Kangiella spongicola sp. nov., a halophilic marine bacterium isolated from the sponge Chondrilla nucula

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A novel halophilic bacterium of the genus *Kangiella* was isolated from a marine sponge collected from the Florida Keys, USA. Strain A79T, an aerobic, Gram-negative, non-motile, rod-shaped bacterium, grew in 2–15 % (w/v) NaCl, at a temperature of 10–49 °C and at pH 4.5–10. Phylogenetic analysis placed strain A79T in the family *Alcanivoraceae* in the class *Gammaproteobacteria*. Strain A79T showed 98.5 % 16S rRNA gene sequence similarity to *Kangiella japonica* KMM 3899T, 96.6 % similarity to *Kangiella koreensis* DSM 16069T and 95.6 % similarity to *Kangiella aquimarina* DSM 16071T. The major cellular fatty acids were iso-C11 : 03-OH, iso-C11 : 0, iso-C15 : 0, iso-C17 : 0 and iso-C17 : 1 \( \alpha9c \) and the G+C content of the genomic DNA was 44.9 mol%. On the basis of physiological, chemotaxonomic and phylogenetic comparisons, strain A79T represents a novel species in the genus *Kangiella*, for which the name *Kangiella spongicola* sp. nov. is proposed. The type strain is A79T (ATCC BAA-2076T = DSM 23219T).

Marine sponges are filter-feeders, and, although microorganisms are a major component of the sponge diet, several studies have shown that sponges also harbour a diverse array of bacterial species, many of which have yet to be cultivated (Hill, 2004; Hentschel et al., 2006; Taylor et al., 2007). Sponges are highly diverse and are found in nearly every aquatic habitat and play important ecological roles (Wörheide & Erpenbeck, 2007). Hentschel et al. (2006) found great differences in the density and diversity of sponge microbial communities compared with those of the immediate surrounding seawater, indicating selection for particular bacterial species by the sponge animal. Studies of the symbiotic relationship between bacteria and marine sponges suggest that many associated micro-organisms aid in the homeostasis of the sponge (Hentschel et al., 2006; Hill, 2004; Lee et al., 2001). The precise roles of the sponge microbiota, although intimately linked to the host animal, have not been clearly determined.

In this study, a novel bacterium, strain A79T, was isolated from a *Chondrilla nucula* sponge, collected from the Florida Keys, USA (Carolina Biological Supply Company), and delivered to the laboratory in plastic bags in seawater within a few days of collection. Sponge tissue was aseptically cut and ground with a mortar and pestle. The cell extract was inoculated into marine broth 2216 (37.4 g l\(^{-1}\); BD Difco; MB) and incubated at 28 °C for 7 days. A 100 μl aliquot of a 10\(^7\) dilution of this enrichment culture was spread-plated on marine agar, pH 7.6 (MA; BD Difco). Individual colonies were isolated and purified through sequential streak plating. One of the isolates, designated strain A79T, was selected (based on preliminary identification) for further analysis. Bacterial stocks were stored at −80 °C in MB containing 40 % glycerol.

Colony and cell morphology of strain A79T were observed after growth at room temperature and at 28 °C on MA. Gram staining was determined following the protocols of Murray et al. (1994). Cell morphology was observed by using a phase-contrast microscope (BX60; Olympus). The temperature range for growth was analysed after incubation for 10 days on MA at 4, 10, 27, 36 and up to 50 °C. NaCl tolerance was measured in MB (approx. 2 % NaCl) by adding up to 25 % (w/v) additional NaCl. The pH range for growth was determined in MB adjusted to pH 4.0–10.0 by addition of NaHCO\(_3\) and HCl or NaOH. Growth under anaerobic conditions was determined by incubation of strain A79T on MA and on MA with nitrate in an anaerobic chamber with an atmosphere of H\(_2\)/N\(_2\) (2 %: 98 %). Catalase activity was determined by gas production upon...
exposure to 3 % (v/v) hydrogen peroxide solution. Oxidase activity was determined by the oxidation of 1 % tetramethyl-p-phenylenediamine dihydrochloride. Hydrolysis of casein, starch, gelatin, tyrosine, aesculin, Tween 20 and Tween 80 was tested following protocols by Smibert & Krieg (1981) and Lelliott & Stead (1987). Growth in minimal media with glycogen and fructose was tested with or without 2 g L−1 peptone. Additional carbohydrate metabolism was tested by examining acid production using API 50CH tests (bioMérieux) and enzymic tests were carried out using API ZYM kits (bioMérieux) according to the manufacturer’s instructions. The medium used for these tests was artificial seawater, as used previously by Romanenko et al. (2010) for the study of Kangiella japonica. Artificial seawater, as per an original recipe from Lyman & Fleming (1940), was prepared using instructions from Kester et al. (1967). Susceptibility to antibiotics (penicillin, chloramphenicol, ampicillin, neomycin, erythromycin, tetracycline, vancomycin and gentamicin) was tested on MA by using BBL Sensi-Disc (BD Difco) antibiotic susceptibility test discs on lawns of strain A79T with Escherichia coli used as a comparison.

