

Simidua agarivorans gen. nov., sp. nov., a marine, agarolytic bacterium isolated from shallow coastal water from Keelung, Taiwan

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A Gram-negative, heterotrophic, agarolytic, marine bacterium, designated strain SA1^T, was isolated from a seawater sample collected in the shallow coastal region of Keelung, Taiwan. Cells were straight to slightly curved rods. Nearly all of the cells were non-motile and non-flagellated during the exponential phase of growth in broth cultures; a few cells (<1%) were motile and were considered to have monotrichous flagella. The isolate required NaCl for growth and grew optimally at 30–35 °C and 2–3% (w/v) NaCl. It grew aerobically and was incapable of anaerobic growth by fermentation of glucose or other carbohydrates. However, anaerobic growth could be achieved by reduction of nitrate to nitrite. Polar lipids comprised phosphatidylethanolamine (71.8%), diphosphatidylglycerol (12.7%), phosphatidylglycerol (12.2%) and phosphatidylserine (3.3%). Isoprenoid quinones consisted of Q-10 (87.5%), MK-9 (6.6%) and MK-7 (5.9%). Major cellular fatty acids were C_{16:1ω7c} and/or iso-C_{15:0} 2-OH (28.6%), C_{17:1ω8c} (22.8%), C_{16:0} (14.5%), C_{18:1ω7c} (11.0%) and C_{17:0} (6.4%). The DNA G+C content was 55.6 mol%. Phylogeny based on 16S rRNA gene sequence analysis showed that strain SA1^T formed a distinct lineage within the class *Gammaproteobacteria*. Strain SA1^T was related most closely to *Teredinibacter turnerae*, *Cellvibrio* spp., *Saccharophagus degradans*, *Pseudomonas* spp. and *Microbulbifer* spp., strains of these species sharing <93% 16S rRNA gene sequence similarity with strain SA1^T. The phylogenetic data and those from physiological, morphological and chemotaxonomic characterizations indicated that strain SA1^T represents a novel species and genus, for which the name *Simidua agarivorans* gen. nov., sp. nov. is proposed. The type strain is SA1^T (=BCRC 17597^T=JCM 13881^T).

Agar, a complex polysaccharide extracted from marine red algae, is employed widely as a gelling agent for microbiological culture media. Hydrolysis of this refractory material is a property found only among the domain *Bacteria*. Hydrolysis of agar is indicated by the development of depressions, softening or, in some cases, complete liquefaction of the agar surrounding colonies of agarolytic bacteria.

A novel agarolytic isolate, strain SA1^T, was recovered in our laboratory from a seawater sample collected in the shallow coastal region of Keelung, Taiwan, during a survey of the diversity of heterotrophic marine bacteria. Data from the

present polyphasic study indicate that this isolate represents a novel species and genus.

A seawater sample collected from the shallow coastal region of Keelung, Taiwan, was diluted 10-fold with sterile NaCl/Tris buffer [30 g NaCl, 0.24 g Tris (I deionized water)⁻¹; pH 8.0]. Aliquots (0.1 ml) of the 10-fold dilutions (10⁻¹ to 10⁻³) were spread on polypeptone/yeast extract (PY) plate medium (Shieh *et al.*, 2000) in triplicate. The plates were incubated at 25 °C in the dark for 7 days under aerobic conditions. An apparently agarolytic strain, SA1^T, was isolated from one of the plates and was subsequently purified by successive streaking on PY plates. Maintenance of this strain in our laboratory was performed repeatedly at an interval of 3–4 months by inoculating early stationary-phase cultures grown in PY broth into 7/10-strength seawater at a ratio of 2:50 (v/v). Maintenance cultures were kept at 20 °C under aerobic conditions. The isolate has also been deposited in both the Japan Collection of

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SA1^T is EF617350.

A maximum-likelihood tree showing the phylogenetic relationship between strain SA1^T and related bacteria in the class *Gammaproteobacteria* is available with the online version of this paper.

Micro-organisms (JCM) and the Bioresource Collection and Research Center (BCRC) as lyophilized cultures.

