Silicibacter pomeroyi sp. nov. and Roseovarius nubinhibens sp. nov., dimethylsulfoniopropionate-demethylation bacteria from marine environments

José M. González,¹ Joseph S. Covert,² William B. Whitman,³ James R. Henriksen,³ Frank Mayer,⁴ Birgit Scharf,⁵ Rüdiger Schmitt,⁵ Alison Buchan,² Jed A. Fuhrman,⁶ Ronald P. Kiene⁷ and Mary Ann Moran²

¹Departamento de Microbiología y Biología Celular, Facultad de Farmacia, Universidad de La Laguna, 38071 La Laguna, Tenerife, Spain
²,³Departments of Marine Sciences² and Microbiology³, University of Georgia, Athens, GA 30602, USA
⁴Institut für Mikrobiologie, Universität Göttingen, 37077 Göttingen, Germany
⁵Institut für Biochemie, Genetik und Mikrobiologie, Universität Regensburg, D-93040 Regensburg, Germany
⁶Department of Biological Sciences, University of Southern California, Los Angeles, CA 90089, USA
⁷Department of Marine Sciences, University of South Alabama, Mobile, AL 36688, USA

Three Gram-negative, rod-shaped, aerobic bacteria that were capable of degrading dimethylsulfoniopropionate (DMSP) were isolated from marine waters. These isolates (DSS-3⁷, DSS-10 and ISM⁷) exhibited the ability to demethylate and cleave DMSP, as well as to degrade other sulfur compounds related to DMSP that are cycled in marine environments. Intracellular poly-β-hydroxybutyrate inclusions, surface blebs and one polar, complex flagellum that rotated exclusively in the clockwise direction were observed for DSS-3⁷. The outer membrane of ISM⁷ was separated from the cytoplasm at the poles in a toga-like morphology. The primary fatty acid in both strains was C₁₈ : 1₉₇c. DNA G+C contents for the isolates were 68.0 ± 0.1, 68.1 ± 0.1 and 66.0 ± 0.2 mol% for DSS-3⁷, DSS-10 and ISM⁷, respectively. 16S rRNA gene sequence analyses placed these organisms within the Roseobacter lineage of the α-Proteobacteria. Closely related species were Silicibacter lacuscaerulensis and Ruegeria atlantica (DSS-3⁷ and DSS-10) and Roseovarius tolerans (ISM⁷). Neither DSS-3⁷ nor ISM⁷ exhibited 16S rRNA similarity > 97 % or DNA–DNA hybridization values > 45 % to their nearest described relatives. Genotypic and phenotypic analyses support the creation of two novel species: Silicibacter pomeroyi sp. nov. with strain DSS-3⁷ (=ATCC 700808T =DSM 15171T) as the type strain, and Roseovarius nubinhibens sp. nov. with strain ISM⁷ (=ATCC BAA-591T =DSM 15170T) as the type strain.

INTRODUCTION

Dimethylsulfoniopropionate (DMSP) is an organic osmoregulator, synthesized by marine algae, which is ubiquitous in oceanic surface waters. Degradation of DMSP by marine bacteria is one of the primary routes for formation of dimethylsulfide (DMS), a volatile sulfur compound that contributes significantly to the global atmospheric sulfur pool and influences climate regulation through backscatter of solar radiation and cloud formation (Charlson et al., 1987; Simó, 2001). However, degradation of DMSP by marine bacteria does not always lead to DMS formation (Kiene, 1996; Ledyard & Dacey, 1996; van Duyl et al., 1998). Along with a ‘cleavage’ pathway that produces DMS, DMSP may be metabolized via a ‘demethylation/demethiolation’ pathway that produces 3-methylmercaptoproprionate and then methanethiol (MeSH), or via a ‘double demethylation’
pathway that produces 3-mercaptopropionate (MPA) (Fig. 1) (Visscher et al., 1992). The relative importance of these alternate pathways in the marine environment has major implications for global control of DMS release from the ocean (Kiene et al., 2000).

