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Genetic Diversity and Antibiotic Resistance of *Enterococcus faecalis* Isolates from Traditional Korean Fermented Soybean Foods

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Eighty-five *Enterococcus faecalis* isolates collected from animals (40 isolates), meju (a Korean fermented soybean product; 27 isolates), humans (10 isolates), and various environmental samples (8 isolates) were subjected to multilocus sequence typing (MLST) to identify genetic differences between samples of different origins. MLST analysis resulted in 44 sequence types (STs), and the eBURST algorithm clustered the STs into 21 clonal complexes (CCs) and 17 singletons. The predominant STs, ST695 (21.1%, 18/85) and ST694 (9.4%, 8/85), were singletons, and only contained isolates originating from meju. None of the STs in the current study belonged to CC2 or CC9, which comprise clinical isolates with high levels of antibiotic resistance. The *E. faecalis* isolates showed the highest rates of resistance to tetracycline (32.9%), followed by erythromycin (9.4%) and vancomycin (2.4%). All isolates from meju were sensitive to these three antibiotics. Hence, MLST uncovered genetic diversity within *E. faecalis*, and clustering of the STs using eBURST revealed a correlation between the genotypes and origins of the isolates.

Keywords: *Enterococcus faecalis*, multilocus sequence typing, meju, antibiotic resistance, diversity

Introduction

Enterococci, belonging to the group of genera collectively termed lactic acid bacteria, are widely distributed in plants, soil, animals, and food products, including fermented foods. Recent studies have shown that enterococci contribute to the ripening and aroma development of fermented foods such as cheese and sausages [1], although the presence of enterococci in cooked food products has long been considered an indication of poor sanitary conditions. In addition, they are used as starter cultures for the fermentation of food products, especially cheese [2]. Many enterococcal strains have interesting biotechnological traits [2, 3], such as bacteriocin production, as well as probiotic features. *Enterococcus faecium* SF68 (Cerbios-Pharma SA, Switzerland) and *Enterococcus faecalis* Symboflor 1 (SymbioPharm, Germany) are commercially used as probiotics to improve human or animal health.

Enterococci are also important nosocomial pathogens, causing diseases such as bacteremia, endocarditis, and urinary tract infections in humans [4]. Pathogenic isolates are often multidrug resistant, and display virulence factors such as hemolysis. Although *Enterococcus* species have very few intrinsic virulence traits, they readily acquire virulence factors, especially antibiotic resistance genes, when exposed to selective antibiotic pressure [5]. Notably, the prevalence of vancomycin-resistant enterococci has steadily increased over the past two decades. The National Healthcare Safety Network (https://www.cdc.gov/nhsn/) reported that 99.5% and 91.9% of clinical *E. faecium* and *E. faecalis* strains, respectively, showed resistance to vancomycin [6], although antibiotic resistance is far less common among enterococci of food origin [7]. Vancomycin resistance is not a direct virulence factor, but enhances the virulence of pathogenic enterococci [8, 9].

Recently, we used culture-dependent methods to understand

changes in the living bacterial community diversity during the production of doenjang (a fermented soybean paste) from meju (fermented soybeans) [10]. Enterococci, especially *E. faecalis* and *E. faecium*, were the predominant species in meju fermentation, together with bacilli and coagulase-negative staphylococci. *Enterococcus* species can grow in medium containing up to 7% (w/v) NaCl, and exhibit protease and lipase activities, as well as acid production. In particular, *E. faecalis* strains demonstrated higher protease and lipase activity than *E. faecium* strains under 6% and 4% NaCl conditions [10]. Thus, we concluded that *E. faecalis* could be a suitable starter culture for enhancing flavor development through proteolysis and lipolysis in meju fermentation. However, the use of *E. faecalis* as a starter should be carefully considered from a safety standpoint, as virulence traits have been identified in clinical isolates [4].