Growth and other phenotypic properties used for physiological and morphological characterization of strain SA1^T were determined following established procedures described previously (Jean *et al.*, 2006a), with modifications and additional tests as described below. Haemolysis was tested as described by Chiu *et al.* (2007). Hydrolysis of chitin and cellulose was tested by growth of strain SA1^T on modified PY plate media containing either colloidal chitin (Hobel *et al.*, 2005) or cellulose, both at 2 g l⁻¹. Congo red was added (0.2 g l⁻¹) to the cellulose-containing plate medium to enhance the detection of bacterial colonies that were able to hydrolyse cellulose (Hendricks *et al.*, 1995). Positive reactions for hydrolysis of chitin and cellulose were indicated by the appearance of clear zones around the colonies on the plates. All of the test cultures were incubated aerobically at 30 °C in the dark for 7 days unless stated otherwise.

Cells grown in PY broth at 30 °C for 4 days were harvested by centrifugation. Polar lipids in the cells were extracted, purified and analysed by using the methods described by Lin & Shieh (2006). Isoprenoid quinones in the cells were extracted, purified and analysed according to Nishijima *et al.* (1997), by using an HPLC apparatus equipped with a reversed-phase column (4.6 × 250 mm; Waters model 5C₁₈-AR-II). A mixture of methanol/isopropyl-alcohol (7:3, v/v) was used as the mobile phase, and quinones were detected at 270 nm. Fatty acids in the whole cells grown on PY plate medium at 30 °C for 4 days were extracted, saponified and esterified, followed by GC analysis of the fatty acid methyl esters according to the instructions of the MIDI system (Sasser, 1997). This work, together with DNA G + C content determination by HPLC analysis (Shieh & Liu, 1996), was performed by the BCRC, Food Industry Research and Development Institute, Taiwan.

Cells grown in PY broth at 30 °C for 3 days were harvested by centrifugation. Extraction and purification of total genomic DNA from the cells and PCR amplification of the 16S rRNA gene were performed according to the methods described previously (Jean *et al.*, 2006a). Sequencing of the 16S rRNA gene, alignment and comparison of the resulting sequence with reference sequences available in GenBank, calculation of distance matrices for the aligned sequences and reconstruction of phylogenetic trees based on the neighbour-joining, maximum-parsimony and maximum-likelihood methods were performed as described by Shieh *et al.* (2004) and Jean *et al.* (2006a). The stability of clusters was evaluated by a bootstrap analysis of 1000 resamplings.

The nearly complete 16S rRNA gene sequence (1431 nt) of strain SA1^T was determined. Preliminary 16S rRNA gene sequence comparisons with those in GenBank indicated that strain SA1^T belonged to the class *Gammaproteobacteria*. This new isolate showed no apparent relationship with other bacteria on the basis of 16S rRNA gene sequence similarity. It showed ≤93% 16S rRNA gene sequence similarity to strains

of any recognized bacterial species. Strain SA1^T showed highest levels of 16S rRNA gene sequence similarity to the type strains of *Teredinibacter turnerae* (92.9%), *Cellvibrio japonicus* (92.9%), *Cellvibrio mixtus* (92.4%), *Cellvibrio fulvus* (92.1%), *Pseudomonas flavescens* (91.7%), *Pseudomonas pachastrellae* (91.5%), *Saccharophagus degradans* (91.5%), *Microbulbifer salipaludis* (91.5%), *Pseudomonas argentinensis* (91.4%), *Pseudomonas elongata* (91.3%), '*Microbulbifer arenaceus*' (91.3%), *Microbulbifer hydrolyticus* (91.0%), *Pseudomonas pseudoalcaligenes* (90.8%) and *Marinobacter excellens* (90.8%). The distant relationship between strain SA1^T and these bacteria was also evident in the neighbour-joining tree; strain SA1^T may represent a novel lineage in the tree, in which this isolate was an outgroup with respect to a clade that contained the type strains of *T. turnerae* and *S. degradans* (Fig. 1). A similarly distant relationship was also found in the maximum-parsimony tree (not shown). However, in the maximum-likelihood tree (see Supplementary Fig. S1, available in IJSEM Online), strain SA1^T formed a clade only with the type strain of *T. turnerae*, being located next to a sister clade comprising the type strain of *S. degradans* as well as species of the genus *Cellvibrio*. The low levels of 16S rRNA gene sequence similarity to all recognized bacterial species (<93%), together with the phylogenetic data from the three tree-making algorithms employed, suggested that strain SA1^T may represent a novel species and genus.

