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Genomic and metabolic features of *Tetragenococcus halophilus* as revealed by pan-genome and transcriptome analyses

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ABSTRACT

The genomic and metabolic diversity and features of *Tetragenococcus halophilus*, a moderately halophilic lactic acid bacterium, were investigated by pan-genome, transcriptome, and metabolite analyses. Phylogenetic analyses based on the 16S rRNA gene and genome sequences of 15 *T. halophilus* strains revealed their phylogenetic distinctness from other *Tetragenococcus* species. Pan-genome analysis of the *T. halophilus* strains showed that their carbohydrate metabolic capabilities were diverse and strain dependent. Aside from one histidine decarboxylase gene in one strain, no decarboxylase gene associated with biogenic amine production was identified from the genomes. However, *T. halophilus* DSM 20339^T produced tyramine without a biogenic amine-producing decarboxylase gene, suggesting the presence of an unidentified tyramine-producing gene. Our reconstruction of the metabolic pathways of these strains showed that *T. halophilus* harbors a facultative lactic acid fermentation pathway to produce L-lactate, ethanol, acetate, and CO_2 from various carbohydrates. The transcriptomic analysis of strain DSM 20339^T suggested that *T. halophilus* may produce more acetate via the heterolactic pathway (including D-ribose metabolism) at high salt conditions. Although genes associated with the metabolism of glycine betaine, proline, glutamate, glutamine, choline, and citrulline were identified from the *T. halophilus* genomes, the transcriptome and metabolite analyses suggested that glycine betaine was the main compatible solute responding to high salt concentration and that citrulline may play an important role in the coping mechanism against high salinity-induced osmotic stresses. Our results will provide a better understanding of the genome and metabolic features of *T. halophilus*, which has implications for the food fermentation industry.

1. Introduction

Tetragenococcus halophilus (formerly known as *Pediococcus halophilus*) is a gram-positive, non-motile, non-spore-forming, facultative anaerobic, and moderately halophilic lactic acid bacterium with a coccus shape and relatively low G + C content (Justé et al., 2012). As its name implies, the members of this species are halophilic and osmotolerant, capable of growing in environments with up to 25% salt or 66% sucrose concentrations; thus they have been frequently identified from highly salted traditional fermented foods or sugar-thick juices (Guan et al., 2011; Justé et al., 2012; Jung et al., 2016a; 2016b; 2017; Lee et al., 2015; Udomsil et al., 2010). It has been reported that *T. halophilus* may play important roles in the production of organic acids, amino acids, and flavoring compounds during the fermentation of salted foods (Lee et al., 2018; Udomsil et al., 2010, 2017). In addition, some health beneficial effects of *T. halophilus*, such as their immunomodulatory effect (Masuda et al., 2008) and amelioration of atopic diseases (Ohata et al., 2011), have been reported. Therefore, the use of *T. halophilus* as a starter culture for improving the flavoring and taste characteristics, safety, and health-promoting effects of fermented salted foods has been suggested (Kuda et al., 2012; Udomsil et al., 2011, 2017). However, there are also speculations that *T. halophilus* may be a major causative agent of undesirable biogenic amines (e.g., histamine, cadaverine, putrescine, and tyramine) during the fermentation of salted foods and for the quality degradation of sugar-thick juices (Jung et al., 2016b; Justé et al., 2008a; Kobayashi et al., 2016; Satomi et al., 2011, 2014; Sitdhipol et al., 2013).

Most studies on *T. halophilus* strains have focused on their profiling or monitoring during food fermentation and their effects as a starter culture on food fermentation (Amadoro et al., 2015; Kuda et al., 2012, 2014; Udomsil et al., 2011, 2016), whereas little is known about their metabolic and fermentative features. Despite some recent genome

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sequencing studies on *T. halophilus* strains (Nishimura et al., 2017) and investigations on their responses to high salinity through metabolite, proteome, and transcriptome analyses (He et al., 2017a, 2017b; Lin et al., 2017; Liu et al., 2015), the diversity and features of the genomes and metabolites among different strains of this species remain unclear. Such knowledge is necessary for a clearer understanding about the fermentation of salted foods that contain predominantly *T. halophilus*.

A pan-genome analysis can provide profound insights into the genomic and metabolic diversity and features of phylogenetic lineages, given that a pan-genome represents all the possible metabolic and physiological repositories of strains of a particular species (Deng et al., 2010). To be best of our knowledge, the genomic and metabolic features of the *T. halophilus* strains have not yet been comprehensively explored using pan-genome analysis. Therefore, in this study, we conducted such an analysis on all *T. halophilus* strains, using data available on the Clusters of Orthologous Groups (COG), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Basic Local Alignment Search Tool (BLAST) databases. In addition, the metabolic pathways of various carbohydrates and of some intracellular organic compounds (including compatible solutes) in *T. halophilus* strains were reconstructed and their metabolic features were examined through transcriptome and metabolite analyses. Our results should lead to a better understanding of the fermentative features of *T. halophilus*, the strategies used by the strains to cope with high salinity stress, and the contribution of this species to the fermentation of salted foods, such as soybean paste, soy sauce, and sea foods.

2. Materials and methods

2.1. Collection and sequencing of T. halophilus genomes

All publicly available genomes of *T. halophilus* strains and of the type strains of *Tetragenococcus solitarius* (NBRC 100494^T) and *Tetragenococcus muriaticus* (DSM 15685^T) were retrieved from GenBank. In addition, the genomes of the type strains of *T. halophilus* subsp. *halophilus* (DSM, 20339^T), *T. halophilus* subsp. *flandriensis* (LMG 26042^T), *Tetragenococcus koreensis* (KCTC 3924^T) and *Tetragenococcus osmophilus* (JCM 31126^T) were sequenced as described previously (Kim et al., 2018). In brief, the type strains were cultivated to the stationary phase in 5% (w/v) NaCl-containing MRS (BD, Franklin Lakes, NJ, USA) broth at 30 °C, following which their genomic DNA was extracted, according to standard procedures, including phenol-chloroform extraction and ethanol precipitation (Sambrook and Russell, 2001). The genomic DNA was then sequenced by a combination of PacBio RS single-molecule real-time sequencing based on a 10-kb library, and Illumina HiSeq 2500 sequencing (101 bp) with paired ends at Macrogen (Seoul, Korea). *De novo* assembly was performed through a hierarchical genome assembly process using the PacBio sequencing reads, and the genomes derived from the de novo assembly were error-corrected by mapping them to the Illumina sequencing reads.

The relatedness among the genomes was evaluated through average nucleotide identity (ANI) and *in silico* DNA–DNA hybridization (DDH) analyses, using a stand-alone program (http://www.ezbiocloud.net/sw/oat; Lee et al., 2016) and the server-based genome-to-genome distance calculator (ver. 2.1) (http://ggdc.dsmz.de/distcalc2.php; Meier-Kolthoff et al., 2013), respectively, as described previously (Chun et al., 2017).

2.2. Phylogenetic analyses based on 16S rRNA gene sequences and core-genomes

The phylogenetic relatedness among the *T. halophilus* strains and the type strains of the other *Tetragenococcus* species was investigated using their 16S rRNA gene and core-genome sequences to infer their evolutionary relationships. For the 16S rRNA-based analysis, the gene sequences were aligned using the Infernal secondary structure aware aligner available on the Ribosomal Database Project website (http://rdp.cme.msu.edu/; Nawrocki and Eddy, 2007). A phylogenetic tree with 1000 replicate bootstrap values was constructed using the neighbor-joining algorithm in the MEGA program (ver. 7) (Kumar et al., 2016). For the genome-based analysis, core-genome sequences were extracted from the genomes of all the *Tetragenococcus* strains used in this study and *Melissococcus plutonius* ATCC 35311^T (as an out-group), using the USEARCH program (ver. 9.0) (Edgar, 2010) available in the Bacterial Pan-Genome Analysis (BPGA) pipeline (ver. 1.3), with a 50% sequence identity cut-off (Chaudhari et al., 2016). The concatenated amino acid sequences of the core-genomes were aligned using the MUSCLE program (ver. 3.8.31) (Edgar, 2004), and a phylogenetic tree with 1000 replicate bootstrap values was constructed using the neighbor-joining algorithm in the MEGA program.