Previous studies have examined the diversity of *E. faecalis* sequence types (STs) of strains collected from animals, fecal samples from healthy humans, and from patients with bloodstream infections [11–15]. These results showed that specific clonal complexes (CCs) are associated with different strain origins and antibiotic resistance patterns; for example, *E. faecalis* strains belonging to CC2 were isolated from hospitalized patients and showed vancomycin resistance [15]. However, no specific genetic type differences have been correlated with *E. faecalis* from fermented foods. The genetic relatedness of *E. faecalis* strains has mostly been researched using two typing methods: pulsed-field gel electrophoresis (PFGE) [16] and multilocus sequence typing (MLST) [17]. Although PFGE is a standard and efficient typing method with a high degree of discrimination [18], MLST has the significant advantage of worldwide comparison using exchangeable data based on DNA sequence results [19]. In addition, MLST has been used to study the long-term epidemiology of bacterial species, and has provided insights into population structure and patterns of evolutionary descent [20]. Therefore, our goal was to investigate genetic differences among *E. faecalis* isolates from fermented foods, animals, and the environment using MLST, and to evaluate their potential for use as starter cultures. Additionally, antibiotic resistance profiles of each of the isolates was examined to understand the relationship with genetic diversity.

Materials and Methods

Bacterial Strains and Culture Conditions

Eighty-five *E. faecalis* isolates collected in Korea were used for MLST: 27 isolates from meju [10], 8 isolates from environmental samples, 10 isolates from humans [21], 13 isolates from chickens, 13 isolates from cows, and 14 isolates from pigs. The isolates from environment samples (catheter, aquarium, leather wastewater, and rice bran), humans, and farm animals (chickens, cows, and pigs) were kindly provided by the Korea Environmental Microorganisms Bank (http://knrrb.knrrc.or.kr), Kangwon National University, and the Animal and Plant Quarantine Agency of Korea (http://www.qia.go.kr), respectively. The taxonomic identities of all isolates were confirmed by sequence analysis of near-complete 16S rRNA gene regions [10], and all showed >99.9% identity to the type strain ATCC 19433. All *E. faecalis* isolates were cultured in de Man-Rogosa-Sharpe medium (MRS; Difco, USA) at 30°C for 24 h.

MLST

MLST of *E. faecalis* isolates was performed according to a previously published *E. faecalis* MLST protocol [17] using seven housekeeping genes: *gdh* (glucose-6-phosphate dehydrogenase), *gyd* (glyceraldehyde-3-phosphate dehydrogenase), *pstS* (phosphate-ATP binding cassette transporter), *gki* (glucokinase), *aroE* (shikimate 5-dehydrogenase), *xpt* (xanthine phosphoribosyltransferase), and *yqiI* (acetyl-CoA acetyltransferase).

Genomic DNA used as a template for MLST was extracted using a DNeasy Tissue Kit (Qiagen, Germany) following cell wall lysis by incubation with lysozyme at 37°C for 30 min. Housekeeping genes for MLST were amplified using a T3000 Thermocycler (Biometra, Germany). The amplicons were purified using a PCR purification kit (SolGent, Korea), and sequenced by GenoTech (Korea). Sequence similarities were identified by BLASTX analysis of the GenBank database on the National Center for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov/).

MLST Data Analysis

The sequences of the seven amplified genes from each isolate were proofread using SeqMan (DNASTAR, USA), and high-quality double-stranded sequences were used for further analyses. The rate of occurrence of genetic diversity and the ratio of non-synonymous (dN) to synonymous (dS) substitutions per nucleotide site were calculated using MEGA ver. 6.06 [22], according to the Nei and Gojobori method [23]. Allele numbers were assigned by comparing the sequence at each locus to known alleles in the *E. faecalis* MLST database (http://pubmlst.org/efaecalis/). The ST was determined through a combination of the seven alleles. New STs identified in the present study were deposited in the MLST database. Phylogenetic analysis based on the STs was performed using the maximum likelihood method. STs were analyzed to determine CCs using the eBURST algorithm to identify single-locus variants, double-locus variants, and singletons [19]. Singletons were defined as STs with at least two allelic mismatches with all other STs.

