Systematic and Applied Microbiology 42 (2019) 302–308

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: http://www.elsevier.com/locate/syapm

Oenococcus sicerae sp. nov., isolated from French cider

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

ARTICLE INFO

Article history:
Received 26 June 2018
Received in revised form 5 December 2018
Accepted 14 December 2018

Keywords:
Lactic acid bacteria
Oenococcus sicerae
Cider
Apple
Fermented beverage

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).

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 sep-

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.

* Corresponding author at: ER MALIM, EA 4651 ABTE, Esplanade de la paix, 14032 Caen cedex 05, France.
E-mail addresses: fabien.cousin@unicaen.fr (F.J. Cousin), rozenn.le.guellec@live.fr (R. Le Guellec), caroline.chagnot@unicaen.fr (C. Chagnot), didier.goux@unicaen.fr (D. Goux), marion.dalmasso@unicaen.fr (M. Dalmasso), jean-marie.laplace@unicaen.fr (J.-M. Laplace), marina.cretenet@unicaen.fr (M. Cretenet).

https://doi.org/10.1016/j.syapm.2018.12.006
0723-2020/

arate genetic group from the other known *Oenococcus* species, which is here proposed as *O. 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 5 mM 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 2 ml 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 (70–100 %), 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

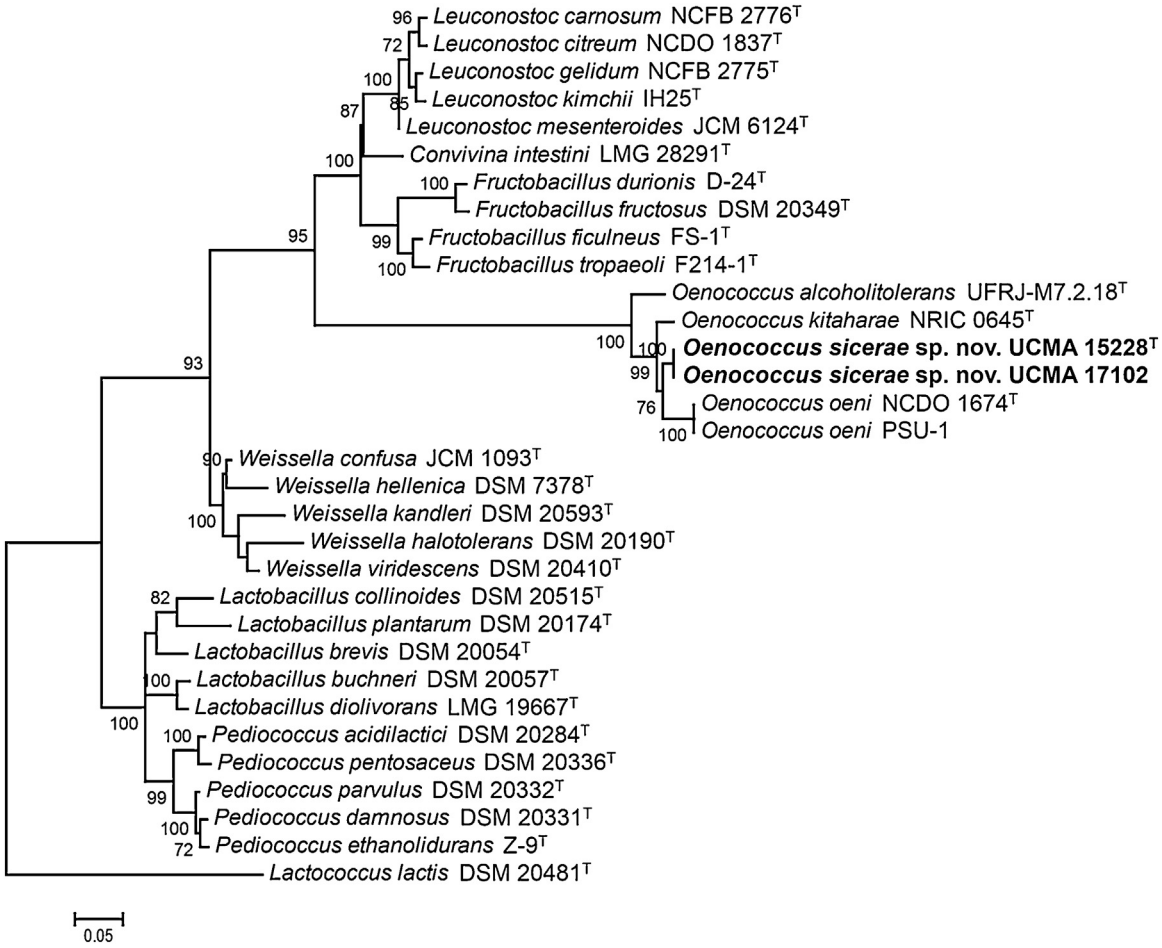

Fig. 1. Phylogenetic tree showing the position of the *Oenococcus sicerae* sp. nov. strains based on the 16S rRNA gene sequences (1463 bp). The tree was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model [8] with MEGA7 [9]. Bootstrap values (>70 %) based on 1000 repetitions are shown. The sequence of *Lactococcus lactis* DSM 20481^T was used as outgroup. Bar 5 % estimated sequence divergence.

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

Species	Strain	*O. sicerae* sp. novUCMA15228^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				
O. oeni	NCDO1674^T	73.72[a]	100			
		73.35[b]				
		17.7[c]				
O. oeni	PSU-1	73.56	99.66	100		
		73.34	99.63			
		17.8	97.9			
O. kitaharae	NRIC0645^T	72.62	72.15	71.88	100	
		71.99	71.61	71.51		
		17.5	17.6	17.6		
O. alcoholitolerans	UFRJ-M7.2.18^T	70.08	69.79	69.99	70.08	100
		69.62	69.59	69.81	69.73	
		19.5	20.6	21.9	20.3	

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

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 (Fig. 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 macro-

