Molecular characteristics of *optrA*-carrying *Enterococcus faecalis* from chicken meat in South Korea

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ABSTRACT The purpose of this study was to identify the genetic environment of *optrA* gene in linezolid (**LZD**)-resistant *Enterococcus faecalis* from chicken meat and to describe the probable mechanism of dissemination of the *optrA* gene through plasmid or chromosomal integration. Whole genome sequencing and analysis revealed that all 3 *E. faecalis* isolates confirmed as LZD- and chloramphenicol-resistant carried *fexA* adjacent to the *optrA* gene as well as a variety of resistance genes for macrolides, tetracyclines, and aminoglycosides, simultaneously. But, the other genes conferring LZD resistance, *cfr* and *poxtA*, were not detected in those strains. Two isolates harboring the *optrA* gene in their chromosomal DNA showed >99% similarity in arrangement to the transposon Tn*6674* and the transposase genes, *tnpA*, *tnpB*, and *tnpC* and were located in the first open reading frame for transposase. One isolate harboring an *optrA*-carrying plasmid also showed >99% similarity with the previously reported pE439 plasmid but had 2 amino acid changes (Thr96Lys and Tyr160Asp) and a higher minimum inhibitory concentration against LZD of 16 mg/L than that of pE439 (8 mg/L). Mobile genetic elements such as transposons or plasmids flanking the *optrA* gene conduct a crucial role in the dissemination of antimicrobial resistance genes. Further investigations are required to identify the way by which *optrA* is integrated into chromosomal DNA and plasmids.

Key words: *optrA*, *fexA*, *Enterococcus faecalis*, antimicrobial resistance, linezolid-resistance

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INTRODUCTION

Enterococci are significant pathogens in that they may transfer their antimicrobial resistance genes to other animals or humans via the food chain (Ogier and Serror, 2008). In particular, animal-origin *Enterococcus faecalis* seems to be a zoonotic threat as it has been reported to express similar phenotypes in animals and humans (Hammerum, 2012; Hasan et al., 2018). The ability to acquire and transfer plasmids and transposons carrying antimicrobial resistance genes and virulence genes of *E. faecalis* has enabled them to act as multidrug-resistant pathogens, which are of significant concern in many countries (Freitas et al., 2017; Tyson et al., 2018b).

Linezolid (**LZD**) is a member of a class of oxazolidinones that is used for the treatment of infections in human caused by vancomycin-resistant enterococci, methicillin-resistant *Staphylococcus aureus*, and multidrug-resistant gram-positive bacteria including enterococci (Bozdogan and Appelbaum, 2004; O'Driscoll and Crank, 2015). Although LZD is not used in food-producing animals, the appearance of LZD-resistant isolates in animals has been reported in China (Wang et al., 2015), South Korea (Tamang et al., 2017), the United States (Tyson et al., 2018a), Europe (De Jong et al., 2019), and Africa (Elghaieb et al., 2019). The most common mechanisms associated with oxazolidinone resistance among enterococci include mutations in the central loop of domain V of the 23S rRNA (Arias et al., 2010) and the plasmid-mediated antimicrobial genes such as *cfr,* which contributes resistance to oxazolidinones, phenicols, lincosamides, and streptogramin A (Long et al., 2006). Recently, a transferable oxazolidinone resistance gene, *optrA*, from *E. faecalis* of human and animal origins was reported worldwide such as in China (Wang et al., 2015), Italy

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(Brenciani et al., 2016), Spain (Ca'Mara et al., 2019), and Ireland and Malaysia (Mendes et al., 2014, 2016). The *optrA* gene encodes for an ATP-binding cassette F protein mediating resistance to both phenicols and oxazolidinones through target protection (Sharkey and O'Neill, 2018), which contributes antimicrobial resistance against LZD, tedizolid, and phenicols (Wang et al., 2015). Moreover, the location of the *optrA* gene on the plasmids of *E. faecalis* was revealed to be adjacent to the phenicol resistance gene, *fexA*, based on sequence analysis of *optrA*-positive *E. faecalis* (He et al., 2016; Kang et al., 2019). The *optrA-fexA* genes have also been reported to be present of chromosomal DNA (He et al., 2016). Although oxazolidinone resistance mediated by plasmids and transposons has been previously described (Bender et al., 2018; Chen et al., 2019), the genetic environment of chromosomal DNA or plasmids harboring *optrA* in Korea has not been reported.