Antibiotic Susceptibility Test

Antibiotic minimum inhibitory concentrations (MICs) were determined using the broth microdilution method according to

the guidelines of the Clinical and Laboratory Standards Institute [24]. *E. faecalis* exhibits relatively high levels of resistance to erythromycin, tetracycline, and vancomycin [25–27], and thus all three antibiotics were selected to test antibiotic susceptibility. Two-fold serial dilutions were prepared for each antibiotic in deionized water, with final concentrations ranging from 0.5 to 2,038 mg/l. *E. faecalis* isolates were cultured in MRS broth and matched to a McFarland 0.5 turbidity standard (bioMerieux, France). The cultured isolates were further diluted (1:100) in cation-adjusted Mueller-Hinton broth (Oxoid, UK) containing 2% (v/v) horse blood (KisanBio, Korea) to achieve the desired inoculum concentration. The final inoculum density in each well was 5×10^5 colony forming units/ml. Microplates were then incubated at 35°C for 20 h. The MIC of each antibiotic was determined as the lowest concentration at which no turbidity was observed in the wells. Resistance to a particular antibiotic was defined as when the MIC value of a tested antibiotic was higher than the recommended breakpoint value for *E. faecalis* as defined by the European Committee on Antimicrobial Susceptibility Testing (http://mic.eucast.org). All of the experiments were conducted in triplicates on separate days.

Results and Discussion

Allelic Variation of the MLST Target Genes

Seven housekeeping gene fragments from all 85 *E. faecalis* isolates were sequenced, and the allelic variation of each gene sequence was determined. The number of polymorphic sites within each gene ranged from 8 (*gyd*) to 27 (*gki* and *aroE*) (Table 1). The most variable locus was *gki*, with 30 polymorphic sites, and *gyd* was the least variable, with only five polymorphic sites (Table 1). The average d_N/d_S ratio across all MLST genes was 0.4248, and it was assumed that these genes were not under positive selective pressure (*i.e.*, selection is against amino acid changes).

Forty-four different STs were identified for the 85 isolates, and 17 STs consisted of a single isolate (Table 2). Eleven new STs (ST695, ST696, ST697, ST698, ST699, ST700, ST703, ST704, ST705, ST706, and ST707) were identified as a result of new alleles for six of the seven genes (all except *gyd*): *gdh* (2 alleles), *pstS* (2), *gki* (3), *aroE* (5), *xpt* (1), and *yiqL* (5), whereas 12 new STs (STK1–STK12) were identified on the basis of a new combination of the existing alleles (Table 2). The predominant ST was ST695 (21.1%, 18/85 isolates), followed by ST694 (9.4%, 8/85). These two major STs only comprised isolates from meju. Fourteen of the STs (excluding the two major STs) comprised at least two isolates. Eight STs (ST277, ST314, ST387, ST403, ST694, ST695, ST699, and STK9) contained isolates originating from only one origin, whereas the remaining six STs contained isolates from two or three origins (Table 2).

A maximum likelihood phylogenetic tree was generated on the basis of the ST data (Fig. 1). The phylogenetic tree revealed three different clusters; however, isolates were not clustered on the basis of origin, although a few branches contained isolates from the same origin. The 27 isolates from meju were allocated into three STs (ST648, ST694, and ST695), which were located on distinct branches, and were clustered with isolates from other origins. Therefore, we assumed that the current MLST data for *E. faecalis* was descriptive at the strain level, but could not be used to infer a correlation between the genetic backgrounds and origins of the isolates.

Identification of Clonal Groups Based on STs

The eBURST algorithm clustered the 44 STs into 21 CCs and 17 singletons using the default stringent definition for groups, which assigned STs with shared alleles at six of the seven loci to the same group (Fig. 2). The dominant clonal group CC16 contained seven isolates from two STs, whereas CC4, CC21, CC165, CC648, and CC698 each contained two STs and comprised three, two, two, three, and three isolates, respectively.

