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Genetic and physiological diversity of *Tetragenococcus halophilus* strains isolated from sugar- and salt-rich environments

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Tetragenococcus halophilus is known to flourish in extreme salt environments. Recently, this halophilic bacterium also appeared as the dominant microflora during storage of sugar thick juice, an intermediate product of beet sugar production. Although *T. halophilus* can cause degradation of thick juice, dominance of this bacterium does not always result in degradation. In this study *T. halophilus* strains from high-salt and high-sugar environments, and in particular from degraded and non-degraded thick juice, were compared in detail. Both physiological and genetic characterization using Biolog, repetitive extragenic palindrome PCR fingerprinting (REP-PCR) and random amplified polymorphic DNA (RAPD) technology, revealed clear differences between *T. halophilus* strains isolated from salt- and sugar-rich environments. However, no strain pattern could be specifically and systematically associated with degraded or non-degraded thick juice. Remarkably, halophilic *T. halophilus* strains were not able to grow in sugar thick juice. Irrespective of the differences between the strains from high-salt or high-sugar environments, DNA–DNA hybridization grouped all strains within the species *T. halophilus*, except one isolate from sugar thick juice that showed different physiological and genetic characteristics, and that may represent a new species of *Tetragenococcus*.

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INTRODUCTION

At present, the genus *Tetragenococcus* comprises four species: *T. halophilus* (Collins *et al.*, 1990), *T. muriaticus* (Satomi *et al.*, 1997), *T. solitarius* (Ennahar & Cai, 2005) and *T. koreensis* (Lee *et al.*, 2005). The species *T. halophilus* and *T. muriaticus,* both homofermentative lactic acid bacteria, play an important role in halophilic fermentation processes such as the production of soy sauce, soy paste, brined anchovies, fish sauce, Japanese fermented puffer fish ovaries, Indonesian 'terasi' shrimp paste and fermented mustard (Ito *et al.*, 1985; Villar *et al.*, 1985; Röling *et al.*, 1994; Röling & van Verseveld, 1996; Kobayashi *et al.*, 2000, 2003; Thongsant *et al.*, 2002; Chen *et al.*, 2006). However, recently, Willems *et al.* (2003) detected *T. halophilus* in concentrated sugar thick juice, an intermediate in the production of beet sugar. Subsequently, *T. halophilus* was reported as the dominant microflora in this sugar-rich environment and as a probable cause of thick juice degradation, which is characterized by a reduction in pH from pH 9 to 5–6 and, typically, an increase in reducing sugar content resulting in economic losses (Sargent *et al.*, 1997; Willems *et al.*, 2003; Justé *et al.*, 2008b). Nevertheless, the dominance of this bacterium in equally conditioned tanks of thick juice does not always result in degradation,

Abbreviations: RAPD, random amplified polymorphic DNA; REP-PCR, repetitive extragenic palindrome PCR fingerprinting.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this work are given in Fig. 2.

suggesting that different strains of *T. halophilus* might be responsible for the different effects during storage (Justé *et al.*, 2008b). Consequently, a more detailed characterization, based on the genetic and physiological diversity of the *T. halophilus* strains isolated from degraded and non-degraded thick juice was undertaken to elucidate this controversy and provide options for effective control of thick juice degradation.

In this study, we characterized 25 isolates of *Tetragenococcus* obtained from thick juice using random amplified polymorphic DNA (RAPD) fingerprinting. Based on these results, an extensive genetic and physiological characterization of nine *Tetragenococcus* strains isolated from both degraded and non-degraded thick juice (further referred to as 'osmophilic tetragenococci') was performed, in comparison with seven strains isolated from a high-salt environment (further referred to as 'halophilic tetragenococci'). At the genetic level, strains were further compared by 16S rRNA gene sequencing, repetitive extragenic palindrome PCR fingerprinting (REP-PCR), and DNA–DNA hybridization. The physiological characterization included substrate utilization patterns and growth in salt-rich and sugar-rich media.

METHODS

Bacterial strains. Twenty-five representative strains (Table 1) were selected from a large collection of thick juice tetragenococci (Justé *et al.*, 2008a, b) for use in this study. In order to ensure a wide diversity, the strains were isolated from different geographical origins, and from degraded and non-degraded thick juice samples to which hop acids had been added or not. One strain was identified as *T. muriaticus*, the others as *T. halophilus* based on a species-specific PCR (Justé *et al.*, 2008a). *T. halophilus* strains from salt-rich environments were purchased from the BCCM/LMG Bacteria Collection (Ghent, Belgium), the Japanese Collection of Micro-organisms (JCM; Hirosawa, Japan) or the Institute of Applied Microbiology (IAM; University of Tokyo, Japan) (Table 1). Strains were cultured on Tryptone Soy Agar (TSA) at 30 °C. Stock cultures were maintained in 15 % (v/v) glycerol at –80 °C.

DNA extraction. Genomic DNA was extracted using the phenol/chloroform extraction method as described before (Lievens *et al.*, 2003). For DNA–DNA hybridization, DNA extractions were performed by the method of Wilson (1987) with minor modifications (Cleenwerck *et al.*, 2002).

