Thalassobius mediterraneus gen. nov., sp. nov., and reclassification of Ruegeria gelatinovorans as Thalassobius gelatinovorus comb. nov.

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A Gram-negative, slightly halophilic, non-pigmented, strictly aerobic, chemo-organotrophic bacterium was isolated from sea water off the western Mediterranean coast near Valencia (Spain). This strain was able to grow on several organic acids and amino acids added to a minimal medium as carbon sources, but used few carbohydrates or yielded slight growth when sugars were used. Phylogenetic analysis based on an almost complete 16S rRNA gene sequence revealed that strain XSM19T was a member of the Roseobacter group within the ‘Alphaproteobacteria’, with its closest phylogenetic neighbour being Ruegeria gelatinovorans (97·6 % sequence similarity). Following a polyphasic approach, it was concluded that strain XSM19T represents a new genus and novel species, for which the name Thalassobius mediterraneus sp. nov. is proposed. The type strain is XSM19T (= CECT 5383T = CIP 108400T = CCUG 49438T). It is also proposed that Ruegeria gelatinovorans (Rüger & Höfle 1992) Uchino et al. 1999 is reclassified as Thalassobius gelatinovorus comb. nov.

The Roseobacter group, classified within the order ‘Rhodobacterales’ in the last release of the Taxonomic Outline of the Prokaryotes (Garrity et al., 2004), includes 33 genera and almost 100 species from a large number of marine environments (Mediterranean coastal lagoon, Atlantic Ocean central gyre, Black Sea, North Sea and Antarctic sea ice among others). Novel taxa are continuously arising within this group. Thus, since that review of names, 16 proposals of novel species and nine new genera of Proteobacteria have been published in the International Journal of Systematic and Evolutionary Microbiology (Abraham et al., 2004; Adachi et al., 2004; Cho & Giovanoni, 2004; Lau et al., 2004; Lee et al., 2004; Martinez-Cánovas et al., 2004; Shieh et al., 2004; Van Trappen et al., 2004; Wagner-Döbler et al., 2004; Labrenz et al., 2005; Macián et al., 2005a, b; Pujalte et al., 2005) and a few others are currently in press. A concentration of papers and independent studies on the same bacterial group implies that exhaustive care has to be taken with the criteria for proposing novel taxa and their arrangement into higher rank units. This paper deals with the description of a new genus, Thalassobius, with Ruegeria gelatinovorans as the type species and the reassignment of Ruegeria gelatinovorans to the new genus.

In a previous study (Ortigosa et al., 1994), our group isolated aerobic, Gram-negative bacteria associated with oysters (Ostrea edulis) and the surrounding sea water 2–3 miles off the western Mediterranean coast near Valencia, Spain. After characterization and numerical taxonomic analysis, 46 phena were defined but most of them remained unidentified. Now, we present the taxonomic characterization of one of those isolates, strain XSM19T, which was included in phenon 23 of the previous study and whose closest phylogenetic neighbour is Ruegeria gelatinovorans.

Cultures of the Mediterranean isolate XSM19T as well as R. gelatinovorans CECT 4357T were maintained on marine agar (MA; Difco) slants at room temperature and as suspensions in marine broth 2216 (MB; Difco) plus 10 % glycerol at −80 °C. They were routinely grown at 24–26 °C on MA or MB and thoroughly investigated using previously published methods for phenotypic characterization (Macián et al., 2001, 2005a). For some tests, other reference strains were also included: Ruegeria atlantica CECT 4292T, the type species of the genus Ruegeria, and Silicibacter luscaerulensis CECT 5319T, a very close neighbour of R. atlantica in terms of 16S rRNA gene sequence (98·3 %).

