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ORIGINAL RESEARCH

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*s) 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-pyrrolidonyl-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

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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.[5] 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 streptothricin was set at ≥1,024 μg/mL, according to Werner et al.[23] 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.

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^r	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^r	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^r	Our laboratory collection
pUCP20	Cloning vector for the PCR products of *ant6, aph3', ermB, and sat4*, Ap^r	Our laboratory collection
pAM401	Cloning vector for the PCR products of all resistance genes with the promoter regions, CHL^r	Our laboratory collection

Abbreviations: CHL, chloramphenicol; GM, gentamicin; ORFs, open reading frames; ^{r}, resistance; RF, rifampin; AP, ampcillin.

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 electrotransformation, 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 *Kpn*I and *Bam*HI (Takara Biomedical Technology, Beijing Co., Ltd, Beijing, China) and the recombinant plasmids (pAM401-ORFs) were digested with *Xba*I and *Bam*HI (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 2 Primers used in this study

Genes	Primer	Sequence[a] (5′–3′)	Restriction endonuclease	Vector	Amplicon size (bp)	Annealing temperature (°C)
bla	P-*bla*-F	CGGGATCCATGACAGTGTTAAATGAGGA	*Bam*HI	pUCP24	639	52
	P-*bla*-R	GGGGTACCCTATTGTAGTTTAATACATG	*Kpn*I			
ant6	P-*ant6*-F	CGGGATCCATGAGATCAGAAAAAGAAAT	*Bam*HI	pUCP20	909	53
	P-*ant6*-R	GGGGTACCTCACTGTTCCCGCCTCTCTT	*Kpn*I			
ermB	P-*ermB*-F	CGGGATCCATGAACAAAAATATAAAATA	*Bam*HI	pUCP20	738	55
	P-*ermB*-R	GGGGTACCTTATTTCCTCCCGTTAAATA	*Kpn*I			
aph3'	P-*aph3'*-F	CGGGATCCATGGCTAAAATGAGAATATC	*Bam*HI	pUCP20	795	52
	P-*aph3'*-R	GGGGTACCCTAAAACAATTCATCCAGTA	*Kpn*I			
sat4	P-*sat4*-F	CGGGATCCGTGATTACAGAAATGAAAGC	*Bam*HI	pUCP20	291	54
	P-*sat4*-R	GGGGTACCTTAATCTTTAAGCTTATTTT	*Kpn*I			
bla	P-pro-*bla*-F[b]	GCTCTAGATATTTAAAAAGCTACCAAGACGA	*Xba*II	pAM401	847	55
	P-pro-*bla*-R[b]	CGGGATCCCTATTGTAGTTTAATACATGCTC	*Bam*HI			
ant	P-pro-*ant6*-F[b]	GCTCTAGAATGAAACACGCCAAAGTAAACAA	*Xba*II	pAM401	1309	60
	P-pro-*ant6*-R[b]	CGGGATCCTCACTGTTCCCGCCTCTCTTCTAT	*Bam*HI			
ermB	P-pro-*ermB*-F[b]	GCTCTAGATGGTTAACCCTAAAGTTATGGAA	*Xba*II	pAM401	1089	56
	P-pro-*ermB*-R[b]	CGGGATCCTTATTTCCTCCCGTTAAATAATA	*Bam*HI			
aph3'	P-pro-*aph3'*-F[b]	GCTCTAGAATTCAGCAATTGCTTAAGCTGCC	*Xba*II	pAM401	1086	60
	P-pro-*aph3'*-R[b]	CGGGATCCCTAAAACAATTCATCCAGTAAAA	*Bam*HI			
sat4	P-pro-*sat4*-F[b]	GCTCTAGATTCTATGCCATCAATTGTTCAGG	*Xba*II	pAM401	472	55
	P-pro-*sat4*-R[b]	CGGGATCCTTAATCTTTAAGCTTATTTTTTC	*Bam*HI			

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

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*), and KY579372 (the plasmid of *En. faecium* strain F12085).

