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Characterization of *Pseudoalteromonas distincta*-like sea-water isolates and description of *Pseudoalteromonas aliena* sp. nov.

Elena P. Ivanova,[1,2] Nataliya M. Gorshkova,[2] Natalia V. Zhukova,[3] Anatolii M. Lysenko,[4] Elena A. Zelepuga,[2] Nina G. Prokof'eva,[2] Valery V. Mikhailov,[2] Dan V. Nicolau[1] and Richard Christen[5]

Correspondence
Elena P. Ivanova
eivanova@swin.edu.au

[1]Industrial Research Institute Swinburne, Swinburne University of Technology, PO Box 218, Hawthorn, Victoria 3122, Australia

[2]Pacific Institute of Bioorganic Chemistry of the Far-Eastern Branch of the Russian Academy of Sciences, 690022 Vladivostok, Prospekt 100 Let Vladivostoku 159, Russia

[3]Institute of Marine Biology of the Far-Eastern Branch of the Russian Academy of Sciences, 690041 Vladivostok, Russia

[4]Institute of Microbiology of the Russian Academy of Sciences, 117811 Moscow, Russia

[5]UMR6543 CNRS–Université de Nice Sophia Antipolis, Centre de Biochimie, Parc Valrose, F06108 Nice cedex 2, France

Seven melanogenic *Pseudoalteromonas distincta*-like strains, KMM 3562^T, KMM 3536, KMM 3537, KMM 3538, KMM 3539, KMM 3615 and KMM 3629, which expressed tyrosinases were isolated from sea-water samples collected from different locations in Amursky Bay (Sea of Japan, Pacific Ocean) and characterized to clarify their taxonomic position. By 16S rRNA gene sequence analysis, the bacteria were shown to belong to the genus *Pseudoalteromonas*. The G+C content of the DNAs of the strains was 41–43 mol%. The level of DNA similarity among these strains was conspecific (92–97 %), indicating that they represented a single genospecies. However, DNA from the strains isolated from sea water showed only 63–65 % genetic relatedness with the DNA of the type strain *P. distincta*. The novel organisms grew mainly between 4 and 30 °C, were neutrophilic and slightly halophilic (four strains had a narrow range of growth between 3 and 6 % NaCl, w/v), were haemolytic and cytotoxic and were able to degrade starch, gelatin and Tween 80. The predominant fatty acids, including 16 : 0, 16 : 1ω7, 17 : 1ω7 and 18 : 1ω7, were typical of the genus *Pseudolateromonas*. The phylogenetic, genetic and physiological properties of the seven strains placed them within a novel species, *Pseudoalteromonas aliena* sp. nov., the type strain of which is SW19^T (=KMM 3562^T=LMG 22059^T).

Marine, aerobic, heterotrophic bacteria of the genus *Pseudoalteromonas* (Gauthier *et al.*, 1995) have recently received significant levels of attention because they are readily cultivated and are widespread in the marine environment. *Pseudoalteromonas* is one of the largest genera within the γ-*Proteobacteria* and currently comprises more than 30 species (Ivanova & Mikhailov, 2001).

During the course of our studies on the biodiversity of marine *Proteobacteria*, we isolated a collection of *Alteromonas*-like bacteria from the North-West Pacific (Ivanova *et al.*, 1996, 1998, 2000b, 2002). Of these, seven strains were phenotypically similar to *Pseudoalteromonas distincta*. Since the description of the species *P. distincta* was based on a single strain isolated from a marine sponge collected at a depth of 350 m near the Komandorskie Islands, Russia (Romanenko *et at.*, 1995; Ivanova *et al.*, 2000b), detailed study of the novel isolates originating from sea water was of particular interest.

The strains examined in this study were isolated from a few samples of sea water collected in June 1999 at a depth of 0–1 m (salinity, 31 ‰; temperature, 10 °C) in Amursky Bay (near Vladivostok, Russia) of the Sea of Japan. The strains

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The GenBank accession number for the 16S rRNA gene sequence of *Pseudoalteromonas aliena* KMM 3562^T is AY387858.

