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Comparing the Genomes of Multidrug and Vancomycin-Resistant *Enterococcus faecium* from a Florida Wastewater Treatment Plant

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Comparing the Genomes of Multidrug and Vancomycin-Resistant *Enterococcus faecium* from a Florida Wastewater Treatment Plant

by

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List of Abbreviations

Abbreviation	Definition
AMR	Antimicrobial resistance
BUSCO	Benchmarking universal single-copy orthologs
CC	Clonal complex
MDR	Multidrug resistant
MGE	Mobile genetic element
qPCR	Quantitative polymerase chain reaction
ST	Sequence type
VRE	Vancomycin resistant *Enterococcus*
WWTP	Wastewater treatment plant

Abstract

Vancomycin-resistant *Enterococcus* (VRE) are a serious health threat, causing 50,000 infections and 5,000 deaths each year in the United States (CDC, 2019). Multidrug-resistant VRE are becoming more prevalent, and treatment options for infections caused by these organisms are limited (Arias et al., 2010; CDC, 2019). Multidrug resistant VRE have been previously isolated from wastewater in Brazil, Canada, and Portugal (Araújo et al., 2010; de Farias et al., 2022; Sanderson et al., 2019). Thirteen vancomycin-resistant *Enterococcus faecium* isolated from three stages of a Florida wastewater treatment plant were fully resistant to the following antibiotics: vancomycin, ampicillin, ciprofloxacin, erythromycin, tetracycline, and nitrofurantoin, and sensitive to linezolid, fosfomycin, and quinupristin-dalfopristin. The genomes of the thirteen VRE strains were sequenced via 50 paired-end sequencing on an Illumina NovaSeq and analyzed to determine relationships among the genomes and mobile genetic elements. All strains belonged to clonal complex 17, and three sequence types were identified (ST18, ST412, and ST584). Each strain contained a mean of 18 antimicrobial resistance genes and 22 virulence genes, and multiple putative plasmids and genomic islands (clusters of genes that are transferable between organisms) were identified. All strains shared Tn1546, which carries the *vanA* operon, as well as four putative genomic islands. The strains had 99.5% percent genomic identity and were more closely related to other wastewater and clinical VRE strains than to vancomycin-resistant *E. faecium* strains isolated from environmental habits, or vancomycin-susceptible *E. faecium*. The similarity among the strains as well as to clinical strains suggests an origin from hospital sewage.

Chapter 1: Introduction

Section 1: The Threat of Antimicrobial Resistant Bacteria

The World Health Organization (WHO) has called antimicrobial resistance (AMR) the greatest health threat of the 21st century (WHO, 2014). Infections caused by antimicrobial-resistant and multidrug-resistant (MDR) bacterial pathogens such as vancomycin resistant *Enterococcus* spp. (VRE) are associated with more negative health outcomes compared to those caused by antibiotic-susceptible bacteria (Founou et al., 2017; R. R. Roberts et al., 2009; Rossolini et al., 2014; Shrestha et al., 2018). Millions of people in the USA are infected with antimicrobial-resistant bacteria every year and, in 2019, 35,000 people died as a result (CDC, 2019). This number is expected to rise as the frequency of AMR increases (WHO, 2022).

Enterococcus spp. are part of the normal fecal flora of humans and animals and can also occur naturally in the environment, such as in soil or water (Byappanahalli et al., 2012; Lebreton et al., 2014). Some species can act as opportunistic pathogens (Byappanahalli et al., 2012), such as *E. faecalis* and *E. faecium*, which cause 10% or more of all nosocomial (hospital-acquired) infections such as urinary tract infections and bacteremia (Brinkwirth et al., 2021; Byappanahalli et al., 2012; Kao & Kline, 2019; Li et al., 2022; Weiner et al., 2016). *E. durans* and *E. gallinarum* are also opportunistic human pathogens, along with several other *Enterococcus* species (Byappanahalli et al., 2012; Lebreton et al., 2014). *Enterococcus* can become resistant to a wide range of antibiotics, including antibiotics of last-resort such as vancomycin and linezolid (Hollenbeck & Rice, 2012; Huycke et al., 1998; Yadav et al., 2017), via mutation or via mobile

genetic elements (MGEs) that are passed on via horizontal gene transfer (Palmer et al., 2010). *E. faecium* has demonstrated resistance to the largest number of antibiotics of all *Enterococcus* species (Byappanahalli et al., 2012; Kao & Kline, 2019; Li et al., 2022). *E. faecium* are intrinsically resistant to low concentrations of cephalosporins (beta-lactam antibiotics) and to clinically achievable concentrations of aminoglycosides (Gaca & Lemos, 2019; Hollenbeck & Rice, 2012).

E. faecium strains can be separated by sequence type, which is based on single nucleotide polymorphism in seven core genes (*atpA*, *ddl*, *gdh*, *purK*, *gyd*, *pstS*, *adk*) (Homan et al., 2002; Jolley et al., 2018), and by clonal complex, which are comprised of groupings of similar sequence types (Freitas et al., 2009; O'Toole et al., 2023). Clonal complex 17 (CC17) is a phylogenetic group to which the majority of *E. faecium* associated with nosocomial infections belong (O'Toole et al., 2023). CC17 was the first known clonal complex of *E. faecium* to be distributed globally and is associated with ampicillin resistance (Top et al., 2008; Willems et al., 2005). CC17 strains also all contain characteristic mutations conferring quinolone resistance (Leavis et al., 2006; Top et al., 2008) and nitrofurantoin resistance (Zhang et al., 2021), and a correlation with vancomycin resistance has also been observed (Klare et al., 2005; Ochoa et al., 2013).

Vancomycin-resistant *Enterococcus* spp. were identified fifteen years after the first use of the antibiotic, in 1972 (Fisher & Phillips, 2009). Vancomycin inhibits the cell wall synthesis of Gram-positive bacteria; resistance against the antibiotic occurs via a gene that changes the peptidoglycan synthesis pathways (Cetinkaya et al., 2000; Stogios & Savchenko, 2020). Vancomycin molecules bind to the terminal of D-Ala-D-Ala on peptidoglycan precursors in vancomycin-sensitive strains, preventing the cross-linking necessary for cell-wall formation,

which leads to stress on the cell envelope and eventually cell death (Cetinkaya et al., 2000; Stogios & Savchenko, 2020). A main mechanism of resistance to vancomycin is the change in the peptidoglycan cross-linker structure from D-Ala-D-Ala to D-Ala-D-lac, which makes it more difficult for vancomycin to bind to the molecule (Cetinkaya et al., 2000; Stogios & Savchenko, 2020).

Vancomycin-resistant *Enterococcus* spp. are considered by the CDC to be a serious threat to human health. Annual morbidity and mortality caused by VRE in the United States is approximately 50,000 people infected and over 5,000 deaths (CDC, 2019). The prevalence of vancomycin resistance in clinical *Enterococcus* isolates has been estimated at 6% in South America (Panesso et al., 2010), between 8.1% and 23% in Asia (Alevizakos et al., 2017; Shrestha et al., 2021), 21% to 30% in North America (Alevizakos et al., 2017; CDC, 2019), and 26.8% in Africa (Alemayehu & Hailemariam, 2020), . The frequency of MDR VRE is also rising, increasing the threat to human health and limiting treatment options (CDC, 2019). VRE are typically isolated in hospital settings (El Haddad et al., 2021; Karki et al., 2012; O'Toole et al., 2023), but have also been isolated from various other sources including chicken feces (Harwood et al., 2001), marine environments (M. C. Roberts et al., 2009), surface water (Young et al., 2016), and sewage (Araújo et al., 2010; de Farias et al., 2022; Goldstein et al., 2014; Iversen et al., 2002; Sanderson et al., 2020).

Enterococcus spp. can acquire resistance to vancomycin via acquisition of one of many different gene families. The *vanA* and *vanB* operons are the most often observed and best characterized vancomycin resistance genes, and can be carried by a wide range of *Enterococcus*, including both *E. faecium* and *E. faecalis* (Ahmed & Baptiste, 2018; Cetinkaya et al., 2000; Jensen et al., 1998; Werner et al., 2008). Other *van* operons are *vanC*, *vanD*, *vanE*, *vanG*, *vanL*,

vanM, and *vanN* (reviewed in Ahmed & Baptiste, 2018). The *vanA*, *vanB*, and *vanM* genes confer the highest levels of vancomycin resistance, i.e. the minimum inhibitory concentration for *vanA* VRE strains ranges from 256 to 1024 μg/mL (Tenover et al., 1995) for *vanB* VRE strains from 16 to 128 μg/mL (Hanaki et al., 2004; Rahe et al., 2010), and for *vanM*: 128 to >256 μg/mL (Chen et al., 2015).

MGEs such as transposons, plasmids, and insertion sequences are common strategies for sharing antibiotic resistance genes among *Enterococcus* (Hegstad et al., 2010; Palmer et al., 2010). Plasmids generally range from 5-500 kb in size and often carry AMR genes (Hegstad et al., 2010; Werner et al., 2011). Insertion sequences and transposons are generally much smaller than plasmids but can still play an important role in distributing AMR genes (Arthur et al., 1993; Siguier et al., 2014). Two of the vancomycin resistance genes discussed previously, *vanA* and *vanB*, are located on and transferred via transposons: e.g. *vanA* is transferred on Tn1546 (*vanA*) (Arthur et al., 1993; Biavasco et al., 2007; Cetinkaya et al., 2000) and *vanB* is transferred on Tn1547 (Quintiliani_Jr. & Courvalin, 1996), Tn1549 (Bender et al., 2016; Garnier et al., 2000; Launay et al., 2006), or Tn5382 (Lu et al., 2005).

Tn1546, first described in 1993, is a transposon around 10 kb in size (Arthur et al., 1993). The *vanA* operon on Tn1546 usually consists of seven genes (*vanA*, *vanH*, *vanS*, *vanR*, *vanX*, *vanY*, and *vanZ*) (Arthur et al., 1993; Simjee et al., 2002); however, *vanY* and *vanZ* are not required for vancomycin resistance (Arthur et al., 1993; Stogios & Savchenko, 2020). The DNA sequence of vancomycin resistance genes is typically stable, with occasional point mutations. Variation in the rest of the transposon, i.e. the intergenic regions and the transposase (ORF1), occurs more frequently, such as the presence of insertion sequences or deletions (Jensen et al., 1998; Simjee et al., 2002; Willems et al., 1999). Tn1546 is usually located on and transmitted via

a conjugative plasmid (Garcia-Migura et al., 2007; Kohler et al., 2018; Novais et al., 2008; Simjee et al., 2002; Werner et al., 2011), including the transfer of Tn1546 and vancomycin resistance to methicillin-resistant *Staphylococcus aureus* (Arthur et al., 1993; Kohler et al., 2018).

Transposons like Tn1546 may also be located on genomic 'islands', which are clusters of genes within a genome that are transferable between organisms and can contain AMR genes and virulence factors (Li et al., 2021). Genomic islands can range in size from 10 to 500 kb, but smaller genomic 'islets' have also been identified (Juhas et al., 2009; Li et al., 2021). Genomic islands (>10kb) and islets (<10kb) are prevalent in *Enterococcus* spp. (Juhas et al., 2009; Li et al., 2021); e.g., many *E. faecium* CC17 strains possess a pathogenicity island containing the virulence gene *esp* (Top et al., 2008). Genomic islands are often self-mobile, containing mobility-related elements such as conjugative genes, transposons, or insertion sequences that allow them to self-transfer via conjugation or transduction (Juhas et al., 2009; Li et al., 2021).

