Chapter 16. Genus *Tetragenococcus*

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Abstract

In 1990 the genus *Tetragenococcus* was created after reclassification of the halophilic lactic acid bacterium (LAB) *Pediococcus halophilus* as *T. halophilus*. Tetragenococci are typical LAB in that they are Gram-positive, catalase negative, and oxidase negative. Physiologically, tetragenococci are distinguished from other LAB mainly by their high salt tolerance and ability to grow at high pH values. Presently, the genus comprises a limited number of species, including *T. halophilus*, *T. koreensis, T. muriaticus, T. osmosphilus* and *T. solitarius*. Based on both physiological and genetic characteristics as well as on the origin of the strains, the species *T. halophilus* was further subdivided into two subspecies, including *T. halophilus* subsp. *halophilus* and *T. halophilus* subsp. *flandriensis* for strains isolated from salt-rich and sugar-rich environments, respectively. In this chapter, both phenotypical and genotypical characteristics of the genus are outlined, with a detailed description of each species comprising the genus. In addition, emphasis is put on the industrial relevance of the genus.

Keywords: Halotolerance; tolerance to high pH; osmotolerance;; sugar thick juice; salt-rich fermented foods .

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16.1 Introduction

The genus *Tetragenococcus* was created by Collins *et al.* (1990) after reclassification of the halophilic lactic acid bacterium (LAB) *Pediococcus halophilus* as *T. halophilus*. Tetragenococci are typical LAB in that they are Gram-positive, catalase negative, and oxidase negative. The genus *Tetragenococcus* is characterized by its typical cell morphology: non-motile, spherical cells (0.5–0.8 µm), which divide in two planes at right angles to form tetrads (Fig. 1). The cells may also occur separately or in pairs, and even clusters of cells can be observed, especially during early or mid-logarithmic growth (Holzapfel *et al.*, 2006). This cell morphology is also typical for pediococci. Nevertheless, physiologically tetragenococci are distinguished from pediococci (and other LAB) mainly by their high salt tolerance (growth at > 18 % NaCl [w/v]) and ability to grow at high pH values, i.e. up to pH 9.0, but not at pH 5.0 (Holzapfel *et al.*, 2006). Presently, the genus comprises a limited number of species, including *T. halophilus* (Collins *et al.*, *1990*), *T. muriaticus* (Satomi *et al*., 1997), *T. solitarius* (Ennahar & Cai, 2005) and *T. koreensis* (Lee *et al.*, 2005). Additionally, a fifth species called *T. osmosphilus* has been described recently by Justé *et al.* (2012). Table 1 summarizes the phenotypic characteristics of all *Tetragenococcus* species which are further individually outlined in this chapter.

The species *T. halophilus*, *T. muriaticus* and *T. koreensis* are typically isolated from salt-rich environments such as fermented foods (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). On the other hand, *T. solitarius* has been isolated from human ear secretions (Ennahar & Cai, 2005) and *T. osmophilus* has been found in concentrated sugar thick juice, an intermediate in the production of beet sugar (Justé *et al.*, 2012). *T. halophilus* and *T. muriaticus* have also been isolated from this sugar-rich environment (Willems *et al.*, 2003; Justé *et al.*, 2008a, e) in which *T. halophilus* strains were presented as the most

probable cause of thick juice degradation (Justé *et al.*, 2008b). Thick juice degradation results in sugar loss characterized by a reduction in pH from 9 to 5 or 6 and an increase in reducing sugar content (Sargent *et al.*, 1997; Willems *et al.*, 2003). Consequently, the species *T. halophilus* includes strains isolated from both salt rich and sugar rich environments and are further referred to as "halophilic" and "osmophilic" strains, respectively. For these strains, the new subspecies *T. halophilus* subsp. *halophilus* and *T. halophilus* subsp. *flandriensis* have been described, respectively (Justé *et al.*, 2012). Whereas *Tetragenococcus* species are generally associated with beneficial properties such as food preservation (Kobayashi *et al.*, 2004) or generating specific (sour) flavours (Orji *et al.*, 2003; Uchida *et al.*, 2005; Chen *et al.*, 2006), the osmophilic strains are so far only associated with sugar thick juice degradation (Justé *et al*., 2008b). *T. muriaticus* is reported as a possible cause for food-poisoning associated with fish-derived products because of its ability to produce histamine (Kimura *et al.*, 2001).

In this chapter, the phenotypic and genetic diversity within the *Tetragenoccus* genus will be reviewed, including a detailed description of all known *Tetragenococcus* species *anno* 2010. Further, industrial applications of *Tetragenococcus* species are also highlighted.

