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Genomic Insights to Control the Emergence of Vancomycin-Resistant Enterococci

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P.D.R.J. and T.P.S. are joint senior authors.

ABSTRACT **Nosocomial outbreaks of vancomycin-resistant *Enterococcus faecium* (VREfm) are thought to occur by transmission of VREfm between patients, predicting that infection control interventions will limit cross-transmission. Despite implementation of such strategies, the incidence of VREfm infections continues to rise. We aimed to use genomics to better understand the epidemiology of *E. faecium* within a large hospital and investigate the reasons for failure of infection control strategies. Whole-genome sequencing was performed on 61 *E. faecium* (36 VREfm) isolates, predominately from blood cultures collected at a single hospital between 1998 and 2009, and on five *vanB*-positive anaerobic commensal bacteria isolated from human feces. Phylogenomic analysis and precise mapping of the *vanB* gene, which contains the Tn*1549* transposon, showed that at least 18 of the 36 VREfm isolates had acquired the transposon via independent insertion events, indicating *de novo* generation of VREfm rather than cross-transmission. Furthermore, Tn*1549* sequences found in 15 of the 36 VREfm isolates were the same as the Tn*1549* sequence from one of the gut anaerobes. National and international comparator *E. faecium* isolates were phylogenetically interspersed with isolates from our hospital, suggesting that our findings might be globally representative. These data demonstrate that VREfm generation within a patient is common, presumably occurring in the human bowel during antibiotic therapy, and help explain our inability to reduce VREfm infections. A recommendation from our findings is that infection control practices should include screening patients for specific hospital clones of vancomycin-susceptible *E. faecium* rather than just VREfm.**

IMPORTANCE ***Enterococcus faecium* is an increasingly important human pathogen causing predominantly antibiotic-resistant infections in hospitalized patients. Large amounts of health care funding are spent trying to control antibiotic-resistant bacteria in hospitals globally, yet in many institutions around the world, vancomycin-resistant *E. faecium* (VREfm) infections continue to rise. The new findings from this study help explain the failures of our current approaches to controlling *vanB* VREfm in health care institutions. Given the importance of this bacterium as a cause of hospital-acquired infections and the difficulties faced by infection control units in trying to prevent colonization in their institutions, the novel findings from this study provide evidence that a new approach to controlling VREfm in hospitals is required. In particular, more attention should be given to understanding the epidemiology of hospital-adapted vancomycin-susceptible *E. faecium*, and patients at higher risk for *de novo* generation of VREfm need to be identified and optimally managed.**

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Controlling increasing antimicrobial resistance in clinically important bacteria is a key challenge for clinicians and scientists, and there is an urgent need to prevent the emergence and subsequent spread of resistant isolates. *Enterococcus faecium* is a primary example of one such troublesome pathogen; while innately resistant to many classes of antibiotics, this bacterium has demonstrated a remarkable capacity to evolve new antimicrobial resistances. In fact, *E. faecium* has been highlighted by the Infectious Diseases Society of America as one of the key problem bacteria, or ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) pathogens, requiring new therapies (1), and there has been a steadily increasing prevalence of *E. faecium*-related nosocomial infections (2).

Millions of dollars are spent each year by health care systems trying to contain antibiotic-resistant bacteria and prevent cross-

transmission. Nosocomial outbreaks of vancomycin-resistant enterococci (VRE) are thought to occur when a patient already carrying VRE in his/her bowel sheds VRE, which are then transmitted by health care workers or via the environment to other patients (3). This model predicts that interventions based on screening and isolation of VRE-colonized patients, improved hand hygiene, and enhanced hospital cleaning will limit cross-transmission (4). Interestingly, interventions at one U.S. hospital based on modified use of antibiotics have also been associated with reducing VRE transmission (5). At the Austin Hospital in Melbourne, Australia (an 800-bed tertiary referral institution), hand hygiene schemes have resulted in a significant reduction in the incidence of methicillin-resistant *S. aureus* (MRSA); however, the incidence of VRE has continued to rise (6). A similar story has unfolded in other regions, with VRE now being responsible for over 30% of *E. faecium* bacteremias in some European countries (7) and for outbreaks in other European countries, Asia, and the United States (8–11). In the United Kingdom, concern about increasing VRE incidence led to mandatory reporting of rates of VRE bacteremia, and more than 500 cases per year have been reported since 2004 (http://www.hpa.org.uk/; accessed, 3 December 2012).

Clinically significant VRE strains were first reported from Europe and the United Kingdom in 1986 and in the United States soon after (12–14). Glycopeptide resistance in enterococci is mediated predominantly by mobile gene clusters that confer resistance to vancomycin and teicoplanin (*vanA* genotype) or vancomycin only (*vanB* genotype) (15). In the United States, *vanA*-containing VRE dominate, in Europe, a mix of *vanA* and *vanB*-containing VRE is found, while in Australia, *vanB*-containing VRE dominate (16–18). Nonenterococcal species (predominately anaerobes) may carry the *vanB* resistance determinant as part of transposon Tn*1549* (19) in the human bowel, and in Australia, while carriage of vancomycin-resistant *E. faecium* (VREfm) in the general community is rare, up to 50% of healthy adults have detectable *vanB* in their fecal samples as determined by PCR (20, 21). This unexpected finding has also been reported in Canada (22). Transmission of a Tn*1549*-like element from one human fecal anaerobe, *Clostridium symbiosum* MLG101, to *Enterococcus faecium* and *Enterococcus faecalis* in the digestive tracts of mice has been demonstrated, suggesting that the same process may occur in the human bowel (23).

