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Full Paper

Chitiniphilus shinanonensis gen. nov., sp. nov., a novel chitin-degrading bacterium belonging to *Betaproteobacteria*

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A bacterial strain capable of degrading chitin, strain SAY3^T, was isolated from moat water of Ueda Castle in Nagano Prefecture, Japan. The strain was gram-negative, curved rod-shaped, facultatively anaerobic, and motile with a single polar flagellum. It grew well with chitin as a sole carbon source. The cellular fatty acids profiles showed the presence of C16:1 ω7c and C16:0 as the major components. The G+C content of DNA was 67.6 mol% and Q-8 was the major respiratory quinone. A 16S rRNA gene sequence-based phylogenetic analysis showed the strain belonged to the family *Neisseriaceae* but was distantly related (<94% identity) to any previously known species. Since the strain was clearly distinct from closely related genera in phenotypic and chemotaxonomic characteristics, it should be classified under a new genus and a new species. We propose the name *Chitiniphilus shinanonensis* gen. nov., sp. nov. The type strain is SAY3^T (=NBRC 104970^T=NICMB 14509^T).

Key Words——*Betaproteobacteria*; chitin; *Chitiniphilus shinanonensis* gen. nov., sp. nov.; chitinolytic bacterium; *Neisseriaceae*; 16S rRNA gene sequence

Introduction

Chitin is a linear polymer consisting of β-1, 4-linked *N*-acetyl-D-glucosamine residues. It occurs in the exoskeletons of insects, mollusks, and crustaceans as well as in the cell walls of many fungi. In nature, more than 10^{11} metric tons of chitin is synthesized each year in the aquatic biosphere alone, and a corresponding amount is degraded during the same period of time (Yu et al., 1993). A large number of chitinolytic bacteria have been isolated from soil and seawater and their chitin-degrading enzymes have been characterized (Cohen-Kupiec and Chet, 1998; Keyhani and Roseman, 1999), but relatively little is known about those isolated from freshwater.

We have been studying chitinolytic bacteria living in freshwater such as lakes, rivers, and moats to clarify their role in the recycling of chitin-related compounds in freshwater. We already isolated the chitinolytic bacterium *Aeromonas hydrophila* strain SUWA-9 from Lake Suwa, a freshwater lake located in Nagano Prefecture, Japan (Lan et al., 2004). We reported that the strain produced several kinds of endo- and exo-type chitinolytic enzymes in the presence of the substrate, chitin (Lan et al., 2006, 2008).

During the screening of chitinolytic bacteria from moat water of Ueda Castle in Nagano Prefecture, Japan, we isolated a bacterial strain that grew well on synthetic medium containing colloidal chitin as a sole carbon source. A phylogenetic analysis based on 16S rRNA gene sequences showed that this bacterial strain

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named SAY3^T belongs to the family *Neisseriaceae*, but does not form a significant cluster with any species of the known genera of this family. In this paper, we report the taxonomic characteristics of the strain and propose to classify it under a new genus and species with the name *Chitiniphilus shinanonensis.*

Materials and Methods

Isolation of bacterial strains. The bacterial strain SAY3^T used in this study was isolated from moat water of Ueda Castle in Nagano Prefecture, Japan. A nylon bag containing chitin flakes (a gift from Kyowa Technos Co., Ltd., Japan) was soaked in moat water for 14 days, and then the chitin flakes were recovered and washed with tap water. Then the 0.2 g of chitin flakes were inoculated into 100 ml of synthetic medium (0.5% $(NH_4)_2SO_4$, 0.085% KH_2PO_4, 0.015% K_2HPO_4, 0.05% $MgSO_4$, 0.01% NaCl, 0.01% $CaCl_2$) containing 0.2% powder chitin (from crab shells, Nacalai Tesque, Japan) as the sole carbon source. The flask was shaken vigorously at 25°C until the culture showed a significant turbidity caused by cell growth. Then a portion of the culture was transferred into a fresh medium of the same composition. This inoculation step was repeated several times to enrich chitin-degrading bacteria. Finally chitin-degrading bacterial strains were isolated and purified on synthetic agar medium containing colloidal chitin as the sole carbon source.

