

## *Arthrobacter bergerei* sp. nov. and *Arthrobacter arilaitensis* sp. nov., novel coryneform species isolated from the surfaces of cheeses

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Fourteen isolates of two different bacterial species isolated from the surface of smear-ripened cheeses were found to exhibit many characteristics of the genus *Arthrobacter*. The isolates were aerobic, Gram-positive, catalase-positive, non-spore-forming and non-motile. The cell-wall peptidoglycan contained lysine, alanine and glutamic acid. *rrs* sequence analysis indicated that the new isolates Re117<sup>T</sup> and Ca106<sup>T</sup> are closely related to the *Arthrobacter nicotianae* group and showed highest sequence similarity (>98%) to *Arthrobacter nicotianae* and *Arthrobacter protophormiae*. However, DNA–DNA hybridization studies indicated that the strains represented two novel genomic species within the genus *Arthrobacter* and did not belong to *A. nicotianae* or *A. protophormiae* (<43% DNA–DNA relatedness). On the basis of the phylogenetic and phenotypic distinctiveness of the new isolates, these bacteria should be classified as two novel *Arthrobacter* species, for which the names *Arthrobacter bergerei* sp. nov. and *Arthrobacter arilaitensis* sp. nov. are proposed. Type strains have been deposited in culture collections as *Arthrobacter bergerei* Ca106<sup>T</sup> (=CIP 108036<sup>T</sup>=DSM 16367<sup>T</sup>) and *Arthrobacter arilaitensis* Re117<sup>T</sup> (=CIP 108037<sup>T</sup>=DSM 16368<sup>T</sup>).

Representatives of the cheese-surface bacterial flora are isolated from soft or semi-soft cheeses such as Camembert and Munster, from blue-veined cheeses and also from hard cheeses such as Gruyère. Bacterial counts ranging from 10<sup>7</sup> to 10<sup>9</sup> c.f.u. g<sup>-1</sup> are reached within the first 2 weeks of ripening of smear soft cheeses, attaining densities of 10<sup>13</sup> c.f.u. g<sup>-1</sup> for smear Gruyère cheeses within the first 3 weeks of ripening. This flora is composed of two principal Gram-positive groups: coryneform bacteria (irregular rods or cocci, catalase-positive) and staphylococci (cocci, catalase-positive) (Bockelmann & Hoppe-Seyler, 2001; Bockelmann, 2002; Brenann *et al.*, 2002; Place *et al.*, 2002, 2003). These two groups present similar physiological properties permitting their growth on the cheese surface:

they are aerobic, alkaliphilic, mesophilic and salt-tolerant and cannot develop under acid conditions.

Fifteen years ago, in both the dairy industry and the literature, cheese coryneform bacteria were still either classified separately in pigmented groups (orange, yellow, cream, grey, unpigmented) or assigned to groups based on a few, subjectively weighted morphological and staining properties (Eliskases-Lechner & Ginzing, 1995; Piton & Fontanier, 1990; Piton-Malleret & Gorrieri, 1992). To date, only a few taxonomic molecular studies have been carried out on the identification of these bacteria (Brennan *et al.*, 2001a, b, 2002; Hoppe-Seyler *et al.*, 2003) and knowledge of the species composition of the smear cheese is limited.

Members of the genus *Arthrobacter* are Gram-positive, catalase-positive, aerobic and asporogenous bacteria that display a coryneform morphology (Keddie *et al.*, 1986). This genus is phenotypically heterogeneous and over 35 species are currently recognized (Euzéby, 2004; Stackebrandt & Schumann, 2000). Two main groups of species are distinguished within the genus *Arthrobacter sensu stricto* on the basis of their peptidoglycan type and menaquinone composition (Keddie *et al.*, 1986; Schleifer & Kandler, 1972; Stackebrandt *et al.*, 1983; Stackebrandt & Schumann, 2000).

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains Gor104, Ma107, Bres102, Cou102, Epo104, Po101, Po102, Re117<sup>T</sup>, Stp101, Ca106<sup>T</sup>, Ka101, La101 and Re127 are AJ609621–AJ609633, respectively.

A dendrogram based on ribotyping data, an extended 16S rRNA gene-based neighbour-joining tree and detailed DNA–DNA hybridization results are available as supplementary material in IJSEM Online.

