Genomic and phenotypic diversity of *Enterococcus faecalis* isolated from endophthalmitis

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ABSTRACT

Enterococcus faecalis are hospital-associated opportunistic pathogens and also causative agents of post-operative endophthalmitis. Patients with enterococcal endophthalmitis often have poor visual outcomes, despite appropriate antibiotic therapy. Here we investigated the genomic and phenotypic characteristics of *E. faecalis* isolates collected from 13 patients treated at the University of Pittsburgh Medical Center Eye Center over 19 years. Comparative genomic analysis indicated that patients were infected with *E. faecalis* of diverse multi-locus sequence types (STs) previously associated with clinical, commensal, and environmental sources. We identified known *E. faecalis* virulence factors and antibiotic resistance genes in each genome, including genes conferring resistance to aminoglycosides, erythromycin, and tetracyclines. We assessed all isolates for their cytolysin production, biofilm formation, and antibiotic susceptibility, and observed phenotypic differences between isolates. Fluoroquinolone and cephalosporin susceptibilities were particularly variable, as were biofilm formation and cytolysin production. In addition, we found evidence of *E. faecalis* adaptation during recurrent endophthalmitis by identifying genetic variants that arose in sequential isolates sampled over eight-months from the same patient. We identified a mutation in the DNA mismatch repair gene *mutS* that was associated with a hypermutator phenotype in the final isolate from the patient, which was also more resistant to ceftazidime. Overall this study documents the genomic and phenotypic variability among *E. faecalis* causing endophthalmitis, as well as possible adaptive mechanisms underlying bacterial persistence during recurrent ocular infection.

IMPORTANCE

Bacterial endophthalmitis is a sight-threatening infection of the inside of the eye. *Enterococcus faecalis* cause endophthalmitis occasionally, but when they do the infections are often severe.

Here we investigated the genomes, antibiotic susceptibilities, and virulence-associated traits among *E. faecalis* collected from 13 patients with post-operative endophthalmitis. We wondered whether there were common bacterial factors that might explain why enterococcal endophthalmitis is so destructive to ocular tissues. Instead we found that *E. feacalis* isolated from endophthalmitis were genetically and phenotypically diverse; isolates belonged to a variety of genetic lineages and showed varying levels of antibiotic resistance and biofilm formation. We also undertook further characterization of three closely related *E. faecalis* isolates from a patient with recurrent endophthalmitis, and found that a hypermutator strain emerged during persistent infection. Hypermutators have been found in a variety of other infection contexts; here we describe what we believe is the first case of a hypermutator arising during ocular infection.

INTRODUCTION

Enterococci are Gram-positive bacteria that inhabit the gastrointestinal tract of humans and most land animals (1). Two members of this genus, *E. faecium* and *E. faecalis*, account for nearly all clinical enterococcal infections (2). Multidrug-resistant *E. faecalis* are leading causes of healthcare-associated infections, manifesting as bacteremia, endocarditis, and urinary tract, intra-abdominal, surgical site, and device-associated infections (3). The prevalence of enterococci in the modern hospital environment is attributed to their intrinsic resistance to commonly used antibiotics, their ability to survive under harsh and stressful conditions, their propensity to acquire mobile genetic elements carrying drug-resistance and other pathogenicity-enhancing genes, and their ability to form biofilms on indwelling medical devices such as catheters and intraocular lenses (4–6).

Endophthalmitis is characterized by inflammation of the interior of the eye. It is often caused by infection, either as a consequence of intraocular surgery (post-operative endophthalmitis),

58 penetrating injury (post-traumatic endophthalmitis), or spread of bacteria from a distant site of
59 infection to the eye (endogenous endophthalmitis) (7). Most cases of bacterial endophthalmitis
60 are caused by coagulase-negative staphylococci (CoNS), *Staphylococcus aureus* and
61 *Streptococcus viridans* (8). *Enterococcus faecalis* are infrequently isolated from post-operative
62 endophthalmitis, but patients with post-operative enterococcal endophthalmitis have worse
63 outcomes compared with similar infections caused by CoNS and *S. aureus* (5). Bacterial
64 endophthalmitis is frequently managed by pre- and post-operative administration of topical or
65 intravitreal antibiotics, often before identifying and testing the susceptibility of the organism (10,
66 11).

67 The purpose of this study was to characterize the clinical, genomic, and phenotypic features of
68 15 *E. faecalis* isolates from post-operative endophthalmitis collected from 13 patients treated at
69 the University of Pittsburgh Medical Center (UPMC) Eye Center. In addition to assessing the
70 genomic diversity of these isolates, we also analyzed the plasmid, antibiotic resistance gene,
71 CRISPR-Cas, prophage and virulence gene profiles of their genomes. We measured the ability
72 of each isolate to resist antibiotics commonly used to treat enterococcal endophthalmitis, as well
73 as their ability to produce the cytolysin toxin and form biofilms. Where possible, we made
74 connections between these phenotypes and bacterial genotypes. Finally, we identified factors
75 contributing to the persistent colonization of *E. faecalis* in one patient with recurrent
76 endophthalmitis (12).