Cell morphology and motility were examined by optical microscopy. Cells of strain XSM19T are coccolid to rod-shaped, 0·5–0·8 µm wide, 0·5–2·0 µm long and form chains...
of two to three cells, but not rosettes. Cells were not motile when observed on wet mounts. Bacteria grown on MA for 2 days were used for electron microscopy. Samples were examined by Servicio Central de Soporte a la Investigación Experimental (SCSIE; University of Valencia) using a transmission electron microscope (JEM-1010; JEOL) at 60 kV after negative staining with 2% (w/v) phosphotungstic acid at an appropriate pH. Binary division was observed on transmission electron micrographs, but flagella and rosette formation were not observed. Microscopic observations of strain XSM19T showed bright inclusions inside the cells and so polyhydroxybutyrate (PHB) production was investigated by using Nile blue A staining and fluorescence microscopy (Smibert & Krieg, 1994) with cells grown on minimal medium and on MA for 5 days. *Escherichia coli* CECT 101 was used as a negative control. Strain XSM19T, *R. atlantica* CECT 4292 and *S. lacuscaerulensis* CECT 5319 showed the typical bright orange fluorescence of PHB on cells grown on basal medium agar (BMA) with glycerol. *R. gelatinovorans* CECT 4357 showed fluorescence on BMA-fructose-grown cells. In all cases very few cells showed fluorescence when MA was used as the culture medium. We conclude that strain XSM19T and the other *Ruegeria* species studied here are able to produce PHB. Moreover, *S. lacuscaerulensis* is also able to produce and accumulate PHB as shown clearly by the presence of bright orange fluorescence in almost all stained cells. This result is in agreement with the interpretation of Gonzalez et al. (1999) regarding the white inclusion bodies observed in *Silicibacter pomeroyi* and in micrographs of *S. lacuscaerulensis* (Petursdottir & Kristjansson, 1997) and originally believed to be gas vacuoles by the latter authors.

Cells of strain XSM19T grew on MA as unpigmented, regular, opaque colonies that did not swarm or luminesce. Strain XSM19T required sea-water-based media for growth and was unable to grow in salt tolerance agar [STA; 1% (w/v) tryptone, 0.3% (w/v) yeast extract and 1.5% (w/v) agar] with the addition of Na⁺ and K⁺ ions, but showed good growth when divalent ions (Mg²⁺ or Ca²⁺) were present in the medium. The salinity range supporting growth on diluted MA or in MA supplemented with NaCl, as reported in Macián et al. (2005a) was between 1-4 and 8% (w/v) total salts. Thus strain XSM19T is a slight halophile in terms of ionic requirements. Using the same methodology, *R. gelatinovorans* and *S. lacuscaerulensis* were able to grow even with only 1.0% (w/v) total salts, whereas *R. atlantica* only grew when the total salts concentration was greater than 1.8% (w/v). Strain XSM19T did not reduce nitrate to nitrite in nitrate broth and was also unable to grow in Baumann’s denitrification medium (Baumann & Baumann, 1981). *R. atlantica, R. gelatinovorans* and *S. lacuscaerulensis* were originally described as being able to reduce nitrate to nitrite, but other species in these genera such as *Ruegeria algicola* and *S. pomeroyi* were described as unable to reduce nitrate (Rüger & Höfle, 1992; Lafay et al., 1995; Petursdottir & Kristjansson, 1997; Gonzalez et al., 2003). Strain XSM19T was mesophilic, growing from 15 to 37 °C, but not at 4 or 40 °C on solid medium (MA). In contrast, *R. gelatinovorans* grew at 4 and 40 °C and *S. lacuscaerulensis* was unable to grow at 4 and 15 °C.

Isolate XSM19T was oxidase- and catalase-positive and negative for the following enzyme activities: arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, indole production from tryptophan, H₂S production from thiourea and sulfite oxidation. Strain XSM19T did not grow on Tween 80 and no hydrolytic activities were detected on the following substrates: casein, gelatin, starch, alginate and lecinthin. These tests were performed in media supplemented with marine salts [MA or half-strength artificial sea water (ASW)]. In contrast, *R. gelatinovorans* was reported to hydrolyse gelatin and weakly hydrolyse Tween 80, but was negative for casein, starch, alginate and lecinthin. Other enzyme activities of strain XSM19T and reference strains were tested by using API ZYM strips (bioMerieux). Strips were inoculated with a cell suspension prepared in half-strength ASW to the recommended turbidity of MacFarland 6. Results were recorded after 4 h incubation at 26°C. Strain XSM19T showed only three activities, esterase (C4), esterase lipase (C8) and leucine arylamidase, whereas the *R. atlantica* and *R. gelatinovorans* reference strains showed identical profiles with eight positive results: alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, β-galactosidase and α-glucosidase. The same profile was obtained for *S. lacuscaerulensis*, except that the result for esterase lipase was negative.

