Myroides injenensis *sp. nov.*, *a new member isolated from human urine*

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Myroides injenensis sp. nov., a new member isolated from human urine

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Abstract A Gram-negative, yellow-pigmented, rod-shaped bacteria, designated M09-0166^{T}and M09-1053 were isolated from human urine samples. 16S rRNA gene sequence analysis revealed that the isolates belong to the *Myroides* cluster and were closely related to *Myroides phaeus* DSM 23313^T (96.3 %), *Myroides odoratimimus* KCTC 23053^T (96.1 %), *Myroides profundi* KCTC 23066^T (96.0 %), *Myroides odoratus* KCTC 23054^T (95.4 %) and *Myroides pelagicus* KCTC 12661^T (95.2 %). The major mena quinone was identified as MK-6. The major polar lipids were identified as phosphatidylethanolamine, amino lipids, and several unknown lipids, and the major fatty acids as iso-$C_{15:0}$ and iso-$C_{17:0}$ 3-OH. Phenotypic and chemotaxonomic data supported the affiliation of the isolates with the genus *Myroides* and clearly indicated that two isolates represent novel species, for which the name *Myroides injenensis* sp. nov. (type strain, M09-0166^T = KCTC 23367^T = JCM 17451^T) is proposed.

Keywords *Myroides injenensis* · New species · Urine sample

Introduction

The genus *Myroides* of the family *Flavobacteriaceae* was created in 1996 to accommodate "*Flavobacterium*"*odoratum* which was reclassified as *Myroides odoratus* (Vancanneyt et al. 1996). Members of the

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genus *Myroides* are Gram-negative, lacking gliding motility bacteria which form yellow-pigmented colonies due to the presence of a flexirubin-type pigment (Vancanneyt et al. 1996). At the time of writing, the genus *Myroides* comprises eight validly named species: *Myroides odoratimimus* (Vancanneyt et al. 1996), *M. profundi* (Zhang et al. 2008), *M. marinus* (Cho et al. 2011), *M. phaeus* (Yan et al. 2012), *M. pelagicus* (Yoon et al. 2006), *M. odoratus* (Vancanneyt et al. 1996), *M. guanonis* (Tomova et al. 2013), and *M. xuanwensis* (Zhang et al. 2014). Bacteria of this genus were isolated from a variety of sources including clinical samples, terrestrial and marine environments. Here, we apply a polyphasic approach including genotypic and phenotypic properties of two strains, M09-0166^{T}and M09-1053, isolated from human urine sample and present a description of a new species of the genus *Myroides.*

Materials and methods

Sampling, strains, and isolation

A 51-year-old woman was admitted to the emergency room with fever and abruptly reduced drainage from the left kidney. A urine sample was taken in a sterile tube (Corning), placed in an icebox, and transported to the laboratory for immediate analysis. The sample was serially diluted in saline solution (0.85 % NaCl, w/v) and spread onto Tryptic Soy Broth (TSB, BD) solidified with 1.5 % (w/v) agarose plate that were incubated at 30 °C for 48 h. The isolated bacterial strains were designated M09-0166^T nd M09-1053. These strains were sub-cultured several times to confirm their purity.

