A Sustained Hospital Outbreak of Vancomycin-Resistant *Enterococcus faecium* Bacteremia due to Emergence of *vanB* *E. faecium* Sequence Type 203

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**Background.** A significant increase in the rate of vancomycin-resistant *Enterococcus faecium* (VREfm) bacteremia at our health service, despite improved infection control, prompted us to investigate the cause.

**Methods.** *E. faecium* bacteremia (including VREfm) over a 12-year period (1998–2009) was investigated using multilocus sequence typing, antibiotic and antiseptic susceptibility profiles, optical mapping, and whole genome sequencing of historical and recent isolates.

**Results.** For 10 years, the rate of bacteremia due to *vanB* VREfm remained stable and sequence type (ST) 17 was predominant. In 2005, ST203 vancomycin-susceptible *E. faecium* first appeared at our institution, and from March 2007, coinciding with the appearance of a *vanB* VREfm ST203, the rate of VRE bacteremia has increased exponentially. Although we found no difference in antiseptic susceptibility or presence of genes encoding putative virulence determinants (*espEfm*, *hylEfm*, and *fms* genes), comparative genomics revealed almost 500 kb of unique sequence when an ST17 and an ST203 VREfm isolate were compared, suggesting that other genomic factors are responsible for the apparent success of *E. faecium*.

**Conclusions.** The application of multilocus sequence typing has uncovered the emergence of an epidemic clone of *E. faecium* ST203 that appears to have acquired the *vanB* locus and has caused a sustained outbreak of VRE bacteremia.

Clinically significant vancomycin resistance in enterococci was first observed in isolates from the United Kingdom and Europe in 1986 [1, 2], and patients with hospital-acquired infections caused by vancomycin-resistant enterococci (VRE) were detected in the United States soon after [3]. *VanA Enterococcus faecium* rapidly became the major VRE of clinical concern in the United States as prevalence rates increased from 0% in 1989 to 28.5% in 2003 [4], with vancomycin resistance per se shown to be an independent predictor of death [5, 6]. The first clinical isolate of VRE in Australia, also a *vanA* *E. faecium*, was isolated from a liver transplant recipient at our institution, Austin Health, in 1994 [7]. Since then, there have been numerous VRE outbreaks in Australian hospitals, mostly involving colonization rather than clinical infection [8–12]. Interestingly, although the majority of clinical isolates of VRE in Australia are also *E. faecium*, they usually carry the *vanB* operon rather than *vanA* [8]. The rate of VRE colonization in the general community in Australia is very low (0.2%) [11], but in contrast, up to 60% of healthy Australians appear to carry anaerobic bowel flora that harbor the *vanB* operon [13]. A similar observation has recently been reported from Canada [14]. Transposition...
of vanB operons to cocolonizing sensitive enterococci on Tn1549-like elements has been observed in an animal model using vanB-positive anaerobes isolated in Australia [15].

The understanding of molecular epidemiology and bacterial evolution has been assisted by the development of sequence-based typing methods, such as multilocus sequence typing (MLST). MLST has been applied to E. faecium and has revealed a clonal complex (CC), CC17, that is predominant in hospitals [4]. Organisms belonging to CC17 are typically ampicillin-resistant and are enriched for a number of putative virulence factors that may assist with hospital adaptation and spread [4]. These include enterococcal surface protein (encoded by esp), hyaluronidase (encoded by hyl), and cell wall–anchored E. faecium surface proteins (Fms), which have characteristics typical of microbial surface components—recognizing adhesive matrix molecules [16–21].

At Austin Health, clinical VRE infection was rare from 1994 until early 2007, in contrast to a high incidence of methicillin-resistant Staphylococcus aureus (MRSA) infections. Since 2001, after introduction of an institution-wide, multimodal program based around the introduction and promotion of alcohol/chlorhexidine hand hygiene and programs targeting line-related sepsis [22–24], the rate of MRSA bacteremia has decreased substantially [23]. However, despite targeted rectal screening and isolation for patients with VRE colonization, we observed an increase in the incidence of VRE bacteremia, predominantly vanB E. faecium. In fact, by mid-2009 the rate of nosocomial VRE bacteremia at our institution surpassed MRSA bacteremia for the first time. In an attempt to understand this increase in VRE bacteremia, we reviewed all E. faecium bacteremia over the period 1998–2009 and analyzed the molecular epidemiology of blood culture isolates by MLST.

