Arenicella chitinivorans sp. nov., a gammaproteobacterium isolated from the sea urchin *Strongylocentrotus intermedius*

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A strictly aerobic, Gram-stain-negative, rod-shaped, non-motile and yellow-pigmented bacterial strain, designated KMM 6208^T, was isolated from a sea urchin. Phylogenetic analysis based on 16S rRNA gene sequencing revealed that this novel isolate was affiliated to the class *Gammaproteobacteria* and formed a robust cluster with *Arenicella xantha* KMM 3895^T with 98.2 % 16S rRNA gene sequence similarity. Strain KMM 6208^T grew in the presence of 0.5–5 % NaCl and at a temperature range of 4–38 °C. The isolate was oxidase-positive and hydrolysed aesculin, casein, chitin, gelatin, starch and Tweens 40 and 80. The prevalent fatty acids of strain KMM 6208^T were $C_{16:1}\omega7c$, iso-$C_{16:0}$, iso-$C_{18:0}$, $C_{18:1}\omega7c$ and $C_{16:0}$. The polar lipids consisted of phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and an unidentified aminophospholipid, and the major isoprenoid quinone was Q-8. The DNA G+C content of strain KMM 6208^T was 46.3 mol%. The DNA–DNA relatedness value of strain KMM 6208^T with *Arenicella xantha* KMM 3895^T was 5 %. Molecular data in a combination with phenotypic findings strongly suggest inclusion of this novel strain in the genus *Arenicella* as a representative of a novel species for which the name *Arenicella chitinivorans* sp. nov. is proposed. The type strain is KMM 6208^T (=KCTC 12711^T=LMG 26983^T).

The genus *Arenicella* was proposed by Romanenko *et al.* (2010) to accommodate chemo-organoheterotrophic, strictly aerobic, Gram-negative, oxidase- and catalase-positive, rod-shaped, non-motile and yellow-pigmented bacteria. The type and only strain of the sole species *Arenicella xantha*, designated KMM 3895^T, was isolated from a sandy sediment sample collected from the Sea of Japan and formed a distinct evolutionary lineage within the class *Gammaproteobacteria* with 87–89.5 % 16S rRNA gene sequence similarity to the phylogenetic neighbours belonging to the genera *Alcanivorax*, *Kangiella*, *Microbulbifer*, *Nitrincola* and *Spongiibacter*.

In the course of a taxonomic survey of the microbial community of the edible sea urchin *Strongylocentrotus intermedius*, a strictly aerobic, Gram-stain-negative, rod-shaped, non-motile and yellow-pigmented bacterial isolate, designated KMM 6208^T, was obtained. The results of the phylogenetic analysis indicated that its closest relative was *Arenicella xantha* KMM 3895^T with 98.2 % 16S rRNA gene sequence similarity. Other close relatives of the novel isolate were uncultivated bacteria associated with the brown alga *Saccharina japonica* collected from the Sea of Japan with 98.0–98.4 % 16S rRNA gene sequence identity (Balakirev *et al.*, 2012). It is interesting that the adult sea urchins of the genus *Strongylocentrotus* often feed on macrophytes, among these the kelps are prevalent (Lawrence, 2007). The taxonomic position of strain KMM 6208^T was further investigated using a polyphasic approach.

Strain KMM 6208^T was isolated from the sea urchin *Strongylocentrotus intermedius* collected in September 2002

Abbreviation: FAME, fatty acid methyl ester.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Arenicella echinivorans* KMM 6208^T is KC136313.

at the G.B. Elyakov Pacific Institute of Bioorganic Chemistry Marine Experimental Station, Troitza Bay, Gulf of Peter the Great, Sea of Japan by a standard dilution plating method. The sample of tissues (5 g) was homogenized in 10 ml sterile seawater in a glass homogenizer and 0.1 ml homogenate was spread onto marine agar 2216 (MA, Difco) plates. The novel isolate was obtained from a single colony after incubation of the plate at 28 °C for 7 days. After primary isolation and subsequent purification, the isolate was cultivated at 28 °C on the same medium and stored at −80 °C in marine broth (Difco) supplemented with 20 % (v/v) glycerol.