Morphological, physiological, and biochemical characterization

The phenotypic properties of the newly isolated bacteria were determined by subjecting the strains to a range of morphological, physiological, and biochemical analyses, as previously described (Chang et al. 2008; Smibert and Krieg 1994). All tests were performed under relevant conditions using fresh cultures of purified strains. The type strains *M. odoratimimus* KCTC 23053^T, *M. profundi* KCTC 23066^T, *M. odoratus* KCTC 23054^T, and *M. pelagicus* KCTC 12661^T were obtained from the Korean collection for type cultures (KCTC, Republic of Korea) as used as reference species. Unless stated otherwise, the selected reference species were grown under their optimal culture conditions (Yoon et al. 2006; Zhang et al. 2008; Vancanneyt et al. 1996). The Gram reaction was performed using a Gram staining kit (Difco) according to the manufacturer's instructions. Cell morphology was examined under bright field (Nikon Optiphot-2), phase-contrast (Nikon 80i), and electron microscopes. For electron microscopy, cells were fixed in a 2.5 % (w/v) paraformaldehyde/glutaraldehyde mixture and coated with gold in a sputter coater (SC502, Polaron). The prepared cells were then observed under a scanning electron microscope (S4300N, Hitachi). Cells were negatively stained with 1 % (w/v) uranyl acetate and the flagella type was observed under a CM-20 transmission electron microscope (Philips) (Chang et al. 2008). The presence of endospores was observed by phase-contrast microscopy (E600; Nikon) after staining with 5 % malachite green and safranin of cells incubated at 37 °C for 5 days on Tryptic Soy Agar (TSA, Difco) (Chang et al. 2008). The production of flexirubin-type pigments were investigated using the methods recommended by Bernardet et al. (2002). Motility was determined using 0.4 % (w/v) semi-solid TSA as described by Tittsler and Sandholzer (1936). The temperature (10–60 °C) and NaCl requirement (0–15 %, [0.5 % increments] for growth was tested over a period of 3–7 days by incubating cells in TSB. Growth was assessed by measuring the optical density at 600 nm in a spectrophotometer (M-680, Bio-Rad). The pH range for growth was determined by culturing the bacteria in 250 ml buffered (Tris/HCl or NaOH) TSB at 30 °C and pH 4.0–12.0 (increments of 0.5 pH unit) (Smibert and Krieg 1994). Anaerobic growth was tested on TSA plates incubated under strictly anaerobic conditions in an anaerobic glove box (Coy Laboratory Products, Inc., Ann Arbor, MI, USA.) with mixed gases (N_2/CO_2/H_2, 88 %/5 %/7 %, v/v/v). Catalase activity was determined by bubble production in 3 % (v/v) H_2O_2 solution. The activity of oxidase was determined by drop-wise addition of Kovacs oxidase reagent (0.6 g *N*-tetramethyl-*p*-phenylenediamine dihydrochloride and 0.02 g stabilizing agent in 100 ml of dimethyl sulfoxide [DMSO]) (Smibert and Krieg 1994). Other phenotypic and biochemical tests were performed using API 20E and ZYM strips (BioMérieux) according to the

manufacturer's instructions. Carbon source utilization was examined using Biolog GN2 microplates (Biolog) according to the manufacturer's instructions. Other tests, including nitrite reduction, gelatin liquefaction, Voges–Proskauer, blood hemolysis, H_2S and indole production, and digestion of litmus milk, Tween 40 and Tween 80, were performed according to standard protocols (Atlas 2010). *M. odoratimimus* KCTC 23053^T, *M. profundi* KCTC 23066^T, *M. odoratus* KCTC 23054^T, and *M. pelagicus* KCTC 12661^T were used as reference species for all biochemical tests.

Chemotaxonomic analysis

The G+C content of genomic DNA was analyzed by reversed-phase HPLC according to the protocol of Mesbah et al. (1989). Relative values were calculated using *E. coli* KCTC 2441^T DNA (51.2 mol %, HPLC) as a reference (Chang et al. 2008). Cellular fatty acid profiles were determined for the isolates and related species grown on TSB (pH 7.3, Difco) solidified with 15.0 g L^{-1}agar (TSA) at 30 °C for 48 h, according to a standard protocol (Sherlock Microbial Identification System; (MIDI 1999). Fatty acids were separated in an automated GC system (model 6890N attached to a 7683 autosampler; Agilent) and identified using the associated software package (Library TSBA 40, v4.0; MIDI). The isolates and related species were grown on TSB at 30 °C for 48 h, and quinones and cell wall type were determined. Respiratory quinones were examined using TLC and HPLC, as previously described (Komagata and Suzuki 1987). Peptidoglycan structure was examined as described previously (Chang et al. 2008) with a slight modification: two-dimensional TLC on cellulose plates was used instead of paper chromatography. Polar lipids were extracted and separated from 100 mg freeze-dried cellular material using a two-stage method, as previously described (Tindall 1990). Polar lipids were identified by the German collection of microorganisms and cell cultures, Braunschweig, Germany.

