Tessaracoccus rhinocerotis sp. nov., isolated from the faeces of *Rhinoceros unicornis*

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A novel Gram-stain-positive, non-spore-forming, irregular rod-shaped, non-motile and facultatively anaerobic actinobacterium, designated strain YIM 101269^T, was isolated from the faeces of *Rhinoceros unicornis* living in Yunnan Wild Animal Park, Yunnan province, south-west China. The isolate grew at 10–35 °C, at pH 6–12 and with 0–9 % (w/v) NaCl. The cell-wall peptidoglycan of the organism contained LL-diaminopimelic acid as the diagnostic diamino acid. The polar lipids detected were diphosphatidylglycerol, phosphatidylglycerol, three unidentified polar lipids, one unidentified aminophospholipid and three unknown glycolipids. The major cellar fatty acid was anteiso-$C_{15:0}$.MK-10(H_4) was the predominant menaquinone. The DNA G+C content was 69.5 mol%. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain YIM 101269^T belonged to the genus *Tessaracoccus*, closely related to *Tessaracoccus flavescens* DSM 18582^T (97.4 % similarity). Based on the evidence from the present study, strain YIM 101269^T is considered to represent a novel species of the genus *Tessaracoccus*, for which the name *Tessaracoccus rhinocerotis* sp. nov. is proposed. The type strain is YIM 101269^T (=DSM 27579^T=CCTCC AB 2013217^T).

The genus *Tessaracoccus* was erected by Maszenan *et al.* (1999) and was characterized as comprising facultatively aerobic, non-motile, oxidase-negative, catalase-positive, non-spore-forming, coccoid-shaped bacteria. At the time of writing, the genus comprised five recognized species, *Tessaracoccus bendigoensis* (Maszenan *et al.*, 1999), *Tessaracoccus flavescens* (Lee & Lee, 2008), *Tessaracoccus lubricantis* (Kämpfer *et al.*, 2009), *Tessaracoccus oleiagri* (Cai *et al.*, 2011) and *Tessaracoccus lapidicaptus* (Puente-Sánchez *et al.*, 2014).

Strain YIM 101269^T was isolated from the faeces of *Rhinoceros unicornis* living in Yunnan Wild Animal Park, Yunnan province, south-west China. Fresh faeces of *Rhinoceros unicornis* was collected into a sterile bag immediately after defecation and brought back to the lab. The samples were dried at 28 °C for 1 week, then heated at 80 °C for 1 h and ground lightly with a sterile pestle. The sample was dissolved with 18 ml of 0.1 % $Na_4P_2O_7$ and shaken for 1 h. Finally, the sample was disrupted for 40 s using an ultrasonic wave device. The ultrasonic wave machine used was 160 W and 50 KHz. Aliquots (100 µl) of the serial diluent of the samples were spread onto YIM 6 agar plates. The isolation medium (YIM 6) contained 1 % soluble starch, 0.03 % casein, 0.2 % KNO_3, 0.2 % NaCl, 0.2 % KH_2PO_4, 0.002 % $CaCO_3$, 0.005 % $MgSO_4 . 7H_2O$, 0.001 % $FeSO_4 . 7H_2O$ and 1.3 % agar, pH 7.2, plus 50 mg $K_2Cr_2O_7$ l^{-1}. The plates were incubated at 28 °C for 10 days. The purified strain was routinely cultivated on YIM 38 agar plates (0.4 % glucose,0.4 % yeast extract, 0.5 % malt extract, 3.7 mg vitamin mixture, 1.3 % agar, pH 7.2) at 28 °C and stored as aqueous glycerol suspensions (20 %, v/v) at −20 °C.

Morphological observations under a light microscope (model BH2; Olympus) and scanning electron microscope (Quanta 200, FEI) were observed following the procedure described by Cheng *et al.* (2013). Gram-staining was

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM 101269^T is KT215777.

One supplementary table and two supplementary figures are available with the online Supplementary Material.