2.3. Analyses of T. halophilus pan-genomes and core genomes using COG and KEGG

The pan-genomes and core genomes of the *T. halophilus* strains were analyzed using the BPGA pipeline, with a 50% sequence identity cut-off. Sequencing errors during genome sequencing are frequently generated by base over- or under-calls, which can lead to normal genes being incorrectly described as pseudogenes or non-genes in genome annotation (Chun et al., 2017). Therefore, in this study, the core genome of *T. halophilus* included all normal genes, pseudogenes, or gene homologs commonly identified from all strains through BlastN searches, while the accessory genome included all genes, pseudogenes, or gene homologs identified from less than 14 genomes through BlastN searches.

The COG category assignment of functional genes derived from either the core or accessory genomes of the *T. halophilus* strains was performed using the USEARCH program within the BPGA pipeline against the COG database, where the portions of genes assigned to each COG category were expressed as relative percentages.

The predicted protein sequences derived from the genomes of the *T. halophilus* strains were submitted to BlastKOALA (http://www.kegg.jp/blastkoala/; Kanehisa et al., 2016) for functional annotation based on KEGG Orthology (KO), and the KO numbers of the functional genes were then used to generate the metabolic pathways of the strains using the iPath (ver. 3) module (https://pathways.embl.de/; Darzi et al., 2018). The metabolic pathways of the core or accessory genomes of the *T. halophilus* strains in the KEGG pathways were displayed by different colors and line thickness on the basis of the numbers of strains harboring genes with the same KO numbers. The presence or absence of genes associated with the production of thiamine (vitamin B_1), bacteriocin, and biogenic amines in the *T. halophilus* strains was bioinformatically investigated through BLASTN analyses against their genomes, using all corresponding gene sequences available in the UniProt database as reference sequences.

2.4. Transcriptome analysis of T. halophilus DSM 20339^T at different NaCl concentrations based on KEGG pathways

The transcriptional expression levels of the genes in *T. halophilus* DSM 20339^T at 0%, 9%, and 18% (w/v) NaCl concentrations were analyzed to investigate the metabolic features of *T. halophilus* at different salt concentrations. In brief, strain DSM 20339^T was anaerobically cultivated to the early stationary phase in MRS broth supplemented with 5% NaCl at 30 °C without shaking, following which 1% (v/v) of the cell culture was inoculated into MRS broths supplemented with 0%, 9%, and 18% NaCl, respectively. The cells were cultivated at 30 °C without shaking, harvested by centrifugation (15,000 rpm, 5 min) during their exponential phase (Supplementary Fig. S1), and stored at −80 °C until RNA extraction. Total RNA was extracted from the frozen cells using the Ambion™ TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. The

rRNA was removed from the total RNA, and the resultant purified mRNA was sequenced using an Illumina HiSeq 2500 system at Macrogen.

Metabolic mapping of the mRNA sequencing reads against all coding sequences (CDS) of strain DSM 20339^T was quantitatively performed using the Burrows–Wheeler Alignment tool (Li and Durbin, 2009), which is based on the best-match criteria of a 90% minimum identity and 20-bp minimum alignment. RPKM values (read numbers per kilobase of each CDS, per million mapped reads) for quantification of the relative gene expression levels were calculated on the basis of all mRNA sequencing reads that were mapped to all CDS of strain DSM 20339^T. The transcriptional expression levels of genes in the respective metabolic KEGG pathways at 0% NaCl concentration were displayed by color density and line thickness, on the basis of the RPKM values of genes corresponding to the metabolic KEGG pathways, as described previously (Chun et al., 2017). The indicated transcriptional expression levels of genes in the metabolic KEGG pathways at 9% and 18% NaCl were based on their fold changes relative to the expression levels at 0% NaCl.

2.5. Reconstruction and transcriptome analysis of the metabolic pathways for carbohydrates and compatible solutes of T. halophilus

The metabolic pathways for carbohydrates and some intracellular organic compounds (including compatible solutes) of the *T. halophilus* strains were reconstructed on the basis of the predicted KEGG pathways and Enzyme Commission (EC) numbers. The abilities of three *T. halophilus* strains (DSM, 20339^T, LMG 26042^T, and MJ4) to metabolize various carbohydrates were tested using the API 50 CHL system (bioMérieux, Capronne, France), according to the manufacturer's instructions. The results were also used as reference information for reconstructing the carbohydrate metabolic pathways of the *T. halophilus* strains. The presence or absence of the metabolic genes in each *T. halophilus* strain was manually confirmed through BLASTN analyses against their genomes, using available reference gene sequences of other closely related bacteria.

To investigate the metabolic activities of the reconstructed metabolic pathways on a transcriptional level, the mRNA-sequencing reads of *T. halophilus* DSM 20339^T cultured at different NaCl concentrations were mapped to the strain's own genome using the Burrows–Wheeler Alignment tool. The transcriptional expression levels of each metabolic gene according to NaCl concentrations in the reconstructed metabolic pathways for carbohydrates and compatible solutes were visualized as heatmaps, using RPKM values based on the functional genes of strain DSM 20339^T.

2.6. Analysis of biogenic amines and intracellular organic compounds

The abilities of the *T. halophilus* strains to produce histamine, tyramine, cadaverine, and putrescine from their corresponding precursors (viz., histidine, tyrosine, lysine, and ornithine, respectively) were assessed through *in vitro* tests. The type strains of *T. halophilus* subsp. *halophilus* (DSM, 20339^T) and *T. halophilus* subsp. *flandriensis* (LMG 26042^T) were used as representative *T. halophilus* strains for the test, and the type strain of *T. muriaticus* (KCTC 21008^T) (harboring a histidine decarboxylase gene) was used as a positive control. The concentrations of biogenic amines produced from their precursors were analyzed by HPLC (Shimadzu, Japan) equipped with a reverse-phased Hypersil ODS C_{18} column (250 × 4.6 mm; Thermo Fisher Scientific, USA) and a fluorescence detector (RF-10AXL; Shimadzu, Japan), according to the procedures described previously (Kim et al., 2017, 2019).

To investigate which intracellular organic compounds of *T. halophilus* respond to high salinity, cells of strain DSM 20339^T were grown in MRS broth supplemented with 0%, 9%, or 18% (w/v) NaCl and harvested by centrifugation during the exponential phase (Fig. S1). The concentrations of intracellular organic compounds including compatible solutes from the harvested cells were analyzed using ^{1}H NMR spectroscopy, according to previously described procedures (Kim et al., 2017).

3. Results and discussion

3.1. Collection and sequencing of T. halophilus genomes

At the time of writing of this manuscript (June 2018), a total of 15 genomes belonging to *T. halophilus* strains were publicly available in GenBank as draft or complete genomes. Although the genomes of the type strains of *T. halophilus* subsp. *halophilus* and *T. halophilus* subsp. *flandriensis* (two subspecies with validly published names of *T. halophilus*) were also publicly available, they were sequenced again in this study because their sequence qualities were relatively low. In addition, the genus *Tetragenococcus* currently includes four other species with validly published names: *T. koreensis*, *T. osmophilus*, *T. solitarius*, and *T. muriaticus*. However, because the genomes of the type strains of *T. koreensis* and *T. osmophilus* were not publicly available, they were also sequenced in this study for the genome-based analysis. The general features of the genomes of the 15 *T. halophilus* strains and type strains of the other *Tetragenococcus* species used in this study are presented in Table 1.

Because ANI and *in silico* DDH analyses have been generally used as standard methods for the delineation of prokaryotic species, pairwise ANI and *in silico* DDH values were calculated for the various genomes and used to display their relatedness of the strains (Supplementary Fig. S2). The analyses showed that the 15 *T. halophilus* genomes shared ANI and *in silico* DDH values that were higher than the 95–96% ANI and 70% *in silico* DDH thresholds for prokaryotic species delineation (Rosselló-Móra and Amann, 2015). Moreover, the 15 strains were clearly differentiated from the other *Tetragenococcus* species, with low ANI (< 82%) and *in silico* DDH (< 27%) values, suggesting that the *T. halophilus* strains form a unique phylogenetic lineage that is distinct from other *Tetragenococcus* species. The general genomic features of the *T. halophilus* strains were relatively similar (Table 1), with an average size and a total gene number of approximately 2.47 ± 0.11 Mb and 2425 ± 110, respectively, and a G + C content range of 35.6%–36.3%. The complete genome sequence information of the *T. halophilus* strains suggested that they harbor no or only one plasmid.

