Yersinia similis sp. nov.

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Strains originally identified phenotypically as members of the species *Yersinia pseudotuberculosis* were subjected to a more detailed classification employing 16S rRNA gene sequence analysis, DNA–DNA hybridization, determination of the DNA base composition and phenotypic characterization. The quinone system, consisting of the predominant compound ubiquinone Q-8 and minor amounts of menaquinone MK-8, the major components of the polar lipid profile, as well as the polyamine pattern, with putrescine as the major compound, supported the assignment of the strains to the genus *Yersinia*. Based on DNA–DNA relatedness, a specific 16S rRNA gene sequence type, absence of melibiose fermentation and a polar lipid profile lacking phosphatidylmonomethylethanolamine and two aminolipids, the strains were identified as members of a novel species for which the name *Yersinia similis* sp. nov. is proposed. The type strain of *Yersinia similis* sp. nov. is strain Y228^T (=CCUG 52882^T=LMG 23763^T).

On the basis of DNA–DNA hybridization, *Yersinia pseudotuberculosis* has been considered to be a molecularly homogeneous species (Brenner *et al.*, 1976; Bercovier *et al.*, 1980). Phenotypic characterization of *Y. pseudotuberculosis* strains by means of a sophisticated serotyping scheme (Tsubokura & Aleksic, 1995; Nagano *et al.*, 1997) and restriction endonuclease analysis of virulence plasmids (REAP), has discriminated pathogenic and non-pathogenic clones (Fukushima *et al.*, 1998). Apart from European O:3 melibiose-negative strains, melibiose non-fermenters are only rarely found in other serogroups (<0.5%; Fukushima *et al.*, 2001). A PCR assay targeting the 16S rRNA gene of *Yersinia pestis* and *Y. pseudotuberculosis* reacted negatively when it was used on a number of strains phenotypically assigned to *Y. pseudotuberculosis* (Neubauer *et al.*, 2000a). Several studies have demonstrated the applicability of 16S rRNA gene sequence analysis for the identification of species within the genus *Yersinia* and its use to determine distinct species-specific markers, for example for *Yersinia aleksiciae* and *Yersinia enterocolitica* subsp. *enterocolitica*/*Yersinia enterocolitica* subsp. *paleartica* (Ibrahim *et al.*, 1993; Neubauer *et al.*, 2000b; Sprague & Neubauer, 2005). The aim of the present study was to investigate whether

isolates of *Y. pseudotuberculosis* displaying different 16S rRNA gene sequences could be classified as representatives of a novel species.

Six *Yersinia* isolates that had been originally phenotyped as *Y. pseudotuberculosis* by the former German *Yersinia* Reference Centre, Hamburg, Germany, were extensively investigated by molecular and biochemical techniques (Tables 1 and 2). Type strains were purchased from the ATCC, Rockville, Maryland, USA, with the exception of *Y. enterocolitica* subsp. *paleartica* Y11^T (=DSM 13030^T). Other type strains of species of the genus *Yersinia* included in the study were *Y. pseudotuberculosis* ATCC 29833^T, *Yersinia ruckeri* Y81^T (=ATCC 29473^T) and *Yersinia kristensenii* Y110^T (=ATCC 33638^T). Strains were maintained on standard nutrient agar I (Oxoid) at 4 °C. Subcultivation was performed on blood agar overnight at 28 °C. Broth culture was carried out overnight in Luria–Bertani medium (Difco) at 28 °C and 150 r.p.m. in controlled environment shakers (New Brunswick). Cells were harvested by centrifugation (1200 g for 30 min) and stored in 70% 2-propanol at 4 °C until used for DNA–DNA hybridization experiments.

Chemotaxonomic analyses were carried out with strain Y228^T, *Y. enterocolitica* subsp. *paleartica* Y11^T and *Y. pseudotuberculosis* ATCC 29833^T. The strains were grown on 3.3 × PYE [1% peptone from casein (Roth); 1% yeast extract (Merck), pH 7.2] and polyamines were extracted and analysed as described by Busse & Auling (1988) and Stolz *et al.* (2007). Quinone systems and polar lipids were

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains Y36, ATCC 29833^T, Y117, Y228^T, Y233 and Y252 are AM182400, AM182401, AM182402, AM182404, AM182405 and AM182406, respectively.

