# Oenococcus sicerae sp. nov., isolated from French cider

Fabien Cousin, Rozenn Le Guellec, Caroline Chagnot, Didier Goux, Marion Dalmasso, Jean-Marie Laplace, Marina Cretenet

Version of Record: https://www.sciencedirect.com/science/article/pii/S0723202018302613
Manuscript_f6ca6791767ea0fc9ec8f93cb5ba7151

# *Oenococcus sicerae* sp. nov., isolated from French cider

**Authors:**

Fabien J. COUSIN[a], Rozenn LE GUELLEC[a], Caroline CHAGNOT[a], Didier GOUX[b], Marion DALMASSO[a], Jean-Marie LAPLACE[a], Marina CRETENET[¶ a,c]

[a] Normandie Univ, UNICAEN, UNIROUEN, ABTE, 14000 Caen, France

[b] Normandie Univ, UNICAEN, SF ICORE 4206, CMAbio3, F-14032 Caen, France

[c] Normandie Univ, UNICAEN, SF ICORE 4206, CBM, F-14032 Caen, France

**E-mail addresses:**

fabien.cousin@unicaen.fr

rozenn.le.guellec@live.fr

caroline.chagnot@unicaen.fr

didier.goux@unicaen.fr

marion.dalmasso@unicaen.fr

jean-marie.laplace@unicaen.fr

marina.cretenet@unicaen.fr

**[¶] Corresponding author:**

Mailing address: ER MALIM, EA 4651 ABTE, Esplanade de la paix, 14032 Caen cedex 05, France

Phone: +33 (0)2 31 56 57 85

Fax: +33 (0)2 31 56 61 79

E-mail: marina.cretenet@unicaen.fr

**ABSTRACT**

Two Gram-stain-positive, small ellipsoidal cocci, non-motile, oxidase- and catalase-negative, and facultative anaerobic strains (UCMA15228$^T$ and UCMA17102) were isolated in France, from fermented apple juices (ciders). The 16S rRNA gene sequence was identical between the two isolates and showed 97 % similarity with respect to the closest related species *Oenococcus oeni* and *O. kitaharae*. Therefore, the two isolates were classified within the genus *Oenococcus*. The phylogeny based on the *pheS* gene sequences also confirmed the position of the new taxon. DNA–DNA hybridizations based on *in silico* genome-to-genome comparisons (GGDC) and Average Nucleotide Identity (ANI) values, as well as species-specific PCR, validated the novelty of the taxon. Various phenotypic characteristics such as the optimum temperature and pH for growth, the ability to metabolise sugars, the aptitude to perform the malolactic fermentation, and the resistance to ethanol and NaCl, revealed that the two strains are distinguishable from the other members of the *Oenococcus* genus. The combined genotypic and phenotypic data support the classification of strains UCMA15228$^T$ and UCMA17102 into a novel species of *Oenococcus*, for which the name *O. sicerae* sp. nov. is proposed. The type strain is UCMA15228$^T$ (= DSM107163$^T$ = CIRM-BIA2288$^T$).

**KEY WORDS**

Lactic acid bacteria; *Oenococcus sicerae*; Cider; Apple; fermented beverage

## ABBREVIATIONS

ANI: Average Nucleotide Identity

DDH: DNA–DNA Hybridization

GGDC: Genome-To-Genome Distance Calculator

LAB: Lactic Acid Bacteria

MLF: MaloLactic Fermentation

MLO: Medium for *Leuconostoc oenos*

RAPD: Randomly Amplified Polymorphic DNA

UCMA: Université de Caen Microbiologie Alimentaire

## INTRODUCTION

French cider is produced from apple juice, which is usually fermented by indigenous yeasts and bacteria, and is produced mainly in the Normandy and Brittany regions in France [4]. The abundance and diversity of microorganisms during the fermentation processes are widely recognised to contribute to the quality of the final product [4]. French ciders can be bottled for direct consumption or distilled in order to produce spirit (i.e. calvados). *Saccharomyces* yeasts are principally responsible for the alcoholic fermentation of apple juice, and predominate at the beginning of the cider process [4]. Non-*Saccharomyces* yeasts and lactic acid bacteria (LAB) are also present. The major LAB isolated from cider are part of the *Lactobacillus* sp., *Pediococcus* sp., *Oenococcus* sp. and *Leuconostoc* sp. genera [4]. They are particularly involved in the malolactic fermentation (MLF) during the second step of the cider fermentation process, and especially the *Oenococcus* genus.

The *Oenococcus* genus currently comprises three species, *O. oeni* [5], *O. kitaharae* [6] and *O. alcoholitolerans* [1]. *Oenococcus oeni* is, by far, the most studied species in the genus, especially for its ability to perform MLF during wine and other alcoholic-food related fermentation processes [2,18]. Only *O. kitaharae* is not able to perform MLF due to a point mutation resulting in a premature stop codon in the *mleA* gene encoding the malolactic enzyme [3].

Two LAB strains, UCMA15228$^T$ and UCMA17102, were isolated from fermenting ciders, produced in Normandy (France). These strains differed from the currently documented *Oenococcus* species based on the 16S rRNA gene sequences. *Oenococcus* members are usual inhabitants of fermenting apple juice during cider production, which makes cider a potential source for revealing new biochemical and genomic traits of the genus. The aim of this study was to perform the taxonomic characterisation of these new *Oenococcus* strains. They were found to form a separate genetic group from the other known *Oenococcus* species, which is here proposed as *Oenococcus sicerae* sp. nov., with the type strain UCMA15228$^T$ (= DSM107163$^T$ = CIRM-BIA2288$^T$). In our

genomic taxonomic approach, we analysed a complete genome sequence of the type strain UCMA15228$^T$ in order to determine genomic and phenotypic features of the new species.

## MATERIAL AND METHODS

### Bacterial strains and growth conditions

The novel strains, UCMA15228$^T$ and UCMA17102, were isolated from two distinct cider samples during the fermentation process, originating from two separate localities in Normandy (France). The UCMA15228$^T$ strain has been isolated from a cider intended to be bottled whereas the UCMA17102 strain has been isolated from a cider intended to be distilled for spirit production. The isolation media used were modified medium for *Leuconostoc oenos* (MLO) supplemented with 10 g.L$^{-1}$ L-malic acid, and Rogosa SL agar (Rogosa SL agar, Conda) for UCMA15228$^T$ and UCMA17102 strains, respectively. The agar plates were supplemented with 7 mg.L$^{-1}$ cycloheximide and 100 mg.L$^{-1}$ pimaricin, and incubated at 10°C under anaerobic conditions (Anaerogen, Thermo Scientific) for a month. After isolation, bacteria were routinely cultured on MRS (Difco) at pH 5.5, supplemented with 5 g.L$^{-1}$ fructose and 0.5 g.L$^{-1}$ cysteine, and incubated at 30°C with 5 % $CO_2$.

