Molecular characterization of vancomycin-resistant *Enterococcus faecium*

isolates collected from the Dallas, Texas area

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Abstract

Molecular characterization was performed for 46 vancomycin-resistant *E. faecium* (VREfm) isolates and one vancomycin-sensitive comparator obtained during routine fecal surveillance of high-risk patients from a Dallas, Texas area hospital system. Hybrid assemblies of long (Oxford Nanopore Technology) and short (Illumina) sequence reads enabled the generation of 31 complete and 16 draft high quality genome sequences. The VREfm isolates possessed up to 12 plasmids each. A total of 251 closed plasmid sequences, assigned to 12 previously described and 9 newly defined *rep* family types, were obtained. Phylogenetic analysis clustered the Dallas isolates into genomic Clade A1, including 10 different sequence types (STs), the most prevalent ones being ST17 and ST18. VREfm isolates with the novel sequence type ST1703 were also identified. All but three of the VREfm isolates encoded *vanA*-type vancomycin resistance within Tn*1546*-like elements on a pRUM-like plasmid backbone. New variants of the Tn*1546* were described that harbored a combination of 7 insertion sequences (IS) including 3 novel IS elements reported in this study (IS*Efa16*, IS*Efa17* and IS*Efa18*). The pRUM-like plasmids carrying the Tn*1546* were grouped into 4 sub-groups based on the replication and stability modules of the plasmid backbone. Overall, we conclude that the VREfm isolates analyzed in our collection are representative of other global VanA-type VREfm in that they belong to the Clade A1 lineage, but they possess novel arrangements of the Tn*1546*-like elements and the *vanA* operon, which have evolved independently of the pRUM-like backbone.

Importance

Vancomycin is a broad-spectrum antibiotic used to treat infections caused by multi-drug resistant Gram-positive bacteria. However, vancomycin resistance is predominant for *Enterococcus faecium* and therefore infections with VREfm have few treatment options. The vancomycin resistance genes are encoded on a transposon, which can be disseminated by different plasmids with different host ranges and transferring efficiencies. The presence of resistance genes within a broad host range plasmid can generate a wide variety of resistant bacteria very quickly. Therefore, surveillance of resistance plasmids is critical. This study provided a comprehensive analysis of VREfm collected from the Dallas, Texas area, with particular focus on the mobile elements associated with vancomycin resistance genes. We find that a single plasmid family, the pRUM-like family, is associated with vancomycin resistance in the majority of isolates sampled.

Introduction

Originally described as a commensal of the healthy human gut, the enterococci attracted attention in the 1990s due to their alarming multi-drug resistance[1]. Increasing cases of vancomycin-resistant enterococci (VRE), first reported in 1988 in Europe[2] and then in USA in 1989[3] became a major concern for nosocomial infection[4]. In recent years, vancomycin-resistant *E. faecium* (VREfm) is considered to be the most prominent VRE species, and hence is widely studied[4].

Whole genome-based phylogenetic analysis and multi-locus sequence typing (MLST) of *E. faecium* isolates has provided valuable information regarding their origin and evolution. Phylogenetic analysis of *E. faecium* strains collected from sources including healthy human volunteers, animals, and hospital infections revealed the presence of at least two major clades, referred to as Clade A and B, within this species[5, 6]. Clade B *E. faecium* strains are mostly commensals of the healthy human gut. Clade A strains adapted to colonize the animal gut approximately 3000 years ago, coincident with human urbanization, hygienic behavior, and animal domestication[5]. Clade A *E. faecium* strains diverged further into Clades A1 and A2 around 75 years ago, coinciding with widespread usage of antibiotics[5]. The multi-drug resistant *E. faecium* strains, responsible for hospital outbreaks, typically belong to Clade A1, whereas animal gut commensals typically belong to Clade A2[5].

MLST analysis, which pre-dates whole genome-based phylogenetic analyses, classifies VREfm isolates by nucleotide sequence variations occurring in 7 housekeeping genes (*adk, ddl, gdh, purK, pstS, atpA,* and *gyd*)[7]. Isolates are assigned to different sequence types (ST) based on the allelic variants. Most of the reported hospital outbreaks of VREfm emerged from a single genetic lineage of clonal

complex-17 (CC17) that was founded by the ST17[8]. The VREfm isolates involved in

hospital outbreaks in different parts of the world, within the time period 1986-2012,

represented 26 STs[9]. The most prominent VREfm isolates reported were ST17

(reported in Spain, China, Brazil), ST18 (reported in Canada, Poland, South America,

Turkey, Serbia) and ST78 (reported in Turkey, Paraguay, Belgrade, Italy). In USA,

VREfm of ST16 and ST17 were responsible for clonal outbreaks from 1990 to 2005.

Generally, a single ST of VREfm is associated with an outbreak in a certain

geographic region, while globally, outbreaks are caused by multiple different STs. For

example, ST25 (France, 1987), ST114 (Brazil, 1998), ST18 (UK, 1992) and ST16

(Netherland, 2002) were reported outbreak strains from different parts of the world[9].

Epidemiological differences in diverse geographical areas[9] reiterate the importance of

tracking VREfm genotypes.

Vancomycin resistance genes are encoded within mobile elements in VREfm, and

can be horizontally disseminated[10]. The most commonly occurring vancomycin

resistance type worldwide, currently, is VanA-type resistance, which is typically

encoded within the element Tn*1546*[11]. Broadly speaking, the Tn*1546* may be

chromosomally integrated or plasmid-borne. Different plasmid families carrying

Tn*1546* including Inc18, pRUM, and pLG1 have been reported worldwide. Hybrids of

pRUM-Inc18, Inc18-pHTβ, pRUM-pLG1 family plasmids carrying *vanA* have been

reported as well[9]. Structural variations of Tn*1546* due to the presence of insertion

sequences (IS) like IS*1251* (USA, Australia, Europe)*,* IS*1216* (Europe)*,* IS*1485* (Saudi

Arabia) and IS*Ef1* (Germany, Portugal, Poland) have also been reported. Previous

studies determined the Tn*1546* structural variation by PCR mapping and then

sequencing of the overlapping fragments[9,12,13]. However, in the absence of a

completely closed genome assembly, conclusive links between *vanA*, Tn*1546*

variants, and specific plasmid backbones in VREfm is difficult to achieve. Surveillance

of resistance elements is important because changes in host range and transfer

frequency can evolve, which could impact clinical care. Utilization of whole genome

sequencing for epidemiologic study provides an opportunity for multilevel molecular

analysis that includes detection of novel mobile genetic elements (MGE) and

monitoring changes in resistance gene vectors. The advent of long-read next

generation sequencing (NGS) by Oxford Nanopore Technology (ONT) made it

possible to resolve long repeat regions and generate genome assemblies of a large

number of bacterial isolates[14] in a cost effective manner. The combination of long

(ONT) and short (Illumina) reads generates high quality completely closed genome

assemblies that gives access to fully assembled mobile genetic elements[15].