The ability to use several compounds as the sole carbon and energy source was analysed on BMA [50 mM Tris/HCl, pH 7.5, 19 mM NH₄Cl, 0.33 mM K₂HPO₄, 33 mM K₂HPO₄, 0.1 mM FeSO₄·7H₂O and 1-3% (w/v) purified agar (Oxoid) on half-strength ASW; Baumann & Baumann, 1981]. BMA was supplemented with 0.1 g yeast extract l⁻¹. Carbohydrates were added at 2 g l⁻¹ while the remaining compounds were added at 1 g l⁻¹. Incubation was prolonged for 14 days. Positive control plates were prepared with 5 g yeast extract l⁻¹ while negative control plates consisted of BMA plus 0.1 g of yeast extract l⁻¹. Growth was scored as negative when it was equal to or less than that found in the negative control plates. The detailed results of the nutritional tests are listed in the species description section and compared with the reference strains in Table 1. In general, strain XSM19T grew best and fastest on organic acids and some amino acids rather than on carbohydrates, with the exception of glycerol, which yielded abundant and rapid growth. A similar profile was observed in our laboratory for *R. gelatinovorans*, whereas *R. atlantica* was unable to utilize any amino acid.

In parallel, the substrate oxidation profile was performed using the Biolog system (GN2 plates) according to the manufacturer’s recommendations, apart from the preparations of the inoculum. Strains were grown for 2 days on MA and the inoculating fluid was half-strength ASW. Plates were incubated at 26 °C for 2 days. In general, the response of strain XSM19T was very poor, with only one positive result after 48 h of incubation. In contrast, *R. gelatinovorans* CECT
Table 1. Phenotypic characteristics that differentiate Thalassobius mediterraneus gen. nov., sp. nov. from Thalassobius gelatinovorus comb. nov. and other phylogenetic neighbours

Taxa: 1, Thalassobius mediterraneus XSM19^T; 2, Thalassobius gelatinovorus CECT 4357^T; 3, Ruegeria atlantica CECT 4292^T; 4, Ruegeria algicola ATCC 51440^T (data from Lafay et al., 1995); 5, Silicibacter lacuscarludensis CECT 5319^T; 6, Silicibacter pomeroyi ATCC 700808^T (data from Gonzalez et al., 2003); 7, Oceanicola batsensis ATCC BAA-863^T (data from Cho & Giovannoni, 2004).

+ Positive; w, weakly positive; (+), positive after 14 days incubation; −, negative; NG, no growth; ND, not determined. Data obtained in this study unless stated otherwise.

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<td>64</td>
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*aData obtained from other studies are indicated by: a, Uchino et al., 1998; b, Rüger & Höfe, 1992; c, Labrenz et al., 1998; d, Petursdottir & Kristjansson, 1997.

4357^T yielded eight positive results: Tween 40, Tween 80, D-fructose, β-hydroxybutyric acid, DL-lactic acid, L-proline, inosine and 2,3-butanediol. It is noteworthy that substrates yielding good growth of strain XSM19^T when tested as sole carbon and energy sources (see species description) are apparently not oxidized in Biolog plates. We have observed this phenomenon in other strains of marine origin characterized in our laboratory. Therefore researchers should take care when considering results obtained using different methodologies even though the substrates examined are the same.

The cellular fatty acid content of strains XSM19^T and R. gelatinovorans CECT 4357^T was determined by GLC at the DSMZ using a previously described method (Kämpfer & Kroppenstedt, 1996). In total, only six fatty acids with percentages above 1 % were detected for strain XSM19^T. The major fatty acid was 18:1ω7c (84.6 %) followed by 12:1 3-OH (3.97 %), ECL 11:798 (3.60 %), 16:0 (3.04 %), 10:0 3-OH (1.9 %) and 18:0 (1.1 %). The major fatty acid of R. gelatinovorans CECT 4357^T was 18:1ω7c (68.8 %) followed by 18:1ω7c 11-methyl (7.5 %), 12:0 3-OH (5.8 %), 10:0 (3.5 %), 12:0 (3.1 %), 16:0 (2.9 %), 19:0 cyclo (2.3 %), 18:0 (1.9 %) and 10:0 3-OH (1.5 %). These results confirm the affiliation of XSM19^T to the Roseobacter group and improve the description of R. gelatinovorans (Uchino et al., 1998).

DNA G + C content was determined by HPLC at the DSMZ according to Mesbah et al. (1989). Strain XSM19^T has a DNA G + C content of 57 mol %, a value close to that of R. gelatinovorans (59 mol %). The DNA G + C content of the closest phylogenetic neighbours of these organisms is within the range 55–68 mol % (Table 1).

