Zhang et al. Standards in Genomic Sciences (2015) 10:35
DOI 10.1186/s40793-015-0021-1

Standards in Genomic Sciences

SHORT GENOME REPORT Open Access

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

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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, bacteraemia 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

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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.

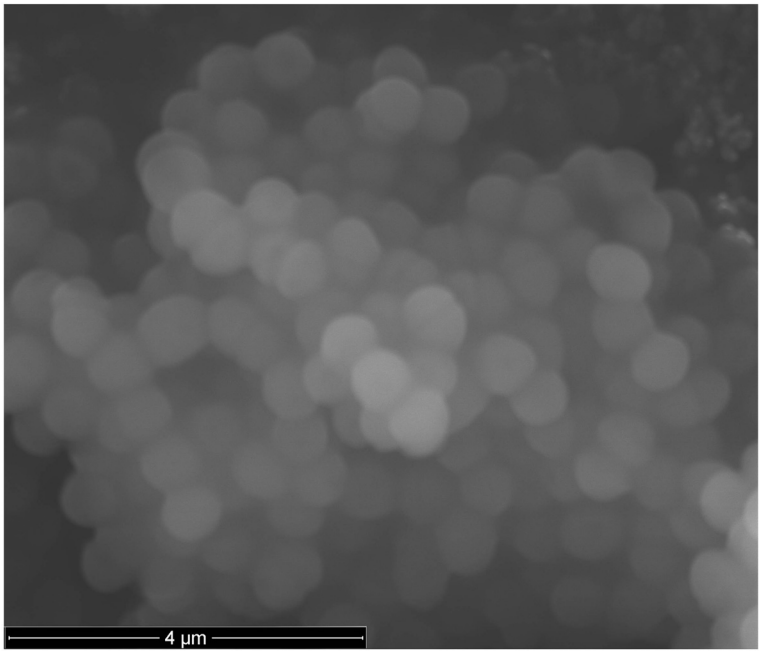

Fig. 1 Phylogenetic tree highlighting the position of the six *E. faecalis* strains relative to phylogenetically closely related type strains within the genus *Enterococcus*. The sequences were aligned using Clustal W, and the neighbor-joining tree was constructed based on kimura 2-parameter distance model using MEGA 6.0. Bootstrap values above 50 % are shown obtained from 1,000 bootstrap replications. Bar, 0.02 substitutions per nucleotide position. GenBank accession numbers are displayed in parentheses. Large triangles represent the six *Enterococcus* strains sequenced in this study

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

MIGS ID	Property	Term	Evidence code[a]
	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

[a]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]

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. RNAmmer 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

