Possible origin of a vanB-type Enterococcus faecium causing a multicentre outbreak in Sweden

Audun Sivertsen[1], Hanna Billström[2], Öjar Melefors[2,] Barbro Olsson Liljequist[2], Karin Tegmark Wisell[2], Måns Ullberg[3], Arnfinn Sundsfjord[1,4], Kristin Hegstad[1,4*]

[1] Research group for Host-Microbe Interactions, Faculty of Health Sciences, University of Tromsø – The Arctic University of Norway, Tromsø, Norway

[2] Division of antibiotics and infection control, Swedish Institute for Communicable Disease Control, Solna, Sweden.

[3] Department of Clinical Microbiology, Karolinska University Hospital, Huddinge, Sweden.

[4] Reference Centre for Detection of Antimicrobial Resistance, Department of Microbiology and Infection Control, University Hospital of North-Norway, Tromsø, Norway

*Corresponding author: Kristin.hegstad@uit.no

Abstract

Dissemination of vancomycin-resistant *Enterococcus faecium* (VREfm) with similar pulsed-field gel electrophoresis patterns in three Swedish hospitals between 2007 and 2011 prompted molecular characterization to reveal the possible origins and features of the strain. A representative subset of collected isolates (VREfm (n=18) and vancomycin-susceptible *Enterococcus faecium* (VSEfm) (n=2)) reflecting the spread in time and location was subjected to Multi Locus Sequence Typing, antibiotic resistance testing, virulence gene screening, characterization of mobile genetic units carrying the resistance gene and their ability to transfer. In addition, 3 outbreak strains and 1 isolate collected prior to the outbreak was whole-genome sequenced. The isolates were predominantly ST192, considered to belong within a high-risk lineage, and concordantly harbored at least eight virulence genes associated with high-risk genotypes, as well as were geno- and phenotypically resistant to ampicillin, gentamicin, ciprofloxacin and vancomycin, with susceptibility to teicoplanin. The vancomycin resistance was of *vanB2*-type, and this gene cluster was part of the conjugative transposon Tn*1549*/Tn*5382*. PFGE analysis with S1 nuclease restriction as well as filter mating experiments indicated that *vanB2*-Tn*1549*/Tn*5382* was placed in a 70 kb sized pRUM replicon, which readily transferred between *E. faecium*. The plasmid also contained an *axe-txe* toxin-antitoxin stability module capable of securing persistence within the bacterial host. The two VSEfm were similar by PFGE and MLST and harbored a 30 kb smaller pRUM plasmid lacking the *vanB2*-Tn*1549*/Tn*5382*.

In conclusion, the obtained results indicate introduction of *vanB2*-Tn*1549*/Tn*5382* into a pRUM plasmid harbored in a pre-existing high-risk clone. Afterwards, the resulting VRE containing the pRUM-*vanB2*-Tn*1549*/Tn*5382*-*axe-txe* plasmid successfully disseminated in the three hospitals.

Introduction

Enterococci, *Enterococcus faecium* in particular, have undergone a genomic transition from harmless gut commensals to leading causes of multidrug resistant hospital infections since the introduction of antimicrobial agents (1). *E. faecium* is frequently causing urinary tract infections, endocarditis, infections in indwelling catheters and septicaemia (2-4). Recent trends show a pronounced increase (more than 19 % per year) in bacteraemias caused by *E. faecium* in Europe (4). Their ability to gain antibiotic resistance and virulence factors by horizontal gene transfer and the emergence of strains in which these traits concentrate, might be attributable to this rise (1).

E. faecium recombines frequently, and the use of Multi Locus Sequence Typing (MLST) has been considered a standard method for global epidemiological surveillance (5), while pulsed-field gel electrophoresis (PFGE) is the preferred method for surveillance of *E. faecium* seen in local outbreaks. The clonal complex (CC) 17 has shown to pool *E. faecium* strains involved in hospital-associated infections and the CC17 genogroup has a high rate of recombination, displays a broad resistance profile as well as a concentration of genes mediating virulence (6-8). Newer insights pertained with another type of population structure analysis; Bayesian Analysis of Population Structure (BAPS) of strains in CC17, show a divergent origin of sequence type (ST) lineages within CC17. The isolates in CC17 could largely be found and divided in two BAPS groups, 2-1 (ST78), and 3-3 (ST17 and ST18), with the corresponding MLST ancestry nodes in parenthesis (9).

Globally, resistance to widely used antibiotics such as ampicillin, gentamicin, ciprofloxacin and vancomycin is increasing in prevalence. Vancomycin resistant enterococci (VRE) gain resistance by acquisition of one of eight resistance gene clusters, *vanA,B,D,E,G,L-N* (10-13). The *vanA* genotype is the most prevalent globally, but VRE infections with the *vanB* genotype are predominant in Australia and on the rise in many European countries (14-19). The *vanB* gene cluster confers inducible low- to high-level resistance to vancomycin and susceptibility to teicoplanin and has 3 gene sequence subdivisions; *vanB1-3* (20). The predominant subtype *vanB2* is an integral part of an Integrative Conjugative Element transposon family Tn*1549*/Tn*5382* which might infer conjugative dissemination of *vanB2* (21). Several replicons representing plasmids associated with glycopeptide resistance as well as stabilizing toxin-antitoxin systems have been linked to CC17 strains. Several possible virulence genes

have been associated with *E. faecium* and it is hypothesized that their phenotypes might work in concert to establish invasion of a host (2).

The prevalence of vancomycin resistant *E. faecium* (VREfm) in Sweden remained low until 2007 when a large hospital associated outbreak occurred (15). Three different hospitals in separate counties of Sweden were involved in the transmission of VREfm harbouring the *vanB* gene. This multicentre outbreak was not declared over until 2011.

The aim of the present study was to explore the origins of the outbreak strain by performing molecular characterization of different isolates of the outbreak strain and comparing them with consecutive invasive *E. faecium* isolates from the same time period and location. The selected strains were characterized with regard to clonal relatedness and in-depth molecular analysis.

Materials and methods

Bacterial isolates

In Sweden, all cases of vancomycin-resistant enterococci are mandatorily reported to the Swedish Institute for Communicable Disease Control (SMI) and the respective isolates are collected for verification of resistance and for epidemiological typing at SMI. In 2007, an increasing number of notified cases were seen in Stockholm County, related to an *E. faecium* with *vanB* gene. This strain subsequently caused clonal dissemination also in two other geographically separate counties (Västmanland and Halland) (15). During the autumn 2008, 17 isolates of the outbreak strain from these three counties were selected for further studies. They consisted of isolates found early and late during the outbreak period, and also isolates with different resistance profiles (Figure 1, Collection A). In addition, four blood isolates from Collection B (see below) were included.

In an attempt to reveal the origin of the *E. faecium vanB* outbreak strain, all consecutive *E. faecium* blood isolates from 1st of January 2006 to 31st of August 2009 (n=191) diagnosed at the Karolinska University Hospital Huddinge where the outbreak started, were collected and analysed using Pulsed-Field Gel Electrophoresis (PFGE). A total of 45 (2006), 32 (2007), 71 (2008) and 43 (2009) isolates were investigated (Figure 1, collection B). Out of these blood isolates, four were selected for further analysis using 454 full genome sequencing. The selection criterion was based on PFGE patterns, where the first vancomycin susceptible (VSE1036), the first vancomycin resistant (VRE1044) and the most recent vancomycin

resistant (VRE1261) isolate with identical or closely related patterns to the outbreak strain, were chosen. In addition, one vancomycin resistant isolate (VRE576) from 2006 was chosen because of its divergent PFGE pattern (Figure 1). For the filter mating experiments *E. faecium* 64/3 (22), BM4105RF and BM4105-Str (23) were used as recipients.

Antimicrobial susceptibility testing

The minimum inhibitory concentration (MIC) was determined using Etest (BioMerieux). The methodology and clinical breakpoints applied were according to the European committee of Antimicrobial Susceptibility Testing (EUCAST) (www.eucast.org/). For ciprofloxacin a tentative breakpoint was used classifying isolates with MIC >32 mg/L as high level resistant (39).

PFGE

For *Sma*I-digestion, the protocol adapted by Saeedi *et al.* (24) was used with 5U/mL lysozyme added in the lysis buffer. The bands were separated with the following program: Block I switch time 3 to 26,5s for 14 hours and 50 minutes. Block II: switch time 0,5 to 8,5s for 6 hours and 25 minutes. Total run time 21 hours and 15 minutes at 6V with 120°. The PFGE patterns were analysed and compared using BioNumerics software (version 6.6, Applied Maths). The Dice coefficient was used for pair-wise comparison of patterns, and the un-weighted pair group method with arithmetic mean (UPGMA) for pattern grouping. Isolates clustering above 97% were considered identical and isolates with identity > 90% closely related.

