Molecular characteristics and comparative genomics analysis of a clinical Enterococcus casseliflavus with a resistance plasmid

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Purpose: The aim of this work was to investigate the molecular characterization of a clinical Enterococcus casseliflavus strain with a resistance plasmid.

Materials and methods: En. casseliflavus EC369 was isolated from a patient in a hospital in southern China. The minimum inhibitory concentration was found by means of the agar dilution method to determine the antimicrobial susceptibilities of the strains. Whole-genome sequencing and comparative genomics analysis were performed to analyze the mechanism of antibiotic resistance and the horizontal gene transfer of the resistance gene-related mobile genetic elements.

Results: En. casseliflavus EC369 showed resistance to erythromycin, kanamycin, and streptomycin, but was susceptible to vancomycin, ampicillin, and streptothricin and other antimicrobials. There were six resistance genes (aph3′, ant6, bla, sat4, and two ermB) carried by a transposon identified on the plasmid pEC369 and a complete resistance gene cluster of vancomycin and a tet (M) gene encoded on the chromosome. This is the first complete plasmid sequence reported in clinically isolated En. casseliflavus. The plasmid with the greatest sequence identity with pEC369 was the plasmid of Enterococcus sp. FDAARGOS_375, followed by the plasmids of Enterococcus faecium strains F12085 and pRE25, whereas the sequence with the greatest identity to the resistance genes carrying a transposon of pEC369 was on the chromosome of Staphylococcus aureus strain GD1677.

Conclusion: The resistance profiles of En. casseliflavus EC369 might contribute to the resistance genes encoded on the plasmid. The fact that the most similar sequence to the transposon carrying resistance genes of pEC369 was encoded in the chromosome of a S. aureus strain provides insights into the mechanism of dissemination of multidrug resistance between bacteria of different species or genera through horizontal gene transfer.

Keywords: Enterococcus casseliflavus, antimicrobial resistance, transposon, molecular characteristics, comparative genomics analysis

Introduction

Enterococci are gram-positive and facultative anaerobic organisms. They can grow under 6.5% NaCl concentrations and in a high-pH environment, and hydrolyze bile-esculin and L-pyrolidonyl-B-naphthylamide.1 Enterococci are usually characterized by individual, paired, or short-chain gram-positive catalase-negative cocci.2 The Enterococcus genus was considered to belong to Lancefield group D Streptococcus; however, DNA homology studies have suggested that it is a distinct genus. To date, >40 Enterococcus species have been described and constitute a widespread group of bacteria.3 Enterococci have usually been found in the intestines of humans and animals, on the surfaces of plants, and in dairy products.4 They can persist in the environment
because of their ability to survive under a wide range of harsh conditions, for instance, drying, extreme temperatures, high osmolarity, and the presence of disinfectants. Moreover, enterococci are also used in food production as probiotic products to monitor fecal contamination.6

Enterococcus spp. are an increasingly common cause of nosocomial infections, with Enterococcus faecalis and Enterococcus faecium accounting for the majority of human enterococcus infections.7 Other Enterococcus spp., including Enterococcus casseliflavus, have also been shown to be pathogenic to humans.8 En. casseliflavus was given species status in 1984.9 It can be motile and produces a yellow pigment.10 However, nonpigmented and nonmotile strains may also occur.11 The species En. casseliflavus was formerly thought to be associated primarily with vegetation.12 McGowan found that 9 of 27 (33.3%) tomato samples harbored Enterococcus spp.,13 En. casseliflavus is among the normal flora in human and animal gastrointestinal tracts, unlike other enterococci, such as En. faecium and En. faecalis, which are the predominant conditionally pathogenic bacteria that cause hospital-acquired infections. En. casseliflavus is not frequently isolated from clinical specimens, with the rates being <1.3%.14 It is an opportunistic pathogen, which means it targets persons who are immunocompromised or chronically ill and is sometimes nosocomially acquired.15 Although the infection of En. casseliflavus is not common, it can be seriously invasive if infection occurs. Recently, En. casseliflavus has been increasingly implicated in infections and hospital outbreaks.16 En. casseliflavus is associated with a wide variety of invasive infections in humans, such as endocarditis, bacteremia, endophthalmitis, and spontaneous bacterial peritonitis.17–19

Enterococcus possesses intrinsic or acquired resistance to antimicrobials.20 The acquired resistance is usually mediated by mobile genetic elements such as insertion elements, transposons, and bacteriophages, which carry a pool of resistance genes and transmit the resistance between the bacteria of different species or genera via horizontal gene transfer.21 Enterococci also possess an inherent resistance to antimicrobial agents, for instance, low to moderate levels of resistance to aminoglycosides and low levels of resistance to vancomycin.22 It is possible that enterococci have the potential for resistance to virtually all clinically useful antibiotics. The current study was performed to introduce the molecular characteristics of a clinical En. casseliflavus with a plasmid carrying several resistance genes. Genome analysis has illuminated the extent of resistance genes related to the mobile genetic elements and the evolution of antibiotic resistance.