For analysis of cellular fatty acids, strain A79T was grown on MA at 30 °C for 7 days. Approximately 60 mg cell mass was harvested and fatty acids were saponified, methylated, extracted and analysed by GC following the protocol of the Sherlock Microbial Identification System MIDI (Sasser, 1990). Identification was confirmed by GC-MS with an Agilent Series 6890 GC system and 5973 mass-selective detector as described previously (Ahn et al., 2009).

Chromosomal DNA was isolated by using a phenol/chloroform extraction (Scala & Kerkhof, 1999). The G + C content (mol%) was determined by applying the method of Mesbah et al. (1989) with a Shimadzu LC-10AS HPLC system (Shimadzu Corp.) equipped with a Syngery 4U Fusion RP 80A C18 reverse phase column (Phenomenex). The eluent consisted of 7 % 20 mM ammonium acetate (pH 4.5) and 93 % acetonitrile with a 1 ml min−1 flow rate at 37 °C. The nucleosides were detected at a wavelength of 260 nm. Salmon sperm DNA was used for calibration and E. coli was used as a control.

For 16S rRNA gene sequence analysis, DNA was isolated using a MoBio Ultraclean Microbial DNA isolation kit. Universal primers 27F and 1525R and multiple internal primers (Lane, 1991) were used to amplify the 16S rRNA gene of strain A79T using the conditions described in Ahn et al. (2009). The PCR product was purified using a MoBio PCR Purification kit and sequenced by Genewiz, Inc. A contiguous sequence (1473 bp) was compiled from multiple internal sequencing reactions using CLUSTAL W and manually adjusted to assure accuracy. The 16S rRNA gene sequence of strain A79T was compared with those available in GenBank using the BLAST program (Altschul et al., 1990). Neighbour-joining and maximum-parsimony phylogenetic trees with 1000 bootstrap replications were reconstructed using the MEGA version 4 software package (Tamura et al., 2007) with the maximum composite likelihood model for nucleotide substitutions (Tamura et al., 2007).