16.2 Phenotypic characteristics of the genus *Tetragenococcus*

16.2.1 Isolation and enumeration

Tetragenococci grow under different atmospheric conditions ranging from aerobic to microaerobic and anaerobic conditions. Most tetragenococci cannot grow in standard synthetic media for LAB such as MRS (De Man, Rogosa & Sharpe) agar (Table 1), but rather require glycine betaine and carnitine as specific growth factors (Robert *et al.*, 2000), or the addition of NaCl. All described *Tetragenococus* species grow on Tryptic soy agar (TSA) at 30

°C (Table 1). When hematin is added to the medium of aerobically grown cultures, both the lag phase and generation time are reduced, and an increased growth yield can be observed (Gürtler *et al.*, 1998). Furthermore, the addition of 5 % NaCl enhances growth as well (Justé *et al.*, 2012). Isolation and enumeration of *Tetragenococcus* strains can therefore be performed by incubating at 30 °C on TSA supplemented with 5 % NaCl, with or without blood.

All *Tetragenococcus* species can grow up to >18 % NaCl, but at concentrations of >15 % NaCl, *Tetragenococcus* spp. grow substantially slower. Consequently, *Tetragenococcus* strains are considered slightly halophilic and highly halotolerant, thus enabling selective cultivation by the use of relatively high salt concentrations. Other than *Tetragenococcus*, only a few LAB taxa have been described to date as characteristically halophilic or highly halotolerant. These include *Halolactibacillus halophilus*, *Halolactibacillus miurensis* and *Marinilactibacillus psychrotolerans*, all isolated from marine organisms (Ishikawa *et al.*, 2003; 2005), *Alkalibacterium olivapovliticus* isolated from alkaline edible-olive wash-water (Ntougias & Russell, 2001) and *Weissella halotolerans*, isolated from meat products (Kandler *et al.*, 1983).

16.2.2 Mixed acid fermentation

In general, tetragenococci have been reported as homofermentative LAB. However, a heterofermentative or mixed acid metabolism was recently suggested for *T. osmophilus*, which produces both lactate and acetate during growth in Tryptic soy broth (TSB) (Justé *et al.*, 2012). In addition, the type strains for the other *Tetragenococcus* species were found to produce acetate as well besides a smaller amount of lactic acid (Justé *et al.*, 2012). These findings corroborate the results from both Röling & van Verseveld (1997) and Gürtler *et al.* (1998) who reported a mixed acid fermentation for *T. halophilus*. Therefore, one can assume

that all *Tetragenococcus* species are characterised by mixed fermentation. Gürtler *et al.* (1998) reported that the composition of the metabolic end products was strongly affected by growth conditions. More specifically, anaerobically grown cultures produced lactate, acetate and formate, in contrast to aerobically grown cultures that mainly produced acetate. Furthermore, the fermentation pathway was affected by pH, generating lactate and acetate at extreme pH values of 5.5 and 8.8, while at pH 6.5 and 7.5 formate was formed as well.

16.2.3 Optimal pH

In contrast to most other LAB, the optimal pH for growth of tetragenococci is between 7 and 9 (Table 1), while on Glucose Yeast-extract Peptone (GYP) agar with 10 % NaCl, the optimal pH for *T. halophilus* is 7.0 (Röling & van Verseveld, 1997). However, a low water activity (a_w) and a pH of 9.0 is preferred, regardless of whether the osmotic stress is created by high sucrose concentrations (49 %; a_w = 0.95; 1.97 M) or by high NaCl concentrations (23 %; a_w = 0.83; 3.89 M) (Justé *et al.*, 2008c). Moreover, at 3.89 M NaCl, *Tetragenococcus* strains did not grow at pH 7.0 but did slowly grow at a pH of 9.0. A similar observation was noted for the halotolerant cyanobacterium *Aphanothece halophytica* in which a betaine transporter specifically catalyzes the uptake of the osmoprotectant betaine optimally in an alkaline pH range (Laloknam *et al.*, 2006). In general, H^+ uptake is critical to keeping the cytoplasmic pH neutral. This function can be fulfilled by the Na^+/ H^+ antiporter by which cytoplasmic sodium is removed by the exchange of Na^+ and H^+. To maintain the homeostasis at an alkaline pH level, a reentry route for Na^+ is required (Padan *et al.*, 2005). The Na^+-betaine symporter was suggested to be a reentry route (Laloknam *et al.*, 2006), explaining the unusual enhanced growth at pH 9.0 at high salinity (Padan *et al.*, 2005).

16.2.4 Osmoprotection

Organisms have developed mechanisms to withstand osmotic stress induced by hyperosmolar conditions such as high salinity, high sugar contents, drought or other extreme conditions. These mechanisms work by accumulating and/or synthesizing metabolites, termed 'osmolytes', 'compatible solutes' or 'osmoprotectants' which help in raising the osmotic pressure and thereby maintaining both the turgor pressure and the cell volume. In addition, these metabolites help in maintaining the integrity of enzymes, membranes and other cellular components during stress (Roberts, 2005). There are only a limited number of compounds used by LAB as osmoprotectants, including free amino acids (e.g. proline and glutamate), quaternary amines and their sulfonium analogues (e.g. glycine betaine, carnitine, dimethylsulfonioacetate, dimethylsulfoniopropionate (Baliarda *et al*., 2003). As with most eubacteria subjected to salt stress, glycine betaine is the major effective osmoprotectant used by LAB (Baliarda *et al.*, 2003). Interestingly, osmoprotectants differ according to the genus studied and *T. halophilus* was found to exhibit a larger diversity of osmoprotectants than those of non- or less tolerant LAB. In addition, Balardia *et al.* (2003) suggested that within the group of LAB, restoration of growth by adding the osmoprotectant ectoine under osmotic constraint appears to be specific to the genus *Tetragenococcus.* In most cells where glycine betaine is accumulated, the betaine is actively transported from a complex environment. However, *T. halophilus* is the only LAB reported having a choline-glycine pathway, allowing to the accumulation of glycine betaine through the conversion of its precursor choline (Robert *et al.*, 2000).