A supplementary table detailing DNA–DNA hybridization values between strains of *Yersinia pseudotuberculosis* and strains Y228^T, Y233 and Y252 is available with the online version of this paper.

Table 1. Characterization of the *Yersinia* isolates examined in this study

ND, Not determined; P, 16S rRNA gene sequence type of *Y. pseudotuberculosis* ATCC 29833^T; S, 16S rRNA gene sequence type of strain Y228^T. The P and S 16S rRNA gene sequence types are characterized by the following specific sequence markers: P, 5'-ATTTGGCAGAGATGCTAAA-3'; S, 5'-GATTTGGCAGAGATGCCTTA-3' (Neubauer *et al.*, 2000a). None of the isolates tested positive for the presence of the plasmid fixed pathogenicity marker adhesin gene (Neubauer *et al.*, 2000c).

Strain	Origin	Source	16S rRNA gene sequence type	V-antigen gene*	Serotype
ATCC 29833 ^T	Turkey	ND	P	—	ND
Y36†	Germany	Guinea pig	P	+	III
Y117†	Germany	Mara	P	+	III
Y228 ^T	Germany	Rabbit	S	—	6
Y233‡	Japan	Mole	S	+	5b
Y252‡	Japan	Mole	S	+	5a

*Presence of the chromosomally fixed pathogenicity marker V-antigen (Neubauer *et al.*, 2000c).

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analysed according to the procedures of Tindall (1990a, b) and Altenburger *et al.* (1996) using HPLC apparatus as described by Stolz *et al.* (2007). Molecular and biochemical characterization and identification of *Yersinia* isolates was conducted as described by Neubauer *et al.* (2000a, b, c). Multiple sequence alignments of partial 16S rRNA gene sequences were performed with CLUSTAL W 1.8 and with BOXSHADE 3.21 (available at <http://clustalw.genome.jp/> and http://www.ch.embnet.org/software/BOX_form.html).

The phylogenetic tree based on *rrs* gene sequences (1388 nt) was generated using the online accessible bioinformatics tools from HUSAR (available from <http://genius.embnet.dk/fz-heidelberg.de/menu/w2h/w2hdkfz>). The *rrs* gene sequences were aligned using CLUSTAL W. The phylogenetic tree was constructed from the alignment obtained with CLUSTREE and the Kimura two-parameter model was implemented. Phylogenetic reconstruction was conducted using the neighbour-joining method and 1000 bootstrap resamplings were performed to assess the probabilities of the nodes. Trees were rooted using the 16S rRNA gene sequence of *Escherichia coli* ATCC 43895.

The API 20E (bioMérieux) test was performed as described by the manufacturer and the semi-miniaturized *Yersinia* identification system (Merlin) was used according to Neubauer *et al.* (2000d). All incubations were carried out at 28 °C in order to avoid the failure of metabolic reactions that can occur at 37 °C. DNA extractions, DNA–DNA hybridizations and determination of the G+C contents were carried out at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany, as recently described (De Ley *et al.*, 1970; Cashion *et al.*, 1977; Escara & Hutton, 1980; Huß *et al.*, 1983; Tamaoka & Komagata, 1984; Mesbah *et al.*, 1989; Jahnke, 1992; Martin *et al.*, 1997).

All of the putative *Y. pseudotuberculosis* strains were small, Gram-negative, motile coccoid rods and were catalase- and