Materials and methods

Bacterial strain

En. casseliflavus EC369 was isolated from a bile specimen of a patient in Lishui Hospital, Zhejiang, China. The strain was identified using the Vitek-60 microorganism autoanalysis system (BioMerieux Corporate, Craponne, France). Further verification was performed using homologous comparisons of the sequences of 16S rRNA genes and the whole-genome sequence of the bacteria from the National Center for Biotechnology Information (NCBI) nucleotide database by BlastN and BlastP programs (http://www.ncbi.nlm.nih.gov). The bacteria and plasmids used in this work are listed in Table 1.

Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of the antibiotics for the bacteria were determined by the agar dilution method in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI document M100-S27, 2017). In the evaluation of MICs, resistance to streptomycin was set at ≥1,024 µg/mL, according to Werner et al.21 The MIC was defined as the lowest concentration producing no visible growth. Escherichia coli ATCC 25922 and En. faecalis ATCC 29212 served as the quality control strains for the antimicrobial susceptibility tests.

Whole-genome sequencing

Bacterial DNA was extracted using the Generay Genomic DNA Miniprep kit (Shanghai Generay Biotech Co., Ltd, Shanghai, China) from a single colony subcultured in brain heart infusion broth at 37°C for 16 hours, following the manufacturer’s instructions. Genomic DNA was sequenced with Illumina HiSeq-2500 and Pacific Bioscience sequencers at Annoroad Genomics Technology Co., Ltd (Beijing, China). Reads derived from the HiSeq-2500 sequencing were initially assembled de novo with the SOAPdenovo software to obtain contigs of the genome sequences. Pacific Bioscience sequencing reads of ~10–20 kb in length were mapped onto the primary assembly to scaffold the contigs. The gaps were filled either by remapping the short reads from HiSeq-2500 sequencing or by PCR product sequencing of the gap. Glimmer (http://ccb.jhu.edu/software/glimmer) was used to predict protein-coding genes with potential open reading frames (ORFs) >150 bp. GView was used to construct the basic genome features.24 BlastX (https://blast.ncbi.nlm.nih.gov) was used to annotate the predicted protein-coding genes against a nonredundant protein database with an e-value threshold of 1e−5.
Cloning experiments

The resistance gene sequences were PCR amplified, and the PCR products were then eluted from agarose gel and ligated into suitable vectors (pUCP20, pUCP24, or pAM401). The ORFs of the resistance genes were ligated into the pUCP20 or pUCP24. The resistance genes with the predicted promoter regions (http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb) were ligated into pAM401. We designed the primers (Table 2) by using Primer Premier 5.0 and then synthesized them at Shanghai Sunny Biotechnology Co., Ltd (Shanghai, China). The recombinant plasmids (pUCP20-ORFs) were transformed into Es. coli DH5α via the calcium chloride method, and the bacterial colonies were grown on Luria-Bertani agar plates supplemented with ampicillin (100 µg/mL). The recombinant plasmids (pUCP24-ORFs) were transformed into Es. coli DH5α using the same method, but were grown on Luria-Bertani agar plates supplemented with gentamicin (20 µg/mL). Additionally, the recombinant plasmids (pAM401-ORFs) were transformed into En. faecalis JH2-2 by electrottransformation, and the bacterial colonies were grown on brain heart infusion agar plates supplemented with chloramphenicol (16 µg/mL). The recombinant plasmids (pUCP20-ORFs and pUCP24-ORFs) were extracted and digested with KpnI and BamHI (Takara Biomedical Technology, Beijing Co., Ltd, Beijing, China) and the recombinant plasmids (pAM401-ORFs) were digested with XbaI and BamHI (TaKaRa) to confirm the insert size, and then, the orientation and frame of the ORFs were further verified by sequencing with an ABI 3730 automated sequencer (Thermo Fisher Scientific, Waltham, MA, USA).