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 Paranoid.[27] The sequence retrieval, statistical analysis, and other bioinformatics tools used in this study were applied with Python and Biopython scripts.[28]

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* EC20 (CP004856, 98.0%) and *Enterococcus* sp. FDAARGOS_375 (CP023515, 98.0%). Therefore, we finally grouped the strain into a species of *En. casseliflavus* and named it *En. casseliflavus* EC369.

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. casseliflav*us, similar to most enterococci, showed resistance to a variety of antibiotics, such as glycopeptides, aminoglycosides, macrolides, tetracycline, and beta-lactams.[29–31] 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*s 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 or without the promoter region). 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.

Table 3 General features of *Enterococcus casseliflavus* EC369

Element and characteristics	Chromosome	Plasmid
Size (bp)	3,576,376	91,960
GC content (%)	42.56	34
Predicted ORFs	3,333	95
Average ORF length (bp)	933	805
Known proteins	2,692 (81%)	62 (65%)
Hypothetical protein	641 (19%)	33 (35%)
Protein coding (%)	87	83
tRNAs	63	0
rRNA operons	5*(16s-23s-5s)	0

Abbreviation: ORFs, open reading frames.

It has been reported that *En. casseliflav*us is intrinsically resistant to vancomycin at low levels, and that resistance is mediated by the *vanC-2*-type genes.[32] Some enterococci strains showed high-level resistance to vancomycin and teicoplanin (MIC >256 mg/L), coinciding with the presence of the *vanA* gene.[33] The mechanisms of glycopeptide resistance in enterococci have been sufficiently investigated.[34] 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.[35] This characteristic can be used for species identification.[36]

The *vanC-2* containing vancomycin resistance gene cluster of *En. casseliflavus* consists of five genes. The first three genes of the cluster, *vanC-2*, $vanXY_{C\text{-}2}$, and $vanT_{C\text{-}2}$, are essential for vancomycin resistance. *vanc-2* encodes a D-Ala:D-Ser ligase, $vanXY_{C\text{-}2}$ encodes a protein possessing both D, D-dipeptidase and D, D-carboxypeptidase activities, and $vanT_{C\text{-}2}$ encodes a serine racemase. Expression of the resistance genes is controlled by a two-component regulatory system that is present downstream of $vanT_{C\text{-}2}$ consisting of a response regulator, $VanR_{C\text{-}2}$, and a histidine kinase, $VanS_{C\text{-}2}$.[37] 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