77

78 **METHODS**

79 **Collection of isolates**

80 Bacterial isolates were collected from patients seeking treatment at the UPMC Eye Center
81 between 1993 and 2012. The clinical specimens were processed in the Charles T. Campbell

Ophthalmic Microbiology Laboratory, University of Pittsburgh Medical Center, Pittsburgh, PA. In general, samples of aqueous and vitreous humor of patients with endophthalmitis were cultured for pathogens. The specimens were collected directly by tapping the eye chambers using tuberculin syringes and needles. An approximate volume of 0.2-0.3 mL of intraocular fluid was collected from each patient. Intraocular fluid was placed on two glass slides (for Gram and Giemsa staining) for rapid visualization of microorganisms. The remaining fluid was dispersed on various isolation medium, inlcuding trypticase soy agar supplemented with 5% sheep blood, chocolate agar, enriched thioglycolate broth, a chocolate plate incubated in an anerobic bag, and Sabouraud dextrose medium with gentamicin for fungi. The media were incubated at 37^{O} C in a CO_2 atmosphere. The Sabouraud dextrose medium was incubated in a 30^{O}C air incubator. *Enterococcus faecalis* grew readily on all culture media. The isolates were Gram-positive cocci (coccoidal), catalase-negative, pyrrolidonyl arylamidase-positive, and the colonies were greyish in nature. After laboratory isolation, *E. faecalis* isolates were patient de-identified, and were stored at -80^{O} C in broth medium containing 15% glycerol. The isolates were part of a clinical tissue bank used for testing and susceptibility validation.

Whole genome sequencing and analysis

Genomic DNA from 15 post-operative endopthalmitis isolates was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD) from 1mL bacterial cultures grown in Brain Heart Infusion media. Next-generation sequencing libraries were prepared with a Nextera XT kit (Illumina, San Diego, CA), and libraries were sequenced on an Illumina Miseq using 300bp paired-end reads. Genomes were assembled with SPAdes v3.13.0 (13), annotated with prokka (14), and were compared to one another with Roary (15). A core genome phylogenetic tree was generated using RAxML with the GTRCAT substitution model and 100 iterations (16). Sequence types, antimicrobial resistance genes, virulence factors and plasmid replicons were determined using online tools provided by the Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/).

CRISPR-cas loci and prophage sequences were identified using CRISPRCasFinder (17) and PHASTER (PHAge Search Tool Enhanced Release), respectively (18). Variants were identified in serial isolates from the same patient using CLC Genomics Workbench v11.0.1 (Qiagen, Germantown, MD), using a read depth cut-off of 10 reads and a variant frequency cut-off of >90%. Illumina read data for each isolate have been submitted to NCBI under BioProject PRJNA649986, with accession numbers listed in Table S1.

Antimicrobial Susceptibility Testing

At the time of collection, antibiotic susceptibilities were determined for all isolates to vancomycin, cefazolin, ampicillin, gentamicin, amikacin, ceftazidime, and moxifloxacin using a disk diffusion assay. Additional susceptibility testing to determine the minimum inhibitory concentration (MIC) of selected antimicrobials, including amikacin, benzalkonium chloride, ceftazidime, moxifloxacin, ofloxacin, povidone iodide and vancomycin was carried out by broth microdilution in Mueller Hinton Broth (MHB) (19). Briefly, overnight cultures of *E. faecalis* grown in MHB were diluted to an OD_{600} of 0.1, and were further diluted 1:1000 into fresh MHB. 100µL of this culture was then transferred to 96-well plates containing 100µL of MHB with serial two-fold dilutions of each antimicrobial, yielding approximately 10^5 bacteria per well. Plates were incubated for 24 hours at 37°C under static conditions, and growth in each well was analyzed by both visual inspection and by OD_{600} measurement using a Synergy H1 microplate reader (Biotek, Winooski, VT).

Biofilm assay

Microtiter plate-based biofilm assays were performed as previously described (20). Briefly, overnight cultures of each isolate were diluted 100-fold into BHI broth supplemented with 0.25% glucose. 200µL of each culture was plated into eight replicate wells of a 96-well untreated polystyrene microtiter plate, and plates were incubated for 24 hours at 37°C under static conditions. Planktonic cells were removed and plates were washed three times with 250µL

1xPBS, then wells were stained with 200µL 0.1% crystal violet (CV) in water. After incubation for 30 minutes at 4°C, stained wells were washed twice with 250µL 1xPBS to remove excess stain. Plates were dried and then 250µL of 4:1 ethanol:acetone was added to each well to solubilize CV-stained biofilms. After incubation for 45 minutes at room temperature, the absorbance in each well was measured at 550nm using a Synergy H1 microplate reader (Biotek, Winooski, VT).

Cytolysin activity

The beta-hemolytic activity of the 15 UPMC endophthalmitis isolates was evaluated by streaking each isolate on Brain Heart Infusion agar plates supplemented with 5% defibrinated horse blood. Plates were incubated for 24 hours at 37°C. A clear zone around the streaked bacteria indicated positive beta-hemolysis.

Quantification of spontaneous mutation rate

The rates of spontaneous mutation in the E616, E623, and E633 isolates were determined by a previously described protocol (21). Briefly, 50µL of overnight bacterial culture grown in Brain Heart Infusion broth was plated onto duplicate BHI agar plates, each containing 50µg/mL of rifampin. The initial cell concentration was determined by plating serial dilutions of the overnight culture onto agar with no antibiotic. Colonies were counted after overnight incubation at 37°C. The mutation frequency (µ) was determined by dividing the number of rifampin-resistant colonies by the initial inoculum.

Statistics

Differences in antimicrobial resistance gene content, biofilm formation, and spontaneous mutation rates were assessed with a two-tailed t-test.