In this study, we describe the genetic environments of the *optrA-fexA* genes in LZD-resistant *E. faecalis* isolated from chicken meat in South Korea and describe the probable mechanism involved in the dissemination of the *optrA* gene via plasmid and chromosomal integration.

MATERIALS AND METHODS

Bacterial Strains

Among a total of 345 *E. faecalis* isolates from 200 retail chicken meat samples previously described (Kim et al., 2018), after the disk diffusion test using broth microdilution method, 7 LZD-resistant *E. faecalis* isolates were analyzed to confirm the presence of the *optrA* gene by applying a previously published PCR protocol (Wang et al., 2015). Subsequently, 3 *E. faecalis* were shown to carry *optrA* and were analyzed for this study.

Antimicrobial Susceptibility Testing

Minimum inhibitory concentrations (**MICs**) were determined by using the broth microdilution method in the Sensititre custom panel KRVP2F (TREK Diagnostic Systems, West Sussex, England), in accordance with the manufacturer's instructions. The antimicrobial agents tested were ampicillin, chloramphenicol (**CHL**), ciprofloxacin (**CIP**), daptomycin, erythromycin (**ERY**), florfenicol (**FFN**), gentamicin, kanamycin, LZD, quinupristin/dalfopristin (**SYN**), streptomycin, tetracycline, tylosin tartrate (**TYLT**), tigecycline, vancomycin, and salinomycin. *E. faecalis* ATCC 29212 was used as the quality control strain, and the MIC values were interpreted in accordance with the Clinical and Laboratory Standards Institute (**CLSI**) guidelines (CLSI, 2019). When breakpoints were unavailable in the CLSI guidelines, the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme or the National Antimicrobial Resistance Monitoring System was applied (CDC, 2013; DANMAP, 2017).

Conjugation Experiment

The transferability of plasmids carrying the *optrA* gene was assessed by applying the broth-mating protocol using rifampicin-resistant and fusidic acid–resistant *E. faecalis* FA2-2 as the recipient strain as described previously (Werner et al., 2008; Tamang et al., 2017). Transconjugants were recovered after incubation at 37°C on BHI agar (Becton Dickinson) plates supplemented with 2 μg/mL LZD, 25 μg/mL rifampicin, and 25 μg/mL fusidic acid followed by antimicrobial susceptibility testing and PCR analysis of the *optrA* gene.

Whole Genome Sequencing and Analysis

Genomic DNA of optrA-positive *E. faecalis* was extracted from overnight cultures using the MasterPure Gram Positive DNA Purification Kit (Lucigen, WI) in accordance with the manufacturer's instructions. Genome sequencing, accomplished by using an Illumina HiSeq platform in accordance with standard Illumina protocols, was performed at Macrogen (Seoul, South Korea). The reads were de novo assembled using hierarchical genome assembly process 3. Genome annotation was performed using Rapid Annotation by Subsystem Technology version 2.0. The assembled genomes were initially screened for genes encoding antibiotic resistance, virulence, and multilocus sequence typing (**MLST**) using the *in silico* genomic tools ResFinder 3.1 and LREfinder 1.0, VirulenceFinder 1.5, and MLST 2.0, respectively, that are available through the Center for Genomic Epidemiology (http://www.genomicepidemiology.org). Plasmid content associated with *optrA* was analyzed using the contigs obtained by plasmidSPAdes that were annotated with Prokka v1.12 (http://vicbioinformatics.com). BLAST analysis with default parameters was performed to compare the contigs with known sequences contained in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Artemis tools and DNAPlotter of the Sanger Institute (http://www.sanger.ac.uk/Software/Artemis) were used for drawing the plasmid schemes and comparisons. All representative sequences of the known *optrA* genes described were obtained from GenBank in FASTA format.

