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Genome sequences of copper resistant and sensitive *Enterococcus faecalis* strains isolated from copper-fed pigs in Denmark

Siyu Zhang¹,², Dan Wang¹, Yihua Wang¹, Henrik Hasman⁴, Frank M. Aarestrup⁴, Hend A. Alwathnani⁵, Yong-Guan Zhu²,⁶ and Christopher Rensing¹,⁶*

**Abstract**

Six strains of *Enterococcus faecalis* (S1, S12, S17, S18, S19 and S32) were isolated from copper fed pigs in Denmark. These Gram-positive bacteria within the genus *Enterococcus* are able to survive a variety of physical and chemical challenges by the acquisition of diverse genetic elements. The genome of strains S1, S12, S17, S18, S19 and S32 contained 2,615, 2,769, 2,625, 2,804, 2,853 and 2,935 protein-coding genes, with 41, 42, 27, 42, 32 and 44 genes encoding antibiotic and metal resistance, respectively. Differences between Cu resistant and sensitive *E. faecalis* strains, and possible co-transfer of Cu and antibiotic resistance determinants were detected through comparative genome analysis.

**Keywords:** *Enterococcus faecalis*, Copper resistance, Antibiotic resistance, Genome sequence, Comparative genomics

**Introduction**

Copper is an essential trace element with an ubiquitous cellular distribution and performs several biological functions [1]. It serves as an important structural component or catalytic co-factor for a wide range of different enzymes in various important biochemical pathways in bacteria, plants and animals [2]. Because Cu, among many other micronutrients, is beneficial for growth promotion and feed efficiency of farm animals [3, 4], it is extensively used as an additive in swine feed. Normally, the concentration of Cu used in animal feed is in excess of the nutritional requirements of animals as it is used as an alternative to in-feed antibiotics for prevention of diarrheal disease [5]. Therefore, enteric bacteria, both commensal and pathogenic, in these animals have typically acquired several additional Cu resistance determinants to survive its toxicity [1, 6, 7].

*Enterococci* belong to the gastrointestinal flora of humans and animals, and have been known for more than a century for their pathogenicity to humans, causing urinary tract and surgical wound infections, bacteremia and endocarditis [8]. Currently, more than 30 species within the genus *Enterococcus* have been described, and the two most studied enterococcal species are *Enterococcus faecium* and *Enterococcus faecalis* [9]. Over the last two decades, *E. faecalis* and *E. faecium* have become increasingly important nosocomial pathogens worldwide and are difficult treat due to their increasing multidrug resistance [10]. The intrinsic resistance of *Enterococcus* to many antibiotics and its acquisition of resistance determinants to other antimicrobial agents led to the emergence of *Enterococcus* as a nosocomial pathogen [11, 12]. Recently, the co-selection of MDR isolates by antibiotics, metals and biocides has been reported [13, 14], and the resistance of *Enterococcus* to both Cu and antibiotics has been established [15, 16]. However, few studies have addressed gene transfer and the underlying molecular mechanisms of the various Cu resistance determinants in *E. faecalis* [17]. Herein, we present the genome sequences along with the main features of six *E. faecalis* strains showing...
the differences between Cu resistant and sensitive strains of *E. faecalis*, and suggesting possible co-transfer of Cu and antibiotic resistance determinants in these bacteria.

**Organism information**

**Classification and Features**
Phylogenetic analysis was performed using the 16S rRNA gene sequences on the six strains S1, S12, S17, S18, S19 and S32 and related species. Sequences were aligned using Clustal W, and a phylogenetic tree was constructed using neighbor-joining (NJ) method implemented in MEGA version 6.0. The resultant tree topologies were evaluated by bootstrap analyses with 1,000 random samplings. Phylogenetic analysis based on 16S rRNA gene sequences showed that the six strains clustered together with *E. faecalis* ATCC 29212 and *E. faecalis* SFL with a high bootstrap value (100 %). All the *E. faecalis* are in a distinct branch with the other enterococci, such as *E. casseliflavus*, *E. faecium*, *E. hirae* and the another pig gut *Firmicute*, that is *Streptococcus equinus* NCDO 1037 (Fig. 1). The six strains could be classified as members of the genus *Enterococcus* based on their 16S rRNA gene phylogeny and phenotypic characteristics (Table 1).