Nevertheless, the osmoregulatory machineries used to cope with osmotic stress, either imposed by ions or organic solutes, do not necessarily result in the same outcome. Many xerophilic micro-organisms isolated from foods that are high in sugar are also tolerant to low a_w levels imposed by ions (Grant, 2004). However, the converse is not generally true. For example, micro-organisms isolated from saturated soda lakes (a_w ~ 0.75) as a rule, cannot

grow in media of similar a_w levels imposed by organic solutes (Kushner, 1978). Likewise, this phenomenon is also observed for *Tetragenococcus*, where all *T. halophilus* strains isolated from sugar thick juice (aw ~ 0.82) tolerate extremely high salt concentrations (23 % NaCl). On the contrary, *T. halophilus* strains isolated from salt-rich environments are not capable of growth in sugar thick juice, suggesting that different adaptive mechanisms are used to cope with differentially induced osmotic stress (Justé *et al*., 2008c).

16.2.5 Physiological diversity

16.2.5.1 Physiological diversity among Tetragenococcus species

Tetragenococcus species differ in their growth temperature range on different media and in their carbon metabolism (Table 1). Among all *Tetragenococcus* species, *T. solitarius* is the only species that is able to grow at 40 °C on TSA. In NaCl-GYP broth, also *T. muriaticus* was found to grow up to 40 °C (Satomi *et al*., 1997). Whereas all other tetragenococci are able to grow on TSA at 37 °C, *T. osmophilus* is not able to grow at this temperature. Unlike many LAB, most tetragenococci cannot grow on the standard LAB synthetic medium MRS, but require the addition of specific growth factors (Robert *et al.*, 2000). Only *T. koreensis* and *T. solitarius* are able to grow on MRS (Justé *et al*., 2012; Table 1). Furthermore, *T. muriaticus* is the only *Tetragenococcus* species that does not grow in the absence of NaCl (Satomi *et al.*, 1997).

Differences in carbon metabolism were observed between the *Tetragenococcus* species using both BIOLOG and API 50CH fingerprinting (Röling & van Verseveld, 1996; Kobayashi *et al.*, 2000; Justé *et al.*, 2008c). D-xylose and D-melezitose for example, are only fermented by *T. koreensis*. Additionally, *T. halophilus* is the only species that ferments D-tagatose. *T. osmophilus* is characterized by several negative reactions, including for example D-ribose and amygdaline (Table 1). Remarkably, *T. muriaticus* fermented only a few of the

carbon sources, including D-ribose, D-manitol, salicin and D-trehalose, depending on the strain (Table 1; Justé *et al.*, 2012), D-mannose and fructose (Satomi *et al.*, 1997), D-glucose and arbutin (Kobayashi *et al.*, 2000). As *T. muriaticus* is the only *Tetragenococcus* species that does not grow without salt (Satomi *et al.*, 1997), this may explain the weak and/or negative reactions of this species (Table 1).

No BIOLOG results were obtained for *T. osmophilus* and the *T. halophilus* isolates from thick juice (Justé *et al*., 2008c). By contrast, *T. halophilus* strains isolated from salt environments do produce positive reactions in the BIOLOG GP2 plates (Justé *et al*., 2008c). Generally, the use of a carbon source in the BIOLOG system is indicated by the reduction of the colourless tetrazolium violet (TV) to the purple formazan (Bochner, 1989). It was found that isolates from sugar-rich media were inhibited by TV or cannot reduce it to formazan, explaining the negative BIOLOG results (Justé *et al.*, 2008c).

Tetragenococcus muriaticus is the only species reported as a histamine-forming bacterium in salted and fermented fish products (Kobayashi *et al.*, 2004). After its description as a novel species of histamine-forming halophilic LAB (Satomi *et al.*, 1997), its ability to form histamine has been confirmed at low acidity (pH 5.8), under O_2-limiting conditions, optimal NaCl concentration (5–7 %) and glucose >1 % (Kimura *et al.*, 2001). The histidine decarboxylase, catalyzing the decarboxylation of the amino acid histidine to form histamine, was purified and sequenced by Konagaya *et al.* (2002), and appeared highly similar to other Gram-positive bacterial histidine decarboxylases.