RAPD fingerprinting. To select discriminative RAPD primers to type *Tetragenococcus* strains, initially 10 decamer oligonucleotides, randomly selected from the Operon primer kits (Operon Technologies), were screened on a subset of the strains listed in Table 1. The best three primers that led to the production of clear, distinct, reproducible and polymorphic bands were selected for further analysis. These were RAP1 (CAGCACTGAC), RAP3 (AGGGTCGTTC) and RAP6 (GGGCCGTCT). Amplification was performed in a total volume of 20 µl containing 0.5 µM of the single random primer, 0.15 mM of each deoxynucleoside triphosphate, 1.0 U Titanium *Taq* DNA polymerase (Clontech Laboratories) and 1 ng genomic DNA. Before amplification, DNA samples were denatured at 94 °C for 2 min. Subsequently, 35 cycles were run of 1 min at 94 °C, 1 min at 35 °C, and 2 min at 72 °C, with a final extension step at 72 °C for 10 min. RAPD-PCR products were separated by loading 10 µl of the reaction volume on 1.5 % agarose gels followed by 120 min electrophoresis at 4 V cm^{-1} in 1× Tris/acetate-EDTA (TAE) buffer, stained with ethidium bromide, and visualized with UV light. A 1 kb DNA ladder (Smartladder; Eurogentec) was used as size marker. Gel images were acquired with the BioChemi System (UVP, Upland, CA, USA). Image data were processed by using GelCompar software, version 4.6.1 (Applied Maths). After normalization and background subtraction, the levels of similarity between RAPD-PCR fingerprints were calculated by using the Pearson correlation coefficient. Cluster analysis was performed on three gels, optioned with three different RAPD primers. and was performed by the unweighted pair group method (UPGMA). The different RAPD primers resulted in similar clustering (data not shown). All RAPD reactions were performed in triplicate to check reproducibility.

16S rRNA gene sequence analysis. The nearly complete 16S rRNA gene was amplified by PCR with the universal bacterial primers 27F and 1492R (Lane, 1991). The target DNA (1 ng) was amplified in a total volume of 20 µl containing 0.3 µM of each primer, 0.3 mM of each deoxynucleoside triphosphate, 1 mM $MgSO_4$, 2× *Pfx* amplification buffer, 2× PCR Enhancer and 1.0 U Platinum *Pfx* DNA polymerase (Invitrogen). Before amplification, DNA samples were denatured at 94 °C for 2 min. Subsequently, 30 cycles were run of 15 s at 94 °C, 30 s at 59 °C, and 90 s at 72 °C, with a final extension step at 72 °C for 10 min.

Sequencing was performed on purified PCR products with the same primers 27F and 1492R. Samples were analysed on an Applied Biosystems 373A Automated Sequencer. A search for sequence similarities was performed using the BLAST (Altschul *et al.*, 1997) algorithm to screen GenBank (Benson *et al.*, 2004). All sequences were deposited in GenBank under the accession numbers shown in Fig. 2. Multiple sequence alignment was performed using CLUSTAL_X. Phylogenetic analyses were performed using the neighbour-joining algorithm of the CLUSTAL_X software package. Phylogenetic trees were constructed using CLUSTAL_X and visualized using TreeView.

REP-PCR fingerprinting. To select a good REP-PCR primer (pair) to characterize *Tetragenococcus* species, two single oligonucleotides, BOXA1R (CTACGGCAAGGCGACGCTGACG) and $(GTG)_5$, and one primer pair, REP1R-I (IIIICGICGICGICATCIGGC) and REP2-I (ICGICTTATCIGGCCTAC), were initially tested on a subset of five *Tetragenococcus* strains. PCR conditions were as described by Versalovic *et al.* (1994). In contrast to primer BOXA1R, which generated only 1–3 bands, the $(GTG)_5$ primer and the REP1R-I and REP2-I primer set had good discriminative abilities, yielding 2–13 bands for each isolate. Nevertheless, since some products generated with primer $(GTG)_5$ did not migrate from the slots during gel electrophoresis, the REP1R-I and REP2-I primer set was chosen for further analyses.

REP-PCR analysis was performed in a total volume of 20 µl containing 0.5 µM of each primer, 0.15 mM of each deoxynucleoside triphosphate, 1.0 U Titanium *Taq* DNA polymerase, and 1 ng genomic DNA. Before amplification, DNA samples were denatured at 94 °C for 2 min. Subsequently, 35 cycles were run of 1 min at 94 °C, 1 min at 40 °C, and 4 min at 72 °C, with a final extension step at 72 °C for 10 min. REP-PCR products were separated and visualized as described above, and image data were processed as for the RAPD analyses. All REP-PCR reactions were performed in duplicate to check reproducibility.

DNA–DNA hybridization. DNA–DNA hybridizations were performed according to a modification (Goris *et al.*, 1998; Cleenwerck *et al.*, 2002) of the microplate method described by Ezaki *et al.* (1989). The hybridization temperature was 35 °C and reciprocal reactions (e.g. A × B and B × A) were performed. DNA-binding values reported

Table 1. *Tetragenococcus* strains used in this study, isolated from both thick juice (osmophilic strains) and salt-rich environments (halophilic strains)

Geographical origin	Isolate	Source	Isolation date or culture collection number*	RAPD group†	Nearest match using BLAST analysis	
					Organism	Similarity (%)
			Osmophilic strains			
Belgium						
Tienen	T1‡	Degraded thick juice	2006	I	*T. muriaticus*	98
Tienen	T3‡	Non-degraded thick juice	2005	II	*T. halophilus*	99
Tienen	T4‡	Non-degraded thick juice	2006	II	*T halophilus*	99
Tienen	T5‡	Degraded thick juice	2005	II	*T. halophilus*	99
Tienen	T7‡	Non-degraded thick juice	2006	II	*T. halophilus*	99
Tienen	T9‡	Non-degraded, hop-treated thick juice	2004	II	*T. halophilus*	99
Tienen	T10‡	Degraded thick juice	2007	II	*T. halophilus*	99
Genappe	T52 (ID4406)	Degraded thick juice	1997	II	*T. halophilus*	99
Genappe	T53 (ID4407)	Degraded thick juice	1997	II	*T. halophilus*	99
Germany						
Zeitz	T6‡	Degraded thick juice	2000	III	*T. halophilus*	99
Zeitz	T20	Non-degraded thick juice	2007	III	ND	
Zeitz	T25	Non-degraded thick juice	2007	III	ND	
Zeitz	T26	Non-degraded thick juice	2007	III	ND	
Zeitz	T27	Non-degraded thick juice	2007	III	ND	
Zeitz	T47	Degraded thick juice	2007	III	ND	
Zeitz	T49	Degraded thick juice	2007	III	ND	
France						
Guignicourt	T2‡	Non-degraded thick juice	2006	IV	*T. halophilus*	99
Roye	T29	Degraded thick juice	2007	IV	ND	
Roye	T30	Degraded thick juice	2007	IV	ND	
Guignicourt	T31	Non-degraded thick juice	2007	IV	ND	
Guignicourt	T32	Non-degraded thick juice	2007	IV	ND	
Roye	T33	Non-degraded thick juice	2006	IV	ND	
Roye	T34	Non-degraded thick juice	2006	IV	ND	
Eppeville	T35	Non-degraded thick juice	2006	IV	ND	
Eppeville	T36	Non-degraded thick juice	2006	IV	ND	
			Halophilic strains			
Belgium	T11^T‡	Anchovy pickles	LMG 11490^T (ATTC 33315)	V	*T. halophilus*	99
Belgium	T12^T‡	Squid liver sauce	LMG 18498^T	VI	*T. muriaticus*	99
Japan	T13‡	Fermented soybean mash	IAM 1673 (ATTC 13621)	V	*T. halophilus*	99
Japan	T14‡	Soy sauce brewing mashes	IAM 1674 (NISL 7118)	V	*T. halophilus*	99
Japan	T15‡	Soy sauce brewing mashes	IAM 1675 (NISL 7126)	V	*T. halophilus*	99
Japan	T18‡	Fermented shrimps	JCM 2014	VII	*Pediococcus* sp.	100
Japan	T19‡	Fermented small fish	JCM 2015	V	*T. halophilus*	99