### Phenotypic characterization

Physiological and biochemical characteristics were assessed by standard methods. Briefly, the nitrate reductase test was performed in Nitrate Broth with inverted Durham tubes, and zinc and Griess reagents (Biomérieux, France). The growth of the two strains was tested with 49 different carbon sources using Api 50 CHL strips (Biomérieux, France) according to the manufacturer's instructions. Isomers of lactic acid formed from D-glucose were determined with Enzytec™ D-/L-Lactic Acid kit (r-biopharm), according to the manufacturer's instructions. Fatty acid and peptidoglycan analyses were carried out by the Identification Service of the DSMZ, Braunschweig, Germany.

Sugar and organic acid contents were determined by HPLC (Waters Alliance HPLC system) at 40°C at 0.6 ml/min on a C18 Carbomix H-NP 5 column with 5mM sulfuric acid asmobile phase. Sugars and organic acids were monitored with the FID and the PAD (210 nm) detectors at 30°C, respectively. HPLC data were also used to validate the heterofermentative metabolism of UCMA15228$^T$ and UCMA17102 strains.

The optimal pH and temperature ranges for growth were assessed at pH 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0, and at 5, 10, 15, 20, 22.5, 25, 27.5, 30 and 37°C, respectively. Tolerance towards NaCl (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 % w/v) and ethanol content (2, 4, 6, 8, 10, 12, 14, 16 and 18 % v/v) was also checked. All these tests were performed in tubes containing 3 ml of MRS supplemented with 20 % apple juice and 0.05 % cysteine, and inoculated at 1% (v/v) with fresh cultures for 72 h. For testing the temperature range, assayswere performed under both aerobic and anaerobic conditions. For all the other tests, they were performed at 30°C supplemented with 5% $CO_2$, with no NaCl and ethanol for testing pH, with no ethanol at pH 5.5 for testing NaCl, and with no NaCl at pH 5.5 for testing ethanol.

**Phylogenetic and fingerprinting analyses**

DNA amplification of the 16S rRNA gene was performed directly on colonies in 30 µL containing 1X Phusion High Fidelity (Phusion High-Fidelity PCR Master Mix, Thermo Fisher, United States) and 0.25 µM of both primers 16S_F (5'-AGAGTTTGATYMTGGCTC-3') and 16S_R (5'-GGNTACCTTGTTACGACTT-3'). PCR was carried out as follows: an initial denaturation step at 98°C for 10 min, 35 cycles composed of a denaturation step at 98°C for 10 s, an annealing step at 54°C for 20 s and an amplification step at 72°C for 45 s, and final extension at 72°C for 5 min. The *pheS* gene was also amplified using primers Oeno_PheS_F (5'-TGGGTGGTAACACGATAA-3', targeting the upstream area of the *pheS* gene) and Oeno_PheS_R (5'-CCMARACCAAARGCAAAACC-3', slightly modified from [13]). The PCR conditions were the same as described above except for an annealing temperature of 57°C and an amplification step

reduced to 30 s. PCR product purification and Sanger sequencing were carried out at GATC Biotech (Germany). The closest recognized relatives of the isolates were determined by performing BLAST and Sequence Match (RDP) searches, and the sequences of closely related species were retrieved from the DDBJ database. Multiple alignments and manual cleaning (deletion of the extra beginning and ending sequences to homogenize the alignment) of the sequences and maximum likelihood (ML) phylogenetic trees were carried out with MEGA7 [9] with the appropriate substitution model for the ML option selected [7] and 1,000 bootstrap replications. The 16S rRNA and *pheS* gene sequences of the closest LAB type strains used for the phylogenetic analysis were extracted from the genomes when available (using RNAmmer [10] for 16S rRNA gene sequences) or obtained from the Genbank database.

Species-specific PCR primers Osic_16S_F (5′-TCTTCGGAGTGACGCCTAACT-3′) and Osic_16S_R (5′-GACCGACATGTGTCAGAC-3′) for the novel species were designed based on the 16S rRNA gene sequences. The specificity of the primers was confirmed by PCR using genomic DNA from *Oenococcus* sp. strains (Table S1). Universal primers targeting the 23S rRNA gene of all bacteria (500-bp PCR product) were also used as an internal PCR control (Cousin *et al.*, submitted). PCR amplification was performed using 15 µl of a mixture containing 1× DreamTaq Green PCR Master Mix (Thermo Fisher Scientific), 250 nM of each primer and 5 ng of template DNA. The PCR cycling program consisted of one cycle of 95°C for 3 min; 35 cycles of 95°C for 15 s, 50°C for 15 s and 72°C for 15 s; and a final extension step at 72°C for 5 min.

In addition, genotypic differentiation was assessed by multiplex randomly amplified polymorphic DNA (RAPD) using the Coc and On2 primers as previously described [15], with an annealing temperature of 29°C as previously described [21].

**Genome sequencing**

Genomic DNA was extracted from 10 mL stationary phase cultures using the PowerFood microbial DNA kit (Mobio) with slight modifications from the manufacturer's instructions for the lysis step.

Briefly, the bacterial pellet was resuspended in 450 µl PF1 solution and transferred into a sterile 2ml microtube containing 0.1 mm diameter zirconium beads. Mechanical lysis was performed using a MM200 mixer mill (Retsch) for 5 min at 25 Hz. The amount and quality of isolated genomic DNA was verified using a NanoDrop spectrophotometer, Quant-it PicoGreen dsDNA assay kit and gel electrophoresis.

The genome of strain UCMA15228$^T$ was completely sequenced using a PacBio RSII single-molecule real-time (SMRT) sequencing with a 10 kb library at Genoscreen (Lille, France). *De novo* assembly of sequencing reads was performed through the HGAP (hierarchical genome assembly process) protocol version 2.0 in SMRT Analysis version 2.3.0 (Pacific Biosciences, USA). The genome was annotated by using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) version 4.5.

The average nucleotide identity (ANI) and DNA G + C content values were estimated with orthologous average nucleotide identity (OrthoANI) and original ANI [11], digital DNA-DNA hybridization (DDH) values were estimated using *in silico* genome-to-genome comparison Genome-To-Genome Distance Calculator (GGDC) V.2.1 using the recommended BLAST+ alignment and formula 2 (identities/HSP length) [12]. These values were calculated for UCMA15228$^T$ against *O. oeni* PSU-1, and against the type strains *O. oeni* NCDO1674$^T$, *O. kitaharae* NRIC0645$^T$ and *O. alcoholitolerans* UFRJ-M7.2.18$^T$.

