Characterization of *Tetragenococcus* strains from sugar thick juice reveals a novel species, *Tetragenococcus osmophilus* sp. nov., and divides *Tetragenococcus halophilus* into two subspecies, *T. halophilus* subsp. *halophilus* subsp. nov. and *T. halophilus* subsp. *flandriensis* subsp. nov.

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Most bacteria recovered so far from sugar thick juice during storage represent strains of the species *Tetragenococcus halophilus*. Recently, several Gram-positive, non-motile, non-spore-forming cocci with other physiological and genetic traits were isolated from sugar thick juice samples from different origins. In this study, representative isolates were investigated using a polyphasic taxonomic approach. The 16S rRNA gene sequence similarity between these isolates and their closest relative, *Tetragenococcus muriaticus*, was 97.4 %. The level of DNA–DNA relatedness between isolate T1^T, representing the newly found *Tetragenococcus* isolates, and *T. muriaticus* was 57 %. Isolate T1^T had a DNA G + C content of 36.7 mol%. Phylogenetic data and genomic and phenotypic features demonstrated that the isolates represent a novel species, for which the name *Tetragenococcus osmophilus* sp. nov. is proposed with T1^T as the type strain (=LMG 26041^T = DSM 23765^T). Additionally, *T. halophilus* isolates from high-salt and high-sugar environments showed clear differences in several physiological and genetic characteristics like RAPD fingerprints and 16S rRNA gene sequences. DNA–DNA hybridizations, however, showed 79 to 80 % relatedness between osmophilic and halophilic *T. halophilus* isolates, demonstrating that the different strains belong to the same species. Based on the phenotypic and genotypic differences observed, as well as the different origins of the strains and the industrial relevance of thick juice degradation, two subspecies of *T. halophilus* are described in this manuscript: *T. halophilus* subsp. *halophilus* subsp. nov. for the strains isolated from salt media and *T. halophilus* subsp. *flandriensis* subsp. nov. for the strains isolated from sugar-rich environments, which were first isolated in Flanders, Belgium. The type strains for the subspecies are IAM 1676^T (=LMG 11490^T = DSM 20339^T) and T5^T (=LMG 26042^T = DSM 23766^T), respectively.

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Abbreviations: RAPD, random amplified polymorphic DNA; TV, tetrazolium violet.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequences of strains T170, T179 and T181 are HQ123368–HQ123370, respectively.

The genus *Tetragenococcus* was described by Collins *et al.* (1990) through reclassification of the halophilic lactic acid bacterium *Pediococcus halophilus* as *Tetragenococcus halophilus*. Strains of this species were isolated from habitats rich in salt and proteins such as salted anchovies (Villar *et al.*, 1985), soy sauce mash (Röling & van Verseveld,

1996), and fermented mustard (Chen *et al.*, 2006). In addition, the species was recently found in sugar thick juice, an intermediate product in the refining of beet sugar, where it was found to cause sugar degradation (Willems *et al.*, 2003; Justé *et al.*, 2008a, b). In these man-made environments, *T. halophilus* constitutes a major part of the fortuitous microflora (Gürtler *et al.*, 1998). Classification of isolates as *T. halophilus* was based on physiological and biochemical tests, protein fingerprinting, 16S rRNA gene sequencing and DNA–DNA hybridization (Villar *et al.*, 1985; Röling & van Verseveld, 1996; Justé *et al.*, 2008c). At the time of writing, the genus *Tetragenococcus* encompasses four species, namely *T. halophilus* (Collins *et al.*, 1990), *T. muriaticus* (Satomi *et al.*, 1997), *T. solitarius* (Ennahar & Cai, 2005) and *T. koreensis* (Lee *et al.*, 2005). *T. muriaticus* and *T. koreensis* were isolated from fermented food, squid liver sauce and kimchi (a traditional Korean food), respectively, whereas *T. solitarius* was recovered from a clinical sample. In contrast to the species *T. halophilus* and *T. muriaticus* for which several strains have been isolated, the species *T. koreensis* and *T. solitarius* only comprise a single isolate. All species of the genus *Tetragenococcus* grow at pH 9 and below, but not at pH 5, and all tolerate relatively high NaCl concentrations (Lee *et al.*, 2005). Species specific physiological characteristics include growth on De Man Rogosa Sharpe (MRS) agar (Oxoid), which is positive for *T. koreensis* and *T. solitarius* and negative for *T. muriaticus*. Only *T. solitarius* is able to grow at temperatures of up to 45 °C, while *T. muriaticus* grows up to 40 °C in NaCl-GYP broth (Satomi *et al.*, 1997). *T. halophilus* has been reported to grow up to 35 °C. Furthermore, *T. muriaticus* is the only species of the genus *Tetragenococcus* that does not grow in the absence of NaCl.