DNA extraction, PCR and 16S rRNA gene sequencing were carried out as described previously (Vancanneyt *et al.*, 2006). The 16S rRNA gene sequence of the novel isolate and of sequences of phylogenetically related species retrieved from the GenBank database were aligned against the SILVA reference database (http://www.arb-silva.de) using Mothur v 1.29.2 (Schloss *et al.*, 2009). Empty vertical columns were removed and phylogenetic analyses were performed using the MEGA5 software package (Tamura *et al.*, 2011). Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1985) methods with bootstrap analysis to estimate the reliability of the clusters. The phylogenetic analysis revealed that the novel isolate was a member of the class *Gammaproteobacteria* of the phylum *Proteobacteria* and formed a coherent cluster with *Arenicella xantha* KMM 3895^T (Fig. 1) with a 16S rRNA gene sequence similarity of 98.2 %.

Genomic DNA for DNA G+C content determination was isolated following the method of Marmur (1961). A value of 46.3 mol% was obtained for strain KMM 6208^T by the thermal denaturation method (Marmur & Doty, 1962), which is close to that reported for *Arenicella xantha* KMM 3895^T (48.1 mol%; Romanenko *et al.*, 2010). DNA for DNA–DNA hybridizations was isolated according to a modification (Cleenwerck *et al.*, 2002) of the procedure reported by Wilson (1987). DNA–DNA hybridization between strain KMM 6208^T and *Arenicella xantha* KMM 3895^T was performed in the presence of 50 % formamide at 42 °C according to a modification (Cleenwerck *et al.*, 2002; Goris *et al.*, 1998) of the method described by Ezaki *et al.* (1989). With a DNA–DNA relatedness value of 5 %, the strains were clearly proven to be members of different species of the genus *Arenicella* (Wayne *et al.*, 1987).

For whole-cell fatty acid and polar lipid analysis strain KMM 6208^T and *Arenicella xantha* KMM 3895^T were grown under optimal conditions for 48 h at 28 °C on MA. Cellular fatty acid methyl esters (FAMEs) were prepared according to the methods described by Sasser (1990) using the standard protocol of the Sherlock Microbial Identification System, version 6.0 (MIDI) and analysed using a GC-21A chromatograph (Shimadzu) equipped with a fused silica capillary column (30 m × 0.25 mm) coated with Supercowax-10 and SPB-5 phases (Supelco) at 210 °C. FAMEs were identified using equivalent chain-length measurements and by comparing the retention times to those of authentic standards. FAMEs were also analysed by GC–MS (QP5050A; Shimadzu) equipped with an MDN-5S capillary column (30 m × 0.25 mm), the temperature program ranged from 140 to 250 °C, at a rate of 2 °C min^{-1}. The fatty acid profile of strain KMM 6208^T consisted of $C_{16:1}\omega7c$ (24.7 %), iso-$C_{16:0}$ (16.8 %), iso-$C_{18:0}$ (15.8 %), $C_{18:1}\omega7c$ (11.9 %) and $C_{16:0}$ (6.4 %) as predominant components (Table 1) and was similar to that of *Arenicella xantha* KMM 3895^T, although there were differences in the proportions of some fatty acids. The high resemblance in fatty acid compositions of the two strains supported the inclusion of strain KMM 6208^T in the genus *Arenicella*. Polar lipids of strain KMM 6208^T and *Arenicella xantha* KMM 3895^T were extracted using the chloroform/methanol extraction method of Bligh & Dyer (1959). Two-dimensional TLC of polar lipids was carried out on silica gel 60 F254 (10 × 10 cm; Merck) using chloroform/methanol/water (65 : 25 : 4, by vol.) in the first dimension and chloroform/methanol/acetic acid/water (80 : 12 : 15 : 4, by vol.) in the second dimension (Collins & Shah, 1984). The spray reagents used to reveal the spots were phosphomolybdic acid, ninhydrin and 10 % sulfuric acid in ethanol. Isoprenoid quinones were extracted with chloroform/methanol (2 : 1, v/v) and purified by TLC, using a mixture of *n*-hexane and diethyl ether (85 : 15, v/v) as the solvent. The identified polar lipids of strain KMM 6208^T were phosphatidylethanolamine phosphatidylglycerol and diphosphatidylglycerol, and there was also an unidentified aminophospholipid (Fig. 2). The polar lipid profile of the novel isolate was in line with that of *Arenicella xantha* KMM 3895^T, except that the latter contains an additional unidentified phospholipid. The isoprenoid quinone composition of strain KMM 6208^T was characterized by HPLC (Shimadzu LC-10A) using a reversed-phase type Supelcosil LC-18 column (15 cm × 4.6 mm) and acetonitrile/2-propanol (65 : 35, v/v) as a mobile phase at a flow rate of 0.5 ml min^{-1} as described previously (Komagata & Suzuki, 1987). The column was kept at 40 °C. Ubiquinones were detected by monitoring absorbance at 275 nm. Ubiquinone Q-8 was the major respiratory quinone of strain KMM 6208^T, which is also the case in *Arenicella xantha* KMM 3895^T (Romanenko *et al.*, 2010).

