Salegentibacter holothuriorum sp. nov., isolated from the edible holothurian *Apostichopus japonicus*

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Strain KMM 3524^T was isolated from the holothurian *Apostichopus japonicus* living in the Sea of Japan. The bacterial strain was pigmented, non-motile, Gram-negative, strictly aerobic and oxidase-, catalase- and β-galactosidase-positive. From the results of 16S rDNA sequence analysis, strain KMM 3524^T was found to be related closely to *Salegentibacter salegens* (98·1 %). DNA–DNA homology between strains KMM 3524^T and *S. salegens* DSM 5424^T was 38 %; this showed clearly that the holothurian isolate KMM 3524^T belongs to a novel species of the genus *Salegentibacter* for which the name *Salegentibacter holothuriorum* sp. nov. is proposed, with KMM 3524^T (=NBRC 100249^T=LMG 21968^T) as the type strain.

The genus *Salegentibacter* belongs to the family *Flavobacteriaceae* of the phylum *Cytophaga–Flavobacterium–Bacteroides*. It was created by McCammon & Bowman (2000) to accommodate moderately halophilic, yellow-pigmented, non-gliding bacteria that were isolated from a hypersaline, meromictic lake in Antarctica. At present, this genus comprises a single species, *Salegentibacter salegens*, formerly *Flavobacterium salegens* (Dobson *et al.*, 1993).

An unknown marine bacterium, strain KMM 3524^T, was isolated from the edible holothurian *Apostichopus japonicus* inhabiting the Sea of Japan. The 16S rDNA sequence obtained in this study revealed that strain KMM 3524^T belongs to the genus *Salegentibacter*. DNA–DNA hybridization, phenotypic and chemotaxonomic data indicated clearly that the holothurian isolate represents a novel species of the genus *Salegentibacter*, for which the name *Salegentibacter holothuriorum* sp. nov. is proposed.

Strain KMM 3524^T was isolated aseptically from the holothurian *A. japonicus*, collected in Troitsa Bay, Gulf of Peter the Great, Sea of Japan (Pacific Ocean), from a depth of 8 m (salinity, 33 ‰; temperature, 12 °C), in November 1997. For strain isolation, 0·1 ml tissue homogenate was transferred onto plates that contained marine agar 2216 (Difco). After primary isolation and purification, strains were cultivated at 28 °C on the same medium and stored at −80 °C in marine broth (Difco) supplemented with 20 % (v/v) glycerol.

The 16S rRNA gene sequence of strain KMM 3524^T was determined by PCR amplification and direct sequencing (Hiraishi, 1992). Conditions and reagents used for PCR amplification and sequencing of 16S rDNA were as described previously (Suzuki *et al.*, 2001). The sequence determined was aligned with an alignment based on the secondary-structure model that is maintained by the European small-subunit rRNA database (Van de Peer *et al.*, 2000), by using the profile-alignment program of CLUSTAL W software (Thompson *et al.*, 1994). Evolutionary distances were then computed with the DNADIST program in the PHYLIP package, version 3.572 (Felsenstein, 1995) with the two-parameter model (Kimura, 1980); a phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987). To evaluate phylogenetic

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trees, bootstrap analysis with 1000 sample replications was performed with the SEQBOOT and CONSENSE programs in the PHYLIP package, version 3.572.

Strain KMM 3524^T showed highest 16S rDNA sequence similarity to *S. salegens* DSM 5424^T (98·1 %), indicating that strain KMM 3524^T is a member of the family *Flavobacteriaceae* and belongs to the genus *Salegentibacter* (Fig. 1).

Gram-staining reaction, hydrolysis of elastin and Tweens 20, 40 and 80, nitrate reduction, production of hydrogen sulphide and indole, β-galactosidase, oxidase, catalase and alkaline phosphatase activities were tested according to the methods of Gerhardt *et al.* (1994). The medium of Hugh & Leifson (1953), modified for marine bacteria (Lemos *et al.*, 1985), was used to test for oxidative or fermentative utilization of glucose. Degradation of agar, starch, casein, gelatin, cellulose (filter paper and CM-cellulose), chitin, DNA, urea and alginic acids, flexirubin production, growth at different pH values, production of acid from carbohydrates and susceptibility to antibiotics were tested as described previously (Nedashkovskaya *et al.*, 2003a). Growth at different temperatures and salinities was tested as described by Nedashkovskaya *et al.* (2003b). Carbon-source utilization was tested in a medium that contained 0·2 g $NaNO_3$, 0·2 g NH_4Cl, 0·05 g yeast extract (Difco) and 0·4 % (w/v) carbon source in 1000 ml artificial sea water. Carbon sources tested were arabinose, glucose, lactose, mannose, sucrose, inositol, sorbitol, mannitol, fumarate, citrate and malonate. Spreading growth was observed by cultivation on medium that contained (l^{-1}): 1 g Bacto peptone (Difco), 1 g yeast extract (Difco), 15 g agar and half-strength natural sea water under high-moisture conditions. Gliding motility was determined as described by Bowman (2000).