A phylogenetic tree, a comparison of fatty acid compositions and details of the phenotypic features that distinguish *P. aliena* sp. nov. from most other phylogenetically related species of the genus are available as supplementary material in IJSEM Online.

were isolated by plating (0·1 ml) on agar plates of marine agar 2216 (Difco) and on plates with medium B. Medium B contained 0·2 % (w/v) Bacto peptone (Difco), 0·2 % (w/v) casein hydrolysate (Merck), 0·2 % (w/v) Bacto yeast extract (Difco), 0·1 % (w/v) glucose, 0·02 % (w/v) KH_2PO_4, 0·005 % (w/v) $MgSO_4.7H_2O$, 1·5 % (w/v) Bacto agar (Difco), 50 % (v/v) natural sea water and 50 % (v/v) distilled water at pH 7·5–7·8, as described elsewhere (Ivanova *et al.*, 1996). After initial isolation, the strains were purified on medium B and maintained on the same semi-solid medium B in tubes under mineral oil at 4 °C and stored at −80 °C in marine broth (Difco) supplemented with 30 % (v/v) glycerol. All isolates were streaked on agar plates from broth cultures every 6 months to ensure purity and viability.

The following physiological and biochemical properties were examined: oxidation/fermentation of glucose, denitrification (Azegami *et al.*, 1987), oxidase and catalase activity, gelatin liquefaction, arginine dihydrolase activity, lysine decarboxylase activity, ornithine decarboxylase activity, sodium requirement (NaCl at 0, 0·5, 1, 3, 6, 8 and 10 %, w/v), indole and H_2S production and the ability to hydrolyse starch, Tween 80 and casein (determined using methods described by Smibert & Krieg, 1994). Growth at different temperatures was determined in marine broth 2216 (Difco) and on plates with medium B after 24–72 h at 2, 4, 6, 29, 30, 31, 32 and 33 °C. Degradation of macromolecules by the strains was tested using medium B. Chitin (1 %, w/v) and alginate (sodium salt) (0·1 %, w/v) hydrolysis was determined by assessing the development of clear zones around the colonies. The Biolog GN test kit was also used to examine the primary oxidation of 95 carbon compounds, as described elsewhere (Ivanova *et al.*, 1998). Susceptibility to antibiotics was tested by using the conventional diffusion plate technique, using solid medium B and discs impregnated with antibiotics. Antibacterial activity was determined by using the agar diffusion assay, based on the method described by Barry (1980). Haemolysis was tested on Merck blood agar plates (6·5 % sheep blood). Cytotoxicity on Ehrlich ascites carcinoma cells was tested on butanol extracts of the strains (Sasaki *et al.*, 1985). Bacterial cells from a 100 ml culture were extracted with butanol (100 ml) and evaporated under reduced pressure. The method described by Prokof'eva *et al.* (2003) was employed to study haemolytic activity. Briefly, an erythrocyte suspension was prepared in 66 mM PBS (120 mM NaCl, 4 mM KCl, pH 7·4). A concentration of erythrocytes giving an optical density [OD_{700}; Specord M40 UV spectrophotometer (Zeiss); cuvette path length, 1 cm] of 1·0 for non-haemolysed samples was used. The haemolytic activity of a sample was expressed as the concentration causing 50 % haemolysis.

