Flavobacterium faecale sp. nov., an agarase-producing species isolated from stools of Antarctic penguins

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Taxonomic studies were performed on an agarase-producing strain, designated WV33^T, isolated from faeces of Antarctic penguins. Cells of strain WV33^T were Gram-staining-negative, strictly aerobic, orange and rod-shaped. Strain WV33^T displayed agarase activity and was able to utilize galactose as a sole carbon source. 16S rRNA gene sequence analysis revealed that strain WV33^T was closely related to *Flavobacterium algicola* TC2^T (98.0 % similarity), *F. frigidarium* ATCC 700810^T (96.9 %) and *F. frigoris* LMG 21922^T (96.1 %). The predominant cellular fatty acids were iso-$C_{15:1}$ G, iso-$C_{15:0}$, $C_{15:0}$, $C_{16:0}$ and summed feature 3 (comprising iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$). Menaquinone 6 (MK-6) was the sole quinone identified, and the major pigment was zeaxanthin. The major polar lipid was phosphatidylethanolamine. DNA–DNA relatedness of strain WV33^T with respect to its closest phylogenetic neighbours was 25 % for *F. algicola* NBRC 102673^T, 23 % for *F. frigidarium* DSM 17623^T and 21 % for *F. frigoris* DSM 15719^T. The DNA G+C content of strain WV33^T was 37 ± 0.6 mol%. Based on the phenotypic, chemotaxonomic and phylogenetic data, strain WV33^T is concluded to represent a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium faecale* sp. nov. is proposed. The type strain is WV33^T (=KCTC 32457^T=CECT 8384^T).

The genus *Flavobacterium* (family *Flavobacteriaceae*, phylum *Bacteroidetes*) consists of approximately 100 species (*List of Prokaryotic names with Standing in Nomenclature*: http://www.bacterio.net/). These organisms have been isolated from various environmental sources, including soil, sediment, microbial mats, fresh water, sea ice and diseased fish. According to Bernardet & Bowman (2011), members of the genus *Flavobacterium* are yellow-pigmented, Gram-staining-negative rods, non-motile or with gliding motility, and contain menaquinone 6 (MK-6) as the major or sole respiratory quinone. Some strains of the genus *Flavobacterium* have attracted interest because they express enzymes of biotechnological significance, and therefore have potential for use in bioremediation or wastewater treatment (Miyashita *et al.*, 2010; Touchon *et al.*, 2011; Huang *et al.*, 2013). Recently, research on bioproduction of biofuels and biochemicals from renewable sources has focused on polysaccharide-degrading enzymes such as cellulases and xylanases. These enzymes can convert various types of polysaccharide into hydrolysed saccharides, providing inexpensive carbon and energy sources for microbial growth (Kim *et al.*, 2011). In particular, agarases, which catalyse the hydrolysis of agar, have found wide application as bulky or fine biocatalysts in the food, cosmetics and biotechnology industries (Chi *et al.*, 2012). Agar is a major component of the cell walls of red algae, and agarases have been isolated from different genera of bacteria found in seawater and marine sediments (Macián *et al.*, 2001). The ability of some species of the genus *Flavobacterium* to hydrolyse agar was reported previously (Bernardet & Nakagawa., 2006). Here, we describe a novel agarase-producing strain, designated WV33^T, which was isolated from Antarctic penguin faecal samples.

Faecal samples were collected from penguin colonies near the King Sejong station on King George Island, Antarctica

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain WV33^T is KF214259.

A supplementary figure is available with the online version of this paper.

(62° 14′ 45.4″ S 58° 46′ 36.2″ W). The faecal samples were suspended in sterile saline, and 100 µl of the suspension was spread on marine agar 2216 plates (MA; Difco) and incubated at 16 °C for 5 days (Yi *et al.*, 2005). Strain WV33^T was isolated by picking colonies from the MA plates and restreaking them on fresh MA. *Flavobacterium algicola* NBRC 102673^T, *F. frigidarium* DSM 17623^T and *F. frigoris* DSM 15719^T were used as reference strains for phenotypic and genotypic characterization and fatty acid analysis.

