Comparative analysis of two consecutive genome sequencing results of *Enterococcus faecium* strain EntfacYE

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Running title: Comparing *Enterococcus faecium* EntfacYE genome changes

Abstract

The overuse of antimicrobials in healthcare has driven emergence, persistence, and rapid spread of antimicrobial-resistant pathogens. Vancomycin-resistant enterococci, majorly *Enterococcus faecium*, have recently emerged as multidrug-resistant bacteria worldwide. Therefore, enterococcal infections are more challenging to treat due to their increased multiple-drug resistance. Studying genome of an enterococcal isolate and investigating genome changes over time help researchers better understand antimicrobial resistance development in bacterial isolates. In the present study, *E. faecium* EntfacYE isolate from a human biological sample was assessed. After phenotypic, biochemical and molecular verifications of the bacterial isolate, the bacterial genome was wholly sequenced. In total, the EntfacYE genomic subsystems contained 23 categories with 46 antimicrobial resistance genes. In a previous study by Elahi et al., 59 antimicrobial resistance genes were reported for this isolate. In the current study, 31 antimicrobial resistance genes were reported in the subsystems and 15 genes had no subsystems, while these categories were respectively reported as 49 and ten in the previous study. Genes of tetracycline resistance were reported in this study, unlike the previous study. Despite the short time interval between the two studies, increases in the number and type of antimicrobial resistance genes were recorded in the current study, indicating that bacteria are becoming rapidly resistant to the available antimicrobials. In general, study of antimicrobial resistance genes in bacteria can be effective in better understanding of the resistance patterns and mechanisms, which can lead to find novel protocols for limiting spread of antimicrobial resistance in bacteria.

Keywords: Whole-genome sequencing, *Enterococcus faecium*, Biological samples, Antimicrobial resistance

1. Introduction

Enterococci are a part of the natural intestinal flora of mammals, birds and humans. Of various *Enterococcus* species, *E. faecalis* and *E. faecium* are the most common human species while *E. gallinarum* and *E. casseliflavus* are less common. *Enterococcus* spp. can opportunistically cause fatal endocarditis and urinary tract (UT) infections in humans (1). Excessive use of antimicrobials in human health care has resulted in emergence, and rapid spread of antimicrobial resistance; by which, microorganisms demonstrate resistance to a range of common antimicrobials (multiple-drug resistance) (2). Antimicrobial-resistant bacteria are major causes of healthcare-associated infections (HAIs) worldwide. Infections by multidrug-resistant microorganisms significantly increase morbidity, mortality and treatment costs. Recently, World Health Organization (WHO) has enlisted antimicrobial-resistant priority pathogens with major threats to human health, including *E. faecium* (3, 4). Enterococci are potential bacteria in expression of resistance genes (1). While vancomycin-resistant enterococci (VRE) make threats to the public health, multidrug-resistant enterococci (MDR) act as repositories for the horizontal gene transfer (HGT) of antimicrobial resistance determinants to other pathogenic microorganisms since transmission of *van*A from *Enterococcus* spp. to *Staphylococcus aureus* has frequently been reported (5). In this study, *E. faecium* EntfacYE was re-cultured and exposed to bacteriophages. This bacterial strain was previously isolated from a human biological sample and was wholly sequenced. Then, genome of the isolate was re-sequenced and changes in its antimicrobial resistance determinants, virulence factors, mobile genetic elements (MGEs) and multi-source sequencing patterns within a three-month time period were investigated.

2. Materials and Methods

2.1. Isolation of *E. faecium* EntfacYE and phenotypic identification

The *E. faecium* EntfacYE was previously isolated from a patient's blood in Imam Khomeini Hospital, Tehran, Iran, using conventional microbiological methods (ethics approval no. IR.TUMS.SPH.REC.1397.139) (6). The bacterial isolate was identified using routine methods such as Gram staining as well as oxidase, catalase, NaCl tolerance, PYR hydrolysis and bile esculin tests. Disc diffusion (Kirby) method was used to assess antimicrobial resistance of the bacterial isolate against erythromycin, clindamycin, linezolid, ceftriaxone, cefoxitin and vancomycin.