**PATIENTS AND METHODS**

**Setting.** Austin Health is a 1003-bed teaching hospital on 3 sites, where screening for VRE rectal colonization is undertaken for close contacts of known VRE-colonized patients and weekly for all inpatients in selected high-risk clinical areas (intensive care unit, renal, liver transplantation, haematology/oncology wards). Opportunistic screening is also performed if a fecal sample from an inpatient with diarrhea is submitted ≥48 h after hospital admission. VRE-colonized patients are isolated in a single room or are cohorted with other VRE-colonized patients. Standard contact precautions for VRE (gowns and gloves) were routinely used during the study period [25]. Increasing numbers of VRE screening specimens per year have been processed by the microbiology laboratory at Austin Health (from 2181 specimens in 1998 to 10,271 specimens in 2008). This study was undertaken as an infection control quality improvement project at Austin Health.

**Incidence of E. faecium and MRSA bacteremia.** The microbiology laboratory computer database was searched for isolates of MRSA or E. faecium isolated from blood culture from 1 January 1998 through 30 June 2009. We defined a patient episode of bacteremia by the date of the first MRSA or E. faecium blood culture isolate, unless 14 days passed without a positive blood culture, in which case an additional episode was recorded. Patient-episodes of bacteremia per 6-month periods were converted to rates per 1000 episodes of care with use of all inpatient discharges (“total separations”). For the purposes of this study, 2 periods were defined—period A (January 1998 through December 2006), when VRE bacteremia was uncommon, and period B (January 2007 through June 2009), when the rate of VRE bacteremia increased significantly.

**Antimicrobial utilization.** Antimicrobial utilization was assessed for vancomycin, ceftriaxone, and meropenem from the time accurate data collection was commenced (2004) until the completion of the study. The defined daily dose was defined as 2 g per day for all antibiotics.

**Bacterial isolates and identification.** Presumptive E. faecium were identified by standard methods [26]. The first available isolate from each patient-episode of confirmed vancomycin-resistant E. faecium (VREfm) or vancomycin-susceptible E. faecium (VSEfm) was used in the study, provided the blood culture was not mixed with other pathogens, the patient did not have a history of VRE colonization (for VSEfm cases), and the isolate was still available for testing. The definitive identification and confirmation of VSE and VRE was based on a multiplex polymerase chain reaction for the D-Ala-D-Ala ligase gene [27], and van genotyping [8].

**Susceptibility testing.** Susceptibility testing for penicillin, ciprofloxacin, erythromycin, vancomycin, teicoplanin, tetracycline, fusidic acid, and high-level gentamicin resistance was performed by agar dilution according to Clinical and Laboratory Standards Institute criteria [28]. Minimum inhibitory concentrations (MICs) to Triclosan (Irgasan; Sigma-Aldrich) and chlorhexidine digluconate (Sigma-Aldrich) were determined by adapting Clinical and Laboratory Standards Institute agar dilution methods with use of serial doubling dilutions from 0.001 mg/L to 16 mg/L in Mueller Hinton Agar (BD BBL) [28]. Susceptibility to linezolid and daptomycin was performed by Etest, according to the manufacturer’s instructions.

**MLST.** MLST on E. faecium (VREfm and VSEfm) isolates was performed as described by Homan et al [29]. Unique sequences were submitted to the curator for assignment of a new allelic profile and sequence type (ST), and CCs were determined using eBURST, version 3 ([http://efaecium.mlst.net/](http://efaecium.mlst.net/)). Patient demographic characteristics and isolate details are shown in Table 1.

**Pulsed field gel electrophoresis (PFGE).** PFGE of Small-
digested genomic DNA agarose plugs was performed and analyzed as described elsewhere [30].