Strain SA1^T had a DNA G + C content of 55.6 mol%, a value greater than that for *T. turnerae* (49.0–51.0 mol%) and *S. degradans* 2-40^T (45.8 mol%). The major fatty acids of strain SA1^T were summed feature 3 (28.6% of the total fatty acids; C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; the two fatty acids could not be differentiated in the MIDI system), C_{17:1}ω8c (22.8%), C_{16:0} (14.5%), C_{18:1}ω7c (11.0%) and C_{17:0} (6.4%). Other fatty acids present at levels ≥1% included C_{14:0} (3.6%), C_{10:0} 3-OH (1.4%), C_{12:1} 3-OH (1.2%), C_{18:0} (1.0%) and C_{15:1}ω8c (1.0%). The major fatty acids of *S. degradans* 2-40^T contained iso-C_{16:0} (37.0%), C_{14:0} (15.0%), C_{10:0} 3-OH (11.0%), C_{12:1} 3-OH (6.0%) and C_{10:0} (5.0%) (Ekborg *et al.*, 2005; González & Weiner, 2000), whereas only low levels of these fatty acids (<1.0–3.6%) were detected in strain SA1^T. The fatty acid composition of *T. turnerae* has not been described. Strain SA1^T contained Q-10 (87.5%) as the predominant isoprenoid quinone. MK-9 (6.6%) and MK-7 (5.9%) were present as minor components. The polar lipids of strain SA1^T consisted of phosphatidylethanolamine (71.8%), diphosphatidylglycerol (12.7%), phosphatidylglycerol (12.2%) and phosphatidylserine (3.3%). The polar lipid and isoprenoid quinone profiles of *S. degradans* and *T. turnerae* have not been reported.

Strain SA1^T was mesophilic, halophilic and Gram-negative. It produced circular, convex and non-luminescent colonies surrounded by depressions when grown on marine agar (Difco 2216) and PY plate medium for 3–7 days. Colonies on marine agar and PY plate medium were cream-coloured and off-white, respectively. Clear yellow haloes formed