16S rRNA gene sequence determination and phylogenetic analysis

Nearly complete 16S rRNA gene sequences for isolates were determined according to the method of Chang et al. (2008). Preliminary sequence comparisons were made against 16S rRNA gene sequences of prokaryotic type strains with validly published names retrieved from the EzTaxon-e program (http://www.ezbiocloud.net/) and GenBank databases. Sequences were manually aligned against representatives of closely related genera using a bacterial 16S rRNA secondary structure model (Woese et al. 1980). Only those regions available for all sequences (conserved and variable regions) that showed unambiguous alignment were used to construct phylogenetic trees. Alignment and neighbor-joining tree (Saitou and Nei 1987) analyses were performed using the PHYLIP (Felsenstein 1993) and jPHYDIT programs (Jeon et al. 2005). Phylogenetic trees were also constructed using the maximum-likelihood (Felsenstein 1981) and maximum-parsimony (Fitch 1971) methods. An evolutionary distance matrix for the neighbor-joining tree was generated according to the Kimura two-parameter model (Kimura 1983). The reliability of the resulting trees was evaluated using bootstrap analyses (Felsenstein 1985) based on 1,000 re-samplings. *Sphingobacterium spiritivorum* DSM 2582^T was used as an out group.

Results and discussion

Morphological, physiological, and biochemical characterization

Bacterial cells were found to be Gram-negative, strictly aerobic, and either straight or curved rod-shaped. Cells are lacking flagella and non-motile, similar to previously reported *Myroides* species (Cho et al. 2011; Vancanneyt et al. 1996; Yan et al. 2012; Yoon et al. 2006; Zhang et al. 2008, 2014). Both strains was determine to grow between 10 and 45 °C (optimum, 37 °C), 0 and 6 % (w/v) NaCl (optimum, 1 %), and pH 4.5 and 10.5 (optimum, pH 6). Sodium ions are not essential for the growth of isolates.

Biochemical analysis showed that isolates and the reference strains are positive for catalase and oxidase activity, and negative for indole production. Substrate utilization profiles were determined for isolates and for the reference strains as shown in Table 1. Both strains were found to be able to utilize *N*-acetyl-D-glucosamine, adonitol, pyruvic acid methyl ester, succinic acid mono-methyl-ester, acetic acid, formic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, α-ketoglutaric

Table 1 Differential characteristics of strains M09-0166^{T}and M09-1053 and closely related species

Characteristic	1	2	3	4	5	6
Cell size (μm)	0.4–1.1	0.7–1.2	1.0–2.0	0.7–0.9	1.0–2.0	0.5–1.0
Pigment	Pale yellow	Yellow to brown	Yellow	Pale yellow	Yellow	Yellow to orange
Flexirubin-type pigments	−	−	+	−	−	−
Cell morphology	Rod	Rod	Rod	Spindle-shaped rod	Rod	Short-rod
Growth with/at						
Temperature (°C)	10–45	6–37	15–37	10–37	15–37	15–45
NaCl (w/v, %)	0–6	0–6	0–5	0–5	0–3	0–8
pH	4.5–10.5	5–10	4.5–10.5	5.0–10.0	5.5–10.0	5.0–10.0
Hydrolysis of						
Milk	−	−	+	+	−	−
Tween 80	−	+	+	−	+	−
Activity of						
Esterase	+	+	+	+	+	−
Esterase lipase	+	+	+	+	+	−
Urease	+	+	+	−	+	+
Utilization of						
L-Histidine	−	−	+	−	+	−
Methlpyruvate	+	−	+	−	+	+
α-Hydroxybutyric acid	+	−	+	−	+	−
Succinic acid	+	+	+	−	+	+
Urocanic acid	+	−	+	−	+	−
DNA G+C content (mol%)	32.1	34.3	35.7	33.5	32.8	31.9
Mena quinone	MK-6	MK-6	MK-6	MK-6	MK-6	MK-6
Isolation source	Urinary	Saliva	Wound swab	Deep-sea sediment	Feces of patient	Sea water

All data are from this study, except strain 2. All strains are negative reduction of nitrate and activity of catalase. +, Positive; −, negative