Comparative genomics. To investigate the potential role of genomic variation in the different epidemiological behaviors of the 2 most common MLST STs of VREfm, optical mapping and complete genome sequencing was performed. Optical maps for strains AUS0004 (ST17, isolated February 1998) and AUS0085 (ST203, isolated February 2009) were prepared by OpGen, as described elsewhere [31], with an NcoI digest. The OpGen Mapviewer program was used to align and compare the optical maps for the 2 strains. Genome sequences of AUS0004 and AUS0085 were obtained using Illumina GAII 36 cycle paired-end chemistry. Each isolate was de novo assembled using Velvet [32] and annotated with Prokka (in-house). The putative proteome was clustered into 25 functional groups [33] using Velvet [32] and annotated with Prokka (in-house). The characteristics of the van loci were also compared for the 2 strains. The raw sequence data is available from the National Center for Biotechnology Information Sequence Read Archive via accession numbers SRR031626 (AUS0004) and SRR031627 (AUS0085).

Statistical analysis. Statistical analysis of rates of bacteremia was performed using linear regression curves fitted with Graph Pad Prism, version 5.0 (Graph Pad Software).

RESULTS

Patient episodes of MRSA or E. faecium (Efm) bacteremia. From January 1998 through 30 June 2009, there were 867,771 "separations" at Austin Health. The rates of MRSA, VREfm, and VSEfm bacteremia over this period are shown in Figure 1A. Although the rate of MRSA bacteremia decreased ~3-fold over 12 years, patient-episodes of VREfm bacteremia remained at <0.04 episodes per 1000 separations for period A (January 1998 through December 2006) but then increased ~5-fold during the subsequent period (period B). By linear regression, the decrease in MRSA was statistically significant (P < .001), as was the increase in VREfm (P = .002).

Epidemiological investigation. Eighty-five E. faecium blood culture isolates were included in the study (34 VREfm and 51 VSEfm) (Table 1). Only 1 VREfm isolate was vanA genotype; all others were vanB. Cases of E. faecium bacteremia (VREfm and VSEfm) predominately occurred in patients from our hematology/oncology (n = 28; 14 VREfm, 14 VSEfm), gastroenterology/liver transplantation (n = 16; 9 VREfm, 7 VSEfm), and gastrointestinal surgery (n = 16; 3 VREfm, 13 VSEfm) units, plus 25 cases (8 VREfm, 17 VSEfm) that occurred elsewhere in the hospital. The significant increase in VREfm bacteremia from 2007 onwards correlated with an increase in VRE cases in the hematology/oncology unit.

MLST. Among the 85 isolates investigated, 17 different STs were identified, with 82 (96.5%) of 85 isolates belonging to CC17. The 3 largest ST groups, which represented 68 (80.0%) of the 85 isolates, were ST203 (32 [37.6%] of 85), ST17 (25 [29.4%] of 85), and ST252 (11 [12.9%] of 85). There were only 1–3 isolates in each of the other 14 STs.

There were 36 E. faecium isolates tested from the 8 years prior to 2006 (VSEfm, 28 of 36; VREfm, 8 of 36), and ST17 was predominant (VSEfm ST17, 18 [64%] of 28; VREfm ST17, 3 [37.5%] of 8). However, from January 2006 through June 2009 (3.5 years), there were 49 isolates tested (VSEfm, 23 of 49; VREfm, 26 of 49), and of these isolates, 31 (63.3%) of 49 were ST203 (VSEfm ST203, 12 [24.5%] of 49; VREfm ST203, 19 [38.8%] of 49) (Figure 1B and 1D), indicating a significant switch in dominant ST of both VSEfm and VREfm.

Notably, for all 3 dominant STs found in this study (ST17, ST252, and ST203), the STs first appeared as a VSEfm blood culture isolate and subsequently appeared in a different patient as a VREfm. A single isolate of ST252 VSEfm first appeared at Austin Health in 2003. Subsequently, 2 isolates of VREfm ST252 were isolated in 2005, with a total of 11 ST252 isolates in the study period (VREfm ST252, 5 of 85; VSEfm ST252, 6 of 85; Figure 1C). Similar to ST252, ST203 first appeared in VSEfm blood culture isolates in 2005, then subsequently appeared in VREfm isolates 2 years later (March 2007). However, since 2007, coinciding with the emergence of VREfm ST203, the rate of VREfm bacteremia has increased exponentially, with ST203 accounting for 19 (76%) of 25 VREfm blood culture isolates, whereas ST17 (VSEfm and VREfm) only accounted for 4 (16%) of 25 during the same period. During 2009, ST203 isolates accounted for 10 of 14 E. faecium bacteremia isolates and 9 (81.8%) of 11 VREfm isolates. The other 2 VREfm isolates were ST17 and ST483. However, ST483 is a single locus variant of ST203 and only differs by a single nucleotide change in the gdh allele, which defined a new allele (allele 39). If the VREfm ST203 isolates were excluded from the analysis, there would have been no observable net change in the rate of VRE bacteremia over the entire 11.5-year study period (P = .13, by linear regression with ST203 excluded) (Figure 2).