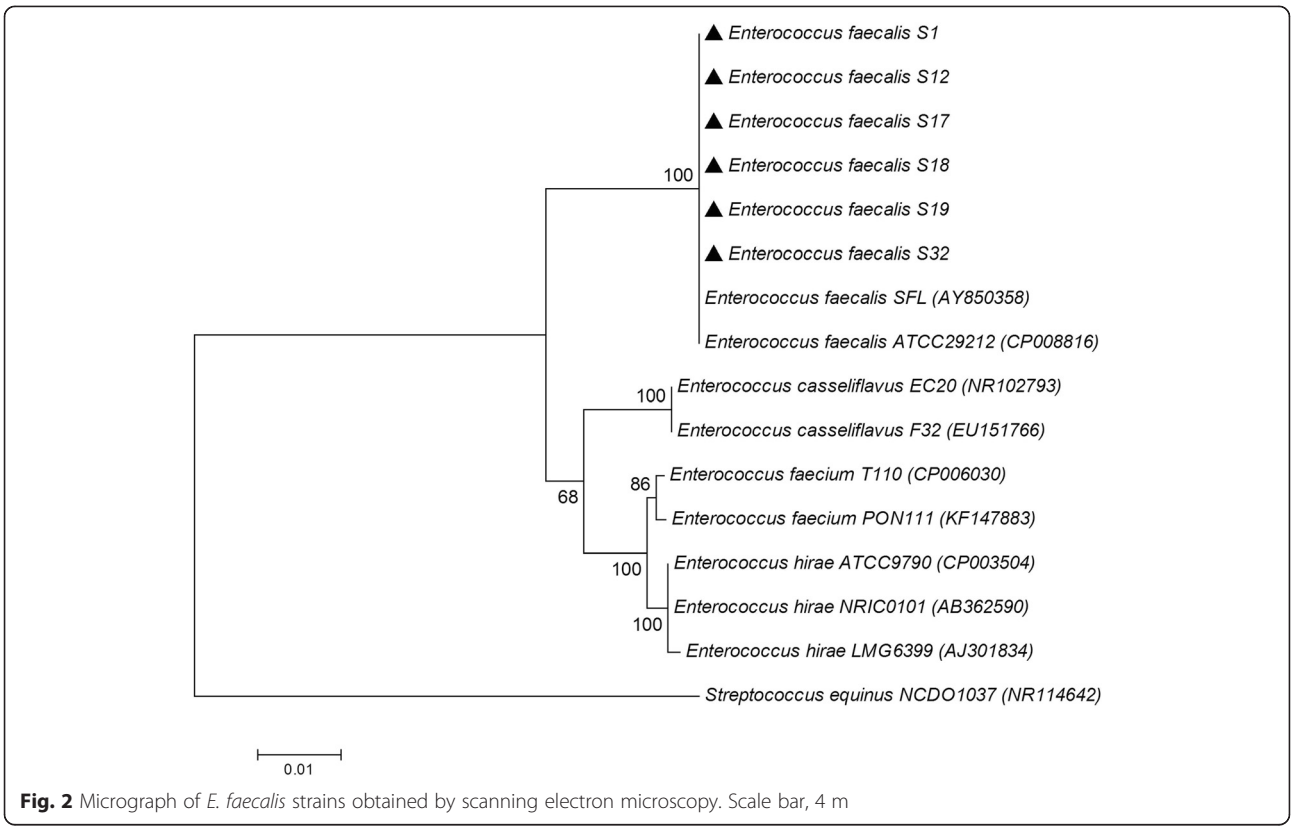

Fig. 2 Micrograph of *E. faecalis* strains obtained by scanning electron microscopy. Scale bar, 4 m

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), 48 (3 rRNA genes and 45 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.

Table 2 Project information

MIGS ID	Property	Term/Strains					
		S1	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)

Table 3 Genome statistics

Attribute	Strain											
	S1		S12		S17		S18		S19		S32	
	Value	%	Value	%	Value	%	Value	%	Value	%	Value	%
Contigs	24	-	57	-	20	-	103	-	34	-	89	-
Genome size (bp)	2,762,808	100	2,896,725	100	2,786,673	100	2,888,656	100	2,969,229	100	3,037,709	100
DNA coding region (bp)	2,443,661	88.45	2,539,142	87.66	2,451,937	87.99	2,539,829	87.92	2,579,002	86.86	2,639,903	86.90
DNA G + C content (bp)	1,038,816	37.6	1,083,375	37.4	1,045,002	37.5	1,080,357	37.4	1,104,553	37.2	1,130,028	37.2
Total genes	2,701	100	2,864	100	2,706	100	2,892	100	2,962	100	3,043	100
Protein-coding genes	2,615	98.09	2,769	98.09	2,625	98.21	2,804	98.15	2,853	98.15	2,935	98.17
RNA genes	52	1.93	54	1.89	48	1.77	52	1.80	53	1.79	55	1.81
Pseudo genes	35	1.30	43	1.50	34	1.26	36	1.24	59	1.99	63	2.07
Genes in internal clusters	1,150	42.58	1,228	42.88	1,127	41.65	1,256	43.43	1,265	42.71	1,313	43.15
Genes with function prediction	2,002	76.56	2,006	72.44	1,949	74.25	2,001	71.36	2,058	72.13	2,073	70.63
Genes assigned to COGs	2,011	76.90	2,024	73.09	1,980	75.43	2,025	72.22	2,049	71.82	2,084	71.01
Genes with Pfam domains	2,268	86.73	2,313	83.53	2,231	84.99	2,282	81.38	2,318	81.25	2,374	80.89
Genes with signal peptides	575	21.99	614	22.17	600	22.86	590	21.04	632	22.15	639	21.77
Genes with transmembrane helices	729	27.88	769	27.77	756	28.80	754	26.89	779	27.30	797	27.16
CRISPR repeats	1	-	1	-	2	-	1	-	2	-	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