2.2. Molecular verification of the bacterial isolate

Sanger sequencing was used for the molecular verification of bacterial isolate. First, bacterial genome was extracted using heating method. Then, PCR was carried out on the genome using specific primers designed for the elongation factor Tu (EF-Tu) encoding gene. Primer sequences included Ent1: 5'-TACTGACAAACCATTCATGATG-3' and Ent2: 5'-AACTTCGTCACCAACGCGAAC-3' (7). Additionally, PCR products were detected in 1% agarose gels with TBE buffer (0.5%) and further investigated under UV. Then, PCR products were used for partial sequencing using Sanger method.

2.3. Complete genome sequencing

First, genome of *E. faecium* EntfacYE was manually extracted using methods of ethanol and propanol and then wholly sequenced using Illumina Hiseq platform (Novogen, China). Sequencing results were assembled using *de novo* technology and SPAdes algorithm. In addition, reference assembly method was applied for the analysis of raw data. The bacterial genome was generally analyzed using Rapid Annotation using Subsystem Technology (RAST) (https://rast.nmpdr.org) and results were annotated in DNA Data Bank of Japan (DDBJ) (www.ddbj.nig.ac.jp).

3. Results

3.1. Phenotypic and molecular verification results

After phenotypic and biochemical assessments on the bacterial isolate, the isolate was verified as *E. faecium*. Sanger sequencing of *tuf* gene approved initial characteristics of the isolated bacteria (DDBJ accession nos. LC580430 and LC580431).

3.2. Complete genome sequencing results

In this study, the complete bacterial genome was analyzed and its structure was studied. The *E. faecium* EntfacYE genome included 3,056,624 bp; of which, 37.5% were GC content. Furthermore, genome included 160 contigs. Totally, number of the subsystems in the bacterial genome was 231 (Table 1).

Table 1. General genomic information of the *Enterococcus faecium* EntfacYE

Genome	***Enterococcus faecium* EntfacYE**
DDBJ accession nos.	BPUK01000001–BPUK01000160
Isolation source	Patient blood sample
Size (bp)	3,056,624
GC content (%)	37.5
Contigs	160
Subsystems	231
Coding sequences	3155
RNAs	60

Results were annotated in DDBJ (accession nos. BPUK01000001–BPUK01000160). In total, *E. faecium* EntfacYE genome subsystems contained 23 categories; from which, carbohydrates, protein metabolism and amino acids and derivatives respectively included the highest and metabolism of aromatic compounds, sulfur metabolism and cell division and cell cycle included the lowest frequencies (Figure 1).