Much of the focus of VREfm genomics has been on infection-derived isolates. There

is a comparative lack of information regarding genotypic diversity of fecal (i.e., non-

infection-derived) VREfm isolates that colonize hospitalized patients in a given

population. These represent reservoirs from which future hospital outbreaks could

emerge. The current study aims to address this knowledge gap by analyzing fecal

VREfm isolates collected from hospitalized patients in Dallas, Texas, using a

combination of ONT and Illumina sequencing.

Materials and Methods

Bacterial strains analyzed in this study. The isolates used in this study (**Dataset

S1A**) were cultured from surveillance rectal swabs of high-risk patients on hospital

admission within a span of two months (August 2015 to October 2015), and their

clinical collection and initial characterization was previously reported[16]. Briefly, rectal

swabs from patients admitted with at least one of the following risk factors

(hospitalization for ≥2 consecutive nights in the preceding 30 days, transferred from

another medical facility, residence in a nursing home or extended/long term care

facility, or the presence of decubitus ulcer or a draining wound) were cultured on

Spectra VRE (Remel) agar as a part of routine infection control surveillance. Instead

of disposal after clinical procedures were complete, cultures positive for presumptive

VRE growth were coded numerically, de-identified, and transferred to the University of

Texas at Dallas for further analysis. Isolates were coded by de-identified patient

number. If multiple colonies were collected from a Spectra VRE plate, this was

indicated by hyphenated numerics. For example, the VRE isolates 124-1, 124-2 and

124-3 were collected from the same patient (de-identified patient #124). In our

previous study of these isolates, presumptive VREfm were confirmed for species by

ddl typing and for the presence of *vanA* or *vanB* genes by PCR[16,17]. All isolates used

in this study, with the exception of 163-1, grew on brain heart infusion (BHI) agar

supplemented with 256 μg/mL vancomycin. The vancomycin MIC of isolate 163-1

was previously determined to be 2 μg/mL by broth microdilution[16].

Genome sequencing and assembly. Methods for DNA isolation, MinION

sequencing, Illumina sequencing, and hybrid genome assembly are described in the

Supplemental Text.

Phylogenetic analyses and MLST. Genome assemblies were annotated with

PROKKA (v 1.12)[18]. A sequence alignment was generated for 1706 core genes by

ROARY (v 3.12)[19] using PRANK where the paralogs were not split. A phylogenetic

tree was built by RAxML (v 8.2.12)[20] with rapid bootstrapping (1000 inferences) using

the GTR+GAMMA model and subsequent ML search on the core gene alignment

where the outgroup was specified to be the reference genome *E. faecium* 1,231,501

(GenBank accession number ACAY01). The phylogenetic tree was visualized with

iTOL (v4)[21]. MLST was determined by the *E. faecium* MLST database

(https://pubmlst.org/efaecium/)[22,7]. The novel sequence type ST1703 and novel allele

number for *gyd* (allele-70) was assigned for 5 VREfm isolates (111, 121, 137, 154-1,

158) upon request from the database.

Plasmid detection and typing. Each contig of <2 Mbp in size in a closed genome

assembly was considered a putative plasmid. *In silico* detection and typing of

plasmids was performed with PlasmidFinder[23] (v 2.1). Putative plasmids in closed

genome assemblies that were not classifiable by PlasmidFinder were analyzed further

(see below).

Definition of pMIX plasmid groups. Putative plasmid sequences that were

unclassifiable by PlasmidFinder were analyzed as follows. Presumptive *rep* genes

were identified from plasmid annotations and analyzed by NCBI Conserved Domain

Analysis (CDS). Confirmed *rep* genes encoded protein domains that matched known

Rep protein families with e-values of e^{-10} to e^{-78}. The Rep sequences were also

scanned by HMMER (v 2.41.1)[24] to analyze pfam domains. Multiple sequence

alignment of the nucleotide and predicted amino acid sequences of *rep* genes were

performed with MUSCLE (v 3.8.424) where the "group sequences by similarity" option

was selected. A neighbor-joining tree was built based on the sequence alignment

using the "Tamura Nei" genetic distance model[25]. The tree was visually scrutinized in

order to sort the plasmids into 9 groups harboring *rep* genes with at least 95% identity

in both nucleotide and predicted amino acid sequence (except for pMI4; see Results).

Each unique *rep* group of plasmids was given a name of format pMIX, where the "X"

is a numeric number from 1 to 9.

Eight closed circular DNA elements of varying sizes (3 to 72 kb) could not be

classified by PlasmidFinder nor by the pMIX *rep* typing scheme described above.

Analysis of these elements is described in the Supplemental Text.

Transcriptional activity of predicted toxin-antitoxin (TA) systems. Transcriptional

activity of the TA systems, TA$_{axe-txe}$ and TA$_{relE}$, was determined by RT-qPCR for the

VREfm isolates 1 and 5, described in the Supplemental Text. The primer sequences

used for qPCR are provided in **Dataset S1B.**

Resistance gene identification. Acquired antimicrobial resistance genes were

detected with ResFinder 3.1[26].

Tn*1546* analysis. The Tn*1546* nucleotide sequence described in *E. faecium* BM4147

(accession number M97297) was used as a reference to identify variations occurring

in the Tn*1546* identified in this study. The Tn*1546* were classified using a previously

described nomenclature[13]. The presence of insertion sequence elements (IS

elements) was denoted with a one-letter code, shown in parentheses: IS*1216* (B)*,*

IS*1251* (C), IS*Efa5* (D), IS*256* (J), IS*Efa16* (K), IS*Efa17* (L), IS*Efa18* (M). The

combination of IS elements within the transposon is described by a two or three letter

code; i.e., group 'BC' possesses one each of IS*1216* and IS*1251*. Arabic numerals,

following the alphabet code, indicate differences due to point mutations or different IS

element insertion sites. For the group 'BC', a previous study[13] described BC1-BC5,

each with specific insertion sites of IS elements. Any novel insertion sites identified in

this present study were therefore numbered from 'BC6' forward. The novel IS*Efa16,*

IS*Efa17,* IS*Efa18* sequences were identified in this study and registered in the

ISFinder public database[27].

Accession numbers. DNA sequences from this study have been deposited under

Bioproject PRJNA682584. Individual accession numbers for all sequence files are

provided in **Dataset S1A**.

Results

VREfm isolates belonging to ST17 and ST18 were prevalent in the collection.

Genome sequencing of 46 VREfm isolates and one vancomycin-sensitive comparator

collected from rectal surveillance of high-risk patients at a Dallas, Texas, hospital was

performed with Oxford Nanopore MinION and Illumina technologies. High quality

genome sequences were generated by hybrid assembly of the sequencing reads from

these two platforms. 31 closed and 16 draft genome assemblies were generated

(**Table S1**). In our previous analysis of these isolates using PCR, all isolates were

found to be *vanA*-positive and *vanB*-negative by PCR, with the exception of the

isolate 163-1, which had neither *vanA* or *vanB* and had a vancomycin MIC of 2 µg/mL

by broth microdilution[16]. We included 163-1 in this study for comparative purposes to

evaluate its relationship to the *vanA*-positive isolates in the collection. All 46 *vanA*-

positive isolates sequenced in this study grew with 256 µg/mL vancomycin in the

growth medium.