Group I species of *Arthrobacter* contain A3 $\alpha$  peptidoglycan variant and include most species of the genus including *Arthrobacter globiformis*, the type species (Stackebrandt *et al.*, 1983). Group II species possess the A4 $\alpha$  peptidoglycan variant and include *Arthrobacter nicotianae*, *Arthrobacter sulfureus*, *Arthrobacter mysorens*, *Arthrobacter uratoxydans* and *Arthrobacter protophormiae* and also two relatively recently described species, *Arthrobacter creatinolyticus* (Hou *et al.*, 1998) and *Arthrobacter rhombi* (Osario *et al.*, 1999). Representatives of the group II species form a separate branch within the phylogenetic cluster of the genus *Arthrobacter* (Osario *et al.*, 1999; Stackebrandt & Schumann, 2000).

The occurrence of yellow-pigmented *Arthrobacter* strains including those identified as *A. nicotianae* in surface-ripened cheeses (Valdès-Stauber *et al.*, 1997) or in mould surface-ripened cheeses, such as Brie and Camembert (Marcellino & Benson, 1992), has been reported. A number of yellow coryneform isolates from several varieties of Austrian cheeses were preliminarily identified as *A. globiformis*, while most *Arthrobacter* strains could not be assigned to any known species (Bockelmann *et al.*, 1997; Eliskases-Lechner & Ginzinger, 1995).

In this paper, we present the results of a polyphasic taxonomic study of 14 strains from the surface of smear-ripened cheeses and propose to classify them as two novel species of the genus *Arthrobacter*, *Arthrobacter bergerei* sp. nov. and *Arthrobacter arilaitensis* sp. nov.

The 14 strains under study were isolated from the surfaces of different smear-ripened cheeses (French cheeses unless stated otherwise): Bres102, from Bresso (a German cheese); Ca106<sup>T</sup>, Camembert; Cou102, Coulommiers; Ep104, Epoisses; Gor104, Gorgonzola (an Italian cheese); Ka101, Kamtohtv (a Russian cheese); La101, Langres; Ma107, Maroilles; Po101 and Po102, Pont l'Évêque; Re117<sup>T</sup> and Re127, Reblochon; Sne104, Saint-Nectaire; Stp101, Saint Paulin. All strains were cultivated on brain heart infusion (BHI) agar and incubated at 28 °C. The following reference strains were used in the comparative study: *A. nicotianae* LMG 16305<sup>T</sup>, *A. nicotianae* CIP 82.22, *A. protophormiae* DSM 20168<sup>T</sup>, *A. protophormiae* ATCC 21040, *A. sulfureus* LMG 16694<sup>T</sup>, *A. uratoxydans* CIP 102367<sup>T</sup>, *A. mysorens* LMG 16219<sup>T</sup>, *A. mysorens* LMG 16125, *A. rhombi* CCUG 38813<sup>T</sup> and *A. creatinolyticus* JCM 10102<sup>T</sup>. Stocks of all strains were prepared in 50 % (w/v) glycerol and stored at -80 °C.

Cell morphology was determined by phase-contrast microscopy of cells grown on BHI agar (Difco), motility by the hanging-drop method and the catalase test by the production of bubbles on the application of 3 % (v/v) H<sub>2</sub>O<sub>2</sub> to a colony. Oxidase activity was determined by using the modified oxidase test of Faller & Schleifer (1981). Salt tolerance (NaCl) and optimal growth temperature range were determined using a multipoint inoculator (Kloos *et al.*, 1974).

The commercially available API CORYNE kit (bioMérieux) was used to determine enzymic activities and some other phenotypic properties and interpreted according to the manufacturer's instructions; strips were read after 24 h incubation. Carbon-source utilization tests were performed with the Biotype 100 system using medium 2 (bioMérieux) after 2, 4 and 6 days incubation. All biochemical tests were performed at 28 °C.

