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Genomic Insight into the Salt Tolerance of *Enterococcus faecium*, *Enterococcus faecalis* and *Tetragenococcus halophilus* S

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To shed light on the genetic basis of salt tolerance in *Enterococcus faecium*, *Enterococcus faecalis*, and *Tetragenococcus halophilus*, we performed comparative genome analysis of 10 *E. faecalis*, 11 *E. faecium*, and three *T. halophilus* strains. Factors involved in salt tolerance that could be used to distinguish the species were identified. Overall, *T. halophilus* contained a greater number of potassium transport and osmoprotectant synthesis genes compared with the other two species. In particular, our findings suggested that *T. halophilus* may be the only one among the three species capable of synthesizing glycine betaine from choline, cardiolipin from glycerol and proline from citrate. These molecules are well-known osmoprotectants; thus, we propose that these genes confer the salt tolerance of *T. halophilus*.

Keywords: *Enterococcus faecium*, *Enterococcus faecalis*, *Tetragenococcus halophilus*, salt tolerance, pan-genome

Introduction

Doenjang, a traditional Korean high-salt fermented soybean paste, is made by mixing meju with a high-salt (~18%) brine, followed by ripening for approximately 2 months. Meju is a naturally fermented soybean block; microorganisms grow spontaneously during the ripening and produce enzymes that degrade macromolecules in the soybean [1, 2]. The microorganisms and enzymes produced enhance the sensory qualities of the product during doenjang production [3–7]. Doenjang is used frequently in Korean cuisine and has thus been the subject of several studies, including of the microbial community. In initial microbial studies, the presence of fungal species belonging to the genera *Mucor*, *Penicillium*, *Scopulariopsis*, *Aspergillus*, *Rhodotorula*, *Torulopsis* (amended to *Candida*), and *Saccharomyces* was confirmed using culture-dependent methods, as was the presence of bacterial species belonging to the genus *Bacillus* and lactic acid bacteria such as *Lactobacillus* sp. [8–10]. Recent culture-independent analysis methods such as pyrosequencing [11] have shown that lactic acid bacteria, including *Enterococcus* and *Tetragenococcus*, are predominant bacteria in doenjang, alongside *Bacillus* [12–16].

The genera *Enterococcus* and *Tetragenococcus* are included in the family Enterococcaceae [17]. *Enterococcus* species have been detected in fermented foods such as dairy foods [18–20], while *Tetragenococcus* species are widely detected in high-salt-fermented food products including fish, soy pastes, and soy sauce, and are considered potential starters for the production of these foods [21]. Although 16S rRNA gene sequences of species from the two genera are very similar [22], *Enterococcus faecalis* and *Enterococcus faecium* cannot grow in medium with NaCl concentrations greater than 7% (w/v), while *Tetragenococcus halophilus* can grow in media with NaCl concentrations of up to 21% (w/v) [23–25].

Recently, we examined the diversity of cultivable bacteria using media supplemented with NaCl to understand changes in the bacterial community during processing from meju to doenjang [23]. *E. faecalis* and *E. faecium*, together with bacilli and coagulase-negative staphylococci, were the predominant bacterial species during meju fermentation. However, the community composition shifted to include

salt-tolerant bacilli, coagulase-negative staphylococci, and *T. halophilus* as the predominant members during doenjang fermentation, which involved inoculation via sea salt during the brining process. These results showed that *Enterococcus* sp. and *T. halophilus* are candidate starter species for meju and doenjang, respectively.

We have also assessed the safety and technological properties of *E. faecium*, *E. faecalis*, and *T. halophilus* strains from fermented soybeans to select functional and safe starter candidates [24–26]. The selected starter candidates (*E. faecium* and *T. halophilus*) were inoculated into sterilized soybeans to assess their contributions to the sensory properties of the products [6]. Our results showed that *E. faecium* and *T. halophilus* produced a similar profile of volatile compounds in soybeans, with no dramatic differences in soybean flavor. However, no comprehensive picture of the cellular components and metabolic pathways involved in the degradation of macromolecules and the development of sensory properties by *E. faecium* and *T. halophilus* during soybean fermentation has been obtained. In addition, currently available genomic data are insufficient to identify the genomic features of various strains that contribute to differences in salt tolerance [22, 27].

In the current study, we performed a comparative genomic analysis of *Enterococcus* and *Tetragenococcus* species to define the scale and scope of the pan-genome and to identify the core genes, as well as clarify the genetic background of strains from different niches, with particular reference to salt tolerance.

Materials and Methods

Comparative Genomics

For comparative genomic analysis, genome sequence data for 10 *E. faecalis*, 11 *E. faecium*, and three *T. halophilus* strains were obtained from the NCBI genome database (http://ncbi.nlm.nih.gov/genomes) (Table 1). *T. halophilus* KUD23 was isolated and selected as a starter candidate for the fermentation of high-salt foods in our previous study [26]. Average nucleotide identity, which provides a robust measurement of genetic distance among bacterial genomes, was used for comparative analysis of the conserved genes among the genomes [28]. Genome sequences of the 24 strains were uploaded to the Rapid Annotations using the Subsystems Technology server for SEED-based automated annotation, whole-genome sequence-based comparative analysis, and Kyoto Encyclopedia of Genes and Genomes metabolic pathway analysis [29]. The efficient database framework for comparative genome analyses using basic local alignment search tool (BLAST) score ratios was used for core genome, pan-genome, and singleton analyses [30]. The genome of *T. halophilus* KUD23 was used as a reference genome for Venn diagram construction. Further comparative analyses were performed for specific regions and genes of interest using the BLASTN, BLASTX, and BLASTP tools.