RESULTS

Clinical presentation and management of patients with enterococcal endophthalmitis, and identification of *E. faecalis*

All 13 patients in the study were examined and treated by the Retina service at the UPMC Eye Center. The mean age of the patients was 92.5 years (range 83-99 years). All patients presented with acute endophthalmitis. The presenting visual acuity was hand motions or worse in all patients. Bacterial isolates were collected as part of routine clinical care. Vitreous and aqueous humor samples were collected during surgery and were sent to the Charles T. Campbell Ophthalmic Microbiology Laboratory for propagation on blood agar, chocolate agar, Sabouraud dextrose agar and thioglycolate broth. All isolates were sensitive to vancomycin and resistant to cefazolin. Clinical management was in the form of vitrectomy and injection of intravitreal antibiotics. The final visual acuity was hand motions or less in all patients.

Genomic features of *E. faecalis* from endophthalmitis

A total of 15 *E. faecalis* isolates collected from 13 endophthalmitis patients treated at the UPMC Eye Center were available for genome sequencing and analysis. A search of the National Center for Biotechnology Information (NCBI) database identified two additional genomes isolated from patients with endophthalmitis in Denmark (Genbank accessions GCA_900205805.1 and GCA_900205785.1) (22), resulting in 17 genomes total. All isolates were sequenced on the Illumina platform, and the data were used to construct a core genome phylogeny (Figure 1). *In silico* multi-locus sequence typing (MLST) of all 17 genomes showed that 11 distinct sequence types (STs) were identified. No single ST was found to dominate; however, multiple isolates belonging to ST2, ST40, ST64, and ST122 were observed. In the case of the ST122 isolates, all three were collected from a single patient with recurrent endophthalmitis (12).

We screened all 17 *E. faecalis* endophthalmitis genomes for acquired antimicrobial resistance genes using the ResFinder tool available through the Center for Genomic Epidemiology (CGE)

(23) (Figure 1). All isolates were predicted to be intrinsically resistant to lincosamides due to the presence of *lsa(A)*, a core gene found in all *E. faecalis* (Table S1). Additionally, five isolates carried the erythromycin resistance gene *erm(B)*, and eight isolates possessed either *tet(M)* or *tet(S)* tetracycline resistance genes. Finally, aminoglycoside resistance-conferring genes *ant(6)-Ia* and *aph(3')-III* were identified in the two ST2 isolates, and *aac(6')-aph(2")* was observed in the single ST103 isolate genome.

Next we screened all genomes for plasmid *rep* genes using PlasmidFinder (24), and identified six different *rep* genes that together were detected in ten (58.8%) isolate genomes (Figure 1). Among these, six isolate genomes had only a single *rep* gene identified, while the remaining four had two or more *rep* genes. The E547 genome, belonging to ST2, encoded four different *rep* genes; this isolate also carried erythromycin and aminoglycoside resistance genes, which are often plasmid-encoded. While we did not observe any correlation between the *rep* families and STs sampled, *rep9* family *rep* genes were detected in eight isolate genomes, making *rep9* the dominant *rep* family in this study.

We then assessed the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and their associated Cas loci, as well as the prophage content, of the 17 *E. faecalis* genomes. All 17 genomes were mined using CRISPRCasFinder (25) and PHASTER (18). We observed that all the isolates in our study had identifiable CRISPR sequences, however E46, E139, E171, E474, and WGS406 encoded orphan CRISPR sequences without any associated Cas proteins (Table S2). CRISPR sequences without adjacent Cas proteins have been previously observed in *E. faecalis* (26, 27). The remaining 12 isolates did have Class 1 and/or Class 2 Cas proteins associated with their CRISPR loci (Table S2, Figure 1). The five genomes lacking an intact CRISPR-Cas locus had more acquired antimicrobial resistance genes on average compared to CRISPR-Cas-positive genomes, but the difference was not statistically significant (mean 1.6 vs. 0.75 genes per genome, P=0.09). Prophage analysis with PHASTER (18) detected intact or

questionable prophages in 14/17 (82%) of the enterococcal genomes in this study. Prophages varied in size from 20-58.9 Kb, and showed homology to previously described *Enterococcus* and other Gram-positive phages (Table S3). We did not observe any correlation between CRISRP-Cas loci and the number of plasmid *rep* genes or prophages identified in the study isolate genomes.

Cytolysin activity

The *E. faecalis* cytolysin is a pore-forming toxin that lyses both bacterial and eukaryotic cells in response to quorum signals (28–31). We detected the cytolysin operon in 5/17 (29%) endophthalmitis isolate genomes (Figure 1). To confirm cytolysin operon activity, we tested the beta-hemolytic capacity of the 15 UPMC isolates on agar plates containing horse blood. As predicted from their genomes, E46 (ST64), E171 (ST64), E286 (ST40) and E474 (ST103) showed beta-hemolysis when grown in the presence of horse blood (32). We were unable to test for cytolysin activity in WGS406 because we only analyzed the genome sequence of this strain.