*E. faecalis* is a Gram-positive, oval-shaped, and often highly pathogenic bacterium classified as a member of the genus *Enterococcus* (Table 1 and Fig. 2) [18, 19]. It is a natural inhabitant of the mammalian gastrointestinal tract and is commonly found in soil, sewage, water and food [8]. *E. faecalis* is quite versatile and able to survive a variety of physical and chemical challenges by the acquisition of diverse genetic elements, which may contribute to their adaption to different hosts and environments [20, 21]. They are able to grow in temperatures ranging from 0 °C up to 50 °C, and can survive in the presence of 6.5 % NaCl and in broth at pH 9.6 [22]. They can also be resistant to heavy and transition metals [17], as well as many different antibiotics [23–25], especially vancomycin [20, 21].

**Genome sequencing information**

**Genome project history**
The *E. faecalis* strains (S1, S12, S17, S18, S19 and S32) were isolated from Cu-fed pigs as part of the Danish Integrated Antimicrobial Resistance Monitoring (DANMAP) surveillance program [23]. The isolates were collected from healthy animals at or just prior to slaughter. Those whole-genome shotgun projects have been deposited in DDBJ/EMBL/GenBank under the accession number JTKS00000000, JTKT00000000, JTKU00000000, JTKV00000000, JTKW00000000 and JTKX00000000. Table 2 presents the project information and its association with MIGS version 2.0 compliance [26]. Cu resistant strains are *E. faecalis* strains S1, S18, S32, while the other three strains are Cu sensitive.
Growth conditions and genomic DNA preparation

*E. faecalis* were streaked on Slanetz agar (BD Difco) plates and grown for 48 h at 42 °C. Each strain was inoculated separately into 25 ml of brain heart infusion broth at 37 °C for 24 h. Genomic DNA was purified from the isolates using the Easy-DNA extraction kit (Invitrogen), and DNA concentrations were determined by the Qubit dsDNA BR assay kit (Invitrogen).

Genome sequencing and assembly

Whole genome sequencing of *E. faecalis* strains S1, S12, S17, S18, S19 and S32 was carried out on an Illumina Miseq platform (Illumina, Inc., San Diego, CA). Genomic libraries were prepared by the Nextera XT DNA sample preparation kit (Illumina, cat. No. FC-131-1024), and then sequenced using v3, 2 × 300 bp chemistry on the Illumina MiSeq platform. Genomic assemblies were constructed using Velvet version 1.1.04, generating 24, 57, 20, 103, 34 and 89 contigs, respectively.

Genome annotation

The resulting contigs were uploaded onto the Rapid Annotation using Subsystem Technology server databases and the gene-caller GLIMMER 3.02 [27, 28] to predict open reading frames. The predicted ORFs were translated and annotated by searching against clusters of orthologous groups using the SEED databases [29], as well as NCBI databases. RNAmer 1.2 [30] and tRNAscan SE 1.23 [31] were used to identify rRNA genes and tRNA genes, respectively. CRISPR repeats were examined using CRISPR recognition tool (CRT) [32].

Genome properties

Whole genome sequencing of *E. faecalis* strains S1, S12, S17, S18, S19 and S32 resulted in 156, 162, 240, 84, 172 and 200 fold coverage of the genomes, respectively. The draft genome sizes were 2,762,808, 2,896,725, 2,786,673, 2,888,656, 2,969,229 and 3,037,709 bp in length, with an average GC content of 37.6, 37.4, 37.5, 37.4, 37.2 and