ND, Not determined.

*ATCC, American Type Culture Collection (Manassas, Virginia, USA); IAM, the Institute of Applied Microbiology (University of Tokyo, Japan); JCM, the Japanese Collection of Micro-organisms (Hirosawa, Japan); LMG, BCCM/LMG Bacteria Collection (Ghent, Belgium).

†RAPD grouping was based on a RAPD analysis using primer RAP3 (Fig. 1).

‡Strains selected for further characterization.

are mean values of at least four hybridization experiments, including the reciprocal reactions.

Carbon source metabolic fingerprint. Utilization of 95 different carbon sources was tested in the Biolog MicroStation System (Bochner, 1989). Strain preparation and cultivation were performed according to the supplier's instructions for Gram-positive bacteria, except that the isolates were grown for 3 days to obtain the required inoculum density. Subsequently a GP2 MicroPlate was inoculated with 150 µl bacterial suspension per well and incubated at 30 °C for 4–8 days.

Salt and sucrose tolerance. Salt and sucrose tolerance was first tested in Tryptone Soy Broth (TSB) with added NaCl [15, 20, 25 and 28.5 % (saturation)] or sucrose [30, 60 and 66 % (saturation)]. Experiments were conducted at pH 7.0, defined as the optimum pH for growth of *T. halophilus* by Röling & van Verseveld (1997), and at pH 9.0, a typical pH of non-degraded thick juice. The experiment was performed in closed 50 ml conical tubes with 15 ml of culture under vigorous stirring at 30 °C. Cells were inoculated at a final concentration of 10^6 c.f.u. ml^{-1} and the ability to grow was judged from visual examination of opacity during 6–18 days incubation. Samples without apparent bacterial growth were plated on TSA for confirmation. To further investigate differences in growth characteristics, all strains were also inoculated in a similar way in standard sterilized thick juice with a Brix index of 69 °Bx and a pH of 9.3. Plate counts were determined on TSA (6 days of incubation at 30 °C) after different lengths of incubation. In addition, for two halophilic strains, $T11^T$ and T15, growth was monitored during 62 days storage at 30 °C in thick juice adjusted to a varying Brix index at both pH 7.0 and 9.0. Adjustment of the pH was done with concentrated HCl.

RESULTS

RAPD fingerprinting

In order to make a comprehensive, but well-considered selection of *Tetragenococcus* strains to study the genetic and physiological differences between different tetragenocci, all strains listed in Table 1 were subjected to a RAPD analysis using the primers RAP1, RAP3 and RAP6. RAPD patterns were obtained with DNA fragment sizes ranging from 100 to 3000 bp, and both the presence and the relative intensities of the bands were found to be reproducible for all primers in different runs (data not shown). RAPD analysis of all studied thick juice strains listed in Table 1 with primer RAP3 resulted in 22 distinct fragments (Fig. 1), while RAP1 and RAP6 resulted in 20 and 15 different fragments respectively. All RAPD primers produced seven clearly distinct RAPD profiles as exemplified in Fig. 1 for primer RAP3.

Cluster analysis performed either on the three individual patterns or on the combined dataset resulted into a dendrogram similar to the one shown in Fig. 1. The halophilic *Tetragenococcus* strains $T11^T$, T13, T14, T15 and T19 were grouped into a single cluster (RAPD group V). However, the other halophilic strains, *T. muriaticus* $T12^T$ and T18, landed in a separate group, RAPD group VI and