Virulence-associated gene profiles and biofilm formation

We evaluated all 17 *E. faecalis* genomes for the presence of virulence-associated genes and operons using the VirulenceFinder database (33), along with manually searches for putative virulence gene sequences that have been previously described in *E. faecalis* (34) (Table S4). We found that fibronectin-binding proteins *efbA* and *efaAfs,* endocarditis and biofilm-associated pili *ebpABC,* sortase A *(srtA),* adhesin to collagen of *E. faecalis (ace),* general stress proteins *gls24 and glsB,* membrane metalloprotease associated with endocarditis *(eep),* thiol peroxidase for oxidative stress resistance *(tpx),* and sex pheromones *cCF10, cOB1, cad* and *camE* were present in the genomes of all isolates, with variable nucleotide identity (97-100% versus the reference sequence). Other virulence-associated genes were variably present, including the quorum-

sensing gene *fsrB*, gelatinase *gelE*, serine protease *sprE*, hyaluronidase *hylAB*, and several genes often found within the *E. faecalis* pathogenicity island (PAI, Table S4).

Biofilm formation is a well described pathogenicity-enhancing feature of many bacteria, including *E. faecalis* (35). We measured the *in vitro* biofilm forming capacity of all 15 UPMC isolates using the crystal violet staining method (20), and observed differences in biofilm formation between isolates (Figure 2). Isolate E286 (ST40) formed the most biofilm of any isolate tested, in contrast with the other two ST40 isolates, which formed only moderate biofilms ($P<0.0001$). Similarly, E171 (ST64) formed more biofilm than E46, another ST64 isolate ($P<0.0001$). Both ST2 isolates showed similar, moderate levels of biofilm formation, and the three ST122 isolates from the same patient were similar to one another in their ability to form biofilms, and were among the weakest biofilm formers of all the isolates tested. To identify candidate genes that might contribute to enhanced biofilm formation in E286 (ST40) and E171 (ST64), we examined the sequences of biofilm-associated genes between these isolates and other isolates of the same STs that formed weaker biofilms (Table S4). The sequence of the collagen adhesin *ace* was intact in E286, but appeared to be disrupted in the other two ST40 isolates (Figure S1). The E286 genome also encoded the PAI-associated locus *ef0500-ef0503*, which was absent in E77 and E139. Separately, the genome of the E46 isolate, which formed less biofilm than the other ST64 isolate (E171), also had a disruption in the *ace* gene (Figure S1). We also examined differences in gene content among the ST40 and ST64 isolates (Table S5). Genes that were differentially present in E286 compared to E77 and E139 included prophage and plasmid-associated genes, while among the genes that were present in E171 but absent in E46 was an Asa373-like protein. This protein contains a BspA domain which has been previously associated with adhesion in Group B streptococci (36), and which has been shown to cause cell aggregation in *E. faecalis* (37).

Antimicrobial resistance phenotypes and connection with genotypes

We investigated the antimicrobial susceptibilities of the 15 endophthalmitis isolates we collected by determining the minimum inhibitory concentrations (MICs) of six antibiotic and antiseptic compounds for all isolates (Table 1). All isolates were susceptible to vancomycin, and all were inhibited by low concentrations of benzalkonium chloride and povidone iodide. Larger susceptibility differences between isolates were observed for amikacin, ceftazidime, moxifloxacin, and ofloxacin. The genomes of the three isolates with the highest amikacin MICs (E374, E474, and E547) encoded aminoglycoside resistance genes (Figure 1). The same three isolates were also resistant to both moxifloxacin and ofloxacin (Table 1). Enterococci are known to develop fluoroquinolone resistance via mutations in the genes encoding DNA gyrase subunit A (*gyrA*) and topoisomerase IV (*parC*) (2). We examined the *gyrA* sequence of all three fluoroquinolone-resistant isolates, and found that all three encoded mutations at amino acid position 84 (Figure 3). The S84R and S84I mutations have both been described before (21, 38), and likely explain the fluoroquinolone resistance observed in these isolates. Finally, ceftazidime resistance was quite variable between isolates, with MICs ranging from 78.125-625µg/mL (Table 1). In general, isolates belonging to the same ST had similar ceftazidime susceptibilities, with MICs falling within two-fold of one another. The one exception was the three ST122 isolates, which were all isolated from the same patient (12). The first two isolates from the patient (E616 and E623) had ceftazidime MICs of 78.125µg/mL, while the final isolate (E633) was eight-fold more resistant (MIC = 625µg/mL).

Emergence of ceftazidime resistance in a hypermutator strain isolated from recurrent endophthalmitis

Genome sequencing revealed that three *E. faecalis* isolates collected from a single patient with recurrent endophthalmitis were closely related to one another (12) (Figure 1). Using the first isolate from the patient (E616) as a reference, we looked for variants that arose in E623 (isolated 16 weeks after E616) and E633 (isolated 20 weeks after E616 and then grown in thioglycolate

broth for three additional months) (12). We identified four variants in the E623 isolate genome compared to E616, including three small insertions and a non-synonymous mutation in a predicted arsenic resistance protein (Table S6). The E633 isolate genome, in contrast, had 46 variants compared to E616 (Table S6). Four of these were the same variants identified in E623, suggesting that E633 derived directly from E623. One of the new variants in E633 was a single base deletion in the DNA mismatch repair gene *mutS*, which was predicted to cause a frame-shift mutation at amino acid position 179 (Table S6). Mutations in *mutS* have been observed in *E. faecalis*, and were previously correlated with an increase in the rate of spontaneous mutation (21). To test whether the *mutS* mutation we observed was similarly associated with increased mutability, we quantified the rate of spontaneous mutation in E616, E623, and E633 using a mutagenicity assay determining rifampin resistance frequency (Figure 4). The median spontaneous mutation rates of E616 and E623 were 6.3×10^{-9} and 7.5×10^{-9} respectively, while the mutation rate of E633 was over 100-fold higher at 1.3×10^{-6} (Figure 4, $P<0.0001$). The increased mutation rate in E633 likely explains the excess of variants identified in this isolate, and may have also contributed to its decreased ceftazidime susceptibility and its ability to persist and cause a recurrent infection. Analysis of the variants accumulated by E633 versus E616 revealed that E633 carried mutations in genes encoding: (i) cell wall-associated proteins, such as a RodA-like rod-shape determining protein and a WxL domain-containing hypothetical protein, (ii) transcriptional regulators such as a LuxR two-component transcriptional response regulator and a Spx regulatory protein, and (iii) membrane-associated proteins such a putative serine/threonine exporter and a phosphotransferase system sugar transporter (Table S6). Any of these, or additional variants identified in the E633 genome, might have contributed to the increased cephalosporin resistance and persistence of this isolate *in vivo*.