Isolation of genomic DNA, amplification of almost full-length 16S rRNA gene fragments and sequencing of PCR products were performed as previously described (Macián et al., 2001). Subsequent sequence analysis was conducted using the ARB program package (Ludwig et al., 2004). Following 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 XSM19^T is phylogenetically most closely related to R. gelatinovorans (97.6 % sequence similarity) (Fig. 1). In the case of R. algicola and Oceanicola batsensis, lower percentages were obtained (96.6 and 95.3 %, respectively), and their position in the tree was highly unstable when different treeing methods were applied. A similar pattern was found with the group of sequences formed by R. atlantica and the two Silicibacter species: all three always clustered together but their position relative to other sequences and clusters of sequences varied. The sequence similarity between these strains and XSM19^T was as low as 94–94.2 %. The conclusion of this analysis is clear: strain XSM19^T can be considered to represent a novel species if its DNA–DNA relatedness value to R. gelatinovorans is less than 70 %. Any other species with a validly published name can be considered to be unrelated to XSM19^T, in terms of phylogenetic distance, at the species level. Interspecific hybridizations were performed by the chemiluminescent-HA method, a modification by Ziemke et al. (1998) of the method of Lind & Ursing (1986). Genomic DNA of strain XSM19^T was double-labelled with DIG-11-dUTP and biotin-16-dUTP (Roche) using a nick-translation kit (Roche). DNA–DNA hybridizations were performed with replicates and the calculated mean value was 23 %, indicating that the strains are not related at the species level.

All data reported here confirm that strain XSM19^T represents a novel taxon in the ‘Rhodobacterales’, at the species level; however, the genus delimitation in the Roseobacter group is complicated. From Fig. 1, or any other phylogenetic tree derived from 16S rRNA gene sequence data from Rhodobacterales. At the species level, Thalassobius mediterraneus and Thalassobius gelatinovorus are not related at the species level.
these organisms, it is very clear that the branching arrangement of the sequences does not match the current genus circumscriptions. In addition, if the topology of trees obtained by applying different methodologies is compared, or if resampling techniques (bootstrap values) are used, it becomes evident that the branching order itself is not stable, except for a few associations. Moreover, the affiliation of some of the species involved might need to be reconsidered. The genus *Ruegeria* was originally described by Uchino et al. (1998) to accommodate previously described marine *Agrobacterium* species and their relatives. The study based its conclusions on 16S rRNA gene sequence analysis and reclassified *Agrobacterium atlanticum*, *Agrobacterium gelatinovorum*, *Agrobacterium meteori* and *Roseobacter algicola* in a new genus, *Ruegeria*, located in the vicinity of the genus *Roseobacter*. The genus *Ruegeria* as presently defined is not a monophyletic group. The type species, *R. atlantica*, clusters with another genus, *Silicibacter*, being only distantly related to the other two species of *Ruegeria* (*R. gelatinovorans* and *R. algicola*). In our study, *R. gelatinovorans* consistently clustered with the sequence of strain XSM19\(^T\) but not to those of *R. algicola* or *R. atlantica*. Given these results, the grouping of these species in a single genus may be considered to be unsatisfactory. It is evident from the phylogenetic analysis and the 16S rRNA gene sequence similarity to *R. atlantica* (94.1 %) and *S. lacuscaerulensis* (94.0 %) that strain XSM19\(^T\) represents a separate taxon amongst the *Roseobacter* group. In addition to the large evolutionary distance that can be inferred from these values, in all trees examined these organisms clustered separately from strain XSM19\(^T\) (Fig. 1).

Taking all of the results presented here into consideration, we conclude that the most appropriate assignment for the novel species represented by isolate XSM19\(^T\) is to a new genus, for which the name *Thalassobius* gen. nov. is proposed. The type species of the new genus, *Thalassobius mediterraneus*, consistently clustered with *R. gelatinovorans* in the phylogenetic analyses. They share a high degree of 16S rRNA gene sequence similarity as well as certain phenotypic traits. In view of the data presented here, we propose that *R. gelatinovorans* also be placed in the new genus as *Thalassobius gelatinovorus* comb. nov.

**Description of Thalassobius gen. nov.**

*Thalassobius* (Tha.las.so’bi.us. Gr. fem. n. thalassa the sea; Gr. masc. n. bios life; N.L. masc. n. Thalassobius life form of the sea).

Gram-negative, strictly aerobic, chemo-organotrophic bacteria. Oxidase- and catalase-positive. Cells are cocccid to rod-shaped. Cells divide by binary fission. Gas vesicles not observed. Accumulate PHB. Slightly halophilic; no growth can be obtained without sea water or the addition of combined marine salts to the medium. Mesophilic. Do not ferment carbohydrates. Preferred carbon sources are organic acids and amino acids. DNA G+C content ranges from 57 to 59 mol%. The genus is affiliated to the ‘Alphaproteobacteria’, order ‘Rhodobacterales’ and currently contains two species. The type species is *Thalassobius mediterraneus*.