Multi Locus Sequence Typing

MLST was performed using the method adapted by Homan *et al.* (5) with the following primers: adk1n, adk2n, atp1n, atp2n, ddl1, ddl2, gdh1, gdh2, gyd1, gyd2, pstS1n, pstS2n, purK1n and purK2n. The whole genome sequencing (see below) also allowed us to extract a sequence type (ST) from the VRE0576 (ST17), VRE1036 (ST192), VRE1044 (ST192) and VRE1261 (ST192) isolates.

Detection of genes by PCR and isolation of bacterial DNA

Extraction of DNA for all PCRs were performed by BioRobot M48® (Qiagen®), according to the manufacturers manual.

The chosen virulence genes are associated with high-risk genotypes, and encode proteins involved in biofilm formation (*esp*) (25), tissue permeability (*hyl*) (26), host tissue attachment (*acm* (27), *efaAfm* (18), *srgA, ecbA, scm*, *orf903/2010/2514* (28, 29) and pili formation (*pilA/B*) (28). The PCRs were conducted as stated in Table 1. The presence of genes was tested by PCR using the JumpStart™ REDTaq® Readymix PCR Reaction mix (Sigma®), with a standard program of 1 min in 95° C followed by 30 cycles at 95 °C for 30 sec, 30 sec of annealing in the temperature given in Table 1, 72 °C for 1 min with a final elongation step at 72 °C for 7 min. Presence of the *vanB* gene in transconjugants was tested by *vanB* consensus PCR by inoculating 1 µl of BHI-broth bacterial culture into the PCR mix and using an initial denaturation period of 10 min at 95 °C.

Southern blotting and hybridization

S1-nuclease digestion was used to analyse the plasmid content. Plugs were made as for *Sma*I digestion, and the digestion was performed as described by Rosvoll *et al.* (30). The Vacugene XL system (Amersham Biosciences) was used for Southern blotting. Consecutive hybridization was performed using rep_{pRUM}, *vanB* and *axe-txe* probes in the mentioned order. Probes were made by amplification using positive controls (see Table 1), and labelled using the PCR DIG synthesis kit (Boehringer Mannheim). The same hybridisation protocol as in Rosvoll *et al.* (30) was used with the following modification: The DNA was purified after the first PCR using the Cycle Pure Kit (zDNA®).

Conjugative transfer of *vanB*

Filter mating was performed according to Bjørkeng *et al.* (18) with some minor modifications, using the *E. faecium* 64/3 and *E. faecium* BM4105-RF as recipient strains for the first filter mating, and BM4015-Str in retransfer. Briefly, the isolates were grown together on MF-Millipore membrane filters for 24h, spotted on selective BHI agar plates containing either vancomycin (8 mg/L), fusidic acid (10 mg/L) and rifampicin (20 mg/L), or all three antibiotics together. The bacterial suspension was serially diluted down to 10^{-9} and incubated at 37 °C for 48h. In the retransfer experiments, the recipients were selected on plates containing 1000 mg/L streptomycin.

454 whole genome sequencing

Chromosomal DNA from the four isolates (VSE1036, VRE1044, VRE1261 and VRE0576) was prepared using the DNeasy Blood and Tissue Kit (Qiagen) with lysozyme (20 mg/mL)

added to the lysis buffer and further treated with RNase. This protocol allowed purification of extra-chromosomal plasmids. Libraries were prepared and used for whole genome shotgun sequencing on a 4-region picotiter plate with the Roche 454 FLX system according to standard protocols (www.454.com). Raw sequencing data were processed with standard filters using the GS Run Processor (v 2.6), generating between 246084 and 310421 reads for each of the strains with average lengths between approximately 307 and 320 nucleotides, corresponding to between 78750175 and 99124035 nucleotides. Reads were assembled de novo with the accompanying GS de novo assembler software (v 2.6) (454 Newbler algorithm) generating between 201 and 302 contigs with a length of more than 100 nucleotides. The GS Reference mapper software (v 2.6) was subsequently used for homology comparisons between the different strains and to find homologies to specific query gene sequences and also used to identify indels and point mutations that separated the different strains. Some small plasmids could be identified by screening for contigs where individual reads mapped to both ends of the contig. The contigs were tentatively linked to each other by a collection of evidence, including comparison with molecular biology data, identification of individual reads mapping to two different contigs and comparisons with published genomes. The tentative gene content of the contigs was analysed with the tBLASTx software tool (NCBI).

Results

PFGE patterns and antimicrobial susceptibility

All *E. faecium* blood isolates from the collection at Karolinska University Hospital Huddinge (n=191, Collection B) were analysed using PFGE, retrospectively. One PFGE pattern dominated and comprised 37 isolates. This pattern was identical or closely related to that of the VRE outbreak strain from the large outbreak 2007-2010 (named SE-EfmB-0701 according to the Swedish nomenclature). Among the blood isolates, this PFGE pattern was observed for the first time in a vancomycin susceptible isolate (VSE) in February 2007 and soon after in two more VSE. During the autumn of 2007 this PFGE pattern was detected in three VRE. It should be mentioned that the first known clinical isolate of this VRE strain (subsequently called the outbreak strain) was found in an abdominal infection in August 2007. In 2008 another eight VRE from blood with the same PFGE pattern were detected, but also in 14 VSE isolates. During the observation period from January until August 2009 no more VRE were detected but still 10 VSE of the SE-EfmB-0701 PFGE type.

Antimicrobial susceptibility testing and PCR for detection of *van* genes performed on these 37 blood isolates showed that a total of 11 (30 %) isolates were resistant to vancomycin and 14 (38 %; 9 VSE and 5 VRE) had high level resistance (HLR) to gentamicin. All 37 were resistant to ampicillin and ciprofloxacin but susceptible to teicoplanin. The *vanB* gene was detected in all eleven vancomycin-resistant isolates.

In collection A, with isolates representing PFGE patterns as diverse as possible at the time of selection (autumn 2008), a total of 9 subgroups (a to i) of the pattern SE-EfmB-0701 were noted (Table 2 and Figure 2). All these PFGE patterns displayed a similarity > 90%, thereby fulfilling the definition of clonality (> 81%) according to Morrison *et al.* (31). The isolates did however not group together consistently in relation to their geographical origin (Figure 2).

The nineteen VRE isolates in this collection had vancomycin MICs ranging from 8 to ≥256 mg/L and were susceptible to teicoplanin, consistent with the *vanB2* genotype. All the isolates were resistant to ampicillin and ciprofloxacin, and seven displayed high-level resistance to gentamicin. The two isolates lacking the *vanB* gene (VSE1036 and VSE1027) were susceptible to both vancomycin and teicoplanin, but otherwise showed the same resistance pattern as the other isolates, with the exception of gentamicin (Table 2).

Molecular characterization

Twenty one isolates were studied in greater detail by MLST and PCR for virulence genes (Table 2). Three isolates of the outbreak strain (according to PFGE) and one unrelated VRE isolate were also whole genome sequenced (see Materials and methods), generating a series of contiguous sequences with high sequence coverage.

A total of 19 isolates, including the two VSE isolates, belonged to ST192. Two isolates were either single locus (VRE0673, ST78) or double locus (VRE0881, ST17) variants of ST192. All except the ST17 isolate belonged to the ST78 lineage. All isolates except VRE0576, a pre-outbreak *vanB*-positive ST192 isolate from 2006, had identical or closely related PFGE pattern, as shown by *Sma*I PFGE (Figure 2). The MLST- and PFGE results concurred well in showing the similarity of the isolates, since the isolates that deviated in MLST profiles (VRE0673 and VRE0881) also deviated the most in their PFGE patterns.