Recently, the first complete genome sequences of *E. faecium* have been reported (24, 25), and draft genomes of additional *E. faecium* isolates have been studied to explore antibiotic resistance mechanisms and phylogenomic relationships (26–29). Hospital-associated ampicillin-resistant *E. faecium* isolates, which are represented by three dominant sequence types (ST17, ST18, and ST78; ST203 is an ST78 single-locus variant [SLV]), are genetically distinct and replace the normal reservoir of ampicillin-susceptible *E. faecium* strains in hospitalized patients (24, 25, 30). These three sequence types have been collectively referred to as clonal complex 17 (CC17); however, the growing realization of the significant impact of recombination on the evolution of *E. faecium* suggests that the CC designation may not be accurate (31, 32) because of the significant genetic variation that has been revealed among these hospital-associated isolates (33). Because of the failure of intense infection control interventions to curtail the rise of VRE, we applied genome sequencing and detailed comparative genomic analysis to a collection of *E. faecium* isolates to better understand the relationship between vancomycin-susceptible *E. faecium* (VSEfm) and VREfm isolates and to test the hypothesis that *de novo* generation of VREfm is a frequent occurrence that is undermining infection control efforts.

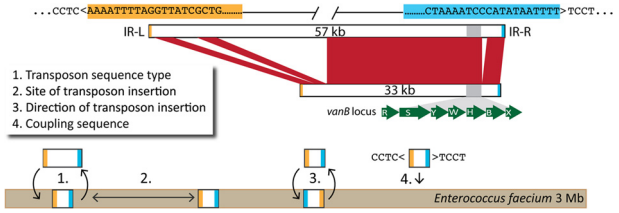


FIG 1 Schematic of the Tn*1549* characteristics of *E. faecium* isolates in this study. Characteristics used to identify unique Tn*1549* transmission events in *vanB*-containing *E. faecium* isolates, including a unique transposon sequence (1), an insertion site (2), the insertion orientation (3), and the flanking coupling sequence (4). Flanking imperfect left and right inverted repeat (IR-L and IR-R) insertion sites are highlighted in orange and blue, respectively. The *vanB* locus contains vancomycin resistance genes (green), which are conserved in all *vanB* sequence types, as well as other genes, which leads to variation in total transposon size, as indicated.

RESULTS

In this study, we sequenced and compared the genomes of 61 *E. faecium* isolates. Fifty-six were bloodstream isolates from unique patients over a 12-year period in a single institute in Melbourne, Australia (32 VREfm and 24 VSEfm isolates), 3 were rectal-screening isolates from Royal Perth Hospital in western Australia (all VREfm), and 2 were clinical isolates from Royal Darwin Hospital, Darwin, Northern Territory, Australia (1 VREfm and 1 VSEfm isolate). Relevant epidemiological information and a summary of sequencing results are presented in Dataset S1 in the supplemental material. The AUS0004 (ST17) (24) and AUS0085 (ST203) (Margaret M. C. Lam, Torsten Seemann, Nicholas J. Tobias, Honglei Chen, Volker Haring, Robert J. Moore, Susan Ballard, M. Lindsay Grayson, Paul D. R. Johnson, Benjamin P. Howden, and Timothy P. Stinear, submitted for publication) genome sequences were used as primary references, and two publicly available international *E. faecium* blood culture isolates were included for comparison: the ST18 DO (TX16) strain isolated from Texas in 1998 (25) and the ST203 strain 1,231,502 from the Broad Institute collection (26). In addition, five anaerobic bacteria previously isolated from human feces and demonstrated to carry Tn*1549* within the *vanB* operon were also subjected to genome sequencing to allow comparison of the *vanB* transposon sequences from these anaerobes and the VREfm isolates. The details and sequencing results for these isolates are summarized in Dataset S2 in the supplemental material.

To address the question of whether patients develop VREfm within their bowel or acquire VREfm from an exterior source, we first conducted a detailed analysis of the characteristics of the Tn*1549* transposon among the *vanB*-positive isolates. In our recent comparison of the ST203 VREfm (AUS0085) and ST17 VRE (AUS0004) whole genomes (Margaret M. C. Lam, Torsten Seemann, Nicholas J. Tobias, Honglei Chen, Volker Haring, Robert J. Moore, Susan Ballard, M. Lindsay Grayson, Paul D. R. Johnson, Benjamin P. Howden, and Timothy P. Stinear, submitted for publication), we identified a number of Tn*1549* features that would permit identification of unique transposon acquisition events, including transposon sequence type, insertion site, insertion direction, and coupling sequence composition (Fig. 1). Analysis of the

