultures of strain 2SM4^T showed a variety of cell morphologies, ranging from coccoid cells to regular rods, tear-shaped cells with polar buds and even very elongated (filamentous) cells (Fig. 1). Cell
division by budding was observed in several cells, yielding almost coccoid forms, but binary fission could also be detected (Fig. 1). On wet mounts, cells were non-motile; flagella were not observed after cell staining with the method of Heimbrook et al. (1989) or by electron microscopy (see supplementary figure available in IJSEM Online). Bright granules were never observed in the cells, suggesting that poly-β-hydroxybutyrate and gas vesicles are not formed by strain 2SM4T.

The cellular fatty acid composition of strain 2SM4T, determined by GLC at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) by using a previously described method (Kämpfer & Kroppenstedt, 1996), confirmed the affiliation of the Mediterranean isolate to the Roseobacter group. In total, only seven fatty acids were detected. The major fatty acid was 18:1ω7c (81.4%), followed by 18:0 (6%), 16:0 (5%), 11-methyl 18:1ω7c (3.3%), 10:0 3-OH (1.9%) and 20:1ω7c (0.8%). In addition, a fatty acid (1.5%) was present that could be identified only as either 19:1ω6c or 19:0 cyclo.

The G+C content (mol%) was determined by HPLC at the DSMZ, according to the procedure of Mesbah et al. (1989). The value for strain 2SM4T was 56 mol%.

The 16S rRNA gene phylogeny of strain 2SM4T was investigated by using procedures that have been reported previously (Macián et al., 2001). Almost full-length 16S

---

**Fig. 1.** Scanning electron micrographs showing the characteristic variations in cell morphology of strain 2SM4T. Modes of cell division can also be discerned. Additional pictures are available as supplementary figures in IJSEM Online. Bars, 2.5 μm (a) and 1 μm (b, c).

---

**Fig. 2.** Maximum-likelihood tree, as implemented in the ARB program package (Ludwig et al., 2004), based on the 16S rRNA gene sequences of *N. ignava* gen. nov., sp. nov. 2SM4T and its closest relatives. For reconstruction of the tree, more than 100 almost complete sequences from type strains of species within the order ‘Rhodobacterales’ were included and later removed to simplify the view. Bootstrap values above 50% are shown at the nodes; percentages are based on 1000 resamplings. Bar, 5% sequence divergence.
rRNA gene sequences were obtained using a LiCor automated sequencer (MWG Biotech) and subsequently analysed using the ARB software package (Ludwig et al., 2004). Automated sequence alignments were inspected by eye and corrected manually using the sequence editor ARB_EDIT. According to the recommendations of Ludwig et al. (1998), alternative treeing methods (maximum parsimony, maximum likelihood and distance matrix) and data subsets were employed, using the appropriate ARB tools to test the robustness of local topologies. Comparative analyses of the sequence obtained in this study established that strain 2SM4T was phylogenetically most closely related to uncultured gall symbionts of the algal genus Prionitis, which clustered together as shown in Fig. 2. The 16S rRNA gene sequence relatedness between strain 2SM4T and the Prionitis gall symbionts ranged from 94–9 to 95–5%. With respect to established species, the most similar 16S rRNA gene sequence was that of Sulfitobacter pontiacus (95–0% sequence similarity). In addition to the large evolutionary distance that can be inferred from this value, this organism clustered in all trees examined separately from strain 2SM4T (Fig. 2). Other species belonging to the ‘Alpha-proteobacteria’ showed even lower levels of relatedness.

Phenotypic and genetic traits that distinguish isolate 2SM4T from its phylogenetic neighbours within the Roseobacter clade are summarized in Table 1. Differential oxidation profiles on Biolig GN plates are available in a supplementary table in IJSEM Online. All these results support the description of a novel genus and species in the ‘Alphaproteobacteria’, for which we propose the name *Nereida ignava* gen. nov., sp. nov.

**Description of *Nereida* gen. nov.**

*Nereida* [Ne.re.i’da. L. fem. n. Nereida (= L. fem. n. Nereis) a Nereid, a sea nymph, referring to the habitat of the bacteria].

Table 1. Phenotypic characteristics that differentiate *N. ignava* gen. nov., sp. nov. from related species