Molecular antimicrobial resistance patterns

The *vanB* genotype of vancomycin resistance is divided into three subtypes where *vanB2*, which is linked to the conjugative transposon family Tn*1549*/Tn*5382*, is the most predominant. All VRE isolates in collection A harboured the *vanB2* gene as an integral part of Tn*1549*/Tn*5382* by the *vanX*$_B$-ORFC-PCR as described by Dahl *et al.* (32). This link was confirmed in the whole genome sequences (WGSs) of the VRE isolates, see Table S1. Analyses of the WGS data show that in VRE576 the *vanB2* transposon shows the same genetic organisation (Figure 3) as well as 99% nucleotide (nt) identity to Tn*1549*. In VRE1044 and VRE1261 the *vanB2* transposon also show the same organisation and 99% nucleotide identity to Tn*1549*, but has an additional 2588 bp inserted between nt 5014 and 5015 of Tn*1549* (Figure 3). Since this insertion is a unique signature of the *vanB* transposon in two SE-EfmB-0701 isolates we are currently working to confirm if the transposon has this signature in the other SE-EfmB-0701 isolates by ICE*slu*van Q8 PCR (Table 1) and sequencing. The 2588 bp insert sequence is 89% identical to the region in *Clostridium saccharolyticum-like* K10 (GenBank Acc. No. FP929037), which encodes a retron-type reverse transcriptase. In line with this, the 2588 bp sequence encodes a putative protein of 610 amino acids (aa) with 99% identity to a putative reverse transcriptase/maturase from *Faecalibacterium prausnitzii* A2-165 (GenBank Acc. No. EEU96266) and a putative group II intron-encoded protein LtrA (reverse transcriptase and RNA maturase) from *Flavonifractor plautii* ATCC 29863 (GenBank Acc. No. EHM54980). The putative protein further shows 43% identity to the group II intron 599 aa multifunctional protein LtrA in *Lactococcus lactis* (GenBank Acc. No. U50902) known to have reverse transcriptase, RNA maturase and site-specific DNA endonuclease activity mediating intron splicing and mobility (33).

The previously described genetic linkage between *pbp5* (involved in high-level ampicillin resistance) and Tn*1549*/Tn*5382* was not detected by PCR in this study (34). The *gyrA* and *parC* genes were extracted from the WGSs and analysed to find possible loci associated with ciprofloxacin resistance. Two mutation events in each gene were found (Table 2), and both amino acid combinations (GyrA Arg83, ParC Ile80 or GyrA Ile83, ParC Arg80) have been described previously in *E. faecium* isolates with ciprofloxacin MICs ≥ 16 mg/L (35-37). Further on, genotypic tetracycline resistance (*tetM*) was also found in the three fully sequenced outbreak isolates, but not in VRE0576. Erythromycin resistance genotype was found in all the fully sequenced isolates (Table S1).

Presence of virulence genes

All isolates of the PFGE type SE-EfmB-0701 harboured *esp*, *sgrA*, *acm*, *scm*, *pilB*, *efaAfm* *orf*2010 and *orf* 2514. Moreover 17 of 20 isolates contained *hyl*. The genes *pilA*, *ecbA* and *orf*903 were not prevalent, with an occurrence in six, one and one of 20 isolates, respectively. The last two genes were found in isolates with unique PFGE subtypes in this collection (VRE0776 and VRE0673). However, the genome sequences showed that *pilA* (VRE1044 and VRE1261) and *ecbA* (VRE1036, VRE1044 and VRE1261) were present albeit with a reduced identity corresponding to a nt match of 1672/1976 (85%) for *pilA* and 2766/3173 (87%) for *ecbA* compared to reference isolates E1162 and TX16 (data not shown). The *pilA* and *ecbA* primers used yielded no good matches in these sequences, thereby concluding that these genes may appear in a form not recognised by the performed PCRs.

vanB presence on pRUM plasmid

The vanB resistance gene co-hybridised with rep_{pRUM} on a ~70kb plasmid

By comparing the VSE1036 isolate with the VRE1044 and VRE1261 isolates the exact AT-rich location of the *vanB2* transposon insertion site could be identified (Figure 4). Moreover, the transposon insertion site was identical for VRE1044 and VRE1261 and the insertion site corresponded with 100% identity to sequences in VSE1036. When comparing the gene content of the whole genome sequenced isolates additional stretches of DNA was observed in isolate VRE1044, all of which contained typical plasmid genes. We could also see that some plasmid-like pieces of DNA were not present in VRE1261, including a stretch containing gentamicin resistance. But as most resistance plasmids are of a large size and typically contain repeated IS-elements it was not possible to generate contiguous sequences of all plasmids. Only in one case a contig could be circularized into a complete plasmid.

To get a better picture of the plasmid composition in the various isolates we compared the results from S1 nuclease PFGE Southern hybridisation analyses with the replication (*rep*) gene specific probes for rep_{pRUM}, rep_{pLG1} and rep_{pRE25}. From the WGSs some additional plasmid replication genes could be identified, as well as a number of plasmid specific relaxases, resistance genes including the *vanB* gene, Tn*1549*/Tn*5382* and *axe-txe* gene sequences (Table S1). According to the WGS data replication genes representative of *rep* group families 2 (rep_{pRE25}), 9 (rep_{pTEF1}), 11 (rep_{pB82}), 17 (rep_{pRUM}), and pLG1 were present in all four isolates, with a slight homology difference between the pre-outbreak isolate (VRE0576) and the three outbreak isolates. Notably, VRE1044 and VRE1261 contained two

rep$_{pRE25}$-sequences with dissimilar homology on different contigs, which reflected the occurrence of two pRE25 plasmids in S1 nuclease PFGE Southern hybridization (Table 3 and S1). The third outbreak isolate VRE1036 had a similar Southern hybridization pattern as the other two isolates, but did not have an identical *rep*$_{pRE25}$-sequence pattern. The pre-outbreak isolate also differed from the outbreak isolates by lacking the *rep* group 14 (*rep*$_{pRI1}$) gene which were present with identical nt identity scores in the outbreak isolates, and by containing a putative *rep*$_{pCIZ2}$ gene absent in the outbreak isolates.

All designated VRE isolates harbour the *vanB* resistance gene on an approximately 70-kb *rep*$_{pRUM}$ replicon (data partly presented in Figure 5 lanes 5, 8 and 11 and Figure 6 lanes 5, 7 and 9). S1 nuclease PFGE hybridisation of selected isolates (Table 3) further revealed the following common pattern for isolates VRE0726, VRE0815, VRE0678, VRE1044, VRE1261, VRE0650, VRE0653, VRE0651 and VRE0673 with *rep*$_{pRE25}$ on a similar sized replicon as *rep*$_{pRUM}$ as well as on a 40-50 kb replicon, *rep*$_{pLG1}$ on a 160-kb replicon as well as an unknown replicon of approximately 100 kb. The *rep*$_{pRE25}$ and *rep*$_{pRUM}$ replicons of approximately 70 kb were difficult to separate by S1 nuclease PFGE (Figure 5 lanes 5, 8 and 11, Figure 6 lanes 5, 7 and 9), because of their similar size. However, all the VRE isolates showed a thick double band around 70 kb that on some gels could be resolved into two bands (Figure 5 lanes 5, 8 and 11, Figure 6 lanes 5, 7 and 9). VRE0673 contained an unknown replicon of around 10 kb (data not shown). The other VRE isolates showed variations of the common pattern showing either a different sized *rep*$_{pRE25}$ plasmid or lacking *rep*$_{pLG1}$ and the 70 kb *rep*$_{pRE25}$ plasmids (Table 3, Figure 5 lanes 5, 8 and 11, Figure 6 lanes 5, 7 and 9, and data not shown). 454 sequence data revealed that VSE1036, VRE1044 and VRE1261 contained a *rep*$_{pRI1}$ replicon of 2.9 kb and a *rep*$_{pB82}$ replicon of 6.1 kb. Furthermore, the two VSE isolates (VSE1027 and VSE1036) belonging to the same clone according to PFGE analysis differed from the common pattern by having a *rep*$_{pRUM}$ replicon that was approximately 30 kb less than that of the VRE isolates (Table 3 and data not shown). This finding was expected since 30 kb is the approximate size of the Tn*1549*/Tn*5382* transposon carrying the *vanB2* vancomycin resistance gene. Notably, the pRUM *repA* was found with 100% homology to the reference sequence in VRE0576, and present in the three outbreak isolates with identical nucleotide differences. Tn*1549*/Tn*5382*-related genes, *axe-txe* and the *vanB2* gene were found within the same contig in the VRE1044 and VRE1261 isolates, thus supporting the S1 nuclease PFGE hybridisation and PCR data showing linkage of *vanB2*-Tn*1549*/Tn*5382* and *axe-txe* genes on the same replicon. The S1-nuclease PFGE hybridisation data further mapping *vanB2*-Tn*1549*/Tn*5382* and *axe-txe* together with pRUM *repA* indicates

these are confined within the same plasmid even though the pRUM *repA* was found in other contigs. WGS analyses of VSE1036 showed that the sequence corresponding to the transposon insertion site of VRE1044 and VRE1261 (Figure 4) mapped to the same contig as *axe-txe* (Table S1). Interestingly, the reference pRUM sequence contains a putative relaxase gene and a mobilization gene both consistently absent from all four WGSs.