Strain WV33^T was tested for growth on R2A agar, MA, nutrient agar, trypticase soy agar, Simmons' citrate agar (all from Difco) and MacConkey agar (Oxoid); growth was assessed after incubation at 16 °C for 3 days under aerobic conditions. Cell morphology was examined by light (Laborlux K; Leitz) and scanning electron (JSM 5410LV; JEOL) microscopy, using cells from exponentially growing marine broth cultures. Anaerobic growth on R2A agar was assessed under anaerobic (with 4–10 % CO_2) conditions by using the GasPak plus system (BBL) at 16 °C for 2 days. Gram staining was performed using the Sigma-Aldrich Gram stain kit, following the manufacturer's instructions. Oxidase activity was tested based on oxidation of 1 % (w/v) tetramethyl *p*-phenylenediamine (Merck) and catalase activity was evaluated by production of oxygen bubbles in 3 % (v/v) aqueous hydrogen peroxide solution. Gliding motility, production of flexirubin-type pigments and adsorption of Congo red by colonies were assessed according to Bernardet *et al.* (2002) and Reichenbach *et al.* (1980). Growth in R2A broth was monitored at 4, 10, 14, 16, 20, 25, 30 and 37 °C by obtaining OD_{600} readings over 2 days. Growth at pH 4–9 (in increments of 0.5 pH units) was investigated using R2A broth that had been adjusted to the correct pH by adding HCl or K_2HPO_4/KH_2PO_4. Growth in R2A broth in the presence of 0–8 % (w/v) NaCl (in increments of 0.5 %) was investigated. The antibiotic susceptibility of strain WV33^T was determined by culture on R2A agar containing kanamycin (30 µg·ml^{-1}), ampicillin (100 µg·ml^{-1}), chloramphenicol (100 µg·ml^{-1}), erythromycin (50 µg·ml^{-1}), tetracycline (30 µg·ml^{-1}) or streptomycin (50 µg·ml^{-1}) at 16 °C for 7 days. Biochemical properties of strain WV33^T were evaluated using the API ZYM, API 20NE and API 20E systems (bioMérieux) incubated at 16 °C for 1 day. Agar degradation was assessed by growing strain WV33^T on agar plates containing 2 % NaCl, 0.1 % K_2HPO_4, 0.1 % NH_4Cl, 3 % NaCl, 0.05 % $MgSO_4$, 0.01 % $CaCl_2$ and 2 % agar. The agar plates were stained by spraying with Lugol's iodine solution (Agbo & Moss, 1979; Hu *et al.*, 2009). Galactose utilization was tested by growing strain WV33^T and the three reference strains in M9 broth and on M9 agar (Sigma) containing 10 % galactose as the sole carbon source. Degradation of casein, CM-cellulose, gelatin, starch, DNA, tyrosine, alginate and Tweens 20 and 80 was examined on R2A agar according to previously described methods (Barrow & Feltham, 1993; Lányi, 1987; Lewin & Lounsbery, 1969; McCammon & Bowman, 2000; Smibert & Krieg, 1981). The phenotypic characteristics of strain WV33^T are stated in the species description and in Table 1.

Genomic DNA was isolated and purified using a genomic DNA extraction kit (Intron). The 16S rRNA gene of strain WV33^T was amplified by PCR from purified genomic DNA using the universal bacterial primer pair 9F and 1512R (Weisburg *et al.*, 1991). The complete 16S rRNA gene sequence (1521 nt) of strain WV33^T was extracted and aligned with reference sequences obtained from the EzTaxon-e server (Kim *et al.*, 2012) by using the CLUSTAL W software (Larkin *et al.*, 2007). Evolutionary distances were calculated using the model of Jukes & Cantor (1969), and a phylogenetic tree (Fig. 1) was reconstructed

Table 1. Differential characteristics of strain WV33^T with respect to closely related members of the genus *Flavobacterium*

Strains: 1, WV33^T; 2, *F. algicola* NBRC 102673^T; 3, *F. frigidarium* DSM 17623^T; 4, *F. frigoris* DSM 15719^T. Data are from this study. +, Positive; W, weakly positive; −, negative; ND, data either not determined or unreliable. All strains are positive for catalase, oxidase, leucine arylamidase and naphthol-AS-BI-phosphohydrolase activities. All strains are negative for gliding motility, production of H_2S, hydrolysis of CM-cellulose, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, α-mannosidase and α-fucosidase activities and assimilation of arabinose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid. All strains are sensitive to ampicillin, chloramphenicol, tetracycline, erythromycin and streptomycin.