urease-positive and oxidase-negative. The strains were positive in tests for rhamnose, glucose, trehalose and L-pyrogutamic acid- β -naphthylamide and showed various negative reactions (Table 2). Strains ATCC 29833^T, Y117 and Y36 all possessed 16S rRNA gene sequence type P and were able to ferment melibiose. By the use of a semi-miniaturized *Yersinia* identification system (Merlin) optimized for *Yersinia* isolates, these strains were clearly identified as *Y. pseudotuberculosis* with a relative probability of 100 % and an absolute probability of <1200:1. Strains Y228^T, Y252 and Y233 all possessed 16S rRNA gene sequence type S and were melibiose-negative. These strains were also identified as *Y. pseudotuberculosis* with a relative probability of >95 % but with an absolute probability of >10 000:1, making this diagnosis doubtful (Neubauer *et al.*, 2000d). The API 20E test system identified all six strains as *Y. pseudotuberculosis*. The type strain of *Y. pseudotuberculosis*, ATCC 29833^T, was negative for tryptophan deaminase and gelatin. However, it showed a number of very untypical reactions when compared with the majority of strains belonging to the species *Y. pseudotuberculosis* by using the semi-miniaturized *Yersinia* identification system, i.e. aesculin, cellobiose, lactose, sorbose and the Voges–Proskauer reaction (Sprague & Neubauer, 2005). In the classical tube testing system, no deviations were observed from the original species description (Bercovier *et al.*, 1980). The anomalous results seen in the semi-miniaturized *Yersinia* identification system can be explained by the formulation of the reagents and the arbitrary choice of cut-off values selected for optimal species and biovar designation within the genus *Yersinia* or *Yersinia enterocolitica*, respectively (Neubauer *et al.*, 2000d). Additionally, positive reactions were observed for γ -glutamyl- β -naphthylamide, L-pyrogutamic acid- β -naphthylamide and proline, but negative reactions were observed for p-nitrophenyl- β -D-xylopyranoside, p-nitrophenyl- β -glucuronide, 5-bromo-4-chloro-3-indolyl butyrate and pyrazinamidase.

Table 2. Biochemical characterization of strains of *Y. pseudotuberculosis* and the proposed species *Yersinia similis* sp. nov. compared with biochemical patterns of other type strains of the genus *Yersinia* (Table modified according to Sprague & Neubauer, 2005)

Taxa: 1, *Y. mollaretii*; 2, *Y. aldovae*; 3, *Y. bercovieri*; 4, *Y. intermedia*; 5, *Y. rhodei*; 6, *Y. enterocolitica* subsp. *enterocolitica*; 7, *Y. enterocolitica* subsp. *paleoartica*; 8, *Y. aleksiciae*; 9, *Y. frederiksenii*; 10, *Y. kristensenii*; 11, *Y. pestis* (n=40); 12, *Y. pseudotuberculosis* (n=36; data from Neubauer *et al.*, 2000d); 13, *Y. similis* sp. nov. (n=3); 14, *Y. similis* sp. nov. strain Y228^T; 15, Y233; 16, Y252; 17, *Y. ruckeri*. +, Positive; -, negative. Values for taxa 11–13 are % positive reactions for the group of strains. All taxa gave negative results for H₂S, for the fermentation of amygdalin, dulcitol and inulin, and for the activities of arginine dihydrolase, p-nitrophenol- β -glucuronide, pyrazinamidase and tryptophan deaminase. All taxa gave positive results for fermentation of glucose.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Fermentation of:																	
Adonitol	-	-	-	-	-	-	-	-	-	-	0	6	0	-	-	-	-
L-Arabinose	+	+	+	+	+	+	+	+	+	+	38	81	100	+	+	+	-
Cellobiose	+	+	+	+	+	+	-	+	+	+	0	0	0	-	-	-	-
D-Xylose	+	+	+	+	+	+	-	+	+	+	10	94	100	+	+	+	-
Glycerine	+	-	-	+	+	+	+	+	+	+	0	89	100	+	+	+	+
Inositol	-	+	-	-	-	-	-	-	+	-	0	0	0	-	-	-	-
Lactose	-	+	+	-	-	-	-	-	-	-	0	0	33	+	-	-	-
Melibiose	-	+	-	+	+	-	-	-	-	-	0	97	0	-	-	-	-
Raffinose	-	-	-	-	+	-	-	-	-	-	0	0	0	-	-	-	-
Rhamnose	-	+	-	+	-	-	-	-	+	-	0	100	100	+	+	+	-
Salicin	-	-	+	+	+	-	-	-	+	-	0	11	0	-	-	-	-
Sorbitol	+	+	+	+	+	+	+	+	+	+	0	0	0	-	-	-	-
Sorbose	+	-	-	+	+	+	+	+	+	+	0	0	0	-	-	-	-
Sucrose	+	-	+	+	+	+	+	-	+	-	0	0	0	-	-	-	-
Trehalose	+	+	+	+	+	+	+	+	+	+	88	94	100	+	+	+	+
5-Bromo-4-chloro-3-indolyl butyrate	-	-	-	-	-	-	+	-	-	-	0	0	0	-	-	-	-
Aesculin	-	-	+	+	+	-	-	-	+	-	100	94	100	+	+	+	-
Citrate	+	+	-	+	+	-	+	-	+	-	5	0	0	-	-	-	-
Indole	-	-	-	+	-	+	-	+	+	+	0	0	0	-	-	-	-
L-Proline- β -naphthylamide	+	+	+	+	+	-	-	+	+	+	73	100	0	-	-	-	+
L-Pyrogutamic acid- β -naphthylamide	+	+	+	+	+	+	+	+	+	+	0	100	100	+	+	+	+
Lysine decarboxylase	-	-	-	-	-	-	-	+	-	-	0	0	0	-	-	-	-
Malonate	-	-	-	-	-	-	+	-	-	-	5	0	0	-	-	-	-
o-Nitrophenyl- β -D-galactopyranoside	+	-	+	+	+	+	+	+	+	+	0	75	0	-	-	-	+
o-Nitrophenyl- β -D-xylopyranoside	-	-	-	-	+	-	-	-	-	-	0	0	0	-	-	-	-
Ornithine decarboxylase	+	+	+	+	+	+	+	+	+	+	0	0	0	-	-	-	+
Urea	+	+	+	+	+	+	+	+	+	+	0	100	100	+	+	+	-
Voges-Proskauer reaction	-	+	-	-	-	-	-	-	-	-	0	0	0	-	-	-	-
γ -Glutamyl- β -naphthylamide	+	+	+	+	+	-	-	-	-	-	0	64	33	+	-	-	-