Transfer of *vanB*

The plasmid containing vanB, axe-txe and rep_{pRUM} is transferable and transferability increases in retransfer experiments after the plasmid has picked up additional genetic material containing rep_{pRE25}

All but 2 (VRE0651 and VRE0673) out of 10 tested VRE isolates produced transconjugants with a low frequency of 10^{-8} to 10^{-11} per donor, close to detection limits with the applied method, when conjugated with the *E. faecium* recipient 64/3 (Table 4). Conjugation of the isolates with *E. faecium* recipient BM4105-RF yielded no transconjugants (data not shown).

S1 nuclease PFGE and subsequent hybridization of the 64/3 transconjugants revealed that the transconjugants had received a *vanB-*rep_{pRUM}*-*rep_{pRE25}-containing plasmid of variable size (110-150 kb), larger than the 70-kb plasmids of their donors. With the exception of VRE0690x64/3 (150 kb) (Figure 6 lane 4) and VRE0776x64/3 (110 kb) (Figure 6 lane 8), all the other transconjugants had a *vanB-*rep_{pRUM}*-*rep_{pRE25} containing plasmid of about 140 kb (Figure 5 lanes 6, 9 and 12 and Figure 6 lane 6 and data not shown). The transconjugants were then tested for susceptibility to streptomycin in order to identify eligible donors for a retransfer. Out of the 8 1st generation transconjugants, only the three originating from donors VRE0726 (ST192), VRE0734 (ST192) and VRE0881 (ST17) were susceptible to streptomycin.

Retransfer attempts succeeded in demonstrating that the plasmids in these transconjugants were stable and readily transferable with transfer rates of 10^{-3}-10^{-5} transconjugants per donor. The following S1 nuclease PFGE and Southern hybridisation revealed that the plasmids in the second generation transconjugants had a stable size at around 140 kb, similar to the plasmid size in their donors. These plasmids also co-hybridized with rep_{pRUM}, *axe-txe*, *vanB* (Figure 5 lanes 7, 10 and 13) and rep_{pRE25} (data not shown).

Discussion

The extensive spread of an *E. faecium* with *vanB* in Sweden from 2007 to 2010 prompted us to further investigate selected isolates from the hospitals where the dissemination took place by an array of molecular methods to get a better insight into the genetic characteristics of this successful outbreak strain, and possibly to find the ancestor. In Sweden, mandatory reporting of VRE according to the Communicable Disease Act includes reporting of all findings of resistant organisms, both from infected and colonised patients. In short, the actual outbreak strain was found mostly in elderly hospitalised patients with underlying diseases, and it was mostly found as a coloniser. Less than 10% of the patients had a clinical infection where VRE was found in blood, urine or wounds (15). Nevertheless, it remains worrisome when a VRE strain is spread among severely ill patients already burdened with several risk factors. In an attempt to identify a probable ancestor of the outbreak strain, we were able to study consecutive blood isolates of *E. faecium* from Karolinska University Hospital, Huddinge, from a four-year period (2006-2009) around the time of appearance of the presumably first clinical isolate of the outbreak VRE strain. Based on the findings of PFGE analysis of all blood isolates, it is highly probable that this strain, as a vancomycin-susceptible variant, already from 2007 was a successful hospital inhabitant and that the acquisition of the *vanB* gene cluster made it even more contagious. VSE but not VRE of the outbreak strain could still be found among invasive isolates in 2009, which could imply parallel development lines for the VRE clone and the VSE clone. Despite efforts it has not been possible to identify a common source which could explain the dissemination of the strain in three different counties.

Molecular characterization of a subset of the isolates done by us has shown that a PFGE pulsotype belonging predominately to ST192 is causative of the observed increased incidence of VRE. This sequence type is a single-locus variant of the ST78 line and is considered to be a high-risk genotype. The MLST- and PFGE results concurred well in showing similarity of the isolates, as the isolates that diverged in the MLST screening (VRE0673/0881) also diverged the most in the PFGE profile. The PFGE subtypes characterized by single- or double band differences did not group together in time or location which could be caused by the length of time (2 years) of sample collection. The isolate VRE0881, considered to be clonal by PFGE, belonged to ST17 which is a single locus variant of ST78 and a double locus variant of ST192. Others (9) have by BAPS concluded that the ST78 and ST17 lineages are not related,

which is paradoxical to our PFGE results which grouped VRE0881 in the SE-EfmB-0701 PFGE strain. Since these data are controversial, both MLST and PFGE were repeated and confirm that these methods disagree whether VRE0881 is related to the other isolates in this clone. The overall presence of high-risk genotypes in this population prior to this outbreak is not known, albeit signs of emergence of high-risk genotypes in Scandinavia have been seen (18, 38, 39)(Lester et al., 2009. Poster P844 ECCMID), and an outbreak of ST192 strains containing *vanB2* has also been seen in Germany in 2008-2009 (17). The isolates in CC17 could largely be found and divided in two BAPS groups, 2-1 (ST78), and 3-3 (ST17 and ST18), with the corresponding MLST ancestry nodes in parenthesis. BAPS 3-3 showed a strong association with hospital-associated isolates whereas BAPS 2-1 was associated with VRE found in animals, thus it is speculated that STs linked to BAPS 2-1 might originate from a farming reservoir (9).

The characterized isolates were both multi-resistant to antibiotics and harboured a range of genes associated with increased invasiveness and virulence, concordant with a high-risk genotype. All isolates had a VanB phenotype resistance, except two VSE1027 and VSE1036 isolates collected at the start of the outbreak period. However, these VSE isolates were clonal to the *vanB*-type resistant *E. faecium* isolated later, and except for vancomycin resistance exerted the same resistance- and virulence profile as the other isolates (Table 2). Both plasmid profile and WGS data (Table 3 and S1) also indicated close relatedness of the isolates within the SE-EfmB-0701 PFGE strain with minor differences in plasmid profile. The WGS data further show that there are clear differences both in content and identity score between the pre-outbreak isolate and the three outbreak isolates.

Our results indicate that internalisation of the *vanB* transposon into the pRUM plasmid is at least a partial explanation for the success of this high-risk clone. The rep_{pRUM} replicon has previously been shown to harbour a segregation stability module encoded by a toxin-antitoxin cassette (*axe-txe*) which may support maintenance of linked antimicrobial resistance genes (40). The tested SE-EfmB-0701 pRUM replicons were shown to contain the *axe-txe* stability module which in the WGSs showed 100% identity to the original pRUM *axe-txe* sequence. The toxin (*txe*) is experimentally shown to be an endonuclease capable of cleaving mRNA one nucleotide downstream from the AUG start codon (41). In an *E. coli* model set up by Boss *et al.*(42), expression of the *axe* and *txe* genes is meticulously regulated by promoters and repressors for both or one of these bi-cistronically transcribed genes to ensure stable toxin

production and further a toxin/antitoxin ratio securing stability of this gene complex in a host. The complete mechanism of how this system operates is still not shown, and it might also change between bacterial species. VSE1027 and VSE1036 contained a 40 kb pRUM-like plasmid which is approximately 30 kb smaller than the pRUM replicons of the VRE isolates. The 30 kb lacking corresponds to the size of the vancomycin resistance carrying transposons Tn*1549*/Tn*5382*. All VanB-type VRE isolates in collection B contained this 70 bp "pRUM-*vanB2*-Tn*1549*/Tn*5382*-*axe*-*txe*-plasmid" with no variance in size by timeline or geographic location. WGS data confirms presence of *vanB2*, Tn*1549*/Tn*5382*-related genes and the *axe*-*txe* system within the same contig while $repA_{pRUM}$ was found in other contigs which does not contradict the hybridisation results since no plasmid of this size, *pRUM* included, was successfully circularized. A pRUM-*vanB2*-Tn*1549*/Tn*5382*-*axe*-*txe*-plasmid of approximately 120-130 kb has previously been described in a polyclonal cluster of *E. faecium* from 2002-2004 in the Swedish county Örebro. This cluster originated from BAPS-group 3-3 (ST17, ST18 and single locus variants of these) (18). The Örebro isolates were however different from the present strain SE-EfmB-0701 according to PFGE analysis (data not shown).

The observation of rep_{pRUM} plasmids with the *vanB2* transposon transferring to a recipient *in vitro* and in the process consistently gaining genetic material is a surprising feature. The "wild-type" plasmid was present only as a 70 kb size, but after conjugation, was considerably larger (110-150 kb) and had fused with a rep_{pRE25} replicon. The WGSs in our study did not contain the putative relaxase and mobilization protein associated with the reference pRUM sequence, explaining the need to fuse with a conjugation system from other intracellular sources in order to be mobilised. Freitas *et al.* (43) have stressed the propensity of independent shuffling and variation of plasmids present in enterococci, thus indicating a high degree of fluidity of plasmid gene content variation. They also described mosaicism and/or merging of plasmids for enhanced host range or other functional benefits as well as the occurrence of several replicons in one plasmid. All in all, fusion with another replicon could account both for the observed plasmid size increase and subsequent gain in transfer frequency. The observed differences in speed of transfer could be explained by HGT "affinity" differences between individual strain backgrounds. Since initial transfer to recipient BM4105-RF did not succeed whereas 2nd generation donors readily transferred the fused plasmid into BM4105-Str which is derived from the same mother strain as BM4105-RF (23), such changes must rather involve difference in the donor than recipient potential.