3.2. Phylogenetic analyses based on 16S rRNA gene and core-genome sequences

To infer the phylogenetic relatedness among the *T. halophilus* strains and *Tetragenococcus* species, phylogenetic analyses based on the16S rRNA gene and core-genome sequences were performed. The 16S rRNA-based analysis showed that all *T. halophilus* strains were tightly clustered together into one phylogenetic lineage with high sequence similarities (> 98.4%) and they were clearly separate from the other *Tetragenococcus* species (Fig. 1A). The analysis also showed that the *T. halophilus* strains were divided into two sublineage groups, one of which contained the type strain of *T. halophilus* subsp. *halophilus* and the other the type strain of *T. halophilus* subsp. *flandriensis* (Fig. 1A). In particular, the type strain of *T. halophilus* subsp. *flandriensis* formed a single cluster with three other *T. halophilus* strains (NISL 7121, FBL3, and NISL 7118), with almost identical 16S rRNA gene sequences, and they were distinctly separate from the sublineage group containing the type strain of *T. halophilus* subsp. *halophilus*. The phylogenetic analysis based on the core-genomes (1471 genes) also showed the distinctiveness of the *T. halophilus* strains from the other *Tetragenococcus* species (Fig. 1B). However, in this analysis, only the type strain of *T. halophilus* subsp. *flandriensis* was separated from the other *T. halophilus* strains, where it did not form a cluster with strains NISL 7121, FBL3, and NISL 7118. The results of these two analyses suggest that *T. halophilus* strains may have independently speciated into a unique phylogenetic group

Table 1
General features of the genomes of the *Tetragenococcus halophilus* strains and the type strains of *Tetragenococcus* species used in this study[a].

Strain name in GenBank (accession no.)	Genome status[b] (no. of contigs)	Total size (Mb)	G + C content (%)	No. of genes	No. of pseudogenes
T. halophilus subsp. *halophilus* DSM 20339^T (PXYA00000000)[c]	D (5)	2.60	36.0	2490	5
T. halophilus KUD23 (CP020017)	C (1)	2.60	36.1	2533	15
T. halophilus MJ4 (CP012047.1)	C (1)	2.39	36.0	2338	9
T. halophilus FBL3 (LSFG00000000)	D (87)	2.42	35.8	2393	75
T. halophilus NISL 7126 (BDEF00000000)	D (214)	2.51	35.7	2509	0
T. halophilus NISL 7116 (BDEB00000000)	D (303)	2.30	35.7	2263	6
T. halophilus NISL 7128 (BDEG00000000)	D (315)	2.31	35.7	2260	2
T. halophilus NISL 7118 (BDEC00000000)	D (281)	2.40	35.7	2331	14
T. halophilus NISL 7125 (BDEE00000000)	D (237)	2.51	35.7	2512	0
T. halophilus NISL 7121 (BDED00000000)	D (235)	2.45	35.7	2429	1
T. halophilus NBRC 12172 (NC_016052)	C (1)	2.56	36.0	2550	0
T. halophilus D10 (BDEH00000000)	D (259)	2.44	35.6	2390	0
T. halophilus D-86 (BDEI00000000)	D (315)	2.46	35.6	2414	4
T. halophilus 11 (BDDZ00000000)	D (311)	2.41	35.6	2330	14
T. halophilus subsp. *flandriensis* LMG 26042^T (CP027768-9)[c]	C (2)	2.72	36.3	2635	48
T. koreensis KCTC 3924^T (CP027786-7)[c]	C (2)	2.73	37.2	2553	61
T. solitarius NBRC 100494^T (BCPY00000000)	D (97)	2.51	36.4	2338	68
T. muriaticus DSM 15685^T (AUIQ00000000)	D (109)	2.08	36.2	1858	122
T. osmophilus JCM 31126^T (CP027783-5)[c]	C (3)	2.38	36.4	2160	46

[a] Bioinformatic analysis of the genomes was carried out using the NCBI prokaryotic genome annotation pipeline (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/).
[b] Genome status: D, draft genome; C, complete genome.
[c] Genomes sequenced in this study.

with genomic and metabolic features that are distinct from those of other *Tetragenococcus* species, and that *T. halophilus* subsp. *halophilus* and *T. halophilus* subsp. *flandriensis* are not distinguished by their 16S rRNA gene sequences only.

3.3. Analyses of T. halophilus pan-genomes and core genomes using COG and KEGG

Pan-genomes and core genomes represent all the genomic repositories of group members in a phylogenetic lineage, and their analyses provide a better understanding of the genomic and metabolic features and diversity among the strains of a particular species (Endo et al., 2015; Vernikos et al., 2015). In the pan-genome analysis, the number of cumulative genes of the *T. halophilus* strains increased with the increase in the number of genomes used for the analysis (Supplementary Fig. S3), suggesting that *T. halophilus* may have an open pan-genome that experiences high evolutionary changes through gene loss and gain or horizontal gene transfer in order to adapt efficiently to new environments (Chun et al., 2019; Vernikos et al., 2015). The pan-genome of the 15 *T. halophilus* strains contained a total of 3488 genes consisting of 1471 genes in the core genome and 2017 genes in the accessory genome (of which 622 were unique genes). The core genome represents the metabolic and physiological features that are common to the *T. halophilus* strains, whereas the accessory genome represents the differential features and may confer competitive fitness or advantages to each strain for their adaptation to different environmental niches (Laing et al., 2017).

The functional analysis of pan-genomes and core genomes may provide valuable information about the metabolic features and diversity of group members in a phylogenetic lineage (Endo et al., 2015). Therefore, the functional genes in the core and accessory genomes of the *T. halophilus* strains were classified into COG categories, where some of the categories showed clear differences in gene abundance (Fig. 2). COG categories associated with housekeeping processes, including cell division and chromosome partitioning (D), nucleotide transport and metabolism (F), lipid transport and metabolism (I), translation, ribosomal structure, and biogenesis (J), cell motility and secretion (N), posttranslational modification, protein turnover, and chaperones (O), and intracellular trafficking, secretion, and vesicular transport (U), were more than two times abundant in the core genome than in the accessory genome. This may support the phylogenetic results (Fig. 1), in that *T. halophilus* strains form a single phylogenetic lineage with similar housekeeping genes. On the other hand, genes associated with carbohydrate transport and metabolism (G) and defense mechanisms (V) were over two times more enriched in the accessory genome than in the core genome, which suggests that carbohydrate metabolism and the defense system against viruses or various stressors are different among the various *T. halophilus* strains.

The metabolic features and diversity of *T. halophilus* were also investigated by cumulatively mapping the functional KEGG genes of the 15 *T. halophilus* strains to the KEGG pathways (Fig. 3). It has been reported that *T. halophilus* may perform homolactic fermentation to produce only lactate from glucose (Justé et al., 2008b). In contrast, another report has suggested that *T. halophilus* may perform heterolactic fermentation, producing lactate, ethanol, and CO_2 from glucose (Justé et al., 2012). In this present study, the KEGG pathway analysis of *T. halophilus* using the pan-genomes and core genomes showed that all 15 strains harbored complete glycolysis and 6-phosphogluconate/phosphoketolase pathways, a lactate dehydrogenase-encoding gene (KEGG gene K00016), and an incomplete tricarboxylic acid cycle, which suggests that *T. halophilus* has the capability to perform both homolactic and heterolactic fermentation processes; that is, facultative lactic acid fermentation. The KEGG pathway analysis also showed that the *T. halophilus* strains harbored common metabolic pathways for various carbohydrates (including glucose, fructose, galactose, mannose, pentose, and amino and nucleotide sugars) and intermediates of purine, pyrimidine, fatty acids, and various amino acids, indicating the metabolic versatility of the species. In addition, *T. halophilus* harbored genes of the thiamine (vitamin B1) biosynthetic pathway (KEGG genes K06949, K00788, K00877, and K03147) in the core genome. However, the gene associated with bacteriocin production that is frequently identified in lactic acid bacteria was not identified in the genomes of the *T. halophilus* strains. The presence of the pathways of facultative lactic acid fermentation, carbohydrate metabolisms, and thiamine production and the absence of bacteriocin-producing gene in *T. halophilus* strains were in common with those in other *Tetragenococcus* species strains genome-sequenced (data not shown), suggesting that they may be common metabolic features of the genus *Tetragenococcus*.