Table 1 Classification and general features of the six *Enterococcus faecalis* strains according to the MIGS recommendations [26]

| MIGS ID | Property | Term | Evidence code* |
|---------|----------|------|----------------|
| Current classification | Domain: Bacteria | TAS [38] |
|  | Phylum: Firmicutes | TAS [39] |
|  | Class: Bacilli | TAS [40] |
|  | Order: Lactobacillales | TAS [41] |
|  | Family: Enterococcaceae | TAS [42] |
|  | Genus: Enterococcus | TAS [18, 19] |
|  | Species: Enterococcus faecalis | TAS [43] |
| Strain: S1, S12, S17, S18, S19, S32 | NAS |
| Gram stain | Positive | TAS [42] |
| Cell shape | Oval cocci | TAS [42] |
| Motility | None | TAS [44] |
| Sporulation | Non-sporulating | TAS [43] |
| Temperature range | 10-45 °C | TAS [22] |
| Optimum temperature | 37 °C | TAS [22] |
| pH range | 4.6-9.9 (Optimum pH at 7.5) | TAS [22] |
| MIGS-6 Habitat | Gastrointestinal tracts of humans and other mammals | TAS [8] |
| MIGS-6.3 Salinity | 0-6.5 % | TAS [22] |
| MIGS-22 Oxygen | Facultatively anaerobic | TAS [44] |
| MIGS-15 Biotic relationship | Commensal bacterium | TAS [8] |
| MIGS-14 Pathogenicity | Highly pathogenic | TAS [43] |
| MIGS-4 Geographic location | Denmark | NAS |
| MIGS-5 Sample collection | 2011 | NAS |
| MIGS-4.1 Latitude | Unknown | NAS |
| MIGS-4.2 Longitude | Unknown | NAS |
| MIGS-4.3 Altitude | Unknown | NAS |

*Evidence codes - TAS: Traceable Author Statement (i.e., a direct exists in the literature); NAS: Non-traceable Author Statement (i.e., not directly observed for the living, isolated sample, but based on a generally accepted property for the species, or anecdotal evidence). These evidence codes are from the Gene Ontology project [45].

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37.2%, respectively, and comprises 2,615; 2,769; 2,625; 2,804; 2,853 and 2,935 protein coding sequences, respectively. Of the protein coding genes, 2,002; 2,006; 1,949; 2,001; 2,058 and 2,073 were genes with function predictions, with 41, 42, 27, 42, 32 and 44 genes responsible for antibiotics and toxic compounds resistant, respectively. There are 52 (4 rRNA genes and 48 tRNA genes), 54 (3 rRNA genes and 51 tRNA genes), 52 (4 rRNA genes and 48 tRNA genes), 53 (3 rRNA genes and 50 tRNA genes) and 55 (5 rRNA genes and 50 tRNA genes) RNA genes for strains S1, S12, S17, S18, S19 and S32, respectively. The properties and statistics for the genome are summarized in Table 3. The distribution of genes into COG functional categories is presented in Table 4 and Fig. 3.

![Fig. 2](Micrograph of *E. faecalis* strains obtained by scanning electron microscopy. Scale bar, 4 μm)

| Table 2 Project information |
|-----------------------------|
| MIGS ID | Property | Term/Strains |
| ST | S12 | S17 | S18 | S19 | S32 |
| MIGS-31 | Finishing quality | High-quality draft |
| MIGS-28 | Libraries used | One paired-end Illumina library |
| MIGS-29 | Sequencing platforms | Illumina MiSeq |
| MIGS-31.2 | Fold coverage | 156 | 162 | 240 | 84 | 172 | 200 |
| MIGS-30 | Assemblers | Velvet version 1.1.04 |
| MIGS-32 | Gene calling method | Glimmer 3.0 |
| Genbank ID | JTKS00000000 | JTKT00000000 | JTKU00000000 | JTKV00000000 | JTKW00000000 | JTKX00000000 |
| Genbank Date of Release | 2014/12/02 |
| Bioproject | PRJNA267758 | PRJNA268957 | PRJNA268240 | PRJNA268137 | PRJNA267759 | PRJNA268241 |
| Project relevance | Environmental |
| MIGS-13 | Source Material Identifier | Strain: 1 Strain: 12 | Strain: 17 | Strain: 18 | Strain: 19 | Strain: 32 |
| Project relevance | Environment, bacteria isolated from copper fed pigs |

