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Genomic characterisation of emerging *Enterococcus faecium vanA* types from 2013 to 2020 in an Australian public hospital

Ronan F. O'Toole[a,b,*], Vitalie Covalciuc[a], Ana Cooke[a], Gaetan Thilliez[a,b], Emma K. Finlay[a,b], Kelvin W.C. Leong[c], Vanessa Farthing[c], Sebastiaan J. van Hal[d]

[a] *School of Biotechnology, Faculty of Science and Health, Dublin City University, Dublin, Ireland*
[b] *Life Sciences Institute, Dublin City University, Dublin, Ireland*
[c] *Department of Rural Clinical Sciences, La Trobe Rural Health School, La Trobe University, Melbourne, Victoria, Australia*
[d] *Department of Infectious Disease and Microbiology, Royal Prince Alfred Hospital, Sydney, New South Wales, Australia*

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ABSTRACT

Objective: High rates of resistance to vancomycin are now being reported among invasive isolates of *Enterococcus faecium*, a major cause of healthcare-associated infections globally. The objective of this study was to generate a better understanding of emerging *vanA* sequence types (ST) of the pathogen.
Methods: A temporal analysis of isolates collected from 2013 to 2020 at the Royal Prince Alfred Hospital, a large Australian hospital, was performed using genome sequencing. Relative frequencies of multi-locus ST, antibiotic resistance markers, and virulence genes were determined.
Results: ST1421 was the dominant *vanA* ST from 2014 to 2018. ST1424, which was not evident in the 2013 and 2014 isolates, emerged in 2016 and became the dominant *vanA* type in 2020 (65% of isolates). *vanA* ST80 was less common among the Royal Prince Alfred Hospital *vanA* isolates. Direct comparison of 120 genomes of each ST revealed significantly higher encoded resistance to aminocyclitols (e.g. spectinomycin) and folate-pathway antagonists (e.g. trimethoprim) in ST1421 and ST1424 compared to ST80. Furthermore, significantly higher carriage of enterococcal virulence genes *ecbA* (*E. faecium* collagen binding protein A) and *hylEfm* (glycosyl hydrolase) was found in ST1421 and ST1424 than in ST80.
Conclusions: Newer *vanA* ST ST1421 and ST1424 harboured several antibiotic resistance loci and virulence genes at significantly higher levels than those observed in ST80. Ongoing genomic surveillance is warranted for the detection of new variants of *E. faecium* and characterisation of their encoded resistance and virulence.

1. Introduction

In its recent point prevalence survey of healthcare-associated infections (HAIs), the European Centre for Disease Prevention and Control (ECDC) estimated that there were 4.8 million (95% CI: 3.1–5.8 million) episodes of HAIs per year from 2022 to 2023 in acute care hospitals in the European Union/European Economic Area (EU/EEA) [1]. HAIs have previously been estimated to annually cause approximately 91,130 deaths (95% UI: 76,117–107,883) in the EU/EEA and an estimated 98,987 deaths in the USA, and cost €7 billion and €6.5 billion in direct healthcare costs per year, respectively [2–4]. In Australia, an estimated 170,574 HAIs occur in adults admitted to public hospitals each year leading to 7583 deaths [5]. In the EU/EEA, the most-frequently reported HAI microorganisms include *Escherichia coli* (12.7%), *Klebsiella* spp. (11.7%), and *Enterococcus* spp. (10.0%) [1]. In the USA, *E. faecalis* and *E. faecium* are responsible for up to 7.4% and 3.7% of all HAIs, respectively [6]. Enterococci can cause a range of illnesses in hospitalised patients including urinary tract infection, hepatobiliary sepsis, endocarditis, surgical wound infection, bacteraemia, and neonatal sepsis [7,8].

Vancomycin is a key antibiotic for the treatment of enterococcal infections, however, the emergence of vancomycin-resistant *E. faecium* (VREfm) in Europe and the USA in the 1980s [9,10], and in Australia in 1994 [11], has contributed to poorer outcomes in patients. A number of European countries are now reporting high rates of resistance to vancomycin among invasive *E. faecium* isolates including Lithuania (67.7% in 2022), Cyprus (56.0%), Greece (49.1%), Slovakia (41.8%), Malta (37.5%), Romania (37.0%), Croatia

* Corresponding author. Mailing address: Ronan F. O'Toole, School of Biotechnology, Faculty of Science and Health, Dublin City University, Dublin D09 K20V, Ireland.
E-mail address: ronan.otoole@dcu.ie (R.F. O'Toole).

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(36.6%), Hungary (35.8%), Italy (30.7%), and Ireland (28.4%) [12]. In Australia, 46.9% of *E. faecium* isolates from enterococcal bacteraemia were resistant to vancomycin in 2022 [13]. In 2019, the Organisation for Economic Co-operation and Development (OECD) and the ECDC noted that: 'Though starting from lower 2015 levels, vancomycin resistance in *E. faecalis* and *E. faecium* is projected to increase in a majority of countries' [14]. Determination of the specific factors that are behind the recent rise in VRE cases is needed for achieving a sustainable reversal in the burden of this organism, classified as a high-priority antibiotic-resistant bacterium by the WHO [15].

Vancomycin resistance in *E. faecium* can be conferred by one of several gene clusters, i.e. *vanA, vanB, vanC, vanD, vanE, vanG, vanL, vanM*, or *vanN* [16]. There has been an expansion of isolates harbouring the *vanA* resistance locus over the past decade or more in Australia. In 2011, vancomycin resistance was mediated by the *vanB* locus in 97.2% of VREfm isolates vs. the *vanA* locus in 1.9% of VREfm isolates [17]. However, by 2019, vancomycin resistance conferred by the *vanA* locus accounted for 49.1% of VREfm isolates [18]. New sequence types (ST) of *vanA* VREfm have also emerged during this time including ST1421 [19]. A number of virulence genes have been identified in pathogenic enterococcal species. These include cell surface adhesin genes *acm* (adhesin of collagen from *E. faecium*), *ecbA* (Efm collagen-binding protein A), and *esp* (enterococcal surface protein), secreted proteins encoded by *hylEfm* (glycosyl hydrolase with β-*N*-acetylglucosaminidase) and *sagA* (peptidoglycan hydrolase), as well as biofilm regulators *ebrB* (enterococcal biofilm regulator B) and *bepA* (biofilm and endocarditis-associated permease A) [20]. In this study, we examined *vanA* VREfm isolates collected over an 8-y period from 2013 to 2020 at a major Australian hospital to better understand bacterial antibiotic resistance and virulence loci that may potentially be involved in the increased prevalence of *vanA E. faecium* among clinical enterococcal isolates.