Section 2: Survival of Fecal Bacteria in Wastewater

Vancomycin-resistant *Enterococcus* are prevalent in sewage; in a study on four WWTPs in the USA, VRE were found 27% of sewage samples (Goldstein et al., 2014). VRE, including MDR VRE, have been isolated from wastewater around the globe, including Brazil (de Farias et al., 2022), Canada (Sanderson et al., 2020), England (Caplin et al., 2008), Portugal (Araújo et al., 2010), South Africa (Ekwanzala et al., 2020), and the United States (Goldstein et al., 2014). Some of these studies isolated VRE at concentrations of vancomycin that correspond to intermediate resistance, though all later confirmed full resistance (Goldstein et al., 2014) or identified vancomycin resistance genes such as *vanA* or *vanB*, which confer full resistance to vancomycin (Caplin et al., 2008; de Farias et al., 2022; Ekwanzala et al., 2020).

Wastewater treatment plants in the United States receive and treat billions of gallons of water every day (USEPA, 2023), but before reaching wastewater treatment, antimicrobial-resistant and MDR pathogens like VRE can enter water bodies via sewage spills or leaking sewage infrastructure. The state of Florida experiences an average of six reported sewage spills/day, and from 2009 to 2019 over one billion gallons of wastewater were released into the environment (Chen et al., 2019). During hurricanes or severe storms, millions of gallons of sewage can enter the environment (Chen et al., 2019; Chesnes, 2022; Rosen, 2024). Potentially pathogenic antibiotic resistant bacteria from sewage can persist in the environment, surviving for several days or more outside of their normal habitat (Mahaney, 2022; Young et al., 2016), thereby increasing the risk of human exposure. For example, VRE were isolated from sediment and water for several days following a sewage spill in Tampa Bay (Young et al., 2016).

Bacterial populations can survive in sewage for extended periods, where they may become naturalized to colonize the sewage environment (Murphy, 2017; Zhi et al., 2016). Naturalization is the process wherein a bacterial strain gradually adapts to be able to persist and replicate in an environment outside of its normal niche. Several studies have demonstrated naturalization within the environment in soil (Chandrasekaran et al., 2015; Ishii et al., 2007; Ishii et al., 2006), in sand (Ishii et al., 2007; Whitman et al., 2014), on plants (Ksoll et al., 2007), and in water (Chandrasekaran et al., 2015), but relatively few studies on naturalization within a WWTP or within sewage pipes have been conducted. Furthermore, most of the studies on naturalization within wastewater focus on *E. coli* (Beattie et al., 2020; Yu et al., 2022; Zhi et al., 2016).

Bacteria can also persist and replicate in wastewater infrastructure by forming biofilms, which are bacterial communities, typically of varying species, that are attached to a surface and

to one another via extracellular polymeric substances (Jensen et al., 2016; Zhao et al., 2023). Biofilms provide a relatively stable environment that is partially protected from stressors such as predation, the immune system, and antibiotics (Jensen et al., 2016; Zhao et al., 2023). Horizontal gene transfer is an important mechanism of gene transfer between bacteria of the same or different species, and can occur within biofilms (Abe et al., 2020; Conwell et al., 2022; Weigel et al., 2007). Biofilms can form on most surfaces, and are an issue in hospital environments due to their propensity to contribute to pathogens' ability to colonize medical devices and cause post-surgical infections (Zhao et al., 2023). They can also form within wastewater collection systems (Skraber et al., 2007), where they may cause corrosion of pipes (Jensen et al., 2016). Most bacteria can participate to some extent in biofilm formation, but virulence factors in a given bacterial genome can play a crucial role in biofilm development (Șchiopu et al., 2023).

The ability of *Enterococcus* to form biofilms is well-studied from a clinical perspective (Ch'ng et al., 2018; Șchiopu et al., 2023). Examples of *Enterococcus* virulence factors include *ebpABC, acm,* and *bopD* (Freitas et al., 2021; Șchiopu et al., 2023). *Ebp* and *acm* are both related to adhesion and aggregation of a biofilm, while *bopD* is related to quorum-sensing, or the ability of bacteria within a biofilm to communicate based on cell density (Freitas et al., 2021; Șchiopu et al., 2023). Enterococcal surface protein gene *esp* is often found within members of CC17 (Heikens et al., 2007; Top et al., 2008), and is important for *Enterococcus* biofilm formation (Ch'ng et al., 2018; Toledo-Arana et al., 2001). Most research on *Enterococcus* biofilm formation has focused on biofilms within a host, but *Enterococcus* has been found in sewage biofilms (Lépesová et al., 2018) as well as sand (Piggot et al., 2012).

Section 3: VRE Strains with Similar Antibiotic Resistance Patterns in Sewage

MDR VRE are a serious health threat, as there are limited options for treatment (Arias et al., 2010; CDC, 2019), so the worldwide detection of VRE with similar AMR patterns (Caplin et al., 2008; de Farias et al., 2022; Ekwanzala et al., 2020; Sanderson et al., 2020) indicates that extra pressure may be put on treatment systems around the world. A study conducted in Canada performed genomic analysis on MDR VRE isolated from WWTPs (Sanderson et al., 2019; Sanderson et al., 2020), which were tested for susceptibility to twelve antibiotics (vancomycin, ampicillin, erythromycin, nitrofurantoin, quinupristin-dalfopristin, linezolid, teicoplanin, doxycycline, levofloxacin, gentamicin, streptomycin, and tigecycline). Eight of the vanR *E. faecium* strains isolated during this study (72%) exhibited a very similar AMR pattern: resistance to vancomycin, ampicillin, erythromycin, teicoplanin, levofloxacin, and streptomycin. The strainsalso shared susceptibility to linezolid, quinupristin-dalfopristin, and tigecycline.

Similar antibiotic resistance patterns have been observed in vancomycin-resistant *E. faecium* in Portugal (Araújo et al., 2010), England (Caplin et al., 2008), Brazil (de Farias et al., 2022), and South Africa (Ekwanzala et al., 2020). Multiple VRE strains were isolated from wastewater that were also resistant to ampicillin, tetracycline, erythromycin, and ciprofloxacin in these studies (Araújo et al., 2010; Caplin et al., 2008; de Farias et al., 2022; Ekwanzala et al., 2020). In Brazil, as in Canada, linezolid susceptibility was confirmed in most isolates (de Farias et al., 2022). Linezolid was not tested in the studies performed in Portugal and England, so the susceptibility of those strains is unknown. Nevertheless, it is clear that there is a world-wide prevalence of a five-antibiotic resistance pattern in vancomycin-resistant *E. faecium*.

Vancomycin-resistant *Enterococcus* spp. were isolated from wastewater samples collected from different treatment stages (all pre-disinfection) of South Cross Bayou WWTP in

St. Petersburg, Florida, and Hampton Roads Sanitation District WWTP in Virginia Beach, Virginia in 2019 during a study funded by the Centers for Disease Control (CDC). Both WWTPs receive millions of gallons of wastewater each day from the metropolitan areas that they serve, including from local hospitals. Both employ primary clarification, activated sludge, and secondary clarification, which at South Cross Bayou WWTP is followed by denitrification and chlorination treatment, and at Hampton Roads Sanitation District WWTP is followed by coagulation/flocculation/sedimentation, ozonation, biologically active filtration/granular activated carbon, UV disinfection, and chlorine disinfection treatment.

Putative *Enterococcus* isolates from the water samples were cultured on mEI agar (USEPA, 2009) amended with 32 μg/mL vancomycin to select for isolates with full resistance to vancomycin (CLSI, 2023). Eighteen VRE isolates were isolated over multiple sampling events from the influent, primary clarification, and aerobic digest. The eighteen isolates were tested for resistance to additional antibiotics: ampicillin, erythromycin, tetracycline, ciprofloxacin, linezolid, and quinupristin-dalfopristin by Kirby-Bauer disc diffusion.

The AMR pattern of fifteen of the isolates was identical when they were tested against the antibiotics above. Fourteen of these isolates were from Florida, and one was isolated from the Virginia WWTP. All fifteen exhibited full resistance to vancomycin, ampicillin, erythromycin, tetracycline, and ciprofloxacin, and susceptibility to linezolid, fosfomycin, and quinupristin-dalfopristin. The fourteen Florida isolates were isolated over two sampling events, thirteen from the first sampling event and one from the second sampling event. The two sampling events took place two months apart in May and July 2019, respectively. Four of the Florida isolates were from the influent, six (five from the first sampling event, and one from the second) from primary

clarification, and four from the aerobic digest, while the Virginia isolate was from the primary clarification stage.

This identical AMR pattern is unlikely to occur by chance in so many VRE isolates from different points within the plant and from different sampling events, as well as in two separate WWTPs. There are at least three plausible explanations for finding phenotypically similar, MDR VRE strains in multiple stages of two WWTPs. The first is a continually and rapidly introduced population, to the extent that it is able to be detected at multiple treatment stages and on different dates. This population may be from a biofilm that has formed within the wastewater collection system or may be introduced from an environment in which VRE are enriched, such as wastewater from a hospital. An introduced population would be comprised of one or several groups of isolates that are genetically similar throughout the treatment train. The second explanation is that a common source, such as a MGE or a genomic island, is the cause of the phenotypic similarities and is distributed in the sewer or WWTP system, where it can be acquired by unrelated *Enterococcus* strains. If one or several MGEs are conferring a similar resistance phenotype to multiple strains, the MGE(s) will be conserved, while the genomes may be relatively dissimilar to one another. The third explanation is that consistent antibiotic pressure within a clinical environment has selected for the same phenotypic antibiotic resistances in bacteria in that environment. Strains impacted by the same antibiotic pressure would exhibit the same AMR pattern, but could be either closely related or unrelated, and may not possess the same AMR genes.

Whole-genome sequencing was chosen to explore these hypotheses through genomic comparison and identification of AMR genes, virulence factors, and MGEs. MDR VRE may persist in wastewater and share their antibiotic resistance genes with other species, increasing the

risk these pathogens pose to human health in the event of a sewage spill. While other studies investigating VRE in WWTPs had broad scopes (Araújo et al., 2010; Goldstein et al., 2014; Sanderson et al., 2020), the focus of this thesis was more focused, analyzing the genomes of thirteen strains from one WWTP that shared an antibiotic resistance pattern and providing a more in-depth comparison of the strains than is typically performed. Analysis of the MDR VRE genomes sequenced in this thesis provided insight to the possible origin of the strains with identical MDR patterns.

Chapter 2: Methods

VRE Isolate History

Thirteen vancomycin-resistant *Enterococcus faecium* strains were recovered from freezer storage out of the 18 strains isolated from the CDC study in 2019. All recovered strains were from the Florida WWTP. Samples were taken from three treatment stages (influent, primary clarification, and aerobic digest) on two different dates in 2019 (Table 1). Samples were filtered onto 0.45-µm mixed ester cellulose membrane filters of 47 mm diameter (Fisher Scientific, Waltham, MA), which were then placed onto 50-mm polystyrene Petri dishes (Pall, Port Washington, NY) containing mEI amended with 32 µg/mL vancomycin to obtain vancomycin-resistant isolates. Four isolates were obtained from the influent, five from the primary clarification (four from the first sampling event and one from the second), and four from the aerobic digest (Table 1).