Processes that determine the ratio of DMS:MeSH production during the degradation of DMSP in sea water are likely to be quite complex; they are influenced by the composition of the microbial community (Stefels et al., 1995), rates of algal grazing by zooplankton (Wolfe & Steinke, 1996) and even depth of the oceanic mixed layer (Simó & Pedrós-Alió, 1999). However, while both algae and bacteria can convert DMSP to DMS, only bacteria are known to express the alternative pathway that leads to MeSH production. Thus, marine bacteria must ultimately play a key role in determining the ratio of DMS:MeSH production during the degradation of DMSP (Kiene et al., 2000). However, relatively little is known about the physiology or ecology of DMSP-degrading bacteria or how the formation of DMS and MeSH is regulated at the cellular level.

Recent research has led to the hypothesis that members of the Roseobacter lineage (a marine clade in the α-Proteobacteria) play a key role in DMSP cleavage and demethylation/demethiolation in sea water. Ledyard et al. (1993) reported the first isolation of a DMSP-degrading roseobacter from the Sargasso Sea. This organism was able to grow on DMSP as the sole carbon source and to convert DMSP to DMS. Subsequently, González et al. (1999) demonstrated the widespread ability of roseobacters, isolated from coastal sea water, to degrade DMSP. Further studies of 16S rRNA gene sequences associated with DMSP-producing algal blooms in the North Sea established that roseobacters dominated the bacterial community in bloom waters (González et al., 2000) and that the growth rate of roseobacter populations was correlated with DMSP turnover in a similar bloom (Zubkov et al., 2001).

Among the DMS-producing Roseobacter isolates studied by González et al. (1999), several strains also demonstrated the ability to degrade DMSP via the demethylation/demethiolation pathway, resulting in the accumulation of MeSH (Kiene et al., 2000). These were the first reports of the presence of both DMSP-cleavage and DMSP-demethylation/demethiolation abilities in the same bacterium. The DMSP-demethylating/demethiolating strains included DSS-3T and DSS-10 (isolated from coastal sea water, Georgia, USA; González et al., 1999) and ISM1 (isolated from surface waters of the Caribbean Sea; Fuhrman et al., 1994). In the current study, we present the phylogenetic and phenotypic characteristics of DSS-3T, DSS-10 and ISM1, and demonstrate that these isolates belong to previously undescribed species in two genera within the Roseobacter lineage. We propose the names Silicibacter pomeroyi sp. nov. and Roseovarius nubinhibens sp. nov., and designate DSS-3T and ISM1, respectively, as the type strains.

**METHODS**

**Isolation.** Strains DSS-3T and DSS-10 were cultured from sea water of coastal Georgia, enriched with DMSP. Filter-sterilized sea water [salinity, 14 p.s.u. (practical salinity units)], amended with 10 μM DMSP, was inoculated with 4% (v/v) sea water that had been filtered through a 1 μm pore-size membrane filter to remove non-bacterial particles. After 2 weeks, aliquots of enrichment culture were spread onto low-nutrient sea-water medium plates: 50 mg peptone, 5 mg yeast extract and 1.5% purified Difco agar (1 aged, sterilized sea water)−1 that contained 10 μM DMSP (González et al., 1999). Colonies were picked from plates after 15 days incubation in the dark. Strain ISM1 was isolated from the Caribbean Sea (22°3′7″N, 74°35′2″W) on sea-water medium plates: 50 mg peptone, 50 mg Casamino acids and 15 g Sigma type 1-A agarose [1.80% (v/v) sea water]−1 (Fuhrman et al., 1994). Plates were incubated at room temperature and colonies were picked after several days incubation.

**Bacterial strains.** Cultures of the type strains of Silicibacter lacus- caeruleus (DSM 11314T), Ruegeria atlantica (DSM 5823T) and Roseovarius tolerans (EL-172T=DSM 11457T) were obtained from the DSMZ (Braunschweig, Germany).

**Media and culture conditions.** Strains DSS-3T, DSS-10 and ISM1 were maintained on half-strength YTSS (1/2 YTSS) plates or broth...
that contained (1−1): 2 g yeast extract, 1.25 g tryptone peptone, 20 g sea salts and (plates only) 15 g agar. Strains were stored in YTSS broth that contained 15% glycerol and 15% DMSO at −70°C. S. lacuscaerulensis, Ruegeria atlantica and Roseovarius tolerans were maintained on Difco Marine Medium 2216 agar and broth.