2. Materials and methods

2.1. VREfm isolate collection

Samples were collected from patients potentially colonised or infected with VREfm at the Royal Prince Alfred Hospital (RPAH) via rectal, faecal, urine, abdominal, or blood specimens and sub-cultured on nutrient agar slopes and sub-cultured on blood heart infusion agar plates. The isolates were tested for the presence of the putative *vanA/B* genotypes by real-time PCR using the LightCycler VRE detection kit (Roche, Germany) and a LightCycler 2.0 instrument (Roche) according to the manufacturer's instructions. Genome sequence analysis was performed on VREfm isolates collected in 2013 + 2014 and every 2 y after that up until 2020.

2.2. Genomic DNA extraction and purification

Cells were harvested by centrifugation at 10,000 × *g* for 8 mins. Cell pellets were resuspended in a mixture of 15 µL lysozyme (VWR, Australia, Cat.# 0663–10 G) and 300 µL phosphate-buffered saline and incubated at 37 °C for 1 h. Following the DNeasy Blood and Tissue Kit protocol (Qiagen, Hilden, Germany), genomic DNA was extracted from 200 µL of lysate. The eluted DNA was then treated with 2 µL of RNase (100 mg/mL) (Qiagen, Hilden, Germany) and incubated at room temperature for 1 h. An amount of 100 µL of the eluted DNA solution was purified with the High Pure PCR Template Preparation Kit (Roche, Basel, Switzerland) according to the manufacturer's protocol to obtain purified DNA in a volume of 50 µL. DNA from each sample was quantified using the Qubit dsDNA (double-stranded DNA) HS (high sensitivity) Quantification Kit and a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). The DNA was then normalised to a concentration of 0.2 ng/µL with purified water.

2.3. DNA library preparation and whole-genome sequencing

DNA libraries were prepared according to the manufacturer's protocol using the Illumina Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, CA, USA). 2.5 µL of input DNA (0.2 ng/µL) were tagmented using the Applied Biosystems Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific, Foster City, CA, USA). The tagmented DNA was then neutralised with 2.5 µL of neutralisation tagmentation buffer (NT buffer). PCR amplification was used to generate a dual-indexed library for each sample by adding Illumina sequencing adaptors and index primers to the tagmented DNA. Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA) were used to remove impurities, such as free index primers, short DNA fragments, and enzymes, from the DNA amplicon library. The cleaned DNA libraries were quantified using Qubit dsDNA HS Quantification kit and a Qubit 2.0 Fluorometer (Life Technologies). The DNA libraries were normalised to 10 µL to form a pooled amplified library at a concentration of 1:1000 dilution of the library as previously described [21]. The concentration was determined by qPCR (quantitative PCR) using the Corbett Rotor-Gene 6000 real-time thermocycler (Qiagen, Hilden, Germany). PCR assays were prepared with the KAPA Library Quantification Kit (KAPA Biosystems Inc., Wilmington, MA, USA) following the manufacturer's recommended protocol. Sequencing of the cleaned pool was carried out on a MiSeq sequencing instrument (Illumina, San Diego, CA, USA) with the MiSeq v2 Reagent Kit (Illumina) to generate 2 × 150-bp paired-end reads.

2.4. Genome assembly

Sequence reads from whole-genome sequencing were mapped to the annotated and publicly-available complete reference genome sequence of the *E. faecium* ST18 DO (TX16, NC_017960.1) strain that was isolated in Texas, USA in 1998. Raw .fastq sequence reads for each VREfm isolate were re-trimmed and mapped with high sensitivity at a maximum of five iterations to NC_017960.1 using the bowtie2 plug-in Geneious Prime (version 2019.2.3, https://www.geneious.com/). The assembled contiguous consensus sequences were exported in .fasta file format and were used for species identification using the NCBI Nucleotide Blast database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) or the Ribosomal Multilocus Sequence Typing via the PubMLST database (https://pubmlst.org/species-id).

2.5. In silico multi-locus sequence typing

The raw sequence reads in .fastq file format were uploaded to the Centre for Genomic Epidemiology (CGE) database (https://www.genomicepidemiology.org/) to generate an ST for each isolate. The MLST tool uses MLST allele sequence and profile data obtained from PubMLST database (https://pubmlst.org/) to generate an ST for each isolate. In cases where the CGE returned an unknown ST, the .fasta file of the assembled contiguous consensus sequence for such isolate was uploaded to the PubMLST database to obtain an *E. faecium* ST output. For *pstS*-negative *E. faecium* isolates, each of the 7 alleles were manually entered into the PubMLST database to generate an ST.

2.6. Antibiotic resistance gene detection

The raw sequence reads in .fastq file format were uploaded to the CGE database for analysis using the ResFinder tool (version 4.4.3) to detect the presence of genes and/or chromosomal mutations associated with antimicrobial resistance.

All available antimicrobial profiles were included, e.g. Aminoglycosides, Beta-lactams, Colistin, Disinfectants, Fluoroquinolones, Fosfomycin, Fusidic Acid, Glycopeptides, MLS (Macrolides, Lincosamides, and Streptogramins), Nitroimidazoles, Oxazolidinone, Phenicols, Pseudomonic Acid, Rifampicin, Sulphonamides, Tetracyclines, and Trimethoprim, with the default settings of 90% for %ID and 60% for minimum length used. The analysis included the detection of the *vanHAX* (VanHAX_M97297) resistance gene locus present in *vanA* VREfm.

2.7. Virulence gene detection

For detecting the presence of known enterococcal virulence genes from the raw .fastq sequence reads for each VREfm isolate, both the 'Enterococcus' database and the 'Enterococcus faecium & Enterococcus lactis' database of the VirulenceFinder 2.0 tool (version 2.0.5) at the CGE were searched [22]. The default thresholds used were 90% for %ID and at 60% for minimum length.