16.2.5.2 Physiological diversity among strains of the same species

Within the species *T. halophilus*, physiological differences were observed between strains isolated from salt-rich environments and strains isolated from sugar-rich environments (Justé *et al.*, 2008c). Based on API 50CH characterization, all osmophilic *T. halophilus* isolates

were able to ferment D-lactose, D-raffinose and D-arabinose and were negative for glycerol, in contrast to the halophilic *T. halophilus* strains which were all negative for D-lactose, D-raffinose, and D-arabinose and all but one positive for glycerol (Table 1; Kobayashi *et al.*, 2000). As previously discussed, no fingerprint was obtained for the osmophilic strains using the BIOLOG GP2 plates, whereas a clear fingerprint was generated for the halophilic strains (Justé *et al.*, 2008c). Among these osmophilic *T. halophilus* strains, a differential response to TV was observed among the isolates tested, as two out of eight isolates were less inhibited by 0.01 % TV and did grow. However, these isolates were not able to reduce the TV to formazan (Justé *et al.*, 2008c).

Remarkably, halophilic *T. halophilus* isolates seriously vary in their carbon utilization pattern (Uchida, 1982; Röling & van Verseveld, 1996; Justé *et al.*, 2008c). Carbon sources that were fermented by only some halophilic isolates include D-mannitol, D-sorbitol, methyl α-D-glucoside, D-melibiose, D-tagatose, D-galactose, D-arabitol (Table 1) and L-arabinose and glycerol (Kobayashi *et al.*, 2000). Intraspecific variation of halophilic *T. halophilus* isolates has been reported for other characteristics as well. For example, a study of Gürtler *et al.* (1998) revealed differences in heme-dependent catalase activity, which was positive for 12 out of 21 strains, including the type strain.

Another physiological difference between halophilic and osmophilic *T. halophilus* strains is tolerance to high-sugar contents. While osmophilic strains can flourish in thick juice of 69 °Bx (% dry matter, mainly sucrose) to concentrations up to 10^6-10^8 cfu ml^{-1}, *T. halophilus* strains isolated from salt environments do not grow in this extreme sugar-rich matrix (Justé *et al.*, 2008c). Nevertheless, further investigation on the behaviour of these strains in sterilised thick juice reveals a gradual adaptation of some strains to high sugar contents, suggesting that certain strains have developed (a) specific mechanism(s) to resist the high osmotic pressure of thick juice (Justé *et al.*, unpublished results).

With regard to intraspecific physiological variation in the other species, only limited information is available, slight differences were found in carbon utilization patterns between strains of *T. muriaticus* and *T. osmophilus* (Table 1). *T. koreensis* and *T. solitarius* comprise only one isolate so far.

16.3 Genotypical characteristics of the genus *Tetragenococcus*

16.3.1 Genetic diversity among Tetragenococcus species

The evolutionary relationships among bacterial species are generally determined by comparing the sequences of 16S rRNA genes, mainly because of their ubiquity and relatively high resolution power (Olsen & Woese, 1993; Stackebrandt *et al.*, 1997). In addition, other housekeeping genes are increasingly being used to extend knowledge of bacterial phylogenies, including the gyrase gene, the RNA polymerase gene, the elongation factor Tu genes, the ATPase β-subunit gene, and the chaperonin Cpn60 gene (Ludwig *et al.*, 1993; Hill *et al.*, 2004; Küpfer *et al.*, 2006; Nicolas *et al.*, 2008), leading to the concept of 'multilocus sequence analysis' in which a combination of several loci is used to reconstruct microbial phylogenies (Rediers *et al.*, 2004; Nicolas *et al.*, 2008). For *Tetragenococcus*, so far only a limited amount of sequence data is publicly available, of which most data represent 16S rRNA gene sequences (Table 2). A phylogenetic tree based on 16S rRNA gene sequences (1349 nucleotides) from all *Tetragenococcus* species as well as some related species revealed a separate cluster for *Tetragenococcus* (Fig. 2). The greatest relatedness was found with *Melisococcus*. Remarkably, the genus *Pediococcus*, which shares physiological properties with *Tetragenococcus* and which was previously considered to be phylogenetically intermixed with *Tetragenococcus* (Stackebrandt & Teuber, 1988), has a relatively long phylogenetic distance to *Tetragenococcus* (Fig. 2; Collins *et al.*, 1990). Within the

290 *Tetragenococcus* cluster, *T. osmophilus* and *T. muriaticus* form a distinct group, separated
291 from the other species. In the latter group of three species, halophilic and osmophilic *T.*
292 *halophilus* isolates differ in three nucleotides, separating them in different subclusters (Justé
293 *et al.*, 2008c). The phylogenetic relationships of *Tetragenococcus* species have been further
294 investigated by DNA-DNA hybridization (Justé *et al.*, 2008c; Table 3), which generally
295 confirmed the 16S rDNA clustering. Currently, no complete genome sequence is yet available
296 for any *Tetragenococcus* isolate (2010-10). However, the PME&BIM research group is in the
297 process of obtaining the whole genome sequences for the type strains of both the halophilic
298 and osmophilic *T. halophilus* subgroups. Such information could reveal specific gene
299 differences between both groups of strains. In addition, it may lead to the discovery of yet
300 unknown genes, leading to new insights into *Tetragenococcus* speciation and potentially new
301 or additional industrial applications of *Tetragenococcus* strains.