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carried out using the standard Gram reaction and was confirmed by the KOH lysis test (Cerny, 1978). Cultural characteristics of the strain were observed after 4 days of incubation under aerobic conditions at 28 °C on trypticase soy agar (TSA; Difco). Growth at various temperatures (5, 10, 15, 20, 25, 28, 30, 35 and 40 °C) and NaCl concentrations (0, 1, 2, 3, 4, 5, 6, 7, 8 and 9 %, w/v, at 28 °C) was tested on TSA. The pH range for growth was tested in trypticase soy broth (TSB, Difco), the pH of which had been adjusted prior to sterilization, from pH 4 to 12 (in intervals of 1 pH unit) using the buffer system described by Xu *et al.* (2005). Milk coagulation and peptonization were determined after incubation on 20 % (w/v) skimmed milk medium at 28 °C. Hydrolysis of starch, casein and Tweens 20, 40, 60 and 80 was tested as described by Williams *et al.* (1989) and Gordon *et al.* (1974). After cells were grown for 4 days at 28 °C on TSA, catalase activity was determined by bubble production in 3 % (v/v) H_2O_2 and oxidase activity was determined by using 1 % (w/v) *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine reagent. Anaerobic growth was tested by using a GasPak EZ Anaerobe Pouch System (Becton Dickinson). Nitrate reductase, H_2S production, indole production, urease and gelatinase were tested by using the API 20E kit (bioMérieux). Other enzyme activities and assimilation of carbohydrates were tested with the APIZYM and 20NE kits (bioMérieux) following the manufacturer's recommendations; for these tests, cells were grown on TSA for 4 days at 28 °C.

Extraction of genomic DNA and PCR amplification of the 16S rRNA gene were performed as described by Li *et al.* (2007). The sequence obtained was compared with available 16S rRNA gene sequences of cultured species using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012). Multiple alignments with sequences of closely related taxa and calculations of sequence similarity were carried out using the CLUSTAL X program (Thompson *et al.*, 1997). Phylogenetic analyses were performed by three tree-making algorithms: the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods using the software package MEGA version 6.0 (Tamura *et al.*, 2013). Kimura's two-parameter model was used to calculate evolutionary distance matrices of the neighbour-joining method (Kimura, 1980). Bootstrap analysis was performed with 1000 replications (Felsenstein, 1985).

The G+C content of the genomic DNA was determined by using the HPLC method (Mesbah *et al.*, 1989). DNA–DNA hybridization was performed between the novel strain and *T. flavescens* DSM 18582^T by the fluorometric micro-well method (Ezaki *et al.*, 1989; Christensen *et al.*, 2000; He *et al.*, 2005). The experiments were set with three replications at the optimal hybridization temperature (47 °C).

Analyses of the diaminopimelic acid isomer and whole-cell sugars were performed as described by Hasegawa *et al.* (1983) and Tang *et al.* (2009), respectively. Polar lipids were extracted and separated by two-dimensional TLC (Minnikin *et al.*, 1979) and analysed using the procedures described by Collins & Jones (1980). Menaquinones were extracted (Collins *et al.*, 1977) and analysed using HPLC (Kroppenstedt, 1982). Cellular fatty acid analysis was performed by using the Microbial Identification System (Sherlock Version 6.1; MIDI database: TSBA6) (Sasser, 1990).

Phylogenetic analyses based on the almost-complete 16S rRNA gene sequence of strain YIM 101269^T indicated that it should be recognized as a member of the genus *Tessaracoccus* with closest sequence similarity to *T. flavescens* DSM 18582^T (97.4 %). Sequence similarities among YIM 101269^T and other species of the genus *Tessaracoccus* were lower than 97.0 %. The neighbour-joining tree also showed that strain YIM 101269^T formed a cluster with species of the genus *Tessaracoccus*, including *T. flavescens* DSM 18582^T and *T. bendigoensis* DSM 12906^T (Fig. 1). The same cluster was found in trees reconstructed with the maximum-likelihood and maximum-parsimony algorithms. Furthermore, in the phylogenetic trees reconstructed with both the neighbour-joining and the maximum-parsimony methods, bootstrap support values for this cluster were above 65 %. However, the level of 16S rRNA gene sequence similarity between YIM 101269^T and *T. bendigoensis* DSM 12906^T was lower than 96.0 %. Thus, *T. flavescens* DSM 18582^T was selected as the sole comparative strain, obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, and was grown under the same conditions as strain YIM 101269^T.

The DNA G+C content of strain YIM 101269^T was 69.5 mol%. *T. flavescens* DSM 18582^T was selected for DNA–DNA hybridization studies with strain YIM 101269^T. The experiment showed that the mean level of DNA–DNA relatedness between YIM 101269^T and *T. flavescens* DSM 18582^T was 48.3±1.4 % (Table S1, available in the online Supplementary Material), which was well below the 70 % cut-off point for recognition of prokaryotic genomic species (Wayne *et al.*, 1987). These results are significant in distinguishing YIM 101269^T from *T. flavescens* DSM 18582^T.