**Bacterial cell morphology**

The bacterial cells were rinsed with physiological water, and fixed with 1% glutaraldehyde in phosphate buffer (0.1 M, pH 7.0) for 5 days at 4°C. During this fixation, the cells were sedimented on Thermanox® coverslip coated with poly-L-lysine. The cells were rinsed in phosphate buffer (0.1 M, pH 7.0) again. The cells were then dehydrated through successive baths of ethanol (70 to 100%), and critical point dried (CPD 030 LEICA Microsystem). The cells were sputtered with platinum and observed with the scanning electron microscope JEOL 6400F. Images were processed with the

public-domain image processing and analysing program Fiji. The measures of bacterial cell size were performed on 20 images of 20 cells each.

**Accession numbers**

The GenBank/EMBL/DDBJ sequence accession numbers are as follows: MH384882 and MH384883 for the 16S rRNA gene sequences of strains UCMA15228$^T$ and UCMA17102, respectively; MH392191 and MH392192 for the *pheS* gene sequences of strains UCMA15228$^T$ and UCMA17102, respectively; and CP029684 for the complete genome of strain UCMA15228$^T$.

## RESULTS AND DISCUSSION

### Phylogenetic analysis

The 16S rRNA gene sequence analysis revealed that the designated type strain UCMA15228$^T$ formed a tight monophyletic branch affiliated to the genus *Oenococcus*, in a robust sub-cluster separated from its closest neighbours (Fig. 1 and S1). UCMA15228$^T$ strain showed 16S rRNA gene sequence identities of 97.4 % and 97.0 % with *O. kitaharae* NRIC0645$^T$ and *O. oeni* NCDO1674$^T$, respectively (Table S2). The 16S rRNA sequence identity of UCMA15228$^T$ strain with its two closest phylogenetic neighbours was close to 97%, the conservative threshold established for a bacterial species delineation [17,19]. The other phylogenetic analysis, based on the *pheS* gene sequence, confirmed this new branch (Fig. 2 and S2). Strain UCMA15228$^T$ shared 74.2 % and 76.5 % identity of the *pheS* gene sequence with *O. kitaharae* NRIC0645$^T$ and *O. oeni* NCDO1674$^T$, respectively (Table S2). These identity scores are comparable to those described for *O. alcoholitolerans*, the last designated species of *Oenococcus* sp. [1]. The 16S rRNA gene sequences of the two strains, UCMA15228$^T$ and UCMA17102 shared 100 % identity. Nonetheless, two SNPs were found between the *pheS* gene sequences of the two strains (Table S2), which also displayed different RAPD (Fig. S3) patterns, suggesting a non-clonal origin of the strains (Fig. S3). This assertion is confirmed by the fact that the two strains have been isolated from different cider

samples, at two distinct producers in Normandy about 50 km apart with no known history of interactions.

**Species-specific PCR for *Oenococcus sicerae* sp. nov.**

A species-specific primer pair (Osic_16S_F/R) was designed from r16S sequence multiple alignments of *Oenococcus* spp. Genomic DNA of several oenococci were used as templates for PCR amplification using universal and species-specific primer pairs (Table S1). The universal primers, used as an internal PCR control, gave a 500 bp positive signal for all the tested bacteria (Fig. S4), as expected. A single species-specific band of 134 bp was observed for *O. sicerae* sp. nov. (Fig. S4), with no cross-reaction against the other species of *Oenococcus* spp. (Fig. S4 and Table S1). This result confirms the divergence of these new members of the genus *Oenococcus*. This PCR method may constitute an efficient tool for their rapid identification in future works.

**General taxonomic genome features of *Oenococcus sicerae* sp. nov. UCMA15228$^T$**

The complete genome sequence of strain UCMA15228$^T$ was obtained using the PacBio RSII. A library, with inserts of 10 kb, was sequenced, and 110,082 polymerase reads were generated. Sequence processing and *de novo* assembly were performed using 205,930 postfiltered subreads with an average read length of 9,560.8 bp, resulting in a unique contig. The complete genome sequence of UCMA15228$^T$ consisted of 1,684,519 bp with an average coverage of 880× and a G+C mol% of 40.35. It contains 1,663 coding genes and 53 predicted RNA genes.

We compared the genome characteristics and sequence of strain UCMA15228$^T$ to other related species (Tables 1 and S3). The orthoANI and original values between *O. sicerae* sp. nov. UCMA15228$^T$ and *O. oeni* NCDO1674$^T$ were of 73.72 % and 73.35 %, respectively (Table 1), which is far below the proposed threshold of 95–96% for species delineation [11,16]. The GGDC value (isDDH) between these strains obtained by the recommended formula 2 (identities/HSP length) was of 17.7 % [15.6–20.0 %], which is also clearly below the boundary of 70 % for species circumscriptions [20]. The calculated probability that isDDH is >70 % was of 0 %. An isDDH

value of 22.6 % was obtained with formula 1 (HSP length/total length) and of 20.8 % with formula 3 (identities/total length), supporting the affiliation of strain UCMA15228$^T$ to a single species. The OrthoANI, original ANI and isDDH values between strain UCMA15228$^T$ and the other *Oenococcus* sp. strains varied from 70.08 to 73.72 %, 69.62 to 73.35 %, and 17.5 to 19.5 % respectively (Table 1), which is clearly lower than the generally accepted cut-off threshold values of 95–96% and 70% for delineation of bacterial species, confirming the authenticity of the novel species.

**Phenotypic and chemotaxonomic characteristics of *O. sicerae* sp. nov.**

Strains are heterofermentative, produce lactic acid, carbon dioxide and ethanol or acetic acid from D-glucose. D- and L-lactic acid are produced in the ratio 9:1. Nitrate is not reduced. The major cellular fatty acids (>10% of the total fatty acids) of UCMA15228$^T$ strain were C 16:0 (39.52%), C 18:1ω9c (40.21%) and C 19:0 cyclo ω10c (12.46%). The overall fatty acid profile of UCMA15228$^T$ strain is presented in Table S4. The total hydrolysate (100 °C, 4N HCl, 16 h) of UCMA15228$^T$ strain contained the amino acids alanine, serine, glutamic acid and lysine, while the analysis of partial hydrolysates (100 °C, 4N HCl, 45 min) revealed the presence of the peptidoglycan type A3α (L-Lys – L-Ser – L-Ala), corresponding to type A11.13 ( www.peptidoglycan-types.info). Growth profiles and phenotypic features of the novel species and its phylogenetically related species are listed in Table 2. In addition, the decarboxylation of L-malate into L-lactate (malolactic fermentation) was investigated by using HPLC. Both *O. sicerae* strains were able to convert L-malate into L-lactate when inoculated in apple juice at pH 4 (Fig. S5). The genes responsible for this malolactic conversion have been found in the genome of UCMA15228$^T$ and are organised in an operon similar to that of *O. oeni* [2]. This operon is composed of three genes involved in the malolactic reaction: *mleA*, encoding the malolactic enzyme (responsible for the conversion of malic acid into lactic acid), *mleP*, encoding the malate permease (responsible for the transport of malate into the cell) and *mleR*, encoding the LysR-type regulatory protein for these two downstream genes.