Histamine, tyramine, cadaverine, putrescine, and beta-phenylethylamine, which are the biogenic amines that can cause adverse health

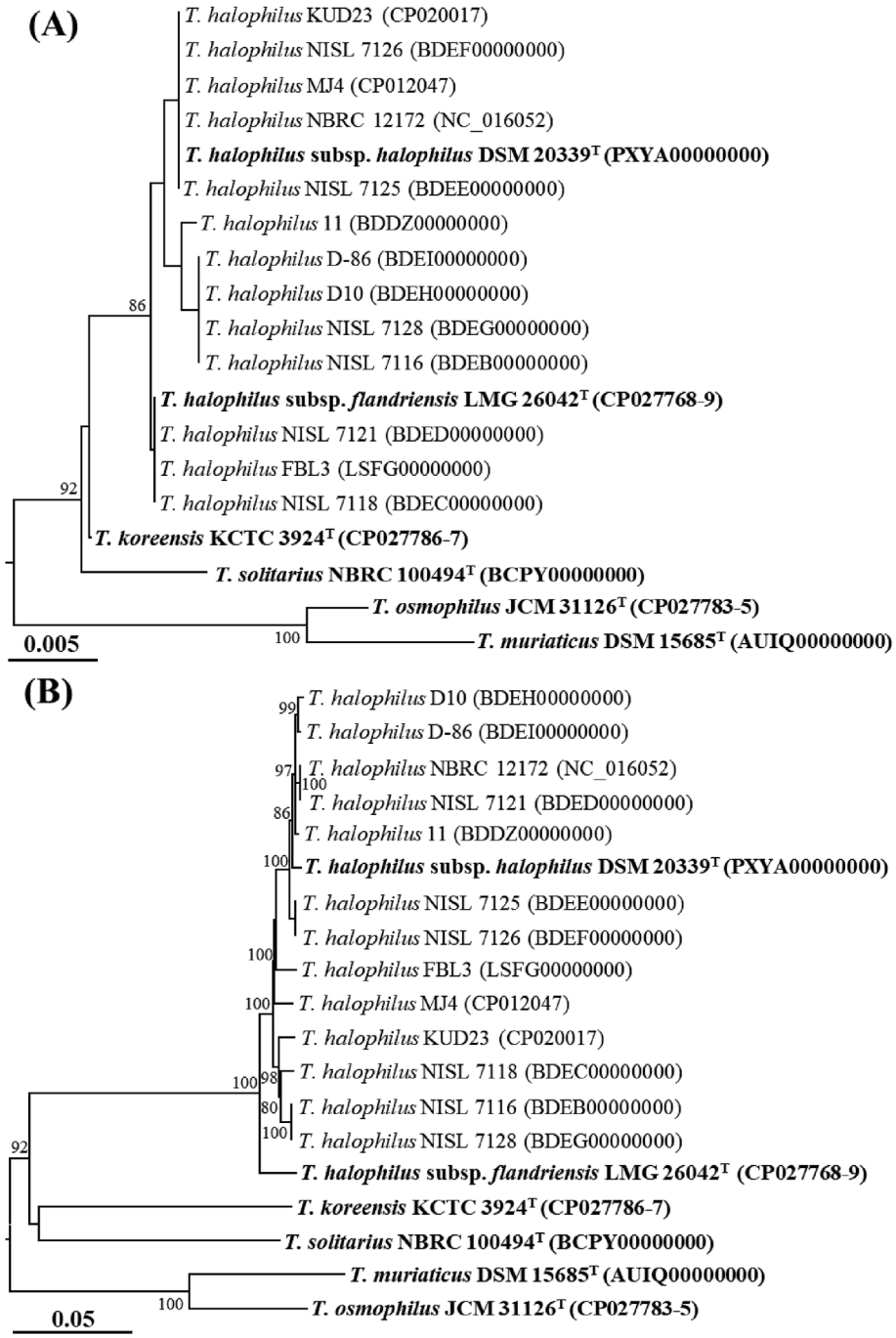

Fig. 1. Phylogenetic trees showing the relatedness among *Tetragenococcus halophilus* strains and type strains of *Tetragenococcus* species, as based on the 16S rRNA gene sequences (A) and the concatenated amino acid sequences of the core-genomes (1471 genes) (B). The 16S rRNA gene (GenBank accession no., AP012200) and genome (GenBank accession no., CP006683-4) sequences of *Melissococcus plutonius* ATCC 35311^T were used as an out-group (not shown) in the 16S rRNA gene- and core-genome-based trees, respectively. The type strains of *Tetragenococcus* species are highlighted in bold. Bootstrap values of over 70% are indicated on the nodes as percentages of 1000 replicates. The bars indicate the numbers of substitutions per sites.

effects, are generally produced through the decarboxylation of their corresponding amino acids or nitrogen compounds (i.e., histidine, tyrosine, lysine, ornithine, and phenylalanine, respectively) during food fermentation (Suzzi and Torriani, 2015). It has been reported that *T. halophilus* strains may be major causative agents for the generation of biogenic amines during food fermentation (Jung et al., 2016b; Jeong et al., 2017; Justé et al., 2008a; Kobayashi et al., 2016; Sitdhipol et al., 2013), and histidine decarboxylase and tyrosine decarboxylase, which produce histidine and tyrosine via decarboxylation, respectively, may be encoded on the plasmids of some *T. halophilus* strains (Satomi et al., 2008, 2011, 2014). Therefore, in this study, all histamine, tyrosine, ornithine, and lysine decarboxylase gene sequences available in the UniProt database were compared against the genomes of the *T. halophilus* strains through BLASTN analyses. As a result, only one histidine decarboxylase gene was identified from the genome of only one strain (*T. halophilus* 11), and no other biogenic amine-related decarboxylase gene was found. However, the biogenic amine production tests using *T. halophilus* subsp. *halophilus* (DSM, 20339^T) and *T. halophilus* subsp. *flandriensis* (LMG 26042^T) showed that strain DSM 20339^T had a clear ability to produce tyramine from tyrosine without any known tyramine-producing decarboxylase gene in its genome (Supplementary Fig. S4). *T. muraticus* KCCM 21008^T, a known histamine producer, harbored a histidine decarboxylase gene in its genome and produced histamine from histidine in the biogenic amine production tests. These results suggest that *T. halophilus* strains may harbor as yet unidentified biogenic amine (tyramine)-producing genes or pathways and further studies on this are therefore warranted.

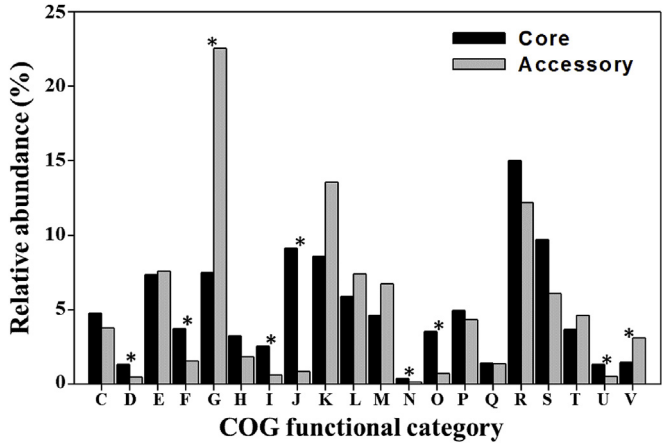

Fig. 2. Comparison of the abundance of core and accessory genomes in the pan-genomes of *Tetragenococcus halophilus* strains in the various COG functional categories. The alphabetical codes represent following the functional categories: C, energy production and conversion; D, cell division and chromosome partitioning; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; G, carbohydrate transport and metabolism; H, coenzyme metabolism; I, lipid metabolism; J, translation, ribosomal structure, and biogenesis; K, transcription; L, DNA replication, recombination, and repair; M, cell envelope biogenesis and outer membrane; N, cell motility and secretion; O, post-translational modification, protein turnover, and chaperones; P, inorganic ion transport and metabolism; Q, secondary metabolite biosynthesis, transport, and catabolism; R, general function prediction only; S, function unknown; T, signal transduction mechanisms; U, intracellular trafficking, secretion, and vesicular transport; and V, defense mechanisms. Asterisks indicate COG categories with a more than two times difference.