Strains are indicated as: 1, Isolates; 2, *M. phaeus* DSM 23313^T (Yan et al. 2012); 3, *M. odoratimimus* KCTC 23053^T; 4, *M. profundi* KCTC 23066^T; 5, *M. odoratus* KCTC 23054^T; 6, *M. pelagicus* KCTC 12661^T

acid, α-ketovaleric acid, D,L-lactic acid, succinic acid, bromosuccinic acid, succinamic acid, L-alaninamide, L-alanylglycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-glutamic acid, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, D-serine, L-threonine, urocanic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, and 2,3-butanediol. As determined by API 20E and ZYM, both strains are positive for urease, gelatinase, alkaline phosphatase, esterase (C4), esterase lipase (C8), acid phosphatase, and naphthol-AS-BI-phosphohydrolase. The strain is negative for lipase (C14), valine arylamidase, leucine arylamidase, crystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, β-galactosidase (ONPG), arginine dihydrolase, lysine decarboxylase, and tryptophan deaminase. Acid is not produced from glucose, mannitol, inositol, sorbitol, rhamnose, saccharose, melibiose, amygdalin, or arabinose. In the GN2 Micro-plate, *N*-acetyl-D glucosamine, adonitol, gentiobiose, pyruvic acid methyl ester, succinic acid mono-methyl-ester, acetic acid, formic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, α-ketoglutaric acid, α-ketovaleric acid, D,L-lactic acid,