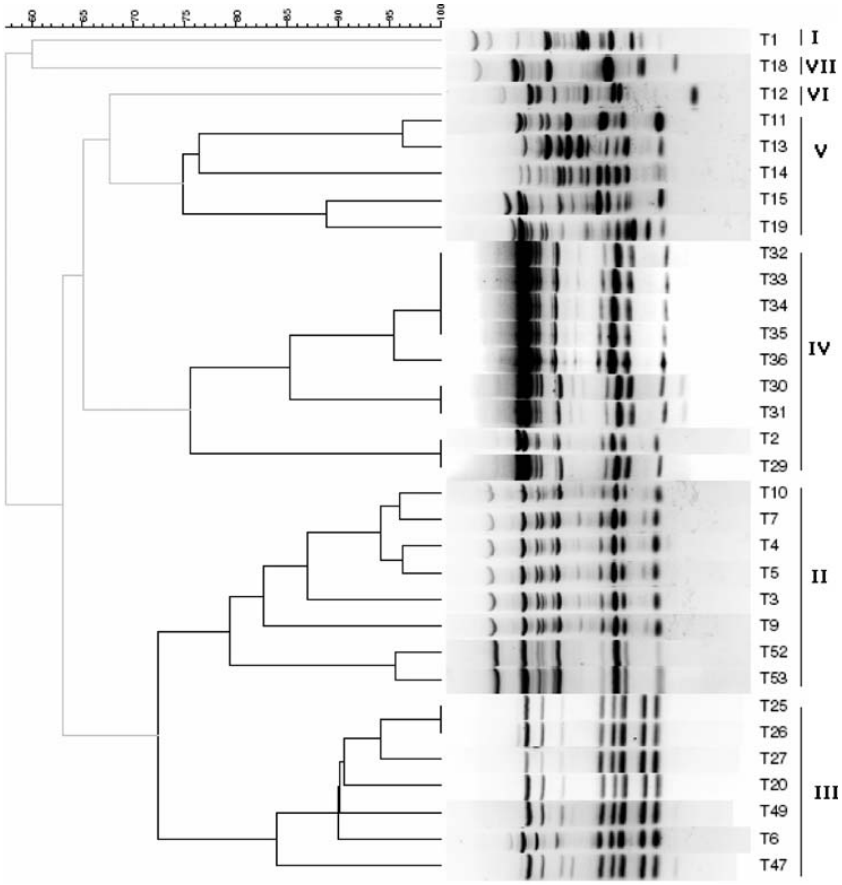

Fig. 1. RAPD fingerprint obtained with primer RAP3 and corresponding dendrogram derived from UPGMA linkage of Pearson correlation coefficients of all *Tetragenococcus* strains listed in Table 1.

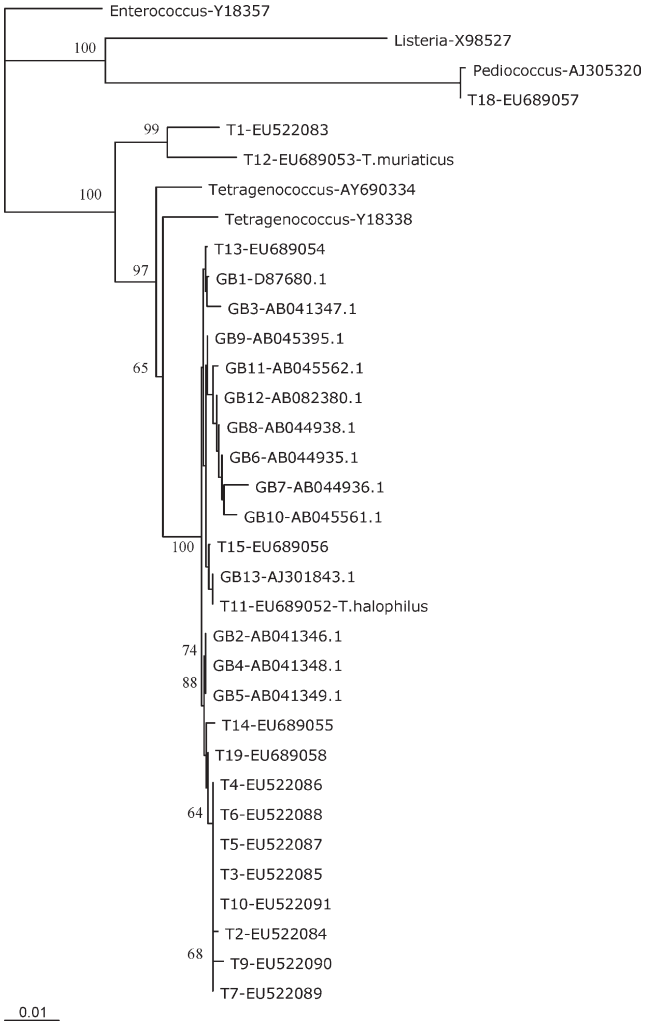

Fig. 2. Phylogenetic analysis of 16S rRNA gene sequences of the selected *Tetragenococcus* isolates recovered from either sugar thick juice (T1–T7, T9 and T10) or from a salt-rich environment (T11^T–T15 and T19), together with all available *T. halophilus* sequences in GenBank (GB1–GB13). Relationships were determined using the neighbour-joining clustering method based on 1077 bp. Bootstrap values (based on 100 replications) higher than 60 are shown at the robust nodes of the tree. Strain numbers are accompanied by their respective GenBank accession numbers.

VII, respectively. The osmophilic strains were grouped depending upon the country of origin, with one cluster for almost all Belgian strains (RAPD group II), all French strains (RAPD group IV) and all isolates from Germany (RAPD group III). Only T1 clustered separately from the other tetragenococci (RAPD group I; Fig. 1). Regardless of the refineries of a given country, for each RAPD primer identical patterns were obtained for the different strains (data not shown). No strain pattern could be specifically and systematically associated with degraded or non-degraded thick juice (Fig. 1, Table 1). Based on this extensive RAPD screening, at least one strain per RAPD group was selected for further genetic and physiological characterization. In order to study the association between specific strains from degraded or non-degraded thick juice, five additional *T. halophilus* strains from the Belgian refinery in Tienen, which correspond to previously published work and as a consequence to well-characterized thick juice samples (Justé *et al.*, 2008a, b), were selected in the final set of test strains. Although these strains had an identical RAPD pattern, they were selected because of their different origin: i.e. isolated from degraded or non-degraded thick juice, isolated from thick juice which was or was not treated with hop biocides, and isolated over a period of several years. In total, nine osmophilic (T1–T7, T9 and T10) and seven halophilic strains (T11^T–T15, T18 and T19) were retained for further experiments.