**Description of Thalassobius mediterraneus** sp. nov.

*Thalassobius mediterraneus* (me.di.ter.ra’ne.us. L. masc. adj. mediterraneus pertaining to the Mediterranean Sea).
Displays the following properties in addition to those given in the genus description. Cells are usually 0.5–0.8 μm wide by 0.5–2 μm long. Unable to reduce nitrate to nitrite or gas. Requires at least 1.4% (w/v) marine salts and tolerates up to 8% (w/v) salts but fails to grow at 9% (w/v). Positive growth from 13 to 37°C. No growth detected at 4 or 40°C. No hydrolysis of casein, gelatin, starch, lecithin, alginate or agar. It does not grow on Tween 80. Tests for arginine dihydrolase, lysine and ornithine decarboxylase, H$_2$S production from thiourea, indole production from tryptophan and sulhide are negative. Enzyme activity in API ZYM is positive for esterase (C4), esterase lipase (C8) and leucine arylamidase. Provided that the medium is supplemented with 0.01% yeast extract (an indication that undetermined growth factors are required), strain XSM19$^T$ utilizes the following compounds as carbon and energy sources after 7 days incubation: D-ribose, D-glucose, salicin, glycerol, propionate, pyruvate, citrate, succinate, fumarate, malate, acetate, lactate, DL-β-hydroxybutyrate, L-glutamate, L-alanine, γ-aminobutyric acid and L-ornithine. Slight growth is detected after 14 days incubation on D-fructose, maltose, D-sucrose, D-mannitol, D-sorbitol, myo-inositol, glycerate, 2-oxoglutarate, L-leucine, L-serine, L-threonine, L-arginine, L-asparginate and putrescine. Growth is negative on D-arabinose, D-xylene, cellobiose, lactose, melibiose, hippurate, lactate, malate, acetate, lactate, DL-β-hydroxybutyrate, L-leucine, DL-serine, L-threonine, L-arginine, L-tyrosine, L-glutamate, L-alanine, γ-aminobutyric acid, L-ornithine, citrulline and L-aspartate. It grows slightly on the following compounds or after 14 days incubation: glycerate, 2-oxoglutarate, glycin, L-histidine and putrescine. The following substrates are not used: trehalose, rhhamnose, lactose, amygdalin, D-glucuronate, D-glucuronate and N-acetylglucosamine. Strain CECT 4357$^T$ contains the following fatty acids: 18:1$\omega_7c$, 18:1$\omega_7c$ 11-methyl, 12:0 3-ΟΗ, 10:0, 12:0, 16:0, 19:0 cyclo, 18:0, 10:0 3-ΟΗ, 17:0 and ECL 11:798.

**Description of Thalassobius gelatinovorus comb. nov.**


The data obtained during this work using strain CECT 4357$^T$ enable the description of the species given by Rüger & Höfe (1992) and Uchino et al. (1998) to be completed. *T. gelatinovorus* accumulates PHB. In API ZYM, enzyme activities are positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, β-galactosidase and α-glucosidase. Utilizes the following compounds as carbon and energy sources after 7 days incubation on BMA supplemented with 0.01% yeast extract: D-ribose, D-glucose, D-fructose, maltose, D-sucrose, melibiose, salicin, glycerol, D-mannitol, D-sorbitol, myo-inositol, propionate, pyruvate, citrate, succinate, fumarate, malate, acetate, lactate, DL-β-hydroxybutyrate, L-leucine, DL-serine, L-threonine, L-arginine, L-tyrosine, L-glutamate, L-alanine, γ-aminobutyric acid, L-ornithine, citrulline and L-aspartate. It grows slightly on the following compounds or after 14 days incubation: glycerate, 2-oxoglutarate, glycin, L-histidine and putrescine. The following substrates are not used: trehalose, rhhamnose, lactose, amygdalin, D-glucuronate, D-glucuronate and N-acetylglucosamine. Strain CECT 4357$^T$ contains the following fatty acids: 18:1$\omega_7c$, 18:1$\omega_7c$ 11-methyl, 12:0 3-ΟΗ, 10:0, 12:0, 16:0, 19:0 cyclo, 18:0, 10:0 3-ΟΗ, 17:0 and ECL 11:798.

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**References**


new marine bacterium isolated from the phycosphere of the toxin-