Copper resistant strains are marked in red (S1, S18 and S32)
| Attribute                              | Strain | S1  | S12 | S17 | S18 | S19 | S32 |
|----------------------------------------|--------|-----|-----|-----|-----|-----|-----|
| Contigs                                |        | 24  | 57  | 20  | 103 | 34  | 89  |
| Genome size (bp)                       |        | 2,762,808 | 2,896,725 | 2,786,673 | 2,888,656 | 2,969,229 | 3,037,709 | 100 |
| DNA coding region (bp)                 |        | 2,443,661 | 2,539,142 | 2,451,937 | 2,539,829 | 2,579,002 | 2,639,903 | 100 |
| DNA G + C content (bp)                 |        | 1,038,816 | 1,083,375 | 1,045,002 | 1,080,357 | 1,104,553 | 1,130,028 | 100 |
| Total genes                            |        | 2,701 | 2,864 | 2,706 | 2,892 | 2,962 | 3,043 | 100 |
| Protein-coding genes                   |        | 2,615 | 98.09 | 2,769 | 98.09 | 2,625 | 98.21 | 2,804 | 98.15 |
| RNA genes                              |        | 52  | 1.93 | 54  | 1.89 | 48  | 1.77 | 52  | 1.80 |
| Pseudo genes                           |        | 35  | 1.30 | 43  | 1.50 | 34  | 1.26 | 36  | 1.24 |
| Genes in internal clusters             |        | 1,150 | 42.58 | 1,228 | 42.88 | 1,127 | 41.65 | 1,256 | 43.43 |
| Genes with function prediction         |        | 2,002 | 76.56 | 2,006 | 72.44 | 1,949 | 74.25 | 2,001 | 71.36 |
| Genes assigned to COGs                 |        | 2,011 | 76.90 | 2,024 | 73.09 | 1,980 | 75.43 | 2,025 | 72.22 |
| Genes with Pfam domains                |        | 2,268 | 86.73 | 2,313 | 83.53 | 2,231 | 84.99 | 2,282 | 81.38 |
| Genes with signal peptides             |        | 575  | 21.99 | 614  | 22.17 | 600  | 22.86 | 590  | 21.04 |
| Genes with transmembrane helices       |        | 729  | 27.88 | 769  | 27.77 | 756  | 28.80 | 754  | 26.89 |
| CRISPR repeats                         |        | 1  | 1  | 2  | 1  | 2  | 1  | 1  |

The total is based on either the size of the genome in base pairs or the total number of protein coding genes in the annotated genome.
| Code | Attribute                                    | S1  | S12 | S17 | S18 | S19 | S32 |
|------|----------------------------------------------|-----|-----|-----|-----|-----|-----|
| J    | Translation, ribosomal structure and biogenesis | 155 | 5.93| 152 | 5.49| 152 | 5.79|
|      |                                              |     |     |     |     |     |     |
| A    | RNA processing and modification              | -   | -   | -   | -   | -   | -   |
| K    | Transcription                               | 172 | 6.58| 178 | 6.43| 174 | 6.63|
|      |                                              |     |     |     |     |     |     |
| L    | Replication, recombination and repair        | 114 | 4.36| 125 | 4.51| 112 | 4.27|
|      |                                              |     |     |     |     |     |     |
| B    | Chromatin structure and dynamics             | -   | -   | -   | -   | -   | -   |
| D    | Cell cycle control, mitosis and meiosis      | 22  | 0.84| 25  | 0.90| 22  | 0.84|
|      |                                              |     |     |     |     |     |     |
| Y    | Nuclear structure                            | -   | -   | -   | -   | -   | -   |
| U    | Intracellular trafficking and secretion      | 24  | 0.92| 25  | 0.90| 25  | 0.95|
| O    | Posttranslational modification, protein turnover and chaperons | 50  | 1.91| 49  | 1.77| 48  | 1.83|
|      |                                              |     |     |     |     |     |     |
| G    | Carbohydrate transport and metabolism        | 269 | 10.29| 282 | 10.18| 264 | 10.06|
|      |                                              |     |     |     |     |     |     |
| E    | Amino acid transport and metabolism          | 173 | 6.62| 172 | 6.21| 169 | 6.44|
|      |                                              |     |     |     |     |     |     |
| F    | Nucleotide transport and metabolism          | 93  | 3.56| 90  | 3.25| 87  | 3.31|
|      |                                              |     |     |     |     |     |     |
| H    | Coenzyme transport and metabolism            | 69  | 2.64| 68  | 2.46| 68  | 2.59|
|      |                                              |     |     |     |     |     |     |
| I    | Lipid transport and metabolism               | 56  | 2.14| 56  | 2.02| 57  | 2.17|
|      |                                              |     |     |     |     |     |     |
| P    | Inorganic ion transport and metabolism       | 118 | 4.51| 115 | 4.15| 110 | 4.19|
|      |                                              |     |     |     |     |     |     |
| Q    | Secondary metabolism biosynthesis, transport and catabolism | 28  | 1.07| 28  | 1.01| 28  | 1.07|
|      |                                              |     |     |     |     |     |     |
| R    | General function prediction only             | 249 | 9.52| 251 | 9.06| 245 | 9.33|
| S    | Function unknown                             | 218 | 8.34| 224 | 8.09| 222 | 8.46|
|      |                                              |     |     |     |     |     |     |
| -    | Not in COGs                                  | 604 | 23.10| 745 | 26.91| 645 | 24.57|