There is a noteworthy reservoir of *vanB* in intestinal anaerobes, and Howden *et al.* (44) noted the possibility of introduction of *vanB*-type vancomycin resistance on Tn*1549*/Tn*5382* in VSE from other co-habitants (mainly Gram-positive anaerobes) in the intestinal environment. By WGS, recipient insertion sites of these transposons as well as a phylogenetic analysis revealed a diversification likely due to a higher grade of *de novo* VRE generation compared to cross-transmission between enterococcal strains than previously believed. They also found increasing incidence of VRE infections intrahospitally despite normal screening and preventive action to limit spread of virulent clones. Acquisition of the *vanB2* transposon into the Swedish outbreak strain is a likely theory of how vancomycin resistance appeared in this strain. The WGSs show that the *vanB* transposon likely was inserted within the *pRUM*-like plasmid of a strain already present, further causing a parallel evolution between VSE clones without, and VRE clones with the pRUM-*vanB2*-Tn*1549*/Tn*5382-axe-txe* arrangement. Notably, the pRUM plasmid replicon has been found in 74% of CC17 strains by Rosvoll *et al.* (26), and this replicon was strongly associated with the toxin-antitoxin gene cassette *axe-txe*.

Several experiments studying bacterial ecology in the intestines during and after antimicrobial therapy point to potentially hazardous effects of prior colonization of VRE, as these bacteria could end up dominating the intestinal flora (45). VRE colonization could persist for months after therapy cessation, and suggested clearance time post-infectionally is in the literature suggested to be 4 years (46). Still, others suggest presence of resistant environmentally adapted VRE capable of inhabiting the intestines in small numbers for even longer periods of time (47, 48). Infections of multi-resistant bacteria seem to add to rather than replace infections by susceptible bacteria, thus creating an additive strain to health services (4). Together, this suggests the presence of VRE in Sweden can become a lasting problem.

MIC values for vancomycin varied from 8 mg/L to larger than 256 mg/L in the tested isolates in our study. Moderate to low vancomycin MIC phenotypes of *vanB*-type VRE is a challenge to phenotypic detection and screening methods. The selective enriched broth used in some laboratories in Sweden before January 2009 with a vancomycin concentration of 32 mg/L was not suitable to screen for *vanB*-type resistance. To address this problem, microbiological laboratories were then advised to reduce the concentration to 4 mg/L (15). However, *vanB*-type VRE may have even lower MICs (17, 49). Moreover, the EUCAST disk diffusion test used by most laboratories in Sweden as the phenotypic vancomycin-susceptibility test method

rely on observing the zone edge quality for identification of low level *vanB*-type resistance, thus having observer experience as a variable (Hegstad *et al.*, in manuscript).

In conclusion, molecular characterization revealed that the disseminated *E. faecium* strain belonged to the high-risk ST192. The strain was resistant to several antibiotics and harboured several virulence genes. A successful pRUM-like plasmid containing a *vanB* transposon that possibly originate from other intestinal species, was present in all the VRE isolates within this strain. This plasmid harboured a toxin-antitoxin stability module capable of ensuring plasmid persistence. In addition the pRUM replicon easily spreads within *E. faecium*, thus making it probable to enter high-risk clones. A feature possibly hampering prevention or limitation of this dissemination of VRE might be the phenotypic screening methods previously and presently used, as low-MIC *vanB*-type VRE might go under the radar.

Acknowledgement

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TABLE 1. Primers used in this article

PCR target:	Primer sequence (5'-3')	Amplicon size (bp)	Annealing temp. (°C)	Positive control	Reference
acm	TGACGAGCGGTGATAAAACAGCTA ATAGGCTGTTCATCTGCTCGTCTTA	636	53	TUH7-15 (20)	**(50)**
efaAfm	GTTCGATAACTTGATGGAAAC CATCTGATAGTAAGAATCTCCTTG	561	53	TUH7-15	**(18)**
esp	AGATTTCATCTTTGATTCTTGG AATTGATTCTTTAGCATCTGG	510	55	TUH7-15	**(25)**
hyl	GTTAGAAGAAGTCTGGAAACCG TGCTAAGATATTCCTCTACTCG	Ca 500	53	TUH7-15	**(26)**
***srgA* (orf2351)**	AATGAACGGGCAAATGAG CTTTTGTTCCTTAGTTGGTATGA	671	50	TUH7-15	**(28)**
***ecbA* (orf2430)**	GCAGTTTACAATGGTGTGAAGCAA CGGCTAATGAGTATTTGTCGTTCC	963	55	TUH7-15	**(28)**
***scm* (orf418)**	CTAACTGGTAACTATGGCTTGT GTCCGTGCTGTCACTTGT	1109	55	TX16 (51)	**(28)**
***pilA* (orf1904)**	AGGCAGATTATGGTGATGTT GGCTGTTGGTTCTTTATCTG	619	55	TX16	**(28)**
***pilB* (orf2569)**	GTGTTTGCAGAGGAGACAGC GACAGAATAATTTACTGGGTCG	1121	55	TX16	**(28)**
orf903 (fms11)	TCAACGGACATACCATACCA CTTACCATCAACGATCTGCC	409	55	TX16	**(28)**
orf2010 (fms14)	GTAGCGAAGAAAATGAGATGG TAACTTGACTGAATCGGTGC	1021	55	TX16	**(28)**
orf2514 (fms15)	AGTTCCAGTTGCGAGTCAGA ATGTAGTCGGATTCGGTGC	989	55	TX16	**(28)**
***vanB* consensus**	CAAAGCTCCGCAGCTTGCATG TGCATCCAAGCACCCGATATAC	484	58	C68 (34)	**(20)**
***vanX*$_B$-ORFC**	ATCAAGGACTCAACCGTAATT TGAGTTGTGGAAGTCGATTAGAG	873	60	C68	**(32)**
pbp5-Tn5382	TCAGCCGATTTGCGACAGGTTATG TGGGGTGGCGGGTATTAGCAGTAT	1079	68	TUH7-15	**(34)**
axe-txe	CTTTAATGGCTCAGGTTTTCCTAA ATGAGGATGCTGAAACACTTATT	351	55	U37	**(30)**
***rep*$_{pRUM}$**	TACTAACTGTTGGTAATTCGTTAAAT ATCAAGGACTCAACCGTAATT	604	52	U37 (52)	**(53)**
***rep*$_{pLG1}$**	TTTAAGGCGGATAGAGTTTACAACG CTGATAGGCTTTTAACAGTGTCGTGT	864	56	TX16	**(38)**
***rep*$_{pRE25}$**	GAGAACCATCAAGGCGAAAT ACCAGAATAAGCACTACGTACAATCT	630	56	RE25 (54)	**(53)**
ICE*slu*van Q8	CAAGTGGTAACGCAGGATGA AAAGATAGCCGTCTGCGTGT	2512/5100		5-F9	**(55)**
ICE*slu*van Q12-15	CTTTGCGAGGGCCAGACCTT GTCTGTTCCATTTGGGCAAG	3006		5-F9	**(55)**

TABLE 2. Demographic data and characteristics including results from MLST- and PFGE analyses as well as antimicrobial susceptibility testing and presence of virulence genes.