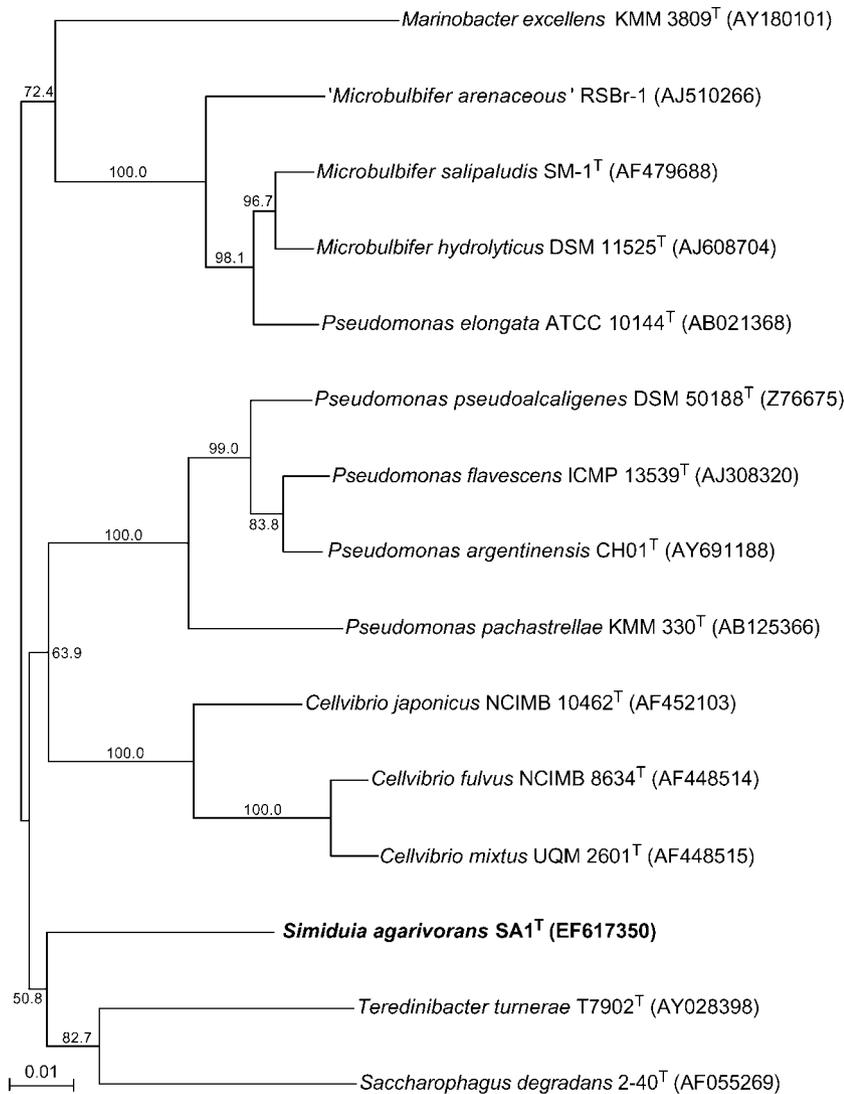


Fig. 1. Neighbour-joining tree showing the phylogenetic relationship between strain SA1^T and related bacteria in the class *Gammaproteobacteria*. GenBank accession numbers are given in parentheses. Bootstrap values (percentages of 1000 replicates) >50% are shown at branch points. Bar, one nucleotide substitution per 100 nt.

around the colonies, in contrast to the purple–brown background when the agar plates were flooded with iodine/potassium iodide solution. This indicated diffusion of agarase out from the colonies and release of reducing compounds during agar hydrolysis. Strain SA1^T hydrolysed agar more rapidly on marine agar than on PY plate medium. The ability to hydrolyse agar was also reported for *S. degradans* (Ekborg *et al.*, 2005), but not for *T. turnerae*. Various other bacterial species belonging to the phyla *Bacteroidetes* (previously known as *Cytophaga–Flavobacterium–Bacteroides*) (Yoon *et al.*, 2007), *Proteobacteria* (Jean *et al.*, 2006b) and ‘*Verrucomicrobia*’ (Scheuermayer *et al.*, 2006; Shieh & Jean, 1998) are also reported to be agarolytic. Cells of strain SA1^T were predominantly straight to slightly curved rods, approximately 2–5 µm long and 0.4–0.6 µm wide, during the exponential phase of growth in PY broth (Fig. 2a). Nearly all of the cells were non-motile and non-flagellated, although a few (<1%) were motile and could be

considered to have monotrichous flagella (Fig. 2b). Most of the cells formed sticky, amorphous aggregations during the stationary growth phase. Cells in the stationary growth phase had a pleomorphic morphology, often appearing as irregular, long rods 5–50 µm in length (Fig. 2c). Blebs and prosthecae were commonly produced in both the exponential and the stationary phases of growth; numerous prosthecae were produced peritrichously on the cells, and these could be branched (Fig. 2c) or network-shaped (Fig. 2a).

Physiologically, strain SA1^T was distinguishable from *S. degradans* and *T. turnerae* in that it grew at 40 °C, and not at pH 6. Other characteristics useful for distinguishing strain SA1^T from *S. degradans* and *T. turnerae* are listed in Table 1.

The data presented herein thus suggest that strain SA1^T represents a novel species and genus, for which the name *Simidiua agarivorans* gen. nov., sp. nov. is proposed.