The members of CC4, CC21, CC165, and CC698 were isolated from animals, whereas CC16 contained isolates from animals, humans, and the environment. CC648 included isolates from meju as well as animals. Again, clustering using eBURST did not present a clear division of

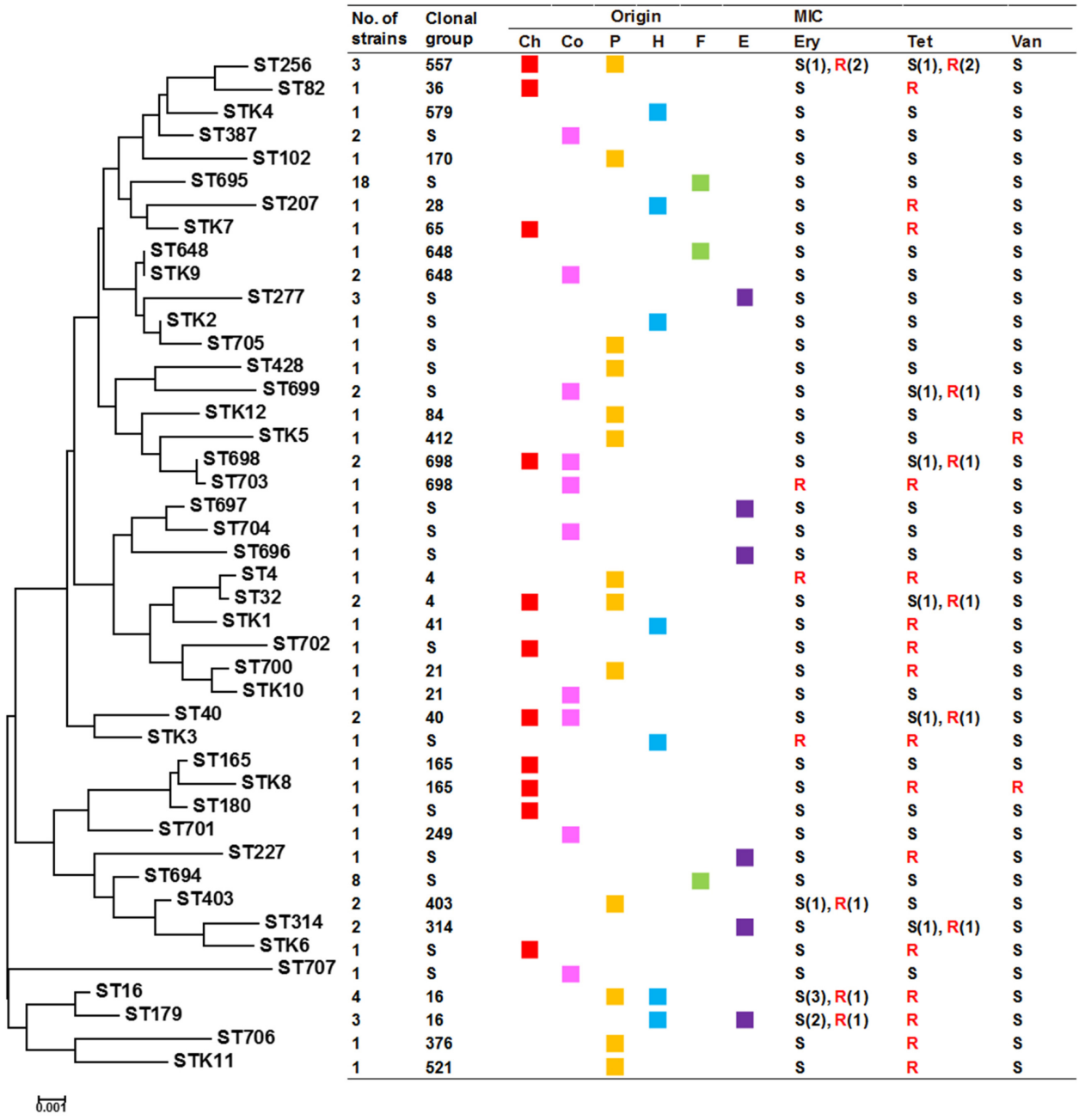
Table 1. Compositional characteristics of genes used in the multilocus sequence typing scheme for *Enterococcus faecalis*.