Conjugation experiments

En. faecalis JH2-2 was used as the recipient in the conjugation experiments to detect the transferable characteristics of the conjugative plasmid of the donor En. casseliflavus EC369 by filter mating, which allows tight cell-to-cell contact, as previously described.25,26 The transconjugants were selected on brain heart infusion plates supplemented with 25 µg/mL.

Table 1 Strains and plasmids used in this work

| Strain or plasmid     | Relevant characteristic(s)                                                                 | Reference or source          |
|-----------------------|-------------------------------------------------------------------------------------------|-----------------------------|
| Strain                |                                                                                           |                             |
| Escherichia coli      |                                                                                           |                             |
| DH5α                  | Es. coli DH5α used as a host for the PCR products cloning of the resistance genes          | Our laboratory collection   |
| ATCC 25922            | Es. coli ATCC 25922 used as the quality control for the antimicrobial test                  | Our laboratory collection   |
| Enterococcus faecalis |                                                                                           |                             |
| JH2-2                 | En. faecalis JH2-2 used as the host for the resistance genes cloning and the recipient for the conjugation experiment, RF' | Our laboratory collection   |
| ATCC 29212            | En. faecalis ATCC 29212 used as the quality control strain for the antimicrobial test       | Our laboratory collection   |
| EC369                 | The wild strain of Enterococcus casseliflavus 369                                          | This study                  |
| Es. coli carrying plasmid |                                                                                       |                             |
| pUCP20-ORFs/DH5α      | DH5α carrying the recombinant plasmids pUCP20-ORFs (ant6, aph3', ermB, and sat4)          | This study                  |
| pUCP24-ORF/DH5α       | DH5α carrying the recombinant plasmid pUCP24-ORF (bla)                                     | This study                  |
| En. faecalis carrying plasmid |                                                                                       |                             |
| pAM401/JH2-2          | JH2-2 carrying vector pAM401, CHL'                                                        | Our laboratory collection   |
| pAM401-ORFs/JH2-2     | JH2-2 carrying the recombinant plasmids of pAM401 cloned with resistance gene ORFs with promoter regions (ant6, aph3', bla, sat4, ermB) | This study                  |
| Plasmid               |                                                                                           |                             |
| pUCP24                | Cloning vector for the PCR products of bla gene, GM'                                      | Our laboratory collection   |
| pUCP20                | Cloning vector for the PCR products of ant6, aph3', ermB, and sat4, Ap'                   | Our laboratory collection   |
| pAM401                | Cloning vector for the PCR products of all resistance genes with the promoter regions, CHL' | Our laboratory collection   |

Abbreviations: CHL, chloramphenicol; GM, gentamicin; ORFs, open reading frames; ', resistance; RF, rifampin; AP, ampicillin.
rifampicin (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China), 50 µg/mL fusidic acid (Sinopharm Chemical Reagent Co., Ltd), and 512 µg/mL kanamycin (Sinopharm Chemical Reagent Co., Ltd). The plasmid DNA was extracted from the transconjugant (pEC369/En. faecalis JH2-2) and verified by PCR of the resistance genes and PCR products sequencing.

### Collection and processing of the plasmids and the resistance gene-related sequences

The plasmid genome sequences for comparative genomics analysis in this study were selected based on the whole-genome sequence (pEC369) comparison against the whole-genome sequences available in the NCBI nucleotide database with coverage of >33%. The accession numbers of the plasmids were CP023514 (a plasmid from Enterococcus sp. FDAARGOS_375), X92945 (pRE25 from En. faecalis), X92945 (pRE25 from En. faecalis), X92945 (pRE25 from En. faecalis), and KY579372 (the plasmid of En. faecium). Orthologous groups of genes from the candidate sequences were identified using BlastP and Paranoïd. The sequence retrieval, statistical analysis, and other bioinformatics tools used in this study were applied with Python and Biopython scripts.

### Results and discussion

#### General features of the En. casseliflavus EC369 genome

*En. casseliflavus* EC369, producing yellow pigment on the plate, had the typical biological characteristics of a gram-positive *Enterococcus*. 16S ribosomal RNA gene homology analysis showed that the genes sharing the greatest nucleotide sequence identities to that of *En. casseliflavus* EC369 were from *En. casseliflavus* LMG10745 (NR114778) and two *Enterococcus gallinarum* strains (LMG 13129, NR104559; NBRC 100675, NR113964). They all showed sequence identities of 99%. The genome sequences sharing the highest identities to that of *En. casseliflavus* EC369 were from *En. casseliflavus* LMG10745 (NR114778) and two *Enterococcus gallinarum* strains (LMG 13129, NR104559; NBRC 100675, NR113964). They all showed sequence identities of 99%. The genome sequences sharing the highest identities to that of *En. casseliflavus* EC369 were from *En. casseliflavus* LMG10745 (NR114778) and two *Enterococcus gallinarum* strains (LMG 13129, NR104559; NBRC 100675, NR113964). They all showed sequence identities of 99%