Characterization of morphology, biochemistry, and phenotypes. Cell morphology was determined with a light microscope (Olympus BX50F4, Tokyo, Japan). Flagellation was observed using a transmission electron microscope (JEM-100 SX, JEOL, Ltd., Tokyo, Japan) after negative-staining with phosphotungstic acid. Utilization of carbon sources and some enzyme activities were determined using API20NE, API 50CH and API ZYM (bioMerieux, Paris, France). Oxidase activity was determined by oxidation of 1% tetramethyl-*p*-phenylenediamine. Catalase activity was determined by bubble production in 3% (v/v) H_2O_2.

Respiratory quinone analysis. Isoprenoid quinones were extracted from freeze-dried cells with chloroform : methanol (2 : 1, v/v), and fractionated with thin-layer chromatography developed with *n*-hexane : diethyl ether (85 : 15, v/v). Quinones were detected under UV light at 275 nm. The ubiquinone fraction was extracted with acetone, dried under a nitrogen gas stream, and analyzed by HPLC (Shimadzu LC-10A) with a Nacalai ODS 5C18 columns (4.6 × 150 mm).

Cellular fatty acid analysis. Cellular fatty acids were prepared according to the protocol of MIDI system. Cells were grown on TSBA medium at 30°C for 48 h. The fatty acids were analyzed by gas chromatography controlled by MIS software (Microbial ID, Inc.). The peaks were integrated and identified by the Microbial Identification software package (Sasser, 1990).

Determination of G+C content. Genomic DNA was prepared according to the method of Sambrook et al. (1989). Total DNA was digested with P1 nuclease using a Yamasa GC kit (Yamasa Shoyu Co., Chiba, Japan). The G+C content of DNA was determined by HPLC method as described by Mesbah et al. (1989).

16S rRNA gene sequencing and phylogenetic analysis. Chromosomal DNA was isolated from the cells and purified by InstaGene Matrix kit (Bio-Rad Laboratories, CA, USA). PCR was performed with PrimeSTAR HS DNA Polymerase (TaKaRa Bio, Inc., Shiga, Japan) with sets of primers encompassing a whole 16S rRNA gene. Sequencing was conducted with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Inc., CA, USA) using the ABI PRISM 3100 Genetic Analyzer System (Applied Biosystems, Inc.). The 16S rRNA gene sequence of the new isolate was determined and compared to those of related taxa retrieved from the DDBJ/GenBank/EMBL databases. For the multiple alignments, ClustalX2 program (Thompson et al., 1997) was used. Alignment positions with gaps and unidentified bases were excluded with the BioEdit program (Hall, 1999). The phylogenetic tree in the neighbor-joining method (Saitou and Nei, 1987) was constructed using the Neighbor program in PHYLIP (PHYLogeny Inference Package). Branching patterns of the tree were evaluated by bootstrapping with 1,000 resamplings, and the evolutionary distance was computed using the Kimura's 2-parameter model (Kimura, 1980). The tree was illustrated using the Tree-Explorer program (Kumar et al., 2001). Maximum-likelihood tree was constructed using the Dnaml program in PHYLIP.

Results

Isolation of the bacterial strain

The chitinolytic bacterial strain was isolated from moat water by collecting bacterial cells tightly bound on the surface of chitin flakes. After enrichment of cells grown in synthetic medium containing chitin powder

as the sole carbon source, one strain growing well and producing a clear halo around the colony was isolated on synthetic agar medium containing colloidal chitin. Preliminary study on the partial sequence of 16S rRNA gene revealed that it did not show identity (more than 97%) to the sequences of the known bacteria available in the public database. This strain named SAY3^T was further investigated for its taxonomic characteristics.

Morphological and physiological characteristics

Strain SAY3^T is a gram-negative, non-spore-forming and curved rod-shaped cell of 0.7–0.8 μm in width and 1.2–1.5 μm in length. The strain formed circular, smooth, pale yellow colonies with rhizoid margins when it grew on LB agar plate at 30°C for 48 h. Cells were motile with single polar flagellum (Fig. 1). The strain was a facultative anaerobic and grew at 15–45°C (optimum 30–37°C) but did not grow at 10°C. It did not grow in the presence of 4% NaCl.