Gene	Length of sequenced fragment (bp)	Number of alleles	Number of polymorphic sites	d_N/d_S ratio
gdh	530	20	17	0.4803
gyd	395	8	5	0.2073
pstS	583	21	26	0.4277
gki	438	27	30	0.5045
aroE	459	27	22	0.4287
xpt	456	20	13	0.5267
yiqL	436	24	26	0.3984

Table 2. Sequence types of the *Enterococcus faecalis* strains used in this study, as well as their corresponding eBURST clonal group and allele numbers.

ST groups	Frequency	Origin	Clonal group	*gdh*	*gyd*	*pstS*	*gki*	*aroE*	*xpt*	*yiqL*
ST4	1	Pig	4	8	7	7	5	4	4	1
ST16	4	Human (3), Pig (1)	16	5	1	1	3	7	7	6
ST32	2	Chicken (1), Pig (1)	4	8	7	9	5	4	4	1
ST40	2	Chicken (1), Cow (1)	40	3	6	23	12	9	10	7
ST82	1	Chicken	36	12	6	28	29	7	10	20
ST102	1	Pig	170	9	1	27	31	31	24	14
ST165	1	Chicken	165	15	2	37	1	17	15	11
ST179	3	Human (2), Environment (1)	16	5	1	1	3	7	1	6
ST180	1	Chicken	S	15	15	37	16	3	15	11
ST207	1	Human	28	4	4	46	3	2	1	3
ST227	1	Chicken	S	25	6	52	56	3	5	51
ST256	3	Chicken (2), Pig (1)	557	4	6	7	29	8	1	20
ST277	3	Environment (3)	S	12	6	53	60	55	47	3
ST314	2	Environment (2)	314	9	6	4	49	11	15	61
ST387	2	Cow (2)	S	9	6	7	13	13	35	8
ST403	2	Pig (2)	403	11	5	4	16	11	13	10
ST428	1	Pig	S	29	6	30	45	60	14	4
ST648	1	Meju	648	12	10	3	17	31	2	5
ST694	8	Meju (8)	S	9	5	4	16	1	11	8
ST695	18	Meju (18)	S	12	2	17	32	88*	79*	4
ST696	1	Environment	S	19	2	15	85*	89*	22	10*
ST697	1	Environment	S	85*	2	7	12	26	1	1
ST698	2	Cow (1), Chicken (1)	698	14	2	2	10	91*	2	12
ST699	2	Cow (2)	S	10	5	83*	86*	90*	22	85*
ST700	1	Pig	21	1	7	9	1	1	1	84*
ST701	1	Cow	249	15	1	2	57	3	35	53
ST702	1	Chicken	S	17	7	28	24	10	20	1
ST703	1	Cow	698	14	2	2	10	91*	1	12
ST704	1	Cow	S	14	2	7	49*	26	1	1
ST705	1	Pig	S	12	7	27	11	60	1	86*
ST706	1	Pig	376	64	1	36	68	92*	25	30
ST707	1	Cow	S	86*	5	78*	53	82	36	83*
STK1	1	Human	41	27	7	11	21	1	4	1
STK2	1	Human	S	12	2	3	11	6	2	3
STK3	1	Human	S	27	2	1	32	7	2	6
STK4	1	Human	579	9	2	7	63	1	11	8
STK5	1	Pig	412	14	1	2	52	67	4	70
STK6	1	Chicken	S	9	6	4	16	11	4	61
STK7	1	Chicken	65	12	7	2	17	3	2	5
STK8	1	Chicken	165	15	2	37	12	39	15	11
STK9	2	Cow (2)	648	12	10	27	17	31	2	5
STK10	1	Cow	21	1	7	7	1	1	10	1
STK11	1	Pig	521	72	2	36	24	75	45	76
STK12	1	Pig	84	14	1	25	27	25	2	19

*Indicates a new allele. S, singleton.