RESULTS

Distribution of Antimicrobial Susceptibility and Genes Conferring Antimicrobial Resistance

Three strains (EFs 116-2, EFs 171-2, and EFs 17-1) were confirmed as LZD- and chloramphenicol-resistant with MICs of 8 to 16 mg/L and >32 mg/L, respectively, and each carried resistance genes for phenicol/oxazolidinone (*optrA* and *fexA*) (Table 1). The *cat* gene, related to phenicol resistance, was detected in only one strain, EFs 171-2. The 3 strains also showed resistance to ciprofloxacin, erythromycin, florfenicol, quinupristin/dalfopristin, tetracycline, and tylosin tartrate, and carried

Table 1. Antimicrobial resistance patterns and genetic features of 3 *optrA*-positive *Enterococcus faecalis* isolates from chicken meat.

Strain	Resistance genes[1]	Virulence genes	MLST	Location of *optrA*	MIC (mg/L)[2]														
					LZD	CHL	AMP	DAP	VAN	ERY	GEN	KAN	STR	SAL	SYN	CIP	TYLT	FFN	TET	TGC
EFs 116-2	***optrA*, *fexA*,** *ant(9)-Ia, aph(3′)-III, erm(A),* ***erm(B)*, *Isa(A)*,** *tet(L)*, ***tet(M)***	***ace***, *agg*, ***cad, camE, cCF10, cOB1, ebpA, ebpB, ebpC, efaAfs,*** *ElrA,* ***hylA,*** *SrtA,* ***tpx***	ST476	Chromosomal DNA	**8**	**>32**	<1	4	<2	**>64**	<128	1,024	<128	4	**16**	**>16**	**>64**	**>32**	**128**	<0.12
EFs 171-2	***optrA*, *fexA*,** *aac(6′)-aph(2″), ant(6)-Ia, ant(9)-Ia, aph(3′)-III, cat, erm(A),* ***erm(B)*, *Isa(A)*,** *tet(L)*, ***tet(M)***	***ace, cad, camE, cCF10, cOB1, ebpA, ebpB, ebpC, efaAfs, hylA, tpx***	ST476	Chromosomal DNA	**8**	**>32**	<1	1	<2	**>64**	**>2,048**	**>2,048**	**>2,048**	<2	**16**	**>16**	**>64**	**>32**	**>128**	<0.12
EFs 17-1	***optrA*, *fexA*, *erm(B)*, *Isa(A)*, *tet(M)***	***ace***, *agg*, ***cad, camE, cCF10, cOB1, ebpA, ebpB, ebpC, efaAfs***, *ElrA, fsrB, gelE*, ***hylA***, *hylB, SrtA,* ***tpx***	ST729	Plasmid	**16**	**>32**	<1	1	<2	**>64**	<128	<128	<128	4	**16**	**>16**	**>64**	**>32**	64	<0.12
transconjugant of **EFs 17-1**	***optrA*, *fexA*, *erm(B)***	-[3]	-	-	**16**	**>32**	<1	1	<2	**>64**	<128	<128	<128	4	4	2	4	**>32**	64	<0.12

Genes detected from all 3 isolates are highlighted in bold.

[1]Resistance genes, *cfr* and *poxtA*, were tested but not detected.

[2]LZD, linezolid; CHL, chloramphenicol; AMP, ampicillin; DAP, daptomycin; VAN, vancomycin; ERY, erythromycin; GEN, gentamicin; KAN, kanamycin; STR, streptomycin; SAL, salinomycin; SYN, quinupristin/dalfopristin; CIP, ciprofloxacin; TYLT, tylosin tartrate; FFN, florfenicol; TET, tetracycline; TGC, tigecycline. MIC values indicating resistance to antimicrobials are highlighted in bold.

[3]Not tested.

resistance genes for macrolides [*erm(B)* and *lsa(A)*] and tetracyclines [*tet(M)*]. Moreover, 2 strains, EFs 116-2 and EFs 171-2, also carried the *erm(A)* gene for macrolides and the *tet(L)* gene for tetracyclines. In particular, one strain, EFs 171-2, showed resistance to gentamicin, kanamycin, and streptomycin with high-level MICs over 2,048 mg/L and carried resistance genes for aminoglycosides such as *aac(6′)-aph(2″)*, *ant(6)-Ia*, *ant(9)-Ia*, and *aph(3′)-III*. Although all 3 strains showed resistance against ciprofloxacin, quinupristin/dalfopristin, and tylosin tartrate, no specific genes related to resistance were detected. Antimicrobial resistance genes, *cfr* and *poxtA*, were investigated but not detected in any strain (Table1).