Physiological, morphological and biochemical characteristics of the strains studied are listed in the species description and in Table 1. Similarities in phenotypic characteristics support the inclusion of strain KMM 3524^T in the genus *Salegentibacter*. However, strain KMM 3524^T clearly differed from strains of *S. salegens* by its inability to grow in 12 % NaCl and to reduce nitrates to nitrites, its maximum growth temperature (37 °C), oxidation of lactose, fucose and *N*-acetylglucosamine, utilization of sucrose and lactose and resistance to streptomycin (Table 1).

To detect whole-cell fatty acid profiles, the strain studied was grown at 28 °C for 48 h on marine agar 2216 (Difco). Analysis of fatty acid methyl esters was performed by using GLC [30 m × 0·25 mm Supelcowax 10 column (Supelco), 205 °C] as described by Svetashev *et al.* (1995). Predominant cellular fatty acids were branched-chain saturated and unsaturated and straight-chain saturated and unsaturated, namely i15 : 0 (26·3 %), i15 : 1 (18·2 %), 15 : 0 (9·6 %), 16 : 1ω7 (10·4 %), i17 : 1 (8·0 %) and i15 : 0 2-OH (7·9 %) and corresponded with the fatty acid composition of *S. salegens* DSM 5424^T, determined under the same conditions (data not shown). Lipids were extracted according to the method of Bligh & Dyer (1959), as modified by Svetashev & Vaskovsky (1972) and Vaskovsky *et al.* (1975). The main polar lipid was phosphatidylethanolamine. Isoprenoid quinones were extracted and analysed by the method of Nakagawa & Yamasato (1993). The major lipoquinone was MK-6, a feature that is characteristic of the *Flavobacteriaceae*.

For determination of DNA G+C content and DNA–DNA binding values, DNA was prepared from cells that had been cultivated on marine agar (Difco) for 24–48 h at 25 °C, according to the DNA extraction protocol of Pitcher *et al.*

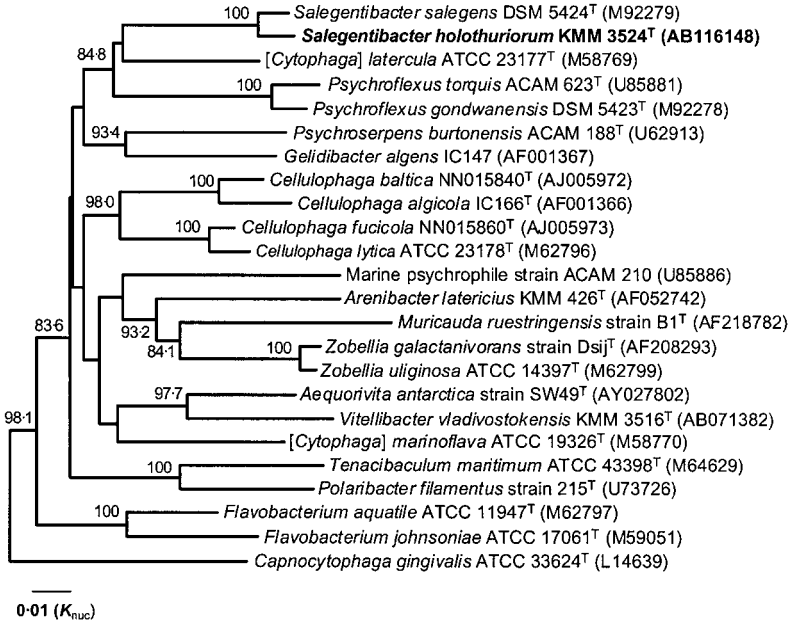

Fig. 1. Phylogenetic relationships between strain KMM 3524^T and marine species of the family *Flavobacteriaceae* on the basis of 16S rDNA sequence comparison. The phylogenetic tree was generated by the neighbour-joining method (Saitou & Nei, 1987). The 16S rDNA sequence of *Capnocytophaga gingivalis* (GenBank accession no. L14639) was used as the outgroup. The number shown next to each node indicates the percentage bootstrap value of 1000 replicates (only values of 70 % or more are shown). Bar, genetic distance of 0·01 (K_{nuc}).