The total is based on the total number of protein coding genes in the annotated genome.
Insights from the genome sequence

All of the six strains contain a four gene operon, copYZAB, encoding a Cu resistance determinant (Table 5), which was initially observed in the Gram-positive bacterium E. hirae [33]. CopA and CopB are P-type ATPases responsible for ATP-dependent Cu⁺ transport across the cytoplasmic membranes. The Cu chaperone CopZ binds two Cu⁺ atoms in a solvent accessible manner, presumably to facilitate their transfer to the transcriptional regulator CopY. Upon binding Cu⁺, CopY undergoes a conformational change and is released from the copA operator allowing expression of the copYZAB operon [1]. A gene encoding the cytoplasmic Cu homeostasis protein CutC was identified in all six strains (Table 5), and CutC has been demonstrated to be involved in Cu homeostasis in E. faecalis [34]. In addition, another possible gene encoding a putative Cu⁺-translocating P-type ATPase, was identified in all six strains named ctpA in this study (Table 5). The genome comparisons of the six E. faecalis strains using E. faecalis S32 as the reference strain by CGview comparison tool [35] indicated that S1 and S18 were more similar to the reference strain S32 than the other three strains.

Fig. 3 Graphical circular map of the genome comparison of E. faecalis S32 with the other five strains. Labeling from the outside to the inside circle: ring 1 and 4 show the protein coding genes on the forward/reverse strand (colored by COG categories); ring 2 and 3 show the denote genes on the forward/reverse strand; ring 5, 6, 7, 8 and 9 show the CDS vs CDS BLAST results of E. faecalis S32 with S1, S18, S12, S19 and S17, respectively; ring 10 shows the G + C content (peaks out/inside the circle indicate values higher or lower than the average G + C content, respectively); ring 11 shows GC skew (calculated as (G - C)/(G + C), peaks out/inside the circle indicates values higher or lower than 1, respectively). Ring 5–9 were arranged based on the CDS BLAST results, with the similarity rank from high to low, that is S1 and S18 were more similar to the reference strain S32 than the other three strains.
**Table 5** Copper and antibiotic resistance genes in *E. faecalis* strains. S1, S18 and S32 represent the three Cu resistant *E. faecalis* strains, and S12, S17 and S19 represent the three Cu sensitive *E. faecalis* strains.