Table 4 Number of genes associated with the 25 general COG functional categories

Code	Attribute	Strain											
		S1		S12		S17		S18		S19		S32	
		Value	%	Value	%	Value	%	Value	%	Value	%	Value	%
J	Translation, ribosomal structure and biogenesis	155	5.93	152	5.49	152	5.79	153	5.46	152	5.33	153	5.21
A	RNA processing and modification	-	-	-	-	-	-	-	-	-	-	-	-
K	Transcription	172	6.58	178	6.43	174	6.63	173	6.17	183	6.41	184	6.27
L	Replication, recombination and repair	114	4.36	125	4.51	112	4.27	127	4.53	127	4.45	132	4.50
B	Chromatin structure and dynamics	-	-	-	-	-	-	-	-	-	-	-	-
D	Cell cycle control, mitosis and meiosis	22	0.84	25	0.90	22	0.84	21	0.75	23	0.81	24	0.82
Y	Nuclear structure	-	-	-	-	-	-	-	-	-	-	-	-
V	Defense mechanisms	56	2.14	45	1.63	51	1.94	46	1.64	46	1.61	54	1.84
T	Signal transduction mechanisms	90	3.44	89	3.21	85	3.24	94	3.35	87	3.05	95	3.24
M	Cell wall/membrane biogenesis	105	4.02	100	3.61	107	4.08	105	3.74	98	3.43	123	4.19
N	Cell motility	10	0.38	10	0.36	11	0.42	9	0.32	12	0.42	12	0.41
Z	Cytoskeleton	-	-	-	-	-	-	-	-	-	-	-	-
W	Extracellular structures	-	-	-	-	-	-	-	-	-	-	-	-
U	Intracellular trafficking and secretion	24	0.92	25	0.90	25	0.95	27	0.96	24	0.84	24	0.82
O	Posttranslational modification, protein turnover and chaperons	50	1.91	49	1.77	48	1.83	48	1.71	49	1.72	48	1.64
C	Energy production and conversion	106	4.05	106	3.83	105	4.00	106	3.78	107	3.75	106	3.61
G	Carbohydrate transport and metabolism	269	10.29	282	10.18	264	10.06	262	9.34	296	10.38	277	9.44
E	Amino acid transport and metabolism	173	6.62	172	6.21	169	6.44	176	6.28	171	5.99	173	5.89
F	Nucleotide transport and metabolism	93	3.56	90	3.25	87	3.31	93	3.32	92	3.22	90	3.07
H	Coenzyme transport and metabolism	69	2.64	68	2.46	68	2.59	72	2.57	66	2.31	72	2.45
I	Lipid transport and metabolism	56	2.14	56	2.02	57	2.17	59	2.10	56	1.96	58	1.98
P	Inorganic ion transport and metabolism	118	4.51	115	4.15	110	4.19	119	4.24	112	3.93	115	3.92
Q	Secondary metabolism biosynthesis, tansport and catabolism	28	1.07	28	1.01	28	1.07	31	1.11	27	0.95	30	1.02
R	General function prediction only	249	9.52	251	9.06	245	9.33	255	9.09	253	8.87	253	8.62
S	Function unknown	218	8.34	224	8.09	222	8.46	220	7.85	235	8.24	238	8.11
-	Not in COGs	604	23.10	745	26.91	645	24.57	779	27.78	804	28.18	851	28.99

The total is based on the total number of protein coding genes in the annotated genome