Cell morphology was analysed with light microscopy (CX41; Olympus) and transmission electron microscopy (Libra 120; Zeiss) using cells grown for 24, 48, 72 and 96 h on MA at 28 °C. Gram-staining was done as described by Gerhardt *et al.* (1994). Oxidative or fermentative utilization of glucose was determined on Hugh & Leifson's medium modified for marine bacteria (Lemos *et al.*, 1985). Catalase activity was tested by the addition of 3 % (v/v) H_2O_2 solution to a bacterial colony and monitoring for the appearance of gas. Oxidase activity was determined by assessing the oxidation of tetramethyl-*p*-phenylenediamine. Degradation of agar, starch, casein, gelatin, chitin,

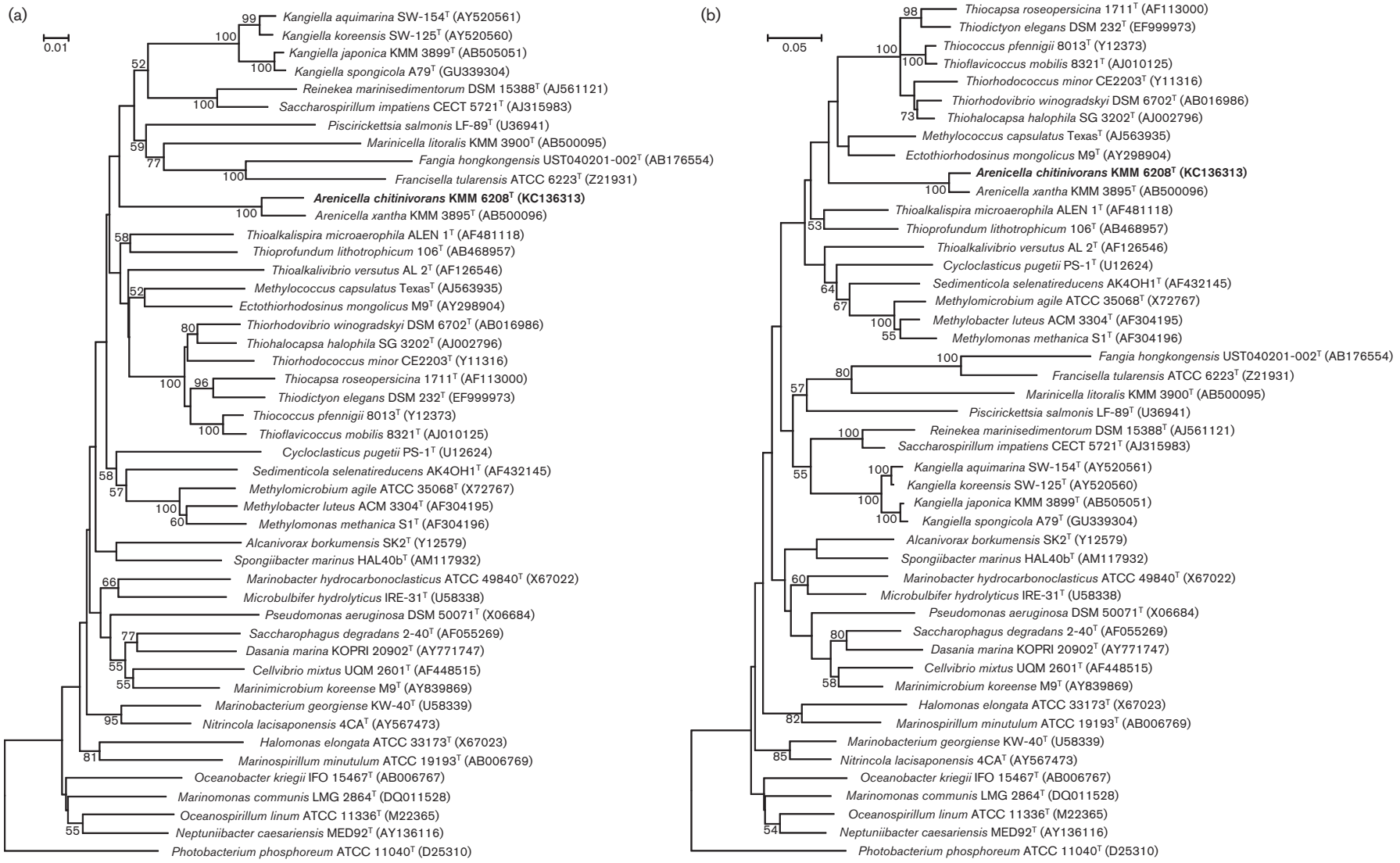

Fig. 1. Neighbour-joining (a) and maximum-likelihood (b) trees based on almost complete 16S rRNA gene sequences showing the phylogenetic position of strain KMM 6208^T among related members of the *Gammaproteobacteria*. Numbers at nodes are bootstrap percentage values based on 1000 resampled datasets; only values >50 % are shown. Bar, 1 nt (a) and 5 nt (b) substitutions per 100 nt.

Table 1. Fatty acid composition of strain KMM 6208^T and *Arenicella xantha* KMM 3895^T

Strains: 1, KMM 6208^T; 2, *A. xantha* KMM 3895^T. All data are from this study. Values are percentages of total fatty acids; those fatty acids for which the mean amount in both taxa was less than 1 % are not given. The predominant fatty acids are indicated by bold type. TR, Trace amount (<1 %).

Fatty acids	1	2
$C_{16:0}$	**6.4**	**8.2**
$C_{17:0}$	1.2	1.0
$C_{18:0}$	1.1	TR
$C_{14:1}\omega5c$	3.0	3.6
$C_{15:1}\omega8c$	1.3	TR
$C_{16:1}\omega7c$	**24.7**	**25.7**
$C_{17:1}\omega8c$	3.7	2.8
$C_{18:1}\omega7c$	**11.9**	**16.7**
iso-$C_{14:0}$	2.1	1.8
iso-$C_{15:1}$	1.1	TR
iso-$C_{16:0}$	**16.8**	**16.6**
iso-$C_{17:1}$	3.7	2.2
iso-$C_{18:0}$	**15.8**	**14.2**
iso-$C_{18:1}$	3.2	1.6
anteiso-$C_{18:1}$	TR	1.4