In a previous study, several *T. halophilus* isolates from both high-salt and high-sugar environments, further referred to as halophilic and osmophilic, respectively, were characterized physiologically and genetically, revealing several clear differences between the two groups of strains (Justé *et al.*, 2008c). Based on these differences, three osmophilic isolates (T2, T5 and T6) and three halophilic isolates (T11^T, T13 and T19) originating from different matrices and/or countries were selected for this study (Table 1). In addition, one deviant isolate, referred to as T1^T, showed traits that differed from those of *T. halophilus* found in thick juice. This strain was isolated in 2006 by direct plating from degraded thick juice from a Belgian sugar refinery after seven months of industrial storage, followed by 20 days of laboratory storage at 30 °C. This thick juice was characterized by 67.9 °Bx and a pH of 8.34 (Justé *et al.*, 2008c). Identical incubation at 30 °C for 20 days of another sample of the same Belgian thick juice resulted in the isolation of additional *Tetragenococcus* strains. All five strains tested showed random amplified polymorphic DNA (RAPD) fingerprints identical to that of strain T1^T (data partially shown in Fig. 1), justifying that only one additional strain, T170, was included for further analyses. The RAPD analysis was performed as described previously

(Justé *et al.*, 2008c). Furthermore, additional isolates with 16S rRNA gene sequences highly similar to that of strain T1^T (>99.4 % sequence identity) were recently (in 2009) recovered from sugar thick juice from a German sugar factory. Five isolates from that factory were therefore subjected to RAPD analyses and their RAPD patterns were highly similar to those of isolates from Belgian thick juice (data partially shown in Fig. 1). In both factories, these *Tetragenococcus* strains dominated the thick juice microflora reaching concentrations of 10⁶ c.f.u. ml⁻¹, which is comparable with earlier observations of *T. halophilus* counts in thick juice tanks (Justé *et al.*, 2008b). For the present study, four of these isolates were randomly selected for further characterization: T1^T and T170 from a Belgian thick juice sample, and T179 and T181 from a German thick juice sample. For *T. halophilus* isolates, three halophilic isolates were selected including the type strain T11^T (=LMG 11490^T), and T13 (=IAM 1673) and T19 (=JCM 1015), two strains from a different matrix. In addition, three osmophilic isolates were characterized, T2, T5 and T6, all originating from sugar companies in different countries. Finally, the type strains of *T. koreensis* (T55^T =LMG 22864^T), *T. muriaticus* (T12^T =JCM 10006^T) and *T. solitarius* (T56^T =DSM 5634^T) were included in this study as well, leading to a total of 13 isolates studied (Table 1). Representative RAPD patterns obtained with primer RAP6 (AGGGCCGTCT) of all selected isolates are given in Fig. 1.

After five days of incubation at 30 °C on tryptone soy agar (TSA; Oxoid), colonies of all isolates studied were smooth, low convex, circular, opaque, matt and white in colour. Growth occurred at NaCl concentrations in the range 0–25 % (w/v) and was enhanced after the addition of 5 % NaCl (w/v). In addition, strains were able to grow in tryptone soy broth (TSB; Oxoid) containing sucrose up to the saturation level of 66 % (w/v). The phenotypic properties of all selected isolates are summarized in Table 2. Remarkably, only *T. solitarius* could grow on TSA at 40 °C. Growth on MRS was observed for *T. solitarius* and *T. koreensis*, while a weak onset of growth was detected for the halophilic *T. halophilus* strains. Standard MRS does not contain any NaCl and has a low pH of 6.2. Addition of 5 % NaCl to the MRS medium resulted in micro-colonies for all species of the genus *Tetragenococcus*. MRS medium with a pH of 7.2 (by addition of 1 M NaOH) resulted in standard *Tetragenococcus* colonies for all strains tested (data not shown). The combination of no NaCl and especially the low pH does not allow growth of most fastidious *Tetragenococcus* strains. Growth in 69 °Bx sugar thick juice was observed only for the osmophilic strains of *T. halophilus*, all isolated from thick juice.