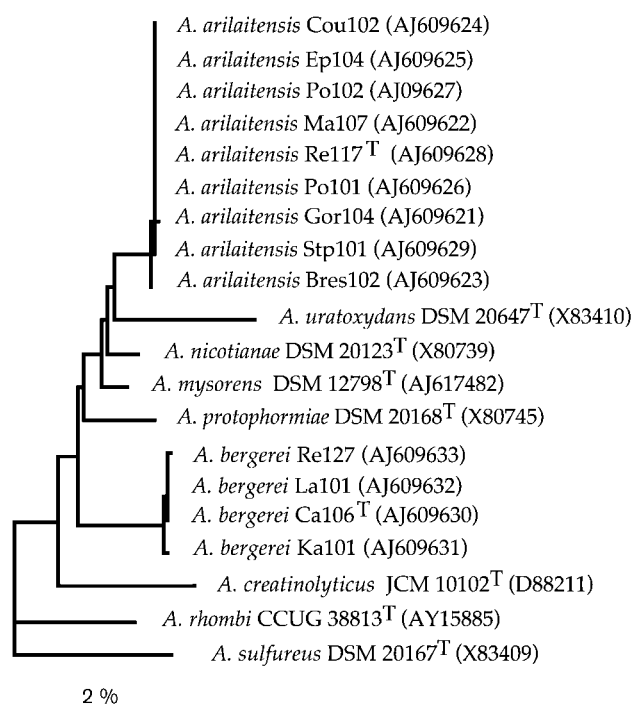
Cell-wall material was prepared and hydrolysed by the method of Keddle & Cure (1977). A 3  $\mu$ l aliquot was spotted onto a high-performance TLC cellulose plate, which was developed by two-dimensional chromatography as described by Brenner *et al.* (1969). Whole-cell sugars were analysed as described by Schaal (1985). Mycolic acids were detected as described by Minnikin *et al.* (1975, 1980).

Genomic DNA was extracted as follows. Strains were grown at 28 °C for 2 days with mixing in a 2 l Erlenmeyer flask containing 500 ml BHI broth. Cultures were harvested at 6000 g for 20 min and washed twice with 50 mM Tris/HCl (pH 8). Next, 15–50 ml (depending on the pellet size) of a solution containing Tris/HCl (50 mM), pH 8, sucrose (100 mM) and 0.5 % (v/v) Triton X-100 was added, mixed and incubated overnight at 30 °C. Subsequently, 250 mg lysozyme and 375  $\mu$ l mutanolysin (5 U  $\mu$ l<sup>-1</sup>) were added, mixed and incubated for 1 h at 37 °C. Next, 3.75 ml proteinase K (20 mg ml<sup>-1</sup>) and 3.7 ml 10 % Sarkosyl were added, mixed and incubated for 24 h at 37 °C. Finally, 3.75  $\mu$ l 25 % SDS was added, mixed and incubated for 1 h at 55 °C.

DNA was purified according to Brenner *et al.* (1982). DNA–DNA hybridization studies were carried out at the Institut Pasteur by the S1 nuclease method following a published procedure (Grimont *et al.*, 1980). DNA–DNA hybridization was carried out at 60 °C. The temperature at which 50 % of the reassociated molecules are dissociated ( $T_m$ ) has been determined (Crosa *et al.*, 1973) when DNA relatedness was between 30 and 80 %. The difference ( $\Delta T_m$ ) between the  $T_m$  of the homoduplex and the  $T_m$  of the heteroduplex was calculated as a measure of divergence (Brenner, 1978).

PCR amplification of *rrs* gene fragments was performed as described by Dauga *et al.* (1997). Amplified fragments of 1500 bp were sequenced by Genome Express. The sequences obtained were assembled and aligned with the Lasergene software (DNASTAR) and compared to the GenBank database ([http://www2.ncbi.nlm.nih.gov/genbank/query\\_form.html](http://www2.ncbi.nlm.nih.gov/genbank/query_form.html)). The *rrs* sequences of 13 strains were aligned with reference sequences (Fig. 1) from the GenBank/EMBL database, using the multiple sequence alignment program CLUSTAL V (Higgins *et al.*, 1992). Phylogenetic distances were determined according to Jukes & Cantor (1969) and a tree was obtained by neighbour-joining (Saitou & Nei, 1987) as implemented by the Lasergene software.

For riboprinting analysis, DNA of each strain was obtained as described in Lefresne *et al.* (2004). Methods for cleavage



**Fig. 1.** Unrooted tree based on 16S rRNA gene sequences showing the phylogenetic relationships of *Arthrobacter arilaitensis* sp. nov., *Arthrobacter bergerei* sp. nov. and other nearest neighbours of the genus *Arthrobacter*. The tree, constructed by the neighbour-joining method, was based on a comparison of approximately 1400 nucleotides. Bar, 2% nucleotide substitution.

of each DNA with either *Bgl*I or *Pvu*II, electrophoresis, hybridization with 16S and 23S rRNA and visualization of ribotypes have been published (Lefresne *et al.*, 2004; Regnault *et al.*, 1997). Ribotypes were interpreted using various programs of the Taxotron package (Institut Pasteur) as described in Brosch *et al.* (1996).