Cells of strain YIM 101269^T were Gram-stain-positive, facultatively anaerobic, oxidase-negative, catalase-positive, non-motile, non-spore-forming, irregular rods (approx. 0.6–0.7 μm wide and 1.2–1.5 μm long), arranged linearly with two, three or four rods (Fig. S1). Colonies were brilliant yellow, circular, opaque and approximately 1–2 mm in diameter after incubation on TSA for 4 days at 28 °C. Growth occurred at 10–35 °C, at pH 6–12 and with 0–9 % (w/v) NaCl, with optimum growth at 28 °C and pH 7–8.

The polar lipid profile of strain YIM 101269^T contained the predominant compounds diphosphatidylglycerol, phosphatidylglycerol, three unidentified polar lipids, one unidentified aminophospholipid and three unknown glycolipids (Fig. S2). Re-examination of the polar lipid profile of *T. flavescens* DSM 18582^T revealed that two unknown glycolipids, with the reported diphosphatidylglycerol and phosphatidylglycerol, comprised major compounds in the

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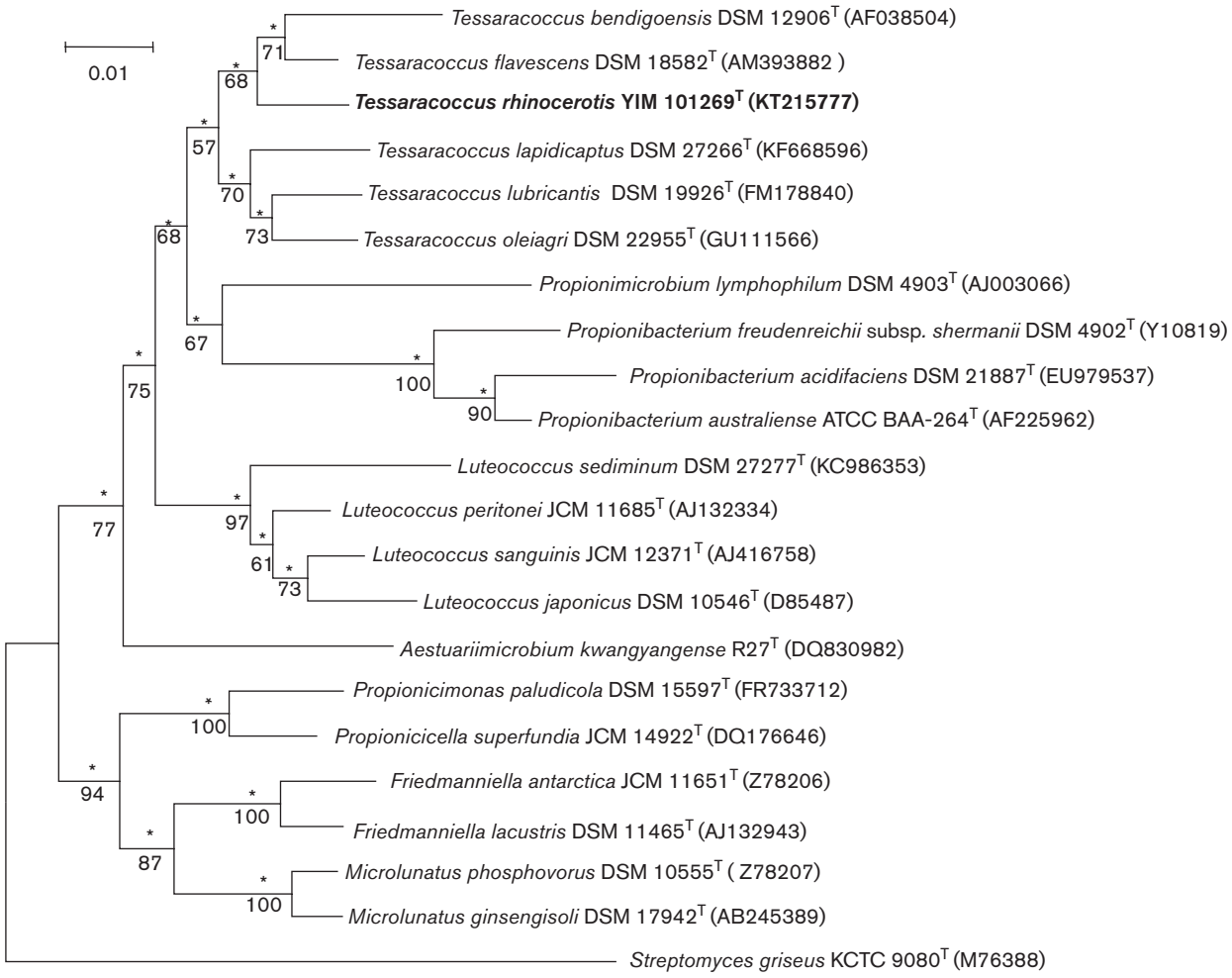