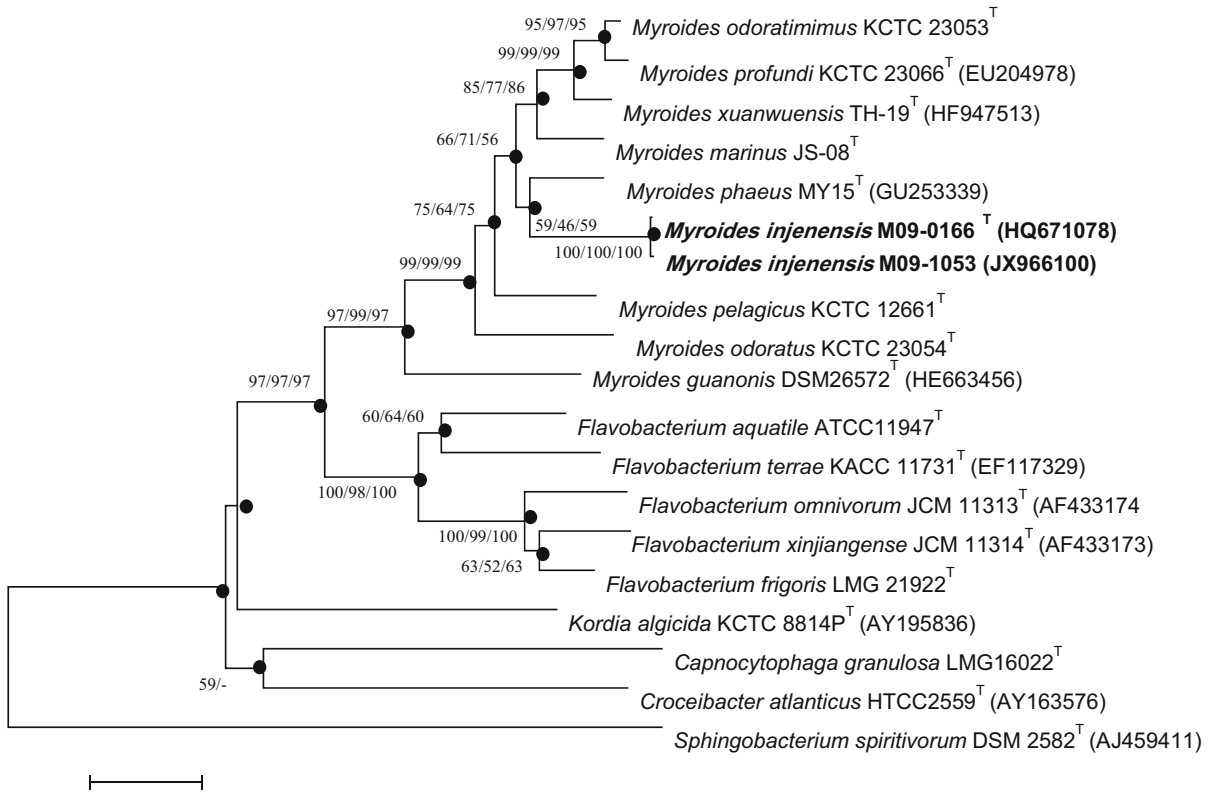

Fig. 1 Phylogenetic tree inferred by neighbor-joining method based on 16S rRNA gene sequences. It shows relationships of isolates between the members of closely related taxa. Bootstrap values are calculated from 1,000 replications and values >50 % are shown at branch points (calculated by neighbor-joining, maximum likelihood, and minimum evolution methods). *Filled circles* indicate that the corresponding nodes were also recovered in the tree generated by the maximum likelihood and minimum evolution algorithms. *Bar* 0.02 changes per nucleotide position

succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, L-alaninamide, L-alanine, L-alanylglycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-glutamic acid, L-leucine, L-ornithine, L-phenylalanine, L-proline, l-pyroglutamic acid, D-serine, L-threonine, urocanic acid, inosine, uridine, thymidine, phenylethylamine, and putrescine are oxidized.

Two isolates, *M. phaeus* DSM 23313^T, *M. odoratimimus* KCTC 23053^T, and *M. profundi* KCTC 23066^T were found to be positive for gelatin liquefaction. All studied species were found to be positive for Voges–Proskauer, and esterase and esterase lipase activity (with the exception of *M. pelagica* KCTC 12661^T), and negative for production of H_2S, hydrolysis of litmus milk, and hemolysis on blood agar. Only *M. odoratimimus* KCTC 23053^T, and *M. profundi* KCTC 23066^T were positive for hydrolysis of litmus milk. Detailed phenotypic characteristics of all studied species are presented in Table 1 and in the species description.

Phylogenetic characteristics

Nearly complete 16S rRNA gene sequences of strain M09-0166^{T}and M09-1053 comprised 1,416 and 1,403 nucleotides each. High sequence similarity (99.9 %) between the two isolates indicated that these strains belong to the same species. Phylogenetic trees based on the 16S rRNA gene sequences were constructed using the neighbor-joining, maximum-likelihood, and minimum evolution methods. Two strains form a robust cluster supported by the high bootstrap values within other species of the genus *Myroides* and within the family *Flavobacteriaceae* (Fig. 1). The closest phylogenetic relative of the isolates were found to be *M. phaeus* DSM 23313^T (96.3 % sequence similarity),

M. odoratimimus KCTC 23053^T (96.1 % sequence similarity), followed by *M. profundi* KCTC 23066^T (96.0 %), *M. odoratus* KCTC 23054^T (95.4 %), and *M. pelagicus* KCTC 12661^T (95.2 %). Since the 16S rRNA gene sequence similarities to any validly described species of the genus *Myroides* were found to be less than 97 %, no DNA–DNA hybridization was required (Stackebrandt and Ebers 2006).

Chemotaxonomic characteristics

The predominant cellular fatty acids (mean value) in the isolates were determined as iso-$C_{15:0}$ (58.2 %) and iso-$C_{17:0}$ 3-OH (9.1 %). The fatty acid profiles of the two isolates were found to be similar to those of other members of the genus *Myroides,* with iso-$C_{15:0}$, iso-$C_{15:0}$ 3OH, and iso-$C_{17:0}$ 3OH being the major components except *M. phaeus* DSM 23313^{T}and *M. pelagicus* KCTC 12661^T. These species was found to containiso-$C_{13:0}$ instead of iso-$C_{15:0}$ 3OH as the major components (Table 2). The quinone system (mean value) of two isolates was determined to be MK-6 (84.1 %) and MK-7 (15.8 %), while the most closely related species *M. phaeus* DSM 23313^T contains MK-6 (>99 %) and MK-7 (<1 %). The polar lipid profiles of the newly isolated bacteria was found to contain phosphatidylethanolamine, aminolipid and unknown lipids. The PE, AL, and L was found to be shared with other studied species, *M. odoratimimus* KCTC 23053^T, *M. profundi* KCTC 23066^T, *M. odoratus* KCTC 23054^T and *M. pelagicus* KCTC 12661^T.