Relatedness of isolates within ST17 or ST203 by PFGE. With use of PFGE, we had not yet recognized the emergence
Figure 1. Patient episodes of methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-susceptible *Enterococcus faecium* (VSEfm) and vancomycin-resistant *E. faecium* (VREfm) bacteremia and multilocus sequence typing (MLST) results for VSEfm and VREfm at Austin Health over a 12-year period. A, MRSA, VSEfm, and VREfm bacteremia rates per 1000 separations for all positive blood cultures over 12 years. B–D, Incidence of MLST sequence type (ST)17, ST252, and ST203 VREfm and VSEfm bacteremia in 85 study isolates over the 12-year study period. For all ST203 bacteremias in period B, hematology/oncology patients accounted for 3 of 8 in 2007, 5 of 9 in 2008, and 4 of 10 in 2009; and for VRE ST203 bacteremias, they accounted for 2 of 4 in 2007, 5 of 6 in 2008, and 4 of 9 in 2009.

of a new clone of VREfm at our institution. Therefore, after designation of the ST of each isolate, the PFGE profiles of the 2 dominant ST types (ST17 and ST203) were reviewed (Figure 3). A distinct difference (<65% similarity) in PFGE profile was demonstrated between the 2 STs. Within ST17 and ST203, some strains had identical banding patterns, including a number of VSEfm ST17 and ST203 isolates; however, a number of PFGE profiles were present.

**Antimicrobial utilization.** Hospital utilization of vancomycin and meropenem did not change over the 5 years from 2004 when corrected for patient bed days; however, ceftriaxone use increased from 35 to 48 defined daily doses per 1000 patient bed days between 2004 and 2009 (Figure 4).

**Antibiotic and antiseptic resistance results.** In general, antibiotic and antiseptic MIC results were not different between different sequence types of either VSEfm or VREfm (Table 1).
The single \textit{vanA} VRE was resistant to vancomycin and teicoplanin, whereas the 30 \textit{vamB} VREfm strains had a vancomycin MIC$_{90}$ of >32 mg/L and were all susceptible to teicoplanin, as expected. The MIC$_{90}$ for penicillin was >16 mg/L, with only 6 penicillin-susceptible isolates (3 non-CC17 strains [2 ST178 and 1 ST495], and 1 ST32, 1 ST71, and 1 ST55 isolate). When read at 24 h, the chlorhexidine MIC$_{90}$ was 8 mg/L (range, 0.5–8 mg/L), whereas the triclosan MIC$_{90}$ was 4 mg/L (range, 4–8 mg/L). An analysis of antiseptic resistance profiles of the dominant STs, ST17 (chlorhexidine MIC$_{90}$, 8 mg/L; triclosan MIC$_{90}$, 4 mg/L) and ST203 (chlorhexidine MIC$_{90}$, 4 mg/L; triclosan MIC$_{90}$, 4 mg/L), did not demonstrate any significant differences.

\textbf{Comparative genomics of ST17 and ST203.} Analysis of the optical map profiles for AUS0004 and AUS0085 demonstrated a chromosomal size of $\sim$3 Mb for both strains (Figure 5). Although ST17 and ST203 are both part of the same MLST CC (CC17), significant genomic variability was distributed across the genomes. Short-read sequencing (Illumina GAII) yielded $\sim$3 Mb of mappable data for each genome and confirmed significant genome variability, with $\sim$136 kb of sequence unique to AUS0004 and 361 kb of sequence unique to AUS0085. Two hundred thirty-one putative coding sequences were present in only AUS0004, and 495 coding sequences were unique to AUS0085. An analysis of functional groups for these unique sequences demonstrated that the majority of unique sequence coded for proteins of unknown function (70.6% in AUS0004 and 65.9% in AUS0085), whereas unique sequence present in AUS0085 also coded for proteins involved in DNA replication, recombination, and repair (7.7%); carbohydrate transport and metabolism (6.9%); and cell envelope biogenesis (2.6%).