2.8. SNP-based phylogenetic analyses

For single nucleotide polymorphism (SNP)-based analyses, .fastq sequence files were first processed to identify low-quality base calls and adapter sequences with the fastp programme (https://github.com/OpenGene/fastp) [23,24]. MultiQC (https://github.com/MultiQC/MultiQC) was used on the .html outputs from fastp to provide a single summary report showing results across all isolate genomes and to remove adapter sequences and reads of low quality [25]. Snippy (https://github.com/tseemann/snippy) was then used on the filtered .fastq reads to identify SNPs and indels in each of the isolate genomes with respect to reference genome *E. faecium* ST18 DO (TX16, NC_017960.1). The Snippy-core function was applied to merge the .vcf outputs for each of the isolate genomes into a core .aln file which was processed with Gubbins (https://github.com/nickjcroucher/gubbins) to distinguish between sequence variations located inside vs. outside of regions of recombination [26]. The .fasta file generated for filtered polymorphic sites was inputted into RAxML-NG (https://github.com/amkozlov/raxml-ng) to produce a maximum likelihood phylogenetic tree using the Generalised Time Reversible substitution model [27]. The RAxML-NG-generated phylogenetic tree was manually rooted in RStudio with the reference genome as the outgroup. FigTree was used to visualise the phylogenetic tree (http://tree.bio.ed.ac.uk/software/figtree/).

2.9. Statistical analyses

To compare the presence or absence of antibiotic resistance loci and virulence genes between isolates of *E. faecium*, pairwise proportion tests were performed in R with the RStudio package (https://posit.co/products/open-source/rstudio/) using the libraries dplyr, tidyverse, and rstatix. RStudio was also used to generate heatmap graphics for the RPAH isolates using the libraries tidyverse, ggplot2, and ggh4x. To combine the RAxML-NG generated phylogenetic tree with the heatmap of antibiotic resistance loci and virulence genes for the 120 each genomes of ST80, ST1421, and ST1424, the libraries ggtree, phytools, ape, ggplot2, dplyr and pheatmap were used. The Inkscape 1.2.2 graphics tool (https://inkscape.org/release/inkscape-1.2.2/) was used for formatting of heatmap outputs from RStudio.

3. Results

3.1. E. faecium *vanA isolates*

The *vanA* locus was detected in 552 patient isolates collected at the RPAH in the years 2013 (n = 8), 2014 (n = 77), 2016 (n = 187), 2018 (n = 195), and 2020 (n = 85). Of these, 530 were confirmed as *E. faecium* using NCBI Nucleotide BLAST analysis of the 16S rRNA gene or Ribosomal Multilocus Sequence Typing via the PubMLST database. All of the 530 *E. faecium* isolates were confirmed as harbouring the *vanHAX* locus associated with *vanA*-based vancomycin resistance using the ResFinder tool at the CGE database. The 530 *vanA E. faecium* isolates were from 2013 (n = 8), 2014 (n = 75), 2016 (n = 171), 2018 (n = 191), and 2020 (n = 85).

3.2. MLST profiles of the RPAH vanA isolates

In silico MLST typing established that 525 of the 530 *vanA* isolates belonged to, in order of abundance, ST ST1421 (n = 283), ST1424 (n = 178), ST80 (n = 29), ST17 (n = 5), ST341 (n = 5), ST78 (n = 4), ST796 (n = 4), ST1422 (n = 4), ST1478 (n = 3), ST117 (n = 2), ST203 (n = 2), ST262 (n = 2), ST555 (n = 2), ST18 (n = 1) and ST789 (n = 1). For the remaining 5 *E. faecium vanA* isolates, they did not match an existing MLST in the database. The breakdown of the *vanA E. faecium* isolates by year and ST is shown in Supplementary Table S1. Of the isolates from 2013, 37.5% belonged to ST1421 (Fig. 1). ST1421 also dominated in 2014 and 2016 making up 73.3% and 63.2% of isolates, respectively (Fig. 1). A newly detected ST, ST1424, accounted for 22.8% of isolates in 2016. In 2018, the predominant STs were ST1421 and ST1424 which accounted for 51.3% and 44.0%, respectively, of *vanA* isolates. ST1424 was the dominant ST in 2020 comprising 64.7% of *vanA* isolates compared to 22.4% for ST1421 (Fig. 1).

3.3. Detection of encoded antibiotic resistance among the RPAH E. faecium *vanA isolates*

The genome sequences of each of the *E. faecium vanA* isolates collected at the RPAH from 2013 to 2020 were analysed using the ResFinder and MLST tools from the CGE and PubMLST databases. As shown in Fig. 2a, most of the isolates exhibited predicted sensitivity to the streptogramin A, oxazolidinone, amphenicol, ionophore, and pleuromutilin antibiotic classes irrespective of MLST ST. Potentially higher levels of predicted sensitivity to both the aminocyclitol and folate pathway antagonist antibiotic classes were apparent for ST80 compared to the ST1421 and ST1424 isolates (Fig. 2a).

3.4. Detection of virulence genes among the RPAH E. faecium *vanA isolates*

The genome sequences of each of the *E. faecium vanA* isolates collected at the RPAH from 2013 to 2020 were analysed using the VirulenceFinder and MLST tools from the CGE and PubMLST databases. As shown in Fig. 2b, most of the isolates carry the *acm* gene irrespective of MLST ST. Potentially lower frequencies of the *hylEfm* and *espfm* genes were observed for ST80 with respect to ST1421 and ST1424 isolates (Fig. 2b).

3.5. Analysis of larger set of ST80 genomes with respect to ST1421 and ST1424

The investigation of the RPAH *E. faecium vanA* isolates highlighted possible differences between ST80 vs. ST1421 and ST1424 with respect to antibiotic resistance gene and virulence gene carriage. As the RPAH collection contained only 29 isolates that belonged to ST80, an assessment was performed of a larger set of ST80 isolates (n = 120) to enable statistical analyses to be performed on comparisons with isolates of ST1421 (n = 120) and ST1424 (n = 120) with additional *vanA E. faecium* genome se-