Table 1. Strain designation of each of the VRE strains, with date and location of sample collection.

Strain	Date Collected	Location
3.1.1	28-May-2019	Influent
3.1.2	28-May-2019	Influent
3.1.4	28-May-2019	Influent
3.1.5	28-May-2019	Influent
3.2.1	28-May-2019	Primary Clarification
3.2.3	28-May-2019	Primary Clarification
3.2.4	28-May-2019	Primary Clarification
3.2.5	28-May-2019	Primary Clarification
3.3.2	28-May-2019	Aerobic Digest
3.3.3	28-May-2019	Aerobic Digest
3.3.4	28-May-2019	Aerobic Digest
3.3.5	28-May-2019	Aerobic Digest
4.2.4	30-July-2019	Primary Clarification

Confirmation of Genus and AMR Pattern

Isolates were confirmed to be *Enterococcus* spp. using the Entero1a assay for the 23S rRNA gene (USEPA, 2015). Three isolated colonies were picked for each isolate and placed into 50 µL of nuclease-free water. The colonies were prepared for qPCR by boiling lysis (100°Cfor 15 minutes). The Entero1a primers and probe were used (forward primer: 5'-GAGAAATTCCAAACGAACTTG, reverse primer: 5'-CAGTGCTCTACCTCCATCATT, probe: [6-FAM]-5'-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-TAMRA), and the PCR master mix and running conditions were as previously described (USEPA, 2015).

The Kirby Bauer disc-diffusion assay (Hudzicki, 2009) was used to determine the susceptibility of each isolate to nine clinically-relevant antibiotics (Table 2). Resistance to the

antibiotics was determined following the Clinical and Laboratory Standards Institute's (CLSI) 2023 Performance Standards for Antimicrobial Susceptibility Testing (CLSI, 2023).

Table 2. Antibiotics used in the Kirby-Bauer disc diffusion assay, with abbreviation and class of each antibiotic, and the antibiotic concentration of each disc.

Antibiotic	Abbreviation	Class	Antibiotic content of disc
Ampicillin	AMP	Penicillins	10 μg
Ciprofloxacin	CIP	Fluoroquinolones	5 μg
Erythromycin	ERY	Macrolides	15 μg
Fosfomycin	FOS	Fosfomycins	200 μg
Linezolid	LZD	Oxazolidinones	30 μg
Nitrofurantoin	NIT	Nitrofurantoins	300 μg
Quinupristin-Dalfopristin	Q-D	Streptogramins	15 μg
Tetracycline	TET	Tetracyclines	30 μg
Vancomycin	VAN	Glycopeptides	30 μg

Minimum Inhibitory Concentration of Antibiotics

The minimum inhibitory concentration (MIC) of the following antibiotics, vancomycin, erythromycin, and ciprofloxacin, was determined for each isolate following the CLSI recommended protocol (Balouiri et al., 2016; CLSI, 2012). The antibiotic concentrations in Table 3 were chosen based on known MIC values for resistant *Enterococcus* (CLSI, 2023). The antibiotic concentrations varied over a two-fold serial dilution (e.g. 32, 64, 128, 256, 512, 1024 μg/mL for vancomycin, Table 3). Each strain was grown overnight in BHI broth at 41°C, and tested in duplicate. 0.2 mL, 96-well microwell plates (Thermofisher) were used for the assay, and

a BioTek Epoch 2 Microplate Spectrophotometer (Agilent) was used to read the OD600 of the wells. Positive growth controls for the media (no antibiotic added) and sterility controls (no culture or antibiotic added) were conducted on for every strain on every plate.

Table 3. Antibiotics and their respective concentrations used for the MIC assay, including the original stock concentration.

Antibiotic	**Manufacturer**	**Solvent**	**Stock concentration**	**Concentrations tested (μg/mL)**							
Ciprofloxacin	Thermo Scientific	0.5 M HCl	5,000 μg/mL	4	8	16	32	64	128	256	512
Erythromycin	Fisher BioReagents	95% Ethanol	10,000 μg/mL	8	16	32	64	128	256	512	1024
Vancomycin (Hydrochloride)	Fisher BioReagents	Sterile Water	25,000 μg/mL	32	64	128	256	512	1024	2048	n/a

Extraction of Genomic DNA for Sequence Analysis

Genomic DNA was extracted from a pure culture of each isolate diluted with sterile BHI broth to 5 x 10^6 CFU/mL using the QIAGEN Blood and Tissue Culture Kit. The protocol was amended for Gram-positive bacteria to include an additional lysis step as recommended by the manufacturer (QIAGEN, 2006). The quality of the extracted genomic DNA was assessed with a Qubit fluorometer to measure the amount of dsDNA, and a Nanodrop to check the 260/280 and 260/230 ratios to ensure the purity of the extracted DNA. The extraction resulted in 400μL of eluted DNA.

16S rRNA and Whole Genome Sequencing

16S rRNA sequencing was performed on 3 μL of DNA from each isolate, which was amplified using the 8F and 1492R primers (Turner et al., 1999), then sent to Eurofins Genomics (Louisville, Kentucky, USA) for sequencing. The resulting chromatograms were analyzed using Geneious 2024.0.5 to check for contamination and species were determined using sequence comparison (*Geneious 2024.0.5*).

Four hundred ng of purified DNA of each isolate was shipped overnight on ice to the Florida State University Next Generation Sequencing Facility (Tallahassee, FL). Library preparation was performed at the Florida State University Next Generation Sequencing facility using a NEB Next Ultra II DNA kit. Whole genome sequencing was performed via Illumina NovaSeq 50bp paired end reads with 50x coverage.

Assembly of Genomes and Plasmids

The reads received from the whole genome sequencing were checked for quality using FastQC (Andrews, 2010). Reads were trimmed based on quality score using BBDuk (Bushnell, 2015). Contigs were assembled via the SPAdes assembler (version 3.15.4) (Prjibelski et al., 2020). The contigs were compared via NCBI BLAST to several VRE genomes available on NCBI to find the genome with the highest similarity to the majority of the strains. That genome (VRE001, isolated from St. Jude Children's Research Hospital, BioSample ID: SAMN06018903) was used as the reference genome for the assembly using Bowtie2 (Langmead et al., 2012). Completeness of the genome was estimated using BUSCO v5 (Manni et al., 2021).

Analysis for Genes of Interest and Genome Alignment

Draft genome sequences of each strain were analyzed for AMR genes using the Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al., 2023), for mutations that cause antibiotic resistance via ResFinder (Florensa et al., 2022), and for virulence factors using the Virulence Factor Database (Chen et al., 2005). Contigs were then analyzed via the Bactopia pipeline to identify plasmids and annotate the genome (Petit_III & Read, 2020). Putative plasmids were identified using MOB-suite (Robertson et al., 2020; Robertson et al., 2018), and coding regions were annotated using Prokka (Seemann, 2014). PlasmidFinder 2.1 (Carattoli et al., 2014) was used to verify plasmid sequences. Annotated genomes were uploaded into IslandViewer 4 to identify potential genomic islands (Bertelli et al., 2017), which are clusters of genes within a genome that are transferable between organisms. Genomic islands in each strain were compared using Island Compare (Bertelli et al., 2022). The sequence type for each strain and the clonal complex to which they belonged was determined via comparison to the PubMLST database (Jolley et al., 2018). The thirteen assembled draft genome sequences were then aligned using progressiveMauve 2.4.0 (Darling et al., 2010).

Phylogenetic Analysis

Single nucleotide variation in the core genome was used to create a phylogenetic tree consisting of 68 genomes. The thirteen VRE genomes sequenced in this thesis were compared to 55 reference genomes from the NCBI GenBank database (Benson et al., 2013): nineteen VRE genomes of WWTP origin, twenty-eight VRE genomes of clinical origin, and eight *Enterococcus faecium* genomes of environmental origin (accession numbers provided in Table A1). The similarity of the strains analyzed in this thesis to the reference genomes was determined via the phylogenetic tree, and used to infer where the strains may have originated as well as the

similarity between the strains from this thesis and other CC17 strains. The genomes of MDR VRE strains from WWTPs with the aforementioned AMR pattern (de Farias et al., 2022; Sanderson et al., 2020) were chosen to compare the similarity of the MDR VRE with similar AMR patterns. Clinical VRE genomes were selected based on the completeness of the genome, and to have genomes from around the world. The genomes of VRE from the environment were chosen as they are not commonly found, while the genomes of vancomycin susceptible *Enterococcus faecium* from the environment were chosen due to the range of sequence types not seen elsewhere in the reference genomes used in the phylogenetic tree. The tree was created using the maximum likelihood method via IQTree 2.2.2.7 (Minh et al., 2020), and bootstrap values calculated using UFBoot (Hoang et al., 2018). The tree was visualized using interactive Tree of Life version v6 (Letunic & Bork, 2024).

Genome Sequencing Data Availability

The draft contig genomes and the assembled draft genome sequences have been uploaded to NCBI GenBank (BioProject PRJNA1104630, in submission).

Chapter 3: Results

The thirteen VRE strains were resistant to six antibiotics (including vancomycin) and susceptible to three antibiotics (Table 4). Vancomycin, erythromycin, and ciprofloxacin were selected for further antibiotic resistance testing using the MIC assay. All strains had an MIC breakpoint for vancomycin of at least 512 μg/mL (meaning that they grew at 256 μg/mL, but not at 512 μg/mL), though one strain (3.3.5) had a breakpoint of 2048 μg/mL (Table 5). All strains had an MIC breakpoint for erythromycin of at least 1024 μg/mL, though five strains grew at the highest tested concentration of erythromycin (1024 μg/mL). Eleven out of the thirteen strains had identical breakpoints for ciprofloxacin (256 μg/mL), while two strains had lower breakpoints (64 and 128 μg/mL). Growth was observed in all positive control wells, and no growth was detected in sterile controls.

Table 4. Antibiotics against which the VRE strains in this thesis were tested for resistance and the outcome. R designates resistance to that antibiotic, while S designates susceptibility.

Antibiotic	Antibiotic Class	Resistance
Vancomycin	Glycopeptides	R
Ampicillin	Penicillins	R
Ciprofloxacin	Fluoroquinolones	R
Erythromycin	Macrolide	R
Nitrofurantoin	Nitrofurans	R
Tetracycline	Tetracyclines	R
Fosfomycin	Fosfosmycins	S
Linezolid	Oxazolidinones	S
Quinupristin-Dalfopristin	Streptogramin	S

Table 5. Antibiotic breakpoints for vancomycin, erythromycin, and ciprofloxacin for each of the thirteen strains. Breakpoint is defined as the lowest antibiotic concentration at which the strain had no detectable growth. Strains with a breakpoint with a > sign grew at all tested concentrations of the antibiotic, with the highest concentration tested listed.