**Microscopy.** Cells used for phase-contrast microscopy were grown to early exponential phase in 1/2 YTSS medium, collected by centrifugation and resuspended in 0-1 vols fresh medium. For ISM1, the cell suspension was then mixed with an equal volume of glycerol to retard motility. Phase-contrast micrographs were acquired with a Princeton Instruments MicroMax CCD camera on a Nikon TE300 inverted microscope; the images were processed by using IP Lab Spectrum and Adobe Photoshop software.

Cells used for electron microscopy were grown overnight in 1/2 YTSS medium, collected by centrifugation (resincoating once with 100 mM HEPES buffer) and resuspended in 100 mM HEPES with 2% gluteraldehyde and 1% formaldehyde. Cells were fixed by incubating at 10°C for 30 min. After incubation, cells were pelleted and resuspended in 100 mM HEPES that contained 1% gluteraldehyde and 0.5% formaldehyde. Electron micrographs were acquired with a Philips model EM 301 instrument, operated at an acceleration voltage of 80 kV.

**Biochemical and physiological characterization.** Gram-staining was determined on overnight cultures. Bacteriochlorophyll a production was assayed by the method of Ledyard et al. (1993), with Roseobacter litoralis and Roseobacter denitrificans as positive controls. Catalase production was assayed by using 0.1% hydrogen peroxide. Oxidase was determined by the method of Kovacs (1956). Motility was examined in semi-solid 1/2 YTSS agar inoculated with a straight needle (Holt & Krieg, 1994).

For analysis of flagellar rotation, DSS-3T was grown in 1/2 YTSS for 2 days, diluted with 2% sea-salt solution to an A600 of 0.05, layered on Bromfield plates (Sournik & Schmitt, 1996) and incubated at 30°C for 20 h until A600 was 0-3-0.5. Swimming cells were analysed with the Hobson BacTracker system (Scharf et al., 2001). Cross-reactivity of the DSS-3T flagellin proteins was determined with whole-cell extracts of DSS-3T that were separated in 10% acrylamide gel. Proteins were transferred to nitrocellulose membranes and probed with purified polyclonal antibodies that had been raised against Rhizobium lupini H13-3 and Sinorhizobium meliloti fla proteins (Scharf et al., 2001). Flagellar filaments were detached from motile cells and purified by differential centrifugation (Scharf et al., 2001).

Carbon source utilization assays were performed in marine basal medium (MBB; Baumann & Baumann, 1981) supplemented with a vitamin solution (González et al., 1997a). Growth on carbon sources was determined by using an inoculum (5%) of overnight cultures that were grown in 1/2 YTSS to visible turbidity. Unless otherwise noted, substrates were added at a final concentration of 10 mM. All utilization assays took place in the dark at 30°C. Liquid cultures were placed in a rotary shaker at 250–300 r.p.m. Tests were considered to give a positive result when turbidity was visible after 3 days. Volume transfers or when changes in turbidity in single-transfer cultures were significant, relative to no-carbon controls. In the latter case, cells from overnight cultures were harvested by centrifugation (12,000 r.p.m. for 1-5 min) and washed three times with MBB without a carbon source, prior to inoculation.

D-Glucose fermentation, denitrification, gelatin hydrolysis and activity of amylase and lipase (Tween 80) were assessed as described by Baumann & Baumann (1981). Xylanase activity was assayed on solid MBB that contained 0.5% birchwood xylan (Sigma) as the sole carbon source. Degradation of cellulose was determined on MBB agar that contained 0-5% Avicel (microcrystalline cellulose, type PH-105; FMC Corporation).

Sodium ion requirement, optimum sodium ion concentration and growth rate at optimum sodium concentration were determined according to the method of González et al. (1997b). Effect of temperature on growth was determined on 1/2 YTSS plates that were incubated at 8, 10, 15, 20, 37, 40, 42 and 45°C for 1-2 weeks.