302 ***16.3.2 Genetic diversity among strains of the same species***

303 Intraspecific genetic diversity has been extensively studied for the species *T. halophilus* (Justé
304 *et al.*, 2008c). Random amplified polymorphic DNA (RAPD) analysis fingerprinting revealed
305 genetic differences between osmophilic isolates from seven different sugar refineries (Justé *et*
306 *al*., 2008c). Isolates from the same sugar refinery were all characterized by identical
307 fingerprints, regardless of the year of isolation or the condition of the thick juice (degraded or
308 not), suggesting the occurrence of a stable "in house" flora (Justé *et al.*, 2008c; Fig. 3).
309 Additional analysis of 14 osmophilic isolates from four other refineries situated in three
310 countries, confirmed this observation (Justé *et al.*, unpublished results). Nevertheless, this "in
311 house" microbiota is not always refinery-specific. Three French refineries, for example, all
312 contained *Tetragenoccus* isolates with identical RAPD patterns, and this for the two
313 successive years that were sampled. Although the three refineries were all situated in the same

part of France, within 100 km of each other, these observations illustrate a wider distribution of this genotype. On the other hand, a slightly different genotype was detected for the tetragenococci from two Belgian thick juice tanks that were only 50 km apart. Regarding halophilic *T. halophilus* isolates, Röling & van Verseveld (1996) reported diverse RAPD patterns for *T. halophilus* strains isolated from different soy sauce manufacturers whereas consistent patterns were found for the same plant over different years. Also for other microbiota, clustering of isolates according to their geographical origin, indicating independent evolutionary origins, has been frequently reported (Dyble *et al.*, 2002; Wong *et al.*, 2004, Kolodziejek *et al.*, 2005; Torrea *et al.*, 2006).

16.3.3 Molecular detection and identification of Tetragenococcus species

Tetragenococcus species are fastidious bacteria and can take up to several days to grow on certain media, *e.g.* TSA or Columbia Blood agar (CBA), before subsequent identification and characterization analyses can be performed. In order to enhance identification, generally 16S rDNA sequences are exploited. For example, Justé *et al.* (2008c) used a BLAST analysis of 16S rDNA sequences for putative species identification of the *Tetragenococcus* strains isolated from sugar thick juice. Instead of gene sequencing, restriction fragment length polymorphism (RFLP) can be used as an elegant identification. Kobayashi *et al.* (2000), for example, studied 413 *Tetragenococcus* strains isolated from Japanese puffer fish ovaries fermented with rice-bran. Based on their growth on five representative substrates, the isolates were grouped into seven groups. RFLP analysis of the 16S rRNA gene of representative strains of each group revealed two genetically different groups: *T. halophilus* as the most prominent species, while the other group showed the pattern of *T. muriaticus* (Kobayashi *et al.*, 2000).

Currently, molecular tools are increasingly being developed for direct microbial detection and identification, without the need of cultivation (Justé *et al.*, 2008d). Among these, PCR is the most common method used today. In the sugar industry, sugar thick juice is regularly spoiled or degraded by *T. halophilus*. Consequently, the sugar industry is highly interested in early detection and rapid identification of this contaminant since it allows client-specific advice and reduced economic losses. Based on 16S rDNA sequences, a PCR has been developed to accurately detect and identify *T. halophilus* from diverse environments (Justé *et al.*, 2008a). Nowadays, this PCR assay is routinely used during the sugar beet campaign in Belgium, The Netherlands and Germany as a screening tool to inform and advise the sugar companies on the presence of this bacterial species in their thick juice storage tanks. Beside this species-specific PCR, a DNA array has been developed, which, in addition to *Tetragenococcus* and *T. halophilus* detection, allows for the simultaneous detection and identification of all other major thick juice bacterial contaminants, including *Bacillus* spp., *Staphylococcus* spp., *Aerococcus viridians*, *Kocuria rhizophila* and *Leuconostoc mesenteroides* (Justé *et al.*, 2008e; 2011). Using these methods, thick juice samples have been screened for *T. halophilus*, which has been detected in all sugar factories that store thick juice (17 factories so far tested from 4 different countries). Moreover, the developed DNA array has the potential to expand detection to other or new important microbial species, e.g. *T. osmophilus* (Justé *et al.*, 2011).

16.4 Industrial relevance of the genus *Tetragenococcus*

Tetragenococcus strains are currently used in several industrial applications, especially as a starter culture in fermentation processes (Röling & van Verseveld, 1996; Holzapfel *et al.*, 2006). Due to their halophilic nature, *Tetragenococcus* species are typically associated with food processes with high salt concentrations.