In contrast to the other isolates tested, T1^T, T170, T179 and T180 were not able to grow at a temperature of 37 °C (Table 2). The strains grew on trypticase soy agar at 30 °C, aerobically and in an anaerobic jar, but not on standard MRS agar at 30 °C under either aerobic or anaerobic conditions. In addition, the strains grew optimally in TSB

Table 1. *Tetragenococcus* isolates selected for this study with corresponding 16S rRNA gene sequence information

Data for strains T1^T, T2, T5, T6, T11^T, T13 and T19 are presented by Justé *et al.* (2008c).

Isolate	Sample origin	Taxon	GenBank accession no.	Nearest 16S rRNA gene sequence match using BLAST analysis		
				Organism	GenBank accession no.	Nucleotide similarity (%)
T1 ^{T*}	Degraded thick juice, Belgium, 2006		EU522083	<i>Tetragenococcus muriaticus</i>	D88824	97
T170*	Degraded thick juice, Belgium, 2006		HQ123368	<i>Tetragenococcus muriaticus</i>	D88824	97
T179*	Non-degraded thick juice, Germany, 2009		HQ123369	<i>Tetragenococcus muriaticus</i>	D88824	97
T181*	Non-degraded thick juice, Germany, 2009		HQ123370	<i>Tetragenococcus muriaticus</i>	D88824	97
T2†	Non-degraded thick juice, France, 2006		EU522084	<i>Tetragenococcus halophilus</i>	AB041346	99
T5†	Degraded thick juice, Belgium, 2005		EU522087	<i>Tetragenococcus halophilus</i>	AB041346	99
T6†	Degraded thick juice, Germany, 2007		EU522088	<i>Tetragenococcus halophilus</i>	AB041346	99
T11 ^T (=LMG 11490 ^T)	Anchovy pickles	<i>Tetragenococcus halophilus</i>	EU689052	<i>Tetragenococcus halophilus</i>	D88668	99
T13 (=IAM 1673)	Fermented soybean mash	<i>Tetragenococcus halophilus</i>	EU689054	<i>Tetragenococcus halophilus</i>	AB041346	99
T19 (=JCM 2015)	Fermented small fish	<i>Tetragenococcus halophilus</i>	EU689058	<i>Tetragenococcus halophilus</i>	AB041346	99
T12 ^T (=JCM 10006 ^T)	Fermented squid liver sauce	<i>Tetragenococcus muriaticus</i>				
T55 ^T (=LMG 22864 ^T)	Kimchi, traditional korean fermented food	<i>Tetragenococcus koreensis</i>				
T56 ^T (=DSM 5634 ^T)	Human ear exudate	<i>Tetragenococcus solitarius</i>				

**Tetragenococcus osmophilus*.

†*Tetragenococcus halophilus* subsp. *flandriensis*.

Fig. 1. Random amplified polymorphic DNA (RAPD) fingerprint of the selected *Tetragenococcus* isolates generated with primer RAP6 (AGGGCCGTCT). T1^T, T170, T179, T180: *T. osmophilus*; T2, T5^T, T6: *T. halophilus* subsp. *flandriensis* subsp. nov.; T11^T, T13, T19: *T. halophilus* subsp. *halophilus* subsp. nov.; T12^T: *T. muriaticus*; T54^T: *T. koreensis*; T56^T: *T. solitarius*; L, smartladder.

at pH 8.0. Cells of the four related strains were tested for their Gram reaction (Brenner *et al.*, 2005), cell shape and cell size after three days of growth on TSA at 30 °C. Oxidase activity was tested using the Oxidase DrySlide testkit (Becton Dickinson). Catalase activity was determined by evaluating bubble production with a 3% (v/v) hydrogen peroxide solution (Cappuccino & Sherman, 2002). *T. halophilus* IAM 1676^T (oxidase-negative and catalase-negative) and *Staphylococcus epidermidis* LMG 10474^T (oxidase-negative and catalase-positive) were used as controls. All isolates were identified as Gram-positive cocci with a diameter of less than 1 µm. Cells occurred in pairs or in tetrads and were oxidase- and catalase-negative.