Utilization of carbon sources and some enzyme activities were determined using API 20NE, API 50CH and API ZYM (Tables 1, 2 and 3). The strain utilized a narrow range of carbon sources, such as D-glucose, D-mannose, *N*-acetyl-D-glucosamine, D-ribose, amygdalin, esculin ferric citrate, and salicin. It reduced nitrate and hydrolyzed esculin, but not starch or gelatin. Acid production from glucose was negative. Both catalase and oxidase activities were positive. The strain produced activities of esterase, leucine arylamidase,

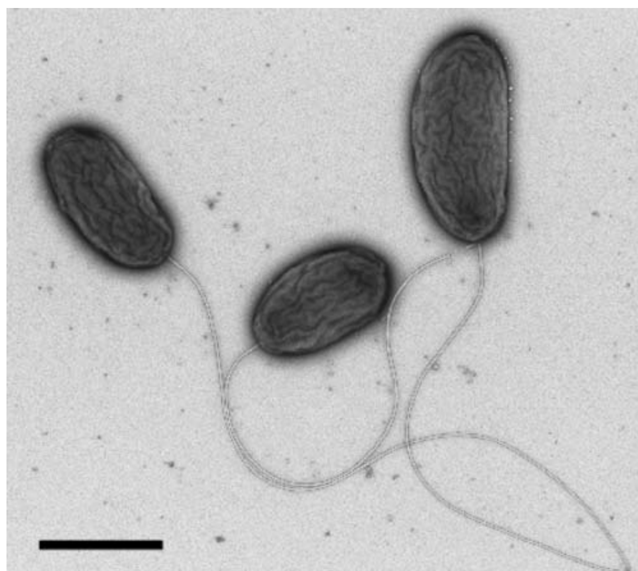

Fig. 1. Transmission electron micrograph of the strain SAY3^T.
Scale bar = 1 μm.

Table 1. Utilization of carbon compounds by the strain SAY3^T.

Carbon compounds	Result
Glycerol	−
Erythritol	−
D-Arabinose	−
L-Arabinose	−
D-Ribose	+
D-Xylose	−
L-Xylose	−
D-Adonitol	−
Methyl-β-D-xylopyranoside	−
D-Galactose	−
D-Glucose	+
D-Fructose	+w
D-Mannose	+
L-Sorbose	−
L-Rhamnose	−
Dulcitol	−
Inositol	−
D-Mannitol	−
D-Sorbitol	−
Methyl-α-D-mannopyranoside	−
Methyl-α-D-glucopyranoside	−
N-Acetyl-glucosamine	+
Amygdalin	+
Arbutin	−
Esculin ferric citrate	+
Salicin	+
D-Cellobiose	+w
D-Maltose	−
D-Lactose	−
D-Melibiose	−
D-Sucrose	−
D-Trehalose	−
Inulin	−
D-Melezitose	−
D-Raffinose	−
Starch	−
Glycogen	−
Xylitol	−
Gentiobiose	+w
D-Turanose	−
D-Lyxose	−
D-Tagatose	−
D-Fucose	−
L-Fucose	−
D-Arabitol	−
L-Arabitol	−
Gluconate	−
2-Keto-gluconate	−
5-Keto-gluconate	−

Cells grown on R2A agar at 30°C for 2 days were suspended in API CHB/E medium and incubated for 24 h at 30°C.
+, positive; −, negative; w, weak utilization.

Table 2. Enzyme activity of the strain SAY3^T.

Enzyme	Result
Alkaline phosphatase	+w
Esterase (C4)	+
Esterase lipase (C8)	++
Lipase (C14)	−
Leucine arylamidase	++
Valine arylamidase	−
Cystine arylamidase	−
Trypsin	+w
α-Chymotrypsin	−
Acid phosphatase	+
Naphthol-AS-BI-phosphohydrolase	+
α-Galactosidase	−
β-Galactosidase	−
β-Glucuronidase	−
α-Glucosidase	+++
β-Glucosidase	−
N-Acetyl-β-glucosaminidase	++
α-Mannosidase	−
α-Fucosidase	−

Cells grown on R2A agar at 30°C for 2 days were suspended in 0.85% NaCl solution and incubated for 4 h at 30°C.

+, positive; −, negative; w, weak activity.

Table 4. Cellular fatty acid composition of the strain SAY3^T.