	MIC breakpoint (μg/mL)		
Strain	**Vancomycin**	**Erythromycin**	**Ciprofloxacin**
3.1.1	1024	>1024	256
3.1.2	512	>1024	256
3.1.4	1024	>1024	256
3.1.5	1024	>1024	256
3.2.1	1024	1024	256
3.2.3	1024	1024	256
3.2.4	512	1024	256
3.2.5	512	1024	128
3.3.2	512	1024	256
3.3.3	512	1024	256
3.3.4	512	1024	256
3.3.5	2048	1024	64
4.2.4	1024	>1024	256

The thirteen genomes were sequenced and assembled into contigs; a summary of the sequencing statistics can be found in Table 6. The length of each genome ranged from 2.93 Mbp to 3.20 Mbp, which is consistent with other *Enterococcus* and *E. faecium* genomes (Zhong et al., 2017). The GC percentage was very similar between all strains (37.7%), and the number of genes and number of protein coding sequences (CDSs) also varied little among strains (Table 6). The completeness of the genomes was estimated to be 98.4% via analysis of the benchmarking universal single-copy orthologs (BUSCOs) in the genome via BUSCO v5 (Manni et al., 2021). Each strain had 0.8% and 1.6% of duplicated BUSCOs and fragmented BUSCOs, respectively, out of 124 total BUSCOs.

Table 6. Genome information for thirteen *E. faecium* genomes, including strain name, size of the genome in base pairs (based on the size of the contig draft genome), %GC content, number of genes, and number of CDSs (protein coding sequences).

Strain	Size (bp)	# of Contigs	%GC	Genes	CDSs	Completeness
3.1.1	3,002,332	1,004	37.7	2,907	2,863	98.4%
3.1.2	2,967,126	922	37.7	2,852	2,805	98.4%
3.1.4	2,998,347	1,010	37.5	2,909	2,858	98.4%
3.1.5	3,003,877	1,008	37.7	2,905	2,861	98.4%
3.2.1	3,007,266	1,016	37.7	2,903	2,860	98.4%
3.2.3	2,954,165	1,029	37.7	2,854	2,808	98.4%
3.2.4	3,020,233	1,049	37.7	2,889	2,844	98.4%
3.2.5	3,018,545	988	37.7	2,888	2,841	98.4%
3.3.2	2,965,561	1,115	37.7	2,846	2,802	98.4%
3.3.3	3,195,770	1,114	38.0	2,945	2,853	98.4%
3.3.4	2,951,254	985	37.7	2,828	2,785	98.4%
3.3.5	2,925,342	1,060	37.8	2,795	2,749	98.4%
4.2.4	2,987,456	903	37.6	2,891	2,845	98.4%

The allele profiles for each strain were identified based on variation within the gene loci, which was compared to the PubMLST database (Jolley et al., 2018), and used to determine the multi-locus sequence type (MLST) for each strain (Table 7). Three sequence types were found among the thirteen strains: eight strains were sequence type 412, four were sequence type 584, and one (4.2.4) was sequence type 18 (Table 7). All three sequence types belong to clonal complex 17 (CC17).

Table 7. The multi-locus sequence type for each strain. The seven allele profiles that identify each sequence type are shown. All strains belong to clonal complex 17 (CC17).

Strain	Sequence Type	Allele Profiles						
3.1.1	412	atpA(15)	ddl(1)	gdh(1)	purK(44)	gyd(1)	pstS(20)	adk(1)
3.1.2	412	atpA(15)	ddl(1)	gdh(1)	purK(44)	gyd(1)	pstS(20)	adk(1)
3.1.4	412	atpA(15)	ddl(1)	gdh(1)	purK(44)	gyd(1)	pstS(20)	adk(1)
3.1.5	412	atpA(15)	ddl(1)	gdh(1)	purK(44)	gyd(1)	pstS(20)	adk(1)
3.2.1	412	atpA(15)	ddl(1)	gdh(1)	purK(44)	gyd(1)	pstS(20)	adk(1)
3.2.3	584	atpA(1)	ddl(1)	gdh(1)	purK(44)	gyd(1)	pstS(1)	adk(1)
3.2.4	584	atpA(1)	ddl(1)	gdh(1)	purK(44)	gyd(1)	pstS(1)	adk(1)
3.2.5	584	atpA(1)	ddl(1)	gdh(1)	purK(44)	gyd(1)	pstS(1)	adk(1)
3.3.2	412	atpA(15)	ddl(1)	gdh(1)	purK(44)	gyd(1)	pstS(20)	adk(1)
3.3.3	412	atpA(15)	ddl(1)	gdh(1)	purK(44)	gyd(1)	pstS(20)	adk(1)
3.3.4	412	atpA(15)	ddl(1)	gdh(1)	purK(44)	gyd(1)	pstS(20)	adk(1)
3.3.5	584	atpA(1)	ddl(1)	gdh(1)	purK(44)	gyd(1)	pstS(1)	adk(1)
4.2.4	18	atpA(7)	ddl(1)	gdh(1)	purK(1)	gyd(5)	pstS(1)	adk(1)

The finished draft genomes, assembled using a reference sequence (VRE001, SAMN06018903), were aligned to one another to determine their similarity (Figure 1). The genomes were very similar (99.5% identity in the core genome), with some rearrangement of small locally colinear blocks (LCBs). Within the sequence types, strains belonging to ST412 were very similar, sharing 99.92% of the core genome. Strains belonging to ST584 shared 99.5% of their core genome sequences.

Figure 1. The alignment of the thirteen assembled genomes using progressiveMauve version 2.4.0. Colored blocks represent locally colinear blocks (LCBs), which are areas of similarity. Lines are drawn between LCBs to link their position on each genome. The order of the strains is shown by the phylogenetic tree to the left, with the reference genome at the top. Closely related strains belonging to Group A and Group B are shown within the red and blue boxes, respectively.

Two sets of strains with nearly identical core genomes were identified, all belonging to ST412. Strains 3.1.1, 3.1.5, and 3.2.1 were very similar, with less than 16 single nucleotide polymorphisms (SNPs) difference in their core genomes. Strains 3.3.2, 3.3.3, and 3.3.4 were the most similar, with nearly identical genomes separated by only six SNPs. These two groups of strains will be referred to as Group A (3.1.1, 3.1.5, and 3.2.1), and Group B (3.3.2, 3.3.3, and 3.3.4). In Figure 1, minimal rearrangement has occurred within Group A, with the exception of some small LCBs. Two of the strains in Group B also appear to be nearly identical, but it appears that some rearrangement occurred within strain 3.3.3 that did not occur in the other members of Group B, nor any other strain sequenced. However, the rearrangement is minor, with

the only rearrangement of smaller blocks of the genome, and may be an artefact of the separate sequencing of that strain.

The relationships of genomes of the VRE isolated in this study on the phylogenetic tree were determined by sequence type, as expected (Figure 2). The ST412 and ST584 strains were most closely related to the reference genome VRE001 and three additional genomes from clinical isolates. The one strain in ST18 (4.2.4) was more closely related to a group of genomes of WWTP and clinical origin than it did to the strains from the Florida WWTP. These genomes (WWTP strains from Canada and two clinical strains) were all part of a sister clade belonging to ST18. Environmental strains formed a clade along with the vancomycin-susceptible WWTP strains, though more divergence was observed within their core genomes compared to the clinical strains, the VRE WWTP strains, and the strains sequenced in this study. These vancomycin-susceptible strains did not belong to CC17, to which all of the clinical and most of the WWTP strains belonged.

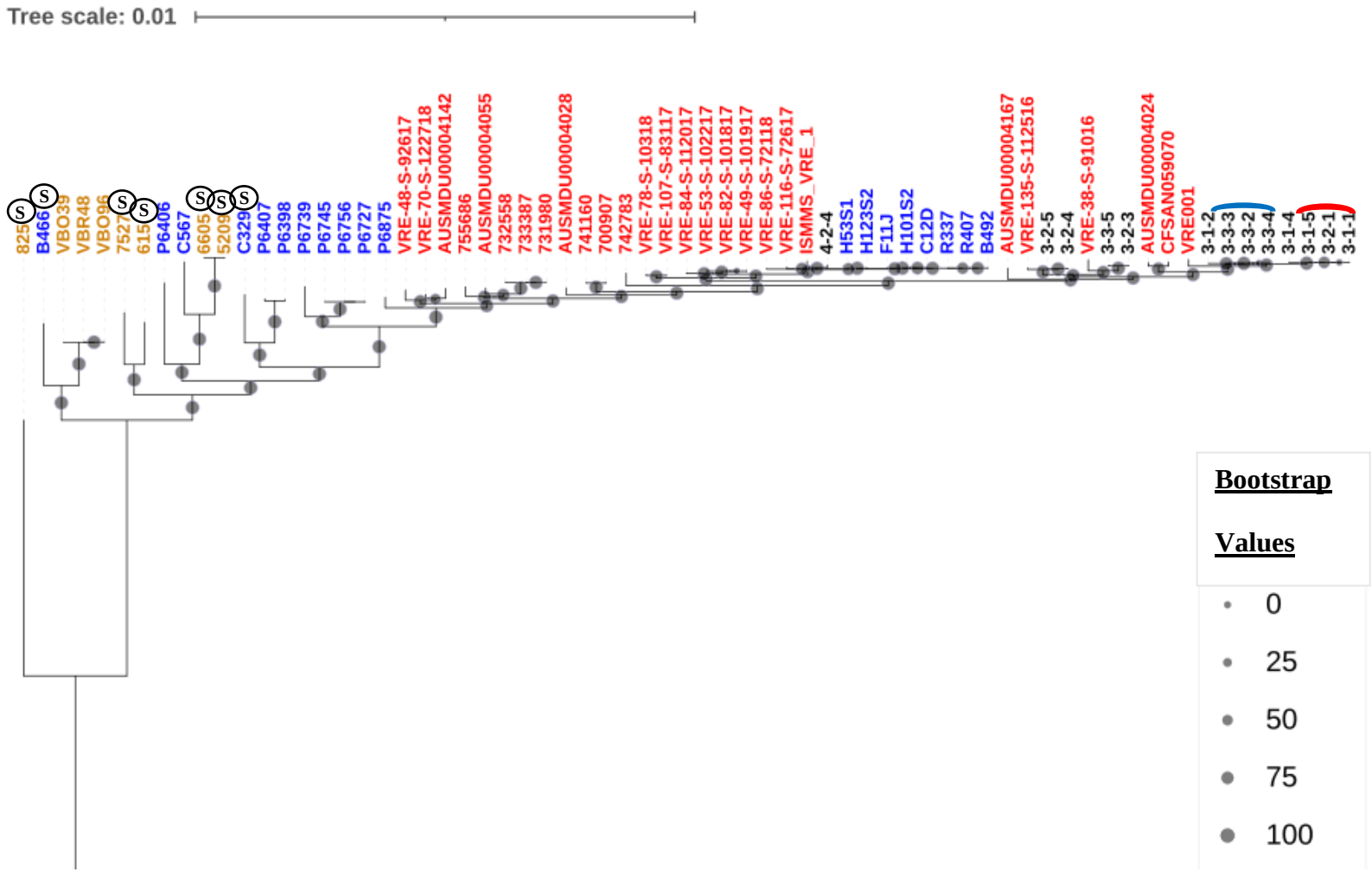

Figure 2. Phylogenetic tree of *E. faecium* genome sequences from this study (in black text) and complete genome sequences from the NCBI GenBank database (Table A1) based on variation in the whole genome. Scale bar in top left corner denotes substitutions per site, and the tree is rooted at *Enterococcus faecium* 825, a vancomycin-susceptible environmental strain. Blue text indicates genomes of wastewater origin, red text indicates genomes of clinical origin, and brown text indicates genomes of environmental origin. "S" marks vancomycin-susceptible strains. Tree was created using the maximum likelihood method via IQTree. Bootstrap values are indicated by the size of the circle on each node, following the legend in the bottom right corner.