DISCUSSION

In this study, we characterized the genetic diversity and variability of antimicrobial resistance and virulence phenotypes among *E. faecalis* isolated from post-operative endophthalmitis. We found isolates belonging to well-known genetic lineages associated with clinical infections, as well as isolates resembling commensal enterococci lacking mobile genetic elements and other pathogenic traits. The presence of genetically diverse *E. faecalis* from endophthalmitis is in agreement with previous studies showing the absence of specific lineages that colonize the ocular environment (39). The sequence types found in multiple endophthalmitis patients, however, included ST2, ST40, and ST64, which have all been previously found to cause infections (40). A similarly diverse array of genetic lineages, with a subset being found more frequently, has also been observed in other Gram-positive bacteria isolated from ocular infections (41).

We found a high degree of variability in mobile genetic element content in the genomes of the isolates that we studied. The variable antimicrobial resistance genes we identified, including *erm*(B), *tet(M), tet(S), ant(6)-Ia*, *aph(3')-III,* and *aac(6')-aph(2")*, are among the most commonly observed resistance genes in *E. faecalis* (42–44). These resistance genes are often plasmid-encoded (45), and indeed nearly all genomes encoding acquired resistance genes also encoded one or more plasmid *rep* genes. Because we did not fully resolve the plasmid sequences in this study, however, we cannot formally connect resistance genes to the mobile elements carrying them. Cytolysin operon presence, CRISPR-Cas loci, and prophages were similarly variable among *E. faecalis* from endophthalmitis. Similar to prior studies (46, 47), we detected more acquired antimicrobial resistance genes in the genomes of isolates lacking a functional CRISPR-Cas locus. The difference was not statistically significant, however, likely due to the limited number of genomes sampled.

Virulence factors previously shown to contribute to enterococcal pathogenicity during endophthalmitis include the *E. faecalis* cytolysin (48), the fsr quorum-sensing system (49), and secreted proteases including gelatinase (GelE) and serine protease (SprE) (50). Cytolysin

operons were detected in only five of the 17 genomes analyzed here, while fsr quorum-sensing genes were detected in 13 genomes and GelE/SprE were detected in 16 genomes. Of the other virulence factors identified, some were found in all isolates while others were variably present, and when virulence genes were identified they often showed differences in nucleotide sequence compared to one another. Detailed clinical information about the patients these isolates were collected from was not available, thus we were unable to assess the relative severity of the infections or look for correlations between virulence factor presence and clinical outcomes.

The ability to form biofilms allows bacteria to persist at sites where they might normally be cleared. Biofilm formation on intraocular lenses has been suggested to contribute to bacterial endophthalmitis (51), and has been hypothesized to play a role in persistent and recurrent ocular infections (12, 52). When we measured *in vitro* biofilm formation in the endophthalmitis isolates we collected, we observed differences across isolates, including those of the same ST. Because same-ST isolates are often more closely related than isolates belonging to different STs, we examined the ST40 and ST64 isolate genomes to identify genetic differences that could account for increased biofilm formation, and focused our analysis on previously described biofilm and attachment factors. We found evidence that disruption of the collagen adhesin gene *ace* was associated with diminished biofilm formation in both ST40 and ST64 isolates. Ace mediates *E. faecalis* adherence (53), and contributes to pathogenicity in animal models of infective endocarditis and urinary tract infection (54, 55) . We also identified an Asa373-like aggregation factor gene that was only present in the ST64 isolate that made more biofilm, and which is predicted to encode a surface adhesin that contributes to *E. faecalis* aggregation (37). Further experiments such as targeted gene disruption or complementation would be required to validate the role of either one of these genes in biofilm formation; this will be a focus of our future work in this area.

We observed differences in bacterial susceptibility to antibiotics between the isolates we tested, and in most cases, we were able to correlate resistance phenotypes to genotypes. All isolates were vancomycin susceptible, and all lacked Van operons. Aminoglycoside resistance genes were found in the isolates with the highest amikacin MICs, and previously described *gyrA* mutations were found in the three fluoroquinolone-resistant isolates. While we observed variability in ceftazidime MICs between isolates, it is likely that multiple genes and/or mutations contribute to cephalosporin susceptibility in these isolates. This was also apparent when considering the mutations present in the hypermutator isolate E633, which was more resistant to ceftazidime compared to closely related isolates from the same patient taken at earlier infection time points. Although the E633 isolate genome encoded mutations in cell wall proteins, membrane proteins and transcriptional regulators, they were unrelated to known mechanisms of cephalosporin resistance such as the CroRS signal transduction pathway (56), the serine/threonine kinase IreK (57), and MurAA (58). Overall, from these data we can conclude that vancomycin remains an appropriate treatment for suspected enterococcal endophthalmitis, however the use of aminoglycosides, cephalosporins, and fluoroquinolones could be ineffective due to pre-existing resistances in the infecting strain.