The 14 isolates exhibited the general characteristics of the genus *Arthrobacter*. They were Gram-positive, non-spore-forming, non-motile, not acid-fast and displayed a rod-coccus growth cycle. They produced a cream or yellow pigment depending on the medium. The cell wall of all 14 strains contained L-lysine as the principal amino acid, galactose, glucose, ribose and mannose as whole-cell sugars and no mycolic acids. The 16S rRNA gene-based phylogenetic analysis showed that all 14 strains fall into a separate phylogenetic cluster (Fig. 1; see also Supplementary Fig. A in IJSEM Online) encompassing seven *Arthrobacter* species that have peptidoglycan variation A4 $\alpha$  (Osario *et al.*, 1999; Stackebrandt & Schumann, 2000). It also indicated that the cheese isolates constituted two different phylogenetic groups and showed highest sequence similarity (>98%) to *A. nicotianae*, *A. mysorens* and *A. protophormiae* (Fig. 1).

Ribotypes with *Pvu*II and *Bgl*I restriction digests of cheese strains showed that three groups, composed respectively of

five strains (Ma107, Po101, Po102, Re117<sup>T</sup>, Bres102), four strains (Cou102, Ep104, Stp101 and Gor104), subsequently referred to as *Arthrobacter arilaitensis* sp. nov., and five strains (Sne104, Re127, Ka101, La101, Ca106<sup>T</sup>), subsequently referred to as *Arthrobacter bergerei* sp. nov., could be distinguished by ribotyping (Supplementary Fig. B in IJSEM Online). The *rrs* sequences of *A. arilaitensis* and *A. bergerei* each showed more than 99% within-group similarity, demonstrating their homogeneity.

DNA of the four strains Re117<sup>T</sup>, Po101, Ep104 and Ca106<sup>T</sup>, representing different ribotypes, was hybridized against that from all 14 cheese isolates and the type strains of phylogenetically neighbouring species. DNA–DNA reassociation values showed *A. arilaitensis* and *A. bergerei* to constitute genomic species with 78–100% within-species relatedness and 23–28% between-species relatedness (for detailed results see Supplementary Table in IJSEM Online). According to DNA–DNA hybridization studies, *A. arilaitensis* (represented by strains Re117<sup>T</sup>, Po101, Ep104) was less than 43, 34 and 18% related to the respective type strains of *A. nicotianae*, *A. protophormiae* and *A. uratoxydans*. *A. bergerei* (strain Ca106<sup>T</sup>) was less than 18% related to *A. protophormiae* DSM 20168<sup>T</sup>. A previous study at the Institut Pasteur (R. Brosch and P. A. D. Grimont, unpublished data) had shown *A. nicotianae* LMG 16305<sup>T</sup> (source of labelled DNA) and *A. mysorens* LMG 16219<sup>T</sup> (source of unlabelled DNA) to constitute a single DNA relatedness group (74% DNA–DNA reassociation). Therefore, *A. arilaitensis* and *A. bergerei* are discrete genomic species.

Support for the distinctiveness of the novel species also came from phenotypic evidence (Table 1). On the basis of the results of physiological, chemical and molecular genetic analyses, it was concluded that the *Arthrobacter* isolates described should be classified as two novel species of the genus *Arthrobacter*, for which we propose the names *Arthrobacter arilaitensis* sp. nov. and *Arthrobacter bergerei* sp. nov.

### Description of *Arthrobacter arilaitensis* sp. nov.

*Arthrobacter arilaitensis* (a.ri.lai.ten'sis. N.L. masc. adj. *arilaitensis* of Arilait, arbitrary name formed to honour Arilait-Recherches, a research association that coordinates the collective research programmes of the professional French dairy federations).

The description given below was based on the study of nine strains. Cells are aerobic, Gram-positive, catalase-positive, oxidase-negative, non-spore-forming, non-motile and exhibit a rod-coccus growth cycle. Colonies on BHI agar are yellow, round, smooth, convex and 2 mm in diameter. Grows between 10 and 30 °C and tolerates up to 10% (w/v) NaCl. Gelatinase,  $\beta$ -galactosidase, pyrazinamidase, pyrrolidonyl arylamidase, phosphatase and  $\alpha$ -glucosidase are produced. Urease and aesculin are not hydrolysed. Nitrate is not reduced. Glucose, ribose, xylose,

**Table 1.** Characteristics that differentiate *Arthrobacter bergerei* sp. nov. and *Arthrobacter arilaitensis* sp. nov. from their seven closest phylogenetic relatives

Species: 1, *A. bergerei* sp. nov. ( $n=5$ ); 2, *A. arilaitensis* sp. nov. ( $n=9$ ); 3, *A. nicotianae* ( $n=2$ ); 4, *A. protophormiae* ( $n=2$ ); 5, *A. uratoxydans* ( $n=1$ ); 6, *A. mysorens* ( $n=2$ ); 7, *A. sulfureus* ( $n=1$ ); 8, *A. creatinolyticus* ( $n=1$ ); 9, *A. rhombi* ( $n=1$ ). +, Positive; -, negative; d, reaction differs among strains. All strains were positive for assimilation of D-gluconate, protocatechuate and tyrosine. All strains were negative for assimilation of rhamnose and myo-inositol. Utilization of sole carbon sources was determined with the Biotype 100 system.