16S rRNA gene sequencing and phylogenetic analysis

To examine the genetic similarity between the selected osmophilic (T1–T7, T9 and T10) and halophilic strains (T11^T–T15, T18 and T19) and to confirm their identity, their 16S rRNA gene sequences were determined and compared with those from GenBank (Table 1). Based on a BLAST analysis, all strains except T18 and T1 were identified as *T. halophilus*. Isolate T18, which was originally isolated from fermented shrimps, appeared identical with the 16S rDNA sequence from a *Pediococcus* species (GenBank accession no. EU147315.1). T1, which was isolated from degraded sugar thick juice from a Belgian factory, showed most homology to the 16S rDNA of *Tetragenococcus muriaticus* (GenBank accession no. D88824.1; Table 1).

Furthermore, the sequences of all selected tetragenococci and all available (13) *T. halophilus* sequences from GenBank (labelled as 'GB') were aligned (1077 bp) and a phylogenetic tree was constructed (Fig. 2). All selected halophilic *T. halophilus* strains (T11^T, T13–T15, T19) clustered within the GenBank sequences in a single group (100 % bootstrap support). The osmophilic strains clustered as a subgroup of the *T. halophilus* cluster (64 % bootstrap support). The 16S rDNA sequences from T3, T4, T5, T6, T7 and T10 are identical, while some subtle nucleotide differences were observed with strains T2 and T9. In addition, T1 and T12^T, the type strain of *T. muriaticus*, were clearly separated from the other isolates. Nevertheless, both strains still differed in 25 nucleotides (2 %). All sequences obtained in this study were deposited in GenBank under the accession numbers shown in Fig. 2.

REP-PCR fingerprinting

REP-PCR fingerprints were generated using the primer set REP1R-I and REP2-I. Cluster analysis clearly separated the osmophilic strains from the halophilic *T. halophilus* strains (Fig. 3). In contrast to the 16S rDNA phylogeny, strain T1 did not cluster together with the type strain of *T.*

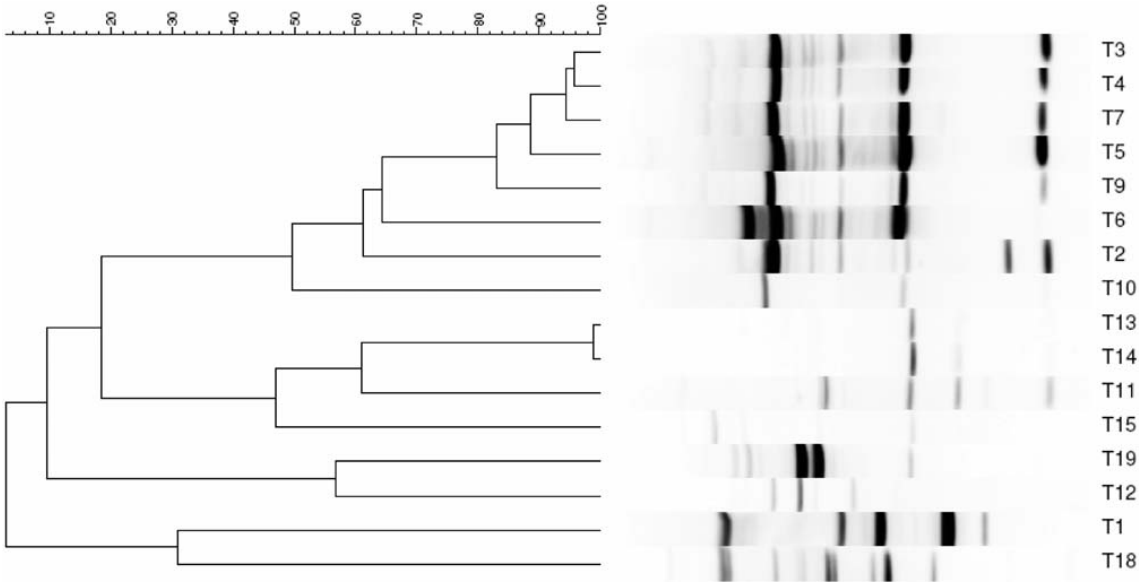

Fig. 3. REP-PCR fingerprint generated using the primer set REP1R-I and REP2-I, and corresponding dendrogram derived from UPGMA linkage of Pearson coefficients of the 16 selected *Tetragenococcus* strains, isolated either from sugar thick juice (T1–7, T9 and T10) or from a salt-rich environment (T11^T–T15, T18 and T19).

muriaticus T12^T, but clustered separately. Similar to the RAPD analyses, no strain pattern could be specifically associated with degraded or non-degraded thick juice. Remarkably, the REP-PCR fingerprint generally produced weaker signals for the halophilic strains than for the osmophilic strains (Fig. 3). Nevertheless, since for all DNA samples (except T10) similar amounts of PCR products were generated using the 16S rDNA universal primers 27F and 1492R (data not shown), differences in DNA quality may be excluded. Nevertheless, the separate clustering of T10 might be due to the relatively low intensity of the bands obtained (e.g. by a less efficient PCR). Indeed, since clustering was done with the Pearson coefficient, importance is given to the relative intensity of the densitometric curves and not only to presence and/or absence as for Dice correlation coefficients. Clustering with the Dice coefficient showed a similar cluster pattern except that T10 clustered together with T3, T4, T5, T7 and T9 (data not shown). In both cases, the isolates from the refineries from Germany (T6) and France (T2) clustered separately from the isolates obtained from the Belgian refinery [T3, T4, T5, T7, T9 (and T10)] (Fig. 3), in accordance with the RAPD results (Fig. 1).

DNA–DNA hybridization

Apart from the above-mentioned fingerprinting techniques targeting random or specific DNA sequences in the genome, genetic similarity between the different strains was also measured by DNA–DNA hybridization. Only strains with a different RAPD pattern were selected for this analysis, including the osmophilic strains T1 (RAPD group I), T2 (RAPD group IV), T5 and T52 (RAPD group II), and T6 (RAPD group III), and the halophilic strains T11^T (RAPD group V) and T12^T (RAPD group VI). All strains that were hybridized with the *T. halophilus* type strain T11^T (strains T2, T5, T6 and T52) showed more than 70 % DNA homology with each other (Table 2). As a DNA homology of 70 % is generally accepted as a limit for species delineation (Wayne *et al.*, 1987), it can be concluded that T2, T5, T6 and T52 all belong to the species *T. halophilus*. Although some of the hybridization results showed variations outside the limits of this method (20–25 units, Goris *et al.*, 1998), these values do not influence the conclusion as presented by the good hybridization values. T1, which shared a similar 16S rDNA sequence with *T. muriaticus* (Table 1, 98 %) but revealed a clearly different RAPD and REP-PCR pattern, showed less than 70 % DNA homology with the *T. muriaticus* type strain T12^T (Table 2). Consequently, T1 may represent a novel species of the genus *Tetragenococcus*.