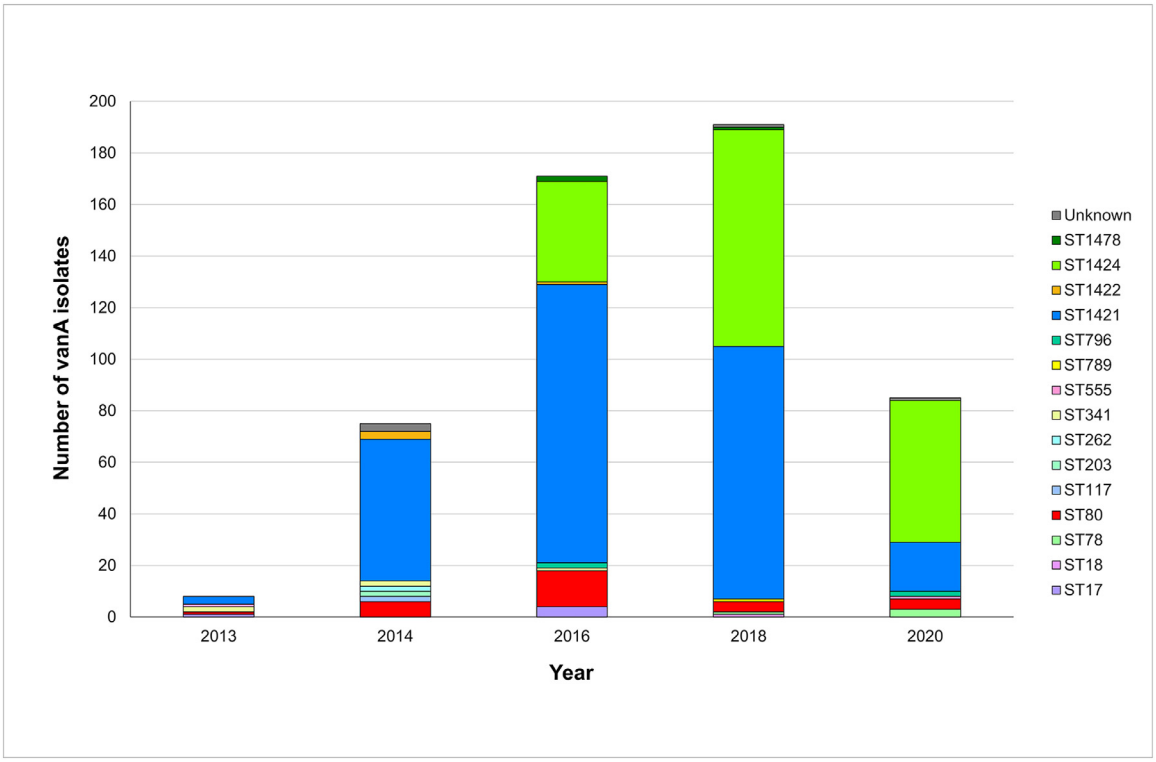

Fig. 1. Multi-locus sequences types of *Enterococcus faecium vanA* isolates by year. MLST was determined for *vanA E. faecium* isolates (n = 525) collected from 2013 to 2020 at the Royal Prince Alfred Hospital, NSW, Australia using the Multi-Locus Sequence Typing (MLST) tool from the Centre for Genomic Epidemiology (CGE) and PubMLST databases. Of these, 520 isolates belonged to 15 distinct MLSTs. Unknown refers to the remaining 5 isolates for which an MLST could not be generated.

quences obtained from the European Nucleotide Archive (https://www.ebi.ac.uk/ena/browser/home). The additional genomes included in the analysis were selected randomly from studies conducted in other geographical locations. The ST1424 genomes were from studies in Scotland (PRJNA877253, n = 40) and Australia (PRJNA1126832 (this study) and PRJNA856406, n = 80). The ST80 genomes were from studies in Ireland (PRJNA734127, n = 40), Scotland (PRJNA877253, n = 40), and Australia (PRJNA1126832 (this study) and PRJNA856406, n = 40). The available ST1421 genome sequences were all from Australia (PRJNA1126832 (this study), n = 120). A list of the *vanA E. faecium* genomes analysed is available in Supplementary Table S2. A heatmap illustrating the presence or absence of an antibiotic resistance locus or virulence gene in ResFinder and VirulenceFinder, respectively, for each *E. faecium vanA* genome was generated (Fig. 3). This indicated differences between ST80 and ST ST1421 and ST1424 with respect to presence or absence of antibiotic resistance loci ant(9)-la and *dfrG* which encode resistance to aminocyclitols and folate pathway antagonists, respectively. This was in agreement with the earlier findings with the 29 ST80 isolates from the RPAH.

Resistance to aminocyclitols was detected in just 1% of ST80 isolates as opposed to 98% of ST1421 and 99% of ST1424 isolates (P < 0.0001) (Fig. 4a). Resistance to folate pathway antagonists was predicted in 18% of ST80 isolates compared to 99% of ST1421 and 100% of ST1424 isolates (P < 0.0001) (Fig. 4a). In contrast, pairwise proportion tests found no significant differences between ST80 and ST ST1421 and ST1424 in terms of resistance to aminoglycosides, beta-lactams, glycopeptides, lincosamides, macrolides, quinolones, streptogramin B and tetracyclines.

In terms of virulence genes, differences were found between ST80 and ST ST1421, and ST1424 with respect to presence of the *hylEfm* virulence gene in accordance with the results obtained with the 29 RPAH ST80 isolates. The *hylEfm* gene was found in 9% of ST80 isolates as opposed to 98% of ST1421 and 98% of ST1424 isolates (P < 0.0001) (Fig. 4b). In addition, the examination of the larger genome set revealed that the *ecbA* virulence gene was present in only 1% of ST80 isolates compared to 88% of ST1421 and 99% of ST1424 isolates (P < 0.0001) (Fig. 4b).

4. Discussion

Among isolates of *E. faecium* in Australia, there has been an increase in vancomycin resistance encoded by the *vanA* locus over the past decade [17,18]. This has included the appearance of new *vanA* ST of VREfm at a number of Australian hospitals [28,29]. To investigate this, samples of *vanA* VREfm from the RPAH between 2013 and 2020 were collected and genome sequenced. The genomes of the isolates were analysed with regard to MLST distribution, antibiotic resistance patterns, and presence of genes encoding known enterococcal virulence factors.

MLST typing established that the 525 *vanA* isolates belonged to 15 different ST. As the numbers of *vanA* isolates rose across the study period, it was apparent that this coincided with an increasing number of isolates belonging to recently-designated ST ST1421 and ST1424 rather than older ST such as ST80. *vanA*-containing ST80, first described in Australia in 2011, became a dominant ST associated with the *vanA* locus among invasive infections until the identification and expansion of *vanA* ST1421 and ST1424 in Australia from 2016 onwards [13,17,18,30–33]. While not detected at the RPAH until 2016, ST1424 became the dominant ST at 64.7% of *vanA* isolates by 2020 (Fig. 1). This raised the question as to whether any of the newer ST had genetic factors that may assist in their persistence in the healthcare environment. We therefore performed an analysis of the genomes of ST80, ST1421, and ST1424 to look for any differences in encoded antibiotic resistance or virulence.