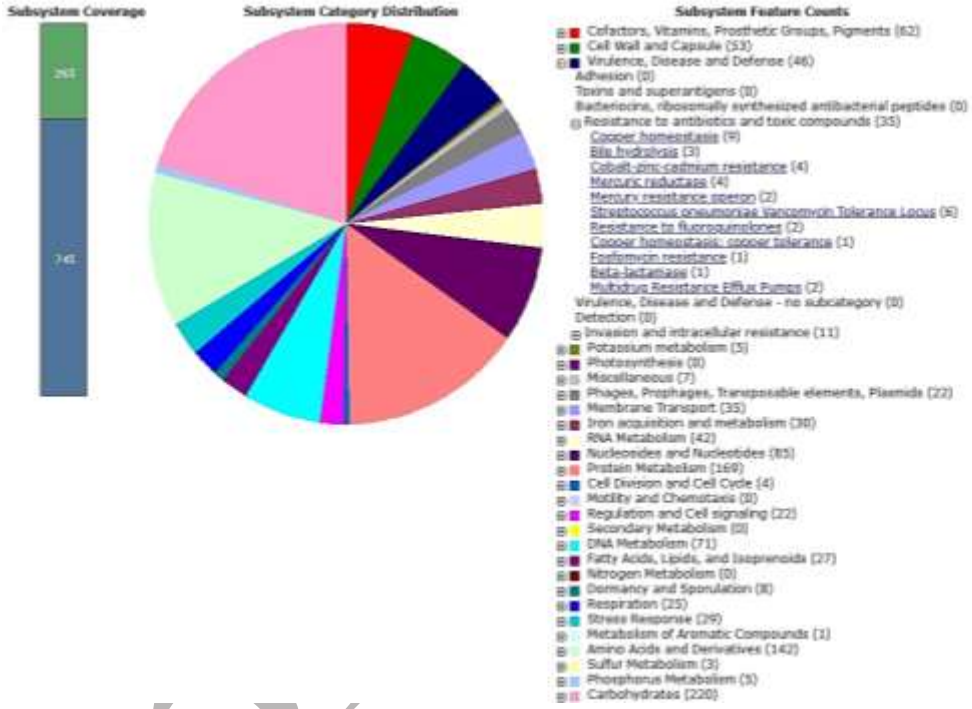

Figure 1. Genome subsystem of the *Enterococcus faecium* EntfacYE analyzed through Rapid Annotation using Subsystem Technology

3.3. Antimicrobial resistance assessment

Results of the antimicrobial resistance assessments revealed that the bacterial isolate was resistant to common antimicrobials such as vancomycin, clindamycin, erythromycin, ceftriaxone and cefoxitin. Bacterial resistance genes contained two main groups with and without subsystems. The subsystem group included 24% and the non-subsystem group included 74% of the bacterial genome. In total, 31 genes of antimicrobial resistance were located in specific subsystems and 15 genes were located in no specific subsystems (Tables 2

and 3). In addition, resistance genes of cadmium, cobalt, copper, zinc and mercury were identified in this study.

Table 2. Antimicrobial-resistance subsystems from genomic analysis of *Enterococcus faecium* EntfacYE

No.	Subsystem	Feature
1	Copper homeostasis	Negative transcriptional regulator-copper transport operon
1	Copper homeostasis	Copper-translocating P-type ATPase (EC 3.6.3.4)
1	Copper homeostasis	Copper chaperone
1	Bile hydrolysis	Choloylglycine hydrolase (EC 3.5.1.24)
1	Cobalt-zinc-cadmium resistance	Cobalt-zinc-cadmium resistance protein
1	Cobalt-zinc-cadmium resistance	Probable cadmium-transporting ATPase (EC 3.6.3.3)
1	Cobalt-zinc-cadmium resistance	Transcriptional regulator, MerR family
1	Mercuric reductase	PF00070 family, FAD-dependent NAD(P)-disulfide oxidoreductase
1	Mercuric reductase	Mercuric ion reductase (EC 1.16.1.1)
1	Mercury resistance operon	Mercuric ion reductase (EC 1.16.1.1)
1	*Streptococcus pneumoniae* vancomycin tolerance locus	Sensor histidine kinase VncS
1	*Streptococcus pneumoniae* vancomycin tolerance locus	ABC transporter, ATP-binding protein Vex2
1	*Streptococcus pneumoniae* vancomycin tolerance locus	Two-component response regulator VncR
1	*Streptococcus pneumoniae* vancomycin tolerance locus	ABC transporter membrane-spanning permease, Pep export, Vex1
1	*Streptococcus pneumoniae* vancomycin tolerance locus	ABC transporter membrane-spanning permease, Pep export, Vex3
1	Resistance to fluoroquinolones	DNA gyrase subunit B (EC 5.99.1.3)
1	Resistance to fluoroquinolones	DNA gyrase subunit A (EC 5.99.1.3)
1	Copper homeostasis: copper tolerance	Cytoplasmic copper homeostasis protein CutC
1	Fosfomycin resistance	Fosfomycin resistance protein FosX
1	Beta-lactamase	Metal-dependent hydrolases of the beta-lactamase superfamily I
1	Multidrug Resistance Efflux Pumps	Multidrug resistance efflux pump PmrA
1	Multidrug Resistance Efflux Pumps	Multi antimicrobial extrusion protein (Na(+)/drug antiporter), MATE family of MDR efflux pumps
1	*Mycobacterium* virulence operon involved in protein synthesis (SSU ribosomal proteins)	SSU ribosomal protein S7p (S5e)
1	*Mycobacterium* virulence operon involved in protein synthesis (SSU ribosomal proteins)	Translation elongation factor G
1	*Mycobacterium* virulence operon involved in protein synthesis (SSU ribosomal proteins)	Translation elongation factor Tu