Phenotypic analysis showed that all isolates were essentially identical to each other, Gram-negative, strictly aerobic, oxidase- and catalase-positive, did not produce H_2S or indole and were negative for denitrification. The novel isolates did not show antibacterial activity, but demonstrated a cytotoxic effect on tumour cells [inhibitor concentration needed to inhibit viability of 50 % of tumour cells (IC_{50}) = 80–100 $\mu g\ ml^{-1}$]. All of the novel strains grew well between 4 and 29 °C, in contrast to *P. distincta*, which grew between 6 and 33 °C. The novel organisms were also less halophilic, tolerating only limited salinity for growth; four of seven isolates grew on nutrient media containing only 3–6 % (w/v) NaCl, whereas *P. distincta* tolerated up to 10 % (w/v) NaCl (though, according to the original description, *P. distincta* only tolerated NaCl concentrations of up to 6 %, w/v). Optimum growth occurred at 23 °C on nutrient media containing 3 % (w/v) NaCl. Unlike *P. distincta*, the seven organisms studied were haemolytic, cytotoxic and hydrolysed starch and casein. In addition, the novel isolates and *P. distincta* had different antibiotic-susceptibility patterns. For example, *P. distincta* was susceptible to kanamycin and neomycin but not susceptible to carbenicillin – the opposite of the pattern for the sea-water strains. The results of examination of the morphological and physiological properties are shown in Table 1 and in the species description (see also the Supplementary Table available in IJSEM Online).

To study the cellular fatty acid composition, all strains were grown on solid medium B (described above) at 28 °C for 24 h. Fatty acids were analysed by GLC as described previously by Svetashev *et al.* (1995). The fatty acid patterns of the five strains studied were very similar and were also very like those found for other *Pseudoalteromonas* species (Ivanova *et al.*, 2000a). The levels of the major fatty acids ranged from 4·8 to 7·4 % for 15 : 0, from 7·2 to 13·8 % for 16 : 0, from 29·2 to 28·8 % for 6 : 1(n-7), from 19·7 to 23·5 % for 17 : 1(n-8) and from 1·1 to 1·6 % for 18 : 1(n-7). Cluster analysis based on cellular fatty acid profiles was performed by using STATISTICA for Windows, release 5.1 (StatSoft). An unweighted pair group averages method was used for cluster analysis, and a diagram was drawn in Euclidian distance scale as recommended by MIDI (Sasser, 1997). The novel isolates, *P. distincta* and other species of the genus *Pseudoalteromonas* formed a few clusters at Euclidian distances from 10 to 15, which related them at the 'genus' level (see Supplementary Fig. A in IJSEM Online). The novel sea-water isolates grouped within a lower Euclidian distance, indicating that they belong to the same species.

Production of pigment was studied in media B and BT (medium B supplemented with 0·1 % L-tyrosine, w/v). Synthetic medium of the following composition (w/v) was also used: 0·05 % KH_2PO_4, 0·05 % K_2HPO_4, 0·005 % $MgSO_4.7H_2O$, 0·06 % $CuSO_4$, 0·005 % $CaCl_2$, 0·002 % mg L-methionine, 0·002 % DL-tryptophan, 0·001 % α-asparagine, 0·001 % DL-phenylalanine, 0·2 % yeast extract, 0·1 % $(NH_4)_2SO_4$ (pH 7·6); ST medium had the same composition but with the addition of L-tyrosine at 0·1 % (w/v). BT-basal medium was also used with inhibitors of melanogenesis (L-cysteine, EDTA, ascorbic acid). All inhibitors were added at a final concentration of 1–10 mM. Solubility of pigments was tested with 0·5 ml

Table 1. Phenotypic characteristics of strains of *P. distincta* and *P. aliena* sp. nov.

All strains are Gram-negative, are positive for oxidase, catalase, lipase and gelatinase activity, grow at 6–29 °C and in 3–6 % (w/v) NaCl, do not utilize D-galactose, D-arabinose, D-ribose, D-sorbitol, glycerol, xylose, melibiose, adonitol, rhamnose or *myo*-inositol and are not susceptible to benzylpenicillin (10 U), lincomycin (15 μg) or tetracycline (30 μg). +, Positive; −, negative; W, weak reaction.