3.4. Transcriptome analysis of *T. halophilus* DSM 20339^T at different NaCl concentrations based on KEGG pathways

To investigate the metabolic features of *T. halophilus*, the type strain DSM 20339^T was cultivated in the presence of 0%, 9%, or 18% NaCl and the transcriptome was analyzed. According to the growth curves, strain DSM 20339^T grew the most rapidly at 9% NaCl. Although its lag phase was longer at 18% NaCl than at 0% NaCl, it eventually grew faster at 18% NaCl (Fig. S1). The metabolic versatility and halophilic property of *T. halophilus* may be important reasons why its members have been frequently identified in many different types of fermented salted foods, such as fermented sausage, doenjang (soybean paste), ganjang (soy sauce), and jeotgal and nam-pla (fish sauces) (Amadoro et al., 2015; Jung et al., 2016a, 2016b; Kim and Park, 2014; Lee et al., 2015).

The metabolic features of *T. halophilus* at different NaCl concentrations were investigated by mapping the transcripts of strain DSM 20339^T grown at 0%, 9%, and 18% NaCl to the KEGG pathways (Fig. 4). The transcriptome profiles at the different NaCl concentrations were generally in accordance with those of previous studies (Lin et al., 2017; Liu et al., 2015). The transcriptome analysis showed that the metabolic pathways associated with lactic acid fermentation and the metabolism of carbohydrates, nucleotides, fatty acids, and various amino acids were highly expressed regardless of the salt concentration. The analysis also revealed that the thiamine biosynthetic pathway was relatively highly expressed regardless of the salt concentration, suggesting that *T. halophilus* may produce thiamine during the fermentation of various salted foods. The transcriptional expression levels of most genes in strain DSM 20339^T were relatively similar regardless of the salt concentration. However, the expression of metabolic pathways associated with the

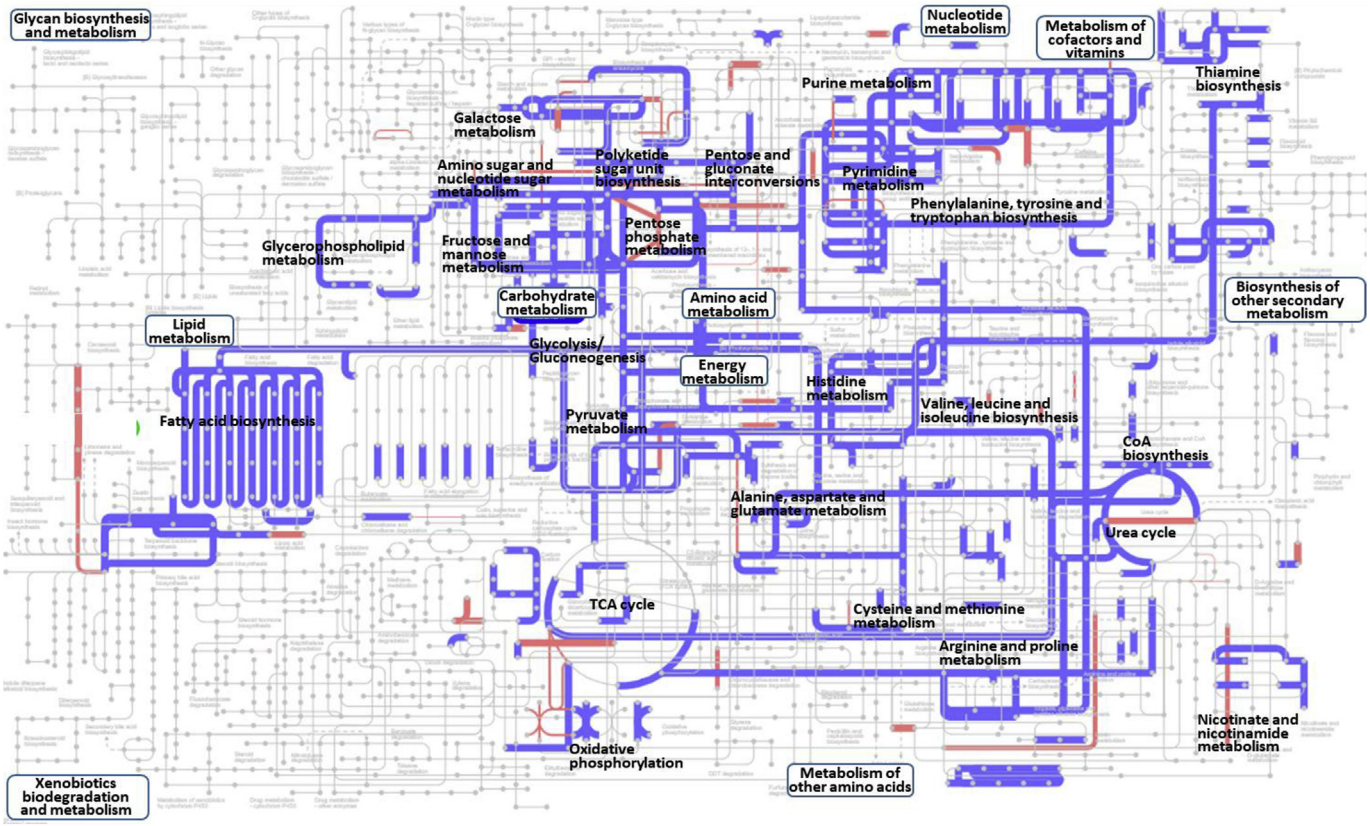

Fig. 3. Metabolic pathways of *Tetragenococcus halophilus* strains. The pathways were generated using the iPath (ver. 3) module and are based on KEGG Orthology numbers of genes identified from the genomes of 15 *T. halophilus* strains. Metabolic pathways identified from the core- and accessory-genomes are depicted in blue and red, respectively. The thickness of the red lines is proportional to the number of *T. halophilus* strains harboring the metabolic pathways. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