**Oxidation of inorganic sulfur compounds.** Isolates were screened for thiosulfate oxidation in solid and liquid media, according to protocols modified from those described by González et al. (1999). For the solid-medium assays, strains were grown on MBM agar supplemented with Fe-EDTA, vitamins, 5 mM arginine and filter-sterilized Na2S2O3 at a final concentration of 10 mM; negative controls were prepared with the same medium, but lacking Na2S2O3. After 2 days incubation, plates were flooded with Ellman’s reagent. Colourless plates, which lacked yellow product from the reaction between Ellman’s reagent and thiosulfate, indicated utilization of thiosulfate by the bacterial strain.

For assays in liquid medium, strains were grown in 10 ml MBM medium that contained Fe-EDTA, vitamins, 5 mM arginine and 10 mM thiosulfate. Daily samples (1 ml) were taken over a 5 day incubation period, centrifuged to pellet cells and then frozen until analysis (<7 days). Ellman’s reagent (20 μl) was added to 350 μl supernatant and samples were incubated for 30 min for colour development. A412 was measured by using a Beckman DU 640 spectrophotometer.

**DMSP degradation.** Ability to convert DMSP to MeSH and DMS was determined according to the method of González et al. (1999). Cells were grown overnight at 30°C in liquid MBM with 5 mM glucose, arginine, acetate or succinate, to visible turbidity. Cell suspension (5 ml) was transferred to a 30 ml serum bottle and capped with a Teflon-faced septum. DMSP was added to each serum bottle at a final concentration of 10 μM. Assays were run in triplicate with heat-killed samples (70°C, 1 h) that served as negative controls.

Samples of the headspace (100 μl) were collected just after the addition of DMSP and then at 30 min intervals for 4–8 h. Formation of DMS and MeSH was monitored by GC (González et al., 1999).

**Cell membrane fatty acid analysis.** Fatty acid methyl ester analysis was performed by Microbial ID (Newark, DE, USA) for strains DSS-3T, DSS-10 and ISM1, as well as for the type strains of S. lacuscaerulensis, Ruegeria atlantica and Roseovarius tolerans.

**DNA G+C content.** DNA base composition was determined by HPLC (Mesbah et al., 1989).

**DNA–DNA hybridization.** Overnight cultures of the strains were grown on 1/2 YTSS liquid medium. Cell pellets were harvested by centrifugation (10,000 r.p.m. for 10 min) and stored in 50% (v/v) 2-propanol. DNA extraction and pairwise DNA–DNA hybridization were carried out by the DSMZ, using the method of De Ley et al. (1970).

**16S rDNA and internal transcribed spacer (ITS) sequencing and comparative analysis.** Complete 16S rRNA gene sequences of strains DSS-3T and ISM1 were determined previously (González & Moran, 1997). To determine the 16S rRNA gene sequence of DSS-10, DNA was extracted according to the method of Tasi & Olson (1991) as modified by González et al. (1996) and the 16S rRNA gene was amplified with primers 10F (Takeuchi et al., 1994) and 1522R (Giovannoni, 1991). Primers 10F, 338R, 338F (Stahl & Amann, 1991), 926R, 926F (Lane et al., 1985) and 1522R were used to sequence overlapping regions of 16S rDNA at the University of Georgia Molecular Genetics Instrumentation Facility (Athens, GA, USA).

Sequence alignments were performed with Genetics Computer Group.
software (version 10.0, 1999). Phylogenetic trees were inferred and bootstrap analyses (100 replicates) were performed with the PHYLIP package (Felsenstein, 1989), using Jukes–Cantor evolutionary distances and the neighbour-joining method. The resulting tree was compared with that inferred by the parsimony (100 bootstrap replicates) and maximum-likelihood methods by using the fastDNAML program (Felsenstein, 1981; Olsen et al., 1994). Only aligned positions for which >50% of sequences shared the most common base were considered. The region analysed spanned Escherichia coli positions 62–1370.

Interstrain similarity between DSS-3T and DSS-10 was investigated by sequence analysis of the ITS region, using methods adapted from Normand et al. (1996). PCR and sequencing were carried out with primers 1522F and 23S-R (5'-CCGGGTTTCCCCATTCGG-3'; Normand et al., 1996).