Characteristic	***P. distincta* KMM 638$^{\mathrm{T}}$**	***P. aliena* sp. nov.**						
		KMM 3562$^{\mathrm{T}}$	**KMM 3536**	**KMM 3537**	**KMM 3538**	**KMM 3539**	**KMM 3615**	**KMM 3629**
Production of melanin-like pigments	+	+	W	+	+	+	+	+
Growth at:								
4 °C	−	+	+	+	+	+	+	+
30 °C	+	+	+	+	−	−	−	+
31 °C	+	−	+	+	−	−	−	+
32 °C	+	−	−	−	−	−	−	+
33 °C	+	−	−	−	−	−	−	−
Growth in NaCl at:								
0·5 % (w/v)	+	−	+	+	−	−	−	+
1 % (w/v)	+	−	+	+	−	−	−	+
8 % (w/v)	+*	−	−	−	−	−	−	−
Production of:								
Caseinase	−*	+	−	−	+	+	+	+
Amylase	−	+	+	+	+	+	+	+
Haemolysis	−	+	+	+	+	+	+	+
Assimilation of:								
Sucrose	−	+	−	−	+	+	+	−
Maltose	−	W	W	W	W	W	W	W
D-Glucose	−	W	−	−	W	W	W	−
D-Mannitol	−	W	−	−	W	W	W	−
N-Acetylglucosamine	−	W	W	W	W	W	W	W
Susceptibility to:†								
Ampicillin (10 μg)	0	0	15	0	12	0	0	0
Gentamicin (10 μg)	19	15	23	17	20	18	13	15
Kanamycin (30 μg)	13	0	0	0	0	0	0	0
Carbenicillin (100 ED)	0	0	20	11	20	0	0	0
Neomycin (30 μg)	17	0	0	0	11	0	0	0
Oleandomycin (15 μg)	0	15	15	20	20	18	15	14
Polymyxin (300 ED)	10	11	12	15	13	10	11	10
Streptomycin (30 μg)	18	15	22	18	20	18	13	15
G+C content (mol%)	43·6	41·1–42·5‡						

*Data obtained in this study.

†Values are diameters of the growth inhibition zone (in mm).

‡Range for the seven strains shown.

culture fluid in 2 ml aliquots of various solvents. Pigments were designated soluble if the solvent was coloured after agitation, and insoluble otherwise. A distinctive feature of the type strain of *P. distincta* is its ability to produce a melanin-like pigment. The isolated strains appeared to synthesize a diffusible brown/black pigment when grown on common media such as marine agar 2216 or medium B. We examined the formation of pigment by the type strain on tyrosine-containing media that are optimal for melanogenesis, and in the same media in the presence of inhibitors of melanogenesis, namely cysteine, EDTA and ascorbic acid. Pigment extracted from cells with a 0·5 M solution of NaOH in water formed a brown solution. The alkaline pigment solution reacted qualitatively as melanin: it became colourless upon the addition of H_2O_2; addition of $KMnO_4$ gave a green solution. The absorption maximum of the pigment, i.e. 225 nm, was close to that for synthetic melanin (Sigma), which is 223 nm, confirming the melanin-like nature of the pigments tested.

Tyrosinase activity was tested according to the method of Pomerantz & Murphy (1974) in crude enzyme extracts, as described elsewhere (Ivanova *et al.*, 2003), then estimated spectrophotometrically by measuring the rate of formation of dopachrome from L-tyrosine at 35 °C under the following conditions: L-tyrosine, 5 μmol; 3,4-dihydroxy-L-phenylalanine, 0·5 μmol; sodium phosphate buffer (pH 6·8), 30 μmol; enzyme, 0·001–0·05 U; total volume, 1·00 ml. One unit of enzyme activity was estimated as the amount of enzyme required to produce 1 μmol dopachrome min^{-1} from tyrosine under the above conditions. The molar absorbance coefficient of dopachrome is 3700 at 475 nm. It appeared that an active melanogenesis took place when the strains studied had reached stationary phase in complex or chemically defined media containing tyrosine. The tyrosinase activity was quantitatively determined in cells and cell supernatants (Table 2). The results obtained indicated that the novel isolates showed significant enzyme activity that ranged between 30 and 107 U mg^{-1} and was nearly 2·5 times greater than that of *P. distincta*. In general, tyrosinase production was highly dependent on culture medium composition. For example, the presence of ascorbic acid induced both enzyme synthesis and 3,4-dihydroxy-L-phenylalanine production. The possible physiological role of melanin pigments, tyrosinases and related enzymes as important factors in resistance against stress factors such as UV radiation and oxidative damage, the degradation of high-molecular-mass compounds of phenolic nature and the regulation of enzyme activities by environmental signals have been discussed elsewhere (Lucas-Elío *et al.*, 2002).