No. of strains	Clonal group	Origin						MIC		
		Ch	Co	P	H	F	E	Ery	Tet	Van
3	557	■		■				S(1), R(2)	S(1), R(2)	S
1	36	■						S	R	S
1	579				■			S	S	S
2	S		■					S	S	S
1	170			■				S	S	S
18	S					■		S	S	S
1	28				■			S	R	S
1	65	■						S	R	S
1	648					■		S	S	S
2	648		■					S	S	S
3	S						■	S	S	S
1	S				■			S	S	S
1	S			■				S	S	S
1	S			■				S	S	S
2	S		■					S	S(1), R(1)	S
1	84			■				S	S	S
1	412			■				S	S	R
2	698	■	■					S	S(1), R(1)	S
1	698		■					R	R	S
1	S						■	S	S	S
1	S		■					S	S	S
1	S						■	S	S	S
1	4			■				R	R	S
2	4	■		■				S	S(1), R(1)	S
1	41				■			S	R	S
1	S	■						S	R	S
1	21			■				S	R	S
1	21		■					S	S	S
2	40	■	■					S	S(1), R(1)	S
1	S				■			R	R	S
1	165	■						S	S	S
1	165	■						S	R	R
1	S	■						S	S	S
1	249		■					S	S	S
1	S						■	S	R	S
8	S					■		S	S	S
2	403			■				S(1), R(1)	S	S
2	314						■	S	S(1), R(1)	S
1	S	■						S	R	S
1	S		■					S	S	S
4	16			■	■			S(3), R(1)	R	S
3	16				■		■	S(2), R(1)	R	S
1	376			■				S	R	S
1	521			■				S	R	S

Fig. 1. Phylogenetic analysis based on sequence typing data, strain origins, and antibiotic susceptibility profiles.
The data were compared using simple matching coefficients, and were clustered using the maximum likelihood method. Branches with bootstrap values <50% have been collapsed. The scale of the diagram is the pairwise distance expressed as the percentage dissimilarity. Colors represent the origins of the isolates: chicken (red), cow (pink), pig (yellow), human (blue), meju (green), and environment (purple). Ery, erythromycin; Tet, tetracycline; Van, vancomycin.

isolates according to their origin, but revealed a correlation between the CCs and the origins of the isolates.

Until now, there have only been five representative *E. faecalis* CCs: CC2, CC9, CC16, CC21, and CC40 [13, 17, 28]. Clinical and animal isolates commonly belong to CC2, CC9, or CC16. In particular, CC2 and CC9 isolates play a predominant role in the spread of antimicrobial resistance in hospitals, and contribute to higher resistance rates in some countries [13, 29]. Isolates belonging to these CCs tend to have high levels of antibiotic resistance and