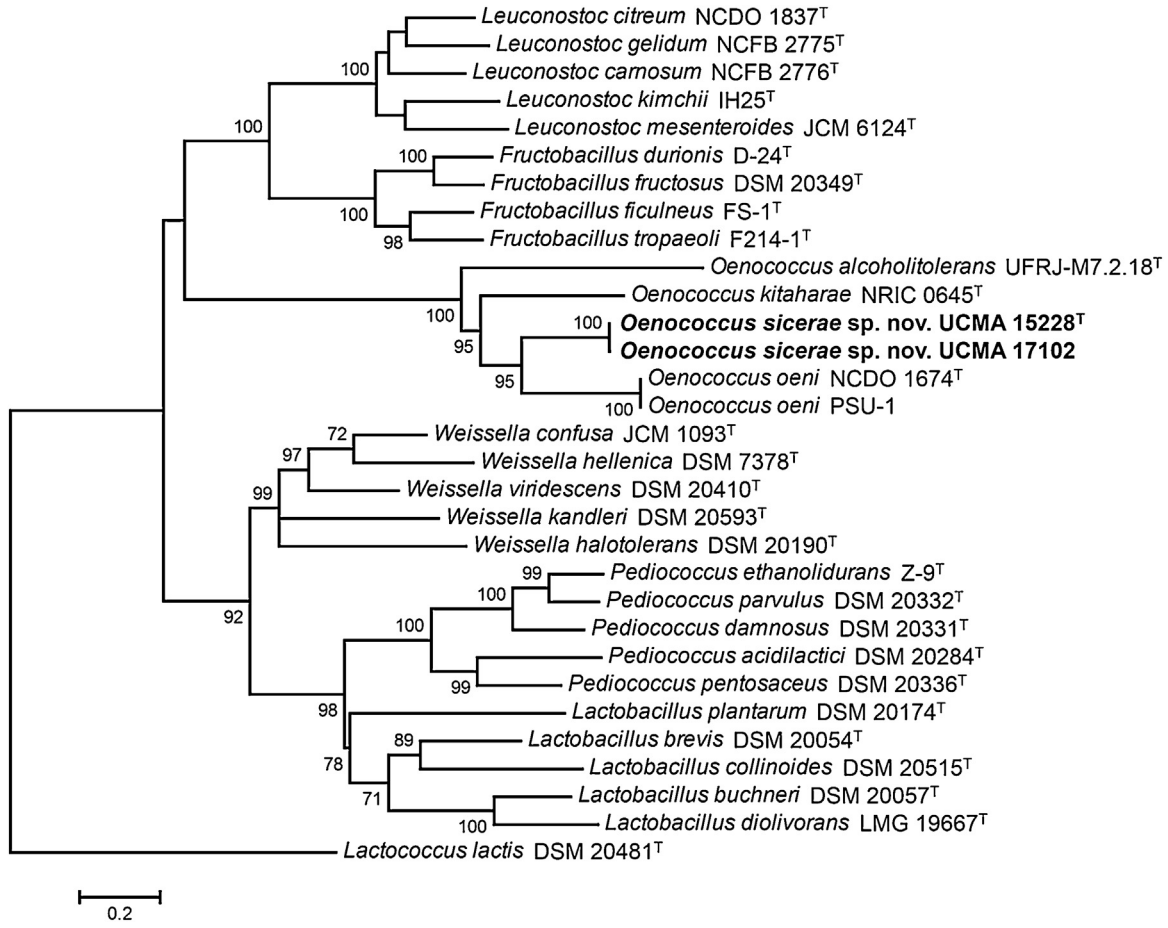

Fig. 2. Phylogenetic tree showing the position of the *Oenococcus sicerae* sp. nov. strains based on the *PheS* gene sequences (1069 bp). The tree was inferred by using the Maximum Likelihood method based on the General Time Reversible model [14] with MEGA7 [9]. Bootstrap values (>70 %) based on 1000 repetitions are shown. The sequence of *Lactococcus lactis* DSM 20481^T was used as outgroup. Bar 20 % estimated sequence divergence.

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[c]	+	+	+	+	+
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.
[c] 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 - pH 5.5 - 30 °C in air with 5% CO2 - 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 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 cells)	None
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 O_2	Facultative aerobe
O_2 conditions for strain testing	Air supplemented with 5 % CO_2 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

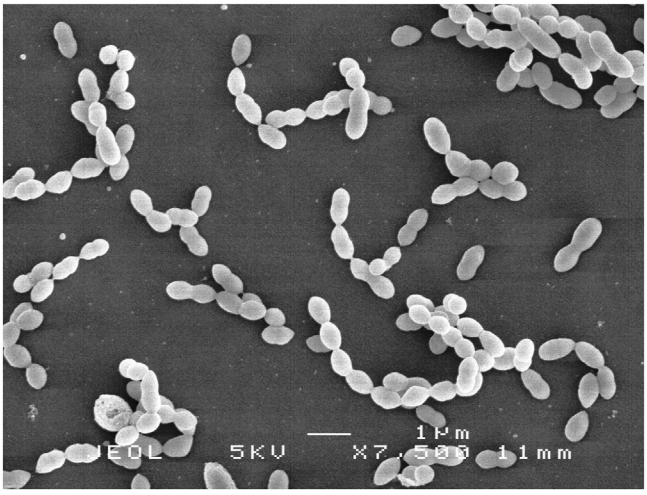

Fig. 3. Photograph taken by SEM of *O. sicerae* sp. nov. UCMA15228^T cultured in MRS supplemented with 20 % apple juice, pH 5.

scopic 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.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.syapm.2018.12.006.

References

[1] Badotti, F., Moreira, A.P.B., Tonon, L.A.C., de Lucena, B.T.L., Gomes, F. de C.O., Kruger, R., Thompson, C.C., Morais de, M.A., 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), 1259–1267, http://dx.doi.org/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), 174–187, http://dx.doi.org/10.1111/j.1755-0238.2005.tb00286.

[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), http://dx.doi.org/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, http://dx.doi.org/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), 395–397, http://dx.doi.org/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), 2345–2348, http://dx.doi.org/10.1099/ijs.0. 64288-0.

[7] Hall, B.G. (2013) Building phylogenetic trees from molecular data with MEGA. Mol. Biol. Evol. 30 (5), 1229–1235, http://dx.doi.org/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), 111–120.

[9] Kumar, S., Stecher, G., Tamura, K. (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33 (7), 1870–1874, http://dx.doi.org/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), 3100–3108, http://dx.doi.org/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), 1100–1103, http://dx.doi.org/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, http://dx.doi.org/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), 2141–2150, http://dx.doi.org/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), 344–353, http://dx.doi.org/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), 19126–19131, http://dx.doi.org/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), 846–849, http://dx.doi.org/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, http://dx.doi.org/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), 407–438.

[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), 463–464, http://dx.doi.org/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), 325–332, http://dx.doi.org/10.1111/j.1574-6968.1998. tb13908.x.