Congruence between 16S rRNA similarity and phylogeny of the marine Roseobacter lineage was assessed by using cophenetic correlation analysis (Keswani & Whitman, 2001). Similarities of complete 16S rRNA gene sequences within this lineage were determined by using aligned sequence data to create a similarity matrix with the DNADIST program in the PHYLIP package (Felsenstein, 1989). 16S rRNA similarity matrices were then used to calculate cophenetic matrices with the UPGMA method, by using the NEIGHBOR program in the PHYLIP package. Cophenetic correlation coefficients for groups of taxa in the Roseobacter lineage were calculated from linear regressions of corresponding values from the similarity and cophenetic matrices.

**Nucleotide sequence accession numbers.** 16S rRNA gene sequences for strains DSS-3T and ISM7 have been previously deposited in GenBank under accession numbers AF098491 and AF098495, respectively (González & Moran, 1997). The 16S rRNA gene sequence for DSS-10 was deposited in GenBank under accession number AF434674.

### RESULTS AND DISCUSSION

**Isolation of bacteria, morphology and motility**

The strains described in this study were isolated from seawater, either with (DSS-3T and DSS-10) or without (ISM1) prior enrichment with DMSP. All three strains were short, Gram-negative rods (Fig. 2).

Electron micrographs of DSS-3T and DSS-10 indicated the presence of blebs associated with the outer membrane (Fig. 2d). Blebs have been observed in previously described marine isolates and may be associated with the degradation of insoluble substrates (González et al., 1997a, b). Apparent poly-β-hydroxybutyrate (PHB) inclusion bodies were also observed in isolates DSS-3T and DSS-10 (Fig. 2e); this is consistent with S. lacuscaerulensis, where similar structures have been observed (Petursdottir & Kristjansson, 1997). Blebs and PHB bodies may allow DSS-3T and DSS-10 to survive in oligotrophic waters by facilitating uptake and storage of nutrients.

Separation of the outer membrane from the cytoplasm at the poles was observed in ISM7, producing a toga-like morphology (Fig. 2f). This separation of membrane and cytoplasm has been observed previously for the genus Thermotoga and its relatives (Huber & Stetter, 2001).

All strains exhibited motility on semi-solid 1/2 YTSS agar. Electron microscopy indicated that DSS-3T had a polar, complex flagellum that did not cross-react with antibodies for proteins from the complex flagella of Rhizobium lupini.

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**Fig. 2.** Phase-contrast micrographs of (a) DSS-3T; (b) DSS-10; (c) ISM7. Transmission electron micrographs of: (d) DSS-3T, arrow indicates surface blebs; (e) DSS-10, arrow indicates inclusion bodies; (f) ISM7, arrow indicates separation of cytoplasm at the poles. (g) Differential interference contrast micrograph of DSS-3T. Scanning electron micrographs of: (h) DSS-3T; (i) close-up of DSS-3T flagellum. Bars, 10 μm (a, b, c and g); 250 nm (d, e, f, h and i).
H13-3 and S. meliloti. The major component of the flagellum was found to have an apparent molecular mass of 31 kDa; two putative minor components (that comprised <5 % of the total) had apparent molecular masses of 31 and 42 kDa. Like other complex flagella, that of DSS-3T rotated exclusively in the clockwise direction.

Culture and growth characteristics

Growth was typically observed for all three strains after 3–5 days on 1/2 YTSS plates when incubated at 25–30 °C. Colonies of strains DSS-3T and DSS-10 were circular, cream-coloured and convex. Older colonies of DSS-3T and DSS-10 developed a brownish-orange margin. Colonies of ISMT were circular with a cream-coloured margin and a raised dark orange-brown centre. All strains had a requirement for NaCl, with optimal growth of DSS-3T at 100–400 mM NaCl and ISMT at 200–400 mM NaCl (both at 30 °C). Temperature range for growth of the three strains was 10–40 °C. Strains DSS-3T and ISMT had similar growth rates (0.42 and 0.39 day⁻¹, respectively) when grown in MBM at 30 °C and their optimal NaCl concentration.