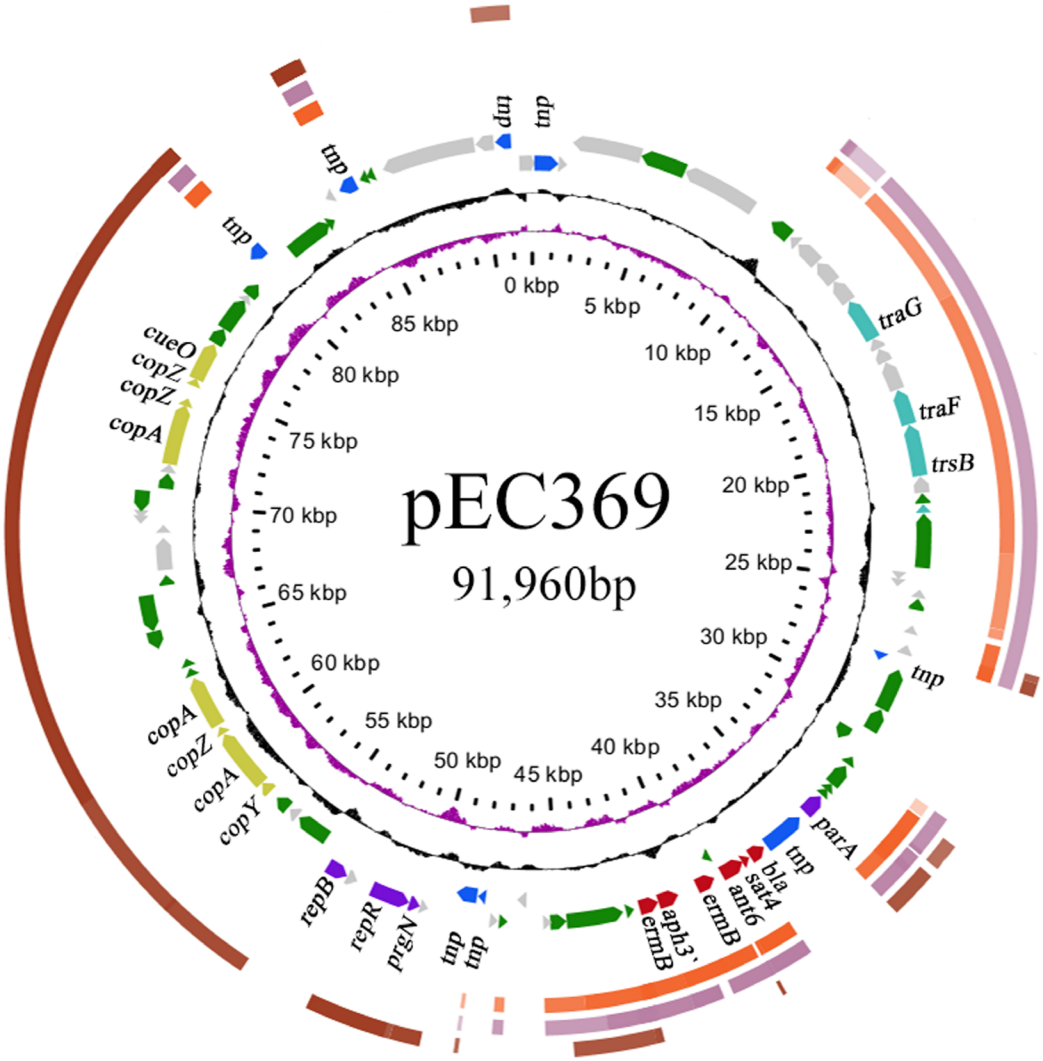

Figure 1 The circular map of the pEC369 genome and comparative genomic analysis of the plasmids.
Notes: Figuring from outside to inside: 1, the plasmid of *Enterococcus* sp. FDAARGOS_375; 2, the plasmid of pRE25; 3, the plasmid of *Enterococcus faecium* strain F12085; 4, the genes encoded on pEC369 with the hypothetical ORFs in brackets; 5, GC content with the G+C content >50% toward the outside rather than inside; 6, GC skew (G–C/G+C) with a positive GC skew toward the outside and a negative GC skew toward the inside; and 7, the most inner circle, scale in bp.
Abbreviation: ORFs, open reading frames.

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 *van*A 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

Table 4 MIC values of antibacterial drugs for all strains (μg/mL)

Strains	AMP	SM	KAN	VAN	ERY	ST	PB	TGC	FFC	CHL	TEC	NOR	CFX
DH5α	4	4	2	>32	128	>256	–	–	–	–	–	–	–
pUCP20/DH5α	>1,024	4	2	>32	128	>256	–	–	–	–	–	–	–
pUCP24/DH5α	4	8	4	>32	128	>256	–	–	–	–	–	–	–
pUCP20-*ant6*/DH5α	>1,024	16	2	>32	128	>256	–	–	–	–	–	–	–
pUCP20-*aph3'*/DH5α	>1,024	4	64	>32	64	>256	–	–	–	–	–	–	–
pUCP20-*ermB*/DH5α	>1,024	4	2	>32	1,024	>256	–	–	–	–	–	–	–
pUCP24-*bla*/DH5α	2	2	2	>32	128	>256	–	–	–	–	–	–	–
pUCP20-*sat4*/DH5α	>1,024	4	2	>32	128	>256	–	–	–	–	–	–	–
pAM401/JH2-2	1	128	128	2	<0.5	<0.5	–	–	–	–	–	–	–
pAM401-*ant6*/JH2-2	1	>1,024	128	2	<0.5	<0.5	–	–	–	–	–	–	–
pAM401-*aph3'*/JH2-2	1	128	>1,024	2	<0.5	<0.5	–	–	–	–	–	–	–
pAM401-*ermB*/JH2-2	1	128	128	2	>1,024	<0.5	–	–	–	–	–	–	–
pAM401-*bla*/JH2-2	1	128	128	2	<0.5	<0.5	–	–	–	–	–	–	–
pAM401-*sat4*/JH2-2	1	128	128	2	<0.5	<0.5	–	–	–	–	–	–	–
JH2-2	1	128	128	2	<0.5	<0.5	–	–	–	–	–	–	–
EC369	<0.5	>1,024	>1,024	1	>1,024	<0.5	>8	0.25	4	4	<1	2	<1
pEC369/JH2-2	1	>1,024	>1,024	2	>1,024	<0.5	–	–	–	–	–	–	–
ATCC 25922	4	8	4	>32	64	<0.5	–	–	–	–	–	–	–
ATCC 29212	1	128	32	4	1	<0.5	>8	0.25	4	4	<1	<0.5	<1