**SEM morphology of the new species *O. sicerae***

Strains UCMA15228$^T$ and UCMA17102 cells were ellipsoidal cocci, in couples, chains or piles (Figs. 3 and S6). In comparison, *O. oeni* NCDO1674$^T$ appeared almost exclusively in pairs in our study. The “pile” phenotype of *O. sicerae* was also visible at a macroscopic scale where cellular mass were observable in broth cultures, especially for strain UCMA17102.

Based on the phenotypic, genomic and phylogenetic data obtained in this study we propose the new species *Oenococcus sicerae* for which the type strain UCMA15228$^T$ (= DSM107163$^T$, = CIRM-BIA2288$^T$) is designated.

The formal proposal of the species *Oenococcus sicerae* is given in Table 3 with the taxonumber TA00560.

**Acknowledgments**

This work was supported by the region Basse-Normandie through the grant "Retour Jeune Chercheur" 14P06493. The authors also thank all the cider producers for providing the cider samples during the sample collection in Normandy, France, 2016.

## REFERENCES


[1] Badotti, F., Moreira, A.P.B., Tonon, L.A.C., Lucena, B.T.L. de., Gomes, F. de C.O., Kruger, R., Thompson, C.C., Morais, M.A. de., Rosa, C.A., Thompson, F.L. (2014) *Oenococcus alcoholitolerans* sp. nov., a lactic acid bacteria isolated from cachaça and ethanol fermentation processes. Antonie Van Leeuwenhoek 106(6), 1259–67, Doi: 10.1007/s10482-014-0296-z.

[2] Bartowsky, E.J. (2005) *Oenococcus oeni* and malolactic fermentation – moving into the molecular arena. Aust. J. Grape Wine Res. 11(2), 174–87, Doi: 10.1111/j.1755-0238.2005.tb00286.x.

[3] Borneman, A.R., McCarthy, J.M., Chambers, P.J., Bartowsky, E.J. (2012) Functional divergence in the genus *Oenococcus* as predicted by genome sequencing of the newly-described species, *Oenococcus kitaharae*. PLoS ONE 7(1), Doi: 10.1371/journal.pone.0029626.

[4] Cousin, F.J., Le Guellec, R., Schlusselhuber, M., Dalmasso, M., Laplace, J.-M., Cretenet, M. (2017) Microorganisms in fermented apple beverages: current knowledge and future directions. Microorganisms 5(3), 39, Doi: 10.3390/microorganisms5030039.

[5] Dicks, L.M.T., Dellaglio, F., Collins, M.D. (1995) Proposal to reclassify *Leuconostoc oenos* as *Oenococcus oeni* [corrig.] gen. nov., comb. nov. Int. J. Syst. Evol. Microbiol. 45(2), 395–7, Doi: 10.1099/00207713-45-2-395.

[6] Endo, A., Okada, S. (2006) *Oenococcus kitaharae* sp. nov., a non-acidophilic and non-malolactic-fermenting oenococcus isolated from a composting distilled shochu residue. Int. J. Syst. Evol. Microbiol. 56(10), 2345–8, Doi: 10.1099/ijs.0.64288-0.

[7] Hall, B.G. (2013) Building phylogenetic trees from molecular data with MEGA. Mol. Biol. Evol. 30(5), 1229–35, Doi: 10.1093/molbev/mst012.

[8] Kimura, M. (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16(2), 111–20.

[9] Kumar, S., Stecher, G., Tamura, K. (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33(7), 1870–4, Doi: 10.1093/molbev/msw054.

[10] Lagesen, K., Hallin, P., Rødland, E.A., Stærfeldt, H.-H., Rognes, T., Ussery, D.W. (2007) RNAmmer: consistent and rapid annotation of ribosomal RNA genes. Nucleic Acids Res. 35(9), 3100–8, Doi: 10.1093/nar/gkm160.

[11] Lee, I., Ouk Kim, Y., Park, S.-C., Chun, J. (2016) OrthoANI: An improved algorithm and software for calculating average nucleotide identity. Int. J. Syst. Evol. Microbiol. 66(2), 1100–3, Doi: 10.1099/ijsem.0.000760.

[12] Meier-Kolthoff, J.P., Auch, A.F., Klenk, H.-P., Göker, M. (2013) Genome sequence-based species delimitation with confidence intervals and improved distance functions. BMC Bioinformatics 14, 60, Doi: 10.1186/1471-2105-14-60.

[13] Naser, S.M., Thompson, F.L., Hoste, B., Gevers, D., Dawyndt, P., Vancanneyt, M., Swings, J. (2005) Application of multilocus sequence analysis (MLSA) for rapid identification of *Enterococcus* species based on *rpoA* and *pheS* genes. Microbiology 151(7), 2141–50, Doi: 10.1099/mic.0.27840-0.

[14] Nei, M., Kumar, S. (2000) Molecular evolution and phylogenetics. Oxford University Press, Oxford, New York.

[15] Reguant, C., Bordons, A. (2003) Typification of *Oenococcus oeni* strains by multiplex RAPD-PCR and study of population dynamics during malolactic fermentation. J. Appl. Microbiol. 95(2), 344–53, Doi: 10.1046/j.1365-2672.2003.01985.x.

[16] Richter, M., Rosselló-Móra, R. (2009) Shifting the genomic gold standard for the prokaryotic species definition. Proc. Natl. Acad. Sci. U. S. A. 106(45), 19126–31, Doi: 10.1073/pnas.0906412106.