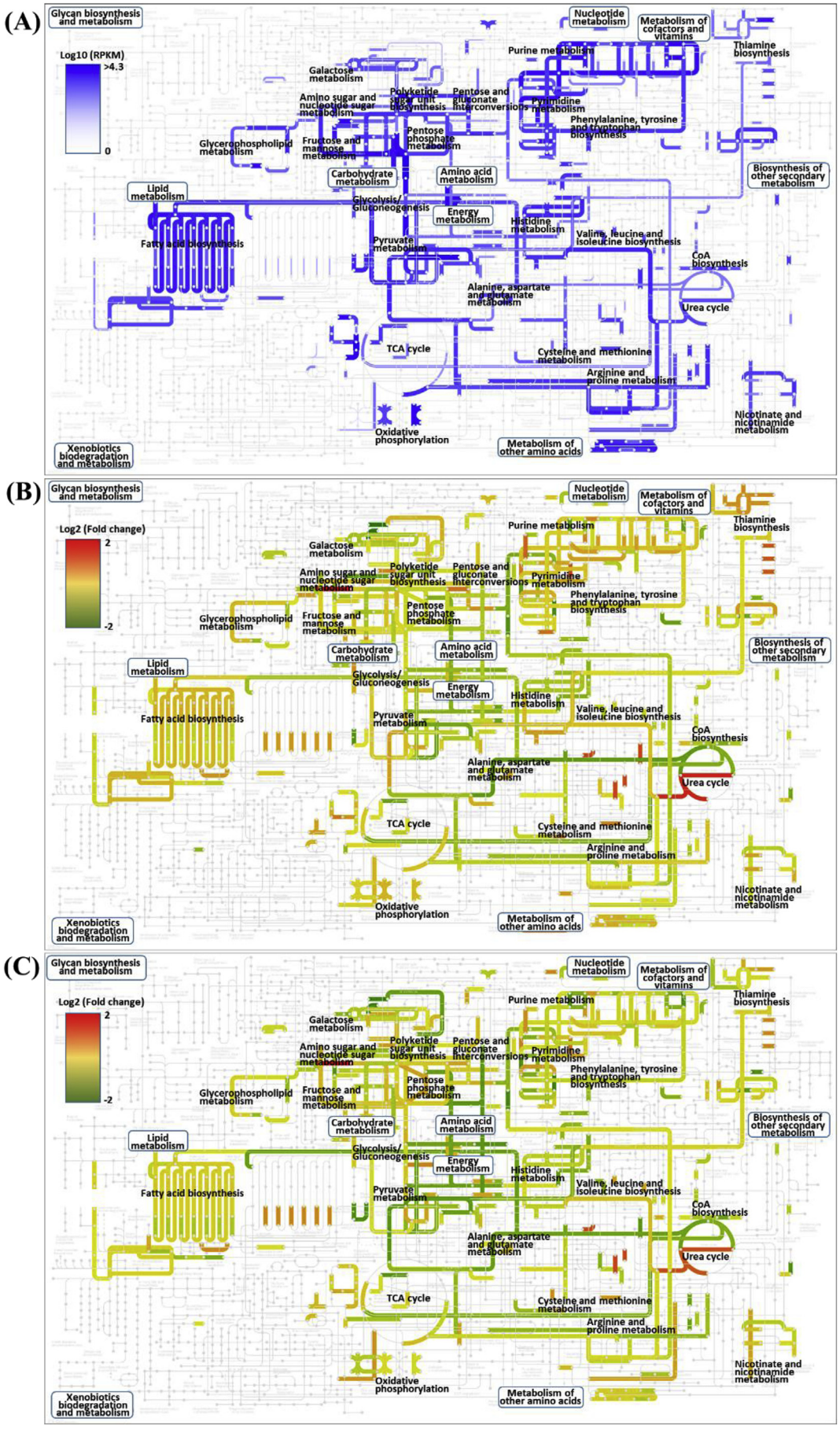

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Fig. 4. Transcriptional expressions of the metabolic pathways of *Tetragenococcus halophilus* DSM 20339^T grown in MRS broth supplemented with 0%, 9%, or 18% NaCl. The metabolic pathways were generated using the iPath (ver. 3) module and are based on gene KEGG Orthology numbers of strain DSM 20339^T. Panel (A) indicates the transcriptional expression of metabolic pathways in strain DSM 20339^T grown in MRS broth without the addition of NaCl (0%); and the expression levels are proportional to the color density and line width. Panels (B) and (C) indicate the differential gene expression levels (fold changes) at 9% NaCl and 18% NaCl relative to that at 0% NaCl, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

metabolism of carbohydrates and amino acids was slightly decreased at high NaCl concentrations compared with that at 0% NaCl (Fig. 4B and C). In contrast, genes related to citrulline production (arginine deaminase, K01478; ornithine carbamoyltransferase, K00611), which are parts of the urea pathway, were much more highly expressed at 9% and 18% NaCl than at 0% NaCl. However, the urea pathway of the *T. halophilus* strains was incomplete owing to the lack of the arginase gene (EC 3.5.3.1), which suggests that *T. halophilus* does not produce urea. Citrulline has been reported to be increased in some lactic acid bacteria (e.g., *Tetragenococcus*, *Lactobacillus*, and *Pediococcus*) at high salt conditions (He et al., 2017b; Vrancken et al., 2009; Zhang et al., 2014) and it may be a potential compound for coping with some oxidative stressors, such as hydroxyl radicals (Akashi et al., 2001; Held and Sadowski, 2016; Kusvuran et al., 2013). This suggests that *T. halophilus* may produce citrulline as a compatible solute or protective compound for coping with the oxidative stress caused by high salinity.

3.5. Reconstruction and transcriptome analysis of the carbohydrate fermentative pathways of T. halophilus

For a closer scrutiny of the metabolic features of *T. halophilus*, the fermentative pathways for various carbohydrates were reconstructed on the basis of the KEGG pathways of the *T. halophilus* strains and through manual curation of the core and accessory genomes of *T. halophilus* by BLASTN (Fig. 5). The capability of *T. halophilus* for metabolizing various carbohydrates was confirmed through carbohydrate assimilation tests conducted on strains DSM 20339^T, LMG 26042^T, and MJ4, using the API 50 CHL system (Supplementary Table S1). According to the reconstructed metabolic pathways, *T. halophilus* strains have the capabilities to metabolize diverse types of carbohydrates. The metabolic genes as well as transport systems of various carbohydrates, including D-glucose, D-ribose, D-fructose, D-mannose, maltose, trehalose, cellobiose, lactose, melibiose, raffinose, stachyose, galactitol, D-sorbitol, D-mannitol, L-arabinose, and D-xylose, were identified from the core or accessory genomes of the *T. halophilus* strains. The presence or absence of metabolic genes and transport systems in the genomes of the three tested strains were in good accordance with their carbohydrate assimilation abilities in the API 50 CHL system. The sucrose, D-galactose, D-maltose, L-xylulose, D-tagatose, and glycerol transport systems were not identified in the bioinformatic analysis of the respective genomes of the *T. halophilus* strains, despite that their metabolic genes were present in the core or accessory genomes, which suggests that there may be as yet unidentified carbohydrate transport systems in the core or accessory genomes. The carbohydrate assimilation test also indicated the consistency of the presence of the metabolic genes of sucrose, D-galactose, D-maltose, L-xylulose, D-tagatose, and glycerol in the three strains with their carbohydrate assimilation abilities, even though the carbohydrate transport genes were not identified by the bioinformatic analysis. The reconstructed metabolic pathways also showed that all *T. halophilus* strains had the capability to metabolize D-glucose, D-ribose, D-fructose, D-mannose, D-maltose, trehalose, cellobiose, galactitol, sucrose, D-galactose, and D-tagatose, whereas the metabolism of lactose, melibiose, raffinose, stachyose, D-sorbitol, D-mannitol, L-arabinose, D-xylose, L-xylulose, and glycerol was strain dependent.

According to the reconstructed fermentative pathways, all genes involved in the heterolactic (producing lactate, ethanol, and CO_2) and homolactic (producing only lactate) fermentation pathways were present in the core genome of *T. halophilus*, but the tricarboxylic acid cycle was not complete, confirming that facultative lactic acid fermentation is the common metabolic feature of *T. halophilus* (Fig. 5). The *T. halophilus* strains harbored only one copy of L-lactate dehydrogenase (EC 1.1.1.27) in the core genome for converting pyruvate to L-lactate with the regeneration of NAD^+, which suggests that *T. halophilus* produces L-lactate as a major fermentation product. The reconstructed metabolic pathways showed that besides its conversion into L-lactate, pyruvate could also be converted into acetyl-P and acetyl-CoA in the *T. halophilus* strains. Acetyl-P, which is formed from the heterolactic fermentation pathway or the oxidation of pyruvate by pyruvate oxidase (EC 1.2.3.3), is converted into either ethanol (with the regeneration of NAD^+) or acetate (with the production of ATP), suggesting that the fermentation products of *T. halophilus* strains are different depending on the reduction potentials (NADH concentrations) (Jeong et al., 2018). Diacetyl and/or acetoin, produced from pyruvate through the activities of acetolactate synthase, acetolactate decarboxylase, and diacetyl reductase, are important flavoring compounds in lactic acid fermentation (Chun et al., 2017; Gaenzle, 2015). However, the reconstructed metabolic pathways showed that *T. halophilus* strains did not harbor the corresponding genes, and only a gene encoding acetolactate decarboxylase (EC 4.1.1.5, interconverting 2-acetolactate and 2-acetoin), was identified.

The fermentative features of *T. halophilus* at different NaCl concentrations were investigated through analysis of the transcriptome of strain DSM 20339^T (Fig. 5). The results showed that the fermentation of carbohydrates was relatively similar regardless of the NaCl concentration. However, genes associated with the D-ribose metabolic pathway (ribokinase, EC 2.7.1.15; RbsU) and heterolactic fermentation pathway (including phosphogluconate dehydrogenase (EC 1.1.1.343), ribulose-phosphate 3-epimerase (EC 5.1.3.1), and phosphoketolase (EC 4.1.2.9)) were more highly expressed at high NaCl concentrations. In addition, the expression of acetate kinase (EC 2.7.2.1, converting acetyl-P to acetate) was also increased at high NaCl concentrations, whereas the expression of acetaldehyde dehydrogenase and alcohol dehydrogenase (in the pathway that converts acetyl-CoA to ethanol) was decreased, suggesting that D-ribose may be a more readily metabolizable carbon source for *T. halophilus* at high salt concentrations and the bacterium may produce more acetate through the heterolactic fermentation pathway. The transcription of genes associated with glycerol metabolism (glycerol dehydrogenase, EC 1.1.1.6; glycerone kinase, EC 2.7.1.29; phosphoenolpyruvate-glycerone phosphotransferase, EC 2.7.1.121) was also upregulated at high NaCl concentrations, which suggests that glycerol may also be a more readily metabolizable carbon source for *T. halophilus* at high salt conditions.