Characteristic	1	2	3	4
Assimilation of (API 20NE):				
D-Glucose	−	+	+	+
Maltose	−	+	+	+
D-Mannose	−	+	+	+
Degradation of:				
Starch	+	+	−	−
Gelatin	−	−	+	−
Alginate	−	+	−	−
Agar	+	−	−	−
DNA	+	−	+	−
Casein	−	−	+	+
Tyrosine	−	−	−	+
Tweens 20 and 40	+	ND	ND	ND
Enzyme activity (API ZYM)				
β-Galactosidase	+	−	−	−
Alkaline phosphatase	+	+	−	+
Esterase C4	+	−	−	W
Valine arylamidase	−	−	+	+
α-Glucosidase	−	−	−	+
N-Acetyl-β-glucosaminidase	−	+	−	−
Acid phosphatase	−	+	−	+
Lipase C14	+	−	−	W
Utilization of D-galactose in medium M9	+	−	−	−
DNA G+C content (mol%)	37±0.6	34±1.2	35±0.8	34±0.7

using the neighbour-joining method (Saitou & Nei, 1987). The resulting tree was subjected to bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. The closest phylogenetic neighbours of strain WV33^T were *F. algicola* NBRC 102673^T (98.0 % sequence similarity), *F. frigidarium* DSM 17623^T (96.9 %) and *F. frigoris* DSM 15719^T (96.1 %) (Stackebrandt & Ebers, 2006). Trees with essentially identical topologies were recovered using the maximum-likelihood and maximum-parsimony algorithms (not shown), supporting the phylogenetic position of strain WV33^T within the genus *Flavobacterium*.

For analysis of cellular fatty acids, strain WV33^T and the three reference strains were grown on R2A agar for 4 days at 30 °C, after which methyl esters were prepared and analysed by GC-MS (models 7890, 5975; Agilent Technologies), using the NIST database (http://www.nist.gov/). To determine quinone content, quinones were extracted from strain WV33^T and analysed using a C_{18} reversed-phase column attached to an Agilent 1200 HPLC system (Agilent Technologies) equipped with a photodiode array detector (Kong & Lee, 2011). To determine the carotenoid content of strain WV33^T, carotenoids were extracted, purified and analysed using the same HPLC system (Kim *et al.*, 2010; Kim & Lee, 2012), and a liquid chromatography-mass spectrometer (LC/MS, Agilent 6150) equipped with an atmospheric pressure chemical ionization ion source (Agilent Technologies) (Heo *et al.*, 2013). For structural analysis, the carotenoids were identified using a combination of HPLC retention times, UV–visible absorption spectra and mass fragmentation spectra (Song *et al.*, 2013). Polar lipids of strain WV33^T grown on R2A broth for