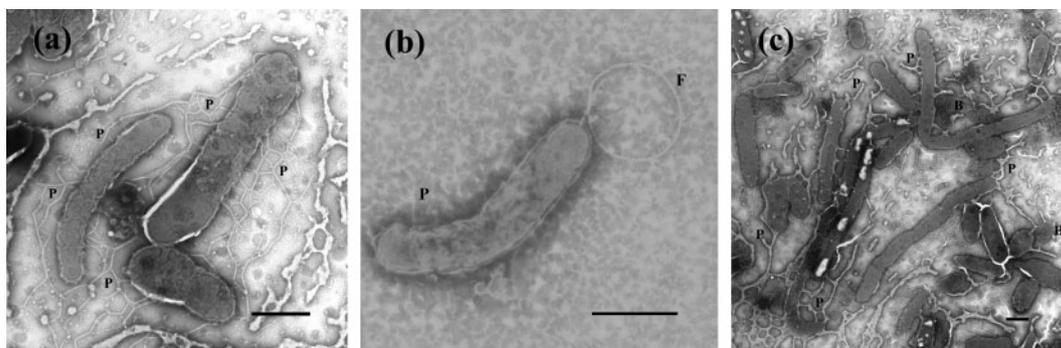


Fig. 2. Electron micrographs of negatively stained preparations of cells of strain SA1^T, showing blebs (B), prosthecae (P) and flagella (F). Cells used for analysis were grown in PY broth at 30 °C and harvested during either the exponential (a, b) or stationary (c) phase of growth. Bars, 1 µm.

Description of *Simidiua* gen. nov.

Simidiua (Si.mi'du.ia. N.L. fem. n. *Simidiua* named after Usio Simidu, a Japanese microbiologist, to honour his work in marine microbiology).

Cells are Gram-negative rods. Chemo-organotrophic; capable of respiratory, but not fermentative, metabolism. Mesophilic and halophilic. Oxidase- and catalase-positive. The major isoprenoid quinone is Q-10. The major polar lipid is phosphatidylethanolamine. Predominant fatty acids include summed feature 3 (*C*_{16:1}ω7*c* and/or iso-*C*_{15:0} 2-OH), *C*_{17:1}ω8*c*, *C*_{16:0} and *C*_{18:1}ω7*c*. Phylogenetically, the genus is affiliated to the class *Gammaproteobacteria*. The type species is *Simidiua agarivorans*.

Description of *Simidiua agarivorans* sp. nov.

Simidiua agarivorans (a.ga.ri.vo'rans. N.L. n. *agarum* agar; L. part. adj. *vorans* devouring, destroying; N.L. part. adj. *agarivorans* agar-devouring).

Has the following characteristics in addition to those given for the genus. Cells are predominantly straight to slightly curved rods, approximately 2–5 µm long and 0.4–0.6 µm wide, during the exponential phase of growth in broth cultures. Nearly all cells are non-motile and non-flagellated; a few cells (<1%) exhibit motility and monotrichous flagella. Most of the cells form sticky, amorphous aggregations during the stationary phase of growth. Cells in the stationary growth phase are pleomorphic, often irregular, long rods 5–50 µm in length. Blebs and prosthecae are produced; prosthecae are peritrichous and can be branched or network-shaped (Fig. 2a, c). Capable of anaerobic growth by reducing nitrate as the terminal electron acceptor, but not by fermenting glucose or other carbohydrates as substrates. Colonies produced on marine agar and PY plate medium are circular, off-white to cream-coloured, convex, non-luminescent and agarolytic; marine agar is hydrolysed more readily than PY plate medium. Growth occurs between 15 and 40 °C, with optimum growth at 30–35 °C; no growth occurs at 4–10 or 45 °C.