The \textit{esp}$_{\text{efm}}$ gene that encodes a putative surface protein was present in both strains, whereas \textit{hyl}$_{\text{efm}}$ was absent from both. All known \textit{fms} genes were present in both strains. Using oligo sequences to scan for \textit{fms} genes, all 14 genes were detected in appropriate clusters (\textit{ebp} operon, \textit{fms} 11–19–16, \textit{fms} 14–17–13, and \textit{fms} 21–20); however, oligo sequences from Galloway-Pena et al. [16] failed to detect \textit{pilA}. Despite this, a gene with 98% sequence homology to \textit{E. faecium} E1165 pilin 1 was present upstream of \textit{fms} 20 in both strains. In both strains, the \textit{van} locus was part of a Tn1549-like element (99% sequence homology to \textit{Enterococcus faecalis} Tn1549 [GenBank accession number AF192329.1]), with only 3 bases differences between AUS0004 and AUS0085 in the $\sim$8.5 kb of sequence surrounding the \textit{van} operon.

\textbf{DISCUSSION}

We performed our study to investigate reasons for an increase in rates of VREfm bacteremia despite improved infection control generally, as indicated by decreased rates of MRSA bacteremia. MLST analysis of clinically significant VREfm isolates revealed a clear switch in the dominant vancomycin-resistant \textit{E. faecium}, which largely explained the recent outbreak. ST203 VREfm first appeared in 2007, rapidly became dominant, and has now replaced the previous ST17 VREfm that had been present at a low level for several years. Additionally, by investigating VSEfm isolates as well as VREfm bacteremia isolates, we observed that each time a new \textit{E. faecium} ST appeared it was observed first as VSEfm before subsequent detection as VREfm, suggesting lateral gene transfer of the \textit{vanB} locus into colonizing VSEfm, prior to the onset of clinically apparent bacteremia.

The hand-hygiene product used at Austin Health contains 0.5% chlorhexidine and 70% isopropyl alcohol with an emollient [23]. In our study, we observed similar MICs to chlorhexidine and triclosan to that previously found for enterococci, with previously reported MIC$_{90}$ ranging from 2 to 8 mg/L for chlorhexidine and 3 to 8 mg/L for triclosan [34–36]. More importantly, there were no differences in susceptibility to chlorhexidine or triclosan, or to non-glycopeptide antibiotics, between VRE and VSE isolates or between STs that could explain the success of ST203, compared with ST17, or the increase in VRE bacteremia, compared with MRSA. We also believe that reduced susceptibility to alcohol in our local hand-hygiene product is unlikely to explain the phenomenon of decreasing MRSA but increasing VRE prevalence, because both MRSA and enterococci are highly susceptible to isopropyl alcohol in vitro [37, 38], although this has not yet been formally studied.

Most clinical strains of \textit{E. faecium} belong to MLST CC17, and it has been proposed that CC17 is an example of continued evolution of a founder strain in the process of adapting to the conditions in a modern hospital [16]. Clinical isolates from CC17 have been shown to be more likely than others to bind to fibronectin and laminin, which may explain their increased virulence [39]. Other surface-expressed proteins linked to virulence in VRE include pilin and microbial surface components—recognizing adhesive matrix molecules [40], as well as the proteins encoded by \textit{esp} and \textit{hyl} [41]. ST203 is itself a member of CC17 and is a double locus variant from the original ST17 founder. However, when comparative genomics of AUS0004 (ST17, isolated in 1998) and AUS0085 (ST203, isolated in 2009)
Figure 3. Pulsed field gel electrophoresis (PFGE) patterns for all vancomycin-resistant *Enterococcus faecium* (VREfm) and vancomycin-susceptible *E. faecium* (VSEfm) study isolates that were multilocus sequence type 17 or 203.
was performed, we identified almost 500 kb of unique sequence, indicating that the outbreak strain is very different from its predecessor at the genomic level, but we could find no differences in the presence of known putative virulence factors thought to favor hospital adaptation. In both sequenced isolates, the \textit{vanB} was observed within \textit{Tn1549}-like elements that have been detected in gut anaerobes from our region [30]. Although VRE\textit{fm} ST203 has recently been identified as an epidemic VRE clone in hospitals in Germany, China, and Korea [42–46], this has been almost exclusively \textit{vanA}-containing VRE; hence, this is the first report of a significant ST203 \textit{vanB} \textit{E. faecium} outbreak.