1	*Mycobacterium* virulence operon involved in protein synthesis (SSU ribosomal proteins)	SSU ribosomal protein S12p (S23e)
2	*Mycobacterium* virulence operon involved in DNA transcription	DNA-directed RNA polymerase beta subunit (EC 2.7.7.6)
1	*Mycobacterium* virulence operon involved in protein synthesis (LSU ribosomal proteins)	LSU ribosomal protein L35p
1	*Mycobacterium* virulence operon involved in protein synthesis (LSU ribosomal proteins)	Translation initiation factor 3
1	*Mycobacterium* virulence operon involved in protein synthesis (LSU ribosomal proteins)	LSU ribosomal protein L20p

Table 3. Non-subsystem antimicrobial resistance genes from genomic analysis of *Enterococcus faecium* EntfacYE

Type	Length (bp)	Subsystem	Function
CDS	399	Uncharacterized	Tetracycline resistance, MFS efflux pump => TetA(P)
CDS	1170	Uncharacterized	Tetracycline resistance, ribosomal protection type => TetB(P)
CDS	402	Uncharacterized	Mercuric resistance operon regulatory protein MerR
CDS	531	Uncharacterized	Uncharacterized protein YacP, similar to C-terminal domain of ribosome protection-type Tc-resistance proteins
CDS	321	Uncharacterized	Small multidrug resistance family (SMR) protein
CDS	1713	Uncharacterized	Heterodimeric efflux ABC transporter, multidrug resistance => LmrC subunit of LmrCD
CDS	1773	Uncharacterized	Heterodimeric efflux ABC transporter, multidrug resistance => LmrD subunit of LmrCD
CDS	1920	Uncharacterized	Tetracycline resistance, ribosomal protection type => Tet(M)
CDS	900	Uncharacterized	Cobalt/zinc/cadmium resistance protein CzcD
CDS	372	Uncharacterized	glyoxalase/bleomycin resistance protein/dioxygenase superfamily protein
CDS	486	Uncharacterized	Teicoplanin resistance protein VanZ
CDS	1170	Uncharacterized	Tetracycline resistance, ribosomal protection type => TetB(P)
CDS	939	Uncharacterized	Tetracycline resistance, MFS efflux pump => TetA(P)
CDS	969	Uncharacterized	D-lactate dehydrogenase VanH, associated with vancomycin resistance (EC 1.1.1.28)
CDS	609	Uncharacterized	D-alanyl-D-alanine dipeptidase (EC 3.4.13.22) of vancomycin resistance => VanX

3.4. Comparative analysis of the two whole-genome sequencing sets

In a study by Elahi et al. (2021), whole-genome sequencing of *E. faecium* EntfacYE was carried out. They reported a genome size of 3,624,552 bp, which was 567,928 bp longer than that of the present study. Naturally, differences in the chromosome size and presence/absence of plasmids (as for other MGEs) could cause changes in genome size of a bacterial species. In

the current study, GC content decreased by 1.5%, compared to that in the previous study. Elahi et al. (2021) report included one contig, compared to 160 contigs of the current study. Technically, repetitive and insertion genetic elements complicate assembly, resulting in changes in the number of contigs. Number of the subsystems reported in *E. faecium* EntfacYE genome by Elahi et al. (2021) was 242, which was 11 subsystems greater than that reported the isolate genome by the present study. In the two sequencing sets, genomes totally included 23 various categories; from which, carbohydrates, amino acids and protein metabolism categories contained the most-frequent subsystems. In the two studies, sulfur metabolism, cell division and cell cycle were the least frequent categories with a reportable difference that metabolism of aromatic compounds was reported in the current study while phosphorus metabolism was reported in the previous study. Elahi et al. (2021) reported similar results in bacterial resistance to vancomycin, erythromycin, clindamycin, cefoxitin and ceftriaxone. They also reported that subsystem group included 23% of the bacterial genome, which was 3% smaller than that of the present study. The non-subsystem group contained 77% of the bacterial genome, with a 3% increase in the current study. In the current study, 31 antimicrobial resistance genes with specific subsystems and 15 genes without specific subsystems were reported while these categories were respectively reported as 49 and ten in the previous study. In addition, resistance genes of cobalt, cadmium, zinc, copper and mercury were reported in the two sequenced *E. faecium* EntfacYE genomes (Table 4).