The AMR genes detected in the genomes were largely similar among each of the strains (Figure 3). All strains carried the *vanA* operon, which was comprised of the seven *vanA* genes typically seen on the operon (*vanA*, *vanH*, *vanS*, *vanR*, *vanX*, *vanY*, and *vanZ*) (Figure 3). The strains also had resistance genes for tetracycline, trimethoprim, and aminoglycosides, as well as the resistance genes *ermB* (lincosamide/macrolide/streptogramin resistance) and *msrC* (macrolide/streptogramin) (Figure 3). Some strains also had heavy metal resistance genes; 3.1.2, 3.3.2, 3.3.3, and 3.3.4 all had cadmium resistance genes, and all strains also had an imperfect match (81% identity) to *copB*, a copper/silver resistance gene (Figure 3).

The virulence genes were also largely consistent among the genomes (Figure 3), with an average of 22 virulence factors per strain found. Eleven to twelve virulence genes in each strain encoded adhesins, and others contributed to biofilm formation (*bopD*), surface protein anchoring (*lgt*), and capsule formation (*cpsA*/*uppS* and *cpsB*/*cdsA*). All strains had virulence genes related to immune evasion, though the number and identity of the genes varied (Figure 3). A few virulence genes were only detected in some strains, including *fcl*, an antigen virulence factor, and *bsh*, a bile resistance factor only found in one strain (4.2.4) (Figure 3).

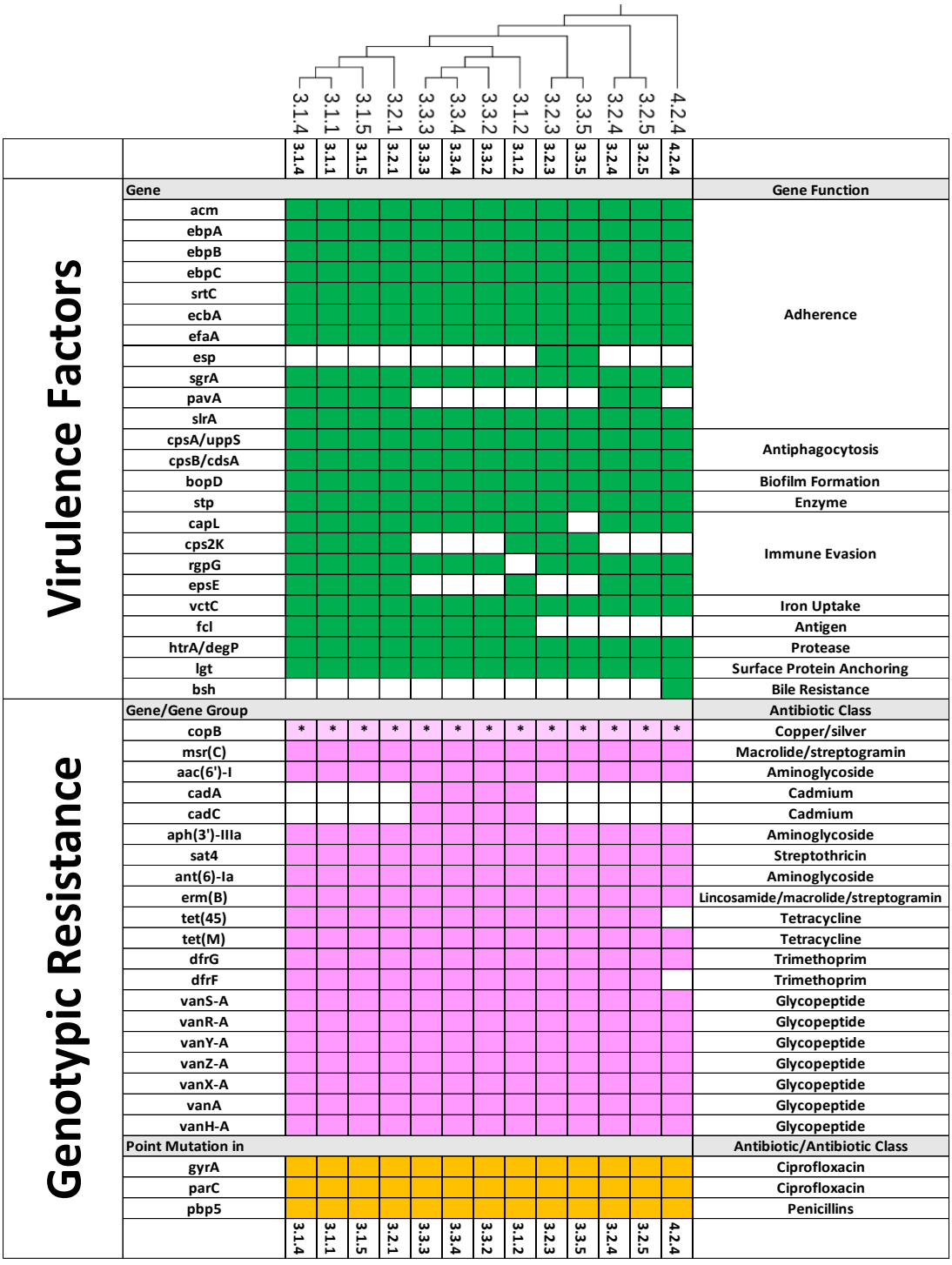

Figure 3. Virulence genes and antimicrobial resistance genes identified in each genome, as well as the gene function and antimicrobial class to which each gene corresponds. Colored cells indicate that the gene is present, while empty cells indicate absence. Strains are not organized by location and date, but by the phylogenetic tree at the top of the figure which shows the relatedness of each organism (branches are not to scale). Names of the strains are included at the bottom of the figure for reference.

Multiple putative MGEs were identified in each genome, including Tn1546 and other transposons, plasmids, and genomic islands. An average of 7 putative plasmids were found in each genome (Table 8), including one plasmid identified as conjugative and 2-3 plasmids identified as mobilizable. All other plasmids were identified as non-mobilizable. Many possible genomic islands (>10kb) and islets (<10kb) were also detected, with an average of 12 putative genomic islands and 3.6 putative genomic islets per strain (Table 8). Annotation of the genome via Prokka (Seemann, 2014) identified between 30-45 transposases in each strain (Table 8), several of which were place on genomic islands by analysis with IslandViewer 4 (Bertelli et al., 2017).

Plasmids identified via MOB-suite carried the *vanA* operon on Tn1546 in all VRE, and a suite of four genes (*ermB*, *SAT-4*, and two aminoglycoside genes, *aac(6')-I* and *aph(3')-IIIa*). The suite of four genes were always located together, and the identity of the plasmid was not consistent in all strains though the most common was plasmid pRUM (Table 9). Tetracycline resistance in most strains was mediated by both *tet(M)* and *tet(45)*, though only *tet(45)* was consistently detected on a plasmid (mobilizable plasmid pBC16) in all strains where the gene was present (Table 9). Tn1546 was detected on a plasmid in all strains, though the identity of the plasmid varied among strains (Table 9). Additionally, not all genes of the vanA operon were detected on the same putative plasmids identified via MOB-suite. As all vanA genes are known to be present and on Tn1546, this is most likely either an issue with sequencing or the software used.

Table 8. Number of mobile genetic elements (plasmids, genomic islands, and transposases) identified in each strain. The conjugative plasmid in each strain is shown in parentheses in the "Conjugative" column.

Strain	Plasmids			Genomic Islands			Transposases
	Total Number	# Conjugative	# Mobilizable	Total Number	>10kb	<10kb	Total Number
3.1.1	7	1 (ER04562.3A.5)	3	15	12	3	36
3.1.2	5	1 (pEFA-99d7)	2	16	10	6	35
3.1.4	6	1 (ER04562.3A.5)	2	16	13	3	35
3.1.5	7	1 (ER04562.3A.5)	3	16	12	4	34
3.2.1	7	1 (ER04562.3A.5)	3	16	13	3	34
3.2.3	4	1 (p3)	2	11	10	1	30
3.2.4	4	1 (p3)	2	17	11	6	44
3.2.5	4	1 (p3)	2	15	12	5	42
3.3.2	6	1 (p3)	3	17	13	4	35
3.3.3	6	1 (pEFA-99d7)	3	16	13	3	45
3.3.4	6	1 (p3)	3	15	13	2	35
3.3.5	7	1 (ISMMS_VRE_p1)	3	16	13	3	31
4.2.4	6	1 (ER04562.3A.5)	2	15	11	4	35

Table 9. Location of each antibiotic resistance gene in each strain (chromosome or plasmid). Plasmid names were identified either from GenBank (those with a P at the beginning and also Plasmid vanA) or by their primary cluster ID (identified via MOB-Suite).