Growth in the Presence of NaCl

E. faecalis KCTC 2011, *E. faecium* KCCM 12118, and *T. halophilus* KUD23 were analyzed for growth in the presence of 3.5%, 7%, or 14% (w/v) NaCl. In addition, the three strains were assayed for growth on tryptic soy agar (TSA; Difco, USA) containing 7% (w/v) NaCl and 0.25% (w/v) choline, citrate, glycerol, or glycine betaine. Strains were cultured in tryptic soy broth (TSB; Difco), normalized to a turbidity of 1.0 at an optical density of 600 nm, and then diluted 1:10 in fresh TSB. A 10-μl aliquot of diluted sample was then dropped onto the surface of a TSA plate supplemented with NaCl at concentrations of 3.5%, 7%, or 14% (w/v). Cell growth was then assessed following incubation at 30°C for 48 h.

Results and Discussion

Genome Summary and General Features

The general features of the genomes of the 24 strains are summarized in Table 1. The average genome sequence lengths of the 10 *E. faecalis*, 11 *E. faecium*, and three *T. halophilus* strains were 3,018,574, 3,045,285, and 2,517,102 bp, respectively. Among the strains, *T. halophilus* strain MJ4 from the high-salt fermented food myeolchi-jeot (pickled anchovy), had the smallest genome (2,389,470 bp), while *E. faecium* strain 6E6 from human feces had the largest genome (3,397,850 bp). The average G+C content percentages of the *E. faecalis*, *E. faecium*, and *T. halophilus* genomes were 37.44%, 37.97%, and 36.3%, respectively. All *T. halophilus* genomes displayed a similar G+C content, which was low compared with the other two species.

To allow a coherent comparative analysis, we performed consistent open reading frame (ORF) predictions for the complete genome sequences of the 24 strains. An average of 2,987, 3,042, and 2,393 ORFs were identified in the *E. faecalis*, *E. faecium*, and *T. halophilus* genomes, respectively (Table 1). Notably, BLAST-based functional *in silico* prediction was achieved for 86.2%, 85.5%, and 96.3% of the identified ORFs in *E. faecalis*, *E. faecium*, and *T. halophilus*, respectively.

Analysis using Clusters of Orthologous Groups (COG) functional categorization and SEED subsystem categorization predicted the existence of an average of 2,576 coding sequences (CDSs) and 2,056 CDSs, respectively, in *E. faecalis*, 2,599 and 1,885 CDSs, respectively, in *E. faecium*, and 2,305 and 1,747 CDSs, respectively, in *T. halophilus*. Based on COG functional categorization, genes involved in carbohydrate transport and metabolism (average 10.2%) were the most

Table 1. General genomic and specific phenotypic features of 24 lactic acid bacterial strains.

Species	Strain	Ref No.	Size (bp)	Chromosome size (bp)	G+C content %	No. of plasmids	COG	SEED	No. of rRNAs	No. of tRNAs	Other RNAs	Contigs	Origin	Country	Accession No.	Status
Enterococcus faecalis	62	A1	3,130,820	2,988,673	37.36	3	2,667	2,071	12	54	4	5	Healthy Norwegian infant	Norway	CP002491	Complete
	ATCC 29212	A2	3,048,130	2,939,972	37.36	2	2,571	2,078	12	61	4	3	*Homo sapiens* urine	USA	NZ_CP008816	Complete
	CLB21560	A3	3,243,540	3,111,017	37.08	2	2,737	2,112	12	66	4	3	*Homo sapiens* clinical	USA	NZ_CP019512	Complete
	D32	A4	3,062,500	2,987,450	37.44	2	2,594	2,037	12	54	4	3	Pig feces	Germany	NC_018221	Complete
	DENG1	A5	2,961,043	2,961,043	37.50	-	2,581	2,061	12	62	4	1	Sputum of 54-year-old male	China	NZ_CP004081	Complete
	KB1	A6	3,026,009	3,026,009	37.20	-	2,526	2,024	12	59	4	1	*Mus musculus*	Germany	NZ_CP015410	Complete
	LD33	A7	2,803,429	2,803,429	37.60	-	2,459	2,047	12	61	4	1	Traditional dairy product	China	NZ_CP014949	Complete
	OG1RF	A8	2,739,625	2,739,625	37.80	-	2,330	1,950	12	58	4	1	Patient with endocarditis	USA	NC_017316	Complete
	Symbioflor 1	A9	2,810,675	2,810,675	37.70	-	2,469	2,013	12	63	4	1	Healthy human adult	Germany	NC_019770	Complete
	V583	A10	3,359,970	3,218,031	37.35	3	2,822	2,167	12	59	4	4	Blood of a hospitalized patient	USA	NC_004668	Complete
Enterococcus faecium	2014-VREF-41	I1	3,280,730	3,009,007	37.59	4	2,749	1,893	18	68	4	5	*Homo sapiens* rectal swab	Korea	NZ_CP019208	Complete
	64/3	I2	2,572,333	2,572,333	38.20	-	2,205	1,859	18	68	4	1	*Homo sapiens*	Germany	NZ_CP012522	Complete
	6E6	I3	3,397,850	2,966,909	37.60	2	2,874	1,893	18	75	4	3	*Homo sapiens* feces	USA	NZ_CP013994	Complete
	ATCC 700221	I4	3,151,410	2,859,123	38.10	3	2,843	2,031	18	72	4	4	*Homo sapiens* feces	USA	CP014449	Complete
	Aus0004	I5	3,019,780	2,955,294	38.34	3	2,565	1,895	18	47	4	4	*Homo sapiens* blood	Australia	NC_017022	Complete
	E39	I6	3,108,030	2,790,106	37.82	5	2,611	1,772	18	70	4	6	Blood of a hospitalized patient	USA	NZ_CP011281	Complete
	ISMMS_VRE_1	I7	3,259,290	3,130,373	37.67	5	2,807	1,997	18	72	4	6	*Homo sapiens* blood	USA	NZ_CP012430	Complete
	NRRL B-2354	I8	2,849,890	2,635,572	37.85	1	2,438	1,882	18	48	4	2	Dairy utensils	USA	NC_020207	Complete
	T110	I9	2,737,960	2,693,877	38.46	1	2,299	1,838	18	65	4	2	Medical	India	NZ_CP006030	Complete
	UW8175	I10	2,878,190	2,598,959	38.02	3	2,472	1,808	18	66	4	4	*Homo sapiens*	Germany	NZ_CP011828	Complete
	VRE001	I11	3,242,670	2,933,966	37.81	3	2,731	1,871	18	72	4	4	*Homo sapiens* blood	USA	NZ_CP018071	Complete
Tetragenococcus halophilus	KUD23	T1	2,599,117	2,599,117	36.10	-	2,390	1,795	15	62	4	1	Doenjang	Korea	NZ_CP020017	Complete
	MJ4	T2	2,389,470	2,389,470	36.00	-	2,173	1,689	15	62	4	1	Myeolchi-jeot	Korea	NZ_CP012047	Complete
	NBRC 12172	T3	2,562,720	2,562,720	36.00	-	2,351	1,756	15	62	4	1	Soy sauce brewing	Japan	NC_016052	Complete