3.6. Reconstruction and transcriptome analysis of the metabolic pathways of compatible solutes of T. halophilus

Compatible solutes, such as glutamate, glutamine, glycine betaine, trehalose, proline, ectoine, β-hydroxyectoine, mannitol, and *trans*-4-hydroxy-L-proline, are polar and highly water-soluble intracellular organic compounds that halophilic or halotolerant microorganisms accumulate to protect themselves from high salinity, where the types accumulated are different depending on the microorganism and salt concentrations (Kim et al., 2017; Roberts, 2005). Glycine betaine, ectoine, choline, L-carnitine, proline, citrulline, *N*-acetyltryptophan, and dimethylsulfonioacetate have been suggested as possible compounds that *T. halophilus* uses to cope with salt stress under high salt conditions (He et al., 2017b), but clear evidence of their metabolic pathways has not been presented. Therefore, in this study, the metabolic pathways for

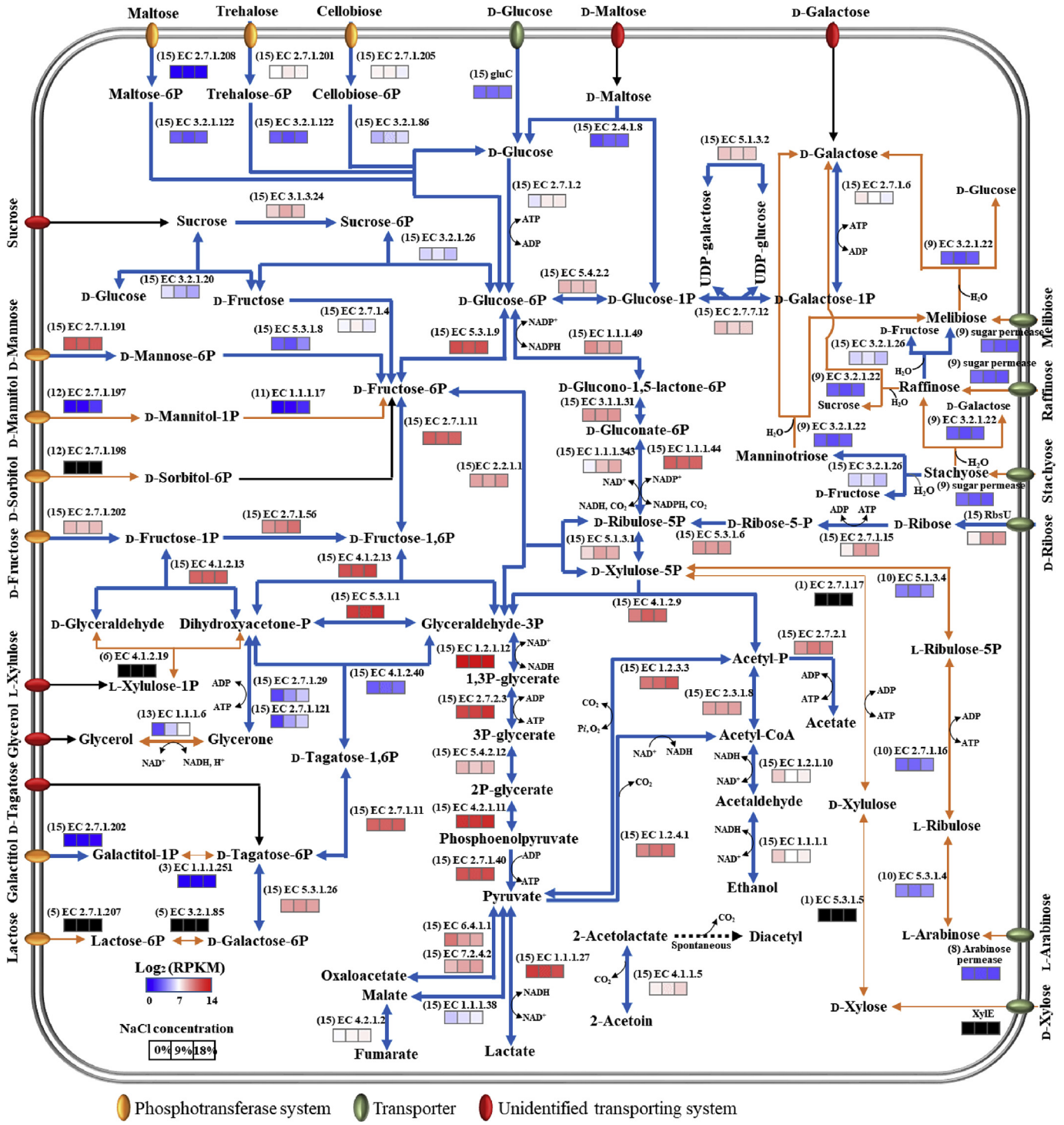

Fig. 5. Proposed fermentative pathways for various carbohydrates and their transcriptional expression in *Tetragenococcus halophilus* strains grown at 0%, 9%, and 18% NaCl. Metabolic pathways in the core- and accessory-genomes are depicted in blue and orange, respectively. The line thickness is proportional to the number of *T. halophilus* strains harboring the genes, which are indicated in parentheses. The black arrows indicate unidentified genes that may be present in *T. halophilus*, as inferred from the presence of the metabolic pathways and assimilation abilities (API 50 CHL system) for the corresponding carbohydrates. The transcriptional expression levels were visualized using heatmaps based on the RPKM values of each metabolic gene in *T. halophilus* DSM 20339^T. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

these compounds in *T. halophilus* strains were reconstructed and their transcriptional expression was analyzed.

The reconstructed metabolic pathways identified genes associated with the metabolism and transportation of glycine betaine, proline, glutamate, glutamine, and choline from the core genome of *T. halophilus* strains, as well as genes associated with the citrulline biosynthetic pathway (Fig. 6). Analysis of the transcriptome of strain DSM 20339^T showed that the osmoprotectant uptake gene (*opuA*), which is a known glycine betaine transporter gene in *Lactobacillus* (Romeo et al., 2003), was highly expressed regardless of the NaCl concentration. However, the analysis also clearly showed that at high NaCl concentrations, the glycine betaine uptake system gene (*busA*) associated with glycine