An initial examination of VREfm isolates from the RPAH revealed potential differences between the ST with regard to the

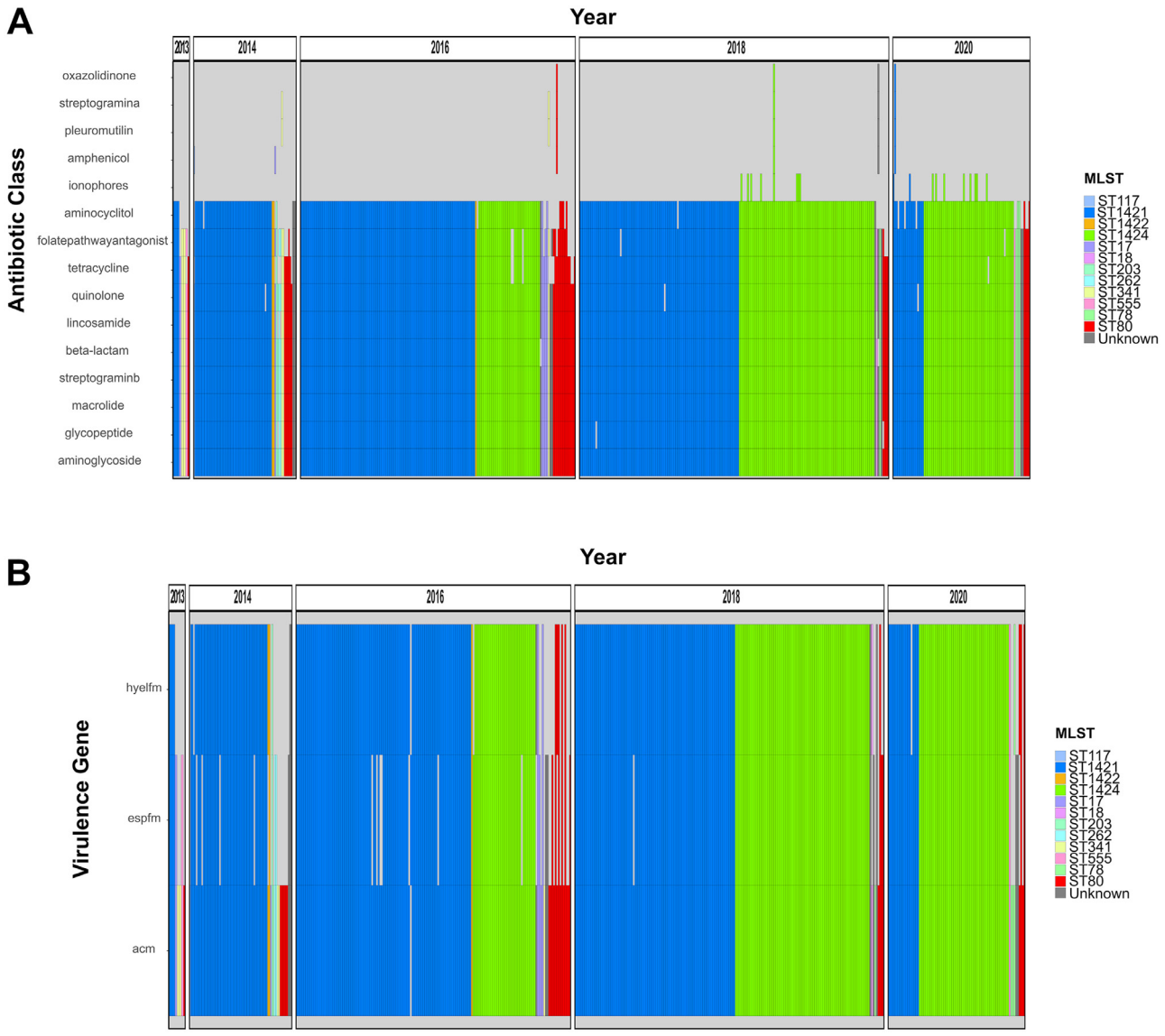

Fig. 2. Profile of antibiotic resistance and virulence loci across the RPAH *Enterococcus faecium vanA* isolates with respect to sequence type and year. (a) The genome sequences of the *E. faecium vanA* isolates collected at the RPAH from 2013 to 2020 were analysed using the ResFinder and MLST tools from the Centre for Genomic Epidemiology (CGE) and PubMLST databases. Profile was plotted using RStudio with encoded resistance expressed as resistance to respective antibiotic class. Predicted sensitivity of an isolate to a particular antibiotic class is illustrated in light grey. Encoded resistance of an isolate to a particular antibiotic class is illustrated with designated colouring for respective MLST sequence type. Sensitivity to the streptogramin A, oxazolidinone, amphenicol, ionophore, and pleuromutilin classes is apparent for most isolates irrespective of sequence type. Potentially higher levels of predicted resistance to aminocyclitol and folate pathway antagonists are indicated for ST1421 and ST1424 with respect to ST80 isolates. (b) The genome sequences of the *E. faecium vanA* isolates collected at the RPAH from 2013 to 2020 were analysed using the 'Enterococcus' database of the VirulenceFinder and MLST tools from the Centre for Genomic Epidemiology (CGE) and PubMLST databases. Profile was plotted using RStudio with detected virulence genes shown. Predicted absence of a virulence gene in an isolate is illustrated in light grey. Predicted presence of a particular virulence gene in an isolate is illustrated with designated colouring for respective MLST sequence type. Presence of the *acm* gene is apparent for most isolates irrespective of sequence type. A potentially lower frequency of the *hylEfm* gene is indicated for ST80 with respect to ST1421 and ST1424 isolates.

presence of loci encoding resistance to aminocyclitols and folate pathway antagonists (Fig. 2a). Furthermore, differences were also detected with regard to the presence of a number of virulence genes (Fig. 2b). Therefore, we examined this in more detail using a larger set of genomes from ST80 ($n = 120$) and performed statistical analysis of comparisons with ST1421 ($n = 120$) and ST1424 ($n = 120$) genomes.