Strains DSS-3T and DSS-10 did not have any specific vitamin requirements, although enhanced growth was observed in defined medium in the presence of vitamin solution. ISMT had a specific requirement for nicotinic acid in defined medium.

Biochemical and physiological characteristics

Strains DSS-3T and DSS-10 were oxidase-positive, whereas strain ISMT was variable for oxidase production. All three strains were positive for catalase production. None of the strains was able to ferment glucose or reduce nitrate. Strains DSS-3T and DSS-10 utilized various organic acids, amino acids and other compounds for growth (acetate, ethanol, DL-β-hydroxybutyrate, glucose, succinate, acryl acid, cysteic acid, glycerol, citrate, pyruvate, Casamino acids, L-alanine, L-serine, L-taurine, L-methionine, DMSP and glycine betaine). Strain ISMT utilized fewer of the compounds tested than DSS-3T, but it still used a variety of osmolytes, organic acids and amino acids (acacetate, ethanol, DL-β-hydroxybutyrate, glucose, succinate, Casamino acids, L-alanine, L-arginine, L-serine, L-taurine and DMSP). DSS-3T and DSS-10 hydrolysed gelatin, but ISMT did not. None of the strains was able to hydrolyse cellulose, starch, Tween 80 or xylan. Previous studies have also indicated that DSS-3T and ISMT are able to degrade aromatic compounds (Buchan et al., 2000, 2001). None of the three isolates tested positive for production of bacteriochlorophyll a when grown in liquid 1/2 YTSS medium in the dark; strains were not tested in the light.

DNA G+C contents of strains DSS-3T and DSS-10 were 68.0 ± 0.1 (n = 3) and 68.1 ± 0.1 (n = 6) mol%, respectively. These values were close to that of S. lacuscaerulensis (66.2 mol%; Petursdöttir & Kristjansson, 1997), but substantially different from that of Ruegeria atlantica (55–58 mol%; Uchino et al., 1998). The DNA G+C content of ISMT was 66.0 ± 0.2 mol%, compared to 62.2–63.8 mol% for Roseovarius tolerans (Labrenz et al., 1999).

Oxidation of inorganic sulfur compounds

When grown on solid and liquid media, DSS-3T and DSS-10 oxidized thiosulfate in the presence of 5 mM arginine. ISMT did not oxidize thiosulfate.

Demethylation of DMSP

DSS-3T, DSS-10, ISMT, S. lacuscaerulensis, Ruegeria atlantica and Roseovarius tolerans all produced MeSH from 10 μM DMSP during exponential-phase growth (Fig. 3). All strains except S. lacuscaerulensis also produced DMS from DMSP. In stationary-phase cultures of DSS-3T and DSS-10, net accumulation of MeSH declined and was always several-fold lower than DMS accumulation (data not shown).

DMSP-demethylating bacteria have been cultured previously from marine environments (Vischer et al., 1992), but DSS-3T, DSS-10 and ISMT are the first characterized strains that possess both competing pathways for DMSP degradation that are thought to dominate in sea-water bacterial communities; demethylation/demethiolation to MeSH and cleavage to DMS (González et al., 1999). Regulation of these two pathways may be driven by DMSP availability or growth rate (González et al., 1999); differential production of DMS versus MeSH during stationary-phase growth by cultures of DSS-3T and DSS-10 supports this idea. As the sulfur moiet of MeSH can be incorporated into cellular protein by these and other marine roseobacters, cellular sulfur requirements may also be a key regulatory factor (Kiene et al., 1999).

Cellular fatty acid profiles

Fatty acid profiles for all three strains were dominated by C₁₈:₁ω₇c (>74 %) and C₁₆:₀ (7–10 %) (Table 1). The profiles of DSS-3T and DSS-10 had minor amounts of C₁₆:₀ 2-OH (3–4 %) and C₁₂:₀ 3-OH (5 %). No differences in major (>10 % of total recovered fatty acids) or minor (1–10 %) fatty acids were observed between DSS-3T and DSS-10. However, C₁₆:₀ 2-OH was found in DSS-3T, DSS-10 and Ruegeria atlantica but not in S. lacuscaerulensis (Table 1). Larger differences in the fatty acids that were common to ISMT and Roseovarius tolerans were observed: several fatty acids, accounting for 10–15 % of the total, were found in ISMT but not in Roseovarius tolerans, including C₁₂:₀ (3 %), cyclo C₁₉:₀ω₈c (5 %) and C₁₆:₁ω₇ciso C₁₅:₀ 2-OH (1 %) (Table 1). Fatty acids found in Roseovarius tolerans, but not ISMT, were C₁₈:₁ 11-CH₃ (5–5 %) and C₁₂:₁ 3-OH (3 %).