Biochemical characterization

Using the Biolog system, the carbon source utilization (SU) pattern of the 15 selected tetragenococci, including nine osmophilic (T1–T7, T9, T10) and six halophilic strains (T11–T15, T19), was analysed. Following the standard protocol for Gram-positive cocci, the five halophilic *T. halophilus* strains (T11^T, T13–T15, T19) were found to metabolize several substrates, generally corresponding to the findings of Röling & van Verseveld (1996). In particular, all halophilic isolates were able to utilize

Table 2. Levels of DNA–DNA reassociation between several *Tetragenococcus* strains

The type strains *T. halophilus* LMG 11490^T and *T. muriaticus* LMG 18498^T represent the strains T11^T and T12^T, respectively. The numbers in parentheses are the variation between two hybridizations. Technical data with high variations are presented in italics.

Strain	Percentage relatedness to labelled DNA from						
	T1	LMG 18498^T	T2	T5	T6	T52	LMG 11490^T
T1	100						
T. muriaticus LMG 18498^T	57 (13)	100					
T2			100				
T5			77 (11)	100			
T6			88 (14)	85 (24)	100		
T52			79 (23)	*97 (49)*	84 (15)	100	
T. halophilus LMG 11490^T			*80 (28)*	*79 (41)*	*80 (66)*	79 (5)	100

α-D-glucose, D-fructose, D-mannose, *N*-acetylglucosamine, maltose, methyl α-D-glucoside, D-ribose, salicin, methyl pyruvate, pyruvic acid and glycerol. Utilization of D-piscose showed day-to-day variation for isolate T11^T and T14. The isolates were unable to utilize α-cyclodextrin, β-cyclodextrin, glycogen, inulin, mannan, L-fucose, D-galacturonic acid, D-gluconic acid, *myo*-inositol, melezitose, methyl β-D-galactoside, 3-methylglucose, L-rhamnose, sedoheptulosan, xylitol, D-xylose and L-malic acid, as well as the amino acids and other organic acids in the Biolog plates. Substrates not mentioned were differentially utilized, giving rise to four distinct SU patterns for the five tested isolates (Table 3). Strains T11^T and T14 gave identical SU patterns. Although these results generally correspond well with the findings of Röling & van Verseveld (1996), a few differences were observed between the two studies. One example is the positive reaction for all our strains for D-glucose, D-fructose and D-mannose while Röling & van Verseveld (1996) obtained sometimes surprisingly negative results for these carbon sources. In addition, D-piscose was used by T11^T and T14, while previously all *T. halophilus* strains were reported not to use this carbon source. On the other hand, the carbon sources arbutin, cellobiose, D-galactose, gentobiose and D-mannitol were not metabolized by any of our strains, in contrast to the observations of Röling & van Verseveld (1996). In addition, both the growth medium and the incubation conditions used by Röling & van Verseveld (1996) differed from the protocol we used, and this could also explain the differences. Nevertheless, when we used the protocol of these authors, using TSB + 5 % NaCl instead of the advised Biolog Universal Growth (BUG) plates with blood, far fewer positive reactions were obtained. In particular, all weak reactions obtained with our protocol were negative when TSB + 5 % NaCl was used as growth medium.

Table 3. Differential substrate utilization patterns by *T. halophila* isolates

+, Positive reaction; −, negative reaction; w, weak or variable reaction.

Substrate	T11 and T14	T13	T15	T19
Arbutin	+	+	−	−
Cellobiose	+	+	−	−
D-Galactose	+	+	−	−
D-Mannitol	−	+	+	−
D-Piscose	w	−	−	−
Trehalose	+	+	−	−
Gentobiose	+	+	−	−
Maltotriose	+	−	−	−
N-Acetyl-D-mannosamine	+	+	−	w

In contrast to the halophilic *T. halophilus* strains, T12^T, the type strain of *T. muriaticus*, was only able to use a single carbon source, namely glycogen. The osmophilic isolate T1 metabolized a limited number of carbon sources: sucrose, D-fructose, α-D-glucose, *N*-acetyl-D-glucosamine, D-mannose and trehalose. The other eight osmophilic isolates produced a negative test result for all carbon sources. Furthermore, an additional French isolate, T30, and German isolate, T20, were analysed and gave identical results. Generally, the use of a carbon source in the Biolog system is indicated by 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-positives, we tested the sensitivity of the tetragenococci for TV by streaking them on TSA plates containing 0.01 % TV and incubating for 8 days at 30 °C. All halophilic strains showed inhibited growth (growth only in densely inoculated zones; no single colonies), but still reduced TV. Most osmophilic strains, however, showed an even more severe inhibition of their growth, and stayed nearly white, illustrating a high sensitivity of these bacteria to TV. Remarkably, osmophilic strains T2 and T6 were not inhibited, but did not reduce TV either.