Feature Author	Various categories	The most-frequent subsystems	The least-frequent subsystems	Resistance drug	Antibiotic resistance genes with specific subsystems	Genes without specific subsystems	Resistance genes
Elahi et al.	23	Carbohydrates, amino acids and protein metabolism	Sulfur metabolism, cell division and cell cycle and phosphorus metabolism	Erythromycin vancomycin, clindamycin, ceftriaxone and cefoxitin	49	10	Cobalt, cadmium, zinc, copper and mercury
Yazdani et al.	23	Carbohydrates, amino acids and protein metabolism	Metabolism of aromatic compounds, sulfur metabolism and cell division and cell cycle	Erythromycin, vancomycin, tetracycline, clindamycin, ceftriaxone and cefoxitin	31	15	Cobalt, cadmium, zinc, copper and mercury

Table 4. Comparison of the whole genome sequencing results

4. Discussion

Antimicrobial resistance is a serious concern of human and veterinary medicines, limiting treatment options and complicating infection controls. Selective pressures by antimicrobial uses have resulted in rapid prevalence of resistant bacterial strains. Investigating genetic basis of antimicrobial resistance is crucial for a better understanding of its transmission and persistence. Extensive and continuous application of antimicrobials in medicine and specifically in animal breeding has been a critical factor in evolutionary development of the antimicrobial resistance in bacteria (8). Enterococci are inherently resistant to several antimicrobial classes. In the past few decades, significant increases have been reported in the level of acquired antimicrobial resistance capability of enterococci, especially *E. faecium*. Recently, WHO has published a list of the preferred bacterial pathogens that need novel antimicrobials and protocols of treatment. Vancomycin-resistant *E. faecium* is included in this high-priority category (9, 10). In this study, 26% of the genes were covered by various subsystems (Figure 1) that increased by 3%, compared to the previous study (6). Antimicrobial susceptibility assessments showed that the bacterial isolate was resistant to vancomycin, tetracycline, erythromycin, ceftriaxone, cefoxitin and clindamycin. In a similar study, Sun et al. (2020) reported resistance to various antimicrobials, including vancomycin, clindamycin and erythromycin (11). Genomic studies are essential for identifying antimicrobial resistance determinants and assessing their potential spreads. Analyzing bacterial genomes allows researchers to track acquisition of resistance genes and investigate their distribution within various bacterial populations. These insights enhance the current understanding of the mechanisms driving resistance development. Naturally, three specific mechanisms are complicated in resistance to tetracycline. These mechanisms are described as 1) antimicrobial efflux pumps, 2) target modification through ribosomal protection protein (RPP), and 3) antimicrobial inactivation, (12). Elahi et al. reported one type of Tet(M) encoding gene, while no evidence of tetracycline resistance was seen in the bacterial isolate (6). In this study, two types of efflux pump genes of Tet(A), two types of efflux pump genes of Tet(B) and one type of RPP gene of Tet(M) were detected and tetracycline resistance was reported as well. Since Tet encoding genes can horizontally be transferred between the bacteria by the plasmids (13), it can be concluded that Tet encoding genes might be transferred to the bacterial isolate over time. Enterococci are commonly resistant to various drugs, including vancomycin (14). Between 2014 and 2017, vancomycin resistance in *E. faecium* isolates increased from 11.2 to 26.1% (15). Nearly 30% of healthcare-related infections by enterococci are reported as resistant to vancomycin and these VRE are usually resistant to other antimicrobials as well (14). In this study, resistance of the isolated EntfacYE to vancomycin was due to the Van (VanZ, VanH