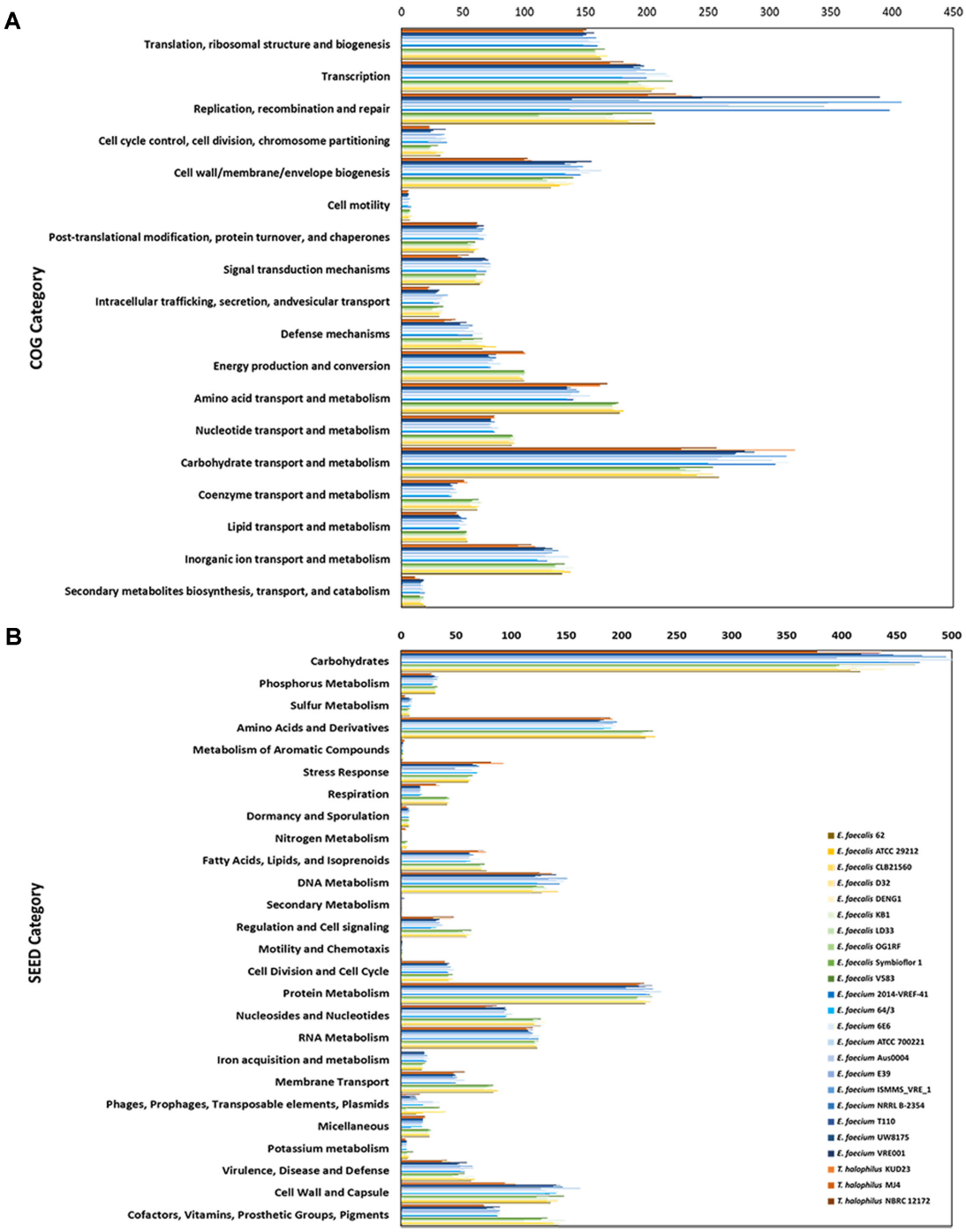

Fig. 1. Average numbers of genes in functional categories in 10 *E. faecalis*, 11 *E. faecium*, and three *T. halophilus* genomes based on Clusters of Orthologous Groups (COG) (**A**) and SEED (**B**) analyses.