Physiological characterization: salt and sucrose tolerance

To examine physiological differences between the different *Tetragenococcus* strains isolated from both salt- and sucrose-rich environments, tolerance to these specific osmolytes at different pH values was tested for a subset of both halophilic ($T11^T$, T15 and T18) and osmophilic strains (T1, T5 and T9) (data not shown). All strains were able to grow in TSB containing up to 66 % sucrose, at both pH 7.0 and pH 9.0, except T18, which was already inhibited at 25 % sucrose. In addition, all strains could grow in TSB with up to 25 % NaCl at pH 7.0 and 9.0, although growth was delayed by NaCl in a concentration-dependent manner (data not shown). The sensitivity to increasing concentrations of NaCl was greater at pH 7.0 than at pH 9.0, and this led to a remarkable shift in the optimum pH for growth at the highest NaCl concentrations (25 % and 28.5 %). At 25 % NaCl, growth was observed at pH 7.0 and 9.0 after respectively 12 and 8 days. At the saturation level (28.5 % NaCl), growth only occurred at pH 9.0. In TSB without extra NaCl or with NaCl concentration up to 19 %, the *Tetragenococcus* strains grew faster at pH 7.0, in agreement with the reported optimum pH for growth of these bacteria (Röling & van Verseveld, 1997).

To further investigate the physiological differences between all studied strains, the osmophilic and halophilic strains were grown to saturation in TSB and inoculated in sterilized thick juice of pH 9.3 and 69 °Bx (Table 4). After 7 days, all strains showed a marked reduction in plate count from the initial inoculum of 10^6 c.f.u. ml^{-1} to less than 6×10^3 c.f.u. ml^{-1}. Apparently, an adaptation period was necessary for the cells before they started growing in the thick juice. For strain T18, the count even dropped below the detection limit of 10 c.f.u. ml^{-1}. After 21 days, the counts of all the osmophilic strains, except T2 and T10, had slightly increased. After 47 days, concentrations of higher than 10^5 c.f.u. ml^{-1} were reached, except for strain T10, which reached 1.2×10^4. In contrast, the counts of all the halophilic isolates continued to decline over the entire duration of the experiment, although this did not occur at the same rate for all strains. The counts after 47 days had dropped below the detection threshold for type strain $T11^T$ and strains T18 and T19, and did not exceed 10^3 c.f.u. ml^{-1} for all the other halophilic strains (Table 4).

Strains $T11^T$ and T15 were chosen to further characterize the growth of the halophiles in thick juice with different solids contents and pH values. As shown in Table 5, the counts of both strains declined over the entire period of 62 days when the thick juice had a solids content of 65 °Bx or above. The decline was stronger at pH 7.0 than at pH 9.0, but did generally not significantly differ between the strains or between thick juice of 65 °Bx, 67 °Bx or 69 °Bx, except at 69 °Bx with pH 9. At 60 °Bx, in contrast, both strains were able to grow, reaching a count of $>10^5$ c.f.u. ml^{-1} at the end of the experiment. However, at pH 7.0, this growth phase was preceded by an initial decline at day 7, after which growth resumed, whereas at pH 9.0, both strains reached $>10^5$ c.f.u. ml^{-1} already at day 7.

Table 4. Growth and survival of halophilic and osmophilic *T. halophilus* strains after inoculation at 10^6 c.f.u. ml^{-1} in thick juice of 69 °Bx and pH 9.3

Strain	Counts for *T. halophilus* (c.f.u. ml^{-1})* after storage at 30 °C for		
	7 days	21 days	47 days
T1	2.6×10^3	1.4×10^4	$>10^5$
T2	2.0×10^3	3.3×10^2	$>10^5$
T3	4.3×10^3	7.8×10^3	$>10^5$
T4	3.0×10^3	1.2×10^4	$>10^5$
T5	5.6×10^3	1.1×10^3	$>10^5$
T6	2.0×10^3	8.0×10^3	$>10^5$
T7	1.3×10^3	9.0×10^3	$>10^5$
T9	1.7×10^3	1.2×10^3	$>10^5$
T10	2.0×10^3	1.0×10	1.2×10^4
T11	1.0×10^3	<10	<10
T13	3.0×10^3	<10	1.0×10
T14	3.9×10^3	2.5×10^3	1.0×10^3
T15	2.2×10^3	5.8×10^3	1.8×10^2
T18	<10	<10	<10
T19	7.7×10^2	2.4×10^2	<10

*On TSA.

DISCUSSION

In this study *T. halophilus* strains from high-salt and high-sugar environments, and in particular from degraded and non-degraded thick juice, were compared in detail. Both physiological and genetic characterization using RAPD technology, REP-PCR, Biolog and growth tolerance revealed clear differences between *T. halophilus* strains isolated from salt- and sugar-rich environments. However, no strain pattern could be specifically and systematically associated with degraded or non-degraded thick juice.

RAPD fingerprinting of 25 thick juice isolates revealed similar patterns for all isolates from the same refinery, regardless of the year of isolation, the condition of the thick juice (degraded or not and hop-treated or not), indicating the existence of a stable in-house flora in each location. This in-house flora was not always producer-specific. All three refineries from France, for example, contained isolates with identical RAPD patterns for 2 years in a row. Röling & van Verseveld (1996), however, observed very different RAPD patterns for *T. halophilus* strains isolated from different soy sauce manufacturers. As for our results, these patterns were consistent over different years. Also for other microflora, clustering of isolates according to their geographical origin, indicating independent

Table 5. Growth and survival of the halophilic strains T11^T and T15 after inoculation at 10^6 c.f.u. ml^{-1} in standard thick juice of varying Brix index and pH

pH	Strain	Brix (°Bx)	Counts for *T. halophilus* (c.f.u. ml^{-1})* after storage at 30 °C for			
			7 days	13 days	24 days	62 days
7	T11	60	<10	7.0×10	2.0×10	$>10^5$
	T15		2.0×10^3	4.0×10^3	$>10^5$	$>10^5$
7	T11	65	<10	1.0×10	<10	<10
	T15		<10	8.0×10	<10	<10
7	T11	67	<10	<10	<10	<10
	T15		<10	5.0×10	3.0×10	<10
7	T11	69	<10	<10	<10	<10
	T15		<10	1.0×10	<10	<10
9	T11	60	$>10^5$	$>10^5$	$>10^5$	$>10^5$
	T15		$>10^5$	$>10^5$	$>10^5$	$>10^5$
9	T11	65	2.0×10^3	2.0×10^3	1.0×10^3	2.0×10
	T15		2.0×10^3	2.0×10^3	2.0×10^3	1.0×10^2
9	T11	67	2.0×10^3	1.5×10^3	7.0×10^2	7.0×10^2
	T15		2.0×10^3	2.0×10^3	2.0×10^3	5.0×10^3
9	T11	69	<10	<10	<10	<10
	T15		2.0×10^3	4.0×10^3	3.0×10^3	1.0×10^2

*On TSA.

evolutionary origins for the different clusters, has been frequently reported (Dyble *et al.*, 2002; Wong *et al.*, 2004).