Phylogenetic analysis revealed that strain A79T was a member of the family Alcanivoraceae in the class Gammaproteobacteria, with K. japonica, Kangiella aquimarina and Kangiella koreensis as its closest relatives (Fig. 1). The separate lineage of strain A79T in the genus Kangiella was supported by high bootstrap values in both the neighbour-joining and maximum-parsimony analyses. Strain A79T showed 98.5 %, 96.6 % and 95.6 % 16S rRNA gene sequence similarity with the sequences of K. japonica, K. aquimarina and K. koreensis, respectively.
The phenotypic characteristics of strain A79\textsuperscript{T} were consistent with the description of the family Acanivoraceae (Golyshin et al., 2005). Cells of strain A79\textsuperscript{T} were non-motile, Gram-staining-negative, short rods with general dimensions of 0.5–1.0 \textmu m (width) and 2.0–2.5 \textmu m (length) in 5-day-old cultures grown at 28 °C on MA. The cell morphology of the novel strain was similar to that of the three recognized species of the genus Kangiella. Colonies were beige- to cream-colored, smooth, circular and raised after 5 days of growth on MA. Strain A79\textsuperscript{T} was observed over a pH range of 4.5–10 and over a temperature range of 10–49 °C, in contrast to \textit{K. japonica}, which grew at 4 °C and at up to 42 °C. All species examined had different maximal growth temperatures (Table 1; Romanenko et al., 2010; Yoon et al., 2004). Species of the genus \textit{Kangiella} are NaCl tolerant, with weak growth of strain A79\textsuperscript{T} observed at 15 % (w/v) NaCl. The recognized species of the genus exhibited growth only up to 13 % (w/v) NaCl (Romanenko et al., 2010; Yoon et al., 2004). Anaerobic growth in the presence of nitrate was observed for strain A79\textsuperscript{T} and has also been reported for \textit{K. koreensis} and \textit{K. aquimarina}, but not for \textit{K. japonica}. Strain A79\textsuperscript{T} required peptone for growth and acid production was not observed using single carbon sources with the API 50CH test system. Strain A79\textsuperscript{T} was positive in tests for the enzymes alkaline phosphatase, leucine arylamidase, valine arylamidase, \textit{x}-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase, and distinct differences were observed compared with \textit{K. koreensis}, \textit{K. aquimarina} and \textit{K. japonica} (Table 1). The DNA G+C content of strain A79\textsuperscript{T} was 44.9 mol%, compared with 45.8 mol% for \textit{K. japonica} and 44.0 mol% for both \textit{K. koreensis} and \textit{K. aquimarina}. The predominant cellular fatty acids of strain A79\textsuperscript{T} were iso-C\textsubscript{11} : 0, iso-C\textsubscript{11} : 0 3-OH, iso-C\textsubscript{15} : 0, iso-C\textsubscript{17} : 0 and iso-C\textsubscript{17} : 1\textit{\textsuperscript{10}9c}, which followed a similar pattern to those of \textit{K. japonica}, \textit{K. koreensis} and \textit{K. aquimarina} grown under identical conditions; however, distinct differences were observed (Table 2).

On the basis of physiological, chemotaxonomic and phylogenetic comparisons, strain A79\textsuperscript{T} represents a novel species of the genus \textit{Kangiella}.
species in the genus *Kangiella*, for which the name *Kangiella spongicola* sp. nov. is proposed.

**Description of *Kangiella spongicola*, sp. nov.**

*Kangiella spongicola* [spon.gi’co.la. L. n. *spong.* -i sponge; L. suff. -cola (from L. *n. incola*) inhabitant; N.L. n. (nominative in apposition) spongicola inhabitant of a sponge].

Cells are non-motile, Gram-negative-staining, short rods with general dimensions of 0.5–1.0 μm (width) and 2.0–2.5 μm (length) in 5-day-old cultures grown at 28 °C on MA. Colonies are beige- to cream-coloured, smooth, circular and raised after 5 days of growth on MA. Abundant growth occurs in the presence of 2–10% (w/v) NaCl, and weak growth is observed at 15% NaCl. Growth is observed at 10 °C and up to 49 °C; growth occurs at pH 4.5–10. Nitrate is reduced under anaerobic conditions. Positive for oxidase and catalase and for hydrolysis of casein, tyrosine, Tween 20 and Tween 80. Negative for gelatin, aesculin and starch hydrolysis. When assayed with API ZYM (bioMérieux), alkaline phosphatase, leucine arylamidase, valine arylamidase, z-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase are present, while esterase (C4), esterase lipase (C8), lipase (C14), cystine arylamidase, trypsin, a-galactosidase, b-galactosidase, b-glucuronidase, a-glucosidase, b-glucosidase, N-acetyl-b-glucosaminidase, mannosidase and z-fucosidase are absent. Acid production is not observed with metabolism of single carbon sources provided by the API 50CH system (bioMérieux). Requires peptone and/or a complex carbohydrate source for growth. Susceptible to penicillin, chloramphenicol, ampicillin and erythromycin. The predominant fatty acids are iso-C<sub>11:0</sub> 3-OH, iso-C<sub>15:0</sub>, iso-C<sub>17:0</sub> 3-OH and iso-C<sub>17:1</sub> 10:9c.

The type strain, strain A79<sup>T</sup> (=ATCC BAA-2076<sup>T</sup> = DSM 23219<sup>T</sup>), was isolated from a *Chondrilla nucula* sponge from the Florida Keys, USA. The genomic DNA G+C content of the type strain is 44.9 mol%.

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**References**