For the comparative genomics analysis of the resistance gene-related transposons, similar sequences were also obtained from the NCBI nucleotide database using the resistance gene-encoded region of pEC369 as the query. The resulting sequence was filtered and the sequence with an identity of >95% and coverage of >80% was retained. Moreover, we searched a plasmid pVEF1 (39 kb in length) that was free of the resistance gene related transposon, but shared about 15.6kb sequence with pEC369 which accounted for 40% (15.6/39) of the genome of pVEF1. The accession number of the plasmid was AM296544 (pVEF1 from *En. faecium*). Orthologous groups of genes from the candidate sequences were identified using BlastP and Paranoïd. The sequence retrieval, statistical analysis, and other bioinformatics tools used in this study were applied with Python and Biopython scripts.

### Table 2 Primers used in this study

| Genes | Primer | Sequence* (5’-3’) | Restriction endonuclease | Vector | Amplicon size (bp) | Annealing temperature (°C) |
|-------|--------|-------------------|--------------------------|--------|--------------------|---------------------------|
| bla   | P-bla-f | CGGGATCCATGACAGTGTAAATGAGGA | BamHI | pUCP24 | 639 | 52 |
|     | P-bla-R | GGGGTAACCTATTAGTATTATATGATG | KpnI | | | |
| ant6  | P-ant6-f | CGGGATCCATGACAGTGTAAATGAGGA | BamHI | pUCP20 | 909 | 53 |
|     | P-ant6-R | GGGGTAACCTATTAGTATTATATGATG | KpnI | | | |
| ermB  | P-ermB-f | GGGGATCCATGACAGTGTAAATGAGGA | BamHI | pUCP20 | 738 | 55 |
|     | P-ermB-R | GGGGTAACCTATTAGTATTATATGATG | KpnI | | | |
| aph3* | P-aph3*-f | GGGGATCCATGACAGTGTAAATGAGGA | BamHI | pUCP20 | 795 | 52 |
|     | P-aph3*-R | GGGGTAACCTATTAGTATTATATGATG | KpnI | | | |
| sat4  | P-sat4-F | GGGGATCCATGACAGTGTAAATGAGGA | BamHI | pUCP20 | 291 | 54 |
|     | P-sat4-R | GGGGTAACCTATTAGTATTATATGATG | KpnI | | | |
| bla   | P-pro-bla-F | GCTCTAGATATTTAAAAGCTACCAAAGCAGA | XbaI | pAM401 | 847 | 55 |
|     | P-pro-bla-R | GGGGATCCATGACAGTGTAAATGAGGA | BamHI | | | |
| ant6  | P-pro-ant6-F | GCTCTAGATATTTAAAAGCTACCAAAGCAGA | XbaI | pAM401 | 1309 | 60 |
|     | P-pro-ant6-R | GGGGATCCATGACAGTGTAAATGAGGA | BamHI | | | |
| ermB  | P-pro-ermB-F | GCTCTAGATATTTAAAAGCTACCAAAGCAGA | XbaI | pAM401 | 1089 | 56 |
|     | P-pro-ermB-R | GGGGATCCATGACAGTGTAAATGAGGA | BamHI | | | |
| aph3* | P-pro-aph3*-F | GCTCTAGATATTTAAAAGCTACCAAAGCAGA | XbaI | pAM401 | 1086 | 60 |
|     | P-pro-aph3*-R | GGGGATCCATGACAGTGTAAATGAGGA | BamHI | | | |
| sat4  | P-pro-sat4-F | GCTCTAGATATTTAAAAGCTACCAAAGCAGA | XbaI | pAM401 | 472 | 55 |
|     | P-pro-sat4-R | GGGGATCCATGACAGTGTAAATGAGGA | BamHI | | | |

**Note:** The underlines represent the restriction enzyme sites and their protective bases; *The primers with the predicted promotor regions.