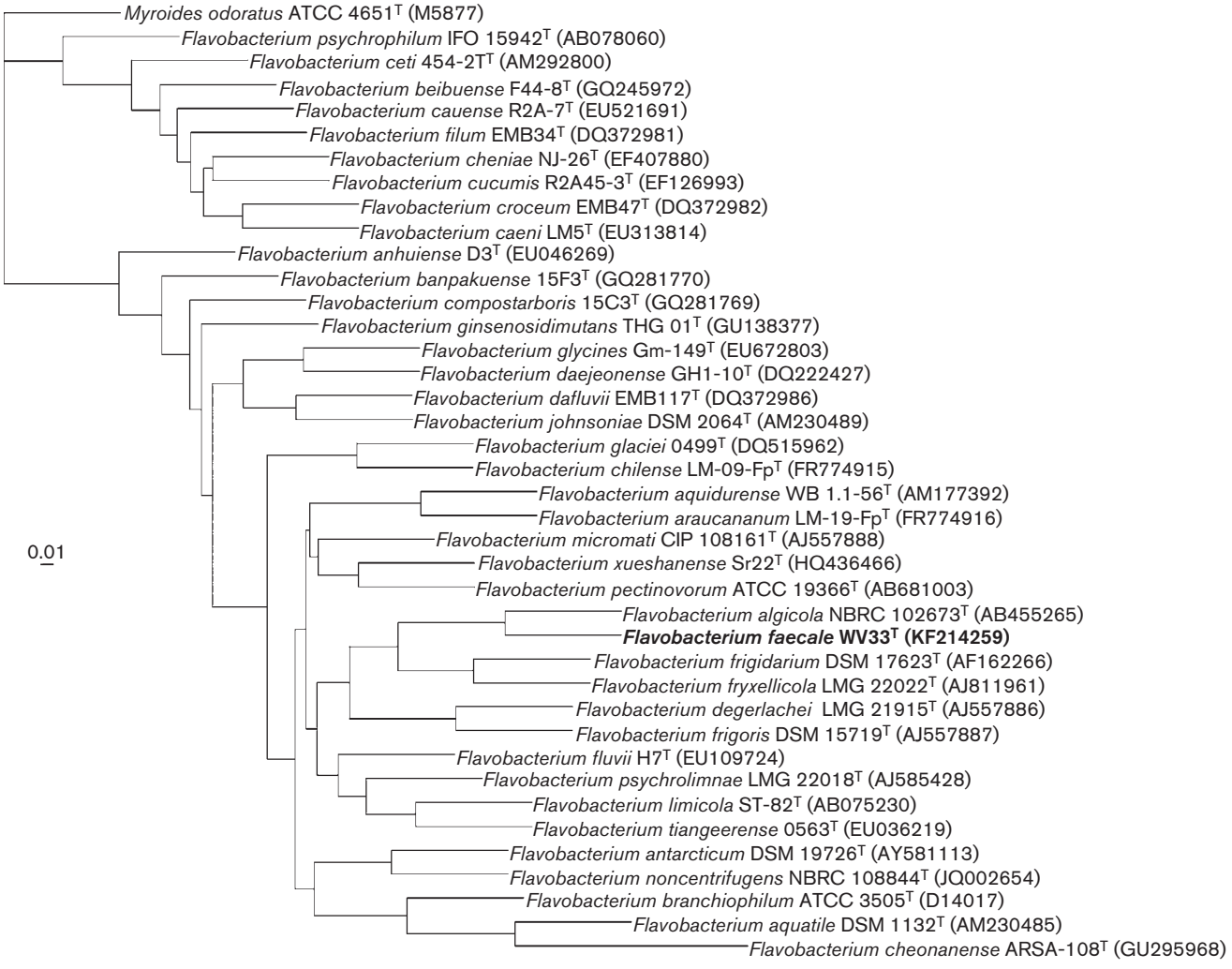

Fig. 1. Neighbour-joining phylogenetic tree showing relationships of strain WV33^T to related members of the genus *Flavobacterium*, based on 16S rRNA gene sequence analysis. Percentages at nodes are levels of bootstrap support (>70 %) from 1000 resampled datasets. *Myroides odoratus* ATCC 4651^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

2 days at 16 °C were extracted and analysed by two-dimensional TLC using Kieselgel 60 F_{254} plates (Merck). Polar lipids were separated in the first direction with a mixture of chloroform/methanol/water (65 : 25 : 4, by vol.), followed by chloroform/acetic acid/methanol/water (80 : 15 : 12 : 4, by vol.) in the second direction. Total lipids were detected by spraying the TLC plates with 5 % (w/v) ethanolic molybdophosphoric acid and other reagents were used to reveal polar lipids with specific functional groups (Minnikin *et al.*, 1977; Sheu *et al.*, 2013; Yokota *et al.*, 1993).

DNA–DNA hybridization of strain WV33^T with the three reference strains was performed at 40 °C using photobiotin-labelled probes, as described by Ezaki *et al.* (1989) and McInnes *et al* (1990). Five replicate experiments were performed for each sample; the highest and the lowest values obtained were excluded and the mean of the remaining three values was considered to be the DNA–DNA relatedness value. To determine the DNA G+C content of strain WV33^T, isolated genomic DNA was degraded enzymically into nucleosides and analysed by performing HPLC, by the method of Tamaoka & Komagata (1984) and Mesbah *et al* (1989). The samples were analysed four times; genomic DNA of *Escherichia coli* K-12 was used as the control.

Based on 16S rRNA gene sequence similarity, the closest phylogenetic neighbours of strain WV33^T were the type strains of *F. algicola*, *F. frigidarium* and *F. frigoris*. Strain WV33^T was distinguishable from the three reference strains on the basis of several phenotypic characteristics (Table 1). Strain WV33^T utilized D-galactose as a sole carbon source in medium M9, in contrast with the three reference strains. Notably, strain WV33^T also displayed agarase activity, in contrast with the three reference strains: the yellow–orange colonies were located in shallow craters on agar plates (Fig. 2a) and staining with Lugol's iodine solution revealed a clear halo surrounding each colony (Fig. 2b).