Isolate ID	County	Material	Year of isolation	MLST type	PFGE type[a]	*vanB2*-Tn*5382*	Antimicrobial susceptibility[b]					Mutations involved in CIP resistance		Virulence genes[c]
							VAN MIC	TEI MIC	GEN	AMP	CIP	GyrA	ParC	
VRE0673	Halmstad	Faeces	2008	78	SE-EfmB-0701g	Pos	8	0,5	R	R	HLR	NT	NT	*hyl, orf903*
VRE0726	Halmstad	Wound	2008	192	SE-EfmB-0701d	Pos	≥256	0,5	R	R	HLR	NT	NT	*hyl*
VRE0734	Halmstad	Faeces	2008	192	SE-EfmB-0701c	Pos	64	1	R	R	HLR	NT	NT	*hyl, pilA*
VRE0762	Halmstad	Faeces	2008	192	SE-EfmB-0701c	Pos	≥256	1	R	R	HLR	NT	NT	*hyl, pilA*
VRE0815	Halmstad	Faeces	2008	192	SE-EfmB-0701c	Pos	16	0,5	R	R	HLR	NT	NT	*hyl*
VRE0651	Stockholm	Abdomen	2007	192	SE-EfmB-0701b	Pos	32	0,5	HLR	R	HLR	NT	NT	*hyl*
VRE0678	Stockholm	Faeces	2007	192	SE-EfmB-0701a	Pos	16	0,5	R	R	HLR	NT	NT	*hyl*
VRE0683	Stockholm	Faeces	2007	192	SE-EfmB-0701c	Pos	16	1	R	R	HLR	NT	NT	*hyl*
VRE0688	Stockholm	Faeces	2008	192	SE-EfmB-0701f	Pos	32	1	HLR	R	HLR	NT	NT	*pilA*
VRE0690	Stockholm	Faeces	2008	192	SE-EfmB-0701e	Pos	16	0,5	HLR	R	HLR	NT	NT	
VSE1036	Stockholm	Blood	2007	192	SE-EfmB-0701a	Neg	1	1	HLR	R	HLR	S83I	S80R	*hyl*
VSE1027	Stockholm	Blood	2007	192	SE-EfmB-0701a	Neg	2	1	R	R	HLR	NT	NT	*hyl, pilA*
VRE1044	Stockholm	Blood	2007	192	SE-EfmB-0701a	Pos	32	1	HLR	R	HLR	S83I	S80R	*hyl*
VRE1261	Stockholm	Blood	2008	192	SE-EfmB-0701a	Pos	16	1	R	R	HLR	S83I	S80R	*hyl*
VRE0576	Stockholm	Blood	2006	192	EfmB Unique	Pos	64	0,5	R	R	HLR	S83R	S80I	*hyl*
VRE0650	Västerås	Urine	2008	192	SE-EfmB-0701a	Pos	32	0,5	R	R	HLR	NT	NT	*hyl*
VRE0653	Västerås	Urine	2008	192	SE-EfmB-0701a	Pos	16	0,125	R	R	HLR	NT	NT	
VRE0654	Västerås	Wound	2008	192	SE-EfmB-0701a	Pos	16	1	R	R	HLR	NT	NT	*hyl, pilA*
VRE0776	Västerås	Faeces	2008	192	SE-EfmB-0701h	Pos	≥256	0,25	HLR	R	HLR	NT	NT	*hyl, pilA, ecbA*
VRE0881	Västerås	Faeces	2008	17	SE-EfmB-0701i	Pos	32	0,5	HLR	R	HLR	NT	NT	*hyl, pilA*
VRE0892	Västerås	Faeces	2008	192	SE-EfmB-0701a	Pos	≥256	0,25	R	R	HLR	NT	NT	*hyl*

VAN, vancomycin; TEI, teicoplanin; GEN, gentamicin; CIP, ciprofloxacin; AMP, ampicillin; R, resistant; HLR, high level resistant; NT, not tested; S, serine; I, isoleucine; R, arginine.

[a] A 97% threshold similarity value of Dice dendrogram was used to designate PFGE subtype (small letter).

[b] Breakpoints for resistance were as follows: VAN, MIC>4 mg/L; TEI, MIC>2 mg/L; GEN HLR, MIC>128 mg/L; AMP, MIC>8 mg/L; CIP HLR MIC>32 mg/L.

[b] All isolates were positive for *esp, srgA, efaAfm, acm, scm, pilB, orf2010* and *orf2514* in addition to the virulence gene results showed in this table.

TABLE 3. Replicon size estimated from S1 nuclease PFGE hybridisation with *rep* probes on selected isolates and whole genome sequences (WGSs)

	Replicon sizes estimated by S1 nuclease PFGE				**Replicon size by WGS**	
Isolate ID (PFGE subtype) (unique ST)	*rep*$_{pRUM}$	*rep*$_{pRE25}$	*rep*$_{pLG1}$	Unknown replicon[a]	*rep*$_{pB82}$	*rep*$_{pRI1}$
VRE0726 (d)	70-kb	50-kb, 70-kb	160-kb	100-kb		
VRE0734 (c)	70-kb	25-kb, 70-kb	160-kb	100-kb		
VRE0815 (c)	70-kb	45-kb, 70-kb	160-kb	100-kb		
VRE0678 (a)	70-kb	50-kb, 70-kb	160-kb	100-kb		
VRE0688 (f)	70-kb	50-kb, 70-kb		100-kb		
VSE1036 (a)	40-kb	50-kb, 70-kb	160-kb	100-kb	6.1-kb	2.9-kb
VSE1027 (a)	40-kb	50-kb, 70-kb	160-kb	100-kb		
VRE1044 (a)	70-kb	50-kb, 70-kb	160-kb	100-kb	6.1-kb	2.9-kb
VRE1261 (a)	70-kb	50-kb, 70-kb	160-kb	100-kb	6.1-kb	2.9-kb
VRE0650 (a)	70-kb	50-kb, 70-kb	160-kb	100-kb		
VRE0653 (a)	70-kb	50-kb, 70-kb		100-kb		
VRE0892 (a)	70-kb	25-kb, 70-kb	160-kb	100-kb		
VRE0651 (b)	70-kb	50-kb, 70-kb	160-kb	100-kb		
VRE0690 (e)	70-kb	50-kb		100-kb		
VRE0673 (g) (ST78)	70-kb	50-kb, 70-kb	160-kb	10-kb, 100-kb		
VRE0776 (h)	70-kb	40-kb		100-kb		
VRE0881 (i) (ST17)	70-kb	25-kb, 70-kb	160-kb	100-kb		

[a] *rep*$_{pTEF1}$ hybridisation will be performed to try reveal the unknown *rep* of the 100-kb plasmid.

TABLE 4. Transfer frequencies between donors and recipients after filter mating

Primary matings		**Transfer frequency**	
Donor (PFGE subtype) (ST)	**Recipient**	**Transconjugants/donor**	**Transconjugants/recipient**
VRE0653 (a) (192)	64/3	6×10^{-9}	4×10^{-10}
VRE0651 (b) (192)	64/3	$<6 \times 10^{-9}$	$<2 \times 10^{-9}$
VRE0683 (c) (192)	64/3	8×10^{-9}	5×10^{-11}
VRE0734 (c) (192)	64/3	2×10^{-11}	2×10^{-12}
VRE0726 (d) (192)	64/3	1×10^{-9}	3×10^{-11}
VRE0690 (e) (192)	64/3	2×10^{-9}	1×10^{-10}
VRE0688 (f) (192)	64/3	7×10^{-9}	6×10^{-9}
VRE0673 (g) (78)	64/3	$<2 \times 10^{-8}$	$<1 \times 10^{-9}$
VRE0776 (h) (192)	64/3	1×10^{-10}	5×10^{-8}
VRE0881 (i) (17)	64/3	2×10^{-8}	7×10^{-11}
Secondary matings			
VRE0726 x 64/3	BM4105-Str	7×10^{-5}	2×10^{-3}
VRE0734 x 64/3	BM4105-Str	1×10^{-3}	2×10^{-3}
VRE0881 x 64/3	BM4105-Str	3×10^{-4}	1×10^{-3}