Abbreviations: AMP, ampcillin; CFX, cefoxitin; CHL, chloramphenicol; ERY, erythromycin; FFC, florfenicol; KAN, kanamycin; MIC, minimum inhibitory concentration; NOR, norfloxacin; PB, polymyxin B; SM, streptomycin; ST, streptothricin; TEC, teicoplanin; TGC, tigecycline; VAN, vancomycin.

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 *En. 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 fromed 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 (KY579372, 73 kb), and both had ~30 kb (33%, 30/91.96) sequences similar to that of pEC369. The regions of the two plasmids pRE25 and the plasmid of *En. faecium* strain F12085, similar to that of pEC369, were different from the plasmid from *Enterococcus* sp. FDAARGOS_375. The two plasmids shared nearly the same similar region, which spanned 10–45 kb of the pEC369, including the sequences of the drug resistance-related transposon and the genes related to conjugation (Figure 1). Although the plasmids with the greatest sequence similarity were the above three plasmids, the sequence most similar to that of resistance genes (transposon) was on the *S. aureus* chromosome, which suggested that exchanges of resistance genes may occur between these bacteria.

Conclusion

In this work, we sequenced the whole genome of the clinical isolate *En. casseliflavus* EC369. In addition to a resistance

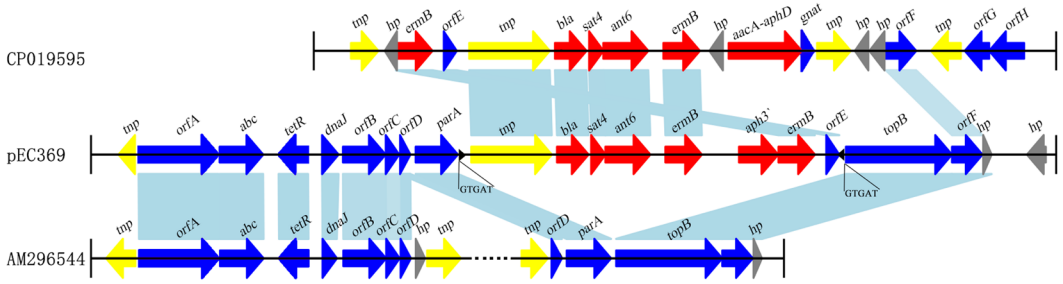

Figure 2 Comparative genomics analysis of the transposon-related region of pEC369.
Notes: The sequence with the highest identity to the resistance gene-related region of pEC369 was that of the chromosome sequence of *Staphylococcus aureus* strain GD1677, and the sequence with the highest identity to the flanking regions of the transposon was the sequence of pVEF1. Black triangles represent the IRs at both ends of the transposon. Nucleotide letters under the black triangles represent DRs. The identical sequence regions are connected with light blue bars. The predicted proteins without direct gene names are illustrated as: *orfA,* tetronasin resistance protein; *orfB,* zeta-toxin; *orfC,* antitoxin; *orfD* and *orfE,* peptide-binding proteins; *orfF,* resolvase; *orfG,* FAD-dependent thymidylate synthase; and *orfH* dihydrofolate reductase.
Abbreviation: DRs, direct repeats; IRs, inverted repeats.

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 *ermB*s) 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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