DNA and urea together with production of acid from carbohydrates, hydrolysis of Tweens 20, 40 and 80, nitrate reduction, production of hydrogen sulphide and indole were tested according to standard methods (Gerhardt *et al.* 1994). The temperature range for growth was assessed in MA. Tolerance to NaCl was assessed in medium A containing 5 g Bacto Peptone (Difco), 2 g Bacto Yeast Extract (Difco), 1 g glucose, 0.2 g KH_2PO_4 and 0.05 g $MgSO_4 . 7H_2O$ l^{-1} distilled water with 0, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12 and 15 % (w/v) NaCl. The pH range for growth was determined at pH 4.0–10.0 (at intervals of 0.5 pH units) in MB. Physiological and biochemical properties of strain KMM 6208^T were also tested using standardized API 20E, API 20NE, API 50CH and API ZYM galleries (bioMérieux) with incubation at 28 °C according to the manufacturer's instructions, except that cells were suspended in 2 % (w/v) NaCl solution. Carbon source utilization was tested using commercial API 20E, API 20NE and API 32GN (bioMérieux) identification strips and using a medium that contained 1 g $NaNO_3$, 1 g NH_4Cl, 0.5 g yeast extract (Difco) and 0.4 % (w/v) carbon source l^{-1} artificial seawater that contained 27.5 g NaCl, 5 g $MgCl_2$, 2 g $MgSO_4 . 7H_2O$, 0.5 g $CaCl_2$, 1 g KCl and 0.01 g $FeSO_4 . 7H_2O$ l^{-1} distilled water. Susceptibility to antibiotics was examined by the routine diffusion plate method. Discs were impregnated with the following antibiotics (μg per disc unless otherwise stated): ampicillin (10), benzylpenicillin (10 U), carbenicillin (100), cefalexin (30), cefazolin (30), chloramphenicol (30), erythromycin (15), doxycycline (10), gentamicin (10), kanamycin (30), lincomycin (15), oleandomycin (15), nalidixic acid (30), neomycin (30), ofloxacin (5), oxacillin (10), polymyxin B (300 U), rifampicin (5), streptomycin (30), tetracycline (5) and vancomycin (30). *Arenicella xantha* KMM 3895^T was also included as a reference strain in the phenotypic analysis.