[17] Stackebrandt, E., Goebel, B.M. (1994) Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Evol. Microbiol. 44(4), 846–9, Doi: 10.1099/00207713-44-4-846.
[18] Sternes, P.R., Borneman, A.R. (2016) Consensus pan-genome assembly of the specialised wine bacterium *Oenococcus oeni*. BMC Genomics 17, Doi: 10.1186/s12864-016-2604-7.
[19] Vandamme, P., Pot, B., Gillis, M., de Vos, P., Kersters, K., Swings, J. (1996) Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol. Rev. 60(2), 407–38.
[20] Wayne, L.G., Brenner, D.J., Colwell, R.R., Grimont, P.A.D., Kandler, O., Krichevsky, M.I., Moore, L.H., Moore, W.E.C., Murray, R.G.E., Stackebrandt, E., Starr, M.P., Truper, H.G. (1987) Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Evol. Microbiol. 37(4), 463–4, Doi: 10.1099/00207713-37-4-463.
[21] Zapparoli, G., Torriani, S., Dellaglio, F. (1998) Differentiation of *Lactobacillus sanfranciscensis* strains by randomly amplified polymorphic DNA and pulsed-field gel electrophoresis. FEMS Microbiol. Lett. 166(2), 325–32, Doi: 10.1111/j.1574-6968.1998.tb13908.x.

## FIGURE LEGENDS

**Fig. 1.** Phylogenetic tree showing the position of the *Oenococcus sicerae* sp. nov. strains based on the 16S rRNA gene sequences (1,463 bp). The tree was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model [8] with MEGA7 [9]. Bootstrap values (>70 %) based on 1,000 repetitions are shown. The sequence of *Lactococcus lactis* DSM 20481$^T$ was used as outgroup. Bar 5 % estimated sequence divergence.

**Fig. 2.** Phylogenetic tree showing the position of the *Oenococcus sicerae* sp. nov. strains based on the *PheS* gene sequences (1,069 bp). The tree was inferred by using the Maximum Likelihood method based on the General Time Reversible model [14] with MEGA7 [9]. Bootstrap values (>70 %) based on 1,000 repetitions are shown. The sequence of *Lactococcus lactis* DSM 20481$^T$ was used as outgroup. Bar 20 % estimated sequence divergence.

**Fig. 3.** Photograph taken by SEM of *O. sicerae* sp. nov. UCMA15228$^T$ cultured in MRS supplemented with 20 % apple juice, pH 5.

**Table 1**. Ortho Average Nucleotide Identity (OrthoANI), original ANI and GGDC values (%) between *Oenococcus sicerae* sp. nov. and the other *Oenococcus* species

| **Species** | **Strain** | *O. sicerae* sp. nov UCMA15228$^T$ | *O. oeni* NCDO1674$^T$ | *O. oeni* PSU-1 | *O. kitaharae* NRIC0645$^T$ | *O. alcoholitolerans* UFRJ-M7.2.18$^T$ |
|---|---|---|---|---|---|---|
| *O. sicerae* sp. nov. | UCMA15228$^T$ | 100 | | | | |
| | | 73.72[a] | | | | |
| *O. oeni* | NCDO1674$^T$ | 73.35[b] | 100 | | | |
| | | 17.7[c] | | | | |
| | | 73.56 | 99.66 | | | |
| *O. oeni* | PSU-1 | 73.34 | 99.63 | 100 | | |
| | | 17.8 | 97.9 | | | |
| | | 72.62 | 72.15 | 71.88 | | |
| *O. kitaharae* | NRIC0645$^T$ | 71.99 | 71.61 | 71.51 | 100 | |
| | | 17.5 | 17.6 | 17.6 | | |
| | | 70.08 | 69.79 | 69.99 | 70.08 | |
| *O. alcoholitolerans* | UFRJ-M7.2.18$^T$ | 69.62 | 69.59 | 69.81 | 69.73 | 100 |
| | | 19.5 | 20.6 | 21.9 | 20.3 | |

[a] OrthoANI values; [b] Original ANI values; [c] GGDC values.

330 **Table 2**. Differential phenotypic characteristics of the *O. sicerae* sp. nov. strains and the other *Oenococcus* species

| **Characteristics** | ***Oenococcus oeni*** [5] | ***O. kitaharae*** [6] | ***O. alcoholitolerans*** [1] | ***O. sicerae* sp. nov.** | |
|---|---|---|---|---|---|
| | | | | **UCMA15228$^{T}$** | **UCMA17102** |
| **Growth** | | | | | |
| T °C range | 15-30[b] | 20-30 | 20 - 40 | 5 - 30 | 5 - 30 |
| Optimum growth T °C | 22 | 30 | 30 | 25 | 25 |
| pH range | 3.0 - 6.5[b] | 5.0 - 7.5 | 4.0 - 7.5 | 4.0 - 7.0 | 4.0 - 7.0 |
| Optimum growth pH | 4.8 | 6.0 - 6.8 | 6.0 - 6.5 | 5.5 - 6.0 | 5.5 - 6.0 |
| Max ethanol (%) | 10.0 | 5.0 | 12.0 | 6.0 | 6.0 |
| Max NaCl (%) | 3.5[b] | 1.0 | 2.5 | 2.0 | 2.5 |
| **Malolactic fermentation** | + | – | + | + | + |
| **Acid production[a] from:** | | | | | |
| L-arabinose | +/– | – | +/– | – | – |
| D-ribose | ND | + | +/– | + | + |
| D-xylose | +/– | – | +/– | + | + |
| D-galactose | +/– | + | +/– | – | – |
| D-glucose * | + | + | + | + | + |
| D-fructose | + | + | + | + | + |
| D-mannose | +/– | + | +/– | + | – |
| Salicin | +/– | +/– | +/– | – | – |
| D-cellobiose | +/– | +/– | +/– | – | – |

| | | | | | |
|---|---|---|---|---|---|
| D-maltose | – | + | +/– | – | – |
| D-lactose | – | – | +/– | – | – |
| D-melibiose | +/– | + | + | + | – |
| Sucrose | – | – | + | + | + |
| D-trehalose | + | + | – | + | – |
| D-raffinose | – | + | + | + | – |
| Gluconate potassium | + | + | – | + | + |