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The whole genome of *En. casseliflavus* EC369 consisted of a chromosome (CP032739) of 3.58 Mb in length encoding 3,333 ORFs and a circular plasmid (pEC369, CP032740) of 91,960 bp in length encoding 95 ORFs. Of the ORFs encoded on the plasmid, 65% (62/95) were predicted to encode proteins with known functions, including a transposon carrying six antimicrobial genes (*aph3′*, *ant6*, *bla*, *sat4*, and two *ermB* genes), two clusters of copper resistance genes, and so on (Table 3; Figure 1). At present, there are ~29 genome sequences of *En. casseliflavus* available in the NCBI GenBank. Most of these 29 genome sequences are the incomplete genome sequences. In addition to the complete genome sequence of *En. casseliflavus* EC369 in this work, only one strain, *En. casseliflavus* EC20 (CP004856), had the complete genome sequence. Even though a variety of complete plasmid sequences have been reported in other species of the *Enterococcus*, such as *En. faecium* and *En. faecalis*, no complete plasmid sequence, however, from *En. casseliflavus* was available in the database.

The resistance genes and their functions in *En. casseliflavus* EC369

*En. casseliflavus*, similar to most enterococci, showed resistance to a variety of antibiotics, such as glycopeptides, aminoglycosides, macrolides, tetracycline, and beta-lactams. The resistance mechanisms are related to both intrinsic and acquired resistance genes. The results of MIC detection of several antibiotics showed that it was resistant to kanamycin, streptomycin, and erythromycin and susceptible to vancomycin, ampicillin, streptothricin, and other antimicrobials (Table 4). Six resistance genes (*aph3′*, *ant6*, *sat4*, *bla*, and two *ermB* genes with the same sequences) were identified on the plasmid pEC369. Among them, the resistance genes *aph3′*, *ant6*, and *ermB* were functional, but *sat4* and *bla* were not (the cloned genes with *ermB* were not functional and the reason for this remains a question). The cloned ORFs of the *ant6* and *aph3′* genes exhibited 2- and 5-fold increases in the MIC levels to streptomycin and kanamycin, respectively, compared with those of the controls (DH5α or DH5α carrying the vector pUCP20). The gene *ermB* exhibited 4-fold increases in the MIC levels to erythromycin. When the ORFs of the resistance genes (*ant6*, *aph3′*, and *ermB*) were cloned with their predicted promoter regions and transformed into the *En. faecalis* JH2-2 recipients, they exhibited at least 4-fold increases in MIC levels to streptomycin, kanamycin, and erythromycin, respectively, compared with those of the control pAM401/JH2-2 (Table 4). The results of the function detection of the cloned resistance genes were in accordance with the resistance phenotypes of the host strain *En. casseliflavus* EC369.

It has been reported that *En. casseliflavus* is intrinsically resistant to vancomycin at low levels, and that resistance is mediated by the vanC-2-type genes. Some enterococci strains showed high-level resistance to vancomycin and teicoplanin (MIC >256 mg/L), coinciding with the presence of the vanA gene. The mechanisms of glycopeptide resistance in enterococci have been sufficiently investigated. To date, nine vancomycin resistance-related genes (vanA, vanB, vanC, vanD, vanE, vanG, vanL, vanM, and vanN) have been identified, with the acquired genotype vanA being the most common, followed by vanB. VanA is related to resistance to both vancomycin and teicoplanin, whereas VanB is resistant only to vancomycin. VanC is usually encoded by the vanC1 and vanC2/3 genes, which are intrinsic to *En. gallinarum* and *En. casseliflavus*, respectively. This characteristic can be used for species identification.

The vanC-2 containing vancomycin resistance gene cluster of *En. casseliflavus* consists of five genes. The first three genes of the cluster, vanC-2, vanXYC-2, and vanTc,c, are essential for vancomycin resistance. vanC-2 encodes a D-Ala:D-Ser ligase, vanXYC-2 encodes a protein possessing both D, D-dipeptidase and D, D-carboxypeptidase activities, and vanTc,c encodes a serine racemase. Expression of the resistance genes is controlled by a two-component regulatory system that is present downstream of vanTc,c consisting of a response regulator, VanRc,c, and a histidine kinase, VanSc,c. In this work, a cluster of five vancomycin resistance-related genes (vanC-2, vanXY, vanT, vanR, and vanS) were all identified in the chromosome genome. However, EC369 showed a low resistance level to vancomycin with an MIC of 1 µg/mL. We may think that the vancomycin resistance genes in *En. casseliflavus* EC369 are not functional and the reason for this remains a question.