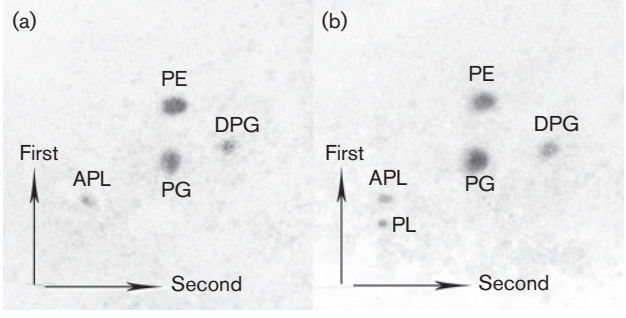

Fig. 2. Two-dimensional TLC of the total polar lipids of strain KMM 6208^T (a) and *Arenicella xantha* KMM 3895^T (b). First dimension, chloroform/methanol/water (65 : 25 : 4, by vol.); second dimension, chloroform/methanol/acetic acid/water (80 : 12 : 15 : 4, by vol.). For detection of the polar lipids, phosphomolybdic acid (for PG, DPG, PE, PL and APL) and ninhydrin (for PE and APL) were applied. DPG, diphosphatidylglycerol, PG, phosphatidylglycerol, PE, phosphatidylethanolamine, PL, unidentified phospholipid, APL, unidentified aminophospholipid.

Morphological, physiological and biochemical characteristics of KMM 6208^T are given in the species description and in Table 2. Cells of KMM 6208^T were Gram-stain-negative, strictly aerobic, non-motile rods and formed yellow-pigmented colonies on MA. Strain KMM 6208^T and its closest relative, *Arenicella xantha* KMM 3895^T, shared many phenotypic features, although they clearly differed from each other by the ability to hydrolyse chitin (positive for strain KMM 6208^T, negative for *Arenicella xantha* KMM 3895^T) and Tween 20 (negative for strain KMM 6208^T, positive for *Arenicella xantha* KMM 3895^T), their utilization of several carbohydrates, a set of enzyme activities and their susceptibilities to antibiotics (Table 2). In addition, strain KMM 6208^T could be distinguished from its closest relative by a higher maximum growth temperature (38 vs 35 °C) and a lower DNA G+C content (46.3 vs 48.1 mol%).

Therefore, based on the results of this taxonomic study using a polyphasic approach, in which significant molecular differences along with phenotypic and genotypic distinctiveness between the sea urchin isolate and *Arenicella xantha* KMM 3895^T were revealed, it is concluded that strain KMM 6208^T represents a novel species of the genus *Arenicella*, for which the name *Arenicella chitinivorans* sp. nov. is proposed.

Description of *Arenicella chitinivorans* sp. nov.

Arenicella chitinivorans (chi.ti.ni.vo′rans. N.L. neut. n. *chitinum* chitin; L. part. adj. *vorans* devouring; N.L. part. adj. *chitinivorans* chitin-devouring).

Table 2. Differential characteristics between strain KMM 6208^T and *Arenicella xantha* KMM 3895^T

Both strains were positive for respiratory type of metabolism; presence of oxidase, catalase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin and β-glucosidase activities; hydrolysis of aesculin, casein, gelatin, starch and Tweens 40 and 80; utilization of arabinose, glucose and L-alanine; susceptibility to cefalexin, chloramphenicol, erythromycin, gentamicin, nalidixic acid, neomycin, ofloxacin, oleandomycin, rifampicin and streptomycin; resistance to cefazolin, doxycycline, kanamycin, lincomycin, polymyxin and tetracycline. Both strains were negative for motility; nitrate reduction; hydrolysis of agar, urea and DNA; acid production from L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, lactose, mannose, melibiose, raffinose, L-rhamnose, ribose, sorbose, sucrose, xylose, *N*-acetylglucosamine, glycerol, inositol, mannitol, sorbitol and citrate; utilization of lactose, raffinose, sorbitol, *N*-acetylglucosamine, L-histidine, L-leucine, DL-methionine, L-phenylalanine, L-tryptophan, adipate, caprate, citrate, gluconate, malate, malonate and phenylacetate; presence of lipase (C14), cystine arylamidase, α-galactosidase, β-glucuronidase, α-glucosidase, *N*-acetylglucosaminidase, α-mannosidase and α-fucosidase activities; H_2S, indole and acetoin production. All data are from this study except where indicated otherwise. +, Positive; −, negative.