Genome sequences of 24 strains were independently uploaded to the COG and SEED viewer servers. Functional roles of annotated genes were assigned and grouped in subsystem feature categories. Colored bars indicate the number of genes assigned to each category.

abundant category in *E. faecalis*, followed by those required for transcription (average 8.5%). Genes required for replication, recombination, and repair (average 12.3%) and carbohydrate transport and metabolism (average 12.5%) were the most abundant categories in *E. faecium* and *T. halophilus*, respectively. Based on the SEED subsystem, a

large number of genes from all three species were allocated to protein metabolism (10.7%–12.5%) and amino acid biosynthesis and utilization (9.9%–10.9%) (Fig. 1). Although the two types of analysis showed different percentages among the three species, the major functional groups were similar.

Comparative Analysis of the *E. faecalis*, *E. faecium*, and *T. halophilus* Genomes

For whole-genome comparison of the 10 *E. faecalis*, 11 *E. faecium*, and three *T. halophilus* strains, we analyzed the core and pan-genomes of the different species. Genes shared across all *E. faecalis* strains decreased with each addition, finally reaching a plateau around 2,007 genes (Fig. 2), while the pan-genome predicted at least 4,818 genes. Analysis of the 11 *E. faecium* strains resulted in core and pan-genomes of approximately 1,652 and 4,461 genes, respectively. The core genome accounted for approximately 63.6%–77.9% of all genes in each genome. Because of the limited number of available genomes, it was not possible to determine core and pan-genomes for *T. halophilus*.

Common strain-specific genes among the pan-genomes of each species included phage transferable elements such as relaxase, genes encoded on plasmids, and genes encoding hypothetical proteins (Table S1). Our findings suggested variability in gene content between species, as well as among strains of the same species. These results once again implied genomic plasticity among species living in different habitats with diverse lifestyles. An open pan-genome is typical of species that colonize multiple environments and have various ways of exchanging genetic material.

We next analyzed the genes shared among three representative species from food—*E. faecalis* LD33 from a traditional dairy product from China, *E. faecium* NRRL B-2354 from dairy utensils, and *T. halophilus* KUD23 from fermented soybean (Fig. 3 and Table S1). The three strains shared 1,084 CDSs within the core genome, corresponding to approximately 44.08%–45.36% of all ORFs. We assume that this core genome is very small because of low genomic similarity among these three representative strains. Most CDSs in the core genome were assigned functions via COG annotation that related to metabolism and the transport of amino acids and carbohydrate utilization. The majority of strain-specific genes were associated with hypothetical proteins, phage proteins, or specific plasmids. Other functional strain-specific genes in the genomes of strains LD33, NRRL B-2354, and KUD23 included streptococcin-encoding genes, tetracycline resistance major facilitator superfamily efflux protein-encoding genes, and CRISPR-associated protein-encoding genes, respectively. Unique genes in *T. halophilus* were related to potassium transport, glycine/betaine transport, an aspartate-alanine antiporter, an anion permease, and electron transport. These *T. halophilus*-specific genes were detected in all three examined *T. halophilus* genomes. These results implied that the higher salt-tolerance of *T. halophilus* compared with the *Enterococcus* species might be associated with these transporters.

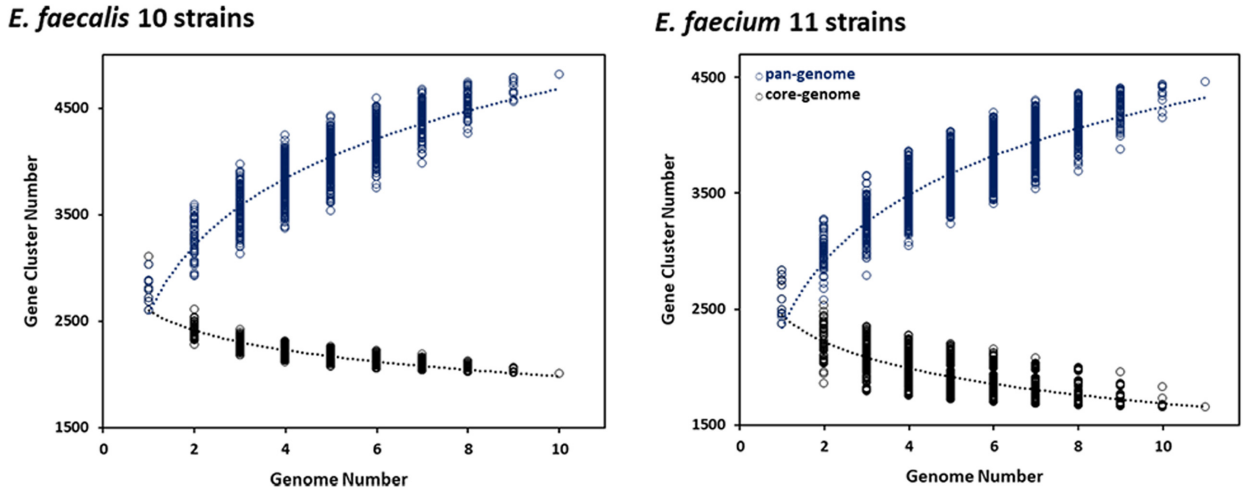

Fig. 2. Sizes of the core and pan-genomes of the 10 *E. faecalis* and 11 *E. faecium* strains.
The black (core genome) and blue (pan-genome) curves were fitted to the decay function ($916.810 \times \exp(-x/3.226) + 2010.866$ for *E. faecalis* and $904.475 \times \exp(-x/4.889) + 1589.834$ for *E. faecium*) and Heap's law function ($2503.183 \times x^{0.236}$ for *E. faecalis* and $2402.594 \times x^{0.463}$ for *E. faecium*), respectively. Each dot shows the gene cluster number of the individual genome.