	3.1.1	3.1.2	3.1.4	3.1.5	3.2.1	3.3.2	3.3.3	3.3.4	3.2.3	3.2.4	3.2.5	3.3.5	4.2.4
	ST412								ST584				ST18
tet(M) ①	Chromosome	Plasmid pBC16	Chromosome	Chromosome	Plasmid pBC16	Chromosome	Plasmid pBC16	Chromosome	Chromosome	Chromosome	Plasmid pBC16	Chromosome	Chromosome
tet(45) ①	Plasmid pBC16	Plasmid pBC16	Plasmid pBC16	Plasmid pBC16	Plasmid pBC16	Plasmid pBC16	Plasmid pBC16	Plasmid pBC16	Plasmid pBC16	Plasmid pBC16	Plasmid pBC16	Plasmid pBC16	n/a
dfrG ②	Chromosome	Chromosome	Chromosome	Chromosome	Plasmid AB117	Plasmid AB117	Plasmid AB117	Chromosome	Plasmid AB117	Plasmid AB117	Plasmid AB117	Chromosome	n/a
dfrF ②	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome
ant(6)-Ia ③	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome
msr(C) ④	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome
erm(B) ④	Plasmid pRUM	Plasmid AB756	Plasmid pRUM	Plasmid pRUM	Plasmid pRUM	Plasmid AB756	Plasmid pEFA-99d7	Plasmid AC731	Plasmid AC731	Plasmid pRUM	Plasmid AB173	Plasmid pRUM	Plasmid AB173
sat4 ⑤	Plasmid pRUM	Plasmid AB756	Plasmid pRUM	Plasmid pRUM	Plasmid pRUM	Plasmid AB756	Plasmid pEFA-99d7	Plasmid AC731	Plasmid AC731	Plasmid pRUM	Plasmid AB173	Plasmid pRUM	Plasmid AB173
aac(6')-I ③	Plasmid pRUM	Plasmid AB756	Plasmid pRUM	Plasmid pRUM	Plasmid pRUM	Plasmid AB756	Plasmid pEFA-99d7	Plasmid AC731	Plasmid AC731	Plasmid pRUM	Plasmid AB173	Plasmid pRUM	Plasmid AB173
aph(3')-IIIa ③	Plasmid pRUM	Plasmid AB756	Plasmid pRUM	Plasmid pRUM	Plasmid pRUM	Plasmid AB756	Plasmid pEFA-99d7	Plasmid AC731	Plasmid AC731	Plasmid pRUM	Plasmid AB173	Plasmid pRUM	Plasmid AB173
vanY-A ⑥	Tn1546: Plasmid AC731	Tn1546: Plasmid AC731	Tn1546: Plasmid AC731	Tn1546: Plasmid AC731	Tn1546: Plasmid AC731	Tn1546: Plasmid vanA	Tn1546: Plasmid vanA	Tn1546: Plasmid vanA	Tn1546: Plasmid AC731	Tn1546: Plasmid AC731	Tn1546: Plasmid AB173	Tn1546: Plasmid AC731	Tn1546: Plasmid AB173
vanZ-A ⑥	Tn1546: Plasmid AC731	Tn1546: Plasmid AC731	Tn1546: Plasmid AC731	Tn1546: Plasmid AC731	Tn1546: Plasmid AC731	Tn1546: Plasmid vanA	Tn1546: Plasmid vanA	Tn1546: Plasmid vanA	Tn1546: Plasmid AC731	Tn1546: Plasmid AC731	Tn1546: Plasmid AB173	Tn1546: Plasmid AC731	Tn1546: Plasmid AB173
vanS-A ⑥	Tn1546	Tn1546	Tn1546	Tn1546	Tn1546	Tn1546: Plasmid vanA	Tn1546: Plasmid vanA	Tn1546: Plasmid vanA	Tn1546: Plasmid AB369	Tn1546	Tn1546	Tn1546: Plasmid AB615	Tn1546
vanR-A ⑥	Tn1546	Tn1546	Tn1546	Tn1546	Tn1546	Tn1546: Plasmid vanA	Tn1546: Plasmid vanA	Tn1546: Plasmid vanA	Tn1546: Plasmid AB369	Tn1546: Plasmid AC731	Tn1546: Plasmid AB173	Tn1546: Plasmid AB615	Tn1546: Plasmid AB173
vanH-A ⑥	Tn1546: Plasmid AC731	Tn1546: Plasmid AC731	Tn1546: Plasmid AC731	Tn1546: Plasmid AC731	Tn1546: Plasmid AC731	Tn1546: Plasmid vanA	Tn1546: Plasmid vanA	Tn1546: Plasmid vanA	Tn1546: Plasmid AC731	Tn1546: Plasmid AC731	Tn1546: Plasmid AB173	Tn1546: Plasmid AC731	Tn1546: Plasmid AB173
vanA ⑥	Tn1546: Plasmid AC731	Tn1546: Plasmid AC731	Tn1546: Plasmid AC731	Tn1546: Plasmid AC731	Tn1546: Plasmid AC731	Tn1546: Plasmid vanA	Tn1546: Plasmid vanA	Tn1546: Plasmid vanA	Tn1546: Plasmid AC731	Tn1546: Plasmid AC731	Tn1546: Plasmid AB173	Tn1546: Plasmid AC731	Tn1546: Plasmid AB173
vanX-A ⑥	Tn1546: Plasmid AC731	Tn1546: Plasmid AC731	Tn1546: Plasmid AC731	Tn1546: Plasmid AC731	Tn1546: Plasmid AC731	Tn1546: Plasmid vanA	Tn1546: Plasmid vanA	Tn1546: Plasmid vanA	Tn1546: Plasmid AC731	Tn1546: Plasmid AC731	Tn1546: Plasmid AB173	Tn1546: Plasmid AC731	Tn1546: Plasmid AB173

1: Tetracycline resistance 2: Trimethoprim resistance 3: Aminoglycoside resistance
4: Macrolide resistance 5: Streptothricin resistance 6: Vancomycin resistance

Chapter 4: Discussion

Antimicrobial Resistance Patterns

The vancomycin-resistant *Enterococcus faecium* strains sequenced here were isolated from a single WWTP and displayed an intriguing similarity of a shared antibiotic resistance pattern (vancomycin, ampicillin, ciprofloxacin, erythromycin, tetracycline, and nitrofurantoin). MDR VRE that share a similar antibiotic resistance pattern, including full resistance to vancomycin, ampicillin, ciprofloxacin, erythromycin, and tetracycline (nitrofurantoin not usually tested) have been isolated from wastewater around the world, including Canada (Sanderson et al., 2020), England (Caplin et al., 2008), Portugal (Araújo et al., 2010), Brazil (de Farias et al., 2022), and South Africa (Ekwanzala et al., 2020), as well as from a WWTP in Virginia in the original study. These MDR VRE strains also all belong to CC17, like the strains sequenced in this thesis. The strains in this thesis and in some similar studies (de Farias et al., 2022; Goldstein et al., 2014; Sanderson et al., 2020) were susceptible to linezolid, indicating that some treatment options are still available.

Minimum Inhibitory Concentrations

The minimum inhibitory concentration assay defines the lowest concentration of an antibiotic that can completely inhibit the growth of a bacterial strain. The resistance breakpoint concentration defined by the CLSI is decided using a variety of clinical and pharmaceutical studies (CLSI, 2023). Differing MIC breakpoints for antibiotics can be associated with the distinct AMR genes in a strain, such as *vanA* or *vanB* (Ahmed & Baptiste, 2018; Cetinkaya et al.,

2000; Jensen et al., 1998; Werner et al., 2008). The MIC of three antibiotics to which the strains in this study were known to be resistant (vancomycin, erythromycin, and ciprofloxacin) were determined to allow a more refined comparison among the strains, as well as to similar clinical studies where MIC testing was performed. Vancomycin was chosen due to its status as an antibiotic of last resort, and due to the varying breakpoints associated with specific vancomycin resistance genes (Hanaki et al., 2004; Tenover et al., 1995). Ciprofloxacin is often used to treat urinary tract infections (CLSI, 2023), and was chosen due to the presence of the point mutations in *gyrA* and *parC* in the sequenced strains that are known to confer quinolone resistance (Dalhoff, 2012; Leavis et al., 2006). Erythromycin was chosen due to its nature as a broad-spectrum antibiotic for a variety of infections caused by *Enterococcus* spp. (CLSI, 2023; Georges et al., 2022), and the identification of two macrolide resistance genes within the genomes.

Differing MIC values to the antibiotics were found among the strains, despite the similarity in AMR genes. The MIC breakpoints for vancomycin, ciprofloxacin, and erythromycin were not divided by sequence type, but the most closely related strains shared the same MIC breakpoints. The vancomycin (Hanaki et al., 2004; Tenover et al., 1995) and ciprofloxacin MIC values (Leavis et al., 2006; Werner et al., 2010) were similar to those reported in clinical studies. Few studies on MDR VRE in wastewater have determined the MIC breakpoints of the antibiotics tested for their strains (Caplin et al., 2008), so while the AMR resistance pattern appears to be shared, it is currently unknown if the strains isolated in other studies are resistant to different levels of antibiotics.

Higher than expected MIC breakpoints were detected for vancomycin in one strain (strain 3.3.5, 2048 μg/mL), and erythromycin (≥1024 μg/mL) in all strains. Vancomycin MIC

breakpoints of 2048 μg/mL have been detected elsewhere (Sahm & Olsen, 1990), but lower values are more often reported (Hanaki et al., 2004; Tenover et al., 1995). The *vanA* operon promoter located in the *vanR* and *vanS* genes (Arthur et al., 1993) in strain 3.3.5 appeared to be identical to those in the other sequenced strains and those described in the literature, so the reason for the increased MIC value is currently unclear. The erythromycin MIC breakpoint in *E. faecium* strains with two macrolide resistance genes, *ermB* and *msrC* (Isogai et al., 2013; Milanović et al., 2019), or with only *ermB* (Lee et al., 2023; Portillo et al., 2000) range from 128 – 256 μg/mL. However, two erythromycin resistance genes, *ermB* and *msrC*, were identified in each strain sequenced in this thesis, yet the MIC for most strains in this thesis was 1024 μg/mL, and four strains were resistant to the highest concentration of erythromycin tested (1024 μg/mL). It is unclear if the same antibiotic concentrations for both vancomycin and erythromycin were tested in other studies as in this thesis, which may explain why higher MIC values were observed in this study.

Multi-locus Sequence Typing

The three sequence types found in this study (ST18, ST412, and ST584) are often found within hospitals (El Haddad et al., 2021; Ochoa et al., 2013; Panesso et al., 2010; Ryan et al., 2015), and ST18 has been detected in sewage in several studies (Caplin et al., 2008; Freitas et al., 2009; Sanderson et al., 2020). Only one strain belonging to ST18 was detected in this study, though it is the most common of the three sequence types and found in both clinical and sewage environments. The majority of strains in this study belong to STs 412 and 584, which have not been detected in other sewage studies on MDR VRE. The identification of multiple sequence types indicates that the shared AMR pattern of the isolates sequenced in this study is not due to a clonal lineage.

Phylogenetic Comparison

The strains sequenced in this thesis were most closely related to all clinical and some WWTP strains. There was limited genetic diversity in this clade compared to the environmental and the other WWTP strains. The WWTP strains which were the most similar to the strains sequenced in this thesis contained the same AMR pattern, and were closely related to clinical strains in their own comparative genomic analysis (Sanderson et al., 2020). *E. faecium* strains isolated from the environment were part of the same monophyletic group, regardless of whether they are vancomycin-resistant or vancomycin-sensitive. Environmental strains represented sequence types that were not found elsewhere in the reference genomes or in the strains sequenced in this study, i.e. 54, 82, 133, and 2477, which do not belong to CC17, and STs 54, 82, and 133 do not belong to any clonal complex. Housekeeping genes used to delineate sequence type are part of the core genome but are not the only genes in the core genome, which is comprised of over 600 genes (Zhong et al., 2017). Therefore, additional changes within the core genome likely affected how the relationships on the phylogenetic tree were calculated.

Strains of the same sequence type tend to form monophyletic clades on phylogenetic trees (El Haddad et al., 2021; Rios et al., 2020; Sanderson et al., 2020). In this study, strain 4.2.4 (ST18) was closely related to multiple reference genomes that originated from WWTPs in Canada, which also belonged to ST18. The strains sequenced in this study belonging to ST412 and ST584 were part of a monophyletic clade on the tree, along with several clinical genomes. The strains are separated by sequence type, but all strains, even the lone ST18, was part of the clinical and CC17 clade of the phylogenetic tree, which indicates that they likely all originate from clinical origin.

Mobile Genetic Elements

Multiple putative genomic islands were found in each of the thirteen strains via IslandViewer 4 (Bertelli et al., 2017). Genomic islands are common in *Enterococcus*, with one study finding an average of 3.2 genomic islands in each genome, and between one to three AMR genes on several of the genomic islands (Li et al., 2021). The strains in this study contained an average of 15.5 putative genomic islands each, four of which were conserved in all thirteen strains. One of these islands was identical in every strain, but primarily contained 30S and 50S ribosomal genes, not a cluster of AMR genes that could explain all of the resistance phenotypes. No AMR genes were detected on any genomic island in the strains sequenced in this thesis.

The strains sequenced in this thesis had an average of 7.3 plasmids identified via MOB-suite (Robertson et al., 2020; Robertson et al., 2018), including one conjugative plasmid in each VRE strain. The genomes of VRE often have several plasmids (de Farias et al., 2022; Flannagan et al., 2003; Gilmore et al., 2013), including a conjugative plasmid that typically carries Tn1546 (Arthur et al., 1993; Top et al., 2008). Several strains (3.1.1, 3.1.4, 3.1.5, 3.2.1, 3.2.4, and 3.3.5) contained the mobilizable plasmid pRUM, which contained several AMR genes (*ermB*, *SAT-4*, and two aminoglycoside resistance genes), but in most strains AMR genes did not cluster on any one putative plasmid. Instead, AMR genes were located across several plasmids in most strains, suggesting that the strains did not acquire the antibiotic resistance genes in one event.