This study had several limitations. The isolates we characterized were from a convenient sampling of strains available at the UPMC Eye Center, and detailed clinical information was not available for most patients. Furthermore, our genomic analysis of virulence factors and correlations with biofilm production were restricted to the STs that were sampled from multiple patients and that showed within-ST differences in biofilm formation. Finally, further investigation is needed to identify the mechanism by which the hypermutator E633 isolate was able to become more ceftazidime-resistant compared to the closely related susceptible isolates from the same patient.

In summary, this study shows that *E. faecalis* causing post-operative endophthalmitis are highly genetically and phenotypically variable. Our functional genomics analysis identified expected associations between antimicrobial resistance and biofilm formation genes, as well as the unexpected occurrence of a hypermutator isolate that arose during recurrent infection. Further work is needed to understand the precise contributions of enterococcal virulence factors such as Ace and Asa-373 to enterococcal growth in the collagen-rich vitreous humor, and to further elucidate the role of hypermutators in ocular infections. Such studies could lead to improved treatment strategies for intra-ocular infections, which would be welcome additions to an antimicrobial treatment arsenal that is under threat from increasing antibiotic resistance.

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FIGURE LEGENDS

Figure 1. Core genome phylogeny of *E. faecalis* endophthalmitis isolates. Single-copy core genome phylogeny of 15 Campbell Lab isolates plus two additional genomes from NCBI (WGS406 and WGS410), all isolated from endophthalmitis. The RAxML tree is built from single nucleotide polymorphisms (SNPs) in 2024 single copy core genes identified with Roary. Tips are annotated with isolate name, multi-locus sequence type (ST), drug resistance-associated genes, plasmid *rep* genes, cytolysin operon, CRISPR-cas loci and prophages. CRISR-cas-positive isolates had Class 1 and/or Class 2 Cas proteins associated with their CRISPR loci. Intact or questionable prophages were identified with PHASTER. lsa(A) = lincosamide resistance, *erm(B)* = erythromycin resistance, *tet(M)* and *tet(S)* = tetracycline resistance, *ant(6)-Ia*, *aph(3')-III* and *aac(6')-aph(2")* = aminoglycoside resistance.

Figure 2. Variable biofilm production among endophthalmitis isolates. *In vitro* biofilm production of 15 *E. faecalis* endophthalmitis strains collected by the Campbell Lab. Biofilm formation was measured as the OD at 550nm using a standard crystal violet-based assay. Isolates are arranged according to their sequence type (ST), and STs with more than one isolate are labeled. Bars shown mean crystal violet absorbance values, and error bars show standard deviation of triplicate experiments, each with eight technical replicates. NC = negative control. ****p<0.0001 by two-tailed t test. ns = not significant.

Figure 3. DNA gyrase A mutations in fluoroquinolone-resistant isolates. Nucleotide sequence alignment of the DNA gyrase subunit A gene at amino acid positions 77-91. The black box marks amino acid position 84, where known resistance-causing mutations occur. Moxifloxacin (MXF) minimum inhibitory concentration (MIC) and gyrase A genotype is noted for each isolate.

Figure 4. The E633 isolate, which carries a frame-shift mutation in the mismatch repair gene *mutS*, has a higher spontaneous mutation rate compared to earlier isolates from the

same patient. Spontaneous mutation rate, measured with a rifampin mutagenicity assay, in isolates E616, E623 and E633, which were all sampled from a patient with recurrent endophthalmitis. Horizontal lines mark sample median values for results from 6 biological replicate experiments (each with 2-4 technical replicates).

Figure S1. E286 and E171 have intact *ace* collagen adhesin sequences, while other isolates of the same ST that form weaker biofilms have truncated *ace* sequences. Nucleotide sequence alignment of collagen adhesin (*ace*) encoding sequences from (A) ST40 : E286, E77 and E139, (B) ST64: E171 and E46. The colored bars indicate the following features: Yellow - Gene, Black – Nucleotide alignment.

584 **Table 1. Minimum Inhibitory concentration (MIC) profiles of *E. faecalis* isolates**