Molecular Characteristics of optrA*-positive* E. faecalis *and Conjugation Experiments*

All 3 strains carried virulence genes related to adhesion (*ace*), biofilm production (*ebpA*, *ebpB*, and *ebpC*), cell wall adhesion expressed in serum (*efaAfs*), protection against oxidative stress (*tpx*), hyaluronidase gene (*hylA*), and sex pheromone-associated genes involved in the transfer of pheromone-responsive plasmids (*cad*, *camE*, *cCF10*, and *cOB1*). In addition, 2 strains, EFs 17-1 and EFs 116-2, also harbored supplementary virulence genes for adhesion (*agg* and *ElrA*) and protease (*SrtA*). Other virulence factors such as hyaluronidase (*hylB*), protease (*gelE*), and quorum sensing–related (*fsrB*) genes were observed only in the EFs 17-1 strain. One strain, EFs 17-1, among the 3 *optrA*-positive *E. faecalis* isolates, successfully cotransferred the o*ptrA* and *fexA* genes. The transconjugant showed resistance against LZD, CHL, ERY, and FFN, but it was susceptible to CIP, TYLT, and SYN unlike the characteristics of the donor strain.

Whole Genome Sequencing Analysis

Based on *in silico* analysis of the assembled genomes, the EFs 116-2 (GenBank accession number SAMN15773991) and EFs 171-2 (GenBank accession number SAMN15773992) strains were identified as ST476, and the *optrA* genes of these strains were located at their chromosomal DNAs. On the other hand, *optrA* gene of the EFs 17-1 strain (GenBank accession number SAMN15773990), belonging to the ST729 lineage, was observed in plasmid DNA. The *optrA*-carrying complete plasmid sequence detected in the EFs 17-1 strain showed >99% identity (query cover, 100%) with the sequence of pE349 (GenBank accession number KP399637) and was deposited under the name of pEFs17-1 (GenBank accession number MT223178). Among the 39 open reading frames (**ORFs**) identified in the plasmid (36,331 bp), 21 encoded hypothetical proteins with no previously reported functions, whereas 18 products matched with proteins that had identified functions in the database such as antimicrobial resistance (*oprA* and *fexA*), replication (*repB* and *repC*), plasmid modulator (*mazF*, *lysS*), and conjugal transfer (*mobA*, *prgI*, *traD*, *traE*, *traG*, and *tnp*) (Figure 1A).

Analysis of regions flanking the *optrA* gene in the chromosomes of 2 strains, EFs 116-2 and EF171-2, revealed that they displayed >99% nucleotide sequence identity (query cover, 100%) with the sequences of Tn*6674* (GenBank accession number MK737778). The transposon genes, *tnpA*, *tnpB*, and *tnpC*, were located in the first ORFs for transposase, similar to that in Tn*6674*. The antimicrobial resistance genes *ant(9)-Ia*, *ermB*, and *fexA* were also located adjacent to the *optrA* gene (Figure 1B).

When the OptrA amino acid sequence was compared with that of *E. faecalis* pE439 (MIC of LZD = 8 mg/L), the 3 strains represented >99% amino acid identity, but pEFs17-1 showed 2 amino acid changes (Thr96Lys and Tyr160Asp) and a higher MIC of LZD (16 mg/L) (Figure 1D).

DISCUSSION

To the best of our knowledge, this study is the first to identify the whole genome sequences of *optrA*-positive *E. faecalis* isolated from retail chicken meat in South Korea. Although only 3 *optrA*-positive *E. faecalis* strains were tested in this study, the results showed that the *optrA* gene could be carried by both chromosomal and plasmid DNAs. When DNA sequences of the *optrA* gene in chromosomal DNA were compared with the available *optrA* sequences, 2 isolates harboring the *optrA* gene in their chromosomal DNA showed >99% similarity to previously reported *optrA* sequences. In particular, the genes adjacent to the *optrA* gene exhibited an analogous arrangement to the transposon Tn*6674* (Li et al., 2019). In addition, the transposase genes, *tnpA*, *tnpB*, and *tnpC*, were conserved in both isolates. The active Tn*6674* can excise from the host DNA and produce circular forms that proceed to the integration of the transposon into a new target sequence (Freitas et al., 2020). This mechanism suggests that *optrA* can be disseminated to a variety of bacteria via transposon mediation (Kehrenberg and Schwarz, 2005; Chen et al., 2019).