Growth occurs at NaCl concentrations of 0.5–7% (w/v), with optimum growth at 2–3% (w/v); no growth occurs without NaCl or in the presence of 8–10% (w/v) NaCl. Able to grow over a pH range of 7–10, but not at pH 6. Nitrate is reduced to nitrite, but not further to N₂O or N₂. Poly-β-hydroxybutyrate is not accumulated as an intracellular product. Not haemolytic. Indole is not produced from tryptophan. Aesculin, alginate, casein, cellulose, chitin, gelatin and starch are hydrolysed, but DNA, lecithin, Tween 80 and urea are not. Arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase are absent. The following constitutive enzyme activities are detected in API ZYM tests: cystine arylamidase, leucine arylamidase, valine arylamidase, esterase (C4), esterase lipase (C8), *N*-acetyl-β-glucosaminidase, naphthol-AS-BI-phosphohydrolase, acid phosphatase, alkaline phosphatase and trypsin. Able to grow on the following compounds as sole carbon and energy sources: cellobiose, D-glucose, maltose, sucrose, acetate, β-hydroxybutyrate, L-alanine, L-arginine, L-glutamic acid, L-lysine and tyrosine. Unable to grow on the following compounds as sole carbon and energy sources: D-arabinose, L-arabinose, cellulose, D-fructose, D-galactose, D-lactose, D-mannose, melibiose, raffinose, ribose, trehalose, D-xylose, salicin, dulcitol, inositol, D-mannitol, citrate, fumarate, lactate, malic acid, malonate, L-aspartic acid, citrulline, L-glutamine, L-glycine, L-histidine, L-isoleucine, L-leucine, L-ornithine, L-phenylalanine, L-serine, L-threonine, L-tryptophan and L-valine. Isoprenoid quinones comprise Q-10 (87.5%), MK-9 (6.6%) and MK-7 (5.9%). Polar lipids comprise phosphatidylethanolamine (71.8%), diphosphatidylglycerol (12.7%), phosphatidylglycerol (12.2%) and phosphatidylserine (3.3%). Cellular fatty acids present at levels ≥1% are summed feature 3 (*C*_{16:1}ω7*c* and/or iso-*C*_{15:0} 2-OH; 28.6%), *C*_{17:1}ω8*c* (22.8%), *C*_{16:0} (14.5%), *C*_{18:1}ω7*c* (11.0%), *C*_{17:0} (6.4%), *C*_{14:0} (3.6%), *C*_{10:0} 3-OH (1.4%), *C*_{12:1} 3-OH (1.2%), *C*_{18:0} (1.0%) and *C*_{15:1}ω8*c* (1.0%). Susceptible to ampicillin (10 µg), carbenicillin (100 µg), chloramphenicol (30 µg), nalidixic acid (30 µg), novobiocin (30 µg), penicillin G (10 U) and tetracycline (30 µg); intermediately susceptible to kanamycin

Table 1. Differential characteristics between strain SA1^T, *Saccharophagus degradans* and *Teredinibacter turnerae*

Taxa: 1, strain SA1^T (data from the present study); 2, *S. degradans* 2-40^T (Ekborg *et al.*, 2005); 3, *T. turnerae* T7902^T (Distel *et al.*, 2002). +, Positive; –, negative; tr, trace amount detected (<1%); ND, no data available. All three taxa are non-fermentative, Gram-negative bacteria that require NaCl for growth, grow at 20–30 °C and pH 7–10, and are positive for oxidase and catalase.

Characteristic	1	2	3
Colony colour	Off-white to cream	Cream	Yellow
Motility/monotrichous flagella	–*	+	+
Blebs/prosthecae	+	+	–
Growth on Difco marine agar	+	+	–
Growth at/in:			
5–7% NaCl	+	+	–
8–10% NaCl	–	+	–
pH 6	–	+	+
4–10 °C	–	+	–
15 °C	+	+	–
40 °C	+	–	–
Hydrolysis of:			
Agar	+	+	–
Gelatin	+	–	ND
Tween 80	–	+	ND
Utilization of:			
Cellulose	–	+ †	+
D-Fructose	–	ND	+
Maltose	+	ND	–
D-Xylose	–	ND	+
DNA G + C content (mol%)	55.6	45.8	49–51
Fatty acids (% of total)			
C _{10:0}	tr	5 ‡	ND
C _{14:0}	3.6	15 ‡	ND
C _{16:0}	14.5	ND	ND
C _{17:0}	6.4	ND	ND
C _{10:0} 3-OH	1.4	11 ‡	ND
C _{12:1} 3-OH	1.2	6 ‡	ND
Iso-C _{16:0}	tr	37 ‡	ND
C _{16:1} ω7c and/or iso-C _{15:0} 2-OH	28.6	ND	ND
C _{17:1} ω8c	22.8	ND	ND
C _{18:1} ω7c	11.0	ND	ND

*Cells were rarely positive.

†Negative as described by González & Weiner (2000).

‡Data from González & Weiner (2000).

(30 µg). Resistant to cephalothin (30 µg), clindamycin (2 µg), colistin (10 µg), erythromycin (15 µg), gentamicin (10 µg), lincomycin (2 µg), neomycin (30 µg), oxacillin (1 µg), polymyxin B (300 U), streptomycin (10 µg) and vancomycin (30 µg). The DNA G + C content is 55.6 mol%.

The type strain, SA1^T (=BCRC 17597^T=JCM 13881^T), was isolated from shallow coastal water from Keelung, Taiwan.

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