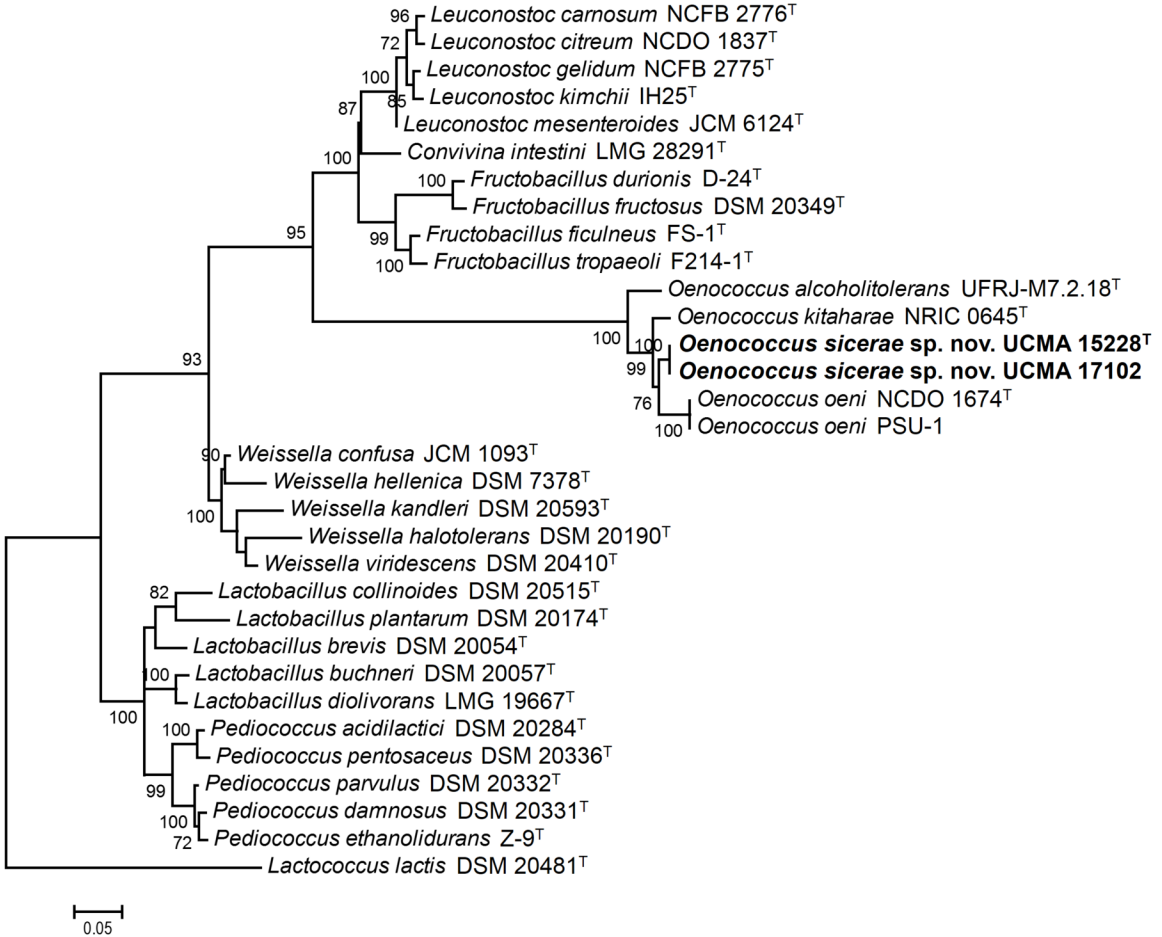
[a] + Acid production, – no acid production, +/- strain dependent, ND not determined. [b] Data obtained in this study. * D-glucose was negative for strain UCMA17102 with API50CHL test but D-glucose was shown to be used by this strain by HPLC analysis.

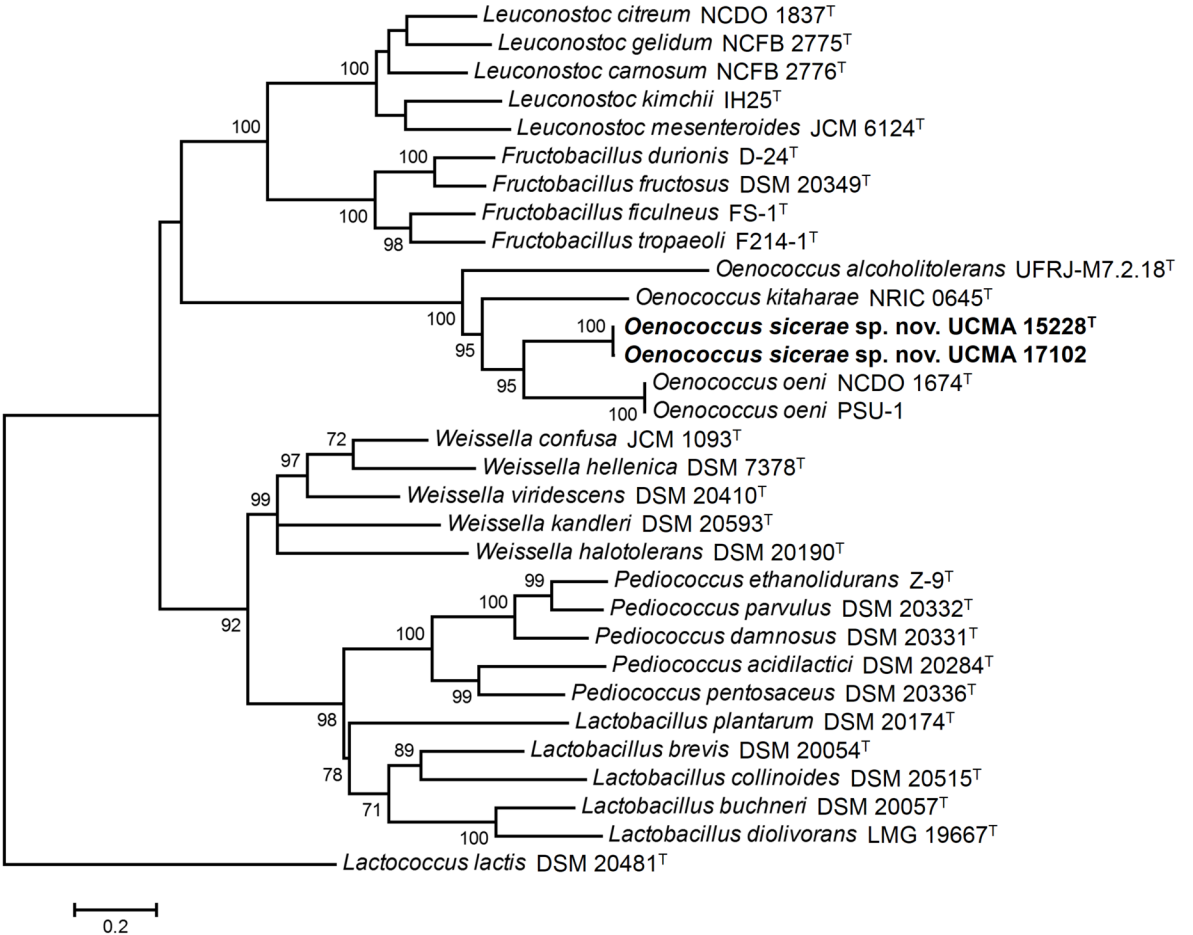
**Table 3**. Description of *Oenococcus sicerae* sp. nov. according to Digital Protologue TA00560 assigned by the www.imedea.uib.es/dprotologue website.