Chemotaxonomic analyses (Table 3) revealed rather similar polyamine patterns for strains Y228^T, Y11^T and ATCC 29833^T with putrescine as the predominant compound. Moderate to minor amounts of spermidine and minor amounts of 1,3-diaminopropane and spermine were detected in the three strains. Only *Y. enterocolitica* subsp. *paleoartica* Y11^T tested positive for the presence of cadaverine. The presence of cadaverine in the polyamine pattern of the type strain of *Y. enterocolitica* subsp. *paleoartica* is in agreement with the results from a previous report (Hamana, 1996) where cadaverine was found to be the predominant compound. This difference could be caused by the use of different media for the growth of biomass. Another explanation for the difference might be that Hamana (1996) harvested cells that were either

growing exponentially or were at the stationary phase, whereas in this study cells were harvested at approximately 70 % of the maximum optical density.

Ubiquinone Q-8 was the predominant quinone compound found in the three strains (Y228^T, Y11^T and ATCC 29833^T). Additionally, minor amounts of Q-9 and minor to moderate amounts of menaquinone MK-8 were detected. Similar quinone systems have been reported for all species of the family *Enterobacteriaceae* examined so far (Yokota *et al.*, 1992). The polar lipid profile of strain Y228^T shared major characteristics with the other two strains (Table 3), but could be distinguished from *Y. pseudotuberculosis* ATCC 29833^T due to the lack of phosphatidylmonomethylethanolamine and two aminolipids. The

Table 3. Chemotaxonomic characteristics of strain Y228^T and the type strains of two *Yersinia* reference species

Components in the polyamine patterns and quinone systems are given in relative amounts (%). All taxa had major amounts of phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG) and moderate amounts of phosphatidylglycerol (PG). ++, Present in moderate amounts; +, present in minor amounts; ND, not detected; PME, phosphatidylmonomethylethanolamine; APL1, unknown aminophospholipid; AL1, AL2, unknown amino lipids.

Strain	Y228 ^T	<i>Y. enterocolitica</i> subsp. <i>paleartica</i> DSM 13030 ^T	<i>Y. pseudotuberculosis</i> ATCC 29833 ^T
Polyamines			
1,3-diamino-propane	0.9	1.8	0.3
Putrescine	91.8	94.2	88.0
Cadaverine	ND	2.6	ND
Spermidine	6.4	1.3	11.4
Spermine	0.9	0.2	0.3
Quinones			
Q-8	89	95	93
Q-9	1	1	1
MK-8	10	4	6
Polar lipids			
PME	ND	ND	+
APL1	++	++	++
AL1	ND	ND	+
AL2	ND	ND	+

chemotaxonomic characteristics of strain ATCC 29833^T were in agreement with those of the two reference strains analysed in this study and thus support the assignment of this strain to the genus *Yersinia*. Differences in the polar lipid profile (absence of phosphatidylmonomethylethanolamine and two aminolipids) compared with that of *Y. pseudotuberculosis* ATCC 29833^T support the view that strain Y228^T is a representative of a novel species not yet described within the genus (Fig. 1).