Characteristic	KMM 6208^T	*A. xantha* KMM 3895^T
Source of isolation	Sea urchin	Sandy sediment
Temperature range for growth (°C)	4–38	5–35
Salinity range (% NaCl)	0.5–5	1–5
Hydrolysis of:		
Chitin	+	−
Tween 20	−	+
Utilization of:		
Galactose, maltose, mannose, rhamnose, sucrose	+	−
Melibiose	−	+
Inositol, mannitol	+	−
Enzyme activity		
α-Chymotrypsin, β-galactosidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase	+	−
Susceptibility to:		
Ampicillin, benzylpenicillin, carbenicillin, oxacillin, vancomycin	−	+
DNA G+C content (mol%)	46.3	48.1

*Data from Romanenko *et al.* (2010)

Cells are 0.5–0.6 μm in diameter and 2.1–3.3 μm in length, Gram-stain-negative, strictly aerobic, rod-shaped and non-motile. On marine agar, colonies are 1–2 mm in diameter, circular, with entire edges, shiny and deep-yellow. Growth occurs at 4–38 °C (optimum, 25–28 °C), at pH 5.5–10.5 (optimum, pH 8.0) and with 0.5–5 % NaCl (optimum, 1.5–3.0 %). Catalase and oxidase activities are present. Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase activities are absent. Aesculin, casein, chitin, gelatin, starch and Tweens 40 and 80 are hydrolysed but agar, urea, DNA and Tween 20 are not. Acid is not produced from L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, mannose, melibiose, raffinose, L-rhamnose, ribose, sorbose, sucrose, D-xylose, *N*-acetylglucosamine, glycerol, inositol, mannitol, sorbitol or citrate. L-Arabinose, cellobiose, D-galactose, D-glucose, maltose, mannose, L-rhamnose, sucrose, inositol and mannitol are utilized, but lactose, melibiose, raffinose, trehalose, D-xylose, sorbitol, *N*-acetylglucosamine, phenylalanine, adipate, caprate, citrate, gluconate, malate, malonate and phenylacetate are not. Growth is observed on L-alanine, L-asparagine, glutamic acid, L-proline, L-threonine, L-tyrosine and L-valine but not on L-histidine, L-leucine, L-phenylalanine, DL-methionine or L-tryptophan. None of substrates of the API 32GN gallery are assimilated. In the API ZYM gallery, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase and β-glucosidase activities are present; but lipase (C14), cystine arylamidase, α-galactosidase, β-glucuronidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are absent. Nitrate is not reduced to nitrite. Hydrogen sulphide, indole and acetoin are not produced. Susceptible to (μg per disc unless otherwise indicated) cefalexin (30), chloramphenicol (30), erythromycin (15), gentamicin (10), nalidixic acid (30), neomycin (30), ofloxacin (5), oleandomycin (15), rifampicin (5) and streptomycin (30); and resistant to ampicillin (10), benzylpenicillin (10 U), carbenicillin (100), cefazolin (30), doxycycline (10), kanamycin (30), lincomycin (15), oxacillin (10), polymyxin B (300 U), tetracycline (5) and vancomycin (30). The prevalent fatty acids are $C_{16:1}\omega7c$, iso-$C_{16:0}$, iso-$C_{18:0}$, $C_{18:1}\omega7c$ and $C_{16:0}$. The polar lipid profile consists of phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and an unidentified aminophospholipid. The major respiratory quinone is Q-8.

The type strain, KMM 6208^T (=KCTC 12711^T=LMG 26983^T), was isolated from the sea urchin

Strongylocentrotus intermedius collected from the Troitsa Bay, Sea of Japan, Pacific Ocean, Russia. The DNA G+C content of the type strain is 46.3 mol%.

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