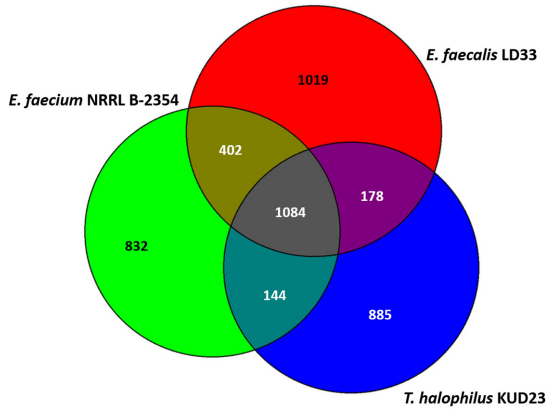

Fig. 3. Venn diagram of *E. faecalis*, *E. faecium*, and *T. halophilus* genomes.

Venn diagram generated using the Efficient Database framework for comparative Genome Analyses using BLAST score Ratios (EDGAR). Overlapping regions represent coding sequences (CDSs) shared between species genomes. The numbers outside the overlapping regions indicate the numbers of CDSs in each genome without homologs in the other species.

Insights into Salt Tolerance

Bacteria respond to osmotic pressure by accumulating or releasing solutes [31]. Relevant solutes include inorganic ions such as K^+, along with organic molecules known as osmolytes. Therefore, we focused our analysis on genes related to the synthesis and transport of these solutes to explain the differences in salt tolerance between the three species.

Inorganic molecules. *T. halophilus* is better able to survive high-salt conditions than many other food-associated bacteria, including *E. faecalis* and *E. faecium* [23–25]. However, the mechanism that allows *T. halophilus* to tolerate such high-salt conditions is not well understood. Potassium (K^+) plays a pivotal role in, and is the most abundant ion in the bacterial cytoplasm [32]. It is required for the activity of intracellular enzymes and is involved in the maintenance of a constant internal pH and membrane potential. Potassium also has an important function as an osmotic solute. Many bacteria, including halophiles, accumulate potassium ions within the cell in response to increases in external NaCl concentrations, although compatible organic solutes are preferred [33, 34]. A rapid import of potassium ions is triggered in Gram-positive bacterium *Bacillus subtilis* in response to osmotic upshock [35]. Holtmann *et al*. also reported that potassium ion concentrations decreased under osmotic downshock [36]. Bacteria usually express multiple specific uptake systems involved in the adjustment of potassium ion concentration.

We hypothesized that the *T. halophilus* genome would contain more genes related to potassium transport than are found in the genomes of the two less salt-tolerant *Enterococcus* species. As shown in Fig. 4, we identified potassium transporter genes in the genomes of all three species. *T. halophilus* contained two potassium transport systems—a Trk potassium uptake family system and potassium transporter YbaL—while *E. faecalis* and *E. faecium* only contained the genes for the Trk system. Based on the pan-genome analysis, *T. halophilus* was predicted to contain a greater number of potassium uptake genes than the other strains (Table S1); however, this was not supported by EC number matching of specific genes and direct gene similarity analysis.

Organic molecules. To achieve salt tolerance, bacterial proteins undergo extensive amino acid substitution with aspartyl, glutamyl, and weakly hydrophobic residues [37]. Solute accumulation stimulates bacterial growth at high osmotic pressure, and solute release allows cells to survive osmotic downshock. Studies of bacterial osmoregulation have focused on enzymes, transporters, and channels mediating solute accumulation and release.

Compatible solutes such as glycine betaine, proline betaine and carnitine accumulation can be accomplished through biosynthesis and/or transport from the environment. Representative transporters of compatible solute in *B. subtilis* are osmoprotectant uptake (Opu) systems [38]. All three species examined in the current study commonly contained three Opu systems, OpuB, OpuC, and OpuD, while only *T. halophilus* contains the additional OpuA system (Fig. 4). While OpuA, OpuB, and OpuC belong to the ATP-binding cassette transporter superfamily, OpuD is a single-component transporter belonging to the BCCT family; however, all Opu import systems are known to be osmotically regulated [38]. Chun *et al*. recently showed that *T. halophilus* contains the OpuA and OpuC systems [39], while Lin *et al*. reported that OpuA (BusA) plays an important role in adaptation to high-salt conditions [40]. In the current study, only the *T. halophilus* strains contained all of the genes required for a functional OpuA system, with the *E. faecalis* and *E. faecium* strains missing the solute-binding, protein-encoding gene (Fig. 4). Overall, our analysis showed that the *T. halophilus* strains possessed four Opu systems (OpuA, OpuB, OpuC, and OpuD), while the *E. faecalis* and *E. faecium* species only contained OpuB, OpuC, and OpuD (Fig. 4). Therefore, we suggest that *T. halophilus* has an advantage in the uptake of compatible solutes, not only glycine betaine but also proline betaine, compared with *E. faecalis* and *E. faecium*.