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences of strain YIM 101269^T and the type strains of closely related species. Bootstrap values (expressed as percentages of 1000 replications) of above 50 % are shown at branch points. Asterisks denote nodes that were also recovered using the maximum-parsimony method. The 16S rRNA gene sequence of *Streptomyces griseus* KCTC 9080^T (GenBank accession no. M76388) was used to root the tree. Bar, 0.01 substitutions per nucleotide position.

profile, and also trace amounts of one unidentified aminophospholipid and two unidentified polar lipids. The predominant menaquinone of YIM 101269^T was MK-10(H_4) (88 %). Additionally, minor amounts of MK-9(H_4) (3 %), MK-10(H_6) (6 %) and MK-10(H_8) (3 %) were detected. By contrast, a test of the quinone composition of *T. flavescens* DSM 18582^T confirmed MK-9(H_4) (95 %) as the major compound, with MK-9(H_2) (5 %) also detected. Differential characteristics between YIM 101269^T and the type strains of recognized species of the genus *Tessaracoccus* are detailed in Table 1.

The major cellular fatty acids (>1 %) of strain YIM 101269^T were anteiso-$C_{15:0}$ (67.8 %), $C_{14:0}$ 2-OH (8.8 %), iso-$C_{15:0}$ (7.4 %), iso-$C_{14:0}$ 3-OH (5.5 %), iso-$C_{16:1}$ H (4.3 %), iso-$C_{16:0}$ (2.2 %), anteiso-$C_{17:0}$ (1.5 %) and summed feature 4 (iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B, 1.7 %). Compared with *T. flavescens* DSM 18582^T, strain YIM 101269^T contained higher amounts of iso-$C_{15:0}$, $C_{14:0}$ 2-OH, iso-$C_{14:0}$ 3-OH and $C_{16:1}$ H and lower amounts of anteiso-$C_{15:0}$. Although anteiso-$C_{15:0}$ was the predominant cellular fatty acid for strain YIM 101269^T and the type strains of all recognized species in the genus *Tessaracoccus*, the compositions of the other cellular fatty acids in these strains were different from each other (Table 2). These results are significant in distinguishing YIM 101269^T from *T. flavescens* DSM 18582^T.

The genotypic and phenotypic features described above suggested that the newly isolated strain could be considered to represent a novel species of the genus *Tessaracoccus*. Therefore, YIM 101269^T was chosen as the type strain of

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Table 1. Differential characteristics between YIM 101269^T and other species of the genus *Tessaracoccus*

Strains: 1, YIM 101269^T; 2, *T. flavescens* 18582^T; 3, *T. bendigoensis* DSM 12906^T; 4, *T. oleiagri* DSM 22955^T; 5, *T. lubricantis* DSM 19926^T; 6, *T. lapidicaptus* DSM 27266^T. Data for *T. bendigoensis* DSM 12906^T, *T. oleiagri* DSM 22955^T and *T. lubricantis* DSM 19926^T were obtained from Cai *et al.* (2011). Data for enzyme activities of *T. lubricantis* DSM 19926^T and data for *T. lapidicaptus* DSM 27266^T were taken from Kämpfer *et al.* (2009) and Puente-Sánchez *et al.* (2014). All strains are Gram-stain-positive, non-motile and non-spore-forming, oxidase-negative, catalase-positive and urease-negative, positive for nitrate reduction, do not produce indole or H_2S, and contain LL-diaminopimelic acid in the cell-wall peptidoglycan. All are positive for assimilation of D-glucose and maltose. +, Positive; W, weakly positive; −, negative; ND, no data.