Table 2 Cellular fatty acid profiles of strains M09-0166^{T}and M09-1053 and closely related species of the genus *Myroides*

Fatty acid	1	2	3	4	5	6
iso-$C_{13:0}$	t	10.5	4.2	2.9	t	7.5
iso-$C_{14:0}$	t	–	t	1.2	t	–
iso-$C_{15:0}$	58.2	51.2	44.3	41.5	52.0	52.9
anteiso-$C_{15:0}$	t	4.4	1.2	t	1.1	0.5
$C_{15:0}$	1.4	–	1.3	2.0	t	0.4
iso-$C_{15:0}$ 3-OH	4.8	4.2	5.3	5.3	4.1	6.1
iso-$C_{16:0}$	1.1	t	1.0	3.5	5.1	0.2
iso-$C_{16:0}$ 3-OH	1.2	t	1.2	1.2	1.1	t
$C_{16:0}$ 3-OH	2.5	6.7	5.1	2.4	1.3	2.7
iso-$C_{17:0}$ 3-OH	9.1	12.9	13.7	10.5	11.0	14.9
Summed feature[a]	1.2	t	7.3	5.1	t	2.6

[a] Fatty acids that could not be separated by GC using the microbial Identification System (Microbial ID) software were considered summed features. Summed feature contains $C_{16:1}$ ω7*c* and/or iso-$C_{15:0}$ 2-OH

Strains are indicated as: 1, isolates; 2, *M. phaeus* DSM 23313^T (Yan et al. 2012); 3, *M. odoratimimus* KCTC 23053^T; 4, *M. profundi* KCTC 23066^T; 5, *M. odoratus* KCTC 23054^T; 6, *M. pelagicus* KCTC 12661^T. All data are from this study, except strain 2. Values are percentages of total fatty acid detected. t trace amount (<1.0 %); –, not detected

The G+C contents of genomic DNA (mean value) from isolates were slightly lower (32.1 %) than that of other type species within the genus *Myroides* (Cho et al. 2011; Yoon et al. 2006 Yan et al. 2012; Zhang et al. 2008, 2014; Vancanneyt et al. 1996).

Taxonomic conclusion

The data obtained in this study showed that the two strains, M09-0166^T and M09-1053, are members of the genus *Myroides*. However, newly isolated bacteria can be differentiated from other species of the genus *Myroides* with regard to phenotypic properties, such as growth temperature, oxygen requirement, and the pattern of carbon sources utilized. Therefore, based on the analysis of the phenol and chemotaxonomic and phylogenetic characteritics we propose that strains M09-0166^T and M09-1053 represent new species within the genus *Myroides*, for which the name *Myroides injenensis* sp. nov. is proposed.

Description of *Myroides injenensis* sp. nov.

Myroides injenensis (in.je.nen'sis. N.L. masc. adj. injenensis, of or belonging to Inje, Republic of Korea, where the organism was first isolated).

Cells are straight or slightly curved rods (0.2–0.4 × 0.8–1.1 μm), Gram-negative, strictly aerobic, non-spore forming, and non-motile. Colonies grown on TSB medium are circular, convex, and pale yellow or ivory, with entire margins. The pigments are diffusible and non-flexirubin type. Cells grow at 10–45 °C (optimum, 37 °C), and at pH 4.5–10.5 (optimum, pH 6). Sodium ions are not required for growth. The strain tolerates up to 6.0 % (w/v) NaCl (optimum, 1.0 % (w/v)). The predominant menaquinone is MK-6. The major fatty acids (>5 % of total fatty acids) are iso-$C_{15:0}$, iso-$C_{15:0}$ 3OH and iso-$C_{17:0}$ 3OH. Phosphatidylethanolamine, aminolipid and unknown lipids were detected as the major polar lipids. Able to utilize gentiobiose, glucuronamide, L-alanine, L-

pyroglutamic acid, and 2-aminoethanol. The G+C content of the type strain is 32.1 mol %.

The type strain M09-0166^T (= KCTC 23367^T = JCM 17451^T) was isolated from the human urine sample of a patient at Inje University, Paik Hospital, Busan, Republic of Korea. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain M09-0166^T is HQ671078.

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