Table 1. Phenotypic characteristics of members of the genus *Salegentibacter*

Strains: 1, *S. holothuriorum* KMM 3524^T; 2, *S. salegens* DSM 5424^T. Both strains gave positive results in tests for the following characteristics: respiratory metabolism; oxidase, catalase, β-galactosidase and alkaline phosphatase activities; hydrolysis of Tweens 20, 40 and 80, gelatin, elastin, starch, alginic acids and DNA; requirement for NaCl for growth; growth at 34 °C and in 8 % NaCl; acid formation from galactose, glucose and maltose; utilization of glucose and mannose; H_2S production; susceptibility to ampicillin, benzylpenicillin, carbenicillin, lincomycin, oleandomycin and tetracycline; resistance to gentamicin, kanamycin, neomycin and polymyxin B. Strains KMM 3524^T and DSM 5424^T both gave negative results results in tests for the following characteristics: motility by gliding; hydrolysis of agar, casein, cellulose (CM-cellulose and filter paper), urea and chitin; acid production from arabinose, cellobiose, melibiose, rhamnose, sucrose, sorbose, xylose, succinate, citrate, glycerol, adonitol, dulcitol, sorbitol, inositol and mannitol; utilization of arabinose, inositol, mannitol, sorbitol, malonate and citrate; indole and acetoin (Voges–Proskauer reaction) production.

Characteristic	1	2
Nitrate reduction	−	+
Growth at/in:		
37 °C	+	−
12 % NaCl	−	+
Acid from:		
Lactose	+	−
Fucose	+	−
N-Acetylglucosamine	+	−
Utilization of:		
Sucrose	−	+
Lactose	+	−
Susceptibility to streptomycin	−	+
DNA G+C content (mol%)	36·8	36·7

(1989) as modified by Leisner *et al.* (2002). The G+C content was determined [by using the HPLC method of Mesbah *et al.* (1989)] to be 36·8 mol%, a value that is analogous to that described for *S. salegens*. DNA–DNA hybridizations were performed by using the microplate method and fluorescence measurements for calculation of binding values, as described by Ezaki *et al.* (1989). Hybridizations between strains KMM 3524^T and *S. salegens* DSM 5424^T were performed at 35 °C in a hybridization mixture (2× SSC, 5× Denhardt's solution, 2·5 % dextran sulphate, 50 % formamide, 100 μg denaturated salmon sperm DNA ml^{-1}, 1250 ng biotinylated probe DNA ml^{-1}) and yielded a relatedness value of 38 %.

We can conclude that the genomic data, supported by phenotypic findings and chemotaxonomic characteristics, clearly classify strain KMM 3524^T in the genus *Salegentibacter* as the type strain of a novel species, for which we propose the name *Salegentibacter holothuriorum* sp. nov.

Description of *Salegentibacter holothuriorum* sp. nov.

Salegentibacter holothuriorum (ho.lo.thu.ri.o′rum. N.L. gen. pl. n. *holothuriorum* of holothurians, sea cucumbers; bacterium isolated from holothurians).

Cells are Gram-negative, strictly aerobic, chemo-organotrophic, non-motile, asporogenic rods, 0·5–0·7 μm wide and 2·7–5·3 μm long. Oxidase-, catalase-, β-galactosidase- and alkaline phosphatase-positive. Colonies are circular, convex, shiny with entire edges and 1–3 mm in diameter on marine agar 2216. Yellow, non-diffusible pigments are produced. No growth is observed without Na^+. Growth occurs in 1–8 % NaCl. Flexirubin pigments are absent. Growth is detected at 4 and 37 °C. Gelatin, starch, alginic acids, DNA and Tweens 20, 40 and 80 are hydrolysed, but agar, casein, cellulose (CM-cellulose and filter paper), chitin and urea are not. Acid is formed from galactose, glucose, lactose, maltose, fucose and *N*-acetylglucosamine, but not from arabinose, cellobiose, melibiose, raffinose, rhamnose, sorbose, sucrose, xylose, adonitol, dulcitol, glycerol, inositol, sorbitol or mannitol. Utilizes glucose, lactose and mannose, but not arabinose, sucrose, inositol, sorbitol, mannitol, citrate or malonate. H_2S is produced. Nitrate is not reduced. No indole or acetoin (Voges–Proskauer reaction) is produced. Susceptible to ampicillin, benzylpenicillin, carbenicillin, oleandomycin, lincomycin and tetracycline. Resistant to kanamycin, neomycin, streptomycin, gentamicin and polymyxin B. Predominant cellular fatty acids are i15 : 0, i15 : 1, 15 : 0, 16 : 1ω7, i17 : 1 and i15 : 0 2-OH. Major isoprenoid quinone is MK-6. Main polar lipid is phosphatidylethanolamine. DNA G+C content is 36·8 mol%.

The type strain is KMM 3524^T (=NBRC 100249^T=LMG 21968^T). Isolated from the holothurian *Apostichopus japonicus* living in the Sea of Japan.

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