The overall fatty acid compositions of the four strains were similar. Strain WV33^T, however, differed from the three reference strains by the absence of iso-$C_{15:0}$ 3-OH and the presence of $C_{14:0}$ and significantly larger proportions of $C_{15:0}$, $C_{16:0}$ and ECL 16.582 (Table 2). The main carotenoid of strain WV33^T was zeaxanthin, a carotenoid pigment already reported in *Flavobacterium akiainvivens* (Kuo *et al.*, 2013) as well as in several other members of the family *Flavobacteriaceae* (Asker *et al.*, 2007a, b; Hameed *et al.*, 2013, 2014). MK-6 was the sole respiratory quinone identified, similar to that observed in other members of the family *Flavobacteriaceae*. The total polar lipids of strain WV33^T were phosphatidylethanolamine, five uncharacterized phospholipids (PL1–PL5) and three uncharacterized aminolipids (AL1–AL3) (see Fig. S1, available in the online Supplementary Material). The major polar lipid was phosphatidylethanolamine, as observed in other members of the family *Flavobacteriaceae* (Bernardet & Bowman, 2011).

The DNA–DNA relatedness of strain WV33^T with respect to its closest phylogenetic neighbours was 25 % for *F. algicola* NBRC 102673, 23 % for *F. frigidarium* DSM 17623^T and 21 % for *F. frigoris* DSM 15719^T. These values are well below the recommended threshold value of 70 % (Wayne *et al.*, 1987), indicating that strain WV33^T indeed represents a distinct species. The DNA G+C content of strain WV33^T was 37 ± 0.6 mol%, which falls within the range reported for other members of the genus *Flavobacterium* (30–41 mol%; Bernardet & Bowman, 2011).

The phenotypic and genotypic data described above confirm the separate taxonomic status of strain WV33^T. Therefore, strain WV33^T is described below as a member of a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium faecale* sp. nov. is proposed.

Description of *Flavobacterium faecale* sp. nov.

Flavobacterium faecale (fae.ca′le. L. n. *faex*, *faecis* dregs, faeces; L. neut. suff. *-ale* suffix denoting pertaining to; N.L. neut. adj. *faecale* pertaining to faeces, faecal).

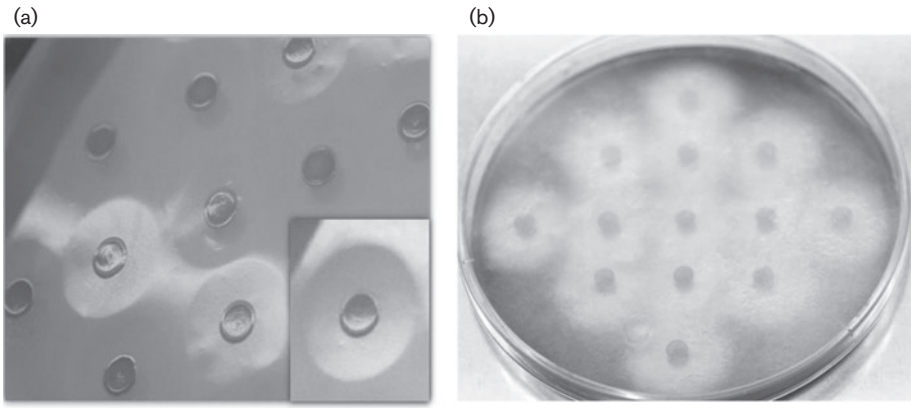

Fig. 2. Agar degradation produces shallow craters around colonies (a) and clear haloes after staining with Lugol's iodine solution (b).

Table 2. Cellular fatty acid compositions of strain WV33^T and type strains of three closely related species of the genus *Flavobacterium*

Strains: 1, WV33^T; 2, *F. algicola* NBRC 102673^T; 3, *F. frigidarium* DSM 17623^T; 4, *F. frigoris* DSM 15719^T. All data were generated in this study. −, Not detected, TR, traces (<1.0 %); ECL, equivalent chain length. Fatty acids amounting to <1.0 % in all strains are not shown.