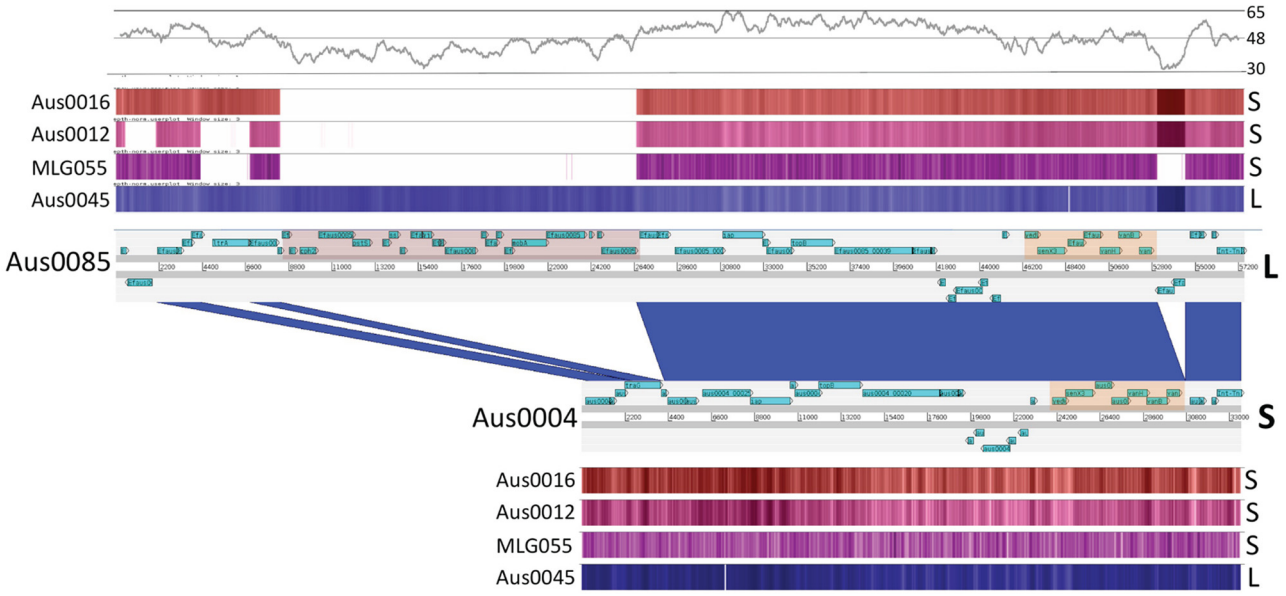


FIG 2 Comparison of Tn*1549* sequences. DNA-DNA comparison of Tn*1549* sequences (33 kb, short version [S]) from AUS0004 and AUS0085 (57 kb, long version [L]), visualized with the Artemis Comparison Tool, showing the location of the *vanB* locus in both elements (orange shading) and the putative novel conjugation locus (pink shading) in the long version of Tn*1549*. Dark-blue shading indicates regions of DNA identity. Percentages of GC content (500-bp sliding window) are shown above, followed by heatmap visualizations of results of read mapping normalized to both AUS0004 and AUS0085 for a representative selection of VREfm and *vanB*-positive bowel anaerobes. Note that changes in read mapping density across Tn*1549* are explained by sequencing biases, as these regions correlate with significant changes in GC content.

36 VREfm isolates and the anaerobic bacteria in this study found that 15 VREfm isolates harbored a larger 57-kb version of Tn*1549* than the other isolates (Fig. 2). The Tn*1549* phylogeny, inferred by comparing the 118 variable nucleotides within this element among all isolates (Fig. 3A), revealed two dominant populations of Tn*1549*, those represented by the sequences from the short, 33-kb element from AUS0069 and those represented by the 57-kb element from AUS0085. Overall, there were five different transposon sequence groups among the 36 VREfm isolates (based on a difference of three or more single nucleotide polymorphisms [SNPs]) (Fig. 3A). Notably, Tn*1549* sequences identified in the anaerobic bacteria were similar to those from VREfm isolates. Remarkably, the Tn*1549* sequence from *Clostridium* sp. strain MLG055, isolated from the feces of a dialysis patient in Melbourne, Australia (20), was identical to Tn*1549* from a number of VREfm isolates from bloodstream infections, indicating that transfer of this element among the bowel microbiota may occur.

The chromosomal insertion site of all Tn*1549*-like elements for the 36 VREfm isolates was investigated, revealing a restricted repertoire of insertions, indicating that Tn*1549* insertion is not random (Fig. 3B). Notably, the VREfm isolates contained only a single copy of Tn*1549*. Seven different chromosomal insertion sites were identified, yet transposons of the same sequence were consistently inserted at the same chromosomal location (Fig. 3A and B). For example, transposons represented by isolate 69 (red in Fig. 3A) all integrated within EFAU004_00592, while transposons represented by isolate 85 (blue in Fig. 3A) all integrated within the signal peptidase I gene (EFAU004_00734). In conjunction with the core genome phylogeny (see below), we then used Tn*1549* sequence type, site of insertion, insertion orientation, and coupling sequence to distinguish inherited from independent Tn*1549* acquisitions among *E. faecium* isolates (Fig. 1 and 3).

To understand the population structure of the *E. faecium* strains included in this study, a core genome *E. faecium* phylogeny was constructed using 14,988 SNPs identified among the 63 isolates (excluding insertions and deletions). Split decomposition analysis produced a reticulate phylogeny, suggesting that recombination contributes to the phylogenetic signal (data not shown). Because recombination would perturb the phylogeny, we employed an iterative algorithm that searched for a high density of SNPs and excluded chromosome regions likely acquired by recombination (Fig. 4). This analysis predicted 297 recombination events, spanning 1.3 Mb (44% of the chromosome) and accounting for 12,849 SNPs (85.7% of all nucleotide variation). The remaining 2,139 SNPs were aligned among all isolates, and a tree was inferred using maximum likelihood based on these vertically inherited base substitutions alone (Fig. 3C).