Characteristic	1	2	3	4	5	6	7	8	9
Urease	-	-	-	-	+	-	+	-	-
Gelatinase	-	3/+*	d	+	+	+	+	+	-
$\beta$ -Galactosidase	+	+	-	-	-	-	-	-	+
Aesculin hydrolysis	-	-	-	-	-	-	-	-	+
Nitrate to nitrite reduction	-	-	+	+	+	-	-	+	-
Utilization of:									
5-Aminovalerate	1/-	1/-	d	+	+	+	-	+	-
Malonate	1/-	2/-	d	-	-	+ <sup>a</sup> †	+	-	+
DL-Glycerate	-	8/+	+	-	+	-	-	-	+
(-)-Quinate	+	-	d	+	+	+	+	-	+
L-Histidine	4/-	7/-	d	+	+	- <sup>b</sup>	+	-	+
$\alpha$ -D(+)-Glucose	+	7/+	+	-	+	+	-	+	+
D-Xylose	4/-	4/+	+	-	-	+	-	-	-
D-Ribose	+	7/+	+	+	+	+	-	-	+
L-Arabinose	+	4/+	+	d	-	+	-	-	+
D-Galactose	4/-	4/+	d	d	-	+	-	-	+
$\alpha$ -Lactose	+	+	-	-	-	-	-	-	+
L-Rhamnose	1/-	-	-	-	-	-	-	-	+
D-Xylitol	-	-	-	-	-	-	+	-	-
Glycerol	+	+	+	+	+	+	-	+	+

\*Number of strains showing positive reaction/reaction of the type strain.

†Discrepant results in earlier studies are indicated by: *a*, negative in Stackebrandt *et al.* (1983); *b*, positive in Stackebrandt *et al.* (1983); *c*, negative in Osario *et al.* (1999).

mannitol, maltose, lactose, sucrose and glycogen are not fermented. In Biotype 100 strips using biotype 2 medium, most strains (>89%) are able to use the following substrates as sole carbon sources: D-glucose, maltotriose, maltose,  $\alpha$ -lactose, D-arabitol, glycerol, 5-keto-D-gluconate, D-gluconate, protocatechuate, 4-hydroxybenzoate, lactate, glycerate and tyrosine. Other substrates are used less frequently (11–88%): D-galactose, sucrose, D-fructose, D-trehalose, D-mannose, lactulose, L-arabinose, D-ribose, D-xylose, malonate, propionate, 2-oxoglutarate, malate, putrescine, succinate, fumarate, D-glucosamine, 3-hydroxybenzoate, 3-hydroxybutyrate, aspartate, glutamate, proline, alanine, L-histidine, serine, methyl  $\beta$ -galactopyranoside, D-cellobiose,  $\beta$ -gentiobiose, aesculin, D-turanose, D-sorbitol, aconitate, citrate, D-glucuronate, 2-keto-D-gluconate,

L-tryptophan, phenylacetate, 4-aminobutyrate, caprylate and 5-aminovalerate. The following carbon sources are not utilized: sorbose, D-melibiose, D-raffinose, methyl  $\alpha$ -galactopyranoside, methyl  $\beta$ -glucopyranoside, palatinose, L-rhamnose, fucose, D-melezitose, L-arabitol, xylitol, dulcitol, tagatose, myo-inositol, D-mannitol, maltitol, adonitol, lyxose, erythritol, methyl  $\alpha$ -D-glucopyranoside, methyl D-glucopyranose, saccharate, mucate, tartrate, tricarballoylate, D-galacturonate, N-acetyl-D-glucosamine, quinate, gentisate, benzoate, 3-phenylpropionate, *m*-coumarate, trigonelline, betaine, histamine, caprate, glutarate, ethanolamine, tryptamine and itaconate.