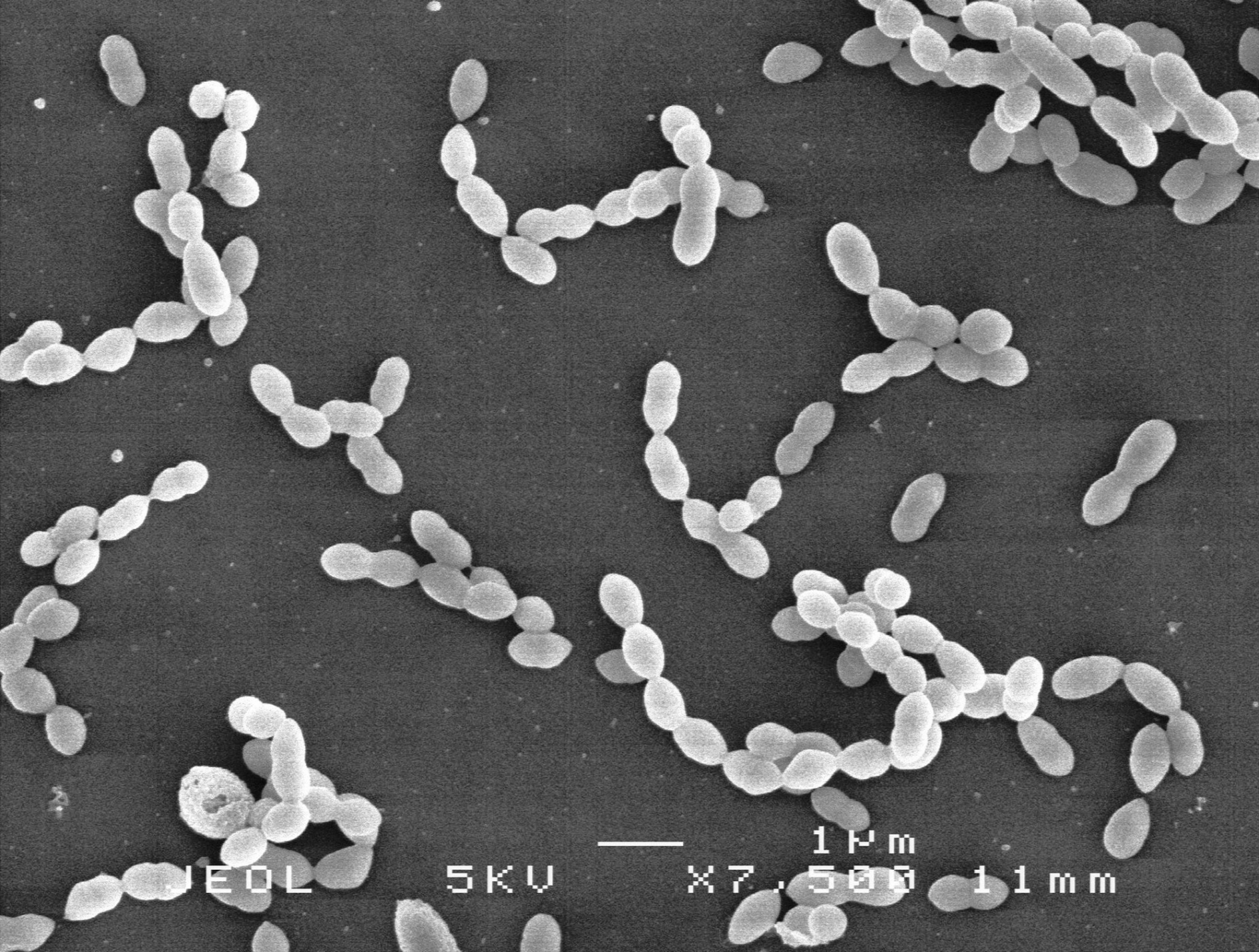
| | |
|---|---|
| **TAXONUMBER** | TA00560 |
| **SPECIES NAME (Give the binomial species name)** | Oenococcus sicerae |
| **GENUS NAME** | Oenococcus |
| **SPECIFIC EPITHET** | sicerae |
| **SPECIES STATUS** | sp. nov. |
| **SPECIES ETYMOLOGY** | si′ce.rae. L. fem. gen. n. sicerae of cider |
| **DESIGNATION OF THE TYPE STRAIN** | UCMA15228 |
| **STRAIN COLLECTION NUMBERS** | DSM107163 = CIRM-BIA2288 |
| **16S rRNA GENE ACCESSION NUMBER** | MH384882 |
| **ALTERNATIVE HOUSEKEEPING GENES:GENE [ACCESSION NUMBER]** | pheS [MH392191] |
| **GENOME ACCESSION NUMBER [RefSeq]** | CP029684 |
| **GENOME STATUS** | Complete |
| **GENOME SIZE** | 1,684,519 |
| **GC mol %** | 40.35 |
| **DATA ON THE ORIGIN OF THE SAMPLE FROM WHICH THE STRAIN HAD BEEN ISOLATED** | |
| **COUNTRY OF ORIGIN** | France |
| **REGION OF ORIGIN** | Normandie, Calvados |
| **DATE OF ISOLATION** | 17/02/2016 |
| **SOURCE OF ISOLATION** | Apple juice in fermentation (cider) |
| **SAMPLING DATE** | 11/02/2016 |
| **GEOGRAPHIC LOCATION** | Calvados |
| **pH OF THE SAMPLE** | 4.05 |
| **NUMBER OF STRAINS IN STUDY** | 2 |
| **SOURCE OF ISOLATION OF NON-TYPE STRAINS** | Apple juice in fermentation (cider) |
| **GROWTH MEDIUM, INCUBATION CONDITIONS [Temperature, pH, and further information] USED FOR STANDARD CULTIVATION** | - MRS (55 g/L, difco BD™ 288130) supplemented with 5 g/L D-Fructose and 0.5 g/L cysteine-HCl<br>- pH 5.5<br>- 30°C in air with 5% CO2<br>- Growth on agar medium is enhanced under anaerobic conditions |
| **ALTERNATIVE MEDIUM 1** | MRS supplemented with 0.5 g/L cysteine-HCl |
| **CONDITIONS OF PRESERVATION** | Liquid medium mixed with 15% (v/v) glycerol and stored at −80°C<br>Freeze-dried |
| **GRAM STAIN** | Positive |
| **CELL SHAPE** | Coccus |
| **CELL SIZE (length or diameter)** | 0.5-0.8 by 0.3-0.6 µm |
| **MOTILITY** | Nonmotile |
| **SPORULATION (resting** | None |