Tn1546 in Sequenced Strains

Tn1546 was detected in all strains, located on a putative plasmid (plasmid cluster IDs AC731 or AB173, or plasmid vanA). Tn1546 contains the *vanA* operon, as detected in this study as well as previously described (Arthur et al., 1993; Biavasco et al., 2007), which points to the

presence of Tn1546 as the most likely cause of vancomycin resistance in the VRE strains sequenced in this study. Three strains had other AMR genes (*ermB, SAT-4*, and aminoglycoside resistance genes) located on the same plasmid as Tn1546, which was a non-mobilizable plasmid (plasmid cluster IDs AC713, AB173, and AB173, respectively). In all other strains, Tn1546 was located on either a conjugative (plasmid cluster ID AC731) or non-mobilizable (plasmid cluster IDs AC713 or AB173) plasmid that did not contain other AMR or virulence genes, indicating that it is unlikely that other resistance genes were acquired on mobile genetic elements common to all the strains. Other studies on vancomycin-resistant *E. faecium* in wastewater have identified Tn1546 in the genomes of their strains (de Farias et al., 2022; Ekwanzala et al., 2020; Novais et al., 2005). However, Tn1546 was not detected in VRE strains from a Canadian WWTP, despite the presence of the *vanA* operon in eight out of eleven *E. faecium* strains, though other transposons that carried antibiotic resistance genes were found (Sanderson et al., 2020). The program that was used (ICEberg) identifies integrative and conjugative elements in DNA, not necessarily plasmids or transposons, which may explain this discrepancy. When ICEberg was run on the strains in this thesis, only two integrative or conjugative elements were detected.

Virulence Factors

Similar virulence genes were found in all thirteen VRE strains sequenced in this thesis, including *acm, ebpABC, ecbA,* and *bopD.* These genes encode virulence factors used for attachment and biofilm formation (Șchiopu et al., 2023). *acm, ebpABC,* and *ecbA* are common among clinically-associated VRE strains in CC17 (Bjørkeng et al., 2011; Nallapareddy et al., 2008; Sillanpää et al., 2009). Two capsule-forming virulence genes (*cpsA*/*uppS, cpsB*/*cdsA*), associated with evasion of the host immune system (Chen et al., 2005; Shridhar et al., 2022), were also detected in all thirteen strains. The *esp* virulence gene, which some research has

suggested is critical for biofilm attachment (Heikens et al., 2007; Toledo-Arana et al., 2001; Top et al., 2008), was found in only two strains (3.2.3 and 3.3.5, both ST584). Most CC17 strains contain a pathogenicity island that contains *esp* (Heikens et al., 2007; Top et al., 2008), so its absence in eleven out of thirteen strains may indicate a loss of the pathogenicity island, or a need for deeper sequencing depth.

Whole Genome Comparison of Sequenced Strains

Two groups, termed A and B, of three strains with extremely similar genomes were identified out of the thirteen sequenced strains. Due to the level of similarity (>99.999% similar) the groups are considered nearly identical (Siranosian et al., 2022). These strains were isolated from samples collected on the same day, but from different WWTP treatment stages. Both groups belong to ST412, and within each group, the strains are ≥99.9995% similar to each other and share the same virulence genes as well as antibiotic MIC values, indicating that the similarities between the strains are across the whole genome, not just the core genome. Strains within each group contained the same number of putative plasmids (Group A: 7, Group B: 6). Group A and Group B are distinct from each other genetically, for example the absence of two immune evasion genes within all three Group B strain that is present within the Group A strains.

Chapter 5: Conclusions

The genomes of thirteen VRE strains isolated from a Florida WWTP that produces recycled water were sequenced due to the similarity of their antibiotic resistance profiles. All thirteen strains were identified as *E. faecium*, and the strains belong to three sequence types (ST18, ST412, and ST584), all part of clonal complex 17. The antibiotic resistance genes within the strains were nearly identical, and virulence factors were largely conserved. Multiple MGEs were identified within the strains, most notably Tn1546, which carries the *vanA* operon, which was located on a plasmid, though plasmid identity varied between strains. Many AMR genes in the strains, including Tn1546 and the *vanA* operon, were located on plasmids, though no discernable pattern was observed. Vancomycin and erythromycin MIC values for some strains were higher than any reported breakpoints in the literature, though the ciprofloxacin MIC values largely aligned with previous observations. Four genomic islands were conserved in all strains, but no antibiotic genes were identified to be located on these islands, eliminating them as a potential route of shared resistance. The strains likely came from the same source upstream of the plant, most likely sewage from a hospital, where they would have been under similar antibiotic pressures. The AMR pattern seen in these MDR VRE has been detected across the globe. The risk that these MDR VRE pose is unclear, but monitoring of MDR VRE and other pathogens in wastewater may prove useful for monitoring the spread of antibiotic resistance.

The strains sequenced in this thesis shared the same AMR genes, yet the mobile genetic elements varied greatly among the strains. The most likely cause for the shared AMR pattern is a

shared lineage. The strains possess antimicrobial resistance mutations characteristic of CC17 and contain Tn1546 which is prevalent in the clonal complex. The strains acquired the tetracycline and erythromycin resistance genes via plasmids. The AMR pattern that these strains possess is spread across the globe, which likely accounts for the additional strain detected at a WWTP in Virginia in the original study. These strains are still susceptible to some antibiotics, e.g. linezolid, fosfomycin, and quinupristin-dalfopristin, but it is possible that further antibiotic resistance genes will be acquired by these similar strains as AMR increases worldwide over the next several decades (Prestinaci et al., 2015; Salam et al., 2023; WHO, 2022). Pan-drug resistant pathogens have already been isolated from clinical sources (Ozma et al., 2022; Souli et al., 2006). The risk that pathogens like MDR VRE pose within wastewater is unknown, but they can enter the environment during sewage spills and persist. As the intensity of storms increases with climate change, the risk that these MDR VRE pose to human health will likely increase as well.

Future Directions

This study was limited in scope, but our findings provide some insight into prevalence and origin of MDR VRE in wastewater. For future comparisons, additional MDR VRE should be isolated from the same WWTP, as well as other WWTPs in the area. In addition, samples should be taken immediately downstream of hospital sewage inputs, as well as from biofilms within the wastewater collection system, if possible. These VRE should be isolated on both media amended with vancomycin, as well as media amended with multiple antibiotics. Strains with similar resistance patterns to the strains sequenced in this thesis, as well as novel AMR patterns of interest should be further investigated via MIC testing and whole genome sequencing.

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Appendix I: Supplementary Tables and Figures

Table A1. List of reference genomes included in phylogenetic tree, including accession number and source of each strain. The year that each strain was submitted to NCBI and the identity of the submitter is included. The sequence type of each strain is listed, and a dash indicates where a sequence type could not be confidently identified. Strains are organized by source in alphabetical order, followed by year submitted to NCBI from oldest to newest, followed by identity of submitter in alphabetical order.

Strain Name	BioSample ID	Source	Year Submitted to NCBI	Identity of Submitter	Sequence Type	VRE?
ISMMS_VRE_1	SAMN03988632	Clinical	2016	Mount Sinai School of Medicine	2272	Yes
VRE001	SAMN06018903	Clinical	2016	St Jude Children's Research Hospital	2272	Yes
CFSAN059070	SAMN06621458	Clinical	2017	FDA	203	Yes
AUSMDU00004142	SAMN08628412	Clinical	2018	University of Melbourne	80	Yes
AUSMDU00004024	SAMN08628409	Clinical	2018	University of Melbourne	203	Yes
AUSMDU00004028	SAMN08628410	Clinical	2018	University of Melbourne	796	Yes
AUSMDU00004055	SAMN08628411	Clinical	2018	University of Melbourne	78	Yes
AUSMDU00004167	SAMN08628413	Clinical	2018	University of Melbourne	1421	Yes
700907	SAMN36411047	Clinical	2023	Polytechnic University of Marche Medical School	-	Variable
741160	SAMN36411052	Clinical	2023	Polytechnic University of Marche Medical School	-	Variable
732558	SAMN36411049	Clinical	2023	Polytechnic University of Marche Medical School	80	Variable
742783	SAMN36411053	Clinical	2023	Polytechnic University of Marche Medical School	117	Variable
755686	SAMN36411054	Clinical	2023	Polytechnic University of Marche Medical School	80	Variable
731980	SAMN36411048	Clinical	2023	Polytechnic University of Marche Medical School	80	Variable

Table A1. Continued.

Strain Name	BioSample ID	Source	Year Submitted to NCBI	Identity of Submitter	Sequence Type	VRE?
733387	SAMN36411050	Clinical	2023	Polytechnic University of Marche Medical School	80	Variable
VRE-38-S-91016	SAMN37146019	Clinical	2023	University of Florida	664	Yes
VRE-48-S-92617	SAMN37146028	Clinical	2023	University of Florida	80	Yes
VRE-49-S-101917	SAMN37146029	Clinical	2023	University of Florida	17	Yes
VRE-53-S-102217	SAMN37146032	Clinical	2023	University of Florida	-	Yes
VRE-70-S-122718	SAMN37146046	Clinical	2023	University of Florida	-	Yes
VRE-78-S-10318	SAMN37146052	Clinical	2023	University of Florida	17	Yes
VRE-82-S-101817	SAMN37146056	Clinical	2023	University of Florida	17	Yes
VRE-84-S-112017	SAMN37146058	Clinical	2023	University of Florida	17	Yes
VRE-86-S-72118	SAMN37146059	Clinical	2023	University of Florida	18	Yes
VRE-107-S-83117	SAMN37145994	Clinical	2023	University of Florida	17	Yes
VRE-116-S-72617	SAMN37146002	Clinical	2023	University of Florida	18	Yes
VRE-135-S-112516	SAMN37146017	Clinical	2023	University of Florida	584	Yes
6605	SAMN07524551	Environment - Agricultural	2017	USDA	54	No
615	SAMN07524546	Environment - Agricultural	2017	USDA	82	No
825	SAMN07524545	Environment - Agricultural	2017	USDA	2477	No
5209	SAMN07524550	Environment - Agricultural	2017	USDA	54	No
7527	SAMN07524549	Environment - Agricultural	2017	USDA	269	No
VBR48	SAMN17922919	Environment - Surface Water	2021	University of Zurich	133	Yes

Table A1. Continued.