Fatty acid	Composition (%)
11:0	0.1
12:0	3.0
3-OH 11:0	0.1
3-OH 12:0	5.3
14:1 ω5c	0.6
14:0	1.4
15:1 ω6c	2.4
15:0	3.1
16:1 ω7c	32.5
16:1 ω5c	0.3
16:0	31.2
17:0 cyclo	6.9
17:0	1.8
18:1 ω7c	9.9
18:0	1.2
19:0 cyclo	0.1

Table 3. Characteristics differentiating the strain SAY3^T from the known bacteria in the family *Neisseriaceae*.

Property	1	2	3	4	5	6	7
Morphology	Curved rods	Curved rods	Rods	Straight rods	Rods	Rods	Rods
Number of polar flagella	One	One	One	One or two	One or two	One or two	One or two
Growth at 40°C	+	−	+	−	+	−	−
Anaerobic growth	+	+	+	−	−	+	−
Catalase	+	−	+	+	+	+	+
β-Glucosidase	−	−	+	−	−	ND	−
Gelatin hydrolysis	−	−	−	+	+	−	+
Chitin hydrolysis	+	−	+	+	+	−	w
Nitrate reduction	+	−	+	−	−	+	+
Acid production from glucose	−	ND	+	+	−	−	−
Utilization of:							
Ribose	+	ND	−	+	−	+	+
Sucrose	−	−	+	−	−	+	−
N-Acetylglucosamine	+	ND	+	+	+	w	+
Cellular fatty acid (% of total):							
C12:0 3-OH	5.3	Tr	−	2.0	4.0	2.5	2.5
C14:0	1.4	−	6.4	0.6	Tr	2.0	8.4
C14:0 3-OH	−	9.5	−	−	−	2.0	−
10-Methyl C19:0	−	−	−	−	1.7	−	−
Major quinine	Q-8	Q-8	Q-8	Q-8	Q-8	Q-8	Q-8
DNA G+C content (mol%)	67.6	59	58	62	56	48.5	51

Taxa: 1, strain SAY3^T; 2, *Formivibrio citricus* DSM6150^T; 3, *Silvimonas terrae* KM-45^T; 4, *Andreprevotia chitinilytica* JS11-7^T; 5, *Chitinibacter tainanensis* BCRC17254^T; 6, *Deefgea rivuli* WB3.4-79^T; 7, *Iodobacter fluviatilis* ATCC33051^T.

+, positive; −, negative; w, weak; Tr, trace; ND, no data.

Data from: 2, Tanaka et al. (1991); 3, Yang et al. (2005); 4, Weon et al. (2007); 5, Chern et al. (2004); 6, Stackebrandt et al. (2007); 7, Logan (1989, 2005).