For maintenance and further analyses, the *Tetragenococcus* strains were grown on TSA at 30 °C with a total concentration of 5.5% NaCl, unless specified otherwise.

The four isolates T1^T, T170, T179 and T181, together with the six *T. halophilus* isolates T2, T5, T6, T11^T, T13 and T19, were subjected to further genetic analyses, i.e. 16S rRNA gene sequencing and DNA–DNA hybridization. 16S rRNA gene sequencing was performed on purified PCR products obtained with the universal bacterial primers 27f and 1492r (Lane, 1991). Resulting sequences were compared to the GenBank database by using the BLAST program located at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>) (Altschul *et al.*, 1997; Benson *et al.*, 2004) to retrieve related sequences for phylogenetic analysis. All sequences were aligned using the CLUSTAL X algorithm. The four *Tetragenococcus* isolates T1^T, T170, T179 and T181 showed identical 16S rRNA gene sequences (analysis of 1192 nt) and were most closely related to *T. muriaticus* JCM 10006^T (97.4% sequence similarity). A

neighbour-joining tree (Saitou & Nei, 1987) was reconstructed and visualized using TreeView (v.1.6.6; Page, 1996; Fig. 2). These four isolates and *T. muriaticus* formed a separate cluster within the genus *Tetragenococcus* (supported by a bootstrap resampling value of 100%). 16S rRNA gene sequence analysis of the six *T. halophilus* strains revealed highly similar sequences (>99.7% sequence identity in a total of 1186 nt) for the osmophilic isolates (T2, T5 and T6), resulting in a subcluster within the species *T. halophilus* (66% bootstrap support). More precisely, there are two bases out of the 1077 bases used for the phylogenetic analysis which are consistently different in isolates from salt-rich environments compared to *T. halophilus* strains isolated from sugar thick juice (from different countries). Both repetitive (rep-) PCR fingerprinting and RAPD fingerprinting confirmed this separate cluster for the sugar thick juice isolates (Justé *et al.*, 2008c).

The genomic DNA–DNA relatedness between isolate T1^T and *T. muriaticus* LMG 18498^T was determined by total genomic DNA–DNA hybridization. Therefore, total genomic DNA was extracted by the method of Wilson (1987), with minor modifications (Cleenwerck *et al.*, 2002). Only high molecular mass DNA with A_{260}/A_{280} and A_{234}/A_{260} absorption ratios of 1.8–2.0 and 0.40–0.60, respectively, was used. DNA–DNA hybridizations were performed according to a modification of the microplate method described by Ezaki *et al.* (1989) (Goris *et al.*, 1998; Cleenwerck *et al.*, 2002). The hybridization temperature was 35 °C and reciprocal reactions were performed. Isolate T1^T showed $57 \pm 6.5\%$ DNA–DNA relatedness with *T. muriaticus* (determined from eight hybridizations), which is below the generally accepted limit for species delineation (70%; Wayne *et al.*, 1987). As a consequence, isolates T1^T, T170, T179 and T181 should be classified as a novel species, for which, because of their osmophilic character, the name *Tetragenococcus osmophilus* sp. nov. is proposed, with T1^T as the type strain of the species.

For determination of the DNA G+C content of isolate T1^T, the same DNA extract was used as for DNA–DNA hybridization. The DNA was enzymically degraded into nucleosides and the DNA base composition was determined using the HPLC technique as described by Mesbah *et al.* (1989). The genomic DNA G+C content of isolate T1^T was 36.7 mol%, which is comparable to the G+C content of previously described species of the genus *Tetragenococcus* (Table 1).

The osmophilic and halophilic *T. halophilus* isolates showed 77 to 88% DNA–DNA relatedness among each other (Justé *et al.*, 2008c). Therefore, both groups belong to a single species, despite their differences.