Strain Name	BioSample ID	Source	Year Submitted to NCBI	Identity of Submitter	Sequence Type	VRE?
VBO39	SAMN17922923	Environment - Surface Water	2021	University of Zurich	133	Yes
VBO96	SAMN17922924	Environment - Surface Water	2021	University of Zurich	133	Yes
R407	SAMN11029776	WWTP	2019	Agriculture and Agri-Food Canada	18	Yes
C12D	SAMN11029416	WWTP	2019	Agriculture and Agri-Food Canada	18	Yes
H101S2	SAMN11029457	WWTP	2019	Agriculture and Agri-Food Canada	18	Yes
C567	SAMN11026090	WWTP	2019	Agriculture and Agri-Food Canada	1216	No
R337	SAMN11029844	WWTP	2019	Agriculture and Agri-Food Canada	18	Yes
F11J	SAMN11029604	WWTP	2019	Agriculture and Agri-Food Canada	18	Yes
H53S1	SAMN11029590	WWTP	2019	Agriculture and Agri-Food Canada	18	Yes
H123S2	SAMN11029491	WWTP	2019	Agriculture and Agri-Food Canada	18	Yes
B492	SAMN11029791	WWTP	2019	Agriculture and Agri-Food Canada	18	Yes
B466	SAMN11025820	WWTP	2019	Agriculture and Agri-Food Canada	672	No
C329	SAMN11025818	WWTP	2019	Agriculture and Agri-Food Canada	40	No
P6398	SAMN18231634	WWTP	2021	Oswaldo Cruz Foundation	168	Yes
P6406	SAMN18231635	WWTP	2021	Oswaldo Cruz Foundation	253	Yes
P6407	SAMN18231636	WWTP	2021	Oswaldo Cruz Foundation	168	Yes
P6727	SAMN18231637	WWTP	2021	Oswaldo Cruz Foundation	32	Yes
P6739	SAMN18231638	WWTP	2021	Oswaldo Cruz Foundation	1894	Yes
P6745	SAMN18231639	WWTP	2021	Oswaldo Cruz Foundation	32	Yes

Table A1. Continued.

P6756	SAMN18231640	WWTP	2021	Oswaldo Cruz Foundation	32	Yes
P6875	SAMN18231641	WWTP	2021	Oswaldo Cruz Foundation	1893	Yes

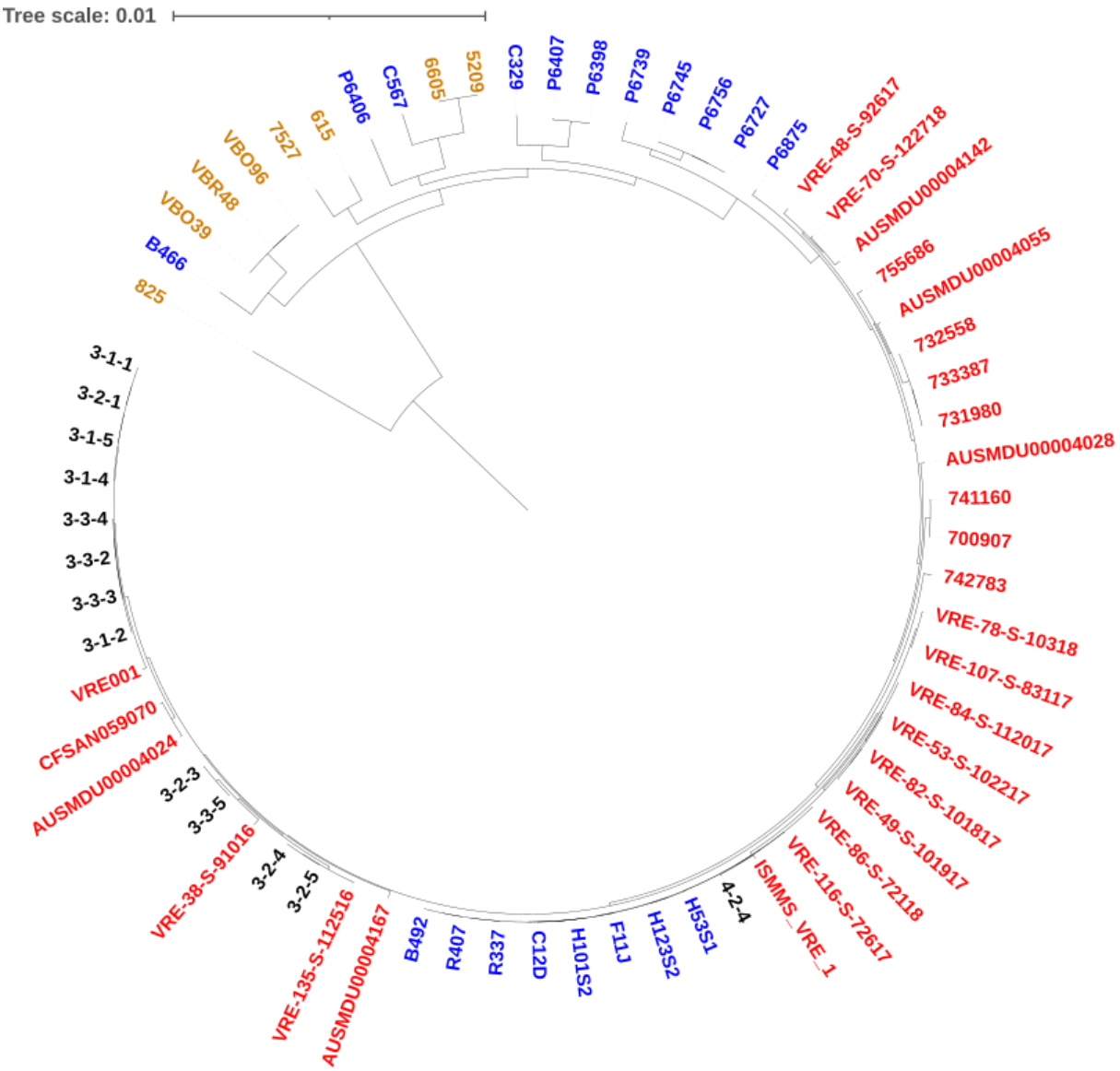

Figure A1. Phylogenetic tree of *E. faecium* genome sequences from this study (in black text) and complete genome sequences from the NCBI GenBank database (Table A1) based on variation in the core genome. Scale bar in top left corner denotes substitutions per site, and the tree is rooted at *Enterococcus faecium* 825, a vancomycin-susceptible environmental strain. Blue text indicates genomes of wastewater origin, red text indicates genomes of clinical origin, and brown text indicates genomes of environmental origin. Tree was created using the maximum likelihood method via IQTree.

Appendix II: MIC Protocol

Minimum Inhibitory Concentration Broth Microdilution Assay Protocol

By Eleanor Brodrick

Following the procedure outlined in the CLSI recommended protocol:

(Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically, Approved Standard, 9th ed., CLSI document M07-A9) (CLSI, 2012)

Procedure

The day prior to the test, streak your strains of interest for isolation. Multiple isolated colonies will be needed, so it is recommended to streak multiple plates for each strain. Incubate at the appropriate temperature for 24 hours.

Prepare enough sterile brain heart infusion or Mueller-Hinton broth for testing (you will likely need a few hundred mL). Mueller-Hinton broth is preferred, but BHI broth will suffice if necessary. Also prepare 0.85% saline (you will likely need ~100 mL). The assay uses 0.2mL 96 well microwell plates, which will need to be sealed with parafilm, and it is recommended to use 200 μL filter tips to prevent contamination.

Antibiotic stocks should be prepared before the assay begins. The antibiotic dilution will proceed twofold from the highest concentration tested to the lowest. The stock will need to be of at least high enough concentration to achieve the desired starting concentration in 50 μL. It is recommended to use a higher concentration stock, and dilute into sterile broth to achieve this.

Pick multiple colonies from each strain and place in 1mL of 0.85% sterile saline. The bacterial suspension is adjusted with additional saline broth if needed for the turbidity to fall within the 0.08-0.12 at 625 nm using a Nanodrop, which is equivalent to the 0.5 McFarland standard (Balouiri et al., 2016). Then the broth culture was diluted 1:150 in sterile brain heart infusion or Mueller-Hinton broth to give a suspension of ~1-2 x 10^6 CFU/mL.

The final volume should be equivalent to 50 μL multiplied by the number of wells needed. Each test will be run in duplicate and include multiple dilutions. For example, when testing against a single antibiotic with seven dilutions (including the growth control), each strain will be pipetted into fourteen wells. Therefore, a minimum of 700 μL is needed, though it is recommended to scale up by at least 10-25%.

Into the first column of a 96-well micro-titration plate, pipet 100 μL of the highest concentration of antibiotic. In all other wells of the plate, pipet 50 μL of sterile BHI broth medium. From the first column, transfer 50 μL was transferred into the second column, to make a 1:2 dilution (e.g. 1024 μg/mL to 512 μg/mL). Repeat for all of the necessary dilutions, with at least two columns of sterile BHI broth left with no antibiotic, one to act as a growth control, and the other to act as a sterile control for the broth (see diagram below).

Once the antibiotic dilution series has been completed, pipet 50 μL of the estimated 10^6 CFU/mL broth culture into each well except for the sterile control column, with each strain

tested in duplicate. The plate is as then sealed via parafilm. It is recommended to either double seal with either more parafilm or with tape. The sealed plate is incubated overnight at 35°C for 18-24 hours. Then, remove the plates from the incubator and read at OD600 using a plate reader.

Interpretation

In the figure below, the results of four strains tested against vancomycin are shown. The first two strains have an MIC breakpoint, defined as the lowest antibiotic concentration where there is no visible growth, of 1024 μg/mL. The bottom two strains have a lower MIC of 512 μg/mL. While the growth in the 256 μg/mL column for the bottom two strains is faint, it is still detectable, which is why 512 is the MIC, since there is no detectable growth in those wells. There is detectable growth in the growth control wells (Column 10), as expected, and the blank control column has a similar OD to any empty wells, indicating that there was no growth in the blank, and therefore no detected contamination.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.042	0.047	0.375	0.577	0.626	0.619	0.705	0.049	0.047	0.215	0.048	0.051
B	0.045	0.047	0.215	0.777	0.776	0.741	0.818	0.048	0.049	0.412	0.048	0.047
C	0.047	0.048	0.951	0.969	0.834	0.751	0.883	0.048	0.046	0.487	0.049	0.049
D	0.047	0.05	0.754	0.996	0.606	0.862	0.913	0.047	0.047	0.721	0.048	0.048
E	0.047	0.051	0.047	0.186	0.637	0.763	0.881	0.047	0.046	0.855	0.047	0.09
F	0.049	0.049	0.05	0.1	0.686	0.761	0.936	0.049	0.046	0.865	0.048	0.094
G	0.048	0.071	0.046	0.132	0.558	0.95	0.974	0.048	0.047	0.678	0.047	0.059
H	0.05	0.047	0.048	0.12	0.708	0.804	0.734	0.047	0.048	0.437	0.047	0.044
	2048	**1024**	**512**	**256**	**126**	**64**	**32**			**GC**		**Blank**

Figure A2. An example result plate for MIC testing, where the highest concentration is in column 1, proceeding in twofold dilutions to the lowest concentration in column 7. The concentrations of each well are listed on the bottom (2048 – 32 μg/mL). In column 10 is the growth control, and in column 12 is the blank. Each strain is tested in duplicate, therefore this plate contains four strains, in rows A-B, C-D, E-F, and G-H.

Controls and Checks

One column of the plate will be a growth control, where 50 μL sterile broth and 50 μL bacterial inoculum are combined. No antibiotic should be pipetted into this column. Growth within the growth control wells will confirm that the conditions for bacterial growth were correct.

One column of the plate will be a blank, with 100 μL sterile broth added, but no antibiotic or bacterial inoculum. Any growth in this column will indicate contamination, either of the broth used in the assay, or via pipet error.

The final concentration of the broth culture should be checked via dilutions in PBS to an estimated 1000, 100, 10, and 1 CFU/mL. These dilutions will then be filtered onto 0.45 nm filters and placed on BHI plates, which are then grown overnight on BHI plates in a 41℃ incubator. Count the plates after 24 hours and back-calculate to determine if the correct concentration was used.