This analysis found that the antibiotic resistance locus ant(9)-la encoding resistance to aminocyclitols (e.g. spectinomycin) was present in only 1% of ST80 isolates compared to 98% of ST1421 and 99% of ST1424 isolates ($P < 0.0001$) (Fig. 4a). The *dfrG* gene encoding resistance to folate pathway antagonists (e.g. trimethoprim) was detected in 18% of ST80 isolates as opposed to 99% of ST1421 and 100% of ST1424 isolates ($P < 0.0001$). Spectinomycin is used for the chemotherapy of *Neisseria gonorrhoeae* infections in cases where there is hypersensitivity or resistance to penicillins [34]. Trimethoprim, in combination with sulfamethoxazole as co-trimoxazole, is used for the treatment of urinary tract infections, prostatic infections, and otitis media in children, as well as the elimination of *Shigella* infections and *Pneumocystis carinii* pneumonia [35]. Although not commonly used in the treatment of enterococcal infections, having resistance to spectinomycin and trimethoprim may give strains of *E. faecium* a survival advantage in healthcare settings by enabling them to persist in the presence of antibiotics used in the therapy of other types of infections. Furthermore, on top of resistance to spectinomycin and trimetho-

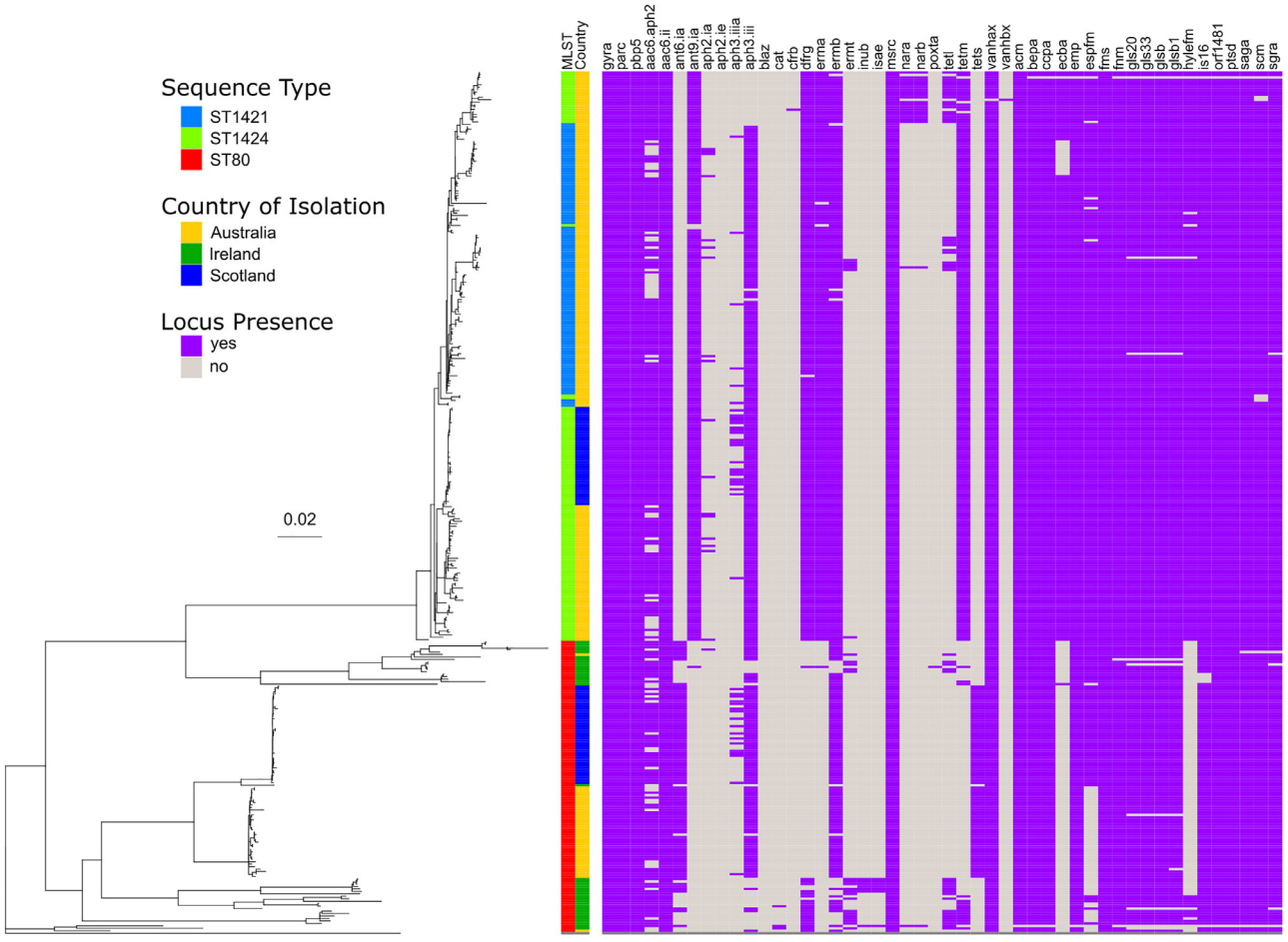

Fig. 3. Maximum likelihood phylogenetic tree of *E. faecium vanA* isolates from the ST80, ST1421, and ST1424. The .fasta file generated from Gubbins for filtered polymorphic sites was inputted into RAxML-NG to produce a maximum likelihood phylogenetic tree using the Generalised Time Reversible (GTR) substitution model. The phylogenetic tree generated was manually rooted against the reference genome (NC_017960.1) as the outgroup in RStudio. The countries in which the ST80, ST1421, and ST1424 isolates were collected are indicated. Adjoining heatmap illustrates the presence or absence of antibiotic resistance loci and virulence genes in each of the *E. faecium vanA* genomes detected using the ResFinder tool and both the 'Enterococcus' and 'Enterococcus faecium & Enterococcus lactis' databases of the VirulenceFinder 2.0 tool (version 2.0.5). Differences are apparent between ST80 and sequence types ST1421 and ST1424 with respect to antibiotic resistance loci (ant(9)-Ia and *dfrG*) and virulence genes (*ecbA* and *hlyefm*) in particular ($P < 0.0001$).

prim, additional resistance to aminoglycosides, beta-lactams, glycopeptides, lincosamides, macrolides, quinolones, streptogramin B and tetracyclines were encoded by the VREfm isolates (Fig. 4a). This adds to the multi-drug resistant nature of VREfm infections for which treatment options may include high dose ampicillin or ampicillin-sulbactam, doxycycline, novobiocin, bacitracin, nitrofurantoin, quinupristin/dalfopristin or linezolid depending on the drug-susceptibility profile of the isolate [36]. For example, a high proportion of the ST80, ST1421, and ST1424 isolates contained at least one of the genes, *tet*(L), *tet*(M), or *tet*(S), for resistance to tetracyclines (e.g. doxycycline). This would disrupt the usefulness of doxycycline in the treatment of VREfm caused by strains of these ST.