For all strains studied, acid production from carbohydrates was determined with the API 50CH microtest system, according to the recommendations of the manufacturer (bioMérieux). All wells were covered with mineral oil to obtain the fermentation pattern of the different tetragenococci (Table 2). For the *T. osmophilus* isolates, T1^T,

T170, T179 and T181, homogeneous carbon utilization patterns were observed. Isolate T1^T did not ferment D-ribose, D-galactose, D-sorbitol, amygdalin or D-tagatose but showed a positive reaction for methyl α -D-glucoside and α -D-mannoside. Isolate T170, which was isolated from the same thick juice as isolate T1^T, showed an identical (but sometimes weaker) carbon source utilization pattern. The selected isolates T179 and T181 from sugar thick juice in Germany showed similar results (Table 2). In contrast to the homogeneous results of the novel species, clear differences can be observed between the *T. halophilus* isolates (Table 2). All osmophilic *T. halophilus* isolates (T2, T5^T and T6) were able to ferment lactose, raffinose and D-arabinose but not glycerol, in contrast to the halophilic *T. halophilus* isolates [T11^T (=IAM 1676^T), T13 (=IAM 1673) and T19 (=JCM 2015)] which were all negative for lactose, raffinose and D-arabinose and positive for glycerol (Table 2). Some carbon sources were fermented by only some halophilic and/or part of the osmophilic isolates. These included D-ribose, D-mannitol, D-sorbitol, methyl α -D-glucoside, melibiose, D-tagatose, D-galactose and D-arabitol (Table 2). Intraspecific variation in fermentation patterns for *T. halophilus* has been reported previously by other researchers (Kobayashi *et al.*, 1995; Röling & van Verseveld, 1996). Furthermore, halophilic isolates showed a clear onset of growth (growth only at the spot of inoculation) on MRS agar when incubated at 30 °C for 5 days, whereas the osmophilic isolates could not grow at all under these conditions.

Remarkably, no carbon source metabolic Biolog fingerprint (Bochner, 1989) could be obtained for the *Tetragenococcus* isolates from thick juice, whereas a clear fingerprint was produced by the halophilic *T. halophilus* strains and *T. muriaticus* using GP2 plates. Generally, the use of a carbon source in the Biolog MicroStation system is indicated by the reduction of the colourless tetrazolium violet (TV) to the purple formazan (Bochner, 1989). However, since TV can be toxic to slowly growing bacteria, and in particular to Gram-positive bacteria (B. Bochner, personal communication), the sensitivity of both the halophilic and osmophilic tetragenococci to TV was evaluated. Strains were grown on TSA containing 0.01 % TV, which corresponds to the TV concentration in the Biolog GP2 assay, followed by an 8 day incubation at 30 °C. All halophilic isolates showed inhibited growth (growth only observed in densely inoculated zones near the initial inoculation spot; no single colonies), but still reduced TV to the purple formazan. Most osmophilic isolates, however, showed a more severe inhibition of their growth and stayed nearly white, illustrating a high sensitivity of these bacteria to TV. Remarkably, two out of the eight osmophilic isolates tested (T2 and T6) were not inhibited, but did not reduce TV either (Justé *et al.*, 2008c). This is the first time that bacterial strains from a single species have shown differing sensitivity for TV (B. Bochner, personal communication).

In addition, acid production was analysed by using HPLC for isolates T1^T, T5, T11^T, T12^T, T55^T and T56^T. Isolates were grown aerobically in TSB for 4 days. Centrifugation

and filtering through an ultrafree-MC tube (0.2 μ m pore size; Millipore) allowed removal of the cells. HPLC analysis of organic acids was performed on a Hitachi Lachrom system (Merck) consisting of an L-7100 programmable pump, an L-7450A DAD detector, an L-7200 programmable autosampler and a D-7000 interface. Separations were carried out on an Aquasil 5 μ m C₁₈ column of 250 mm $\times$ 4.6 mm (Thermo Scientific) with a flow of 1.25 ml min⁻¹. Organic acid detection was performed at 210 nm. The mobile phases were (a) acetonitrile (100 %) and (b) KH₂PO₄ (0.05M), pH 2.8. Isolate T1^T was found to produce acetic acid and lactic acid in approximately equal concentrations (Table 2), and can thus be regarded as heterofermentative. All other strains of the genus *Tetragenococcus* showed these mixed fermentation products as well (Table 2). These results confirm the observations of Gürtler *et al.* (1998) where acetate was reported as the main fermentation product under aerobic conditions for *T. halophilus*.