Isolates generally clustered within their previously defined multilocus sequence types (MLSTs); however, there were also some clear exceptions highlighting the impact of recombination events on MLSTs. Five of the seven MLST loci were impacted by recombination (Fig. 4). For example, isolates 69 (SLV of ST252) and 71 (ST252) clustered among the ST203 isolates based on the nonrecombinant core genome analysis (Fig. 4). As expected, the non-CC17 isolates (isolates 10, 92, and 101) were more distantly related to all other isolates. Significantly, CC17 VSEfm isolates were closely related to, and in some cases essentially indistinguishable from, CC17 VREfm isolates at this core genome level, suggesting that the CC17 VSEfm and CC17 VREfm strains were part of the same circulating population rather than that they evolved independently. Thus, the evolution of VREfm strains from colonizing VSEfm strains, and not just the clonal spread of VREfm strains, explains the epidemiology of invasive VREfm disease observed in this study, suggesting that the epidemiology of VSEfm in

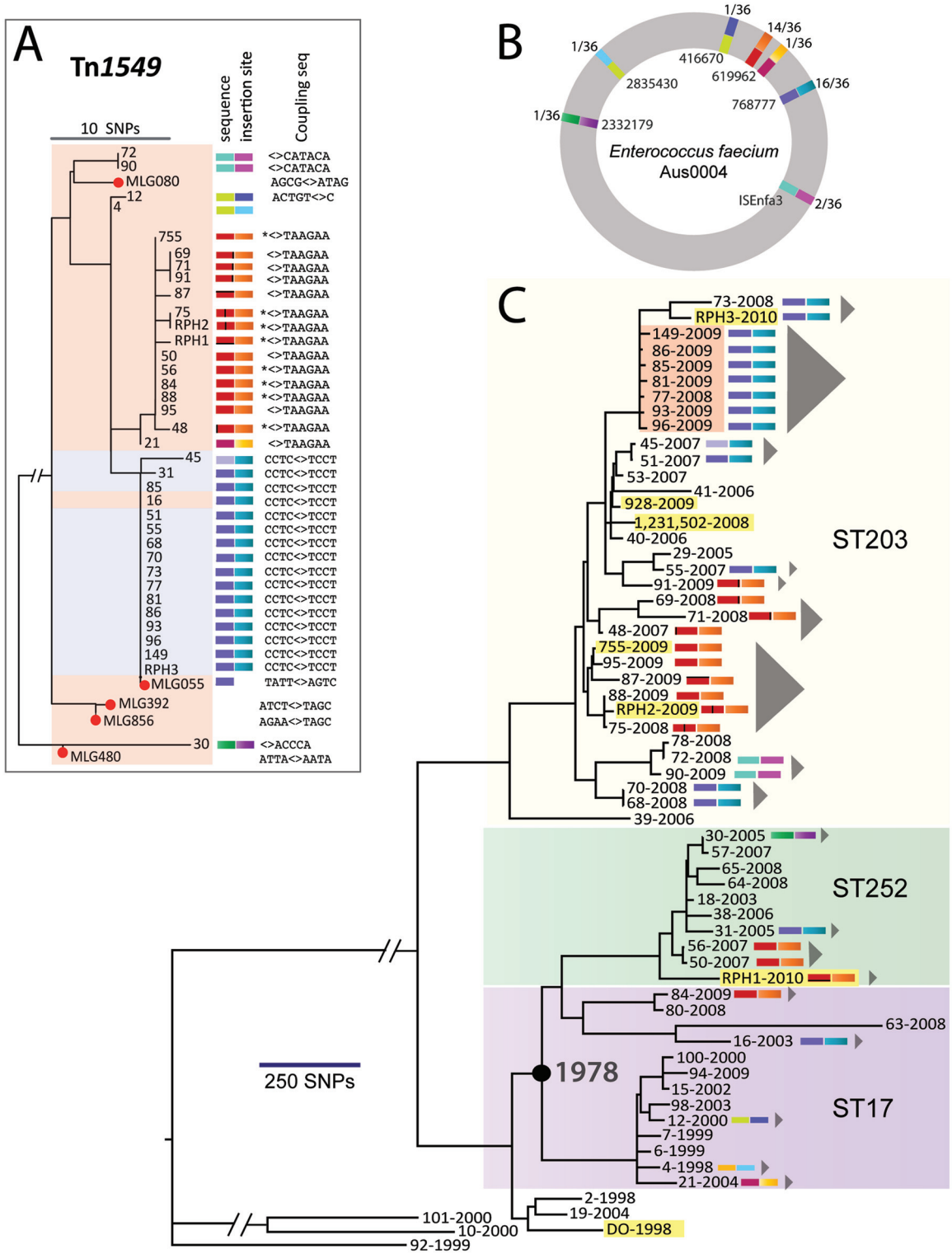


FIG 3 Phylogeny of Tn*1549* elements and *Enterococcus faecium*. (A) SNP-based phylogeny of Tn*1549* elements in *E. faecium* and anaerobes (red dots indicate anaerobes). The color scale represents the Tn*1549* sequence type (based on SNP analysis) as well as the transposon insertion site in the *E. faecium* chromosome, relative to the AUS0004 chromosome sequence. Black bars within the red transposon sequence groups represent single nucleotide differences. The coupling sequence is the 5- to 8-bp sequence introduced from the donor during transposition ("<>" refers to the site of insertion, as shown in Figure 1; * indicates nucleotide deletion). Isolates shaded in pink contain the shorter (33-kb) version of Tn*1549*, while those in blue contain the longer (57-kb) version of Tn*1549*. (B) Overview of the seven unique transposon insertion sites for the 36 VREfm clones mapped to the AUS0004 chromosome, as well as the transposon sequence type. The color scheme follows that described in panel A. Outter numbers indicate numbers of isolates with insertions at that position. Inner numbers indicate the insertion position in the AUS0004 chromosome. (C) Maximum-likelihood phylogeny for *E. faecium* based on SNPs in the core genome, with major multilocus sequence type groups highlighted. Isolates from other institutions are highlighted in yellow and include five isolates from other institutions in Australia (RPH