<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell morphology</td>
<td>Coccus to club-shaped rod</td>
<td>Irregular rod</td>
<td>Ovoid cells to rods</td>
<td>Ovoid cells to rods</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
<td></td>
</tr>
<tr>
<td>Pigment*</td>
<td>N</td>
<td>N</td>
<td>P</td>
<td>P</td>
<td>P†</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Motility</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flagella</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NO₃→NO₂</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>W</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Salinity range (% w/v)</td>
<td>1–4–8</td>
<td>1–7</td>
<td>ND</td>
<td>ND</td>
<td>&lt;1–4</td>
<td>&lt;1–8</td>
<td>1–8</td>
<td>1–12</td>
<td>0–2–8</td>
<td>0.5–8</td>
</tr>
<tr>
<td>Growth with only Na⁺ added</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth with Na⁺, K⁺, Mg²⁺ and Ca²⁺</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth at:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 °C</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>V</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>37 °C</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Amylase</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Gelatinase</td>
<td>NG</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Alginate</td>
<td>−</td>
<td>ND</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Tween 80 hydrolysis</td>
<td>NG</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sulfite oxidation</td>
<td>−</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Utilization of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Melibiose</td>
<td>−</td>
<td>−</td>
<td>ND</td>
<td>ND</td>
<td>W</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>−</td>
<td>W</td>
</tr>
<tr>
<td>Glycerol</td>
<td>−</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>L-2-Amino</td>
<td>−</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>L-Aspartate</td>
<td>−</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>W</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Fatty acids (%)</td>
<td>18:0</td>
<td>6</td>
<td>−</td>
<td>1:3</td>
<td>1:2</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>18:1o7c 11-methyl</td>
<td>3:3</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>G+C content (mol%)</td>
<td>56</td>
<td>60–1</td>
<td>59:6</td>
<td>56–3–58:1</td>
<td>55–56:3</td>
<td>58</td>
<td>60</td>
<td>63:7</td>
<td>59</td>
<td>62:1</td>
</tr>
</tbody>
</table>

*P, Pink; N, none (colonies may appear cream, whitish, beige or brown in colour).
†Delayed.
Gram-negative, strictly aerobic, chemo-organotrophic, slightly halophilic bacteria. Oxidase- and catalase-positive. Cells are coccoïd to elongated rods, most of them being tear-shaped. Usually 0·2–0·3 μm in width by 1–3 μm in length; non-motile. Cells show polar budding. Slightly halophilic; no growth can be obtained without seawater or the addition of combined marine salts to the medium. Mesophilic. Neither gas vesicles nor poly-β-hydroxybutyrate accumulation is observed. Does not ferment carbohydrates and is unable to reduce nitrate to nitrite or N₂. The main cellular fatty acids include 18:1ω7c, 18:0 and 16:0. The genus is affiliated to the ‘Alphaproteobacteria’, order ‘Rhodobacterales’, and so far contains only one species, Nereida ignava, which is the type species. The DNA G+C content of the type species is 56 mol%.

Description of Nereida ignava sp. nov.

Nereida ignava (ig.naˈva. L. fem. adj. ignava lazy).

In addition to the characteristics that define the genus, the species has the characteristics described below. It does not swarm or luminesce. Requires at least 1·36 % (w/v) marine salts and tolerates up to 8 % (w/v) salts, failing to grow at 9 % (w/v). Positive growth at 13 and 28 °C. No growth detected at 4, 37 or 40 °C. Does not hydrolyse casein, starch, lecithin, alginate or agar. Does not grow in gelatin, DNA or Tween 80. Produces negative results in tests for arginine dihydrolase, lysine and ornithine decarboxylase, H₂S production from thiosulfate, indole production from tryptophan and sulfite oxidation. Utilizes the following compounds as carbon and energy sources after 14 days incubation, provided that the medium is supplemented with 0·01 % (w/v) marine salts (an indication that underdetermined growth factors are required): maltose, succinate, fumarate, malate, lactate and sarcosine. In the same conditions, it grows weakly on D-glucose, D-mannose, D-mannitol, pyruvate, propionate, acetate, citrate, α-ketoglutarate, L-serine, L-ornithine, γ-aminobutyric acid and citrulline. The following substrates are not used: D-ribose, D-fructose, L-arabinose, D-xylene, D-galactose, D-trehalose, L-rhamnose, cellobiose, sucrose, lactose, D-melibiose, salicin, amygdalin, D-glucosone, D-glucuronate, D-galacturonate, N-acetylglucosamine, glycerol, D-sorbitol, meso-inositol, glycercate, cis-aconitate, DL-β-hydroxybutyrate, p-hydroxybenzoate, D-saccharic acid, glycine, L-leucine, L-arginine, L-alanine, L-glutamate, L-threonine, L-aspartate, L-lysine, L-tyrosine, L-histidine and putrescine.

The type strain, 2SM4T (= CECT 5292T = DSM 16309T = CIP 108404T = CCUG 49433T), was isolated from Mediterranean sea water off Valencia (Spain).

Acknowledgements

Thanks are due to J. M. López, Colección Española de Cultivos Tipo (CECT), for his assistance with bioinformatic issues. This work was partially supported by the Deutsche Forschungsgemeinschaft and by project AGL-2002-04075-C02-C02 of the Spanish Ministerio de Ciencia y Tecnología. D. R. A. has a contract with the Universitat de Valencia under the Ramón y Cajal program (Ministerio de Ciencia y Tecnología).

References


M. J. Pujalte and others