Altogether, the results of the present study support a clear genotypic and phenotypic differentiation of the four *Tetragenococcus* isolates, T1^T, T170, T179 and T181, isolated from different thick juice samples, from the four currently recognized species of the genus *Tetragenococcus*. These isolates should therefore be classified as a novel species, for which, because of their osmophilic character, the name *Tetragenococcus osmophilus* sp. nov. is proposed with T1^T (=LMG 26041^T = DSM 23765^T) as the type strain of the species.

The osmophilic and halophilic *T. halophilus* isolates, T2, T5, T6, T11^T, T13 and T19, all belong to the same species as shown by the DNA–DNA hybridization data, but they show clear differences on the basis of phenotypic and genotypic characteristics. Because of these differences and the industrial relevance of sugar thick juice degradation, we propose to divide the species *Tetragenococcus halophilus* into two subspecies. More specifically, we propose to classify the halophilic strains as the nominate subspecies *Tetragenococcus halophilus* subsp. *halophilus* with IAM 1676^T (=LMG 11490^T = DSM 20339^T) as the type strain, and the osmophilic strains as the subspecies *Tetragenococcus halophilus* subsp. *flandriensis*, which was first isolated in Flanders, Belgium, with T5^T (=LMG 26042^T = DSM 23766^T) as the type strain.

Description of *Tetragenococcus osmophilus* sp. nov.

Tetragenococcus osmophilus [os.mo.phi'lus. N.L. masc. adj. *osmophilus* (from Gr. n. *ōsmos* impulse and Gr. masc. adj. *philos* friend, loving) osmophile, impulse-loving].

Cells are non-motile, non-spore-forming cocci (approx. 0.6–1 μ m in length). Gram-positive, catalase-negative, oxidase-negative. On TSA agar, colonies are white, round, convex and smooth, and approximately 0.6 mm in diameter after incubation at 30 °C for 5 days. Cells are able to grow on TSA at 30 °C aerobically and in an anaerobic jar, but do not grow on standard MRS agar at 30 °C under either aerobic or anaerobic conditions. Growth is enhanced by the addition of 5 % NaCl (w/v). Growth occurs at NaCl concentrations in the

All data were obtained from this study, except for the DNA G + C content which was taken from Lee *et al.* (2005). For *T. muritaticus*, TSA with a total concentration of 5.5 % NaCl was used instead of the standard TSA with 0.5 % NaCl. Fermentation of sugars was determined with API CH50 strips. None of the isolates hydrolysed aesculin. All isolates were positive for fermentation of D-glucose, D-fructose and D-mannose. All isolates were negative for fermentation of erythritol, L-arabitol, L-arabinose, inulin, D- and L-fucose, amygdalin, D-lyxose, methyl β -D-xyloside, L-xylose, adonitol, L-rhamnose, glycogen, dulcitol, inositol, xylitol and L-sorbose. TV, Tetrazolium violet dye which is used to visualize carbon metabolism in Biolog GP2 plates; +, positive reaction; -, negative reaction; v, variable reaction; ND, no data available.

[illegible]

Table 2. cont.

Characteristic	<i>T. osmophilus</i>				<i>T. halophilus</i>				<i>T. muriaticus</i>	<i>T. koreensis</i>	<i>T. solitarius</i>		
					subsp. <i>flandriensis</i>				subsp. <i>halophilus</i>				
	T1 ^T = DSM 23765 ^T	T170	T179	T181	T2	T5 ^T = DSM 23766 ^T	T6	T11 ^T = LMG 11490 ^T	T13 = IAM 1673	T19 = JCM 2015	JCM 1006 ^T	LMG 22864 ^T	DSM 5634 ^T
Raffinose	—	—	—	—	+	+	+	—	—	—	—	—	—
Gentiobiose	+	+	—	—	+	+	+	+	+	+	—	+	+
Turanose	+	+	+	+	+	+	+	+	+	+	—	+	+
D-Tagatose	—	—	—	—	+	+	+	W	W	—	—	—	—
D-Arabitol	+	+	—	—	+	+	—	W	W	—	—	W	W
DNA G + C content (mol%)	36.7	ND	ND	ND	ND	36.7	ND	36.0	ND	ND	36.5	38.0	38.3

range 0–25 % (w/v) and at 15–30 °C on TSA but not at 4 °C or ≥37 °C. Optimal growth temperature is 30 °C. Optimal pH is 8.0. Able to grow in sugar thick juice of 69 °Bx (and lower). Facultatively aerobic and heterofermentative lactic acid bacterium. Fermentation of methyl α-D-glucoside and α-D-mannoside, but negative API result for D-ribose, D-galactose, D-sorbitol, amygdalin and D-tagatose. The DNA G+C content of the type strain is 36.7 mol%.