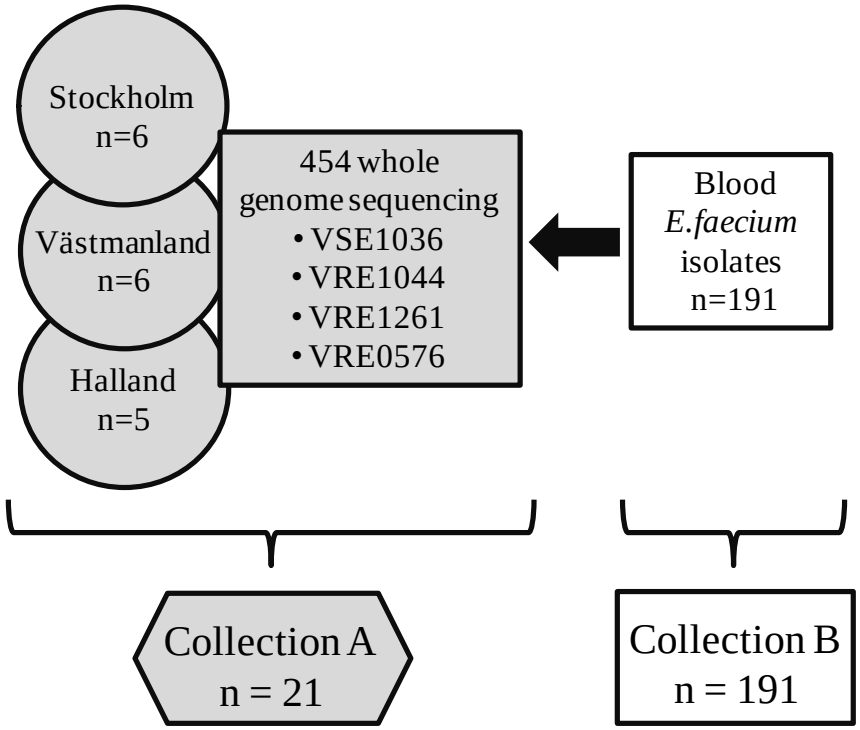
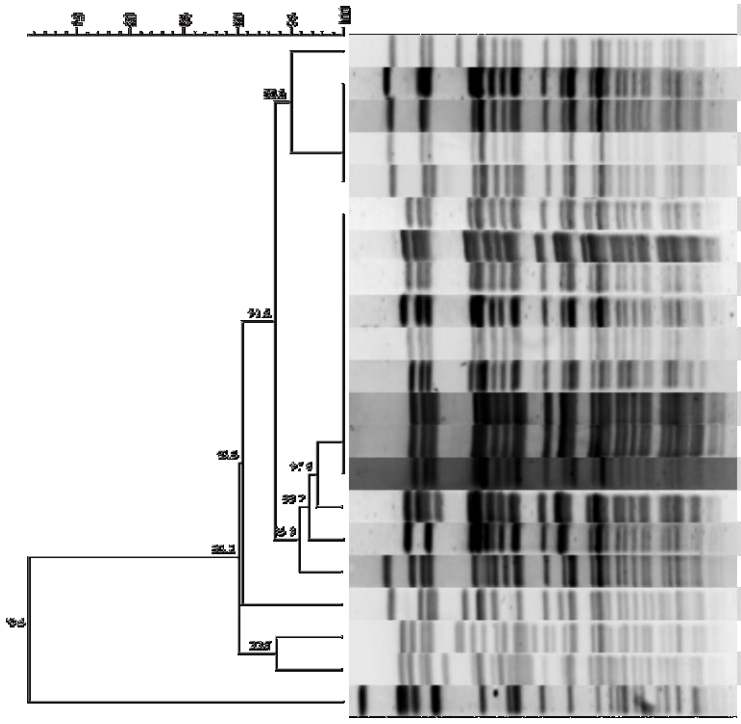


FIG. 1. A schematic figure demonstrating the two different strain collections used in this study. The circles represents non-invasive isolates and the squares represent blood isolates.



Lane	Isolate no.	County	PFGE-type
1	**VRE0726**	**Halland**	**SE-EfmB-0701d**
2	VRE0683	Stockholm	SE-EfmB-0701c
3	**VRE0734**	**Halland**	**SE-EfmB-0701c**
4	VRE0762	Halland	SE-EfmB-0701c
5	**VRE0815**	**Halland**	**SE-EfmB-0701c**
6	VRE0650	Västmanland	SE-EfmB-0701a
7	**VRE0653**	**Västmanland**	**SE-EfmB-0701a**
8	VRE0654	Västmanland	SE-EfmB-0701a
9	**VRE0678**	**Stockholm**	**SE-EfmB-0701a**
10	VRE0892	Västmanland	SE-EfmB-0701a
11	**VSE1027**	**Stockholm**	**SE-EfmB-0701a**
12	VSE1036	Stockholm	SE-EfmB-0701a
13	**VRE1044**	**Stockholm**	**SE-EfmB-0701a**
14	VRE1261	Stockholm	SE-EfmB-0701a
15	**VRE0651**	**Stockholm**	**SE-EfmB-0701b**
16	VRE0690	Stockholm	SE-EfmB-0701e
17	**VRE0688**	**Stockholm**	**SE-EfmB-0701f**
18	VRE0673	Halland	SE-EfmB-0701g
19	**VRE0776**	**Västmanland**	**SE-EfmB-0701h**
20	VRE0881	Västmanland	SE-EfmB-0701i
21	**VRE0576**	**Stockholm**	**EfmB Unique**

FIG. 2. Dendrogram of *Sma*I PFGE of the 20 isolates, showing clonality (lane 1-20) and one divergent isolate (lane 21). The PFGE-type nomenclature is based on the following: SE stands for Sweden, EfmB stands for *E. faecium* with *vanB*, the number 07 represents year 2007 (the year the index was identified) and the last number is a serial number. The letter at the end describes which PFGE-subtype the isolate belongs to.

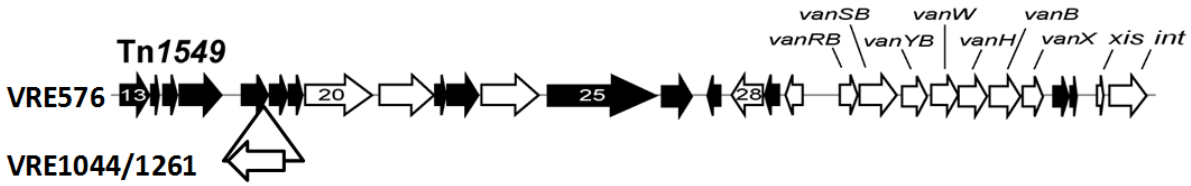


FIG. 3. Genetic organization of the *vanB2*-Tn*1549*/Tn*5382* conjugative transposons in VRE576 versus VRE1044 and VRE1261. Gene identifiers for Tn*1549* are captured from GenBank accession number AF192329 and the black arrows indicate coding sequences (CDSs) with no assigned functions and white arrows CDSs with putative functions. In VRE576 the *vanB2* transposon shows the same genetic organisation and 99% nucleotide (nt) identity to Tn*1549* over nt 1..30810 (VRE576 contig 4) and 30808..33799 (VRE576 contig 8). In VRE1044 and VRE1261 the *vanB2* transposon show the same organisation and 99% nucleotide identity to Tn*1549* over nt 1..15788 (VRE1044 contig 41/ VRE1261 contig 44) and 15795..33799 (VRE1044 contig 36/ VRE1261 contig 49), but has an additional 2588 bp (indicated by triangle) encoding a putative protein (grey arrow) of 610 amino acids inserted between nt 5014 and 5015 of Tn*1549*. The 2588 bp insert sequence is 89% identical to the region covering nt 497155.499743 in the *Clostridium saccharolyticum-like* K10 draft genome (FP929037) which encodes a retron-type reverse transcriptase. We are currently working to close the gap in the *vanB* transposon of VRE1044 and VRE1261 by ICE*slu*van Q12-15 PCR (Table 1) to confirm if the transposon has further deviations from the original Tn*1549*.

FIG. 4. Sequence comparison of the insert regions of Tn*1549/5382* in VRE576 versus VRE1044 and VRE1261 and the corresponding region in VSE1036 (contig00062). Tn*1549*/Tn*5382* left and right end imperfect inverted repeats are shown in bold capital letters. Vertical lines indicate identical nucleotides.