Phylogenetic analysis based on alignment of 1706 core genes allowed us to assess

the epidemiology and diversity of the VREfm isolates (**Figure 1**). Two reference

genomes, *E. faecium* 1,231,501[6] and *E. faecium* ATCC® 700221™[28], were included

as representatives of *E. faecium* clade A2 and A1, respectively. *E. faecium* 1,231,501

is a human bloodstream isolate from the USA and is susceptible to vancomycin. *E.*

faecium ATCC® 700221™ is a USA isolate and VanA-type VREfm control strain used

in antimicrobial susceptibility testing[29]. As expected, the Dallas VREfm isolates

clustered with the clade A1 reference *E. faecium* ATCC® 700221™, although a high

degree of branching within this clade was observed, revealing genetic diversity within

the collection.

MLST is commonly used to study relationships among VREfm isolates worldwide, and

this analysis provides the opportunity to compare the Dallas isolates with other

VREfm strain collections. A total of 10 different sequence types (ST), all belonging to

clonal complex 17 (CC17), were observed among the collection (**Figure 1, Figure 2**).

Isolates belonging to ST17 and ST18 are the most prevalent in the collection,

comprising 50% of the isolates, which clustered together in the core genome tree

(**Figure 1, Figure 2**). Six genotypes (ST18, ST664, ST1703, ST736, ST80 and

ST612) are double locus variants of ST17. The ST262 and ST412 are triple locus

variants and ST1383 is a single locus variant of ST17 (**Dataset S1C**). ST1703, a

double locus variant of ST17 (*pstS* allele-20*;* novel *gyd* allele-70), is a novel ST in the

MLST database and comprised 11% of the collection. Isolate 163-1 branched away

from the rest of the isolates and belonged to ST262, which is still within CC17. Taken

together, these data indicate that *E. faecium* CC17 with varied genotypes colonized

Dallas patients at the time of the study.

Large numbers of plasmids are present in the Dallas isolates. The plasmid

content of the Dallas isolates was analyzed by an *in silico* detection method utilizing

the conserved nucleotide sequences of replication initiation (*rep*) genes[23]. Of the 259

closed circular putative plasmids, 141 were assigned to 10 known *rep* families

described in the existing PlasmidFinder database. The remaining 118 putative

plasmids were categorized into 9 novel plasmid groups (pMI1 to pMI9) and 8 other

elements (discussed in the next section).

The Dallas isolates harbored a variable number of plasmids (n=3 to 12) of sizes

ranging from 1.9 to 294 kb (**Figure 1; Dataset S1D**). The most common elements

were a megaplasmid (126 to 294 kb) and a pRUM-like plasmid (17 to 63 kb), which

were each present in all isolates except 144-1, which lacked the megaplasmid. Both

the reference strains (*E. faecium* 1,231,501 and ATCC® 700221™) harbored the

megaplasmid, but 1,231,501 lacked the pRUM-like plasmid. Each of the

megaplasmids harbored the *rep* gene *repUS15*, encoding a RepA_N protein motif.

The pRUM-like plasmid harbored *rep17* or both *rep17* and *rep18*, which we refer to as

a mosaic plasmid. Isolates collected from the same patient had identical plasmid

profiles in 2 cases (patients 124 and 9) while different plasmid profiles were observed

in 3 other cases (patients 100, 71, and 16).

To determine whether plasmid replicons were enriched among specific lineages, the

percentage presence/absence of specific *rep* genes was quantified among the

isolates belonging to 4 STs (ST17, ST18, ST1703 and ST664) that included ≥5

isolates. The other 6 STs (ST262, ST1383, ST612, ST412, ST80, and ST736) were

not included in this analysis because they were represented by <5 isolates (**Dataset**

S1E). Chromosomally integrated *rep* genes *repUS12* and *repUS43,* encoding the

protein motifs Rep_1 and Rep_trans, were observed in 45% and 22% of the isolates,

respectively, and were absent from isolates belonging to ST17, ST18 and ST1383

(**Dataset S1E**). The *rep14* family was present in 80% and 90% of ST17 and ST18

isolates, respectively, but was absent in ST1703 and ST664 isolates. The *rep18*

family plasmid was present in ST17 (80%) and ST664 (100%) isolates but absent in

ST1703 (0%) and rare in ST18 (30%). In summary, the *rep* genes *repUS12, repUS43,*

rep18 and *rep14* had an ST-specific distribution among the isolates. No conclusion

could be drawn regarding other *rep* genes and STs due to low sample size.

Uncategorized plasmids were sorted into 9 plasmid groups, pMI1 to pMI9.

PlasmidFinder[23] did not identify *rep* genes in a total of 118 circular entities. Of these

118 circular entities, 110 were established to be plasmids based on the presence of

confirmed *rep* genes not currently represented in the PlasmidFinder database (see

Materials and Methods). The remaining 8 circular entities were designated as putative

excision products of genomic islands (**Supplementary text, Dataset S1F-G**)

including a 5 kb *vanA-*encoding circular fragment of Tn*1546* in isolate 111 (discussed

further in a later section).

The 110 plasmids not classifiable by PlasmidFinder were sorted into 9 plasmid

groups, pMI1 to pMI9, based on phylogenetic analyses of the *rep* genes (**Figure S1;**

Dataset S1H). The nucleotide sequences of the *rep* genes and their corresponding

amino acid sequences shared >95% identity within each pMIX plasmid group, except

for pMI4 (**Table 1**). The clustering of the *rep* gene sequences belonging to the pMI4

plasmid group supported their inclusion in a single group, despite lower pairwise

sequence identities (**Figure S1**). A total of 6 Rep protein families (RepA_N, Rep_3,

Rep_1, Rep_trans, Rol_rep_N, and Rep_2) were encoded by the *rep* genes of the 9

pMIX groups (**Dataset S1H**). Specifically, the *rep* genes of pMI1 and pMI3 encoded

protein families RepA_N and Rep_1, respectively. The *rep* genes of pMI2, pMI5, pMI6

and pMI7 encoded the protein family Rep_3. The *rep* genes of pMI4 and pMI8

encoded protein families Rep_trans and Rep_2, respectively. The *rep* of pMI9 was

the only exception, encoding two Rep protein families, Rep_trans and Rol_rep_N

(**Table 1**).

Each of the Dallas isolates harbored 1 to 5 of the pMIX plasmid groups, except the

isolate 83 (ST17), which had none of them (**Figure 1**). Neither of the two reference

strains harbored any of the pMIX plasmid groups. The pMI1 was the most frequently

occurring pMIX plasmid group, identified in 75% of the isolates (**Table 1**). The pMI8

and pMI9 were the rarest ones, found in only isolates 51_1 (ST612) and 5 (ST18),

respectively. The pMIX plasmid groups were not enriched in any specific ST.

Plasmids within a very narrow size range (standard deviation 0.2 to 2.3) were

grouped together (with the exception of pMI1), although plasmid size was not taken

into consideration in assigning plasmid groups. The pMI1 consisted of plasmids size

ranging from 23 to 62 kb (standard deviation 11.2).

To summarize, 9 new plasmid groups, pMI1 to pMI9, were defined for 110 plasmids

based on *rep* gene sequences that encoded 6 different Rep protein families. Most of

the Dallas isolates harbored 1 to 5 plasmids belonging to these newly defined plasmid

groups.