In addition to antibiotic resistance, differences between ST were detected with regard to the presence of virulence genes. Only 9% of ST80 isolates carried the *hylEfm* gene as opposed to 98% of ST1421 isolates, and 98% of ST1424 isolates ($P < 0.0001$) (Fig. 4b). Originally believed to be a hyaluronidase, *hylEfm* has been characterised as a glycosyl hydrolase with β-*N*-acetylglucosaminidase activity [37,38]. Acquisition of a plasmid carrying the *hylEfm* gene has been shown to increase the colonisation of the mouse gastrointestinal tract and decrease the survival of mice in a peritonitis model by *E. faecium* [39,40]. Interestingly, the *hylEfm* gene has been found genetically linked to the *vanA* gene cluster on large plasmids that can readily transfer between *E. faecium* strains in mating experiments [39]. This suggests a direct link between the acquisition of certain virulence determinants and of antibiotic resistance markers in *E. faecium* [39].

The largest difference in virulence gene carriage between the ST was seen for *ecbA* which encodes *E. faecium* collagen binding protein A, an adhesin that belongs to the microbial surface components recognizing adhesive matrix molecule (MSCRAMM) family [41]. The *ecbA* gene was detected in 88% of ST1421 isolates and 99% of ST1424 isolates but in only 1% of ST80 isolates ($P < 0.0001$) (Fig. 4b). The EcbA protein has been shown to bind to collagen type V and the gamma-chain of fibrinogen in a concentration-dependent manner [41]. As fibrinogen is a major coagulation factor in blood clots for wound healing and is deposited on implanted foreign devices, it has been postulated that EcbA may play a role in the pathogenesis of intravascular catheter-related infections [41].

Using the 'Enterococcus' database of the VirulenceFinder tool, the enterococcal surface protein gene of *E. faecium, espfm*, was detected in 53% of ST80 isolates whereas it was observed in 97% of ST1421 and 98% of ST1424 *vanA* isolates ($P < 0.0001$). Esp is believed to promote enterococcal colonisation and evasion of the immune system [20]. Furthermore, disruption of the *esp* gene reduces the ability of enterococci to produce biofilms [20]. However,