Molecular phylogenetic analyses

Phylogenetic analysis of the 16S rRNA gene sequences of DSS-3T, DSS-10 and ISMT indicated affinity with two distinct clusters within the Roseobacter lineage of the α-subclass of
the Proteobacteria (Fig. 4). The 16S rRNA gene and ITS sequences of DSS-3T were identical to those of DSS-10, and had highest similarity to *S. lacuscaerulensis* (97.1%) and *Ruegeria atlantica* (95.8%). DSS-3T and DSS-10 always grouped with *S. lacuscaerulensis* and *Ruegeria atlantica* when different ingroups were included in the phylogenetic analysis, and in both parsimony and maximum-likelihood analyses. This topology was also supported by bootstrap values, in each case above 90%. The 16S rRNA gene sequence of ISMT grouped with that of *Roseovarius tolerans* with 95.8% sequence similarity.

*S. lacuscaerulensis, Ruegeria atlantica, DSS-3T* and DSS-10 formed a subgroup within the Roseobacter lineage, as did *Roseovarius tolerans* and ISMT (Fig. 4). No other taxa clustered clearly with these two groups, either because of low bootstrap values or because groupings were not supported by different treeing methods. A number of other robust clusters within the Roseobacter lineage were also found, namely the *Roseobacter litoralis/Roseobacter denitrificans/Sulfitobacter/Staleya* subgroup, the *Octadecabacter/Ruegeria gelatinovorans* subgroup, the *Sagittula/Antarctobacter* subgroup and the *Ruegeria algicola/Roseobacter galleciensis/Leisingera* subgroup (Fig. 4). These subgroups appeared, no matter which method (neighbour-joining, parsimony or maximum-likelihood) or ingroups were used to construct the phylogenetic tree.

Cophenetic correlation analysis was carried out on 16S rRNA gene sequences of the three new strains and their relatives in the Roseobacter lineage by using approximately 1300 bp of aligned sequence. Using all 16S rRNA gene sequences indicated in Fig. 4, the cophenetic correlation coefficient was 0.95. Similar results were obtained when individual genera were selectively omitted. For instance, when sequences from the genera *Jannaschia* or *Roseovarius* were omitted, the coefficient was also 0.95. This result suggests that 16S rRNA is a reliable phylogenetic marker within the Roseobacter clade and should be highly correlated to DNA hybridization (Keswani & Whitman, 2001).

**DNA–DNA hybridization**

Pairwise comparisons between DSS-3T, DSS-10 and ISMT and their closest described relatives in the Roseobacter clade support the formation of two novel species. DNA
The hybridization levels of DSS-3T to *Ruegeria atlantica* and *S. lacuscaerulensis* were 21 and 43 %, respectively. The hybridization of DSS-3T to DSS-10 was 100 %. The hybridization of ISMT to *Roseovarius tolerans* was 42 %. Both DNA hybridization values and 16S rRNA similarities to previously described species were sufficiently high that placement in novel genera was not warranted.

DSS-3T and DSS-10 were related almost equally to *S. lacuscaerulensis* and *Ruegeria atlantica*. The rationale for their placement in the genus *Silicibacter* was based on higher DNA hybridization and 16S rRNA gene sequence similarity values and a more similar DNA G+C content. Therefore, the name proposed for DSS-3T and DSS-10 is *Silicibacter pomeroyi* sp. nov. and the name proposed for ISMT is *Roseovarius nubinhibens* sp. nov.