Resistance genes were encoded in both chromosomes and plasmids. Different

combinations of 12 resistance genes providing resistance against 5 classes of

antibiotics (aminoglycosides, glycopeptides, macrolides, tetracycline, and

trimethoprim) were identified among the Dallas isolates (**Figure 3**; **Dataset S1I**;

Supplemental text). The most commonly occurring resistance genes were *aac(6')I-1*

(100% of isolates), *msr* (100%), *vanA* (95%), *tetM* (95%), *ermB* (85%), and *aph(3')*

(85%). These resistance genes were encoded in the chromosome and mostly in two

plasmid types, the pRUM-like and the megaplasmid. The pRUM-like plasmids

predominantly encoded 4 of the resistance genes (*ant(6)-Ia, aph(3')III, vanA,* and

ermB) conferring resistance against aminoglycosides, vancomycin and erythromycin

(**Figure 3**)*.* The *repUS7* plasmid (pMG1) was found among only 3 of the Dallas

isolates (66 (ST18), 144-1 (ST18) and 51-4 (ST612)) and encoded *dfrG,* a less

common resistance gene among the Dallas isolates, conferring resistance against

trimethoprim.

All of the Dallas isolates, except for 163-1, encoded VanA-type vancomycin

resistance genes, where *vanA* shared 99.9% (nucleotide) and 99.7% (amino acid)

sequence identity with the reference sequence (GenBank AAA65956.1). The

reference sequence is encoded within the transposon Tn*1546* (GenBank M97297),

originally described in the human fecal isolate *E. faecium* BM4147 collected from

France in 1988[30] (**Dataset S1I**). A classical structure of the *vanA* gene cluster

consisting of *vanHAX* and *vanYZ* encoded within Tn*1546* elements was observed

among the Dallas VREfm isolates, except for the isolate 17-1, which lacked the genes

vanY and *vanZ*. The Tn*1546* structure varied among the isolates in terms of insertion

site (chromosome versus plasmid, IS elements, and point mutations, discussed

further below.

The Tn*1546* is borne on pRUM-like plasmids in the majority of isolates. The

complete Tn*1546* was carried by the pRUM-like plasmid in 42 of 46 *vanA*-positive

isolates (**Figure 4**). The isolate 111 did not encode the entire Tn*1546* in the pRUM-

like plasmid; rather, it encoded *vanRS* in a 17 kb pRUM-like plasmid while *vanHAX*

was encoded within a separate 5 kb circular element (te111_5kb) (**Dataset S1F**).

Tn*1546* was chromosomally encoded in 3 isolates, 53-1, 53-2 and 91, each belonging

to ST17, despite all 3 harboring a pRUM-like plasmid (**Table 2**).

Two toxin-antitoxin (TA) systems, *axe-txe* (TA$_{axe-txe}$) and a *relE/parE* family toxin

associated with an uncharacterized antitoxin component (TA$_{relE}$), were observed

alone or simultaneously on the pRUM-like plasmids (**Table 2**). Both of the TA systems

were actively expressed in two representative VREfm isolates (**Figure S2**).

The 43 pRUM-like plasmids encoding Tn*1546* or a fragment of Tn*1546* (for isolate

111) were categorized into the following four groups based on the presence or

absence of replication (as categorized by PlasmidFinder) and stability modules on

their backbones (**Table 2, Figure 5**). A previous study utilized the term "pRUM

derivatives" where plasmids were characterized by analyzing *rep* and TA systems[12].

i)   Group1 (rep$_{17;}$ rep$_{18}$; TA$_{axe-txe;}$ TA$_{relE}$): The pRUM-like plasmids categorized

393   into group 1 harbored a mosaic replication module *rep$_{17/18}$* as well as both

394   of the stability modules TA$_{axe-txe}$ and TA$_{relE}$. All the group 1 plasmids

395   encoded the *rep18b* (accession number CP018068), except isolate 113,

396   which encoded the *rep18a* (accession number AB158402). A total of 16 of

397   the 43 plasmids belonged to this group. The plasmids had a narrow size

398   range (44 to 48 kbp) and were carried by VREfm isolates from 4 genetic

399   backgrounds (ST17, ST18, ST1383 and ST80).

400   ii)   Group 2 (rep$_{17}$; TA$_{axe-txe}$; TA$_{relE}$): A total of 17 of 43 plasmids harbored the

401   replication module *rep$_{17}$* and both of the stability modules TA$_{axe-txe}$ and

402   TA$_{relE}$, and were categorized into group 2. The group 2 plasmids could be

classified further based on size and genetic background of the isolates

carrying them. Group 2a plasmids (29-45 kb) were carried by isolates

belonging to ST18 (5, 66, 97-1, 124-1, 124-2, 124-3, and 162), ST80 (16-

1, 16-2), ST17 (93-2), and ST612 (51-4), whereas larger (57-63 kb) group

2b plasmids were present in all 6 isolates belonging to ST664 (51-1, 87-1,

93-1, 93-2, 155, and 160). The reference strain ATCC® 700221™

harbored the group 2a pRUM-like plasmid. Sequence analysis revealed

that Group 2b plasmids possess a set of genes that are specific to this

group and present in no other group (**Dataset S1J**), including a ~7.5 kb

region encoding helix-turn-helix XRE-family like proteins (cd00093)

inserted immediately upstream of the *van* gene cluster (**Figure S3**).

iii)　　　Group 3 (rep_{17}; TA_{relE}): The group 3 plasmids possessed the replication

module rep_{17} and the stability module TA_{relE}. A total of 5 of 43 plasmids

were clustered into this group with the size range of 36-42 kb. They were

carried by ST736 isolates (isolates 9-1, 9-2, 52, and 55) and one ST1703

isolate (isolate 121).

iv)　　　Group 4 (rep_{17}): Group 4 plasmids encoded the replication module rep_{17}

and no known stability modules ($TA_{N/A}$). A total of 5 of 43 plasmids with

sizes ranging from 17 to 35 kb belonged to this group. These plasmids

were carried by isolates belonging to the novel genetic background

ST1703 (111, 137, 154-1, and 158) and by a single ST412 isolate (17-1).

It is interesting to note that the isolate 163-1, which belongs to CC17 but lacks

vancomycin resistance genes, harbored a 25 kb pRUM-like plasmid. This pRUM-like

plasmid did not harbor any part of the Tn*1546* and did not belong to any of the

plasmid sub-groups defined above. The presence of the modules *rep17* and $TA_{axe-txe}$

and the absence of both of the modules *rep18* and TA_{relE} was unique among the

Dallas isolates but was described before in the originally described pRUM plasmid in

2003[31].