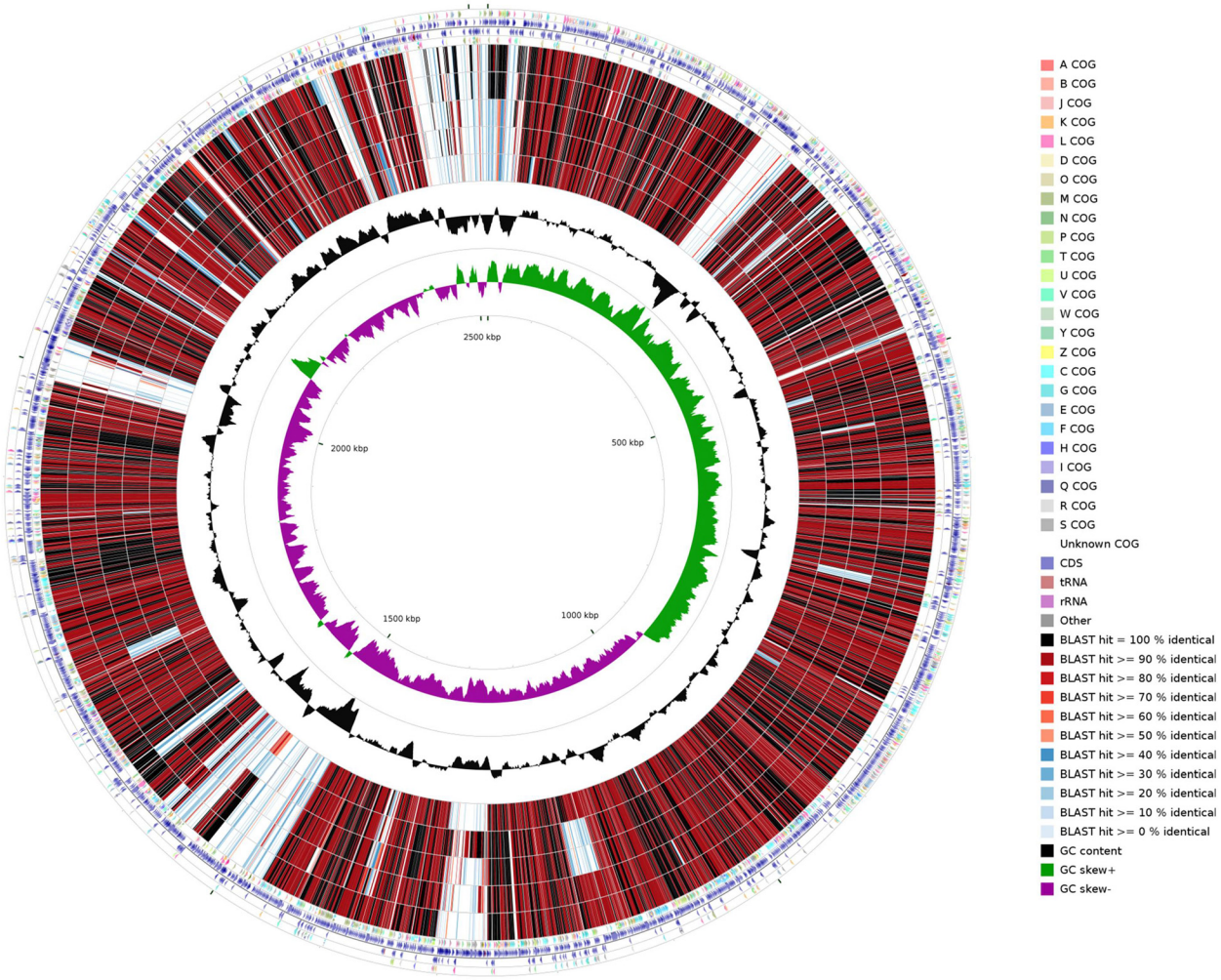

Fig. 3 Graphical circular map of the genome comparison of *E. faecalis* S32 with the other five strains. Labeling from the outside to the inside circle: ring 1 and 4 show the protein coding genes on the forward/reverse strand (colored by COG categories); ring 2 and 3 show the denote genes on the forward/reverse 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

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).