The transposon carrying multiple resistance genes on a conjugative plasmid

The acquisition of foreign resistance genes in *Enterococcus* is often related to transfer of the resistance plasmids that carry
antibiotic resistance genes. Studies have reported that plasmids are abundant in enterococci, as illustrated by the finding of one to seven plasmids in 88 out of 93 En. faecium isolates and high resistance plasmid carriage rates observed in En. casseliflavus isolates.38,39 En. casseliflavus once showed high-level resistance to vancomycin due to the acquisition of plasmid pIP218 carrying the vanA gene.40 The plasmid-mediated aminoglycoside-modifying enzyme gene, aph (2")-Ie, appeared in a strain of high-level gentamicin-resistant En. casseliflavus.41 A conjugative plasmid with transposon Tn6000 related to the tetracycline resistance gene was identified in En. casseliflavus 664.1H1.42 In this work, a cluster of resistance genes including two aminoglycoside resistance genes (aph(3')-III and ant(6)-Ia) and two erythromycin resistance genes ermB was identified encoded on the plasmid. The plasmid (pEC369) was a conjugative plasmid and could be successfully transferred into the recipient cell through conjugation. The MIC levels of the transconjugant to the antibiotics detected were similar to those of the donor cell En. casseliflavus EC369 (Table 4). It demonstrated that the resistance activities of En. casseliflavus EC369 to the antibiotics streptomycin, kanamycin, and erythromycin were mainly related to the genes encoded in the conjugative plasmid pEC369 (Table 3).

Resistance genes can translocate among plasmids or chromosomes through the mobile genetic elements such as transposons, insertion elements, integrons, and phages. The
resistance genes (aph3', ant6, bla, sat4, and two ermB genes) encoded on pEC369 are carried by a transposon of ~7 kb in length. This transposon is characterized by two copies of a 5 bp direct repeat “GTGAT” that precisely borders the transposase gene and the peptide-binding protein gene (Figure 2). Further comparative genomics analysis demonstrated that the sequence with the highest identity to the transposon-carrying resistance genes of pEC369 was located on the chromosome of *Staphylococcus aureus* strain GD1677 (CP019595), and the sequence with the greatest identity to the upstream and downstream sequences of the resistance genes carrying the transposon of pEC369 was pVEF1 (AM296544), a plasmid in *E. faecium*. pVEF1 was 39 kb in length and 52 kb smaller than pEC369, and the region similar to the flanking sequences of the transposon of pEC369 was 15.6 kb in length, consisting of 40% (15.6/39 kb) of the pVEF1 genome. The transposon of pEC369 might have been formed on the basis of the initial transposon of tnp-bla-sat4-ant6-ermB by the transposase (encoded by the tnp gene) of the transposon which captured the other two resistance genes (aph3' and ermB) and the peptide-binding protein gene (orf E) (Figure 2).

**Comparative genomics analysis of the plasmid**

Comparative genomics analysis showed that the plasmid with the greatest sequence similarity to pEC369 was a plasmid (CP023514) from *Enterococcus* sp. FDAARGOS_375. It was 148 kb in length and 57 kb larger than pEC369. The two plasmids had nearly 38 kb (41%, 38/91.96) similar sequences. The similar regions were mainly located between 50 and 80 kb of the pEC369 and included two clusters of copper resistance genes (located in two regions of 57.5–63 kb and 71.5–77.7 kb, respectively), but the plasmid was free of the drug resistance-related transposon. Two other plasmids with higher similarities to pEC369 were pRE25 (X92945, 50 kb) and the plasmid of *En. faecium* strain F12085, similar to that of pEC369, were different from the plasmid from *Enterococcus* sp. FDAARGOS_375.

**Conclusion**

In this work, we sequenced the whole genome of the clinical isolate *En. casseliflavus* EC369. In addition to a resistance
gene cluster of vancomycin, the genome encoded seven other resistance genes, of which a tet (M) was encoded in the chromosome and six resistance genes (aph3’, ant6, bla, sat4, and two ermBs) were carried by a transposon encoded on the plasmid pEC369, which conferred the host high level of resistance to erythromycin, kanamycin, and streptomycin. Although the plasmid with the greatest sequence identity to pEC369 was the plasmid of Enterococcus sp. FDAARGOS_375, followed by the plasmids from the En. faecium strains, the sequence with the greatest identity to the resistance genes carrying the transposon of pEC369 was on the chromosome of S. aureus strain GD1677. This result demonstrates the potential for the spread of multiresistant mobile genetic elements within bacteria of different species or genera, which poses significant challenges for successful clinical treatment and infection control strategies. Therefore, the study of enterococcal biology and genetics is necessary and will undoubtedly contribute to our understanding of bacterial resistance dissemination.

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Disclosure
The authors report no conflicts of interest in this work.

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