Fatty acid	1	2	3	4
$C_{14:0}$	1.9±2.1	TR	TR	TR
iso-$C_{15:1}$ G	15.5±0.3	10.2±0.9	5.5±0.5	5.5±0.4
anteiso-$C_{15:0}$ A	TR	TR	TR	1.2±0.2
iso-$C_{15:0}$	19.4±3.1	18.5±2.3	29.5±1.2	9.5±3,4
anteiso-$C_{15:0}$	5.5±0.4	6.8±0.5	3.4±0.1	19.0±0.3
$C_{15:1}\omega6c$	1.3±0.1	1.3±0.1	1.6±0.1	3.7±0.4
$C_{15:0}$	14.2±3.5	1.2±0.2	3.0±0.1	2.3±0.2
iso-$C_{16:1}$ H	2.6±0.2	−	1.3±0.1	1.4±0.2
iso-$C_{16:0}$	5.0±0.4	TR	1.0±0.1	2.0±0.1
$C_{16:0}$	12.0±6.2	7.3±0.5	2.0±0.1	1.9±0.2
iso-$C_{15:0}$ 3-OH	−	9.9±3.5	11.1±4.2	5.8±3.2
iso-$C_{17:1}\omega9c$	2.1±0.2	2.3±0.2	8.4±5.7	8.5±6.1
$C_{17:1}\omega6c$	−	TR	2.1±0.1	2.8±0.3
iso-$C_{16:0}$ 3-OH	−	−	1.1±0.1	3.1±0.2
$C_{16:0}$ 3-OH	4.7±0.2	5.2±2.9	TR	1.5±0.1
$C_{18:1}\omega5c$	TR	TR	TR	TR
iso-$C_{17:0}$ 3-OH	3.1±0.2	5.7±0.2	11.1±7.3	7.6±5.2
$C_{17:0}$ 2-OH	TR	TR	TR	1.5±0.1
$C_{17:0}$ 3-OH	TR	−	TR	TR
Unknown ECL 16.582	8.1±5.3	1.0±0.1	1.8±0.1	1.1±0.1
Summed feature 3*	12.0±0.6	23.6±6.7	8.0±3.2	12.5±1.3
Summed feature 4*	2.1±0.1	1.0±0.1	1.2±0.1	1.1±0.1

*Summed feature 3 consisted of iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$; summed feature 4 consisted of anteiso-$C_{17:1}$ B and/or iso-$C_{17:1}$ I.

Cells are Gram-staining-negative, strictly aerobic, non-spore-forming rods, 0.4–0.6 μm in diameter and 1.4–1.8 μm long. Gliding motility is not observed. Colonies on MA are circular, yellow–orange and located in shallow craters. The main pigment is zeaxanthin, and flexirubin-type pigments are not detected. Congo red is not adsorbed by the colonies. Growth occurs on MA, R2A agar, nutrient agar, trypticase soy agar, Simmons' citrate and MacConkey agar. The temperature range for growth is 10–30 °C (optimum, 16 °C). Growth in R2A broth is observed at pH 5.0–9.0 (optimum, pH 7.0) and in the presence of 0.5–8 % (w/v) NaCl (optimum, 6 %). Catalase and oxidase activities are present. DNA, starch, agar and Tweens 20 and 80 are degraded, but casein, alginate, gelatin, CM-cellulose and tyrosine are not. On using the API 20NE kit, positive for β-galactosidase activity, but negative for nitrate reduction, indole production, D-glucose fermentation, arginine dihydrolase and urease activities, gelatin and aesculin hydrolysis and assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid. D-Galactose is utilized as a sole carbon source in medium M9. On using the API 20E kit, positive for β-galactosidase activity, but negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, tryptophan deaminase and gelatinase activities, citrate utilization, H_2S, indole and acetoin production and production of acid from D-glucose, mannitol, inositol, D-sorbitol, L-rhamnose, D-sucrose, D-melibiose, amygdalin and arabinose. On using the API ZYM kit, positive for β-galactosidase, alkaline phosphatase, esterase (C_4), lipase (C_{14}), leucine arylamidase and naphthol-AS-BI-phosphohydrolase activities, but negative for esterase lipase (C_8), valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, α-chymotrypsin, α-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, β-glucosaminidase, α-mannosidase and α-fucosidase activities. Agar is degraded. Susceptible to (μg·ml^{-1}) ampicillin (100), chloramphenicol (100), erythromycin (50), tetracycline (30) and streptomycin (50), but not to kanamycin (30). The major polar lipid is phosphatidylethanolamine; significant amounts of five uncharacterized phospholipids and three uncharacterized aminolipids are also present. The predominant cellular fatty acids are iso-$C_{15:1}$ G, iso-$C_{15:0}$, $C_{15:0}$, $C_{16:0}$ and summed feature 3 (comprising iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$). MK-6 is the sole respiratory quinone.

The type strain is WV33^T (=KCTC 32457^T=CECT 8384^T), isolated from penguin faecal samples collected near King Sejong station on King George Island, Antarctica. The DNA G+C content of the type strain is 37±0.6 mol%.

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