The type strain, Re117<sup>T</sup> (=CIP 108037<sup>T</sup>=DSM 16368<sup>T</sup>), utilizes the following substrates as sole carbon sources: D-glucose, D-galactose, D-trehalose, sucrose, maltotriose, maltose, lactose, D-cellobiose, ribose, L-arabinose, D-xylose, D-arabitol, glycerol, turanose, 5-keto-D-gluconate, D-gluconate, protocatechuate, 4-hydroxybenzoate, lactate, glycerate, aspartate, glutamate, alanine, serine and tyrosine. The cell wall contains lysine, alanine and glutamic acid. The whole cell sugars are galactose, glucose, ribose and mannose. The type strain was isolated from the surface of Reblochon cheese.

### Description of *Arthrobacter bergerei* sp. nov.

*Arthrobacter bergerei* (ber.ge're.i. N.L. gen. n. *bergerei* of Bergère, to honour Jean-Louis Bergère, a French microbiologist).

The description given below was based on the results of studies of five strains. Cells are aerobic, Gram-positive, catalase-positive, oxidase-negative, non-spore-forming, non-motile and exhibit a rod-coccus growth cycle. Colonies on BHI agar medium are yellow, round, smooth, convex and 2–3 mm in diameter. Grows between 10 and 30 °C and tolerates up to 7.5% (w/v) NaCl.  $\beta$ -Galactosidase, pyrazinamidase, pyrrolidonyl arylamidase and  $\alpha$ -glucosidase are produced. Urease, phosphatase,  $\beta$ -glucuronidase and gelatinase are not produced. Aesculin is not hydrolysed. Nitrate is not reduced. D-Glucose, ribose, xylose, mannitol, maltose, lactose, sucrose and glycogen are not fermented. In Biotype 100 strips using biotype 2 medium, all strains (100%) are able to use the following substrates as sole carbon sources: D-glucose, fructose, D-galactose, sucrose, maltose, lactose, ribose, L-arabinose, D-xylose, D-glycerol, D-gluconate, quinate, protocatechuate, lactate, aspartate and glutamate. Other substrates are used less frequently (20–80%): lactulose, D-cellobiose, L-rhamnose, D-melezitose, D-mannitol, turanose, D-trehalose, D-mannose, maltotriose, arabitol, methyl  $\beta$ -galactopyranoside, aconitate, citrate, 2-keto-D-gluconate, L-tryptophan, 4-hydroxybenzoate, 3-hydroxybenzoate, phenylacetate, malate, 5-keto-D-gluconate, betaine, 5-aminovalerate, ethanolamine, malonate, 3-phenylpropionate, coumarate, 4-aminobutyrate, benzoate, putrescine, glucosamine, 3-hydroxybutyrate, histidine, L-alanine, serine, propionate,  $\alpha$ -ketoglutarate, proline, D-alanine and tyrosine. The following carbon

sources are not utilized: sorbose, D-melibiose, D-raffinose, methyl  $\alpha$ -galactopyranoside,  $\beta$ -gentiobiose, methyl  $\beta$ -glucopyranoside, aesculin, palatinose, fucose, L-arabitol, xylitol, dulcitol, tagatose, *myo*-inositol, maltitol, D-sorbitol, adonitol, lyxose, erythritol, methyl  $\alpha$ -D-glucopyranoside, methyl D-glucopyranose, saccharate, mucate, D-, L- and *meso*-tartrate, tricarballoylate, D-glucuronate, D-galacturonate, N-acetyl-D-glucosamine, gentisate, trigonelline, histamine, caprate, caprylate, glutarate, glycerate, tryptamine and itaconate.

The type strain, Ca106<sup>T</sup> (=CIP 108036<sup>T</sup>=DSM 16367<sup>T</sup>), utilizes the following substrates as sole carbon sources: D-glucose, fructose, D-galactose, D-mannose, sucrose, lactulose, methyl  $\beta$ -galactopyranoside, maltotriose, maltose, lactose, D-cellobiose, ribose, L-arabinose, D-xylose, D-arabitol, glycerol, D-gluconate, aconitate, citrate, phenylacetate, quinate, protocatechuate, 3-hydroxybenzoate, benzoate, 4-hydroxybenzoate, putrescine, 4-aminobutyrate, lactate, histidine, glucosamine, aspartate, glutamate, 3-hydroxybutyrate, proline, D-alanine, L-alanine, serine, propionate and tyrosine. The cell wall contains lysine, alanine and glutamic acid. The whole cell sugars are glucose, ribose and mannose. The type strain was isolated from the surface of Camembert cheese.

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