The *tcrYAZB* operon was initially identified on the pA17sv1 plasmid in *E. faecium*, which also carried genes encoding resistance to erythromycin (*ermB*) and vancomycin (*vanA*) [17, 36]. High toxic Cu levels could be tolerated due to the presence of *tcrB* in *E. faecium* or

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	+	+	+	+	–	–
ctpA	+	+	+	+	+	+
cueO	+	+	+	–	–	–
cutC	+	+	+	+	+	+
tetM	+	+	+	+	–	–
vanA	–	–	+	–	–	–
Streptothricin acetyltransferase gene	+	+	+	–	–	–
Aminoglycoside adenylyltransferase gene	+	+	–	–	–	–

copYABZ 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; *ctpA*: 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^{+}-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 *tcrYAZB* operon and adjacent *cueO* encoding a multicopper oxidase were only identified in *E. faecalis* S1, S18 and S32 (Table 5). Blasting of the *tcrYAZB* 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,

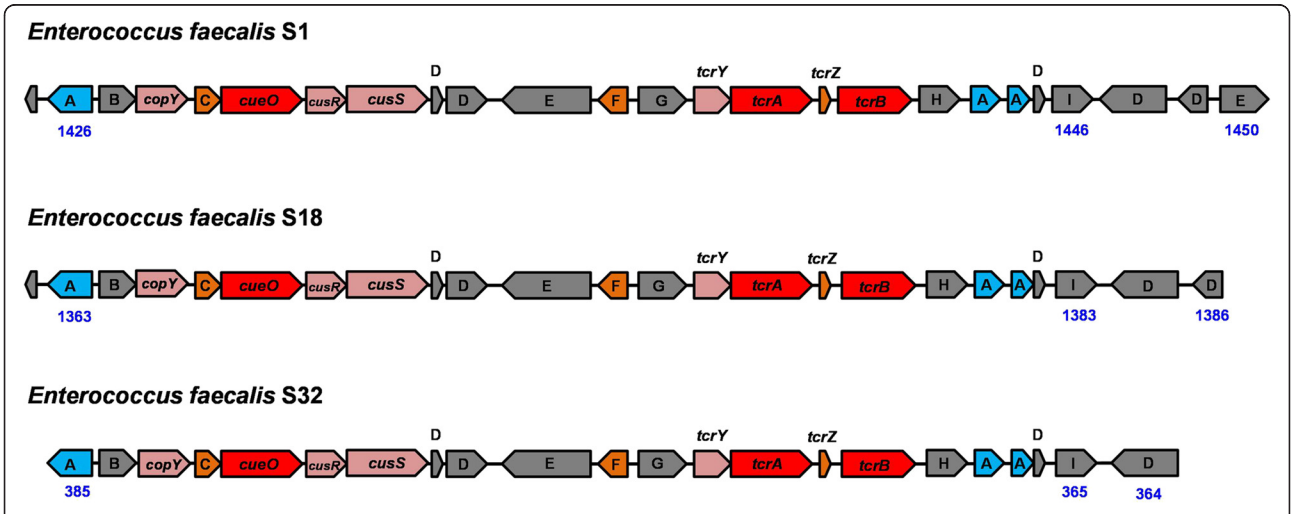

Fig. 4 Cu pathogenicity island in *E. faecalis* S1, S18 and S32. **a**: prolipoprotein diacylglyceryl trPropertyansferase, **b**: intergral membrane protein, **c**: chaperone, **d**: hypothetical protein, **e**:transposase, **f**: disrupted P-type ATPase, **g**: integrase, **h**: adenylate kinase, **i**: resolvase, *copY*: CopY family transcriptional regulator, *cueO*: multicopper oxidase, *cusR*: Cu(I)-sensing regulator, *cusS*: Cu(I)-sensing sensor, *tcrY*: *tcrYAZB* operon regulator, *tcrA*: putative copper-efflux CPx-type ATPase, *tcrB*: Cu^{+}-translocating CPx-type ATPase, *tcrZ*: putative chaperone

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 (*cusR*) and Cu(I)-sensing sensor (*cusS*)) (Fig. 4). Transposase and mobile element protein genes were also identified on this pathogenicity/fitness island next to *tcrYAZB*, indicating mobility. Moreover, genes encoding prolipoprotein diacylglyceryl 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 diacylglyceryl 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. CR organized the study and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This work was supported by the Center for Environmental and Agricultural Microbiology (CREAM) funded by the Villum Kann Rasmussen Foundation.

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Received: 9 February 2015 Accepted: 19 May 2015
Published online: 08 July 2015

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