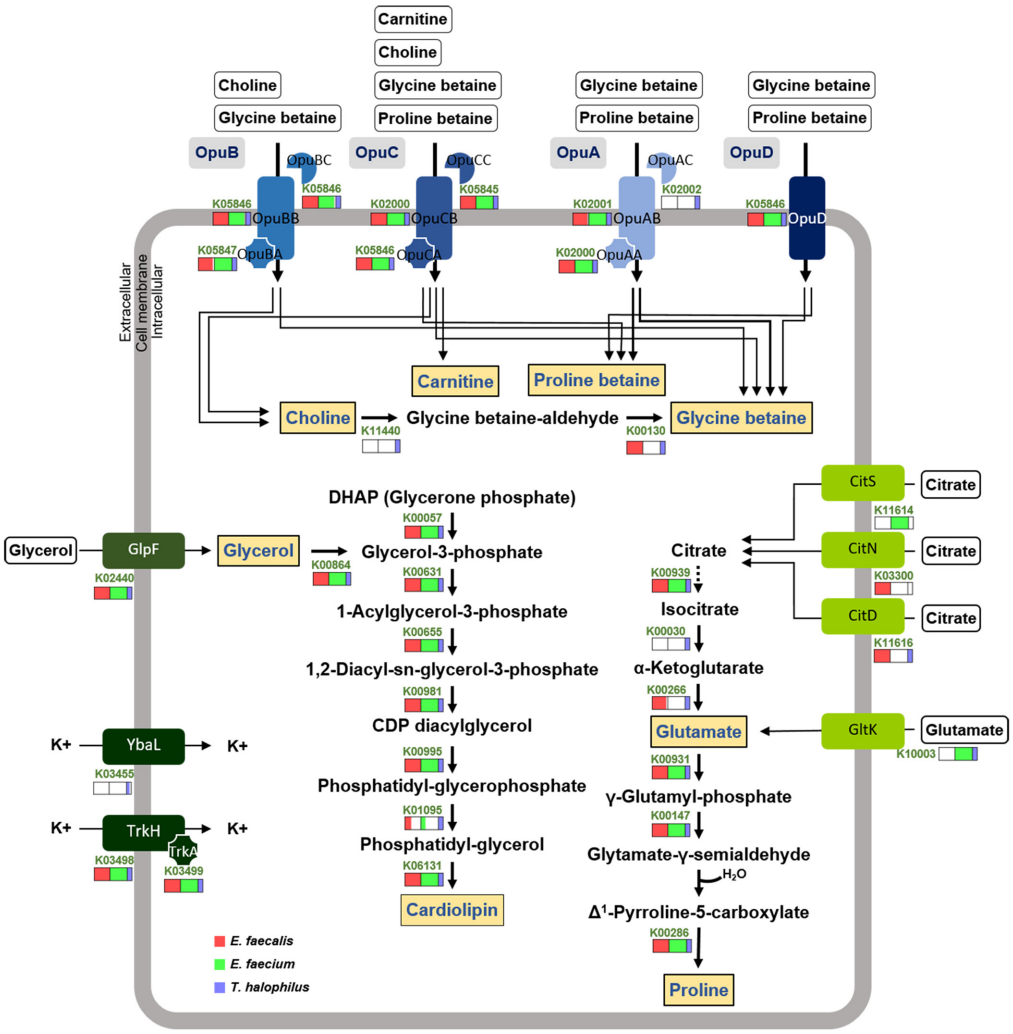

Fig. 4. Predicted membrane transport systems and synthesis pathways for osmoprotectants in the *E. faecalis*, *E. faecium*, and *T. halophilus* genomes.

Enzymes are marked with suggested EC numbers or KEGG numbers. Osmoprotectants are depicted in orange. Black arrows correspond to potential enzymatic reactions catalyzed by gene products encoded by the three genomes.

Glycine betaine is one of the most potent and widely used compatible solutes in nature. Metabolic analysis of *T. halophilus* under high-salt conditions suggested that glycine betaine was the main compatible solute in this species [39]. The precursor of glycine betaine, choline, must be taken up from exogenous sources via the Opu transporters. The abilities of these systems to transport choline have been confirmed previously in *B. subtilis* [38]. The presence of four high-affinity, osmotically-regulated choline and/or glycine betaine transporters therefore gives *T. halophilus* an advantage over the other two species with reference to choline uptake. All of the *T. halophilus* strains also contained genes encoding a soluble type III alcohol dehydrogenase (*gbsB*; K11440) and a glycine betaine aldehyde dehydrogenase (*gbsA*; K00130), both of which are required for the synthesis of glycine betaine from choline [41] (Fig. 4). However, *gbsB* was not identified in any of the *E. faecalis* or *E. faecium* genomes, although choline transporter genes were detected (Fig. 4). Although it appears that glycine betaine cannot be synthesized from choline in *E. faecalis* and *E. faecium*, three glycine betaine transporters were detected in these species, and glycine betaine enhanced the growth of *E. faecalis* and *E. faecium* on TSA plates containing 7% NaCl. Choline also increased the growth of the two species, although to a lesser extent than glycine betaine (Fig. 5).