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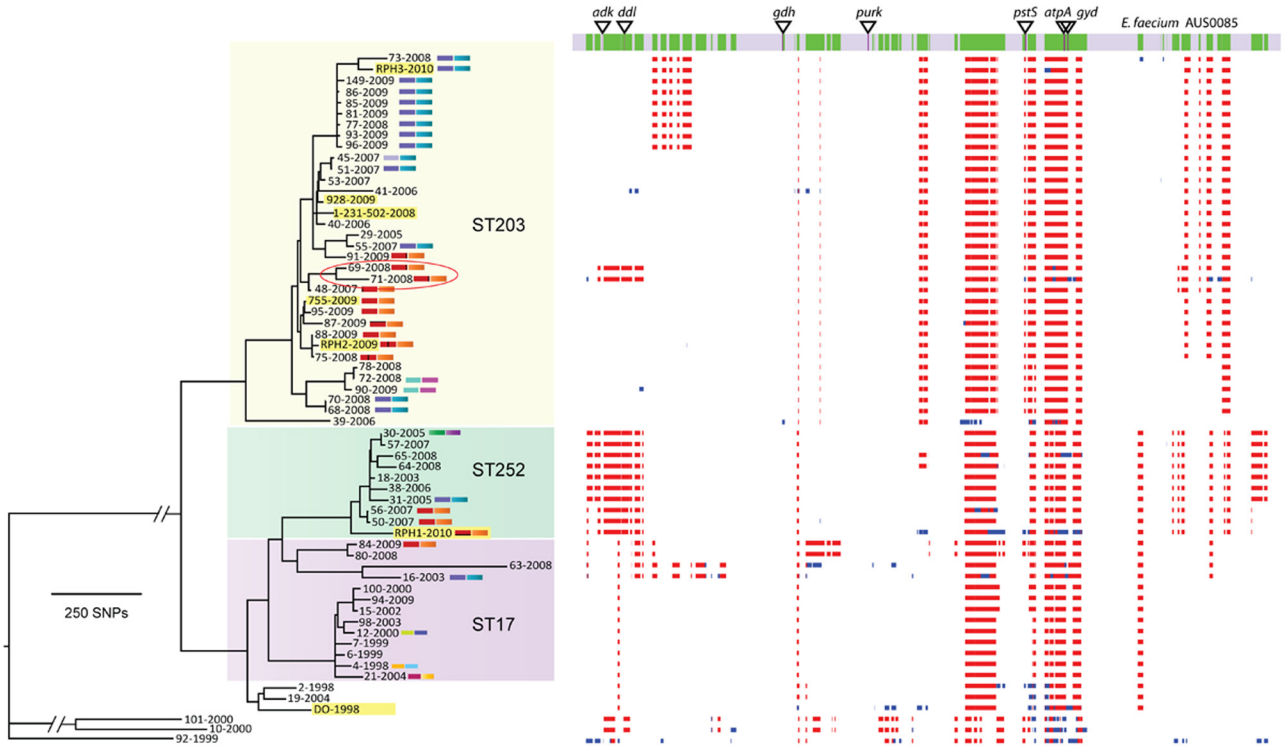


FIG 4 Recombination in *E. faecium*. Areas of predicted recombination in the *E. faecium* chromosome are highlighted, based on SNP density. MLST alleles are indicated by triangles. Only *purK* and *adk* are in regions without predicted recombination. Two isolates (69 and 71, circled) are part of the ST252 complex by MLST but cluster with the ST203 isolates based on core genome SNP analysis. Regions in red are recombinant in multiple isolates in the collection, while regions in blue are unique to one strain.

hospitals is a key factor driving the emergence of clonally related VREfm.

In addition to examining the evolutionary relationship between the isolates based on a core genome, we also assessed an *E. faecium* pan genome which included plasmids, prophages, and other genomic elements (Dataset S3 in the supplemental material and Fig. 5). The pan genome included more than 5 Mb of sequence bearing >5,000 genes and demonstrated that even among isolates that were found to be very closely related based on their core genomes, variability in gene content was detected. For example, isolates 73 and RPH3 had closely related core genomes; however, isolate 73 harbored a unique region (EFPAN04301 to EFPAN04389) that likely represented a plasmid with a large number of hypothetical proteins and plasmid-associated genes. Similarly, isolates 72 and 78 displayed closely related core genomes, with isolate 72 harboring Tn*1549* and isolate 78 lacking this element but possessing a unique prophage (EFPAN02546 to EFPAN 02583).

In some cases, the VREfm phylogeny was suggestive of clonal dissemination of an extant VREfm clone. For example, the ST203 cluster that included isolates 149, 86, 85, 81, 77, 93, and 96 was isolated over a 12-month period from patients in the same hospital (Fig. 3C). Inspection of the pan genomes for these isolates (Fig. 5) suggests that they may have been transmitted directly between patients, except for isolates 86 and 96, where differences in gene content, possibly due to plasmid acquisition, were detected. For example, isolate 86 contained the genes EFPAN04820 to EFPAN04855, encoding a number of hypothetical proteins and a type IV secretion system, while isolate 96 harbored a unique region (EFPAN04913 to EFPAN04927). In contrast, we were able to combine the detailed transposon analysis with the core genome phylogeny to define independent episodes of VREfm evolution (Fig. 3C).