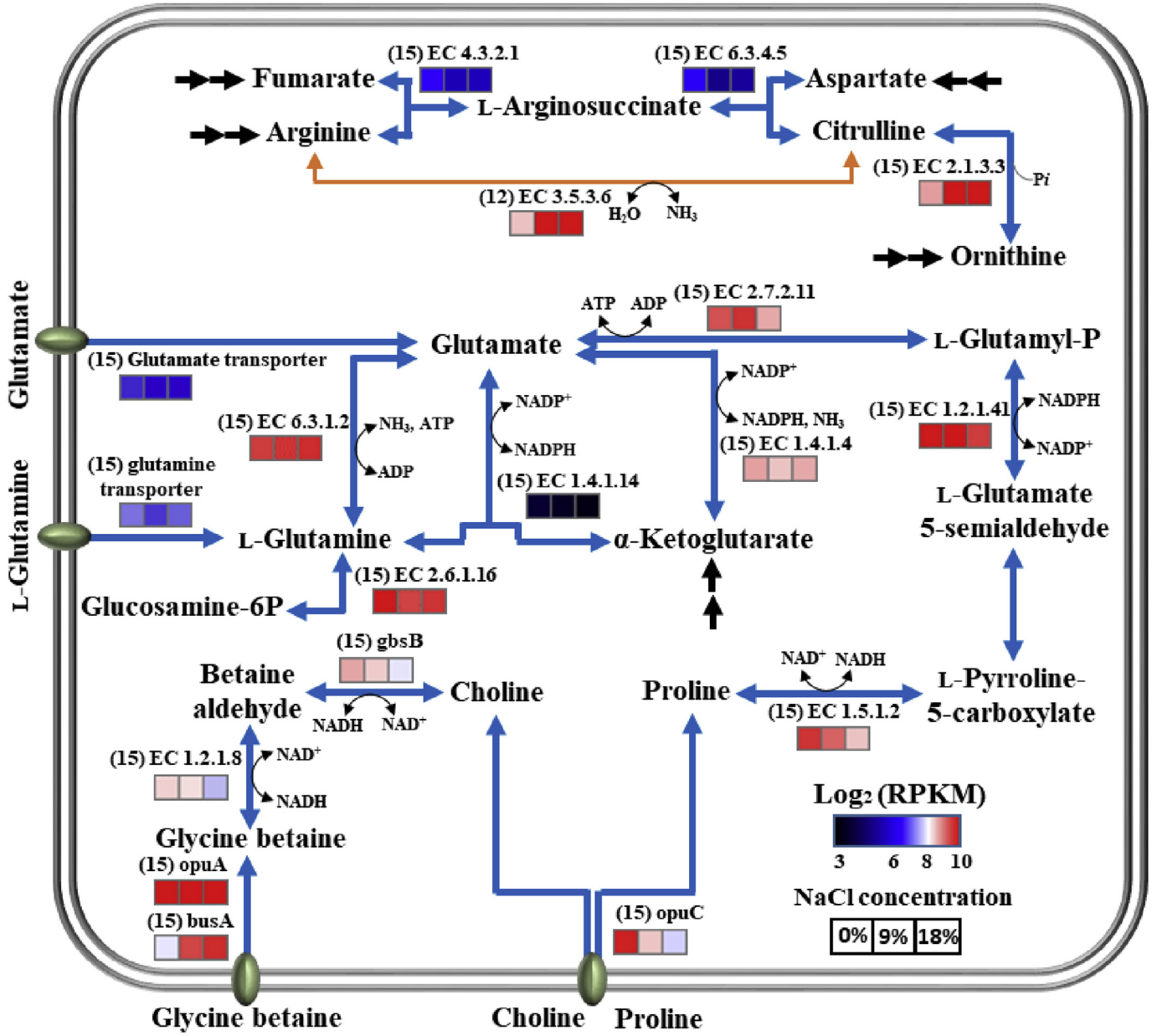

Fig. 6. Proposed metabolic pathways of major intracellular organic compounds (including compatible solutes) in *Tetragenococcus halophilus* and their transcriptional expression levels at 0%, 9%, and 18% NaCl. Metabolic pathways in the core- and accessory-genomes are depicted in blue and orange, respectively. The line thickness is proportional to the number of *T. halophilus* strains harboring the genes, which are indicated in parentheses. The transcriptional expression levels were visualized using heatmaps based on the RPKM values of each metabolic gene in *T. halophilus* DSM 20339^T. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

betaine transport (neighboring with *opuA* in the *T. halophilus* genomes) was highly expressed, whereas the genes for glycine betaine metabolism (betaine-aldehyde dehydrogenase, EC 1.2.1.8; and *gbsB*) were downregulated, which suggests that glycine betaine may be accumulated in *T. halophilus* at high salinity conditions. On the other hand, the transcriptional expression of genes related to the metabolism of other compatible solutes, such as the choline and proline transporter gene (*opuC*) and glutamate metabolic gene (glutamate 5-kinase, EC 2.7.2.11), was decreased at high NaCl concentrations, suggesting that the choline, proline, glutamate, and glutamine levels may be decreased at high salinity conditions. Moreover, the citrulline biosynthetic genes (ornithine carbamoyltransferase, EC 2.1.3.3; arginine deiminase, EC 3.5.3.6) were highly upregulated at the high NaCl concentrations. These results suggest that glycine betaine and citrulline may be important compounds of the *T. halophilus* coping mechanism under high salinity. The genomes of other *Tetragenococcus* species strains also harbored the glycine betaine metabolic pathway, but ornithine carbamoyltransferase gene was not identified from some other *Tetragenococcus* species strains (data not shown), which suggests that the use of citrulline for coping with high salinity may not be a common metabolic feature of the genus *Tetragenococcus*.

The concentrations of these compounds in strain DSM 20339^T cultured at 0%, 9%, and 18% NaCl were analyzed using ^{1}H NMR spectroscopy to confirm the transcriptional expression of their metabolic pathway genes under various salinity conditions (Fig. 7). The profiles of the compounds according to NaCl concentrations were in good accordance with the results of the transcriptome analysis. The concentration of glycine betaine was significantly higher than that of the other organic compounds at all salinity conditions, and increased obviously with the increase in the NaCl concentration, which was in good

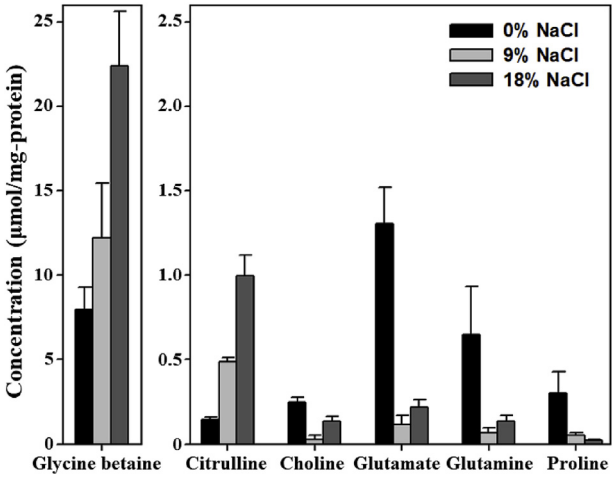

Fig. 7. Concentrations of major intracellular organic compounds (including compatible solutes) in *Tetragenococcus halophilus* DSM 20339^T grown in MRS broth supplemented with 0%, 9%, or 18% NaCl. The measurements were taken by ^{1}H NMR spectroscopy using triplicate samples, and the concentrations of the organic compounds from the ^{1}H NMR peaks were determined using the Chenomx NMR suite (ver. 6.1) with 2,2-dimethyl-2-silapentane-5-sulfonate as the internal standard. The data are given as the average values ± standard deviations.

agreement with the NaCl concentration-dependent transcriptional expression of *opuA*, *busA*, and glycine betaine metabolic genes (betaine-aldehyde dehydrogenase and *gbsB*) (Fig. 6). However, the concentrations of choline, glutamine, glutamate, and proline decreased with increase in the NaCl concentration. These results suggest that glycine betaine is the main compatible solute used by *T. halophilus* to cope with high salinity.

Similar to the transcriptome profile of the citrulline metabolic genes (Fig. 6), the citrulline amount increased with increase in the NaCl concentration, but its overall concentration was much lower than that of glycine betaine. Although citrulline has been reported as one of the intracellular organic compounds (possibly a compatible solute) that is accumulated in *T. halophilus* under high salt conditions (He et al., 2017b), it has generally not been considered as an important compatible solute in microorganisms for achieving osmotic balance (through its intracellular increase) in response to high salinity. This study also showed that the citrulline concentrations in *T. halophilus* were much too low to achieve osmotic balance in response to 9% and 18% NaCl concentrations. It was reported that the high osmotic pressure of salt stress could lead to oxidative stress through the generation of reactive oxygen species (ROS) in bacteria, where genes encoding superoxide dismutase and glutathione synthetase were upregulated to reduce the oxidative stress (He et al., 2017a; Yin et al., 2017). In addition, citrulline could reduce the ROS generation caused by salt stress in plant (Akashi et al., 2001). Therefore, we can infer that *T. halophilus* may use citrulline as an intracellular organic compound to protect against stressors such as the ROS induced by high salinity, rather than as a compatible solute to achieve osmotic balance under high salt conditions.

4. Conclusions

This pan-genome analysis of the 15 *T. halophilus* strains has clearly revealed their phylogenetic distinctiveness from the other *Tetragenococcus* species and their reconstructed metabolic pathways clarified the diverse and strain-specific nature of their carbohydrate metabolic capabilities, which could have important implications in the consideration of their use as starter cultures for various food fermentation processes. Moreover, the genome and biogenic amine analyses of the species indicated that there are still possibly unknown genes involved in the production of some biogenic amines (e.g., tyramine), suggesting further investigations for their identification. In addition, the transcriptome and intracellular organic compound analyses in *T. halophilus* DSM 20339^T grown at 0%, 9%, and 18% NaCl have revealed the main molecules expressed by the strain under high salt conditions, providing a better understanding of the possible coping mechanisms used by *T. halophilus* against the stress caused by high salinity. Overall, this study has given us a deeper insight into the genomic and metabolic features of this important bacterium that is ubiquitous in fermented foods, opening up new possibilities for its manipulation in the food fermentation industry.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.04.009.

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