Characteristic	1	2	3	4	5	6
Cell morphology	Irregular rods	Rods	Cocci	Oval to rods	Rods	ND
Growth temperature (range, optimum) (°C)	10–35, 28	15–40, 28	20–37, 25	4–50, 28	15–36, 25	15–40, 37
Growth with NaCl (range, optimum) (%)	0–9, 1	0–5, 0	0–8, 4	0–11, 4	ND	0–2, 0
Growth pH (range, optimum)	6.0–12.0, 7.0–8.0	6.0–12.0, 7.0–8.0	5.5–9.3, 7.5	6.0–9.0, 7.0	6.5–9.5, ND	6.0–9.0, 8.0
Aesculin degradation	+	+	+	+	ND	+
Glucose fermentation	+	+	+	ND	ND	+
Enzyme activities:						
α-Galactosidase	−	−	+	+	ND	+
β-Galactosidase	+	+	+	−	ND	+
α-Glucosidase	+	+	ND	+	ND	+
β-Glucuronidase	+	−	−	+	ND	−
Valine arylamidase	−	−	+	+	+	−
Leucine arylamidase	+	+	+	ND	ND	+
Cystine arylamidase	−	−	+	+	+	−
Naphthol-AS-BI- phosphohydrolase	−	−	+	+	ND	−
N-Acetyl-β-glucosaminidase	−	+	ND	−	ND	−
Assimilation of:						
D-Arabinose	W	+	+	+	−	+
D-Mannose	+	+	+	+	−	+
Rhamnose	+	+	+	+	+	−
N-Acetyl-D-glucosamine	W	+	+	−	−	−
Acid production from:						
D-Arabinose	+	+	+	+	−	−
D-Galactose	+	+	+	−	+	ND
D-Mannitol	+	+	+	−	−	W
D-Xylose	+	+	+	−	−	−
D-Sorbitol	−	−	+	−		−
Chemotaxonomic data:						
Major menaquinones	MK-10(H_4)	MK-9(H_4)	MK-9(H_4)	MK-9(H_4)	MK-9(H_4)	MK-9(H_4)
Major polar lipids*	DPG, PG, APL, GL, L	DPG, PG, APL, GL, L	DPG, PG, PI, PL	DPG, PG, PI, PL	DPG, PG, L, GL, PI, PL, PI	PGL, PG, L, GL, PI,
Whole-cell sugars†	Rha, Rib, Man, Gal, Glu, Xyl	Rha, Rib, Man, Gal, Glu, Xyl	Rib, Gal, Man, Glu, Xyl	Rha, Rib, Gal, Glu, Man	ND	ND
DNA G+C content (mol%)	69.5	68.4	74.0	67.2	ND	70.3

*DPG, Diphosphatidylglycerol; PG, phosphatidylglycerol; PL, unidentified phospholipid; APL, unidentified aminophospholipid. GL, unknown glycolipid; PGL, unknown phosphoglycolipid; PI, phosphatidylinositol; L, unidentified polar lipid.

†Rib, ribose; Man, mannose; Glu, glucose; Gal, galactose; Rha, rhamnose; Xyl, xylose.

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Table 2. Cellular fatty acid composition (%) of strain YIM 101269^T and other species of the genus *Tessaracoccus*

Strains: 1, YIM 101269^T; 2, *T. flavescens* DSM 18582^T; 3, *T. bendigoensis* DSM 12906^T; 4, *T. oleiagri* DSM 22955^T; 5, *T. lubricantis* DSM 19926^T; 6, *T. lapidicaptus* DSM 27266^T. Data for *T. bendigoensis* DSM 12906^T and *T. oleiagri* DSM 22955^T were taken from Cai *et al.* (2011). Data for *T. lubricantis* DSM 19926^T and *T. lapidicaptus* DSM 27266^T were obtained from Kämpfer *et al.* (2009) and Puente-Sánchez *et al.* (2014), respectively. Data in this study were obtained under identical conditions. Cells were collected after incubation at 28 °C for 4 days on TSA. –, Not detected/not reported.