At least 18 distinct episodes of VREfm evolution of the total 36 VREfm isolates were detected, indicating that *de novo* evolution of VREfm strains is an important source of VREfm infection at our hospital. For example, the ST17 isolates 4 and 21 are closely related at the core genome level, but their transposon sequence and insertion sites are different. Furthermore, the dominant "red-orange" and "blue-aqua" Tn*1549* types are dispersed throughout the *E. faecium* core genome phylogeny and can be explained by instances of independent Tn*1549* acquisition (Fig. 3C). In the ST252 cluster, four VREfm bloodstream isolates were sequenced from

Figure Legend Continued

isolates from Perth; isolates 928 and 755 from Darwin) and two isolates from the United States sequenced by others (DO, 1-231-502). Colored bars indicate transposon sequences and insertion sites as defined for panel A. Gray triangles indicate predicted independent VREfm clones based on Tn*1549* characteristics and core genome phylogeny. The node represented by the black circle shows the estimated divergence date for these ST17 and ST252 isolates, as inferred by Path-O-Gen.

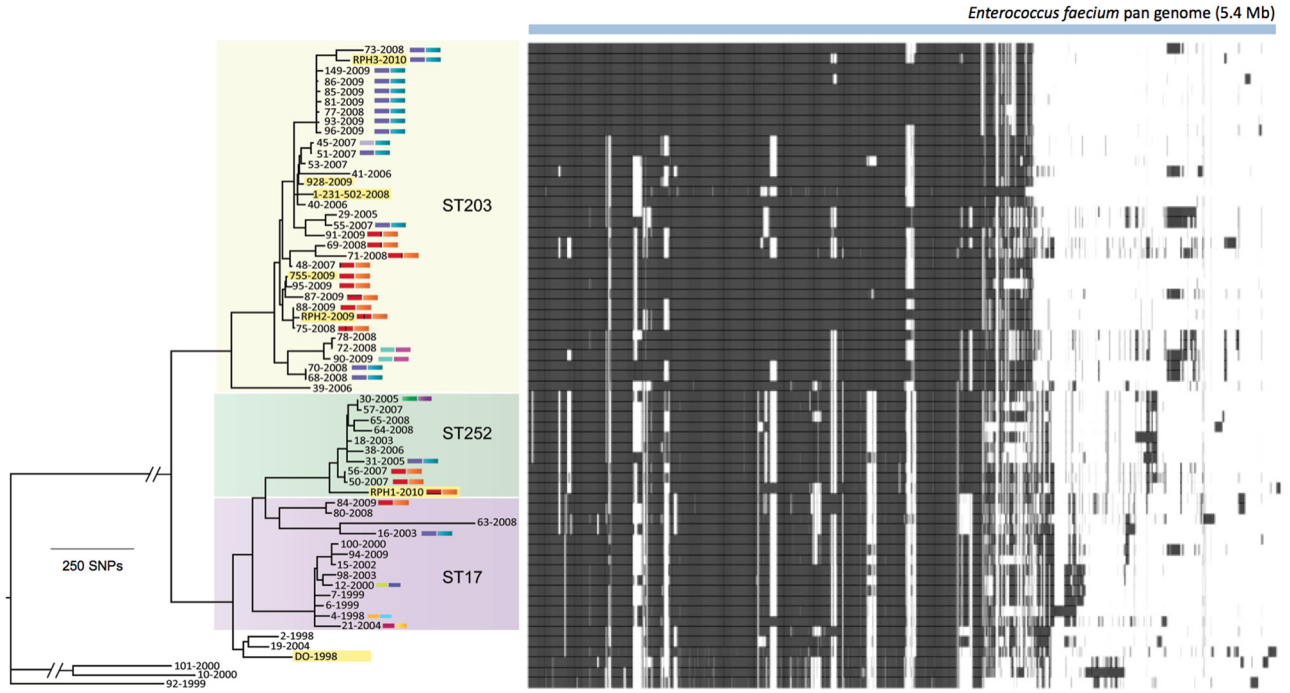


FIG 5 *E. faecium* pan genome. Blue bar indicates the 5.4-Mb *E. faecium* pan genome, including 5,000 annotated genes, detected among genomes analyzed in this study. A heat map shows the presence (black) and absence (white) of the 5,000 genes. Dataset S3 in the supplemental material summarizes the variable regions and pan-genome contents for all isolates and includes EFPAN locus tags for all predicted coding sequences.

our hospital, and at least three of these indicate the independent evolution of VREfm strains.

In the 33-month period from March 2007 to November 2009, we had an outbreak of clinical VRE infection at our institution (6). Based on MLST analysis, it appeared that we had a clonal outbreak of VREfm due to the introduction of a new clone of ST203 VREfm. However, analysis of the ST203 VREfm genome data from these isolates demonstrates that at least 9 unique clones of ST203 VREfm have emerged (out of the 22 sequenced). These unique ST203 VREfm isolates highlight the polyclonal nature of VREfm at our hospital, even within a single MLST cluster that was first detected as late as 2007.

In order to determine if these observations were unusual and restricted to our hospital, we included genome sequences from geographically dispersed *E. faecium* isolates for comparison. The more recent isolates from other Australian cities were closely related to ST203 and ST252 isolates from our institution (highlighted in yellow in Fig. 3C). The older ST18 VREfm isolate from the United States (DO strain, 1998) was more distantly related to the majority of *E. faecium* isolates in this study. The recent VRE isolate 1-231-502 (2008) was very similar to ST203 isolates from our institution, suggesting global dissemination of this *E. faecium* clone.