| | |
|---|---|
| **cells)** | |
| **TEMPERATURE RANGE** | 5-30 |
| **LOWEST TEMPERATURE FOR GROWTH** | 5 |
| **HIGHEST TEMPERATURE FOR GROWTH** | 30 |
| **TEMPERATURE OPTIMUM** | 25 |
| **LOWEST pH FOR GROWTH** | 4 |
| **HIGHEST pH FOR GROWTH** | 7 |
| **pH OPTIMUM** | 5.5 - 6.0 |
| **pH CATEGORY** | Acidophile (optimum < 6) |
| **LOWEST NaCl CONCENTRATION FOR GROWTH** | 0 |
| **HIGHEST NaCI CONCENTRATION FOR GROWTH** | 2.5 |
| **SALINITY OPTIMUM** | 0-1.5 |
| **RELATIONSHIP TO O2** | Facultative aerobe |
| **O2 CONDITIONS FOR STRAIN TESTING** | Air supplemented with 5% CO2 or anaerobiosis |
| **CARBON SOURCE USED [class of compounds]** | Carbohydrates, organic acids |
| **CARBON SOURCE USED [specific compounds]** | D-glucose, D-fructose, D-ribose, sucrose, D-xylose, L-xylose, D-lyxose, potassium gluconate and 5-ketogluconate potassium |
| **CARBON SOURCE NOT USED [specific compounds]** | glycerol, erythritol, D-arabinose, L-arabinose, D-adonitol, methyl-ßD-xylopyranoside, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-αD-mannopyranoside, N-acetylglucosamine, amygdalin, arbutin, salicin, D-cellobiose, D-maltose, D-lactose, inulin, D-melezitose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol and 2-ketogluconate potassium |
| **CARBON SOURCE VARIABLE [specific compounds]** | D-mannose, D-melibiose, D-trehalose, D-raffinose and methyl-αD-glucopyranoside |
| **Positive tests with API** | D-fructose, D-ribose, sucrose, D-xylose, L-xylose, D-lyxose, potassium gluconate and 5-ketogluconate potassium |
| **Negative tests with API** | glycerol, erythritol, D-arabinose, L-arabinose, D-adonitol, methyl-ßD-xylopyranoside, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-αD-mannopyranoside, N-acetylglucosamine, amygdalin, arbutin, salicin, D-cellobiose, D-maltose, D-lactose, inulin, D-melezitose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol and 2-ketogluconate potassium |
| **Variable tests with API** | D-glucose, D-mannose, D-melibiose, D-trehalose, D-raffinose and methyl-αD-glucopyranoside |
| **COMMERCIAL KITS USED?** | API50CHL |
| **ENERGY METABOLISM** | Heterofermentative |
| **OXIDASE** | Negative |
| **CATALASE** | Negative |
| **POSITIVE TESTS** | Gas production, malolactic fermentation, hydrolysis of esculin |
| **NEGATIVE TESTS** | Gas formation with nitrate, reduction of nitrate |
| **MAJOR FATTY ACIDS** | C 16:0, C 18:1ω9c, C 19:0 cyclo ω10c |
| **PEPTIDOGLYCAN TYPE** | A3α (L-Lys – L-Ala – L-Ser) |

| | |
|---|---|
| **BIOSAFETY LEVEL** | 1 |
| **BIOTIC RELATIONSHIP** | Free-living |
| **KNOWN PATHOGENICITY** | None |


96
Leuconostoc carnosum NCFB 2776T
72
Leuconostoc citreum NCDO 1837T
100
Leuconostoc gelidum NCFB 2775T
85
Leuconostoc kimchii IH25T
87
Leuconostoc mesenteroides JCM 6124T
100
Convivina intestini LMG 28291T
100
Fructobacillus durionis D-24T
Fructobacillus fructosus DSM 20349T
95
99
Fructobacillus ficulneus FS-1T
100
Fructobacillus tropaeoli F214-1T
Oenococcus alcoholitolerans UFRJ-M7.2.18T
Oenococcus kitaharae NRIC 0645T
100
100
Oenococcus sicerae sp. nov. UCMA 15228T
99
Oenococcus sicerae sp. nov. UCMA 17102
93
76
Oenococcus oeni NCDO 1674T
100
Oenococcus oeni PSU-1
90
Weissella confusa JCM 1093T
Weissella hellenica DSM 7378T
100
Weissella kandleri DSM 20593T
Weissella halotolerans DSM 20190T
Weissella viridescens DSM 20410T
82
Lactobacillus collinoides DSM 20515T
Lactobacillus plantarum DSM 20174T
Lactobacillus brevis DSM 20054T
100
Lactobacillus buchneri DSM 20057T
Lactobacillus diolivorans LMG 19667T
100
100
Pediococcus acidilactici DSM 20284T
Pediococcus pentosaceus DSM 20336T
99
Pediococcus parvulus DSM 20332T
100
Pediococcus damnosus DSM 20331T
72
Pediococcus ethanolidurans Z-9T
Lactococcus lactis DSM 20481T
0.05


Leuconostoc citreum NCDO 1837T
Leuconostoc gelidum NCFB 2775T
Leuconostoc carnosum NCFB 2776T
Leuconostoc kimchii IH25T
Leuconostoc mesenteroides JCM 6124T
Fructobacillus durionis D-24T
Fructobacillus fructosus DSM 20349T
Fructobacillus ficulneus FS-1T
Fructobacillus tropaeoli F214-1T
Oenococcus alcoholitolerans UFRJ-M7.2.18T
Oenococcus kitaharae NRIC 0645T
Oenococcus sicerae sp. nov. UCMA 15228T
Oenococcus sicerae sp. nov. UCMA 17102
Oenococcus oeni NCDO 1674T
Oenococcus oeni PSU-1
Weissella confusa JCM 1093T
Weissella hellenica DSM 7378T
Weissella viridescens DSM 20410T
Weissella kandleri DSM 20593T
Weissella halotolerans DSM 20190T
Pediococcus ethanolidurans Z-9T
Pediococcus parvulus DSM 20332T
Pediococcus damnosus DSM 20331T
Pediococcus acidilactici DSM 20284T
Pediococcus pentosaceus DSM 20336T
Lactobacillus plantarum DSM 20174T
Lactobacillus brevis DSM 20054T
Lactobacillus collinoides DSM 20515T
Lactobacillus buchneri DSM 20057T
Lactobacillus diolivorans LMG 19667T
Lactococcus lactis DSM 20481T
100
100
100
100
98
100
95
95
100
72
97
99
92
99
100
100
99
98
89
78
71
100
0.2


1μm
JEOL 5KV X7,500 11mm