In this study, one isolate harbored an *optrA*-carrying plasmid and also showed >99% similarity to the previously reported pE439 plasmid (Wang et al., 2015). But, when DNA sequences of the *optrA* gene were compared with that of pE439, the *optrA*-carrying plasmid tested in this study showed 2 amino acid changes (Thr96Lys and Tyr160Asp) and a higher MIC against LZD (16 mg/L) compared to that of pE439 (8 mg/L). Although Bender et al. (2018) reported amino acid substitution in the *optrA* gene was associated with both LZD-resistant and -susceptible *E. faecalis* isolates, Elghaieb et al. (2020) reported that strains could have the same MIC for LZD resistance irrespective of their variable *optrA* gene mutation profiles (Bender et al., 2018; Elghaieb et al., 2020). Therefore, the contribution of these specific amino acid substitutions to LZD resistance remains unclear.

In this study, all 3 strains harboring the *optrA* gene in their chromosomal or plasmid DNAs also carried the *fexA*

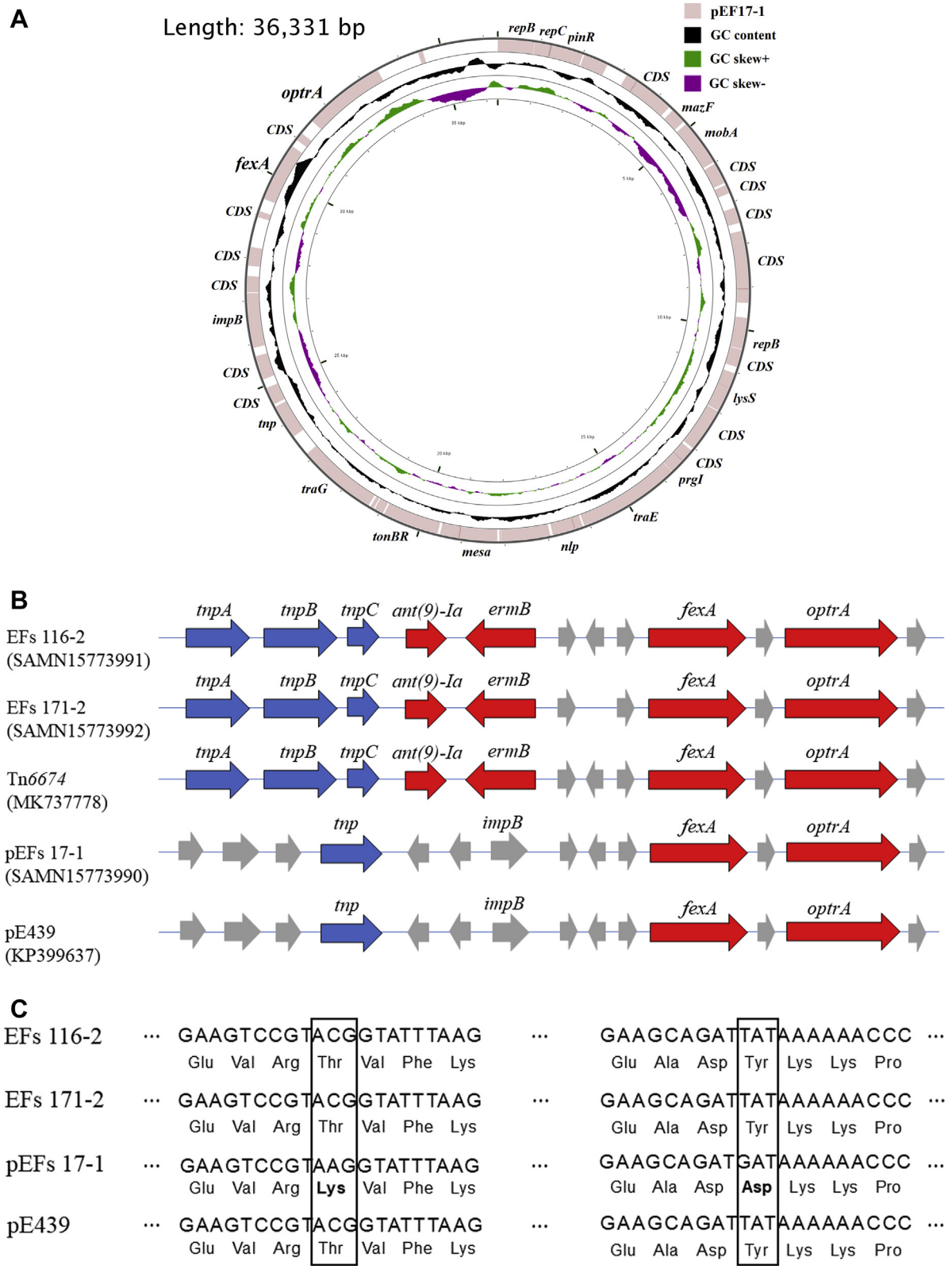

Figure 1. (A) Circular view of the mapped BLAST result of the EFs 17-1 plasmid compared with the originally reported *optrA*-carrying plasmid pE439 sequence. (B) Linear representation of the *optrA* genetic environment in the 3 isolates in this study and the *optrA*-carrying transposon Tn*6674* and the plasmid pE439 (GenBank accession number MK737778 and KP399637, respectively). The GenBank accession numbers are written below the strain name or the plasmid names. The transposase genes, antimicrobial resistance genes, and genes that code for other functions are shown in arrow boxes filled in blue, red, and gray, respectively. (C) Comparison of sequences of the *optrA* gene among the 3 *Enterococcus faecalis* strains and pE439.

gene adjacent to *optrA*. Ca'Mara et al. reported that the presence of both *optrA* and *fexA* in the same isolates occurred at a high frequency (Ca'Mara et al., 2019); moreover, cotransfer of the *fexA* gene with *optrA* in *E. faecalis* has been already shown in many countries such as China (Wang et al., 2015), Spain (Ca'Mara et al., 2019), and the United States (Wardenburg et al., 2019). In this study, the strains with the embedded *fexA-optrA* complex, which is highly similar