The collection of *E. faecium* isolates from a single hospital over a 12-year period allowed us to analyze the evolutionary changes and mutation rate in the isolate collection. For this analysis, we used only ST17 and ST252 isolates from our institution, because they were isolated throughout the 12-year study period. This analysis revealed a strong molecular clock signal (Fig. 6) and allowed estimation of a core genome mutation rate of 1.5×10^{-6} substitutions per site per year or ~5 SNPs per genome per year (excluding SNPs introduced via recombination). This analysis suggested that the ST17/ST252 isolates from the collection arose from a common ancestor that existed in the late 1970s.

DISCUSSION

Using large-scale comparative genomics, we have demonstrated that hospital *vanB* VREfm epidemiology is far more complex than previously recognized and is significantly driven by *de novo* VREfm generation rather than simply nosocomial transmission of extant VREfm strains. We found that many distinct clones of *vanB* VREfm were responsible for bloodstream infection isolates from patients at our institution. In fact, at least 18 of 36 VREfm isolates

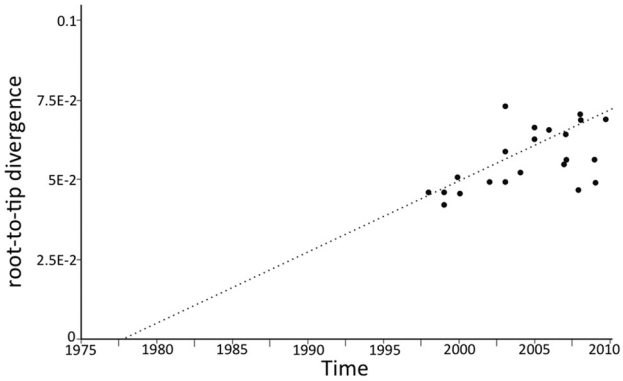


FIG 6 Evolution of *E. faecium* ST17 and ST252 isolates. Estimate of the divergence date for ST17 and ST252 isolates by branch-to-tip analysis using Path-O-Gen and branch lengths inferred by maximum-likelihood analysis (http://tree.bio.ed.ac.uk/software/pathogen/).

sequenced in this study were due to unique VREfm evolution events. This includes a time period where a new sequence type of *E. faecium* (ST203) emerged in our institution and caused what we thought was a clonal outbreak (6). Additionally, circulating clones of VSEfm and VREfm were phylogenetically intermingled. These findings indicate that unique VREfm evolution events are common, presumably occurring in the human bowel, and taken together suggest that control strategies based on screening for VREfm and isolation of colonized patients might not be completely effective.

We identified a number of Tn*1549* features that could be utilized to determine independent VREfm acquisition events (Fig. 1), demonstrating the power of genome sequencing to uncover complex relationships between bacteria. Significantly, the transposon sequences from anaerobic bacteria isolated from human feces were very similar and, in one case, identical to those from VREfm isolates associated with bloodstream infections in humans, suggesting that anaerobic bacteria might form an important component of the Tn*1549* reservoir. If the transposon pool is restricted and the VSEfm population is highly clonal, as occurs in hospitalized patients (30), even unique *in vivo* generation episodes of VREfm may appear like clonal spread of preformed VRE. This issue was highlighted by detailed analysis of three ST203 isolates, 149, 86, and 96. Based on core genome phylogeny and transposon analysis, these isolates appeared clonal; however, analysis of the pan-genome components of these isolates (Fig. 5) demonstrated additional genome diversity, in some cases driven by acquisition of plasmids. While it is possible that individual genome variability evolved in a clonally disseminated preformed VREfm strain, it is also possible that clonally dispersed (but not identical) VSEfm isolates have independently acquired an identical Tn*1549* element. If this is the case, then even more than 18 of the 36 VREfm isolates sequenced in this study may have evolved independently.

While we focused our investigation on a single institution with the primary aim of understanding the persistent VREfm outbreak that we had noted, we included isolates from other Australian cities and the United States. Sequences from these isolates were very closely related to VREfm isolates from our institution in Melbourne, Australia, including the United States isolate (1,231,502). Hospital-hospital transfer of colonized patients may explain this observation in Australia; however, such movements are infrequent between Darwin and Melbourne. In our view, the best model which explains our observations is silent circulation internationally of closely related VSEfm isolates, with local Tn*1549* transposition events, which then lead to the appearance of new clinical cases of VRE colonization and infection in hospitals.

The *E. faecium* genome is known to have high rates of recombination, especially compared to other important hospital pathogens, such as *Staphylococcus aureus* (25, 34, 35). Here we applied whole-genome sequencing and SNP clustering analysis to infer a phylogeny and predict regions of recombination within the 63 *E. faecium* genomes used in this study (Fig. 3 and 4). A total of 1.3 Mb (44%) of the chromosome was affected by recombination. This includes five of the seven MLST loci (Fig. 4), indicating that MLST analysis of *E. faecium* based on the currently selected alleles may give misleading results and must be interpreted with care. We also defined an *E. faecium* pan genome, which includes 1,872 core genes as well as 3,128 accessory genes identified within the 63 *E. faecium* isolates in this study (Fig. 5). This indicates significant plasticity within the *E. faecium* genome, with large accessory elements potentially impacting the clinical behavior of these isolates, as previously suggested (25, 33). Clonal complex 17 *E. faecium* emerged as an important hospital-associated pathogen in the 1980s (36). By analyzing the ST17/ST252 isolates from this study, we found a mutation rate in the core genome of 5 SNPs per genome per year and predicted that the common ancestor strain for *E. faecium* at our institution existed around 1978 (95% confidence interval [CI], 1973 to 1983), in keeping with the appearance of CC17 *E. faecium* and VREfm in the 1980s.