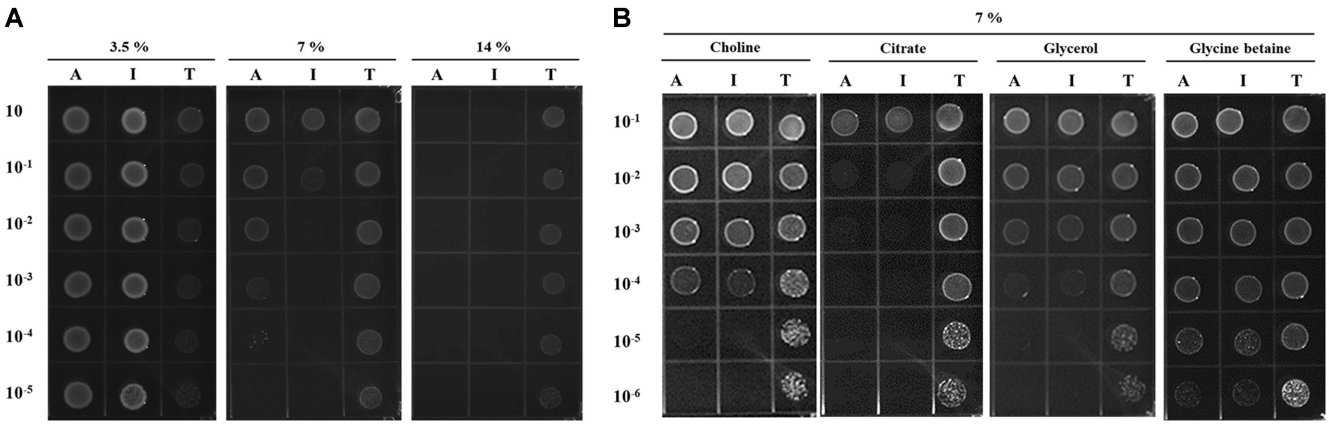

Fig. 5. Growth of *E. faecalis*, *E. faecium*, and *T. halophilus* in tryptic soy agar supplemented with NaCl at different concentrations (**A**) or with various osmoprotectants and NaCl (7%, w/v) (**B**).
Abbreviations: A, *E. faecalis* KCTC 2011; I, *E. faecium* KCCM 12118; T, *T. halophilus* KUD23.

The lipid cardiolipin also plays a role in adaptation to high salinity stress [42, 43], and was shown to be necessary for the prolonged survival of *Staphylococcus aureus* under high-salt conditions [43, 44]. *Staphylococcus equorum* strains containing cardiolipin synthesis genes also showed salt tolerance [45]. Cardiolipin is synthesized from glycerol-3-phosphate (Fig. 4), and our analysis revealed that *T. halophilus*, *E. faecalis*, and *E. faecium* all contained genes allowing the synthesis of glycerol-3-phosphate from glucose and glycerol. The complete cardiolipin synthesis pathway was present in all of the examined *T. halophilus* strains, four *E. faecalis* strains, and three *E. faecium* strains. These results imply that cardiolipin synthesis is strain-specific in *E. faecalis* and *E. faecium* species. To determine whether the addition of glycerol improved the salt tolerance of the three examined bacterial species, potentially indicating the synthesis of cardiolipin, the growth of *E. faecalis* KCTC 2011, *E. faecium* KCCM 12118, and *T. halophilus* KUD23 was assessed on TSA supplemented with 7% NaCl and 0.25% glycerol (Fig. 5B). Glycerol increased the growth of all three strains, suggesting that glycerol was used as an osmoprotectant. However, it remains unclear whether cardiolipin was synthesized from glycerol.

Proline is another compatible solute. *T. halophilus* and the two *Enterococcus* species did not possess a proline transporter, but did contain genes for the biosynthesis of proline from glutamate or citrate (Fig. 4). Glutamate is also a compatible solute, and a specific glutamate transporter was found in *E. faecium* and *T. halophilus*. However, only the *T. halophilus* strains contained a dedicated biosynthesis pathway for the production of glutamate from citrate, although most of the examined strains from all three species possessed a citrate transporter (Table 2). Metabolic analysis of *T. halophilus* under high-salt conditions revealed a decrease in the activity of the glutamate importer [39], assuming that proline may be synthesized from citrate. Accumulation of intracellular proline in *T. halophilus* led to protect cells against salt stress [46]. Therefore, we hypothesize that *T. halophilus* has an advantage over the other two species in the synthesis of proline as a compatible solute. In addition, citrate did not enhance the growth of *E. faecalis* or *E. faecium* compared with that of *T. halophilus* on TSA containing 7% NaCl, although these results are not direct evidence of proline synthesis from citrate (Fig. 5A).

We hypothesized that the *T. halophilus* genome would contain transporter systems and/or compatible solute biosynthesis genes that distinguished it from the *Enterococcus* species. Comparative genomic analysis confirmed that the *T. halophilus* genome contains a greater number of transporter systems and compatible solute biosynthesis genes than are found in the genomes of the *Enterococcus* species. These results suggest that *T. halophilus* has an advantage in the uptake of compatible solutes such as glycine betaine and in the synthesis of solutes such as cardiolipin and proline compared with *E. faecalis* and *E. faecium*, although it did not possess distinctive salt tolerance genes.

Based on comparative genomic analysis, we predicted that *E. faecalis* would be more salt tolerant than *E. faecium*. To test this, we examined the growth of *E. faecalis* KCTC 2011, *E. faecium* KCCM 12118, and *T. halophilus* KUD23 on TSA supplemented with 3.5%, 7%, or 14% (w/v) NaCl (Fig. 5B). Overall, the growth rates matched our predictions based on genomic analysis: *E. faecalis* contained more genes required for the uptake of compatible solutes compared

Table 2. Osmoprotectant synthesis- and transporter system-related genes in the genomes analyzed in this study.