The 16S rRNA gene was amplified and sequenced by MIDI Labs. Primers used for the amplification corresponded to *Escherichia coli* positions 5 and 1540; the details are as described elsewhere (Ivanova *et al.*, 2004). The novel 16S rRNA gene sequence was added, aligned and compared with a database of 80 000 previously aligned sequences. This analysis and BLAST queries against the latest release of the Bacteria division of GenBank allowed us to select the 100 most closely related sequences (see Supplementary Fig. B in IJSEM Online) for further analysis. Finally, 23 sequences, mainly those of type strains, were selected; phylogenetic trees were constructed according to three different methods [BioNJ (neighbour-joining), ML (maximum-likelihood using the GLOBAL option) and MP (maximum-parsimony)]. The BioNJ program from Gascuel (1997) and the ML and MP programs were from PHYLIP (Phylogeny Inference Package, version 3.573c, distributed by J. Felsenstein, Department of Genetics, University of Washington, Seattle, WA, USA). For the NJ analysis, distances were calculated according to the Kimura two-parameter correction Gascuel (1997). Bootstraps were done using BioNJ and 1000 replications. Phylogenetic trees were drawn using NJPLOT (Perrière & Gouy, 1996). Domains used to construct the final phylogenetic trees (positions 86–1396 of KMM 3562$^{\rm T}$) were available for all sequences. All analyses revealed that strain KMM 3562$^{\rm T}$ is included in the clade formed by the genus *Pseudoalteromonas*. The topology of the phylogenetic tree shown in Fig. 1 is that of the bootstrap analysis, as it has been demonstrated that this topology is often better than that of a simple NJ analysis (Gascuel, 1997); see also the discussion in Ivanova *et al.* (2004). Percentages of 16S RNA gene sequence similarity with most similar sequences were calculated by parsing the non-overlapping high-scoring segment pair resulting from

Table 2. Comparative enzyme activities of melanogenic *P. distincta*, *P. aliena* and *P. nigrifaciens* strains

One unit of tyrosinase activity is the amount of enzyme required to produce 1 μmol dopachrome from tyrosine min^{-1}.

Strain	Protein [U (mg wet cells)$^{-1}$]	3,4-Dihydroxy-L-phenylalanine (mg ml^{-1})	Tyrosinase activity*	
			U ml^{-1}	U mg^{-1}
P. distincta				
KMM 638$^{\rm T}$	76	0/0	12/1	9·3/0·9
KMM 638$^{\rm T}$†	98	0·01/1·7	90·1/2·3	12·8/15
***P. aliena* sp. nov.**				
KMM 3562$^{\rm T}$	270	0·027/0·007	135·1/1·09	30·5/10·9
KMM 3562$^{\rm T}$†	320	0·027/0·007	206·6/1·17	82·6/11
KMM 3536	300	0·015/0·01	269·6/2·03	107·84/5·075
KMM 3537	80	0·005/0	71·62/0	45·6/0
KMM 3538	170	0·02/0·12	162·2/1·35	49·9/13·5
KMM 3539	260	0·038/0·008	233·8/0	32·35/0
KMM 3615	70	0·01/0	78·4/0	31·35/0
KMM 3629	250	0·026/0·007	234·5/3·8	97·7/38
P. nigrifaciens				
IAM 13010$^{\rm T}$	0/0	0·01/0	0/0	0/0

*Values give activity in bacterial cells/supernatant.