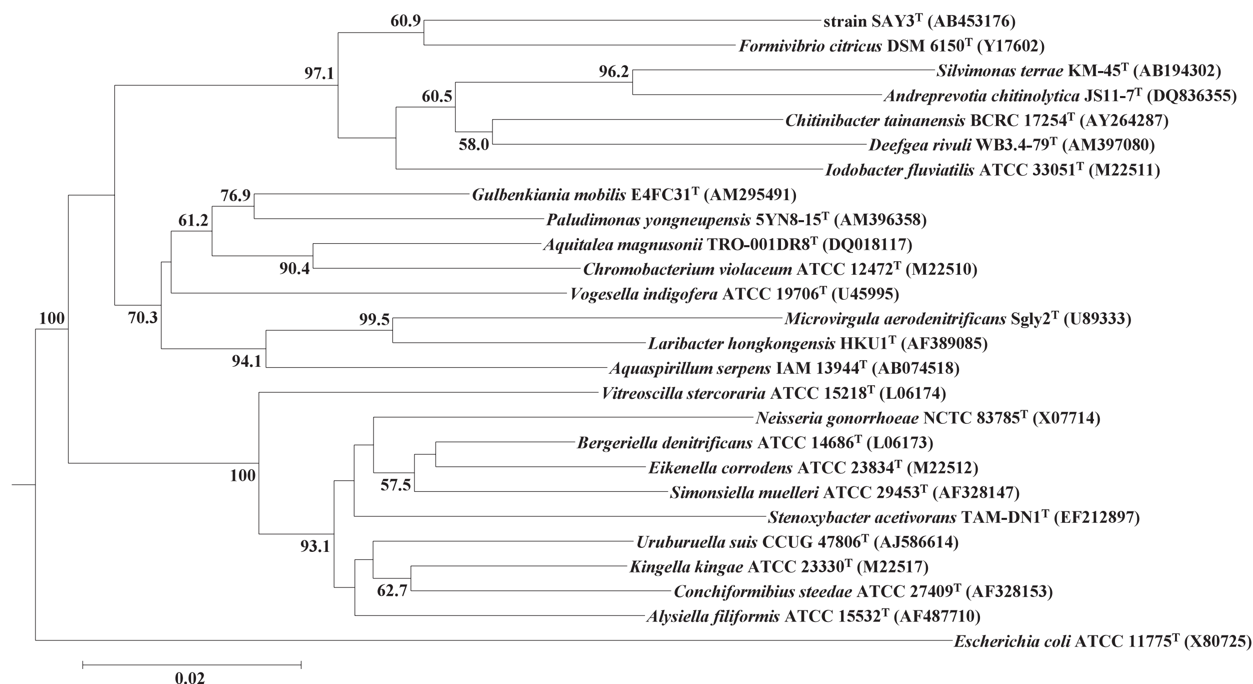

Fig. 2. Rooted neighbor-joining distance matrix tree derived from 16S rRNA gene sequences of strain SAY3^T and related bacteria of the family *Neisseriaceae*.

Accession numbers for the 16S rRNA gene sequences used are given in parentheses behind species and strain names. The sequence of *Escherichia coli* was used as an outgroup. Bootstrap values (%) obtained with 1,000 bootstrap resamplings are shown at branching points; only values >50% are shown. Scale bar represents 2% nucleotide substitution rate according to Kimura's 2-parameter model.

α-glucosidase, *N*-acetyl-β-glucosaminidase, and cytochrome oxidase.

Chemotaxonomic characteristics

The major composition of ubiquinone was Q-8. A composition of cellular fatty acids of the strain SAY3^T was shown in Table 4. The major component was hexadecenoic acid (C16:1 ω7) and hexadecanoic acid (C16:0) (Table 4). The strain contained a significant amount of 3-hydroxy dodecanoic acid (C12:0 3-OH). 2-Hydroxy fatty acids were not detected in appreciable amounts. The G+C content of DNA of the strain SAY3^T was 67.6 mol%.

Phylogenetic analysis

Nearly complete 16S rRNA gene sequence of the isolate, consisting of 1,494 nucleotides, was determined and deposited in the DDBJ/GenBank/EMBL database under the accession number of AB453176. Homology search indicated that the strain SAY3^T had sequence identity of 93.8% to *Formivibrio citricus* as its closest relative, 93.4% to *Silvimonas terrae,* 93.1% to *Chitinibacter tainanensis,* 92.5% to *Andreprevotia chitinolytica*, 92.0% to *Deefgea rivuli*, and 91.6% to *Iodobacter fluviatilis*. All of these species belong to the family *Neisseriaceae* in the class *Betaproteobacteria*. The sequence identity between the SAY3^T and other members of the family *Neisseriaceae* was less than 92%.

Phylogenetic relationships were investigated using sequences of the type strains of the type species of the family *Neisseriaceae.* Twenty-four representative bacteria belonging to the family *Neisseriaceae* were chosen for the phylogenetic analysis, and their 16S rRNA gene sequences were obtained from DDBJ/GenBank/EMBL databases. A neighbor-joining phylogenetic tree inferred from the sequences of the isolate and related organisms is shown in Fig. 2. The tree clearly shows that the isolate belongs to the family *Neisseriaceae* and is distinctively different from related genera. Similar branching patterns on the phylogenetic tree were obtained by the maximum likelihood method (data not shown).

Discussion

The chitinolytic strain SAY3^T was isolated from bacterial cells tightly bound on the surface of chitin flakes soaked in moat water. We clarified several bacterial strains bound on the surface by analyzing 16S rRNA genes that were PCR-amplified using total DNAs prepared from whole bacterial cells bound on the surface (data will be published elsewhere). The 16S rRNA gene sequence identical to that of the strain SAY3^T was found among the microbial cells tightly bound on chitin flakes.