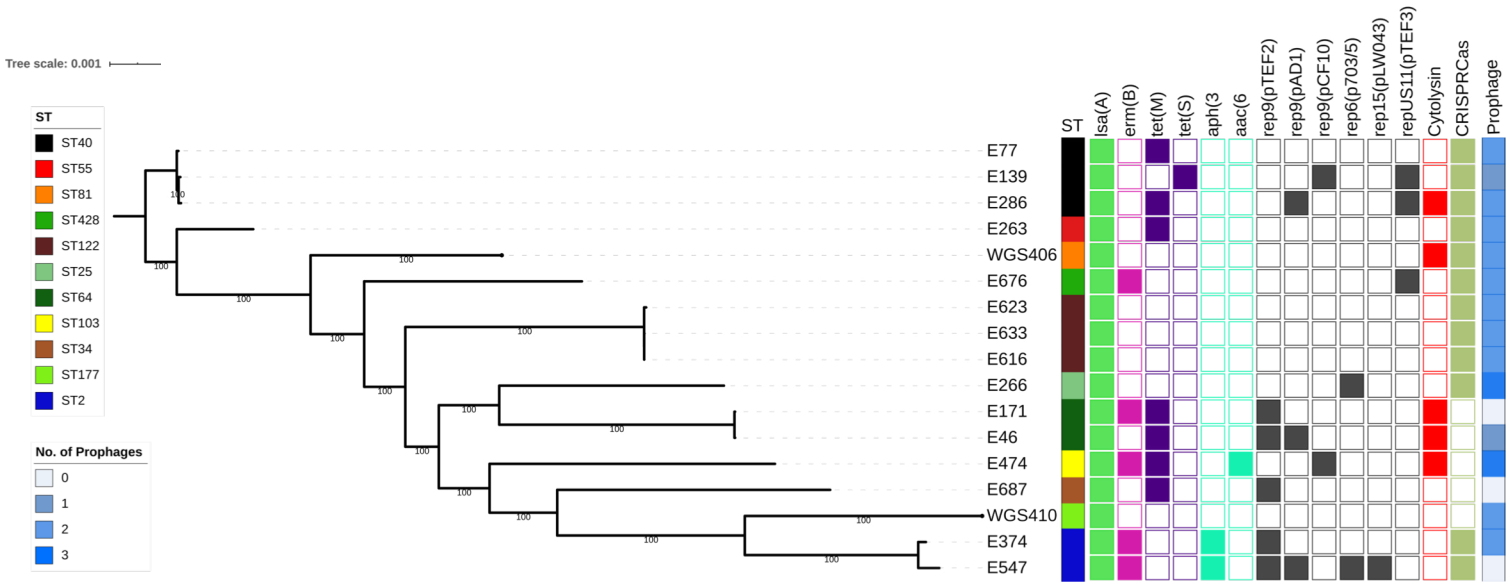
Strain ID	ST	MIC (µg/mL)						
		AMK	BKC	CZA	MOX	OFX	PI[1]	VAN
E374	2	512	2	312.5	16	32	0.31	1
E547	2	512	2	625	16	32	0.31	1
E266	25	128	2	156.25	0.25	4	0.31	0.5
E687	34	64	2	312.5	0.125	2	0.31	2
E77	40	128	2	312.5	0.25	2	0.31	2
E139	40	128	1	625	0.5	4	0.31	2
E286	40	64	2	625	0.5	2	0.31	2
E263	55	128	2	78.125	0.25	2	0.31	2
E46	64	64	2	312.5	0.25	2	0.31	1
E171	64	256	2	625	0.25	2	0.31	1
E474	103	512	2	312.5	32	32	0.31	1
E616	122	128	2	78.125	1	2	0.31	1
E623	122	128	2	78.125	1	4	0.31	1
E633	122	128	2	625	1	2	0.31	1
E676	428	128	2	1250	0.25	4	0.31	1

585

586 AMK = Amikacin, BKC = Benzalkonium Chloride, CZA = Ceftazidime, MOX = Moxifloxacin, OFX

587 = Ofloxacin, PI = Povidone Iodide, VAN = Vancomycin

588 [1]Povidone Iodide MIC is reported as %.



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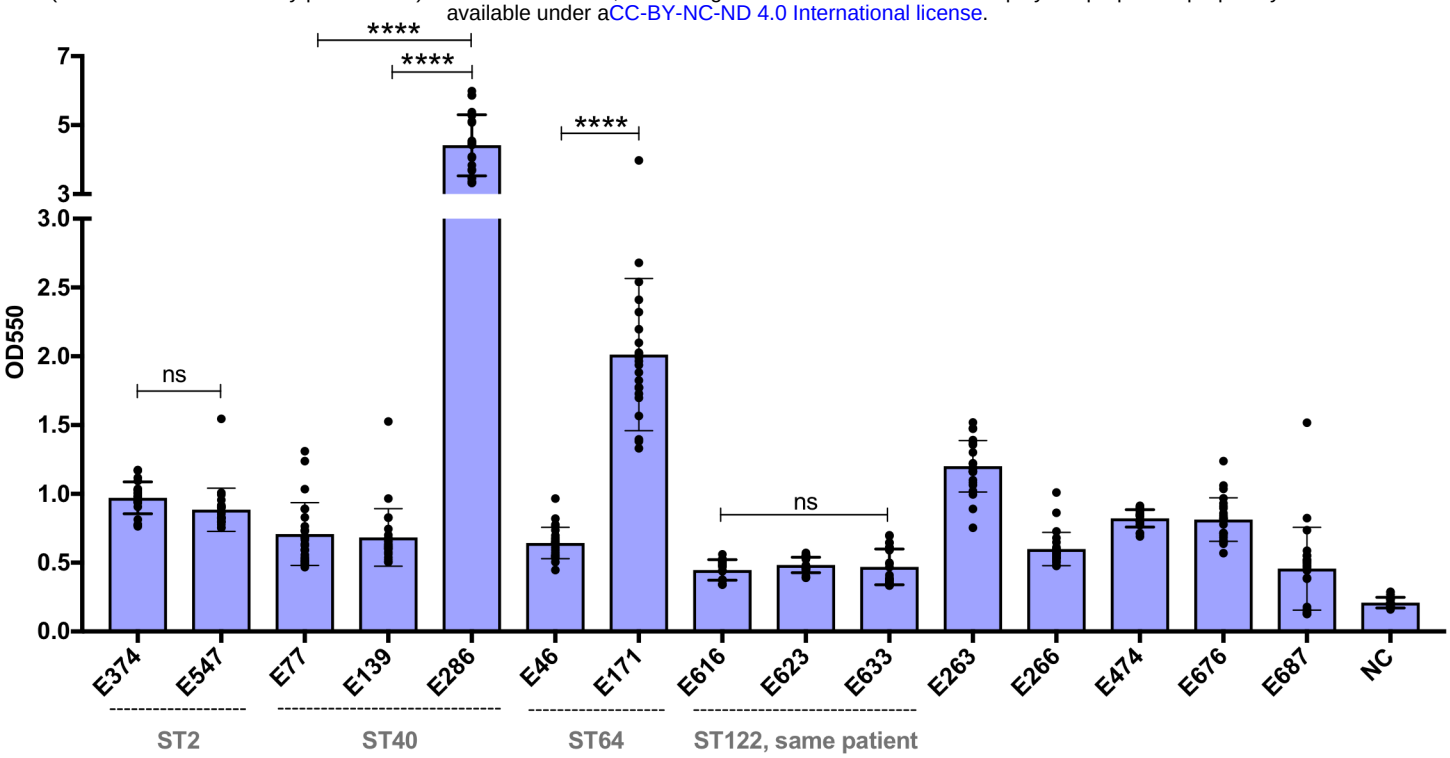