All strains investigated gave a negative result in the plasminogen activator and F1 antigen gene PCR assay specific for *Yersinia pestis*. The 16S rRNA gene sequence type S strains were negative in the *Y. pseudotuberculosis*/*Y. pestis*-specific 16S rRNA gene PCR described by Neubauer *et al.* due to sequence alterations in the sequence of primer PT2 (Neubauer *et al.*, 2000a). Molecular pathogenicity markers were dispersed within all strains investigated (Table 1). A phylogenetic tree constructed from the 16S rRNA gene sequence obtained from this study and the sequences of the type strains of all the other recognized species of the genus *Yersinia* showed the close relatedness of *Y. pestis* and *Y. pseudotuberculosis*. The *Y. pseudotuberculosis*-like strains of 16S rRNA gene type S uniformly grouped into their own cluster (Fig. 2).

Strains possessing 16S rRNA gene sequence type P (strains Y36, ATCC 29833^T and Y117) formed a genomic species

Fig. 1. Two-dimensional TLC of polar lipids of strain Y228^T. For abbreviations see Table 3.

with an intragroup relatedness of >70 % DNA–DNA relatedness. Hence, we consider only strains Y36 and Y117 to represent the species *Y. pseudotuberculosis*. It has to be stressed that this sequence type can also be found in the 16S rRNA genes of other bacteria. As a marker for *Y. pseudotuberculosis*, it can only be used after an isolate has already been phenotypically assigned to the genus *Yersinia*. Strains of 16S rRNA gene sequence type S (strains Y228^T, Y233 and Y252) formed a second genomic species with an intragroup relatedness of >70 % DNA–DNA relatedness, but only 44–66 % relatedness with strains of *Y. pseudotuberculosis* (see Supplementary Table S1 available in IJSEM Online). Members of these genomic species showed no significant degree of DNA–DNA relatedness to *Y. ruckeri* ATCC 29473^T or *Y. kristensenii* ATCC 33638^T (see Supplementary Table S1). The G+C contents of the DNA for strains Y36, Y117, Y228^T, Y233 and Y252 were 48.9 mol%, 48.9 mol%, 48.1 mol%, 47.6 mol% and 49.3 mol%, respectively. Strains characterized by the lack of melibiose utilization and a 16S rRNA sequence of type S were considered to represent a separate novel species, referred from now on as *Yersinia similis* sp. nov.

Y. similis sp. nov. strains showed a high sequence similarity in helix 18 of the 16S rRNA gene sequence [positions 451–480 according to *E. coli* 16S rRNA International Union of Biochemistry (IUB) nomenclature] to the gene sequences of *Y. pestis* and *Y. pseudotuberculosis*. 16S rRNA gene sequences have already been used as markers to identify novel species and subspecies within the genus *Yersinia* (Neubauer *et al.*, 2000b; Sprague & Neubauer, 2005). Thus, in order to differentiate *Y. similis* sp. nov., a new specific sequence 5'-GATTTGGCAGAGATGCCTTA-3' (positions 987–1006; *E. coli* IUB nomenclature) is proposed as a suitable genetic marker. The phenotypic marker for the biochemical differentiation of *Y. pseudotuberculosis* and *Y. similis* sp. nov. is melibiose fermentation which is regularly

Fig. 2. Phylogenetic tree obtained with 16S rRNA gene sequences (1388 nt) using CLUSTREE neighbour-joining analysis. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets. The tree was rooted with the outgrouping sequence of *E. coli* ATCC 43895 (GenBank accession no. Z83205). Bar, 0.01 divergent residues per site.

observed for *Y. pseudotuberculosis* but not for *Y. similis* sp. nov. However, sporadically melibiose-negative strains of *Y. pseudotuberculosis* have been described previously (Fukushima *et al.*, 2001). The strains of *Y. similis* sp. nov. investigated in this study appear to belong to the non-pathogenic, virulence-plasmidless genetic group 4 as described by Fukushima *et al.* (2001). The strains have been isolated from animals with clinically inapparent infections and from the environment, i.e. surface water, but have never been isolated from diseased animals or humans. The occurrence of strains such as *Y. similis* sp. nov. Y228^T in Germany is not surprising as strains of the genus *Yersinia* can be easily introduced into non-endemic areas via food, animals or even human travellers (Fukushima *et al.*, 2001).