Fatty acid	1	2	3	4	5	6
Saturated						
$C_{12:0}$	–	–	0.9	0.7	–	0.8
$C_{14:0}$	–	–	3.4	1.1	1.3	3.3
$C_{14:0}$ 2-OH	8.8	3.7	1.3	2.8	–	–
$C_{16:0}$	–	1.9	4.0	1.6	2.6	5.0
Unsaturated						
$C_{16:1}$ H	4.3	–	–	–	–	–
Branched						
iso-$C_{14:0}$	–	2.3	4.6	4.2	2.0	3.6
iso-$C_{14:0}$ 3-OH	5.5	2.6	1.5	–	–	–
iso-$C_{15:0}$	7.4	4.9	6.5	0.5	3.4	7.5
iso-$C_{16:0}$	2.2	4.2	2.4	3.0	3.8	6.4
iso-$C_{17:0}$	–	1.8	–	–	–	1.9
iso-$C_{18:0}$	–	0.8	–	–	–	0.5
anteiso-$C_{13:0}$	–	0.8	1.7	4.4	–	–
anteiso-$C_{15:0}$	67.8	71.8	58.4	67.1	83.2	55.0
anteiso-$C_{17:0}$	1.5	3.0	2.1	0.9	3.8	2.8
Summed features*						
1	0.9	–	2.2	1.2	–	–
2	–	0.8	4.1	1.9	–	–
4	1.7	1.7	7.4	4.4	–	–

*Summed features 1, 2 and 4 comprised iso-$C_{15:1}$ H/$C_{13:0}$ 3-OH, iso-$C_{16:1}$ I/$C_{14:0}$ 3-OH, and iso-$C_{17:1}$ I/anteiso-$C_{17:1}$ B, respectively.

this novel species, for which the name *Tessaracoccus rhinocerotis* sp. nov. is proposed.

Description of *Tessaracoccus rhinocerotis* sp. nov.

Tessaracoccus rhinocerotis (rhi.no.ce.ro′tis. L. gen. n. *rhinocerotis* of a rhinoceros).

Cells are facultatively anaerobic, Gram-stain-positive, oxidase-negative, catalase-positive, non-spore-forming, non-motile, irregular rods. Colonies are brilliant yellow, circular, opaque and approximately 1.5 mm in diameter after incubation on TSA for 4 days at 28 °C. Tolerates up to 9 % (w/v) NaCl. Growth occurs at 10–35 °C (optimum 28 °C) and pH 6–12 (optimum pH 7–8). Negative for milk peptonization, milk coagulation, H_2S production, indole production and hydrolysis of Tween 60, Tween 80, starch and gelatin. Positive for the Voges–Proskauer reaction, aesculin degradation, glucose fermentation, nitrate reduction and hydrolysis of casein, Tween 20 and Tween 40. The following compounds are assimilated weakly: D-arabinose, D-glucose and *N*-acetyl-β-glucosamine. D-Galactose, D-mannose, melibiose, D-fructose, D-fucose, D-xylose, L-sorbitol, dextran, glycerol, gluconate, malate, maltose, rhamnose and sucrose are assimilated. The following compounds are not utilized: *myo*-inositol, D-mannitol, D-sorbitol, adipate, amygdalin, caprate, cellobiose, citrate, melezitose, inulin, phenylacetate and trehalose. Positive for aesculin, esterase (C4), esterase lipase (C8), leucine arylamidase, α-glucosidase, β-galactosidase, β-glucosidase, β-glucuronidase and β-fucosidase. Negative for acid phosphatase, alkaline phosphatase, arginine dihydrolase, cystine arylamidase, lipase (C14), lysine decarboxylase, *N*-acetyl-β-glucosaminidase, ornithine decarboxylase, valine arylamidase, trypsin, tryptophan deaminase, naphthol-AS-BI-phosphohydrolase, urease, α-chymotrypsin, α-galactosidase and α-mannosidase. LL-Diaminopimelic acid is the diagnostic diamino acid of the cell-wall peptidoglycan. Major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, three unidentified polar lipids, one unidentified aminophospholipid and three unknown glycolipids. The predominant menaquinone is MK-10(H_4). The major cellular fatty acids are anteiso-$C_{15:0}$, $C_{14:0}$ 2-OH, iso-$C_{15:0}$, iso-$C_{14:0}$ 3-OH and iso-$C_{16:1}$ H.

The type strain, YIM 101269^T (=DSM 27579^T=CCTCC AB 2013217^T), was isolated from faeces of *Rhinoceros unicornis* living in Yunnan Wild Animal Park, Yunnan province, south-west China. The G+C content of the DNA of the type strain is 69.5 mol%.

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