The robustness of the *rep*/TA typing system was confirmed using two approaches: a

core genome phylogeny based on sequence variation in 7 pRUM-like core genes

(*rep17,* IS*1216, vanS,* ImpB/MucB/SamB family protein, hypothetical protein, plasmid

partitioning protein, and replication control protein PrgN) (**Figure S5**), and an analysis

of gene presence/absence across 40 pRUM-like plasmids using Roary[19] **(Dataset**

S1J). The Group 2a pRUM-like plasmid from the reference strain *E. faecium*

ATCC® 700221™ was included in the analysis; while 4 of the pRUM-like plasmid

sequences (71-2, 100-2, 124-2, and 124-3) were not included due to sequence

redundancy. The pRUM-like plasmids were clearly divided into two branches in the

phylogenetic tree (**Fig S8**). Interestingly, these two groups corresponded to the

presence/absence of the $TA_{axe-txe}$. Members of the plasmid groups 1, 2a and 2b

clustered together, while those of group 3 and 4 were placed into a separate branch.

The gene presence/absence heatmap (**Dataset S1J**) of 40 pRUM-like plasmids

clearly identified genes specific to certain plasmid groups, other than the two stability

and replication modules.

Dallas VREfm harbored 17 variants of Tn*1546*. The Tn*1546* region of the Dallas

isolates varied from the typical structure consisting of ORF1 (transposase), ORF2

(resolvase) and *van* genes flanked by IR_L (inverted repeat, left) and IR_R (inverted

repeat, right) (**Figure 6**). A total of 17 different sequence variations in the Tn*1546*

were observed among 45 of the Dallas isolates (**Figure 6, Tables 3-4**)*.* These 17

variations were classified using a nomenclature system developed previously[13] based

on the presence of IS elements, point mutations, and deletions relative to the

reference Tn*1546*. VREfm belonging to the same Tn*1546* group shared 99.8%-100%

nucleotide sequence identity in the Tn*1546* region. The isolate 111 was excluded

from this grouping since it harbored a fragmented Tn*1546* in a pRUM-like plasmid and

a circular entity (**Dataset S1F, Figure S2**). The Tn*1546* variation was mostly

associated with the distribution of 7 different IS elements (IS*1251*, IS*1216*, IS*Efa16*,

IS*Efa5*, IS*Efa17*, IS*256*, IS*Efa18*), three of which are novel to the Dallas isolates.

Most of the Tn*1546* groups described in this study are novel, except for group BC2,

which was reported previously in Poland[13]. The most predominant Tn*1546* groups

were BC9 (n=14) and J (n=6). The BC9 variety was observed in ST17 (n=9) and

ST18 (n=5) isolates. The reference strain ATCC® 700221™ harbored the C2

structural variant of Tn*1546* that was described previously[13] but is not present in our

Dallas VREfm isolates.

Identical Tn*1546* groups were observed for isolates collected from the same patient

(for example, all three VREfm isolates from patient 124 harbored Tn*1546* type BCCL),

except patients 51 and 93 (**Table 3**). Isolates 51-1 and 51-4 harbored Tn*1546* type J

and BC2 on the plasmid backbones 2b and 2a respectively. Isolates 93-1 and 93-2

harbored the same Tn*1546* variety J on pRUM-like plasmid group 2b while 93-3

harbored BCM on plasmid group 2a. Interestingly the 51-1 (ST664) and 51-4 (ST612)

isolates belonged to different clades in the phylogenetic tree. The isolates 93-1

(ST664) and 93-2 (ST664) clustered together but 93-3 (ST17) was placed in a

separate clade. It is possible that pRUM-like vancomycin resistance plasmids were

acquired by different *E. faecium* genotypes in two separate events in each of these

patients.

Specific structural variations of the Dallas Tn*1546* elements are described below.

The 3' but not 5' boundary of the Tn*1546* was conserved among the Dallas

isolates. Each of the 17 Tn*1546* groups preserved the IR$_R$ boundary of Tn*1546* but

lacked the IR$_L$ boundary (**Figure 6**). The only exception was the isolate 17-1 (group

BBCD) that lost both of the IR boundaries due to insertion of IS*1216*. The complete or

partial deletion of ORF1 (transposase) and ORF2 (resolvase) was observed in all of

the Tn*1546* groups. This might decrease the likelihood of these Tn*1546* to

disseminate to other isolates without a plasmid as a vehicle.

IS*1251* and IS*1216* were the most common IS elements. IS*1251* was present in

84.4% (38 of 45) of the isolates (**Table 3**). It was inserted at Tn*1546* position 5820 in

the *vanS-vanH* intergenic region, in reverse orientation to the *van* genes (**Figure 6**).

The IS*1251* generated an 8 bp direct repeat (5'-AAAATTAT-3') in the regions where it

was inserted. IS*1216* was inserted at either the 5' end of Tn*1546* in 71.1% (32 of 45)

or in the intergenic region of *vanX-vanY* in 17.8% (8 of 45) of the isolates.

The *van* genes and *vanR* and *vanH* promoters were intact in most isolates,

despite IS element insertions. IS*1251* insertion at Tn*1546* position 5820 did not

disrupt the two 12 bp VanR binding sites located at positions 5846 and 5879 (**Figure**

S4). The 70 bp VanR-interacting region (-110 to -41) from positions 5935 to 6004 is

also intact. The 197 bp long proposed *vanR* promoter region (-173 to +24) is located

at the position 3,803 to 3,999 bp of the Tn*1546*. The isolate 137 (Tn*1546* group BCK)

has an IS*Efa16* inserted at the position 3664 to 3763 that was responsible for a 99 bp

deletion of resolvase at 3' region but the *vanR* promoter region remained

uninterrupted. The *vanR* promoter region including the 12 bp consensus VanR

binding site is disrupted in the isolates 51-1, 87-1, 93-1, 93-2, 155, 160 from group J

because of the insertion of IS*1216* at position 3914. The *vanR* promoter region for

other isolates remained uninterrupted.

Deletion of *vanR* and *vanS* was observed in the isolate 91 (variant group D1).

Expression of the *vanHAX* genes may be constitutive instead of inducible in this

isolate. No mutations were identified in *vanR* and *vanS* in the rest of the groups.

Two point mutations in *vanHAX* were fixed in the population. All isolates

harbored a point mutation (T7658C) in *vanA* that generated a valine to alanine

substitution (**Table 3**) and a second point mutation (G8234T) in *vanX* that generated a

lysine to asparagine substitution. 62.2% (28 of 45) of the isolates harbored a third

point mutation (C9692T) in *vanY* that was also present in the reference strain

ATCC® 700221™. This point mutation generates a proline to leucine substitution in

VanY. Isolates 53-1 and 53-2 (group BC10) had point mutations C6513A and G6514A

in *vanH* that generated an arginine to lysine substitution. Isolate 144-1 harbored a

unique G9566A point mutation in *vanY* that conferred a glycine to glutamic acid

substitution. No mutations in *vanZ* were observed in any isolates.

Discussion

This study provided a snapshot of fecal VREfm genotypes among hospitalized

patients in Dallas, Texas. To our knowledge, this was the first whole-genome

epidemiological analysis of fecal VREfm isolates from patients without an active

infection due to VREfm in this area.