and VanX) encoding genes. Since Elahi et al. (2021) also reported Van encoding genes (6), no changes in resistance of the isolate to vancomycin was reported in the present study. Sanderson et al. (2020) reported vancomycin resistance in nine of 11 *E. faecium* isolates (16). In the present study, results of the antimicrobial susceptibility assessments showed that EntfacYE was resistant to erythromycin. Since active drug efflux mechanism is common for the development of bacterial resistance to macrolides (e.g. erythromycin), genes that encoded efflux proteins might be responsible for the development of this resistance in EntfacYE. Because number and type of the genes encoding efflux proteins were similar in the two studies on *E. faecium* EntfacYE (6), no change in erythromycin resistance was observed. In 2020, Sanderson et al. similarly reported resistance of their isolates to erythromycin (16). In another study by Amachawadi et al., most strains were resistant to erythromycin (17). Clindamycin resistance was seen in the present study. Bozdogan et al. showed that resistance to clindamycin was due to the encoded ribosomal methylase (18). In the present study, resistances to ceftriaxone and cefoxitin were recorded because of beta-lactamase genes; as in the highlighted study by Elahi et al. (6). In a similar study by Edirmanasinghe et al., all isolates were resistant to cefoxitin and ceftriaxone (19). Fosfomycin-resistance genes were detected in the present study as well as a previously published study by Elahi et al. (6). Fosfomycin is an active antimicrobial used against MDR and extensively drug-resistant (XDR) Gram-positive and Gram-negative bacteria (20). Due to the spread of bacteria in the environment, releases of toxic and heavy metals in various forms may lead to increases in antimicrobial resistance of the bacteria. It has been suggested that heavy metals in the environment can develop antimicrobial-resistance bacteria since the resistance genes to both classes of antimicrobials are mostly carried on the same MGEs such as integrons (21). Naturally, bacteria may become resistant to copper, which can be encoded by the plasmids or bacterial chromosomes (22). In this study, a copper homeostasis subsystem was reported; as reported by Elahi et al. (6). Festa et al. reported presence of copper homeostasis in *Streptococcus pneumoniae*, *S. aureus* and *Mycobacterium tuberculosis* (23). In the current study, bile salt hydrolysis gene was investigated in *E. faecium* EntfacYE. Similarly, Elahi et al. detected this gene in their study (6). *Enterococcus* antimicrobial-resistance genes are genetically transferred by transposons and plasmids (24); therefore, presence of genes that are responsible for the resistance to heavy metals such as copper, mercury and cadmium in the genome of bacteria might be seen because of gene transfer by these MGEs. Investigation of heavy metal resistance genes in enterococci can be addressed as an effective way to identify potential antimicrobial-resistant enterococci (25). Genome analysis of antimicrobial-resistant enterococci, as well as study of their heavy-metal resistance,

may be effective in identifying resistant enterococci and providing appropriate methods for their treatment. Increased prevalence of antimicrobial-resistant pathogens highlights the great importance of continuous monitoring and research in this field. Without appropriate surveillance schemes, resistance may continuously evolve, further complicating treatment strategies. Whole-genome sequencing and comparative analyses provide essential data for understanding resistance dynamics and developing effective control measures.

In the present study, genome molecule of the *E. faecium* EntfacYE was sequenced and genes encoding antimicrobial resistance were analyzed and results were compared with those of a similar study by Elahi et al. three months earlier. In most cases, antimicrobial resistance gene schemes were similar in the two studies. Regarding tetracycline resistance genes, number of the genes increased in the present study compared to that number of the genes did in the previous study, which possibly occurred due to the activity of plasmids. Despite the short time interval between the current and previous studies, this increase has revealed that the bacteria become more rapid resistant to antimicrobials than that it was previously thought. The current ability of treating bacterial infections has been challenged seriously by the emergence of antimicrobial-resistant bacteria. Complete sequencing of the bacterial genomes and study of their resistance genes can lead to important insights into effective treatments for the severe infections caused by antimicrobial resistant bacteria.

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Author Contribution

MY, carried out experiments, genetically analyzed data, prepared the primary draft; AASY, advised the project, edited the primary draft; GJS, analyzed data, edited the primary draft; MA, analyzed data, edited the primary draft; RMNF, hypothesized concepts, supervised the project, edited the primary draft.

Conflict of Interest

No conflicts of interest are reported for this study.

Ethics Statement

This study was officially approved by the Ethics Committee of Tehran University of Medical Sciences (ethics approval no. IR.TUMS.SPH.REC.1397.139).

Data Availability

Data availability is verified by the corresponding author.

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