	Gene	KEGG	Product	Strain reference number (refer to Table 1)																						
				A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	I1	I2	I3	I4	I5	I6	I7	I8	I9	I10	I11	T1	T2	T3
Inorganic solute																										
Transporter	*ybaL*	K03455	Potassium transporter																					•		•
	trkH	K03498	Ktr system potassium uptake protein D	•	•	•	•	•	•	•	•	•	•	•	•	•	-	•	•	•	•	•	•	•	•	•	•
	trkH	K03498	Trk family potassium uptake protein	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	trkA	K03499	TrkA family potassium uptake protein	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Organic solute																										
Transporter	*glpF*	K02440	Glycerol uptake facilitator and related aquaporins	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	opuBA	K05847	ABC transporter ATP-binding protein	•		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	opuBB	K05846	ABC transporter permease	•	•	•	•	•	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•	•	•	•
	opuBC	K05846	Glycine/betaine ABC transporter permease	•	•	•	•	•	•	•	•	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•
	opuBC	K05846	HTH-type transcriptional repressor GbsR																						•	•	•
	opuCA	K05846	Glycine/betaine ABC transporter ATP-binding protein	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•		•	•	•	•	•	•
	opuCB	K02000	Glycine betaine/L-proline ABC transporter ATP-binding protein																						•	•	•
	opuCC	K05845	Osmoprotectant ABC transporter substrate-binding protein	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	opuCC	K05845	Osmoprotectant ABC transporter substrate-binding protein																						•	•	•
	opuCC	K05845	Osmoprotectant ABC transporter substrate-binding protein																						•	•	•
	opuAA	K02000	Glycine betaine/L-proline ABC transporter ATP-binding protein	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	opuAA	K02000	Glycine betaine/L-proline ABC transporter ATP-binding protein																							•	•
	opuAA	K02000	Glycine betaine/L-proline ABC transporter ATP-binding protein			•		•		•	•		•												•	•	•
	opuAB	K02001	Glycine/betaine ABC transporter permease	•		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	opuAC	K02002	Glycine/betaine ABC transporter permease																						•	•	•
	opuAC	K02002	Glycine/betaine ABC transporter permease																						•	•	•
	opuAC	K02002	Glycine/betaine ABC transporter permease																						•	•	•
	opuAC	K02002	Glycine/betaine ABC transporter																						•	•	•

Table 2. Continued.

	Gene	KEGG	Product	Strain reference number (refer to Table 1)																						
				A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	I1	I2	I3	I4	I5	I6	I7	I8	I9	I10	I11	T1	T2	T3
	opuAC	K02002	Glycine/betaine ABC transporter substrate-binding protein																						•	•	•
	opuD	K05846	Osmoprotectant transporter system permease protein	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	citS	K11614	Citrate-sodium symporter											•	•	•		•	•	•	•	•	•	•			
	citN	K03300	Citrate-transporter	•	•	•	•	•	•	•	•	•	•														
	citD	K11616	Citrate lyase acyl carrier protein	•	•	•	•	•	•	•	•	•	•												•	•	•
	gltK	K10002	Glutamate/aspartate transport system permease protein											•	•	•	•	•	•	•	•	•	•	•	•	•	•
Synthesis	*gk*	K00864	Glycerol kinase	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	gpsA	K00057	NAD(P)H-dependent glycerol-3-phosphate dehydrogenase											•	•	•	•	•	•	•	•	•	•	•			
	plsY	K00631	Glycerol-3-phosphate 1-O-acyltransferase PlsY	•	•		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	plsY	K00631	Glycerol-3-phosphate 1-O-acyltransferase PlsY	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	plsC	K00655	1-Acyl-*sn*-glycerol-3-phosphate acyltransferase	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	cdsA	K00981	phosphatidate cytidylyltransferase	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	pgsA	K00995	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	pgpA	K01095	Phosphatidlyglycerophosphatase			•					•	•	•	•						•	•				•	•	•
	clsA	K06131	Cardiolipin synthase	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	clsA	K06131	Cardiolipin synthase	•	•	•	•	•	•	•	•	•	•												•		•
	maeN	K11616	Damage-inducible protein CinA																						•	•	•
	AK	K00939	Adenylate kinase	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	IDH3	K00030	Isocitrate/isopropylmalate dehydrogenase family protein																						•	•	•
	gltD	K000266	Glutamate synthase (NADPH), homotetrameric	•	•	•	•	•		•	•	•	•												•	•	•
	proB	K00931	Glutamate 5-kinase	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	proA	K00147	Glutamate-5-semialdehyde dehydrogenase	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	proC	K00286	Pyrroline-5-carboxylate reductase	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	proC	K00286	Pyrroline-5-carboxylate reductase	•	•	•	•	•	•	•	•	•	•														
	gpsB	K11440	NAD-dependent alcohol dehydrogenase																						•	•	•
	gpsA	K00130	Betaine-aldehyde dehydrogenase	•	•	•	•	•	•	•	•	•	•												•	•	•

Strain reference numbers: A1–A10, *E. faecalis*; I1–I11, *E. faecium*; T1–T3, *T. halophilus*.

with *E. faecium*, and *E. faecalis* grew better than *E. faecium* in the presence of 7% NaCl. *T. halophilus* had a faster growth rate than the other two species in the presence of 7% NaCl, and was the only strain to grow in the presence of 14% NaCl.

In conclusion, the genera *Enterococcus* and *Tetragenococcus* cannot readily be distinguished by phylogenetic analyses based on 16S rRNA gene sequences (Fig. S1) but show significant differences in salt tolerance. Here, comparative genomic analysis revealed several species-specific determinants of salt tolerance. The identification of such factors will help in the selection of appropriate starters for applications such as fermentation (*i.e.*, low-salt or high-salt fermentation) used by the food industry.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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