The simplest interpretation of our data is the initial importation of VSE\textit{fm} ST203 as a colonizing strain that was already preadapted in some way, which then locally acquired \textit{Tn1549} containing the \textit{vanB} locus from other VRE\textit{fm} sequence types or cocolonizing anaerobes under antibiotic selection pressure [15, 30]. Repeated acquisition of \textit{vanB} by ST203 VSE\textit{fm} could explain the paradoxical increase in VRE bacteremia at our institution, which has doubled every 8 months since the beginning of 2007, coinciding closely with the first appearance of VRE\textit{fm} ST203. This has occurred in the face of a marked reduction in MRSA bacteremia during the same period, indicating improved infection control conditions, at least with respect to MRSA.

Our study has some limitations. First, as with any retrospective analysis, we cannot be certain that we have identified all potential confounding factors. For instance, ST203 VRE \textit{Ef}m bacteremia increased most markedly during period B in our hematology/oncology ward (11 of 19 ST203 VRE\textit{fm} isolates during period B). However, this is to be expected in this patient population, because gut flora are the usual source of nonnosocomial bacteremia when treatment-associated mucositis develops [47]. There were no specific changes in the management protocols or ward conditions in this or any other of our affected wards that could explain the increased rate of bacteremia. Furthermore, VRE\textit{fm} and VSE\textit{fm} ST203 bacteremia occurred in patients from a variety of hospital units, with <50% occurring in hematology/oncology patients overall. Second, we have not yet identified a phenotypic explanation for why ST203 prevalence has increased so dramatically in our institution and appears to have adapted so well to our hospital environment. Third, our MLST data are confined to blood culture isolates, and we do not know if a shift in ST among fecal enterococcal isolates has also occurred. However, given the usual epidemiology of VRE in which patients who develop infections are usually gut colonized with the same isolate [48], we believe our observations are likely to reflect a true shift in colonizing strains. The fact that this appears to have occurred for both VSE\textit{fm} and then subsequently VRE\textit{fm} is consistent with the possible local acquisition of \textit{vanB} by ST203 VSE\textit{fm} strains. Overall antimicrobial usage did not increase substantially for the years that data were available, suggesting that this alone does not explain the outbreak. Finally, although MLST is a powerful reference method for identifying emergent epidemic clones within a population of bacterial strains [49], the validity of MLST when applied to pathogens with high rates of recombination, such as \textit{E. faecium}, has recently been questioned [50]. Despite this concern, the use of MLST to investigate the outbreak of VRE\textit{fm} bacteremia at our institution has allowed significant insight into this outbreak.

In conclusion, our data provide further evidence for the ongoing lateral transfer of the \textit{vanB} resistance determinant into colonizing strains of VSE\textit{fm}; however, the frequency of this event requires further investigation. The use of MLST uncovered an important switch in the dominant MLST clone at our institution, which was associated with an exponential increase in VRE\textit{fm} bacteremia, but the reasons for the success of ST203

**Figure 4.** Hospital utilization of meropenem, vancomycin, and ceftriaxone from July 2004 through May 2009.

**Figure 5.** Optical map alignment comparison of AUS0004 (sequence type 17 strain isolated in 1998) and AUS0085 (sequence type 203 strain isolated in 2009) with use of NcoI restriction. Regions of similarity are highlighted in blue, whereas regions of difference are white. The regions in red represent likely inversions.
are still not clear. Clinicians should be alert to the emergence of new clones of VRE in their institution and the potential role of MLST to assist outbreak investigations.

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References