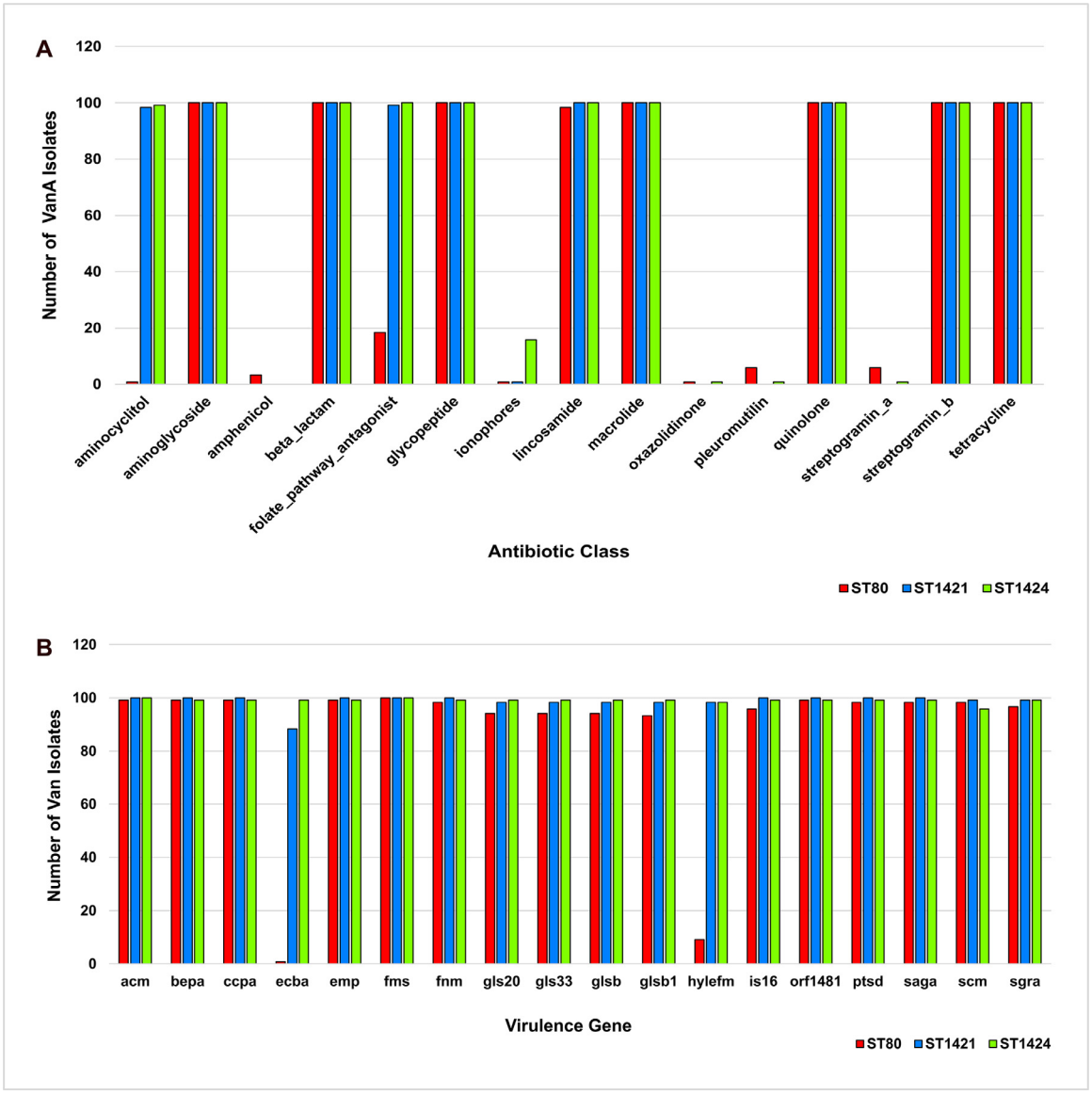

Fig. 4. Encoded antibiotic resistance by class and virulence across *Enterococcus faecium vanA* sequence types ST80, ST1421, and ST1424. A set of genomes was analysed for each *E. faecium vanA* sequence type, ST80 (n = 120), ST1421 (n = 120), and ST1424 (n = 120). (a) Presence or absence of resistance genes from analysis with ResFinder. Resistance to aminocyclitols was detected in 1% of ST80, 98% of ST1421, and 99% of ST1424 isolates ($P < 0.0001$). Resistance to folate pathway antagonists was detected in 18% of ST80, 99% of ST1421, and 100% of ST1424 isolates ($P < 0.0001$). Differences between ST80, ST1421, and ST1424 regarding resistance to aminoglycosides, beta-lactams, glycopeptides, lincosamides, macrolides, quinolones, streptogramin B, and tetracyclines were not observed or were of low significance. (b) Presence or absence of virulence genes from analysis with VirulenceFinder. The *hylEfm* gene was found in 9% ST80, 98% of ST1421, and 98% of ST1424 isolates ($P < 0.0001$). The *ecbA* virulence gene was present in 1% of ST80, 88% of ST1421, and 99% of ST1424 isolates ($P < 0.0001$). Pairwise proportion tests found no significant differences between ST80, ST1421, and ST1424 regarding the other virulence genes shown.

it should be noted that the *espfm* gene has been specifically excluded from the newer '*Enterococcus faecium* & *Enterococcus lactis*' database of VirulenceFinder by Roer and colleagues due to the 'the considerable diversity in both size and structure of this gene' with 'lengths ranging from 112 to 1975 amino acids' [22]. This poses challenges to the selection of representative variants for the *espfm* gene and leaves open the possibility that the *espfm* gene may not be detected in some *E. faecium* genome sequences where their variant of the gene is not represented in the VirulenceFinder database. Therefore, it is difficult to conclude at this stage that carriage of *espfm* is lower in ST80 compared to the ST1421 and ST1424 ST.

It is apparent from the genomic analyses that a higher proportion of the ST1421 and ST1424 isolates analysed harbour the *hylEfm* and *ecbA* virulence genes compared to ST80. Although confirmatory *in vivo* studies would be required, this observed difference in virulence gene carriage may relate to the newer genotypes having a colonisation or infectivity advantage over previous ST of *vanA E. faecium* (Fig. 5). This is of concern given previous reports of the spread of novel ST of VREfm, such as ST1421, across international borders and their causation of outbreaks of enterococcal disease in distant jurisdictions [16].

It should be noted that given the relatively recent emergence of ST1421 and ST1424 in Australia, fewer isolates of these ST have been collected and sequenced in other countries to date. All ST1421 isolates analysed here were collected in Australia whereas genome sequences of ST80 were available from Ireland and Scotland in addition to Australia. This could have contributed to the higher phylogenetic diversity seen for ST80 (Fig. 3). It was possible to analyse genomes of ST1424 collected in Scotland as well as

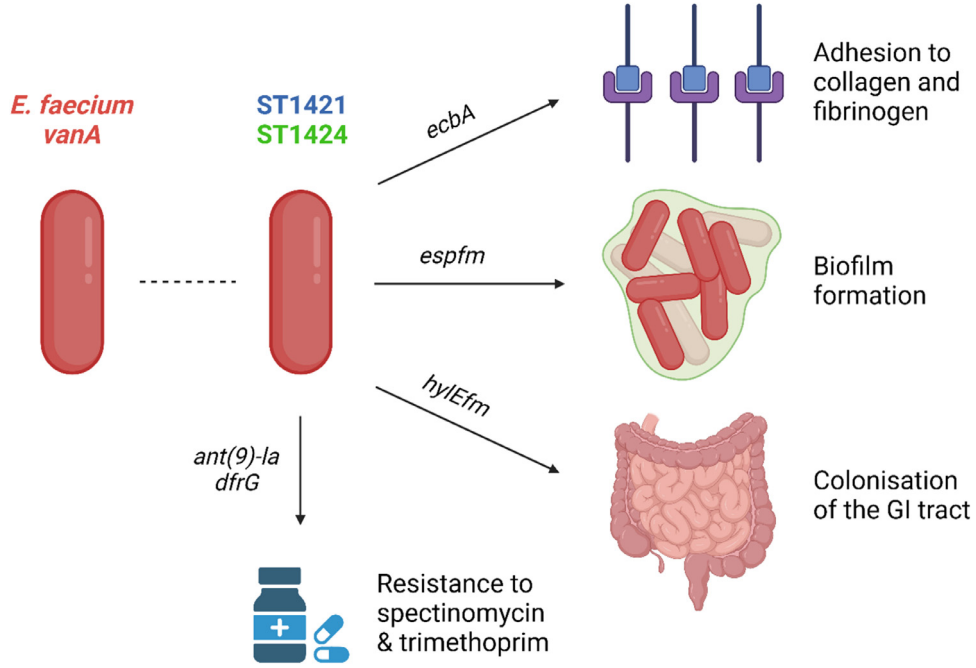

Fig. 5. Model of possible functions provided to *Enterococcus faecium vanA* ST1421 and ST1424 by carriage of antibiotic resistance and virulence genes. Sequence types ST1421 and ST1424 exhibit relatively high carriage levels of antibiotic resistance genes ant(9)-la and *dfrG* and virulence factors *ecbA, espfm* and *hylEfm*. This could potentially provide ST1421 and ST1424 with a greater propensity for survival, colonisation, or pathogenicity in a hospital setting. GI, gastrointestinal. Created with BioRender.com.

Australia, but phylogenetic heterogeneity remained low for this ST (Fig. 3). Therefore, recent evolution of ST1421 and ST1424 may also be a factor in the lower phylogenetic diversity observed for these ST compared to ST80.

5. Conclusion

Our analyses have identified significant differences in antibiotic resistance and virulence gene carriage between distinct ST of *vanA E. faecium*. This could have implications for the control of *vanA* VREfm in healthcare settings as some ST presenting may be more difficult to treat or control than others. Given such differences, ongoing genomic surveillance is needed for the detection and characterisation of new variants of *E. faecium*, particularly those that are associated with outbreaks or international spread of multi-drug resistant enterococcal disease.

Ethical approval

Ethics approval was obtained from the La Trobe University Human Research Ethics Committee, Australia (#HEC20194).

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Data availability

Illumina sequence reads generated in this study have been submitted to the Sequence Read Archive (SRA) at the National Institutes of Health (Bioproject Accession# PRJNA1126832) and will be made available upon manuscript publication.

Declaration of competing interests

None declared.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2025.02.006.

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