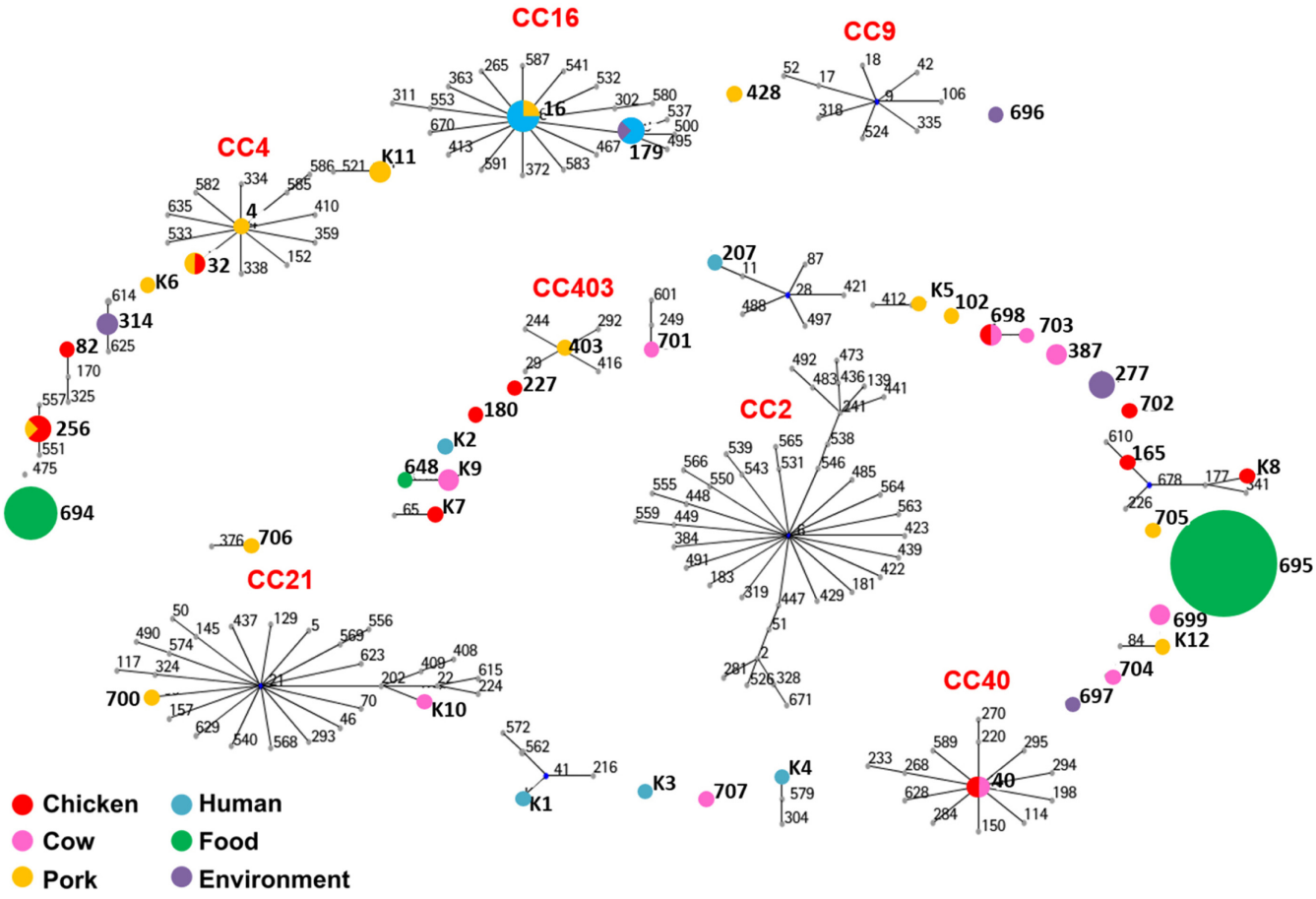


Fig. 2. eBURST analysis showing the clonal assignments of the sequence types (STs) identified in this study, as well as the multilocus sequence typing database reference dataset.

Each ST is represented by a dot, and the lines connect single-locus variants. The sizes of the dots are commensurate with the number of strains in the ST. The colors represent the origins of the isolates: chicken (red), cow (pink), pig (yellow), human (blue), meju (green), and environment (purple).

recombination, and demonstrate increased virulence and transmissibility [30–32]. In addition, CC2 and CC9 isolates have been associated with nosocomial infections [33]. Therefore, CC2 and CC9 are thought to be high-risk *E. faecalis* CCs. However, none of the isolates investigated in the current study belonged to CC2 or CC9.

The two ST403 isolates identified in this study, belonging to CC403, originated from poultry. According to the public MLST database, ST403 single-locus variants (designated ST29, ST244, ST292, and ST416) also tend to be associated with poultry. Thus, we hypothesized that CC403 clones might have become accustomed to poultry hosts. Similarly, ST4 in CC4 appears to be undergoing host expansion through both single- and double-locus variants, which have been isolated from poultry. ST4 has a broad host spectrum and is the predicted subgroup founder of CC4. CC40 appears to have originated from humans, animals, and food [12, 14, 34], and in the current study, ST40 isolates (chicken, n = 1; cow, n = 1) belonged to CC40.