The type strain is T1^T (=LMG 26041^T =DSM 23765^T), isolated from degraded sugar thick juice.

Description of *Tetragenococcus halophilus* subsp. *halophilus* (Mees 1934) Collins *et al.* 1993, subsp. nov.

Tetragenococcus halophilus subsp. *halophilus* [ha.lo.phi'lus. Gr. n. *hals* halos salt; N.L. adj. *philus* -a -um (from Gr. adj. *philos* -ē -on), friend, loving; N.L. masc. adj. *halophilus* salt-loving].

For a detailed description of this subspecies, we refer to the original description of *T. halophilus* by Collins *et al.* (1990). Briefly, cells are non-motile, non-spore-forming cocci (approx. 0.6–1 µm in length). Gram-positive, catalase-negative, oxidase-negative. On TSA agar, colonies are white, round, convex and smooth, and approximately 0.6 mm in diameter after incubation at 30 °C for 5 days. They grow on TSA at 30 °C aerobically and in an anaerobic jar, but show only an onset of growth (growth only at the spot of inoculation) on standard MRS agar at 30 °C under either aerobic or anaerobic conditions. Growth is enhanced by the addition of 5 % NaCl (w/v). Growth occurs at NaCl concentrations in the range 0–25 % (w/v) and at 15–30 °C on TSA agar but not at 4 °C or above 37 °C. Optimal growth temperature is 30 °C. Optimal pH is between 7.0 and 9.0. Cannot grow in sugar thick juice of ≥65 °Bx. Cannot ferment lactose, raffinose or D-arabinose, but positive for fermentation of glycerol. Reduces TV to formazan, resulting in representative fingerprints with Biolog GP2 plates. Facultatively aerobic. Demonstrates heterofermentative production of lactic acid. The DNA G+C content varies between 34 and 36 mol%.

The type strain is IAM 1676^T (=LMG 11490^T =DSM 20339^T), isolated from salted anchovy.

Description of *Tetragenococcus halophilus* subsp. *flandriensis* subsp. nov.

Tetragenococcus halophilus subsp. *flandriensis* (flan.dri.en' sis N.L. fem. adj. *flandriensis* of or belonging to Flandria, the old name for Flanders, Belgium).

Cells are non-motile, non-spore-forming cocci (approx. 0.6–1 µm in length). Gram-positive, catalase-negative, oxidase-negative. On TSA, colonies are white, round, convex and smooth, and approximately 0.6 mm in diameter after incubation at 30 °C for 5 days. Cells are able to grow on TSA at 30 °C aerobically and in an anaerobic jar, but do not grow on standard MRS agar at 30 °C under either aerobic or

Fig. 2. Neighbour-joining tree, based on 16S rRNA gene sequences (1186 nt), showing the phylogenetic position of the *Tetragenococcus* isolates characterized and other related taxa. Sequences used are indicated by the respective GenBank accession numbers. Bootstrap values are expressed as percentages of 1000 replications. *Bacillus subtilis* was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

anaerobic conditions. Growth is enhanced by the addition of 5% NaCl (w/v). Growth occurs at NaCl concentrations in the range 0–25% (w/v) and at 15–30 °C on TSA agar but not at 4 °C or above 37 °C. Optimal growth temperature is 30 °C. Optimal pH is between 7.0 and 9.0. Able to grow in sugar thick juice of 69 °Bx (and lower). All isolates tested ferment lactose, raffinose and D-arabinose and are negative for glycerol. No reduction of TV to formazan, therefore generating no fingerprint with Biolog GP2 plates. Facultatively aerobic and heterofermentative. The DNA G+C content of the type strain is 36.7 mol%.

The type strain is T5^T (=LMG 26042^T =DSM 23766^T), isolated from degraded sugar thick juice.

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