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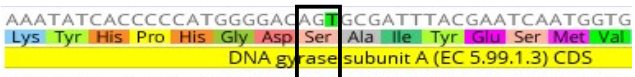
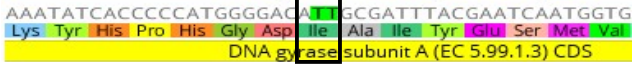
Isolate ID	ST	*gyrA* sequence (amino acids 77-91)	MXF MIC (µg/mL)	Genotype
E286	ST40		0.5	Wild type
E374	ST2		**16**	**S84R**
E547	ST2		**16**	**S84R**
E474	ST103		**32**	**S84I**

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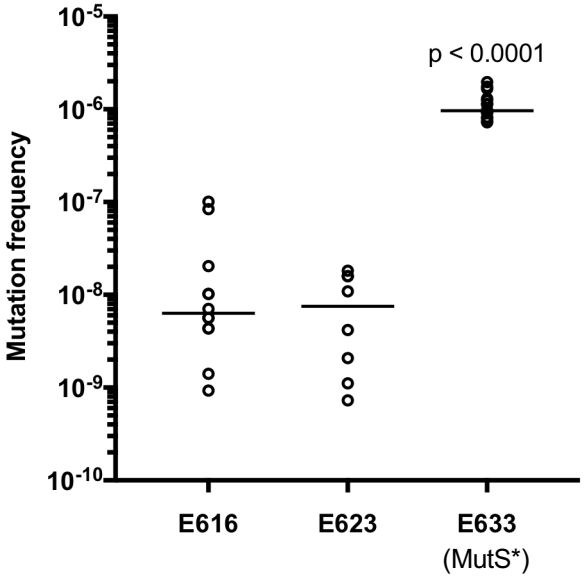

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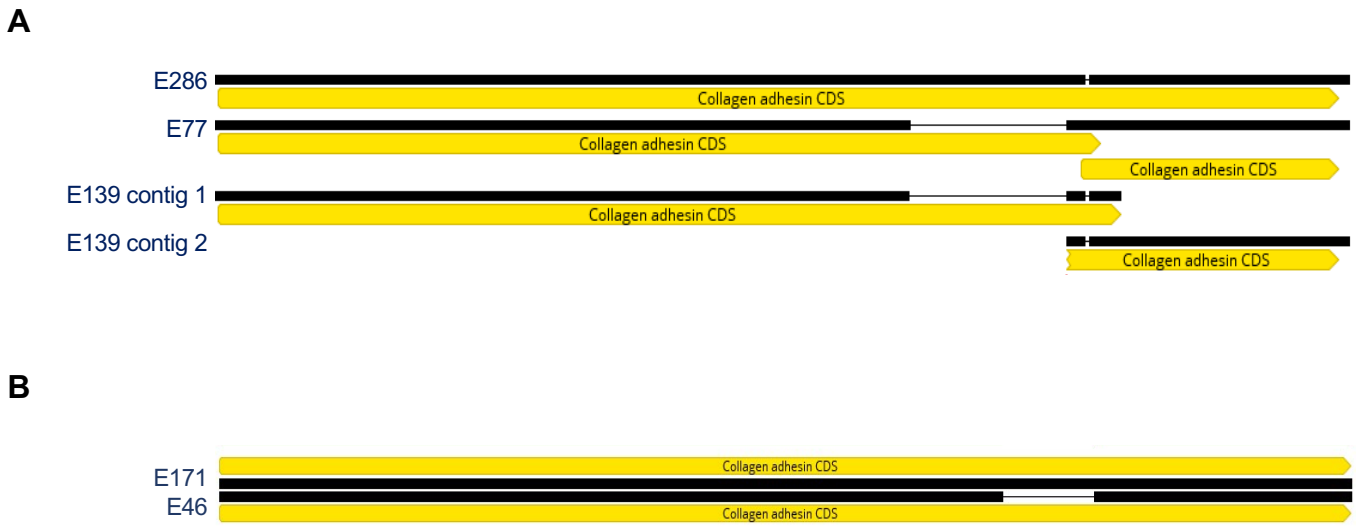

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