362 The main application of *T. halophilus* is its use as a starter culture in oriental
363 fermented products, such as soy sauce, soybean paste (Hanagata *et al.*, 2003; Röling *et al.*,
364 1995; Röling & van Verseveld, 1996), soy cheese (Shi & Fung, 2000), fish sauce (Kimura *et*
365 *al.*, 2001; Uchida *et al.*, 2005), fermented fish (Kuda *et al.*, 2002), shrimp paste (Kobayashi *et*
366 *al.*, 2003), and fermented mustard (Chen *et al.*, 2006). *Tetragenoccus halophilus* is also used
367 in derivatives of soy sauce such as the flavoring agent miso (fermented soybean mash) or the
368 Chinese sufu (soy cheese) (Hesseltine, 1983; Shi & Fung, 2000). In addition, *T. halophilus* is
369 also found in some 'western' products, e.g. anchovy pickles (Villar *et al.*, 1985), sourdough
370 (Gül *et al.*, 2005) and some fermented sausages. In these production processes, *T. halophilus*
371 is thought to be essential for flavour formation and the prevention of undesired microbial
372 contamination through lowering the pH by means of lactic acid production (Abe & Higushi,
373 1998; Shi & Fung, 2000). Traditionally, fermentation was initiated by inoculation with a
374 previously fermented product. Nowadays, fermentation processes have been optimized and
375 standardized to ensure a consistency in flavour and food safety, and to reduce the
376 fermentation time. To achieve this, fermentation reactors and new processes have been
377 developed, enabling the use of standardized and pure *T. halophilus* starter cultures (Luh,
378 1995). *Tetragenococcus halophilus* is also naturally present in the brewing of the African beer
379 Pito, where it is thought to be essential for its characteristic flavour. To achieve industrial
380 production of Pito, *T. halophilus* was examined for its potential as a fermenting
381 microorganism (Orji *et al.*, 2003).
382 *Tetragenococcus muriaticus* has been isolated mainly from fermented fish-derived products,
383 such as fish sauce, fermented small fish, fermented squid liver, and puffer fish ovaries. In
384 these products, *T. muriaticus* is used as a starter culture to improve flavour and shelf-life of
385 the product (Satomi *et al.* 1997; Kobayashi *et al.* 2000; 2003).

Additionally, *Tetragenococcus* species present potential for several other applications that are still under investigation. For instance, a patent application describes the use of *Tetragenococcus* species on wound patches for prevention and treatment of wound infections (CA2673409A1). A similar patent application deals with the treatment of infections with plasma, which was inoculated with *Tetragenococcus* spp. (US2006/0292162A1). A recent study showed that *T. halophilus* possesses immunomodulatory activity that promotes T helper type I immunity, resulting in anti-allergic effects (Nishimura *et al.*, 2009). Oral administration of *T. halophilus* can also reduce the symptoms of perennial allergic rhinitis. The production of double-stranded RNA is thought to have an essential role in this, a finding that is also patented (CA2694354A1; EP2169057A1).

It is clear from the above that *Tetragenococcus* is currently very important in several industrial fermentation processes, in particular in the production of Oriental flavouring agents with high salt concentrations. Furthermore, numerous other patent applications show that the potential of *Tetragenococcus* for industrial use is still largely unexplored, for example in the production of soy sauce (US 6,054,150), fermentation of milk (US 5,962,046), organic waste fermentation processes (US 5,707,856A), or as probiotics in pet food (e.g. US 2005/0281910A1).

16.5 Description of each species comprising the genus

A detailed description for each described *Tetragenococcus* species is given below. For more details we refer to the original manuscripts (Collins *et al.*, 1990; Satomi *et al.*, 1997; Lee *et al.*, 2005; Ennahar & Cai, 2005; Justé *et al.*, 2012).

16.5.1 Tetragenococcus halophilus Collins, Williams and Wallbanks 1990.

(ha.lo.phi'lus. Gr. n. hals halos, salt; N.L. masc. adj. halophilus –a –um, friend, loving; salt-loving).

Originally described as *Pediococcus halophilus* (Mees, 1934), strains belonging to the species were reclassified in 1990 as *T. halophilus* based on 16S rRNA gene sequence information (Collins *et al.*, 1990). Characteristic is their high salt tolerance (up to 26 % NaCl). *T. halophilus* strains have been isolated from salt-rich and sugar-rich environments. Strains from both environments share DNA-DNA relatedness above 70 % (Table 3), and are therefore considered as belonging to the same species. Nevertheless, based on both genetic and physiological differences Justé *et al.* (2008c) recently proposed to subdivide the species into two subspecies, *T. halophilus* subsp. *halophilus* isolated from salted food and *T. halophilus* subsp. *flandriensis* isolated from environments rich in sugar (Justé *et al.*, 2012).