| Genes         | Strain name | S1   | S18 | S32 | S12 | S17 | S19 |
|--------------|-------------|------|-----|-----|-----|-----|-----|
| **copY**     |             | ++   | ++  | ++  | +   | +   | +   |
| **copA**     |             | +    | +   | +   | +   | +   | +   |
| **copB**     |             | +    | +   | +   | +   | +   | +   |
| **copZ**     |             | +    | +   | +   | +   | +   | +   |
| **tcrY**     |             | +    | +   | +   | -   | -   | -   |
| **tcrA**     |             | +    | +   | +   | -   | -   | -   |
| **tcrB**     |             | +    | +   | +   | -   | -   | -   |
| **tcrZ**     |             | +    | +   | +   | -   | -   | -   |
| **cpxA**     |             | +    | +   | +   | +   | +   | +   |
| **cueO**     |             | +    | +   | +   | -   | -   | -   |
| **cutC**     |             | +    | +   | +   | +   | +   | +   |
| **tetM**     |             | +    | +   | +   | -   | -   | -   |
| **vanA**     |             | -    | -   | +   | -   | -   | -   |
| Streptothricin acetyltransferase gene | + | + | + | - | - | - |
| Aminoglycoside adenylyltransferase gene | + | + | + | - | - | - |

*copyYABZ* copper resistance genes in sensitive strains (For S1, S18 and S32, one of the copY is on the Cu resistant island, and the other is on the chromosome.); *tcrYABZ* copper resistance genes in resistant strains; *cpxA*: copper resistance genes; *cueO*: multicopper oxidase genes; *cutC*: genes encoding cytoplasmic copper homeostasis protein; *tetM*: tetracycline resistance genes; *vanA*: vancomycin resistance genes; Streptothricin acetyltransferase gene: streptothricin resistance genes

*E. faecalis* which encodes a Cu\(^{2+}\)-translocating P-type ATPase homologous to CopB encoded on *copYZAB* operon [37]. Comparing these six *E. faecalis* strains against others previously identified with increased Cu resistance, the *tcrYABZ* operon and adjacent *cueO* encoding a multicopper oxidase were only identified in *E. faecalis* S1, S18 and S32 (Table 5). Blasting of the *tcrYABZ* operon against the contigs of the other three strains verified that they were indeed lacking Cu resistance genes. The *cueO* gene identified in putative copper resistant strains encodes a multicopper oxidase that is transported across the cytoplasmic membrane and oxidizes Cu(I) to Cu(II) and so aids protection from high Cu concentrations in *Enterococcus* [9] or other Gram-positive strains [16]. The approximate 20-gene copper pathogenicity/fitness island present in *E. faecalis* S1,
S18 and S32, show cueO is located in close vicinity of tcrYAZB and probably regulated by an adjacent two-component regulator system (Cu(I)-sensing regulator (cusK) and Cu(I)-sensing sensor (cusS)) (Fig. 4). Transposable and mobile element protein genes were also identified on this pathogenicity/fitness island next to tcrYAZB, indicating mobility. Moreover, genes encoding prolipoprotein diacylglycerol transferase, which is responsible for oxidative stress tolerance potentially also caused by Cu+, could be identified on these potential pathogenicity and/or fitness islands as well. For the other three Cu sensitive E. faecalis S12, S17 and S19, tcrYAZB, cueO, cusR, cusS or genes encoding a prolipoprotein diacylglycerol transferase could not be detected.

The antibiotic resistance gene tetM (resistance to tetracycline) could be identified in the three Cu resistant E. faecalis S1, S18, S32, and Cu sensitive E. faecalis S12; vanA (encoding vancomycin resistance) was identified only in Cu resistant E. faecalis S32; streptothricin acetyltransferase gene was identified in the Cu resistant E. faecalis S1, S18, S32; and aminoglycoside adenylyltransferase gene was identified in two Cu resistant E. faecalis S1 and S18 (Table 5).

Conclusions
Since the co-transfer of genes encoding antibiotic resistance along with Cu tolerance genes in one transconjugant has been demonstrated [14], the results in this study might provide valuable information corroborating the co-transfer of genes encoding additional Cu resistance and genes encoding numerous antibiotic resistances. Also, the identified antibiotic resistance gene tetM in all the Cu resistant strains is consistent with the MDR Enterococcus strains observed in the environment [13–16].

Abbreviation
MDR: Multidrug-resistant.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
SZ drafted the manuscript, performed laboratory experiments, and analyzed the data. DW and YW performed the comparative genome analysis. HH, FA and HA sequenced, assembled, and annotated the genome. YZ revised the manuscript. All authors read and approved the final manuscript.

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University, Riyadh, Saudi Arabia. *Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen, China.

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