### Description of *Silicibacter pomeroyi* sp. nov.

*Silicibacter pomeroyi* (po.me.roy'i. N.L. masc. gen. n. pomeroyi of Lawrence R. Pomeroy, marine microbial ecologist who first elucidated the role of bacteria in the marine food web).

Cells are Gram-negative, motile rods with one polar, complex flagellum. Electron micrographs indicate the presence of intracellular inclusion bodies and outer-membrane blebs. Colonies on marine agar 2216 and 1/2 YTSS are circular and cream-coloured. Optimum NaCl concentration is 100–400 mM when grown at 30 °C. Growth occurs at 10–40 °C. Vitamins are stimulatory, but not essential, for growth. Growth occurs on ethanol, glycine betaine, acrylic acid, cysteic acid, DMSP, glycerol, acetate, citrate, DL-β-hydroxybutyrate, glucose, succinate, pyruvate, Casamino acids, L-alanine, L-arginine, L-serine, L-methionine and L-taurine, but not on 3-mercaptopropionate, fructose, mannitol, rhamnose or benzoic acid. Exhibits catalase and peroxidase.

#### Table 1. Fatty acid profiles of strains DSS-3T, DSS-10 and ISMT and their closest described relatives. Values are expressed as a percentage of the total recovered fatty acids.

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</table>

*a*77c.

†cyclo *30c.

‡*30c/15 iso 2-OH.

Fig. 4. Phylogeny of type species in the *Roseobacter* lineage in the α-subdivision of the *Proteobacteria* and representative members outside the group. The tree was generated with the neighbour-joining method. Before analysis, a filter was applied to exclude positions with <50 % conservation within the sequences being aligned. Only positions 62–1370 (E. coli numbering) were considered. *Rickettsia prowazekii* (GenBank number M21789) served as the outgroup. Numbers at nodes indicate bootstrap values above 50 % (100 replicates). Double dots denote robust subgroups that were obtained no matter what treeing method or ingroups/outgroups were used. Bar, 0-05 Jukes–Cantor evolutionary distance.

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oxidase activities. Does not ferment glucose or reduce nitrate. Gelatin is hydrolysed, but cellulose, Tween 80, xylan and starch are not. Capable of demethylation/demethiolation of DMSP to MeSH and cleavage to DMS. Primary cellular fatty acid is C_{18:1} \text{v} \text{v}. DNA G+C content is 68.0 ± 0.1 mol%.

The type strain is DSS-3^T (= ATCC 700808^T = DSM 15171^T). Isolated from sea water in coastal Georgia, USA.

Description of Roseovarius nubinhibens sp. nov.

Roseovarius nubinhibens (nub.in’h.i.bens. L. fem. n. nubes cloud; L. part. adj. inhibens inhibiting; N.L. part. adj. nubinhibens inhibiting clouds).

Cells are Gram-negative and motile rods. Electron micrographs indicate separation of the outer membrane from the cytoplasm at the poles to form a tega. Colonies on marine agar 2216 and 1/2 YTSS are circular with a cream-coloured margin and a raised dark orange-brown centre. Optimal growth occurs at 30 °C in the presence of 200–800 mM NaCl. Growth occurs at 10–40 °C. Requires nicotinic acid for growth. Growth occurs on ethanol, DMSP, acetate, DL-β-hydroxybutyrate, glucose, succinate, Casamino acids, L-alanine, L-arginine, L-serine and L-taurine, but not on 3-mercaptopyrrolpropionate, glycine betaine, acrylic acid, cysteic acid, glyceral, citrate, fructose, mannitol, rhamnose, benzoic acid or L-methionine. Exhibits catalase activity but no amylase activity; oxidase activity is variable. Does not ferment glucose or reduce nitrate. No hydrolysis of cellulose, gelatin, Tween 80 or xylan is detected. Capable of demethylation/demethiolation of DMSP to MeSH and cleavage to DMS. Primary cellular fatty acid is C_{18:1} \text{v} \text{v}. DNA G+C content is 66.0 ± 0.2 mol%.

The type strain is ISM^T (= ATCC BAA-591^T = DSM 15170^T). Isolated from sea water from the Caribbean Sea.

ACKNOWLEDGEMENTS

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REFERENCES


J. M. González and others