Genetic diversity was observed among all of the 47 isolates analyzed. The most

prevalent VREfm isolates, comprising 50% of the population, belonged to ST17 and

ST18. These STs were previously reported to be responsible for outbreaks in

countries including Portugal[12], Spain[32], Ireland[33], Denmark[34], Columbia[35], Brazil[36],

Canada[37]. The other 8 STs, identified among our collection (ST1383, ST612, ST412,

ST80, ST1703 and ST664), were single, double, or triple locus variants of ST17. The

ST1703 was a novel sequence type described in this study. Notably, ST182, which

caused an outbreak in San Antonio, Texas in the 1990s[38], was not detected in our

collection. The isolate 163-1 was identified as a presumptive VREfm from clinical

collection and was co-isolated with a *vanA*-positive VREfm not sequenced in this

study[16]; but was sensitive to vancomycin by broth microdilution and negative for *vanA*

and *vanB* by PCR screening[16]. This isolate was incorporated in our study despite

being sensitive to vancomycin because it provided a reference point to compare with

other Dallas isolates. This isolate generated its own clade, separate from the *vanA*-

positive isolates in the phylogenetic tree, and its genetic background, ST262, has not

been reported as VREfm to our knowledge. However, interestingly, the ST262 is a

triple locus variant of ST17 (*pstS-7, atpA-7,* and *gyd-5*) and still belongs to the CC17.

To conclude, VREfm isolates belonging to a combination of previously described and

new genetic backgrounds colonize Dallas, Texas, patients.

The number of core genes identified among the Dallas isolates was 1706, which is

much higher than the previously reported 1344[5] and 955[39] core genes described by

other groups. The lower number of samples (47 isolates) collected from a specific

source (hospitalized, high-risk patients with fecal VREfm colonization from Dallas,

Texas) is likely responsible for the larger number of core genes in our current study.

The previous studies utilized larger sample sizes (73[5] and 1,644[39]), were collected

from different sources including hospitalized patients, healthy human volunteers, and

animals, and included both VREfm and non-VREfm.

Completely closed plasmid sequences were achieved in this study by producing high

quality genome assemblies. This made it possible to perform comparative analyses of

entire plasmid sequences, which is a significant advantage compared to many

previously employed techniques to analyze resistance plasmid diversity, including

PCR-based typing schemes, restriction fragment length polymorphism (RFLP)

comparisons, and hybridization[12].

All the plasmid-borne Tn*1546* in Dallas isolates were encoded in pRUM-like plasmids

that were clustered into 4 subgroups (1, 2a, 2b, 3, and 4), defined by the presence of

the replication module *rep17* and a combination of other replication and stability

modules. Speculatively, deletion or insertion of $TA_{axe-txe}$ drove the evolution of the

group 3 and 4 pRUM-like plasmids. The vancomycin-sensitive isolate 163-1 also

harbored a pRUM-like plasmid, without Tn*1546,* that did not belong to any of the sub-

groups described in this study. This striking homogeneity in the plasmid backbone

encoding *vanA* was surprising, because previous studies identified a mixture of

different plasmids (pRUM-like, Inc18, mega-plasmid pLG1) as being responsible for

vancomycin resistance in clinical isolates collected from a certain geographic region[12].

However, our study differs from these previous studies in temporal and geographic

features of sample collection. In previous studies, samples were collected over 2 to

12 years[12,13,40] and from different regions of countries including Portugal[12],

Poland[13]and Germany[40].

The patients from whom the isolates in this study were collected resided in different

areas of Dallas and in unconnected facilities including nursing homes and personal

residences. The similarities among them were that they were high-risk patients,

having recently been in a healthcare environment or having antibiotic exposure.

Therefore, the presence of only one type of plasmid backbone carrying *vanA* in the

Dallas isolates is striking. However, the existence of a common source of VRE acting

as a reservoir and transferring a vancomycin resistance plasmid to *E. faecium* of

different genetic background is an unlikely scenario since the pRUM backbone

sequences and Tn*1546* structure varied.

The phylogenetic analysis of pRUM-like plasmids in this study suggested an

evolutionary event driven by either insertion or deletion of the TA$_{axe\text{-}txe}$ which was

originally described in the pRUM plasmid[31]. pRUM-like plasmids lacking TA$_{axe\text{-}txe}$ were

reported previously[12] although the possible implication of absence of TA$_{axe\text{-}txe}$ on

pRUM-like plasmid sub-grouping was not taken into consideration. The current study

suggests a possibility of evolution of the pRUM-like plasmids by deletion of the TA

system *axe-txe* since they have a second active TA$_{relE}$. pRUM-like plasmid groups 3

and 4, defined in this study, may be descendants of groups 1 and 2 in Dallas-area

VREfm (**Figure S5**). Whether this impacts the maintenance of these plasmids in the

absence of antibiotic selection, or their transfer efficiencies, requires further

experimental study.

A total of 17 variations of the Tn*1546* region, defined by the presence of different IS

elements, point mutations, and deletions, were observed among the Dallas isolates.

In absence of a strict guideline for the nomenclature of the Tn*1546* variants, a recent

one described in 2017[13] was followed in this study. A slight variation in Tn*1546*

nomenclature was utilized in 2016 where variants with IS*1251* were designated as

"type F" or "US hospital type"[9], which are designated as group "C" in the current

study. Three newly identified IS elements (IS*Efa16,* IS*Efa17,* and IS*Efa18*) within the

Tn*1546* were observed in this study. Moreover, two point mutations within Tn*1546*,

T7658C and G8234T, which were ubiquitous among the Dallas VREfm isolates, were

reported previously but never in the same Tn*1546* group[13]. The point mutation

T7658C was reported in the Tn*1546* groups BC5, BC1, BC2, BC3, and BC4 while

G8234T was reported in groups A2, C1, C2, BB1, BB2, BC1, BC2, BC3, BC4, BC5,

BBBI1, and BBBI2[13].

The Tn*1546*-borne vancomycin resistance genes were carried by pRUM-like

plasmids, which was categorized into plasmid groups 1, 2a, 2b, 3, and 4 based on

replication and stability modules. Both of the pRUM-like plasmid groups 1 and 2a

harbored the Tn*1546* variation BC9 (**Figure 7**). This was the only example of two

different plasmid groups carrying the same Tn*1546* variation. Therefore, the plasmid

backbone may have diverged into groups 1 and 2a after acquisition of the BC9 type

Tn*1546*. The same variation of Tn*1546*-type transposon was never observed for the

plasmid groups 2b, 3 and 4. Therefore, an independent evolution of Tn*1546* and the

plasmid backbone can be suggested for the rest of the resistance plasmid groups.

The resistance plasmid group 1 harboring the same Tn*1546* variant, BC9, in two

different genetic backgrounds, ST17 (isolate 1, 20, 57, 83, 103, 107-1, 131-2, 148,

and 161) and ST18 (isolate 100-1 and 100-2), was observed. Horizontal transfer of

this specific plasmid between ST17 and ST18 is possible (**Figure 7**). Similarly,

horizontal transfer of plasmid group 1 with Tn*1546* variant BC11 between isolates

belonging to ST18 and ST1383 may have occurred, as well as horizontal transfer of

plasmid group 2a with Tn*1546* variant BC2 between isolates belonging to ST80 and

ST612 (**Figure 7**). No other evidence of horizontal transfer of pRUM-like plasmids

among different VREfm genetic backgrounds was observed. Different plasmid groups

present in isolates with the same genetic background (group 1, 2a in ST17, ST18,

ST80; group 3, 4 in ST1703) suggested independent acquisition events of the

resistance plasmids within those STs. The resistance plasmid groups 1 and 2a were

observed in the common STs 17 and 18. The other resistance plasmid groups 2b, 3

and 4 were carried by the less prevalent STs. So, a combination of both horizontal

transfer and independent acquisition events followed by IS element-driven Tn*1546*

evolution shaped the VanA-type VREfm population in this area.