†Enzyme activities after growth on BT medium containing ascorbic acid.

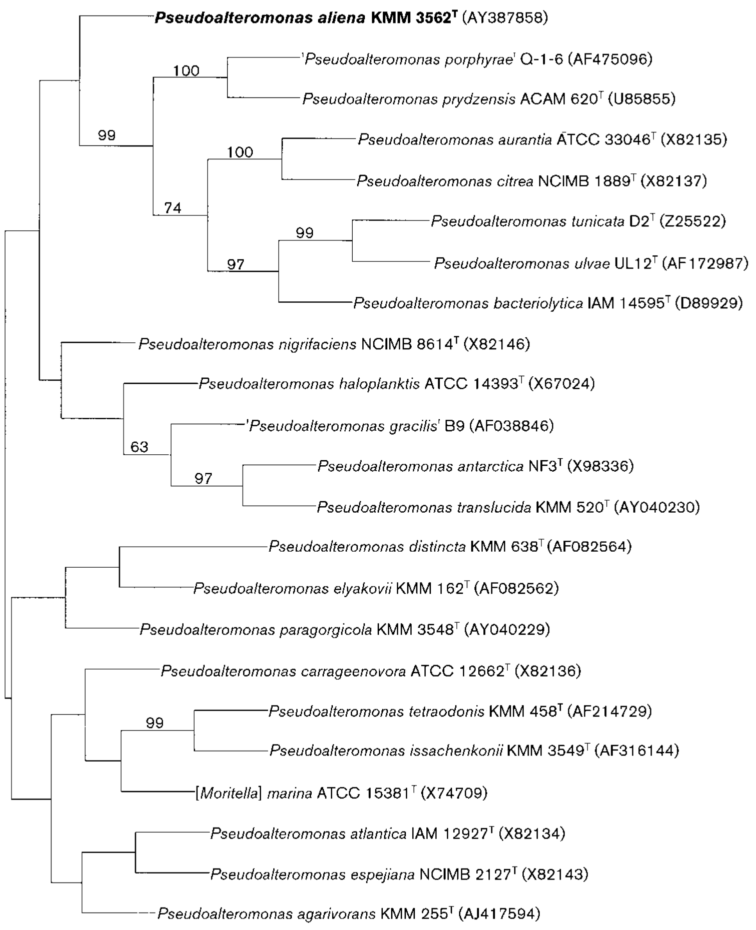

Fig. 1. Phylogenetic position of *P. aliena* sp. nov. according to 16S rRNA gene sequence analysis. The topology shown was obtained using the bioNJ algorithm and 1000 bootstrap replications with a Kimura two-parameter correction for the distances. Bootstrap percentages are indicated only for branches that were also retrieved by maximum parsimony and maximum likelihood ($P<0{\cdot}01$); these branches should be considered as the only robust clusters identified by this analysis.

a BLAST analysis of KMM 3562^{T} on the Bacteria division, with the options no filter and W = 7: they ranged from 98 to 99 % among non-pigmented species of the genus. These results provided additional evidence that 16S rRNA gene sequences can provide only a preliminary assignment of species identity in a genus. The same situation was reported for all other non-pigmented species of the genus *Pseudoalteromonas* (Gauthier *et al.*, 1995) and for some other genera, e.g. *Psychrobacter* (Bowman *et al.*, 1996), *Pseudomonas* (Anzai *et al.*, 2000), etc. The phylogenetic analyses of the 16S rRNA gene sequences also revealed that the novel isolate could not be robustly grouped with any other recognized species including *P. distincta* (shown in Fig. 1).