16.5.1.1 ***Tetragenococcus halophilus* subsp. *halophilus*** Justé, Van Trappen, Verreth, Cleenwerck, De Vos, Michiels, Lievens and Willems 2012, 135[VP]

(ha.lo.phi'lus. Gr. n. hals halos, salt; N.L. masc. adj. halophilus –a –um, friend, loving; salt-loving).

All isolates belonging to this subspecies have been recovered from food products that are rich in salt. Typical habitats are anchovy pickles (Orla-Jensen, 1919), fermented fish (Tanasupawat & Daengsubha, 1983), and soy sauce mashes (Röling & van Verseveld, 1996). Isolates produce a reproducible fingerprint with BIOLOG GP2 plates allowing biochemical characterization. Remarkably, differences have been observed in carbon utilization patterns between different isolates, e.g. for the fermentation of L-arabinose, D-mannitol, D-sorbitol, methyl α-D-glucoside, D-melibiose, D-tagatose, D-galactose, D-arabitol and glycerol. None of the halophilic *T. halophilus* isolates tested so far can ferment D-lactose and D-raffinose (Table 1; Uchida, 1982; Röling & van Verseveld, 1996; Kobayashi *et al*., 2000; Justé *et al.*, 2008c). In contrast to strains isolated from sugar-rich environments, halophilic strains are not able to grow in sugar thick juice with 69 °Bx and do show an onset of growth at the inoculation point when grown on MRS agar at 30 °C.

- *Typical habitat: food products rich in salt*
- *The mol % G+C of the DNA is 36*
- *Type strain: IAM 1676^T, LMG 11490^T, DSM 20339^T*
- *GenBank accession number (16S rRNA gene): EU689052.1*

16.5.1.2 ***Tetragenococcus halophilus* subsp. *flandriensis*** Justé, Van Trappen, Verreth, Cleenwerck, De Vos, Michiels, Lievens and Willems 2012, 135^{VP}

(ha.lo.phi'lus. Gr. n. hals halos, salt; N.L. masc. adj. halophilus –a –um, friend, loving; , salt-loving; flan.dri.en'sis N.L. fem. adj. flandriensis, originating from Flanders, Belgium).

So far, osmophilic *T. halophilus* strains, collectively referred to as *T. halophilus* subsp. *flandriensis*, have only been isolated from sugar thick juice in which they may cause degradation (Justé *et al.*, 2008b). Genetically, they can be discriminated from halophilic *T. halophilus* strains by RAPD fingerprinting (Justé *et al.*, 2008c). Physiologically, they show a different behavior in the fact that they are able to grow in sugar thick juice of 69 °Bx. Moreover, in contrast to halophilic *T. halophilus* strains, the osmophilic strains cannot reduce TV to formazan and therefore do not produce a fingerprint with BIOLOG GP2 plates. Furthermore, they cannot grow on MRS agar. In contrast to *T. halophilus* subsp. *halophilus* isolates, fermentation in API strips is positive for D-arabinose and D-raffinose, but glycerol cannot be used as a carbon source (Table 1). Type strain for this subspecies is T5^T (DSM 23766^T) which was isolated in Belgium from an industrial tank with degraded thick juice.

- *Isolated from sugar thick juice*
- *The mol % G+C of the DNA is 36.7*
- *Type strain:* T5^T, DSM 23766^T

- *GenBank accession number (16S rRNA gene): EU522087*

16.5.2 ***Tetragenococcus muriaticus*** Satomi, Kimura, Mizoi, Sato and Fujii 1997, 835[VP]

Tetragenococcus muriaticus represents the second species that was attributed to the genus (Satomi *et al.*, 1997). As for *T. halophilus* subsp. *halophilus*, *T. muriaticus* has only been isolated from salt-rich fermented food such as squid liver sauce or fermented puffer fish ovaries (Kobayashi *et al.*, 2000). Typically, no growth occurs in the absence of NaCl. *Tetragenococcus muriaticus* prefers NaCl concentrations of 7 to 10 % and tolerates up to 26 % NaCl. Isolates can grow up to 40 °C on 10 % NaCl-GYP agar. Determination of the fermentation pattern with API strips results in many negative reactions (Table 1). This may be explained by its salt requirement for growth. Even at optimal salt concentrations, this species grows slower than the other *Tetragenococcus* species. Similarly to *T. halophilus* subsp. *flandriensis*, (almost) no fingerprint is produced with BIOLOG GP2 plates. No growth is observed in sugar rich thick juice of 69 °Bx. Histamine is produced.

- *Typical habitat: salt-rich fermented food*
- *The mol % G+C of the DNA is 37*
- *Type strain:* JCM 100006[T], LMG18498[T]
- *GenBank accession number (16S rRNA gene): NR_025887*

16.5.3 ***Tetragenococcus solitarius*** Facklam and Collins 1989, 592[VP]

(sol.i.tar.i'us. L. adj. *solitarius* alone, lonely)

Tetragenococcus solitarius comprises so far only a single isolate. The strain was recovered from human ear secretions (Facklam & Collins, 1989). *T. solitarius* represents the most heat-resistant *Tetragenococcus* species described, as it tolerates a temperature of 45 °C. In addition, *T. solitarius* is the only *Tetragenococcus* species which is able to grow on the diverse culture media TSA, MRS agar, and GYP agar. In contrast to most other tetragenocci, the type strain ferments D-arabinose (Table 1), a characteristic which is also shared by *T. halophilus* subsp. *flandriensis* (Table 1).