A molecular subgroup within *Y. pseudotuberculosis* strains (subgroup 4) was identified by Kim *et al.* (2003) by 16S rRNA gene sequencing, REP-PCR, ERIC-PCR and Box-PCR. This subgroup was made up of strains of our 16S rRNA gene sequence type S (GenBank accession nos AF365936, AF365945, AF365948 and AF365950) of serotypes 1c, 6, 9 and 11. These strains can now also be identified as *Y. similis* sp. nov. Strains of *Y. similis* sp. nov. should be grouped into risk class 2 until non-pathogenicity for man has been confirmed.

The 16S rRNA gene sequences of the three *Y. similis* sp. nov. strains were identical (Fig. 2), exhibiting 99.6% similarity with the 16S rRNA gene sequences of *Y.*

pseudotuberculosis and *Y. pestis*. When comparing the 16S rRNA gene sequences of the type strains of the species of the genus *Yersinia*, the sequence similarity never falls below 97.4%.

The mean G + C content of the DNA of the *Y. pseudotuberculosis* isolates was 48.4 mol% and is thus 1.9 mol% above the previously published value (Bercovier *et al.*, 1980). The mean G + C content of the DNA of *Y. similis* sp. nov. is estimated to be 48.3 mol%. This value is slightly higher than the values determined for *Y. pseudotuberculosis* strain IP32953 (47.55 mol%) and several strains of *Y. pestis* (47.63–47.70 mol%) based on their whole genome sequences (<http://cmr.tigr.org/tigr-scripts/CMR/shared/Genomes.cgi>) and 1.8 mol% higher than the value published for *Y. pseudotuberculosis* (Bercovier & Mollaret, 1984). This estimate is within the accepted limits for the genus *Yersinia* of 46–50 mol% (Bercovier & Mollaret, 1984).

The 16S rRNA gene sequence type analysis, the results from DNA–DNA hybridizations, the absence of fermentation of melibiose and p-nitrophenyl- β -D-galactopyranoside and the absence of phosphatidylmonomethylethanolamine and two unknown aminolipids provide evidence that strains Y228^T, Y233 and Y252 are representatives of a novel species which is phenotypically distinguishable from its nearest phylogenetic neighbour *Y. pseudotuberculosis*. The name *Y. similis* sp. nov. is proposed for these three isolates.

The importance of this novel species for ecologists, evolutionary geneticists and clinical microbiologists must

be emphasized. Members of the novel species seem to be well adapted to the environment and do not cause disease. *Y. similis* sp. nov. is distributed in the Far East and in areas where plague is still endemic. *Y. similis* may therefore interfere with classical field diagnostics. The removal of this novel species from the *Y. pestis*/*Y. pseudotuberculosis* complex will also be an important step for future pathogenicity and evolution studies.

Description of *Yersinia similis* sp. nov.

Yersinia similis (si'mi.li.s. L. fem. adj. *similis* similar, resembling, as the strains are similar to those of *Y. pseudotuberculosis*).

Cells are small, Gram-negative and motile coccoid rods. Cells are catalase- and urease-positive, oxidase-negative and reduce nitrate. Rhamnose-, trehalose- and glycine-positive, but melibiose-negative. The specific 16S rRNA gene sequence marker is 5'-GATTGGCAGAGATGCCTTA-3' (position 987–1006; *E. coli* IUB nomenclature). Other characteristics are listed in Tables 2 and 3. The mean DNA G+C content is 48.3 mol% (HPLC). The DNA G+C content of the type strain is 48.1 mol%.

The type strain, Y228^T (=CCUG 52882^T=LMG 23763^T), was isolated from a rabbit in Germany.

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