In the current study, the 27 isolates from meju belonged to three STs: ST648 (1 isolate), ST694 (8 isolates), and ST695 (18 isolates), which did not belong to any of the major clonal groups. In a previous study, 11 of 30 *E. faecalis* isolates from French cheese were associated with CC25 and CC55, which are rarely associated with clinical isolates [35]. These results suggested that the clonal groups of *E. faecalis* from fermented foods might be distinguished from those of pathogenic strains, as CCs appear to depend on strain origin.

Antibiotic Resistance of *E. faecalis* Isolates Grouped by ST

To examine differences between the antibiotic resistance patterns of isolates from fermented foods and other sources, we tested the susceptibilities of all isolates to three

Table 3. Minimum inhibitory concentration (MIC) of three antibiotics against *E. faecalis* isolates.

Antibiotics	No. of isolates	MIC (μg/ml)												Break point (μg/ml)
		0.5	1	2	4	8	16	32	64	128	256	512	1,024	2,048	
Erythromycin	85	30	19	17	11	1			1		1		1	4	4
Tetracycline	85	34	18	3	2		3	12	9	4					4
Vancomycin	85	5	51	26	1				2						4

different antibiotics (Table 3). The antibiotics were selected on the basis of previous reports of a high prevalence of resistance amongst *E. faecalis* strains [7]. None of the *E. faecalis* isolates from meju exhibited resistance to the tested antibiotics (Fig. 1). The highest prevalence of resistance was observed for tetracycline (32.9%), followed by erythromycin (9.4%) and vancomycin (2.4%). Vancomycin resistance was only detected in isolates from animal samples, whereas erythromycin-resistant and tetracycline-resistant isolates were obtained from animals, humans, and environmental samples. As expected, there was a high level of antibiotic resistance amongst animal and human isolates, but antibiotic resistance patterns could not be linked to a classification criterion to define the origin of isolates. However, all isolates belonging to ST16 and ST179 of CC16 exhibited resistance to tetracycline, although they were isolated from three different niches: humans, animals, and the environment (Fig. 1). Similarly, Kuch *et al*. [13] showed that CC16 isolates from humans in Europe exhibited resistance to tetracycline, suggesting that tetracycline resistance may be a characteristic of CC16 strains.

The prevalence of vancomycin-resistant *E. faecalis* isolates worldwide is steadily increasing, and is a significant concern for human health [36]. However, according to the annual report of the Korean Government in 2015, there is generally little vancomycin resistance amongst bacteria from humans and animals in South Korea (http://www.mfds.go.kr; 2016). Only two *E. faecalis* isolates showed resistance to vancomycin in the current study (Table 3). However, 28 isolates (32.9%) demonstrated tetracycline resistance. Tetracycline is the most commonly used antibiotic in Korea, and tetracycline resistance is the most prevalent type of antibiotic resistance demonstrated by bacteria from Korea (http://www.mfds.go.kr; 2016). In the current study, tetracycline resistance was prevalent amongst the *E. faecalis* isolates, and was present in a strain-specific manner. Thus, we assumed that tetracycline resistance may be acquired by *E. faecalis* from the local environment. Interestingly, all isolates from meju were sensitive to the three antibiotics examined. Therefore, we assumed that the STs or CCs of food isolates could be distinguished from those of clinical isolates; however, no clear differences were observed.

This study is the first to use MLST to analyze the genetic diversity within *E. faecalis* isolated from the Korean fermented food meju and from several other sources. We could not identify a correlation between ST and the origin of the isolates, but clustering of the STs by eBURST analysis revealed a correlation between the genetic backgrounds and origins of the isolates, as well as antibiotic resistance patterns. Although the isolates used in this study could not be perfectly classified according to their origin, the applicability of this MLST scheme was proven by the clustering of isolates from meju. In addition, we confirmed that the meju isolates are likely to be safe for use as starter cultures in the food industry because of their antibiotic sensitivity and genetic clustering away from the clonal groups usually associated with pathogenicity.

Acknowledgments

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