- *Isolated from human ear secretions*
- *The mol % G+C of the DNA is 38*
- *Type strain: DSM 5634^{T}, LMG 12890^{T}*
- *GenBank accession number (16S rRNA gene): AF061010*

16.5.4 Tetragenococcus koreensis Lee, Kim, Vancanneyt, Swings, Kim, Kang, and Lee 2005, 1412^{VP}

(ko.re.en′sis. N.L. masc. adj. *koreensis* pertaining to Korea, the origin of the sample of the traditional food, kimchi, from which the type strain was isolated)

Similarly to *T. solitarius*, one isolate has been reported for *T. koreensis*. This isolate has been isolated from kimchi, a traditional Korean food (Lee *et al.*, 2005). In contrast to most other *Tetragenococcus* species (Table 1), *T. koreensis* is able to grow on standard MRS agar at 30 °C. The isolate grows optimally at pH 9. *T. koreensis* is characterized by its diverse carbon metabolism. Unlike other tetragenococci, this isolate can also utilize D-xylose and D-melezitose (Table 1). The type strain is (LMG 22864^{T} = DSM 16501^{T}).

- *Isolated from kimchi*
- *The mol % G+C of the DNA is 38.3*
- *Type strain:, DSM 16501^T, LMG 22864^T*
- *GenBank accession number (16S rRNA gene): AY690334*

16.5.5

Tetragenococcus osmophilus Justé, Van Trappen, Verreth, Cleenwerck, De Vos, Michiels, Lievens and Willems 2012, 133^{VP}(os.mo.phi'lus. N.L. masc. adj. osmophilus, from Gr. n. ōsmos, impulse and Gr. n. masc.adj. philos, friend, loving; osmophile, impulse-loving).

Isolated from sugar thick juice, *T. osmophilus* has recently been described as a new *Tetragenococcus* species (Justé *et al.*, 2012). Physiological characteristics of the species include growth in sugar thick juice of 69 °Bx (and below), growth on TSA at 30 °C, but not at 37 °C, and no reaction in GP2 BIOLOG plates. *T. osmophilus* cells are enhanced in growth by the addition of 5 % NaCl. Growth occurs at NaCl concentrations in the range of 0-25 % and at 15-30 °C on TSA but not at temperatures of 4 °C or ≥37 °C. Optimal growth temperature is 30 °C; the optimal pH is 8.0. *T. osmophilus* is facultatively aerobic and shows mixed fermentation. API strips revealed the fermentation of methyl α-D-glucoside and α-D-mannoside, but showed negative results for D-ribose, D-galactose, D-sorbitol, amygdalin and D-tagatose (Table 1).

- *Isolated from Belgian degraded sugar thick juice*
- *The mol % G+C of the DNA is 36.7*
- *Type strain: T1^T, DSM 23765^T*

- *GenBank accession number (16S rRNA gene): EU522083*

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722

Figure Legends

Fig. 16.1 Phase contrast image of *Tetragenococcus halophilus*. Diameter of the individual cells is 0.7–0.8 µm.

Fig. 16.2 Neighbour-joining tree, based on 16S rRNA gene sequences (1349 nt), showing the phylogenetic position of the different *Tetragenococcus* species (type strains). Used sequences are indicated by the respective GenBank Accession numbers. Bootstrap values, presented on the nodes, are expressed as absolute value of 1000 replications. Bar, 0.01 substitution per nucleotide position. A separate cluster was obtained for the genus *Tetragenococcus*. Although *Tetragenococcus* strains were previously classified as *Pediococcus*, the greatest relatedness was found with *Melisococcus*. Within the *Tetragenococcus* cluster, *T. osmophilus* and *T. muriaticus* form a distinct group, separated from the other *Tetragenococcus* species. In the latter group, halophilic and osmophilic *T. halophilus* isolates differ in three nucleotides, separating them in different subclusters.

Fig 16.3 RAPD fingerprint obtained with primer RAP3 for several *Tetragenococcus* isolates, with the corresponding dendrogram derived from UPGMA linkage of Pearson correlation coefficients (adapted from Justé *et al.*, 2008c). Four RAPD groups were defined according to their geographical origin and/ or isolation matrix (I: high salt habitat; II: thick juice from Germany; III: thick juice from France; IV: thick juice from Belgium). T1: *T. osmophilus*; T4, T5, T9, T10, T21, T25, T26, T29, T31, T32, T40, T50 and T58: *T. halophilus* subsp. *flandriensis*; T11, T13, T14, T15 and T16: *T. halophilus* subsp. *halophilus*; T12: *T. muriaticus*; T17: *Bacillus* sp.