Our findings have implications for the control of VREfm in hospitals, where generation of new VREfm clones is a frequent event. Because a high proportion of hospital patients carry non-enterococcal *vanB*-containing organisms in their bowels (19, 20, 22, 37), we suggest that specific VSEfm clones should also be targets for control in the hospital environment. It remains to be determined if hospital-associated VSEfm is acquired nosocomially by patients, as suggested in European studies, or if in fact these isolates of VSEfm are present in the community in Australia (30, 38). A complete understanding of VSE epidemiology and the biology of the Tn*1549* element will be critical to improving strategies to curtail the VRE epidemic. These data dictate that alternative strategies directed at identifying and isolating patients colonized with high-risk VSEfm clones, identifying patients who harbor *vanB*-containing Tn*1549* and who are likely at increased risk of *in vivo* generation, and identifying and modifying the selective pressures promoting *in vivo* evolution are needed to prevent the ongoing emergence of VREfm as a public health threat.

MATERIALS AND METHODS

Bacterial isolates. Isolates used in this study were vancomycin-susceptible and -resistant *Enterococcus faecium* strains recovered between 1998 and 2009 from blood culture specimens at Austin Health, a tertiary referral hospital in Melbourne, Australia. Additionally, five recent clinical and screening isolates of VSEfm and VREfm from other cities in Australia (Darwin and Perth) were included for comparison, as were five previously identified and characterized human bowel anaerobes containing Tn*1549* within their *vanB* operon that were isolated in Melbourne, Australia (19, 20). All VREfm isolates were found to display the *vanB* genotype, as determined by PCR (17). *Enterococcus faecium* isolates were previously analyzed by MLST and were found to belong predominately to clonal complex 17 (CC17) (6). Epidemiological data, including date and location of isolation, were recorded. A summary of isolates and epidemiological data is provided in Dataset S1 (*E. faecium*) and Dataset S2 (anaerobes) in the supplemental material.

Genome sequencing and analysis. Genomic DNA was extracted, and all isolates were subjected to whole-genome shotgun sequencing using an Illumina HiSeq-2000 sequencing system and 100-bp-paired-end TruSeq chemistry or Ion Torrent single-end 100-bp or 200-bp sequencing chemistry (39) (Datasets S1 and S2). A read mapping approach was used to align the sequences from these isolates with the recently completed *E. faecium* ST203 genome, AUS0085 (Margaret M. C. Lam, Torsten Seemann, Nicholas J. Tobias, Honglei Chen, Volker Haring, Robert J. Moore, Susan Ballard, M. Lindsay Grayson, Paul D. R. Johnson, Benjamin P. Howden, and Timothy P. Stinear, submitted for publication), using SHRiMP v2.0 (40). Single nucleotide polymorphisms (SNPs) in the *E. faecium* core genome were identified using Nesoni v0.93, which aligns the read of each genome to that of the reference to construct a tally of putative differences at each position (http://www.bioinformatics.net.au). In order to exclude regions of suspected recombination from the phylogenomic analysis, regions with high frequencies of polymorphic nucleotides were removed from the alignment, as previously described (41, 42), and a phylogeny was inferred using maximum likelihood with RAxML (43). The temporal signals in the sequence data were analyzed using Path-O-Gen (http://tree.bio

.ed.ac.uk/software/pathogen/). SNPs likely introduced via recombination were identified using the method described in Croucher et al. (41).

To investigate the total *E. faecium* gene content, an *E. faecium* pan genome was defined after *de novo* assembly using Velvet v1.2.04 (44) and alignment of contigs to the AUS0085 genome using MUMmer (45). Unaligned contigs were appended to the AUS0085 genome to construct a pan genome, which was annotated using Prokka (http://bioinformatics.net.au/). The proportion of the length of each annotated gene covered by reads was assessed for each isolate and a map summarizing all variable genes and their distribution in each strain produced.

Variable nucleotide positions in Tn*1549* for all VREfm isolates and anaerobes were also identified, and a Tn*1549* phylogeny was inferred using the neighbor-joining method with uncorrected *P* distances, as implemented in SplitsTree v4.12.3 (46). In addition, the chromosomal Tn*1549* insertion site was determined by aligning the contigs that spanned the left- and right-hand ends of Tn*1549* (obtained from *de novo* assembly of each VREfm clone, as described above) to the AUS0004 reference genome. Other features of the Tn*1549* insertion that were determined included the orientation of insertion within the chromosome and identification of the 5- to 8-bp coupling sequence. Separate clones of VREfm (unique VREfm generation events) were defined as taxa with distinct core genome phylogenies, or if they possessed specific Tn*1549* characteristics, they were defined by including the variation in their transposon sequence, the insertion site, the insertion orientation, or the coupling sequence. Differences in the pan genomes were also used to help define independent VREfm clones.

Nucleotide sequence accession number. The sequence reads for all isolates have been submitted to GenBank under BioProject identifier PRJNA205886.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00412-13/-/DCSupplemental.

Dataset S1, XLS file, 0.1 MB.

Dataset S2, XLS file, 0.1 MB.

Dataset S3, XLSX file, 1.1 MB.

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