To conclude, the molecular diversity of multidrug resistant VREfm isolates in Dallas,

Texas, collected in the year 2015 was documented. VREfm with a novel genetic

background, ST1703, was reported in this study. No clonal spread of any particular

VREfm genotype could be detected in this isolate collection. However, strikingly,

vancomycin resistance genes were borne by pRUM-like plasmids in almost all

isolates despite the diversity of patient characteristics. Diversity of Tn*1546* was

described and new variations involving 3 novel IS elements (IS*Efa16,* IS*Efa17* and

IS*Efa18*) were also described among the VREfm collection. Horizontal transfer of

resistance plasmids between ST17-ST18 and ST80-ST612 was speculated since

VREfm of these genetic backgrounds carried the same variation of Tn*1546* on the

same plasmid backbone. Independent acquisition of resistance was observed in the

rest of the isolates. Therefore, it was concluded that VREfm representatives from

different sources carrying unique Tn*1546* generated the demography of VREfm in

Dallas, Texas.

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817 **Table 1. Features of the pMIX group plasmids**

Plasmid group	No. of isolates with plasmid group[1]	% of isolates harboring this plasmid[2]	Plasmid size range (kb)	stdv of plasmid size within group	*rep* gene information				
					rep gene size (bp)	% identity within group (*rep* DNA)[3]	% identity within group (*rep* aa)[4]	*Rep* encoded Protein Family Id[5]	pfam Accession[6]
pMI1	35	74	23-62	11.2	1086	99.7	98.9	repA_N	PF06970.11
pMI2	32	68	6-8	0.3	951	100	100	Rep_3	PF01051.21
pMI3	17	36	3.6 -4.3	0.2	990	100	100	Rep_1	PF01446.17
pMI4	11	23	4-11	2.3	972	79.4	67.9	Rep_trans	PF02486.19
pMI5	5	11	83-106	8.7	804	95.40	95.9	Rep_3	PF01051.21
pMI6	4	9	4	0	924	100	100	Rep_3	PF01051.21
pMI7	4	9	3	0	720	100	100	Rep_3	PF01051.21
pMI8	1	2	2	N/A	603	N/A	N/A	Rep_2	PF01719.17
pMI9	1	2	70	N/A	1215	N/A	N/A	Rol_Rep_N	PF18106.1
								Rep_trans	PF02486.19

[1]Specifies the total number of isolates among the 47 Dallas isolates harboring the

particular pMIX group plasmids.

[2]The % of isolates harboring the specific plasmid group.

[3,4]The "% identity (DNA)" and the "% identity (aa)" specifies the % identical sites

calculated from the MUSCLE sequence alignment of DNA and amino acid of the *rep*

genes belonging to the same pMIX group, respectively.

[5,6] The *rep* gene encoded protein family (pfam) and the corresponding pfam

accession numbers, respectively.

Table 2. Features of the pRUM-like plasmid groups

plasmid groups	MLST[1]	Isolate	rep17	rep18	axe-txe	relE	size (bp)	Size (kbp) (Avg ±stdev)[4]
Group 1	ST17	1	■		■		44,323	44 ± 1.9
		20	■		■		44,443	
		43-1	■		■		44,730	
		57	■		■		44,421	
		83	■		■		44,442	
		103	■		■		48,758	
		107-1	■		■		41,004	
		131-2	■		■		43,860	
		148	■		■		45,965	
		161	■		■		44,443	
	ST18	100-1	■		■		43,236	
		100-2	■		■		43,236	
		144-1	■		■		47,773	
	ST1383	71-1	■		■		44,420	
		71-2	■		■		44,420	
	ST80	113	■	a	■		41,153	
Group 2a	ST18	5	■		■		45,647	37 ± 4.3
		66	■		■		38,373	
		97-1	■		■		39,713	
		124-1	■		■		37,877	
		124-2	■		■		37,877	
		124-3	■		■		37,877	
		162	■		■		34,583	
	ST80	16-1	■		■		41,600	
		16-2	■		■		33,441	
	ST17	93-3	■		■		34,144	
	ST612	51-4	■		■		29,409	
Group 2b	ST664	51-1	■		■		61,762	59 ± 3
		87-1	■		■		57,116	
		93-1	■		■		63,108	
		93-2	■		■		63,108	
		155	■		■		57,134	
		160	■		■		57,135	
Group 3	ST736	9-1	■			■	38,857	38 ± 2.6
		9-2	■			■	38,857	
		52	■			■	36,284	
		55	■			■	36,284	
	ST1703	121	■			■	42,671	
Group 4	ST1703	111	■				17,982	28 ± 7.4
		137	■				35,487	
		154-1	■				22,729	
		158	■				30,941	
	ST412	17-1	■				33,158	

[1]Multiple locus sequence type (MLST) of the isolates

[2,3]The presence of *rep* and toxin-antitoxin (TA) genes are indicated by blue boxes.

The pRUM-like plasmid from isolate 113 harbored *rep18a* (reference gene Accession

no. AB158402) and is symbolized as "a" in the corresponding box. The *rep18* for rest

of the resistance plasmids were *rep18b* (reference gene Accession no. CP018068).

[4]The average size of the plasmids for each plasmid group type is shown along with

the standard deviation (Avg. ± stdev).