to those of Tn*6674* and pE439, also induced simultaneous resistance to phenicols and oxazolidinones as has been previously described (Wang et al., 2015; Cai et al., 2018; Li et al., 2019). Moreover, mobile genetic elements such as transposons or plasmids flanking the *optrA* gene can be mediated by the horizontal transfer of a variety of resistance genes in the absence of antimicrobial drugs (Morroni et al., 2018; Wardenburg et al., 2019). Mobile genetic elements have frequently carried not only *optrA* but also other resistance determinants such as *fexA*, *ermA*, and *Isa(A)* genes. Freitas et al. (2017) and Tamang et al. (2017) have reported that oxazolidinones-resistant isolates linked to animals in which phenicols, marcolides, and streptogramins may be used might coselect resistance to other antibiotic families (Freitas et al., 2017; Tamang et al., 2017). In this study, 3 strains showed chromosomal- or plasmid-mediated oxazolidinone resistance as indicated by the coresistances to CHL, ERY, SYN, CIP, TYLT, and FFN. Although LZD has not been used in the poultry industry in South Korea, the emergence of an LZD-resistant *E. faecalis* in chickens could be the result of horizontal transfer of resistance genes and coselection of phenicol-resistant strains as has been previously described (Wang et al., 2015; Ca'Mara et al., 2019).

The analysis of 3 *optrA*-positive *E. faecalis* in this study showed the presence of a number of virulence genes in common, which is consistent with the results obtained from other *E. faecalis* strains of food origin (Vidana et al., 2016; Cavaco et al., 2017). Virulence factors contribute to adaptation to different environments and can enable the sharing of various genes such as virulence determinant and antimicrobial resistance genes (Coburn et al., 2007; Vidana et al., 2016). Especially, the expressions of different families of efflux pumps could contribute to the antimicrobial resistance and their pathogenesis (Martinez et al., 2009). As the association of these virulence factors with *optrA* is unclear, further monitoring of food animals is required to ascertain the role of virulence factors affecting the resistances determined by *optrA*.

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