The DNA was isolated according to the method of Marmur (1961) and the G+C content of the DNA was determined using the thermal denaturation method of Marmur & Doty (1962). The G+C contents of the DNAs of the strains were 41–43 mol%. DNA–DNA hybridization was performed spectrophotometrically as described by De Ley *et al.* (1970). DNA–DNA hybridization data revealed the high interspecies levels of DNA relatedness among the six strains (92–97 %). DNA from the type strain *P. distincta* KMM 638^{T} showed a rather high degree of genetic relatedness (63–65 %) with the newly isolated bacteria. Yet these values are lower than 70 %, which is the cut-off value for the definition of genomic species (Wayne *et al.*, 1987).

The novel isolates can be clearly distinguished from other non-pigmented species and, in particular, from *P. distincta* KMM 638^{T} by the combination of phenotypic, genotypic and phylogenetic features, e.g. range of salinity and temperature for growth, presence of haemolytic and cytotoxic activity, ability to hydrolyse starch and casein, different antibiotic-susceptibility patterns (as shown in Table 1 and the Supplementary Table) and by the lack of a robust phylogenetic clade with any species of the genus. Bacteria of the novel species can also be distinguished from phylogenetically related species mainly by salinity tolerance, carbon-source utilization and a number of other phenotypic traits (Supplementary Table). In particular, they are distinct from *Pseudoalteromonas haloplanktis* in terms of the different temperature and salinity ranges for growth, susceptibility to antibiotics and carbon-source utilization, from *Pseudoalteromonas translucida* in terms of tolerance to NaCl, ability to decompose starch and the inability to decompose elastin and alginate, from *Pseudoalteromonas* *prydzensis* and *Pseudoalteromonas issachenkonii* by the inability to decompose chitin and from *Pseudoalteromonas atlantica*, *Pseudoalteromonas nigrifaciens* and *Pseudoalteromonas undina* by susceptibility to antibiotics and by carbon-source utilization. Therefore, we propose that the seven novel isolates be assigned to a novel species, namely *Pseudoalteromonas aliena* sp. nov.

Description of *Pseudoalteromonas aliena* sp. nov.

Pseudoalteromonas aliena (a′li.e.na. L. fem. adj. *aliena* another's, stranger, belonging to another species).

Rod-shaped cells, single, about 0·7–0·9 μm in diameter. Gram-negative. Motile, with a single polar flagellum. Aerobic. Chemorganotroph with respiratory metabolism. Colonies are uniformly round, 2–3 mm in diameter, regular, convex, smooth. A brown, diffusible, melanin-like pigment is produced around colonies after incubation for 48 h on marine agar or medium B. Does not form endospores. Does not accumulate poly-β-hydroxybutyrate as an intracellular reserve product, and has an arginine dihydrolase system. Oxidase- and catalase-positive. Requires Na^+ ion or sea water for growth. Growth occurs in media with 0·5–6 % (w/v) NaCl, and for some strains in media with 3–6 % (w/v) NaCl. Temperature for growth ranges from 4 to 29 °C (some strains grow at 33 °C), with optima at 22–25 °C. No growth is detected at 37 °C. The pH for growth ranges from 6·0 to 10·0, with optima at 7·5–8·0. Cytotoxic and haemolytic. Gelatin, starch, alginate and Tween 80 are decomposed. Some strains decompose casein. A limited range of carbon sources are utilized: some strains utilize sucrose and weakly utilize D-glucose, D-mannitol and maltose. Susceptible to gentamicin, oleandomycin, polymyxin and streptomycin; not susceptible to benzylpenicillin, kanamycin, lincomycin, neomycin or tetracycline. The main cellular fatty acids are 16 : 1(n-7), 16 : 0, 17 : 1(n-8) and 18 : 1(n-7) (approx. 80 %). The G + C content of the DNA is 41–43 mol%.

Isolated from sea-water samples collected from Amursky Bay, Sea of Japan, Pacific Ocean. The type strain is strain SW19^T (= KMM 3562^T = LMG 22059^T).

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