The 16S rRNA gene sequence of the strain SAY3^T showed high similarity to those of bacteria belonging to the class *Betaproteobacteria*, and phylogenetic analysis clearly indicated that the strain formed a cluster at high bootstrap value of 97% with *Formivibrio citricus* DSM6150^T (Tanaka et al., 1991), *Silvimonas terrae* KM-45^T (Yang et al., 2005), *Andreprevotia chitinolytica* JS11-7^T (Weon et al., 2007), *Chitinibacter tainanensis* BCRC17254^T (Chern et al., 2004), *Deefgea rivuli* WB3.4-79^T (Stackebrandt et al., 2007), and *Iodobacter fluviatilis* ATCC33051^T (Logan, 1989). The closest genus, *Formivibrio citricus* DSM6150^T (93.8% identity) is distant enough to warrant placing the strain SAY3^T in a new genus. This view is also supported by comparing phenotypic and chemotaxonomic characteristics among the related genera (Table 3). The strain SAY3^T can be distinguished from the neighboring genera by its significantly higher G+C content (67.6%) and by a high content of 3-hydroxy dodecanoic acids (C12:0 3-OH). Moreover, the SAY3^T clearly differs from all neighbors by a combination of a number of physiological and chemotaxonomical characteristics (Table 3).

In conclusion, the strain SAY3^T should be classified under a new genus and a new species on the basis of morphological, physiological, and chemotaxonomical characteristics together with 16S rRNA gene sequence similarity. Thus we propose the name *Chitiniphilus shinanonensis* gen. nov., sp. nov. belonging to the family *Neisseriaceae* in the class *Betaproteobacteria*.

Descriptions

Description of Chitiniphilus gen. nov.

Chitiniphilus (chi.ti.ni.phi'lus. N.L. n. *chitinum* chitin; Gr. adj. *philos*, loving; N.L. masc. n. *Chitiniphilus* chitin-loving, referring to the substrate used for the isolation of this organism).

Cells are gram-negative, non-spore-forming, curved rods. 0.7–0.8 μm in width and 1.2–1.5 μm in length. Cells are motile by means of a single polar flagellum. Facultatively anaerobic and oxidase- and catalase-positive. Does not produce acid from glucose but positive for nitrate reduction. Major cellular fatty acids (>10%) are 16:1 ω7c (32.5%) and 16:0 (31.2%). Q-8 is the predominant isoprenoid quinone. The DNA G+C content of the type strain of the type species is 67.6 mol%. Phylogenetically belongs to the family *Neisseriaceae* in the class *Betaproteobacteria*.

The type species of the genus is *Chitiniphilus shinanonensis*.

Description of Chitiniphilus shinanonensis sp. nov.

Chitiniphilus shinanonensis (shi.na.no.nen'sis. N. L. masc. adj. *shinanonensis,* pertaining to Shinano, which is the old name of Nagano Prefecture, Japan [the source of water sample from which the type strain was isolated]).

Displays the following properties in addition to those given in the genus description. Colonies are circular with rhizoid margins, smooth, and pale yellow in color. Growth occurs between 15°C and 45°C (optimum 30–37°C) but not at 10°C. It degrades chitin and esculin but not gelatin. Negative for production of indole and urease activity. On the basis of API 50CH test, D-ribose, D-glucose, D-mannose, *N*-acetyl-D-glucosamine, amygdalin, esculin ferric citrate and salicin are utilized. Other substrates are not utilized but weak reactions were detected for D-fructose, D-cellobiose and gentiobiose. With the API ZYM test, positive results are obtained for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and *N*-acetyl-β-glucosaminidase. Negative for lipase (C14), valine arylamidase, cystine arylamidase, α-chymotrypsin, α-galactosidase, β-galactosidase, α-glucuronidase, β-glucosidase, α-mannosidase and α-fucosidase. Major cellular fatty acids (>5%) are 16:1 ω7c (32.5%), 16:0 (31.2%), 18:1 ω7c (9.9%), 17:0 cyclo (6.9%) and 3-OH 12:0 (5.3%). The DNA G+C content of the type strain is 67.6 mol%.

Isolated from moat water of Ueda Castle in Nagano Prefecture, Japan. The type strain of the species is SAY3^T (=NBRC 104970^T=NICMB 14509^T).

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