Table 3. Features of the Tn*1546* groups observed among the Dallas isolates

pRUM-like plasmid group	Tn*1546* Group	Isolate	Identity within Tn*1546* group[1]	No. of Isolates in Tn*1546* group	IS element[2]	vanH (6513, 6514 CG-->AA) Arg-->Lys	vanA (7658 T-->C) Val-->Ala	vanX (8234 G-->T) Lys-->Asn	vanY (9692 C-->T) Pro-->Leu	vanY (9566 G-->A) Gly-->Glu
1	BCL	43-1	N/A	1	IS*1216*, IS*1251*, IS*Efa17*		■	■		
1	BC8	113	N/A	1	IS*1216*, IS*1251*		■	■		
1	BC11	71-1	99.80%	3	IS1216, IS1251		■	■		
1	BC11	71-2					■	■		
1	BC11	144-1					■	■		■
1	BC9	1	99.80%	14	IS1216, IS1251		■	■	■	
1	BC9	20					■	■	■	
1	BC9	57					■	■	■	
1	BC9	83					■	■	■	
1	BC9	100-1					■	■	■	
1	BC9	100-2					■	■	■	
1	BC9	103					■	■	■	
1	BC9	107-1					■	■	■	
1	BC9	131-2					■	■	■	
1	BC9	148					■	■	■	
1	BC9	161					■	■	■	
2a	BC9	5					■	■	■	
2a	BC9	66					■	■	■	
2a	BC9	162					■	■	■	
2a	BC2	16-1	100%	3	IS*1216*, IS*1251*		■	■		
2a	BC2	16-2					■	■		
2a	BC2	51-4					■	■		
2a	BBC	97-1	N/A	1	IS*1216*, IS*1251*		■	■		
2a	BCM	93-3	N/A	1	IS1216, IS1251, IS*Efa18*		■	■		
2a	BCCL	124-1	100%	3	IS*1216*, IS*Efa17*, IS*1251*, IS*1251*		■	■		
2a	BCCL	124-2					■	■		
2a	BCCL	124-3					■	■		
2b	J	51-1	100%	6	IS*256*		■	■	■	
2b	J	87-1					■	■	■	
2b	J	93-1					■	■	■	
2b	J	93-2					■	■	■	
2b	J	155					■	■	■	
2b	J	160					■	■	■	
3	C3	52	100%	2	IS*1251*		■			
3	C3	55					■			
3	BC6	9_1	100%	2	IS*1251*, IS*1216*		■			
3	BC6	9_2					■			
3	BC7	121	N/A	1	IS*1251*, IS*1216*		■			
4	BCB	154-1	100%	2	IS*1216*, IS*1251*, IS*1216*		■			
4	BCB	158					■			
4	BCK	137	N/A	1	IS*Efa16*, IS*1251*, IS*1216*		■			
4	BBCD	17-1	N/A	1	IS*1216*, IS*Efa5*, IS*1251*, IS*1216*		■			
Chromosome integrated Tn*1546*	D1	91-1	N/A	1	IS*Efa5*		■		■	
Chromosome integrated Tn*1546*	BC10	53-1	100%	2	IS1216, IS1251	■	■	■	■	
Chromosome integrated Tn*1546*	BC10	53-2				■	■	■	■	

[1] Nucleotide sequences of the Tn*1546* region for the Tn*1546* groups, including more than one isolate, were aligned and % identity was determined.

[2] The name of the IS elements present in the Tn*1546* group are listed.

[3] Point mutations in the van gene cluster relative to the reference Tn*1546* (accession number M97297). Presence of a point mutation is represented by a colored box.

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Figure 1. Plasmid content of Dallas *E. faecium* isolates. The phylogenetic tree of
49 *E. faecium* strains is based on an alignment of SNPs in 1706 core genes. The
branches colored green, golden and red are supported by a bootstrap value of >70,
40-60, and <30 respectively. The reference genomes *E. faecium* 1,231,501 and
ATCC® 700221™ are labeled in red. Data for MLST, number of presumptive
plasmids (shown as ## for incomplete assemblies), and plasmid *rep* types are shown.
Plasmid replicon conserved domains i) RepA_N, ii) Rep_3, iii) Rep_trans, iv) Rep1, v)
Inc18, vi) Rep_2, vii) Rol_Rep_N **(A)**, *rep* family **(B)**, plasmid size ranges **(C)**, and
reference plasmid names **(D)** present in each strain are shown. A dark purple box
indicates a chromosomally integrated plasmid whose size range was not determined.
The *rep17* and mosaic of *rep17/rep18* are indicated with the gray and light green
boxes, respectively. New plasmid groups identified in this study (pMI1 to pMI9) are
highlighted in light blue color.

Figure 2: Sequence type (ST) distribution of Dallas *E. faecium* isolates. A total of 10 STs were observed across 47 isolates. The frequency of STs (percent of total isolates) is shown.

Figure 3. Distribution of antibiotic resistance genes among Dallas isolates.
Shown are 12 resistance genes (6'-N-aminoglycoside acetyltransferase, (*aac(6')I-i*)*,*
bifunctional enzyme 6'-aminoglycoside acetyltransferase-2"-aminoglycoside
phosphotransferase (*aac(6')-aph(2")*)*,* aminoglycoside nucleotidyltransferase (*ant(6)-*
Ia)*, aph(3')III, vanA, erm(B), erm(T), lnu,* acquired macrolide resistance-like protein
(msr), tet(M), tet(L) and dihydrofolate reductase (*dfrG*))*,* and the antibiotics against
which they provide resistance to. A solid colored box indicates the presence of a
certain resistance gene in the isolate. The boxes are color coded to indicate the
location of the gene (chromosome or a certain type of plasmid).

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Figure 4. Genetic map of a representative Group 2a pRUM-like plasmid. The 39

kb plasmid p97_1_39kb (accession number CP066571) from the Dallas VREfm

isolate 97-1 is shown. Arrows represent genes. The genes are color-coded based on

their predicted activity. Maintenance genes are predicted to be responsible for general

maintenance of the plasmid within the bacterial host. Resistance genes provide

antibiotic resistance.

A.

		Group 1	Group 2a	Group 2b	Group 3	Group 4
rep	*rep18*	+	-	-	-	-
	rep17	+	+	+	+	+
TA	*axe*	+	+	+	-	-
	relE	+	+	+	+	-

B.

953 **Figure 5. Classification and distribution of pRUM-like plasmid groups. A**) pRUM-
954 like plasmids were classified based on the presence or absence (indicated by a + or -,
955 respectively) of 2 replication modules (*rep17* and *rep18*) and 2 stability modules
956 (toxin-antitoxin system; TA$_{axe-txe}$ and TA$_{relE}$). **B)** Frequency of the pRUM-like plasmid
957 groups among 43 Dallas isolates.

Figure 6: Tn*1546* structural variations in the Dallas VREfm isolates. A reference Tn*1546* structure that includes transposase, resolvase and *van* gene cluster flanked by IR$_L$ and IR$_R$ (inverted repeat left and right, respectively) is delineated at the top. The Tn*1546* groups are represented below the reference Tn*1546*, and the name of the group (C3, BC2, BC6, BC7, BC8, BC9, BC10, BC11, BCB, BBC, BCK, BCL, BCM, BBCD, BCCL D1, J) is shown on the left. Red boxes represent deleted regions relative to the reference Tn*1546*. Vertical lines with numbers at the bottom represent the nucleotide position within the reference Tn*1546*. IS elements are represented by downward arrows. Black stars indicate point mutations. The figure is not drawn to scale.

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Figure 7. Evolutionary relationships among pRUM-like plasmid groups, Tn*1546*,
and VREfm genetic background. The resistance plasmid groups 1, 2a, 2b, 3, and 4
are represented on the left side. The Tn*1546* variations encoded in the corresponding
plasmid backbone are displayed on the right side in white boxes. The numbers in
parentheses represent total numbers of isolates harboring this Tn*1546* variant on this
specific plasmid group belong to this genetic background (ST). The Tn*1546* variant BC9
in plasmid groups 1 and 2a is highlighted with pink color to indicate the unique event of
the presence of same *Tn1546* variation in two different plasmid groups. The sequence
type (ST) of VREfm isolates carrying the Tn*1546* variation on the specific plasmid
backbone is indicated with blue ovals. A solid red arrow links the ST and the Tn*1546*
variation carried by the corresponding plasmid backbone. The dotted black arrow
indicates a possible horizontal transfer of resistance plasmid between VREfm isolates
belonging to that genetic background.

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