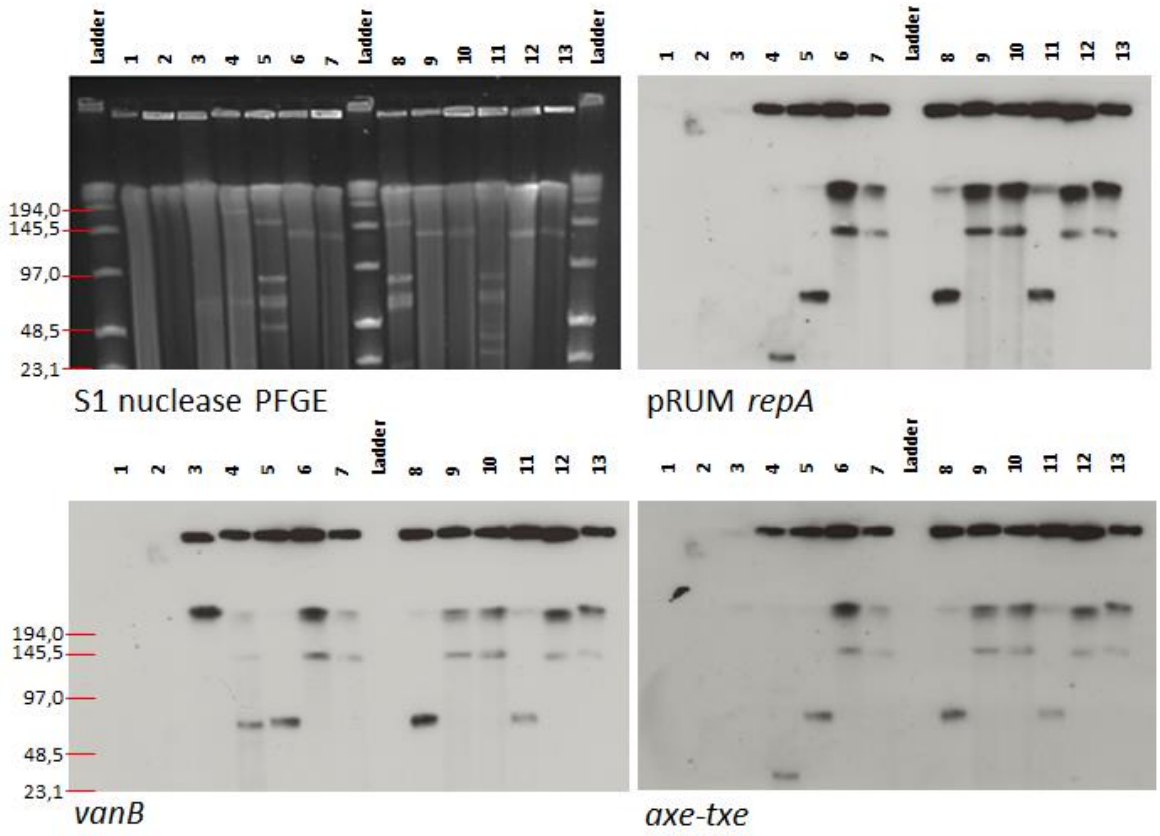


FIG. 5. S1-nuclease PFGE and corresponding Southern hybridisations with rep_{pRUM}, *vanB* and *axe-txe* probes as indicated in the figure illustrated transfer from donors VRE0726/0734/0881 (lanes 5, 8 and 11) of a similar sized plasmid (approximately 140 kb) to 64/3 (lane 1) (1st generation transconjugants shown in lanes 6, 9 and 12) which was subsequently retransferred to BM4105Str (lane 2) (2nd generation transconjugants shown in lanes 7, 10 and 13) when using the 1st generation transconjugants as donors. Lane 3 *vanB* positive control V583, lane 4 rep_{pRUM} and *axe-txe* positive control U37.

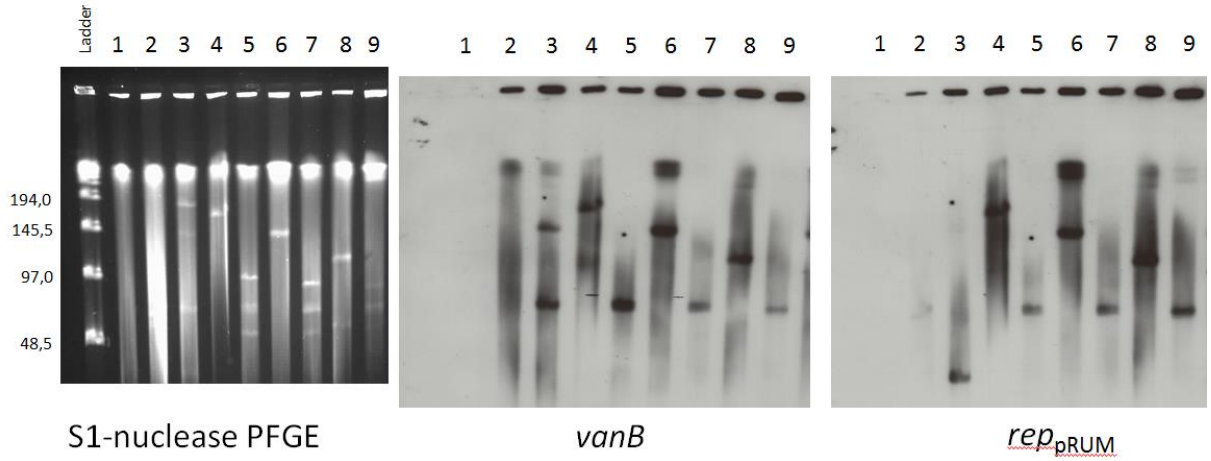


FIG. 6. S1-nuclease PFGE and corresponding Southern hybridisations with *vanB* and rep_{pRUM} probes as indicated in the figure. Donors VRE0690/0653/0776 (lanes 5, 7 and 9) and their respective transconjugants (lanes 4, 6 and 8) illustrate transfer of different sized plasmids co-hybridizing to *vanB* and rep_{pRUM} (≈ 110-150kb) into 64/3 (lane 1). Lane 2 *vanB* positive control V583, lane 3 rep_{pRUM} positive control U37.

TABLE S1. Plasmid replication, resistance, toxin-antitoxin system and conjugative transposon genes found in the WGSs of the pre-outbreak isolate VRE576 and the three outbreak isolates VSE1036, VRE1044 and VRE1261. Gene identity refers to the reference sequence.

Category	Gene	Rep group family	Isolate	Contig	% nt identity	Alignment lengt nt	Reference data		
							Species	GenBank Acc. No.	Locus
Replication genes	CDS1 pRE25	2	VRE0576	contig00101	100.00	1434	*E. faecium*	X92945	33-1526
			VSE1036	contig00084	100.00	1434			
			VRE1044	contig00079	100.00	1434			
				contig00081	89,76	1358			
			VRE1261	contig00085	100.00	1434			
				contig00098	89,76	1358			
	repA -1 pTEF1	9	VRE0576	contig00115	76.29	641	*E.faecalis V583*	AE016833	101-1111
			VSE1036	contig00071	76.44	641			
			VRE1044	contig00086	76.44	641			
			VRE1261	contig00090	76.44	641			
	repA pB82	11	VRE0576	contig00054	83.72	215	*E. faecium*	AB178871	4157-5101
			VSE1036	contig00090	100.00	579			
			VRE1044	contig00087	100.00	579			
			VRE1261	contig00091	100.00	579			
	replication initiation protein pRI1	14	VRE0576				*E. faecium*	EU327398	3161-4114
			VSE1036	contig00113	83.50	976			
			VRE1044	contig00109	83.50	976			
			VRE1261	contig00121	83.50	976			
	Putative *repA* pRUM	17	VRE0576	contig00094	100.00	1041	*E. faecium*	AF507977	20542-21582
			VSE1036	contig00075	97.32	1043			
			VRE1044	contig00070	97.32	1043			
			VRE1261	contig00072	97.32	1043			
	replication-associated protein *repA* pLG1		VRE0576	contig00115	98.6	1041	*E. faecium*	HM565183	40769-41809
			VSE1036	contig00071	99.7	1041			
			VRE1044	contig00086	99.7	1041			
			VRE1261	contig00090	99.7	1041			
	Putative plasmid replication protein pCIZ2	unique	VRE0576	contig00086	99.59	738	*E. faecium*	NC_008259	5690-6427
			VSE1036						
			VRE1044						
			VRE1261						
Resistance genes	vancomycin resistance gene *vanB* (D-alanine:D-lactate ligase) Tn*1549*-like		VRE0576	contig00004	99,71	1029	*E. faecium*	AY655721	4857-5885
			VSE1036						
			VRE1044	contig00036	99,71	1029			
			VRE1261	contig00049	99,71	1029			
	erythromycin resistance transferase		VRE0576	contig00100	100.00	738	*E. faecium*	AF507977	12938-13675
			VSE1036	contig00100	99.46	738			
			VRE1044	contig00095	99.46	738			
			VRE1261	contig00106	99.46	738			
	Tetracycline resistance (*tetM*) Tn*916*		VRE0576				*E. faecalis*	X56353	223-2142
			VSE1036	contig00094	94,62	1357			
			VRE1044	contig00089	94,62	1356			
			VRE1261	contig00096	94,62	1357			
TA-system genes	Antitoxin of *axe-axe* pRUM		VRE0576	contig00161	100.00	270	*E. faecium*	AF507977	5213-5482
			VSE1036	contig00062	100.00	270			
			VRE1044	contig00036	100.00	270			
			VRE1261	contig00049	100.00	270			
	Toxin of *axe-txe* pRUM		VRE0576	contig00161	100.00	258	*E. faecium*	AF507977	4963-5220
			VSE1036	contig00062	100.00	258			
			VRE1044	contig00036	100.00	258			
			VRE1261	contig00049	100.00	258			
Conjugative transposon genes	Integrase Tn*1549*		VRE0576	contig00008	99,92	1194	*E. faecalis*	AF192329	32432-33625
			VSE1036						
			VRE1044	contig00036	99,92	1194			
			VRE1261	contig00049	99,92	1194			
	Excisionase Tn*1549*		VRE0576	contig00008	99	201	*E. faecalis*	AF192329	32148-32348
			VSE1036						
			VRE1044	contig00036	99	201			
			VRE1261	contig00049	99	201			

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