Based on the RAPD results obtained, nine osmophilic strains were selected for further characterization. All genetic analyses, including RAPD fingerprinting, 16S rDNA phylogenetic analysis and REP-PCR, showed a different clustering for the halophilic and osmophilic *T. halophilus* strains. DNA–DNA hybridization, however, revealed more than 70 % similarity between the two groups of strains, indicating that all strains examined should be considered as members of the same species (Wayne *et al.*, 1987). However, the observation of clear biochemical, physiological and genetic differences between strains from a salt- and sucrose-rich environment might defend the proposal of creating two subspecies of *T. halophilus*.

In an attempt to characterize the selected strains biochemically using the Biolog carbon source profiling plates, we found that most osmophilic strains were severely inhibited by tetrazolium violet (TV) in the plates. However, two strains, including T2 and T6, were able to tolerate colourless TV, but did not reduce it to the purple formazan, explaining why no fingerprint was generated. Nevertheless, the clear difference in behaviour of the osmophilic versus the halophilic strains indicated a consistent physiological difference between the two groups.

In addition, the observation that the halophilic strains were not able to grow in sugar thick juice with $\geqslant$65 °Bx confirmed some subtle physiological differences between the osmophilic and halophilic *T. halophilus* strains (Tables 4 and 5). It has not been examined whether the originally inoculated bacteria had died or were in a viable, but non-culturable state. The differences between these strain groups could in theory be explained by the fact that stress imposed by ions (e.g. salt-rich conditions) and organic solutes (e.g. sucrose-rich conditions) is not necessarily the same (Grant, 2004; Kushner, 1978). However, tolerance to high concentrations of sucrose and NaCl in TSB was similar for all osmophilic and halophilic strains tested (data not shown). Another hypothesis is that osmophilic and halophilic strains require different nutritional compounds. It is possible that a growth factor crucial for halophiles is not present in thick juice, while TSB contains a broad range of growth factors enabling all *T. halophilus* strains to grow. An example of such a growth factor in the extreme environment of thick juice could be an osmolyte, and more in particular glycine betaine, since this osmolyte is accumulated in sugar beets under osmotic stress (McCue & Hanson, 1992). On the other hand, Robert *et al.* (2000) demonstrated that osmotic tolerance of halophilic *T. halophilus* strains was enhanced by the uptake of glycine betaine, making this hypothesis implausible. In addition, bacterial adaptation to environmental conditions has the potential to alter the genome in such a way that the organism becomes more resistant to further stress (Johnson & Ogrydziak, 1984; Pérez *et al.*, 2006), which may explain the different behaviour between osmophilic and halophilic strains.

Remarkably, while the optimum pH for *T. halophilus* growth was 7.0, as determined on GYP agar with 10 % NaCl and incubation at 30 °C (Röling & van Verseveld, 1997), at extremely low a_w values pH 9.0 was preferred, regardless ofwhether osmotic stress was created by sucrose or NaCl (data not shown). A similar observation was made

for the cyanobacterium *Aphanothece halophytica* (Laloknam *et al.*, 2006), for which it was found that a betaine transporter specifically catalyses the uptake of the osmoregulator betaine and that uptake activities are high at alkaline pH. In general, H^+ uptake by the Na^+/H^+ antiporter is important to keep the cytoplasmic pH neutral, and the Na^+/H^+ antiporter could extrude Na^+ from the cell. To maintain homeostasis at alkaline pH, a re-entry route for Na^+ is required (Padan *et al.*, 2005). The Na^+/betaine symporter was suggested to be a re-entry route (Laloknam *et al.*, 2006), explaining the unusual enhanced growth at pH 9.0 at high salinity (Padan *et al.*, 2005). Diluting thick juice with sterilized water enables the halophilic strains to grow (better) in thick juice, supporting the hypothesis that an osmolyte enabling the halophilic strains to grow in thick juice is missing. A Brix index of 60 °Bx (or a_w 0.89) enabled halophilic *T. halophilus* strains to grow (Table 5).

In all analyses performed, T1, which was originally isolated from degraded thick juice from a Belgian factory, showed a different behaviour compared to the other *Tetragenococcus* strains. Based on 16S rDNA sequence analysis this strain was closely related (98 %) to the *T. muriaticus* type strain ($T12^T$). However, since the DNA–DNA hybridization level between the two strains was rather low (Table 2), strain T1 is likely to represent a new *Tetragenococcus* species. Description and designation of a species name will be presented elsewhere.

Despite the diversity found among *T. halophilus* strains in this study, no strain could be associated with degraded or non-degraded thick juice. As a consequence, different physiological parameters of the thick juice itself might be responsible for the variable behaviour of *T. halophilus* (Justé *et al.*, 2008b), creating more favourable or unfavourable conditions for thick juice degradation. Moreover, poor storage conditions can occur locally in a storage tank, including a low Brix index or a high temperature (Justé *et al.*, 2008a), or perhaps a critical oxygen concentration (Kanbe & Uchida, 1982) enabling exponential growth of *T. halophilus* and thick juice degradation. Further research on storage of differently conditioned thick juice inoculated with the same strain of *T. halophilus* is necessary to elucidate the different behaviour of dominant populations of *T. halophilus* in sugar thick juice. A fuller characterization of the conditions with and without degradation might facilitate more efficient process control and possibly prevention of thick juice degradation.

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